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K252a is a Potent and Selective Inhibitor of Phosphorylase Kinase

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The inhibition of phosphorylase kinase by a number of protein kinase inhibitors was examined. Both K252a and staurosporine are potent inhibitors of phosphorylase kinase with IC_{50} values of 1.7 nM and 0.5 nM respectively. K252a shows a 300-fold selectivity for this enzyme over protein kinase C whereas staurosporine shows only a 20-fold selectivity for phosphorylase kinase. In contrast, the Roche bis-indolyl maleimides inhibit phosphorylase kinase with IC_{50} values of approximately 1 μM and are highly selective for protein kinase C.

The microbial broth products K252a [1] and staurosporine [2] and the isoquinolinesulphonamide H7 [3] are potent but non-selective inhibitors of protein kinases (Fig. 1). Davis et al [4] have recently reported a series of bis-indolyl maleimide derivatives which were highly selective for protein kinase C (PKC) over c-AMP dependent protein kinase (PKA) and rat brain Ca^{2+}/calmodulin-dependent protein kinase. Meyer et al [5] have also reported that a derivative of staurosporine (CGP41251) showed good selectivity for PKC over PKA, S6 kinase and the tyrosine-specific protein kinase of the epidermal growth factor receptor. However, neither CGP41251 nor staurosporine showed any selectivity for PKC over chicken gizzard phosphorylase kinase (PhK), a key enzyme of the glycolytic pathway. The inhibition of PhK by K252a or by the Roche bis-indolyl maleimides has not yet been reported.

Phosphorylase kinase catalyzes the phosphorylation of phosphorylase b to its active form, phosphorylase a, which, under physiological conditions, triggers the breakdown of glycogen to glucose-1-phosphate, a rate limiting step in glycolysis. PhK itself is activated either through a phosphorylation reaction catalyzed by PKC [6] or through rises in intracellular Ca^{2+} [7,8] and, thus, the enzyme is under both hormonal and neuronal control. Muscle PhK exists as a hexadecamer composed of four types of subunit $\alpha_4\ \beta_4\ \gamma_4\ \delta_4$ [9]. The α and β subunits contain phosphorylation sites for PKA; the γ subunit contains the catalytic site and the δ subunit is a calmodulin. The

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Abbreviations: PKC - Protein kinase C; PKA - cAMP-dependent protein kinase; PhK - phosphorylase kinase; DMSO - dimethyl sulphoxide; ATP - adenosine 5'-triphosphate.

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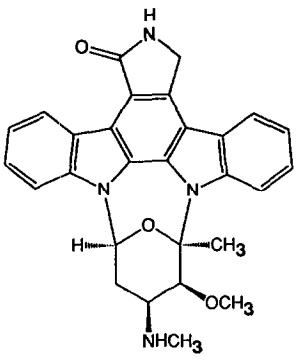
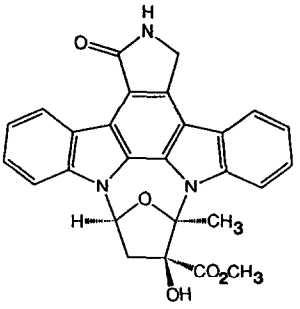
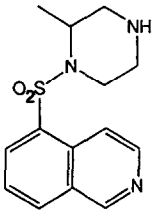


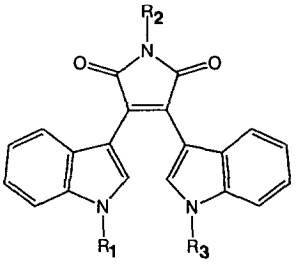
Staurosporine



K252a



H7



Ro 31-	R_1	R_2	R_3
6045	H	CH_3	H
7208	CH_3	H	CH_3
7549	$(CH_2)_3NH_2$	H	CH_3
8161	$(CH_2)_3NC(NNO_2)NH_2$	H	CH_3
8220	$(CH_2)_3SC(NH)NH_2$	H	CH_3

Figure 1

Structures of Protein Kinase Inhibitors.

primary sequence of the catalytic domain shows good homology with those of other protein kinases.

In this study, the profiles of a number of protein kinase inhibitors against PhK are compared and the in vivo implications of PhK inhibition are discussed.

MATERIALS AND METHODS

The following materials were used: DL-dithiothreitol, Ne-2-hydroxyethylene diaminetriacetic acid (HEEDTA), glycerol-2-phosphate. histone VS, adenosine 2,5-cyclic monophosphate (cAMP), phosphorylase b and phosphorylase kinase were obtained from Sigma Chemical Co, Ltd., London; staurosporine and K252a were obtained from Fluka Chemie AG, CH-9470, Bucks. Ro 31-6045, Ro 31-7208, Ro 31-7549, Ro 31-8161, Ro 31-8220 and H7 were synthesised by published procedures [3,10]. All other materials and reagents were Analar R grade.

Rat brain PKC and PKA were partially purified by ion-exchange chromatography of rat brain homogenate as described by Kikkawa et al [11]. Rat brain PKC was assayed as described by Davis et al [4]. 20 µl rat brain PKA were added to a reaction cocktail (80 µl) containing 0.75 mg/ml histone VS, 12.5 µM cAMP, 12.5 µM γ-[^{32}P]-ATP in 50mM Tris-HCl, 10 mM $Mg(NO_3)_2$ pH 7.5. After 10 minutes incubation at 37°C, the procedure thereafter was as described for PKC.

Assay of phosphorylase kinase was based on the method of Cohen [12]. 20 µl of 40 mg/ml phosphorylase b and 80 U/ml rabbit muscle phosphorylase kinase in 25 mM Tris HCl pH 6.8 containing 0.9 mM $CaCl_2$, 0.6 mM HEEDTA, 3.3 mM

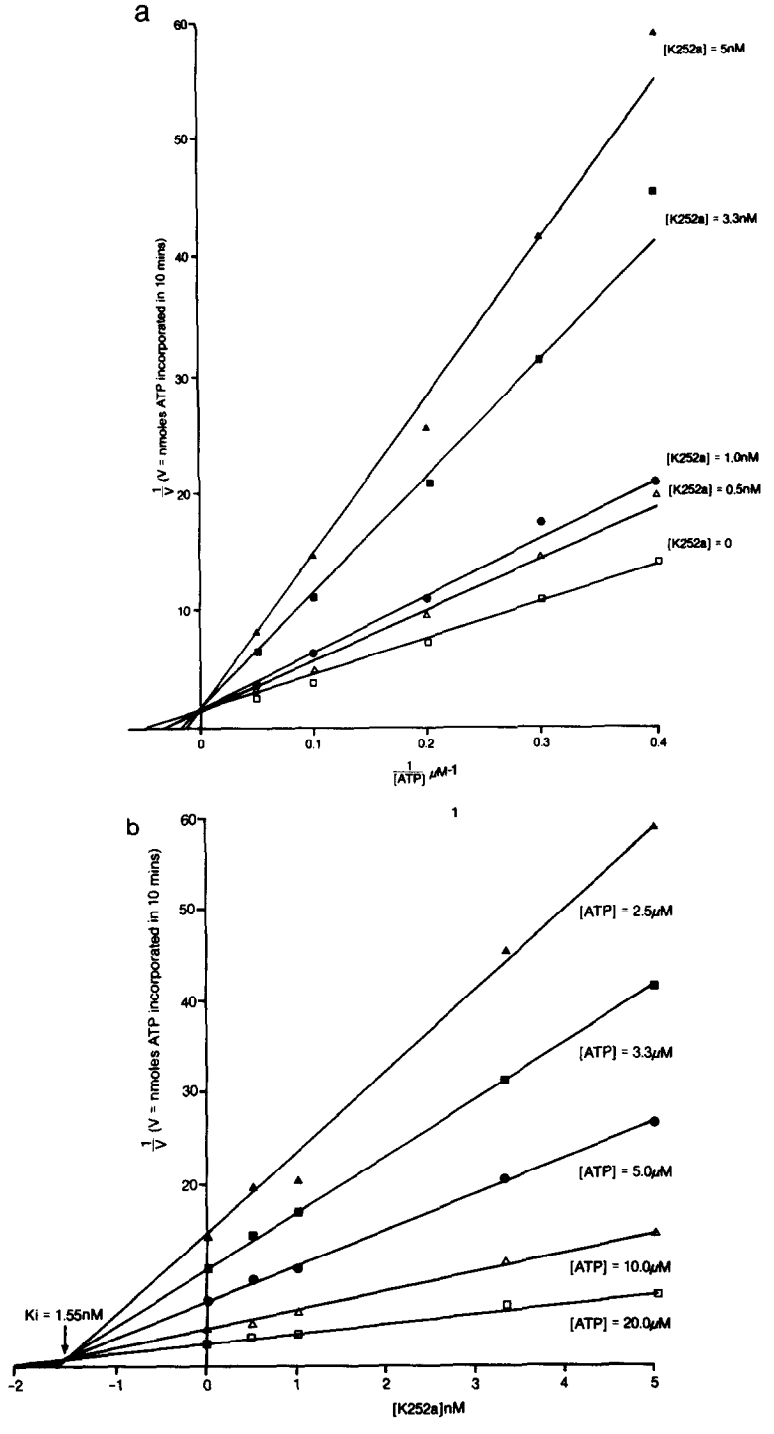


Figure 2

Lineweaver-Burke and Dixon plots are shown respectively for inhibition of phosphorylase kinase by K252a (a,b); staurosporine (c,d) and Ro 31-8220 (e,f).

$Mg(CH_3CO_2)_2$ and 60 mM glycerol-2-phosphate were added to 80 µl of 12.5 µM γ-[^{32}P]-ATP in the same buffer. For Ki determinations, the final assay concentrations of ATP were varied over the range 2.5 - 50 µM ATP. After 4 minutes incubation at 30°C, the reaction was stopped by the 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.

Staurosporine and K252a were prepared as 1 mM stock solutions and stored at -20°C. Ro 31-6045, Ro 31-7208, Ro 31-7549, Ro 31-8161 and Ro 31-8220 were freshly prepared in DMSO. Staurosporine, K252a and the Ro compounds were diluted in DMSO and used at a final DMSO concentration of 10% in the assay. H7 was prepared and diluted into buffer.

RESULTS AND DISCUSSION

Rabbit muscle PhK gave linear progress curves over a range of enzyme and ATP concentrations. The phosphorylation of phosphorylase b was linearly related

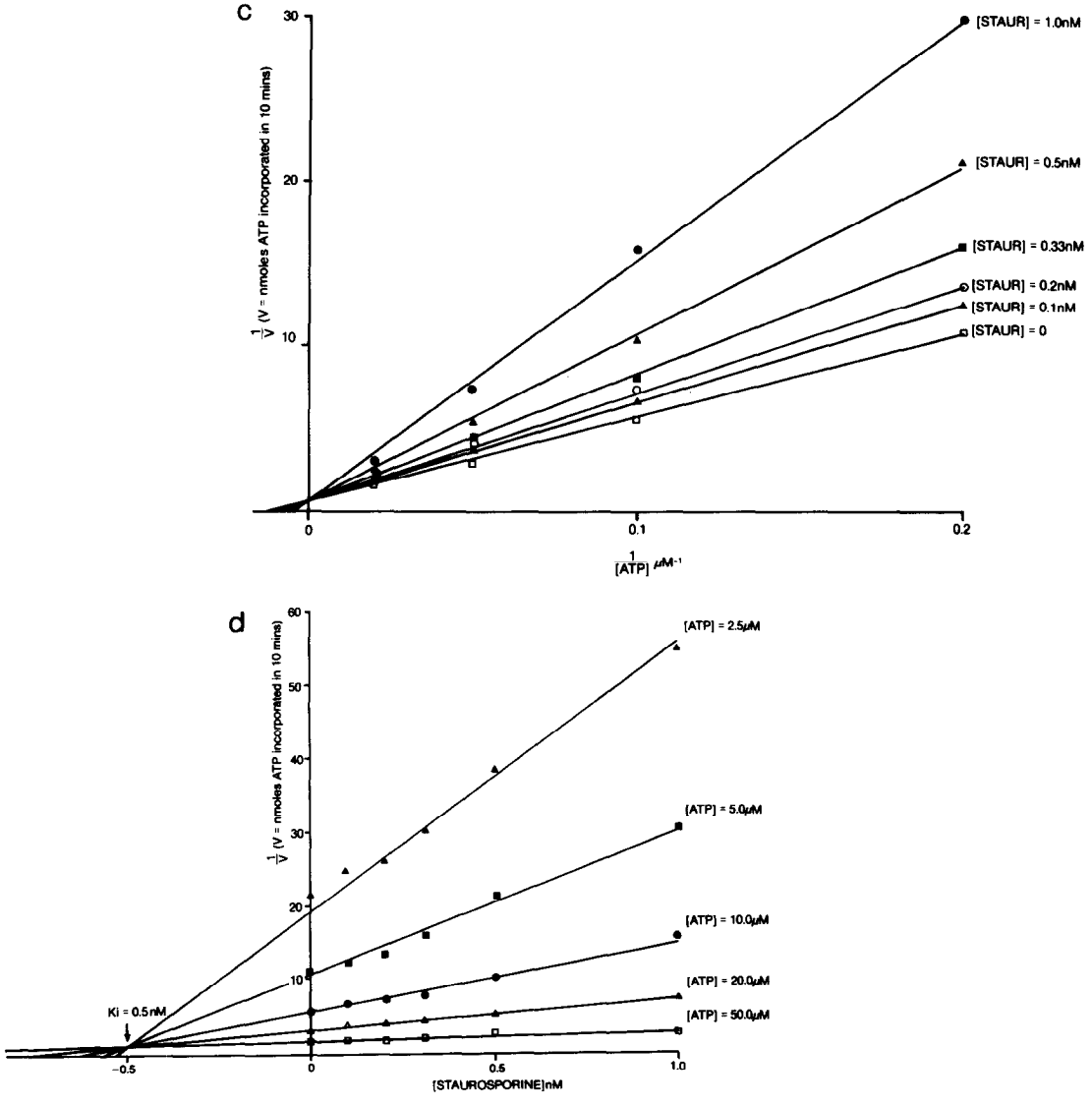


Figure 2 - Continued

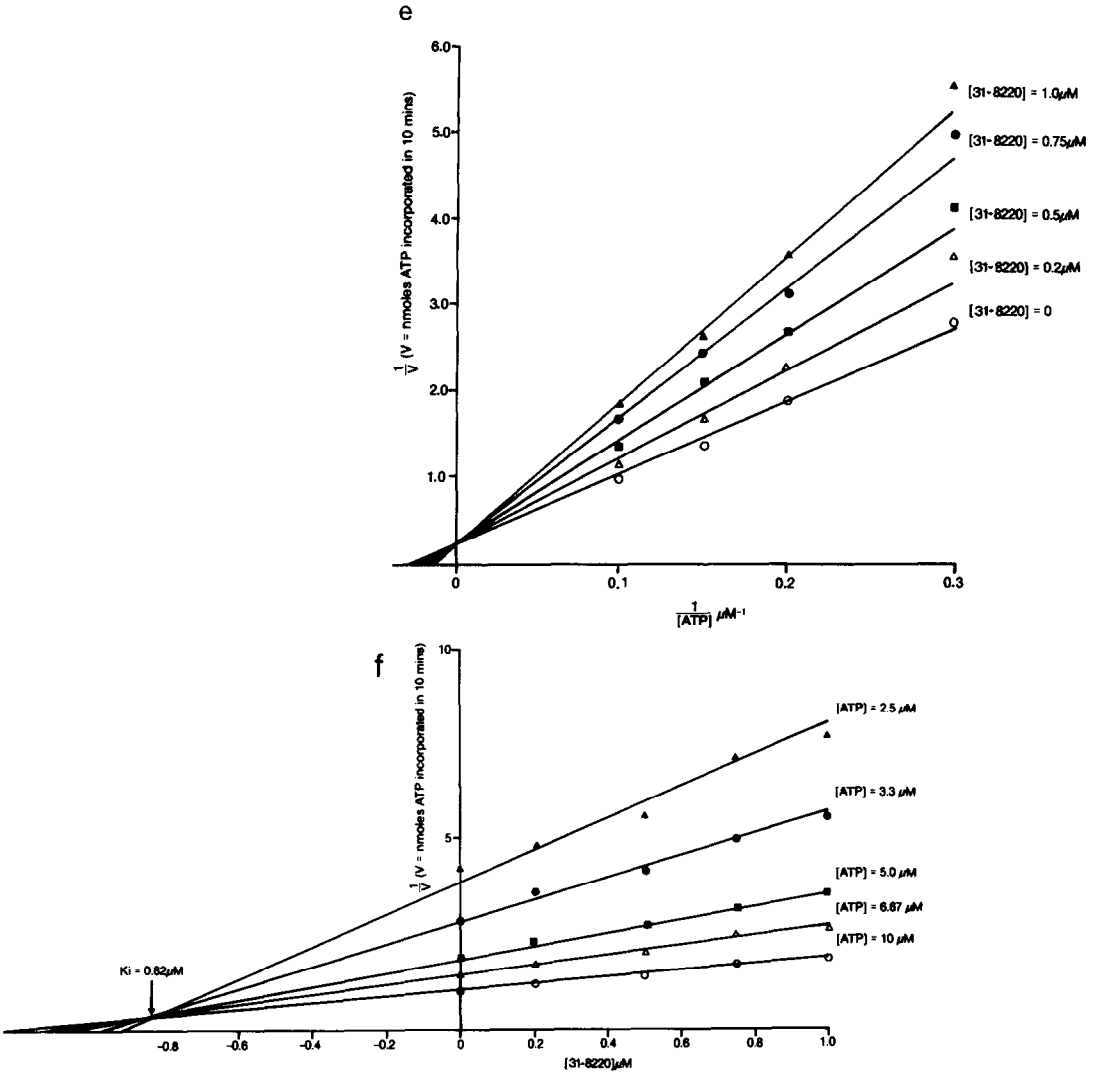


Figure 2 - Continued

to enzyme concentration over the range 2 U/ml to 10 U/ml enzyme. The K_m of PhK for ATP was measured at 77 μM (n=3). This result is in reasonable agreement with the values of 30 μM and 70 μM reported by King et al [13], and by Clerch and Huijing [14], but is somewhat lower than the value of 300 μM observed by Tabatabai and Graves [15].

All the inhibitors examined were fully competitive with ATP for binding to PhK (Figure 2) and this resembles their mode of action against PKC [1, 3, 4, 16], with the possible exception of staurosporine which some workers claim to be non-competitive with ATP [2]. The selectivity exhibited by some of these ATP-competitive protein kinase inhibitors is interesting in view of the high degree of homology in ATP-binding domain sequences in protein kinases.

The bis-indolyl maleimides, Ro 31-7208, Ro 31-7549, Ro 31-8161 and Ro 31-8220 are potent PKC inhibitors which show good selectivity for this enzyme over PKA

Table 1

Inhibition of PhK, PKC and PKA by a selection of Protein Kinase Inhibitors

Compound	IC_{50} value (μM)			IC_{50} PKC / IC_{50} PhK
	PhK	PKC	PKA	
Staurosporine	0.0005 [Ki=0.0005]	0.01	0.04	20
K252a	0.0017 [Ki=0.0016]	0.5	0.1	294
H7	65 [Ki=47]	8	16	0.12
Ro 31-6045	>100	>100	77	-
Ro 31-7208	1.4	0.3	8.9	0.21
Ro 31-7549	0.8	0.07	3.0	0.09
Ro 31-8161	1.6	0.03	3.9	0.019
Ro 31-8220	1.2 [Ki=0.8]	0.01	1.6	0.008

Inhibitor IC_{50} values (μM) against isolated protein kinases assayed at 10 μM ATP are quoted. IC_{50} values quoted are the mean of at least 2 experiments.

and Ca^{2+}/calmodulin dependent protein kinase. These compounds were also highly selective for PKC over rabbit muscle PhK (Table 1). This profile contrasts strongly with that reported for CGP41251 which gave similar potencies against both enzymes.

The high selectivity ratio obtained for staurosporine in the present study (IC_{50} vs PKC/IC_{50} vs PhK = 20) conflicts with that reported previously [5] (IC_{50} vs PKC/IC_{50} vs PhK = 2) and may reflect differences in the assay conditions employed and, in particular, the ATP concentrations used in the two investigations. In contrast to staurosporine, K252a appears to be highly selective for PhK over related protein kinases such as PKC, PKA and Ca^{2+}/calmodulin dependent PK (Table 1). A comparison of Ki values leads to the same conclusion (Ki vs PKC = 25 nM [1], Ki vs PKC/Ki vs PhK = 15).

One possible *in vivo* consequence of PhK inhibition is illustrated by two cases of muscular PhK deficiency: one of an adult man with severe exercise intolerance and cramp [17], the other of a female patient with glycogen storage myopathy [18]. However, one of these patients [17] exhibited normal PhK levels in other tissues. The potent anti-inflammatory and anti-allergic properties reported for K252a *in vivo* [19] do not relate to any obvious way to PhK inhibition.

In conclusion, K252a appears to be a potent and selective inhibitor of PhK whereas the bis-indolyl maleimides are selective for PKC and staurosporine shows little selectivity between different protein kinases.

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