RESEARCH COMMUNICATION

Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C

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The protein kinase C (PKC) family of isoenzymes is believed to mediate a wide range of signal-transduction pathways in many different cell types. A series of bisindolylmaleimides have been evaluated as inhibitors of members of the conventional PKC family (PKCs-α, -β, -γ) and of a representative of the new, Ca^{2+}-independent, PKC family, PKC-ϵ. In contrast with the indolocarbazole staurosporine, all the bisindolylmaleimides investigated showed slight selectivity for PKC-α over the other isoenzymes examined. In addition, bisindolylmaleimides bearing a conformationally restricted side-chain were less active as inhibitors of PKC-ϵ. Most noticeable of these was Ro 32-0432, which showed a 10-fold selectivity for PKC-α and a 4-fold selectivity for PKC-β_I over PKC-ϵ.

INTRODUCTION

The Ca^{2+}- and phospholipid-dependent enzyme protein kinase C (PKC) consists of a family of closely related isoenzymes that mediate a wide range of signal transduction processes. The ten isoenzymes that have been currently identified, PKCs-α, -β_I, -β_{II}, -γ, -δ, -ϵ, -ζ, -η, -θ, -λ, can be divided into three families on the basis of their different requirements for activation [1–4]. Members of the conventional PKC family (PKC isoenzymes PKCs-α, -β_I, -β_{II}, -γ) are Ca^{2+}- and phospholipid-dependent and are activated by diacylglycerols and phorbol esters, whereas enzymes in the new PKC family (PKC isoenzymes PKCs-δ, -ϵ, -η, -θ) are Ca^{2+}-independent. Members of the atypical PKC family (PKC-ζ and PKC-λ) have yet to be fully characterized, but PKC-ζ is insensitive to stimulation by Ca^{2+}, diacylglycerol and phorbol esters [2]. The isoenzyme is, however, activated by phosphatidylinositol 3,4,5-trisphosphate [5].

The functional significance of the existence of so many closely related enzymes is not yet understood. Immunological studies of protein expression have shown that the isoenzymes vary both in distribution between different cell types and in intracellular localization (see for example [6,7]). Increasing evidence suggests that different extracellular signals will generate distinct patterns of lipid activators of PKC (arachidonic acid, diacylglycerols, phospholipids) within the cell. The responses of PKC isoenzymes to these activators [1] suggest that particular isoenzymes may be preferentially activated by distinct extracellular ligands. The composition of lipid activators generated in response to these signals, as well as the intra- and inter-cellular distribution of the PKC isoenzymes, could determine which isoenzymes are involved in mediating particular signal-transduction pathways.

The difficulties in determining the significance and relative importance of the various isoenzymes are, in part, due to the absence of PKC-isoenzyme-specific activators or inhibitors. A series of potent and selective bisindolylmaleimide inhibitors of protein kinase C derived from the structural lead provided by the non-selective protein kinase inhibitor, staurosporine, have recently been described [8]. We report here the inhibition of PKC-α, PKC-β_I, PKC-β_{II}, PKC-γ and PKC-ϵ by a number of these compounds.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP was obtained from Amersham International p.l.c., trypsin from Washington and staurosporine from Fluka. Peptide-γ was supplied by P.J.P. [9]. All other biochemicals were purchased from Sigma Chemical Co. Bisindolylmaleimides were synthesized by Roche Products Ltd.

Purification of rat brain PKC

Rat brain PKC was partially purified by ion-exchange chromatography, as previously described [9].

Purification of PKC isoenzymes

PKC isoenzymes were purified by P.J.P., as previously described [10,11].

Assay of PKC activity

Assay mixtures contained 0.2 mg/ml peptide-γ, 10 μM $MgCl_2$, 0.6 mM $CaCl_2$, 10 μM [γ-^{32}P]ATP, 1.25 mg/ml phosphatidylserine and 1.25 ng/ml phorbol 12-myristate 13-acetate in 20 mM Hepes (pH 7.5), 2 mM EDTA, 1 mM dithiothreitol and 0.02% (w/v) Triton X-100. Peptide-γ is a synthetic peptide, GPRPLFC-RKGSLRQKVV, resembling the PKC-γ pseudosubstrate site, except that a serine residue replaces the pseudosubstrate alanine, converting the peptide from an inhibitor into a substrate [12]. The assays were started by the addition of 2.5 m-units of enzyme, incubated at 30 °C for 10 min and terminated by spotting on to P81 paper (Whatman), followed by extensive washing in 75 mM orthophosphoric acid. The papers were then washed in ethanol, dried, and incorporated radioactivity was determined by liquid-scintillation spectroscopy.

Abbreviation used: PKC, protein kinase C.

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Preparation and assay of protein kinase M

PKC-β_I (3 units) was subjected to limited proteolytic cleavage by 4 μg/ml trypsin in 10 mM Tris/HCl (pH 8.0), 10 mM 2-mercaptoethanol and 0.5 mg/ml BSA at 30 °C for 3 min. The reaction was stopped by the addition of 40 μg/ml trypsin inhibitor and the enzyme diluted down to 0.5 unit/ml in assay buffer. Enzyme activity was measured as described above, except that Ca^{2+}, phospholipid and phorbol ester were omitted from the assay mixture.

PKC inhibitors were dissolved in dimethyl sulphoxide and added to the assay such that the final concentration of solvent was 10 % (w/v). Solvent-only controls were added as appropriate.

RESULTS AND DISCUSSION

The indolocarbazole, staurosporine, is a potent but non-selective inhibitor of a wide range of serine/threonine- and tyrosine-specific protein kinases. Structure/activity relationships among indolocarbazole inhibitors of PKC point to the presence of a cation-binding site that interacts with the 4-aminomethyl substituent in the glycoside ring of staurosporine. Simple bisindolylmaleimides, such as Ro 31-7208 (Figure 1), are less potent but more selective inhibitors of PKC than is staurosporine [10]. Assuming a common binding mode for the indolocarbazole, staurosporine, and the bisindolylmaleimide, Ro 31-7208, a molecular-graphics approach was adopted to place substituents on the bisindolylmaleimide that would interact with the putative cation-binding site on the enzyme [13]. Bisindolylmaleimides carrying a straight-chain alkyl side-chain bearing a cationic substituent, such as Ro 31-7549 and Ro 31-8220 (Figure 1), show a considerable improvement in potency over the simple bisindolylmaleimides (Ro 31-7208). For Ro 31-7549, a further improvement in potency was obtained by correctly conformationally restricting the position of the amine substituent with respect to the rest of the molecule (Ro 31-8425, Ro 32-0432; Table 1) [14]. These compounds block PKC activity in cellular systems and modify a range of cellular responses, in particular those associated with inflammatory and autoimmune responses (A. M. Birchall, J. Bishop, D. Bradshaw et al., unpublished work). Ro 32-0432, when given orally, inhibits an antigen-driven T-cell-mediated arthritis *in vivo*, suggesting that PKC inhibitors may have therapeutic potential in the treatment of autoimmune diseases such as rheumatoid arthritis.

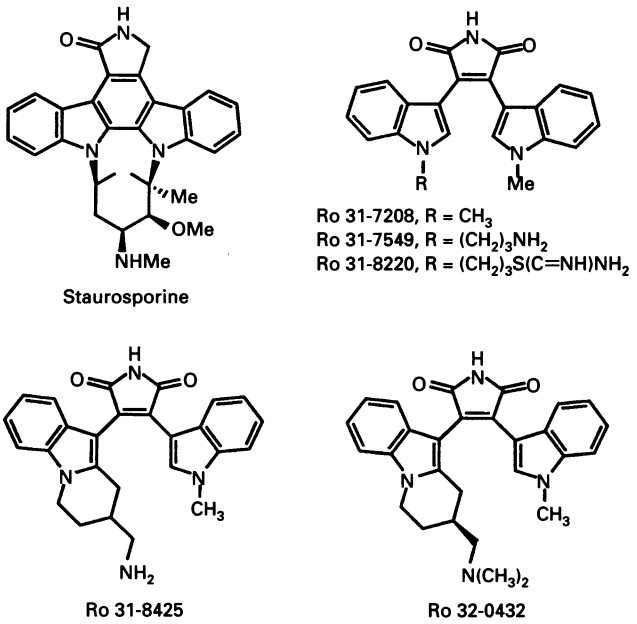

Figure 1 Structure of the indolocarbazole, staurosporine, and some bisindolylmaleimide inhibitors of PKC

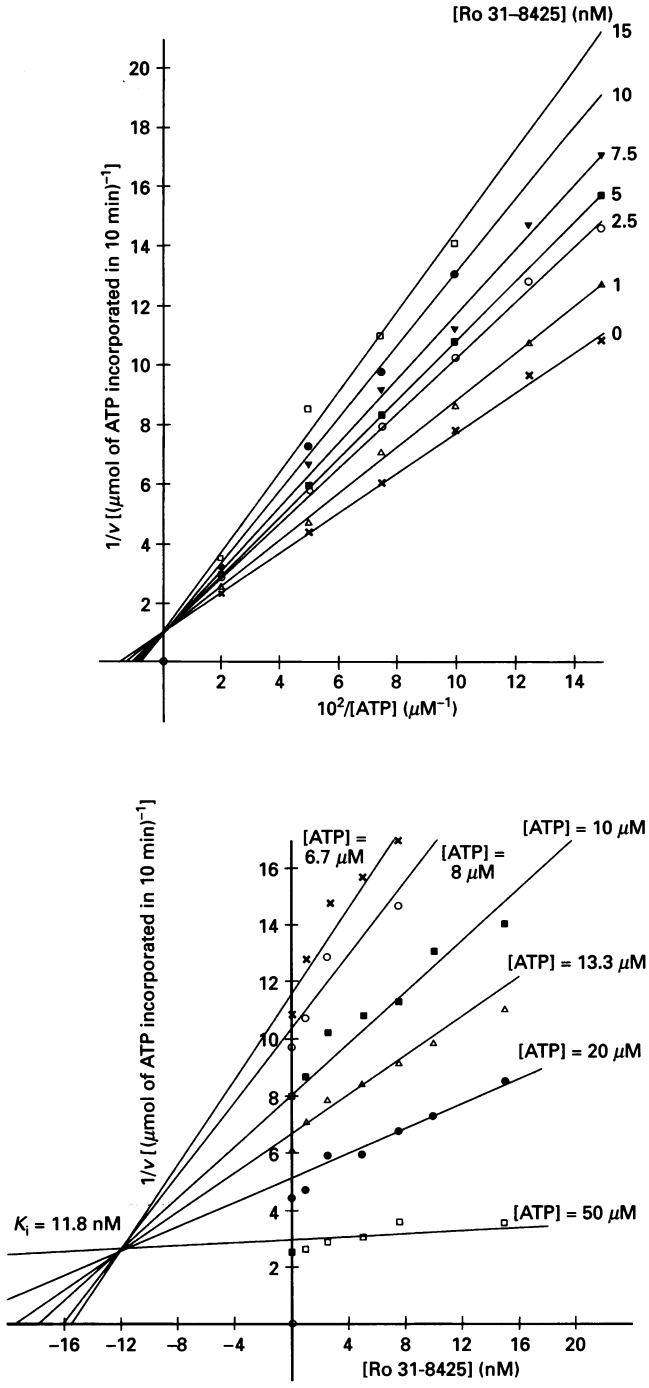

Figure 2 (a) Lineweaver–Burk plot for the inhibition of PKC-β_{II} by Ro 31-8425, and (b) Dixon plot for the inhibition of PKC-β_{II} by Ro 31-8425

These bisindolylmaleimide inhibitors compete with ATP for binding to the enzyme [14,15]. Ro 31-8425 was shown to inhibit

Table 1 Inhibition of PKC isoenzymes by staurosporine and bisindolylmaleimides

For each isoenzyme, a minimum of two analyses were performed; each assay point was in triplicate.

	IC_{50} (nM)					
Inhibitor	Rat brain PKC	PKC-α	PKC-β_I	PKC-β_{II}	PKC-γ	PKC-ϵ
Staurosporine	22	28	13	11	32	25
Ro 31-7208	323	160	310	280	377	330
Ro 31-7549	158	53	195	163	213	175
Ro 31-8220	23	5	24	14	27	24
Ro 31-8425	15	8	8	14	13	39
Ro 32-0432	21	9	28	31	37	108

PKC-β_{II} with a K_i of 11.8 nM (Figure 2). Further evidence that these compounds bind to the catalytic domain of PKC was provided from inhibition studies using protein kinase M, the Ca^{2+}- and phospholipid-independent catalytic fragment of PKC, which is fully functional in the absence of phorbol ester. Protein kinase M was generated by limited proteolytic cleavage of PKC-β_{II} by trypsin. The activity of protein kinase M β_{II} was inhibited by Ro 31-8425 with an IC_{50} value (10 nM $\pm$ 0.58 nM, $IC_{50} \pm$ S.D.; $n = 3$) that was similar to that obtained against PKC-β_{II} (IC_{50} = 14.1 nM $\pm$ 4.2 nM, $IC_{50} \pm$ S.D.; Table 1).

Although PKC isoenzymes have been reported to show distinct enzymological characteristics, their binding affinities for ATP are similar. The K_m values for ATP of rat brain PKC and PKCs-α, -β_I, -β_{II}, -γ and -ϵ were measured as 21.4, 13.8, 27.5, 28.3, 38.2 and 22.7 μM respectively. These data, taken together with the ATP-competitive inhibition of PKC by these bisindolylmaleimides, imply that IC_{50} values can be used to compare the affinities of different PKC isoenzymes for a potential inhibitor.

A range of these PKC inhibitors did not show any high degree of selectivity between PKCs-α, -β_I, -β_{II}, -γ and -ϵ (Table 1). Unlike the indolocarbazole, staurosporine, which displayed 2-fold selectivity for PKC-β over the other isoenzymes examined, all the bisindolylmaleimides investigated showed a slight selectivity for PKC-α over the other isoenzymes. Compounds such as Ro 31-7549 and Ro 31-8220, which carry a straight-chain alkyl side-chain bearing the cationic substituent, were equipotent as inhibitors of PKC-β, PKC-γ and PKC-ϵ. However, conformationally restricted bisindolylmaleimides (Ro 31-8425, Ro 32-0432) displayed a slight selectivity for conventional PKC isoenzymes (PKCs-α, -β, -γ) over the Ca^{2+}-independent PKC-ϵ. This difference was most noticeable for Ro 32-0432, which showed a 10-fold selectivity for PKC-α and a 4-fold selectivity for PKC-β_I over PKC-ϵ.

PKC-α, -β_I, -β_{II}, -γ, -δ, -ϵ and -ζ have all been identified as present in the rat brain [16], although the relative amount of each isoenzyme is not known precisely. The effects of the bisindolylmaleimides on PKC-δ and, perhaps more interestingly, the atypical PKC family member PKC-ζ have yet to be investigated. Furthermore, there may well be other, as yet unidentified, PKC isoenzymes present in the rat brain preparation. The presence of these additional isoenzymes may explain why a comparison of the IC_{50} results obtained against the individual isoenzymes with those obtained for each compound against rat brain PKC does not always result in a constant ratio. However, the temptation to draw too many conclusions from these data should be avoided, since the variations may be largely accounted for by the standard deviations associated with these measurements.

These compounds show little selectivity for the limited range of isoenzymes examined, and, therefore, are not suitable as tools to probe the role of particular isoenzymes in cell function. However, the small differences in IC_{50} values between different isoenzymes may explain variations in the potency of Ro 31-7549 observed against a range of phorbol-ester-induced cellular responses [9,17], although again these differences may not be marked enough to be translated into cellular systems. IC_{50} values would be expected to differ if the various processes are mediated through distinct isoenzymes. The indolocarbazole, K252a has been reported to show a high degree of selectivity for the conventional PKC isoenzymes (PKCs-α, -β, -γ) over PKC-δ and PKC-ϵ [18]. Although K252a, unlike the bisindolylmaleimides described in this paper, is not selective for PKC over other serine/threonine kinases [19], this observation suggests that there is a potential for the design of highly selective bisindolylmaleimide PKC inhibitors of protein kinase C that will also show a high degree of selectivity for particular PKC isoenzymes.

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