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Potent selective inhibitors of protein kinase C

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A series of potent, selective inhibitors of protein kinase C has been derived from the structural lead provided by the microbial broth products, staurosporine and K252a. Our inhibitors block PCK in intact cells (platelets and T cells), and prevent the proliferation of mononuclear cells in response to interleukin 2 (IL2).

Protein kinase C; Selective inhibition; Platelet; T cell; Interleukin 2

1. INTRODUCTION

The isoenzyme family, protein kinase C (PKC) [1], has been implicated as a key element in many signal transduction processes [2]. Involvement of PKC has been inferred by the use of specific activators of the enzyme such as phorbol esters, but clarification of its precise role in cell functions has been hampered by the lack of potent selective inhibitors. This communication describes the derivation of a series of such inhibitors based on the structure of the microbial metabolites, staurosporine [3] and K252a [4], which are potent but non-selective PKC inhibitors. Both staurosporine and K252a have a flat, polyaromatic aglycone which itself inhibits PKC. Modification of the sugar moieties has resulted in some modulation of potency and selectivity [5-7].

We wished to investigate whether activity could be retained in analogues of the aglycone which did not have flat geometries. Compounds were synthesised in which a bond in the aglycone had been formally removed and an extra carbonyl group introduced into the lactam ring. This ensured that, through steric interactions, no low-energy conformation could have an indole group coplanar with the 5-membered ring.

2. MATERIALS AND METHODS

2.1. *Inhibition of PKC in vitro*

10 μl rat brain PKC, partially purified by ion-exchange chromatography [15] were added to a reaction cocktail (90 μl) containing 0.22 mg/ml lysine-rich histone (type IIIs, Sigma), 0.56 mM $CaCl_2$, 44 μg/ml phosphatidylserine, inhibitor in 10 μl DMSO and 11 μM [γ-^{32}P]ATP in 25 mM Tris-HCl, 5 mM $Mg(NO_3)_2$ buffer, pH 7.5 [16]. After 10 min incubation at 30°C, the reaction was stopped by addition of 1 ml ice-cold 10% trichloroacetic acid. Acid-precipitable protein was collected on glass-fibre discs and incorporated radioactivity determined by liquid scintillation spectrometry.

2.2. *Inhibition of PKA in vitro*

10 μl bovine heart PKA (0.1 mg/ml; Sigma) were added to a reaction cocktail (90 μl) containing 0.67 mg/ml histone (type Vs, Sigma), 1.1 μM cAMP, inhibitor in 10 μl DMSO and 11 μM [γ-^{32}P]ATP in 200 mM Tris-HCl, 40 mM $MgCl_2$, pH 8.5. After 10 min incubation at 37°C, the assay procedure thereafter was that described for PKC.

2.3. *Inhibition of Ca^{2+}/calmodulin-dependent protein kinase in vitro*

30 μl Ca/CaM-dependent protein kinase, partially purified from rat brains by ion-exchange chromatography [17], were added to a reaction cocktail (70 μl) containing 0.29 μg/ml myosin light chains, 143 μM $CaCl_2$, 14 μg/ml CaM and 14 μM [γ-^{32}P]ATP, in 10 mM Pipes, 4 mM $MgCl_2$, pH 6.9. The assay procedure thereafter was that described for PKC.

2.4. *Inhibition of PKC in platelets*

100 ml blood from a human volunteer were centrifuged at low speed (140 × *g*) to produce platelet-rich plasma. Plasma was then spun at higher speed (600 × *g*) to deposit platelets as a soft pellet which was resuspended in 2.5 ml 15 mM Tric-HCl containing 140 mM NaCl, pH 7.4. Cells were rested at 37°C for 90 min before addition of 350 μCi carrier-free [^{32}P]orthophosphate in dilute HCl and incubation for a further 60 min. Inhibitors, dissolved in DMSO, were added to aliquots of platelet suspension so that the final solvent concentration was 1%. Cells were preincubated with inhibitor for 20 min at 37°C before stimulation with 1 μM TPA for 3 min. The reaction was terminated by addition of protein disruption buffer followed by boiling for 2 min. Aliquots (30 μl) were subjected to 17.5% SDS/PAGE. Gels were exposed to Fuji-RX X-ray film for 24 h at −80°C. ^{32}P incorporation was assessed by densitometric scanning of the autoradiograph.

2.5. *Inhibition of down-regulation of CD3*

Freshly prepared human peripheral blood mononuclear cells were treated with inhibitors for 15 min prior to addition of PDBu (100 ng/ml). After a further 30 min incubation at 37°C, cells were washed with ice-cold phosphate-buffered saline, incubated 1 h at 4°C

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Abbreviations: PKC, protein kinase C; PKA, c-AMP dependent protein kinase; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PDBu, phorbol 12,13-dibutyrate; DMSO, dimethyl sulphoxide; IL2, interleukin 2; SDS/PAGE, sodium dodecyl sulphate/polyacrylamide gel electrophoresis

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with anti-CD3 (Sera-Lab), washed again and incubated with ^{125}I-labelled anti-murine immunoglobulin (Amersham International). Radiolabelled cells were separated by centrifugation through a cushion of silicone/paraffin oils (24:1, v/v).

3. RESULTS AND DISCUSSION

Imide **1** inhibited PKC and was more selective than the staurosporine-like compounds (table 1). From comparison of the relative inhibitory potencies of staurosporine and K252a analogues it seemed likely that the basic nitrogen of staurosporine contributes significantly to its activity. We assumed a common binding mode of compound **1** and staurosporine and used a molecular graphics approach to design compounds capable of utilising the putative amine binding site. Compounds **2–5** showed improved activity over **1** and were even more selective for PKC over cAMP-dependent protein kinase (PKA) or Ca/calmodulin-dependent kinase. Unlike the amine **2**, the isothiourea **3** and the quaternary amine **5**, the nitroguanidine **4** is not significantly protonated at the pH of the assay and it is possible that the large dipole moment of the nitroguanidine group is a key property for activity of this compound.

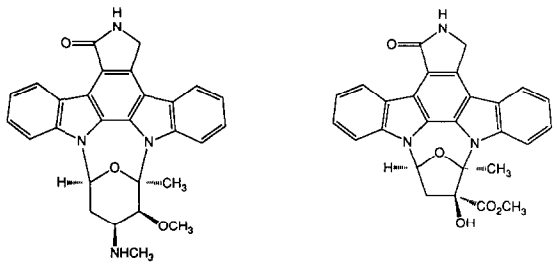
Each of compounds **1–5** was equiactive against PKCs derived from rat brain and human neutrophil (data not shown). The lower potency against PKA was confirmed with enzyme isolated from various sources (bovine heart, rat brain and human neutrophil). The compounds bind to PKC competitively with ATP; a property shared with staurosporine and K252a [5]. This was confirmed directly in the case of compound **3** (Lineweaver-Burke plot; $K_i = 3$ nM) and was inferred for the other inhibitors from the consistent 6–8-fold drop in potency produced by a 10-fold increase in ATP concentration (table 1).

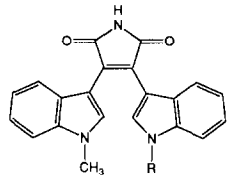
PKC activation in cells is most directly measured by pre-labelling human platelets with [^{32}P]orthophosphate and following the phosphorylation of specific proteins after treatment with the PKC activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA) [8]. Our inhibitors dose-dependently antagonised TPA-induced phosphorylation of a 47 kDa protein in intact human platelets (see fig.2). The function of this protein is not yet established [9], but these experiments provide a useful demonstration of the ability of the inhibitors to penetrate the cell and prevent PKC-mediated phosphorylation. The higher concentrations of ATP in cells [10,11] (mM as opposed to 10 μM in the isolated enzyme assay) probably contribute to the reduced potency of compounds **1–4** in intact cell systems but nevertheless

Table 1

Inhibitor IC_{50} values (μM) against isolated protein kinases

	Rat brain protein kinase C		Bovine heart protein kinase A	Rat brain Ca^{2+}/calmodulin-dependent protein kinase
	10 μM ATP	100 μM ATP		
K252a	0.47	n.d.	0.20	0.30
Staurosporine	0.01	n.d.	0.12	0.04
1	0.30	2.6	16	>100
2	0.08	0.58	5.1	15
3	0.01	0.08	1.5	17
4	0.03	0.20	3.3	14
5	0.09	n.d.	>20	>100





	R
1	CH_3
2	$(CH_2)_3NH_2$
3	$(CH_2)_3SC(=NH)NH_2$
4	$(CH_2)_3NC(=NNO_2)NH_2$
5	$(CH_2)_3\overset{+}{N}(CH_3)_3\ CF_3SO_3^-$

Fig.1. Structures of PKC inhibitors.

Fig.2. Autoradiograph showing the effect of various concentrations of compound **2** on TPA-induced P47 phosphorylation. TPA was present in lanes (b–j) and solvent (DMSO) in lane (a). The concentration of compound **2** in each lane was: a–c, solvent only; d, 100 μM; e, 30 μM; f, 10 μM; g, 3 μM; h, 1 μM; i, 0.3 μM; j, 0.1 μM. The position of molecular weight markers is indicated in the left-hand lane.

Table 2

Inhibitor IC_{50} values (μM) in cellular systems

	Inhibition of TPA-induced P47 phosphorylation in platelets	Inhibition of CD3 down-regulation
K252a	44	20
Staurosporine	0.6	0.2
1	>100	>100
2	4.4	3.1
3	0.7	0.5
4	1.2	2.1
5	>100	>100

these compounds display structure/activity relationships in the platelet system which correlate closely with those obtained against the isolated enzyme. The lack of activity of the quaternary amine (**5**) in the platelet system may be explained by its inability to cross the cell membrane.

The T cell surface protein complex, CD3, which is part of the T cell receptor for antigen is phosphorylated on triggering by antigen [12] and by treatment with phorbol esters [13]. Phosphorylation at serine-126 is associated with down-regulation of CD3 and may be an integral part of the T cell activation process [14]. The PKC inhibitors described in this report inhibited phorbol-induced CD3 down-regulation with potencies closely parallel to those observed in the platelet phosphorylation studies (table 2).

After this demonstration of PKC inhibitory activity in cells, the studies were extended to investigate effects on the response to a physiologically relevant agonist. The involvement of PKC in IL2 signal transduction is unclear. Compounds **2** and **3** do, however, inhibit IL2-induced proliferation of human peripheral blood mononuclear cells with IC_{50}s of 3.2 and 0.8 μM, respectively. The similarity of these values to those in 47 kDa phosphorylation and CD3 down-regulation is consistent with a role for PKC in this proliferative response to IL2.

The potent, selective inhibitors of protein kinase C described in this communication have been shown to block enzyme activity in intracellular environments. Tools have therefore been generated which will facilitate the determination of the physiological importance of PKC in the whole range of cellular activation processes induced by specific agonists. The possibility of using these and related agents for therapeutic intervention in a wide range of diseases including immune disorders, oncology and inflammation will be evaluated further in cellular systems and in animal models of the diseases.

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