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CHARACTERIZATION OF SPECIFIC BINDING SITES FOR [^{3}H]-STAUROSPORINE ON VARIOUS PROTEIN KINASES

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ABSTRACT : Binding of [^{3}H]-staurosporine to different protein kinases was time-dependent, reversible and saturable. Scatchard analysis of saturation isotherms indicated one class of binding sites for [^{3}H]-staurosporine with dissociation constants (K_D) of 9.6, 2.0, 3.0 and 7.4 nM for protein kinase C, cAMP-dependent protein kinase, tyrosine protein kinase and calcium/calmodulin-dependent protein kinase respectively. [^{3}H]- staurosporine binding was fully displaced by unlabelled staurosporine or the related compound K-252a whereas other protein kinase inhibitors (H-7, H-8 and W-7) did not compete with [^{3}H]-staurosporine. These data confirm that staurosporine shows no selectivity for different protein kinases and suggest the putative existence of distinct, specific binding sites for [^{3}H]-staurosporine on these enzymes.

Protein kinases play crucial roles in signal transduction, cellular proliferation and differenciation (1) but the pleiotropic effects of these enzymes in cellular regulation and their involvement in tumor promotion underlines the importance of understanding their mechanism of regulation. Therefore, the discovery and development of specific protein kinase inhibitors will enable the functional roles of each protein kinase in cells to be more clearly defined.

Since the microbial alkaloid staurosporine was first described as the most potent inhibitor of protein kinase C (2), it is now clear that staurosporine inhibits not only protein kinase C but also a variety of other protein kinases *in vitro* (IC_{50} ranging from 3

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Abbreviations used: PKC: Protein kinase C, PKA: cAMP-dependent protein kinase, TPK: Tyrosine protein kinase, Ca^{++}/CM-PK: Calcium/calmodulin-dependent protein kinase.

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to 30 nM) (3). Likewise, the isoquinolinesulfonamides H-7 and H-8 which are often referred to as protein kinase C inhibitors (4) show little selectivity for various protein kinases (5). These compounds are known to act at the ATP binding site of protein kinase C, (2, 5) which shows striking homology with the ATP binding sites of the tyrosine specific kinases and other serine- threonine-specific kinases (6,7). Inhibitors at this site are therefore unlikely to achieve a high degree of selectivity.

This study was designed to investigate how [^{3}H]-staurosporine interacts with different protein kinases, to characterize putative binding sites of staurosporine on various protein kinases and to determine how other types of protein kinase inhibitors interact with these binding sites.

MATERIALS AND METHODS

Chemicals

Staurosporine was purchased from Fluka AG (Germany), [^{3}H]-staurosporine (160 Ci/mmol) was from New England Nuclear (France). H-7, H-8 and W-7 were obtained from Seikagaku Kogyo (Tokyo, Japan) and K-252a was from Kyowa Hakko (Tokyo, Japan).

Enzymes

The purified protein kinase C (PKC) was prepared from rat brain as described by Kikkawa et al. (8). Tyrosine protein kinase (TPK) was extracted and purified from a lymphoma cell line (LSTRA) according to Casnellie et al. (9). Calcium/calmodulin-dependent protein kinase (Ca^{++}/CM-PK) was prepared from rat brain using the method of Nairn et al. (10). cAMP-dependent protein kinase (PKA, from rabbit muscle) was from Sigma (France).

[^{3}H]-staurosporine binding assay

Assays of [^{3}H]-staurosporine binding were carried out in 50 mM Tris/HCl (pH 7.4), $CaCl_2$ 1mM, [^{3}H]-staurosporine (0 -25 nM for saturation experiments or 2 nM for competition studies) and the purified enzymes in a final volume of 250 µl. After 1 hour at 0°C, 4 ml of ice-cold 50 mM Tris/HCl, pH 7.4 were added. The mixture was then rapidly poured onto a glass fibre filter. The filter was washed 3 times with ice-cold buffer and the bound radioactivity determined by scintillation counting. Non-specific binding of [^{3}H]-staurosporine was measured in the presence of staurosporine (1 µM) and was subtracted from total binding to determine the amount of specific binding.

RESULTS AND DISCUSSION

Previous to the binding studies, the time-dependency of specific binding of [^{3}H]-staurosporine to the different protein kinases was determined. Specific binding of

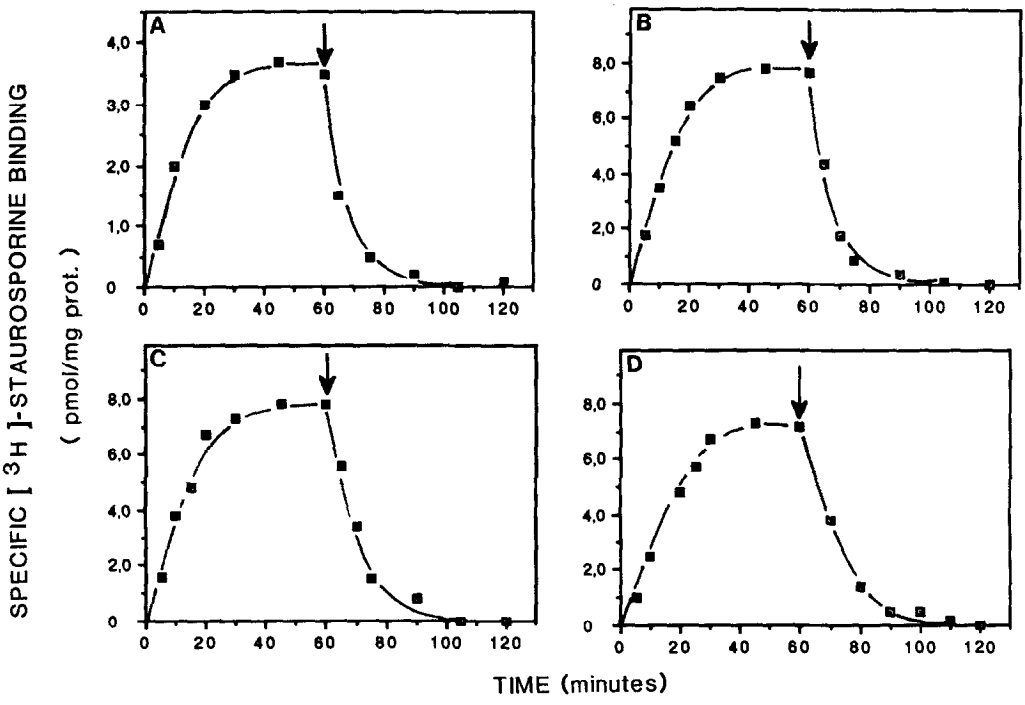

Figure 1: Time course of specific [^{3}H]-staurosporine binding to different protein kinases.
PKC (**A**), PKA (**B**), TPK (**C**) and Ca^{++}/CM-PK (**D**) were incubated at 0°C for various periods of time with 8 nM [^{3}H]-staurosporine. The arrows indicate the time at which staurosporine (1 µM) was added to initiate the dissociation process. Each point is the mean (n = 6) calculated from a typical experiment.

[^{3}H]-staurosporine (8 nM) to protein kinases increased progressively over a 30 minutes incubation period at 0°C whereafter apparent equilibrium of the specific binding was reached (Figure 1). Due to the instability of these protein kinases, when incubated at 25 °C or higher temperatures, the specific binding of [^{3}H]-staurosporine decreased rapidly (not shown). This specific binding was totally reversible since the addition of excess unlabelled staurosporine (1 µM) to the incubation mixture dissociated bound [^{3}H]-staurosporine from protein kinases. Under these experimental conditions, specific binding of [^{3}H]-staurosporine represented 85 - 90% of total binding. Furthermore, total, specific and non-specific binding of [^{3}H]-staurosporine to the different protein kinases increased linearly over protein concentrations ranging from 1 to 200 ug/ml depending on the enzyme (not shown). Therefore, from

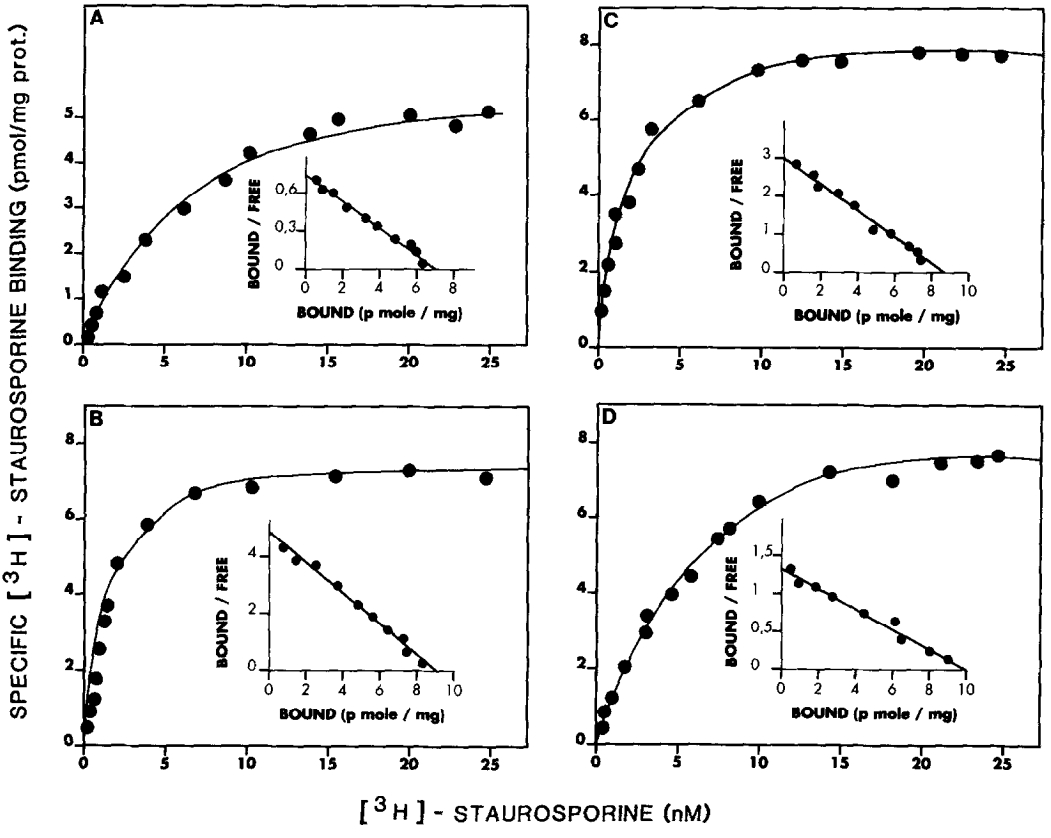

Figure 2: Saturability of the [^{3}H]-staurosporine binding to different protein kinases.

PKC (**A**), PKA (**B**), TPK (**C**) and Ca^{++}/CM-PK (**D**) were incubated for 1 hour at 0°C in the presence of increasing concentrations of [^{3}H]-staurosporine. Data are means of 2 independent experiments, conducted in triplicate. Non-specific binding was measured in the presence of staurosporine (1μM).

Insets: Scatchard analysis of the specific binding of [^{3}H]-staurosporine calculated from saturation isotherms.

these dose-response curves, 100 μg of protein was chosen for all the following experiments.

Studies of saturation binding of [^{3}H]-staurosporine to the different protein kinases revealed that the non-specific binding, as assessed by using 1 μM staurosporine, increased linearly with concentrations of [^{3}H]-staurosporine. In contrast, the specific binding of [^{3}H]-staurosporine exhibited full saturability (figure 2). Scatchard analysis from plotting the bound/free ratio of the labelled staurosporine as a function of the concentration of receptor-bound staurosporine revealed the presence of a single

class of non-interacting binding sites on every protein kinase. The equilibrium dissociation constants (K_D) were 9.6 ± 1.7, 2.0 ± 0.18 , 3.0 ± 0.45 and 7.4 ± 1.5 nM for PKC, PKA, TPK and Ca^{++}/CM-PK respectively, demonstrating that K_D were very similar among individual protein kinases. Similarly, the total number of binding sites (Bmax) were highly comparable between each of the considered enzymes. Bmax values were 7.23 ± 0.1, 9.16 ± 0.05, 8.8 ± 0.08 and 9.95 ± 0.12 pmol/mg protein for PKC, PKA, TPK and Ca^{++}/CM-PK respectively. Hill coefficients (nH) for binding to different kinases were close to 1, indicating again that [^{3}H]-staurosporine was binding to a single class of binding sites in each protein kinase.

Specific [^{3}H]-staurosporine binding to protein kinases was fully displaced by unlabelled staurosporine (Figure 3). The concentrations required to inhibit the specific binding by 50% (IC_{50}) were 0.98 ± 0.03, 1.2 ± 0.09, 1.65 ± 0.08 and 4.5 ± 0.17 nM for PKC, PKA, TPK and Ca^{++}/CM-PK respectively. Hill coefficients (nH) values were close to 1, providing further indication that binding was competitive. K-252a, a microbial product that contains the same indole carbazole system as staurosporine but with a different sugar moiety had comparatively higher IC_{50} values for [^{3}H]-staurosporine binding to different protein kinases than staurosporine (table 1).

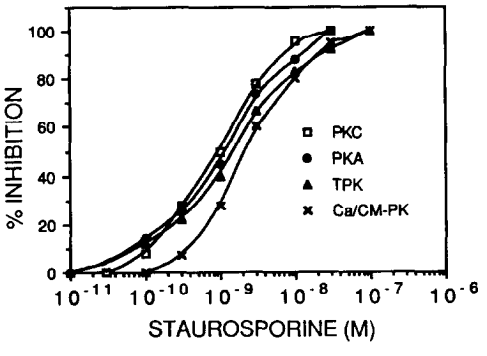

Figure 3: Displacement of specific [^{3}H]-staurosporine binding by unlabelled staurosporine.
PKC (□), PKA (●), TPK (▲) and Ca^{++}/CM-PK (×) were incubated with [^{3}H]-staurosporine (2nM) and increasing concentrations of unlabelled staurosporine for 1 hour at 0°C. Each data point is the average of results from at least 3 independent experimental determinations, performed in triplicate.

Table 1: Effect of various protein kinase inhibitors on [^{3}H]-staurosporine binding to different protein kinases

	PKC	PKA	TPK	Ca++/CM-PK
Staurosporine	0.98 nM	1.2 nM	1.65 nM	4.5 nM
K 252a	3.7 nM	11.1 nM	7.4 nM	13,4 nM
H-7	(5 %)	(0 %)	(7 %)	(7 %)
H-8	(2 %)	(5 %)	(0 %)	(0 %)
W-7	(13 %)	(12 %)	(13 %)	(0 %)
ATP	500 nM	430 nM	330 nM	610 nM

Various protein kinases were incubated with [^{3}H]-staurosporine (2 nM) and increasing concentrations of the compounds to be tested as described under "materials and methods". Results are expressed as IC_{50} in nM determined in triplicate. Numbers in parentheses are percent inhibition at 10^{-5}M.

Isoquinolinesulfonamides (H-7 and H-8) and naphtalenesulfonamides (W-7), 2 classes of non-selective protein kinase inhibitors were shown to bind protein kinases through their catalytic domains (11 , 12). Surprisingly, when tested in our model, these compounds showed almost no ability to antagonize [^{3}H]-staurosporine binding to different protein kinases (table 1). This observation suggests that, although binding at the catalytic domain of the protein kinases, these compounds bind to a site distant from that of [^{3}H]-staurosporine. In these experimental conditions, ATP, the common substrate of these different protein kinases, was a good competitor of [^{3}H]-staurosporine (IC_{50} ranging from 330 to 610 nM).

Thus, our results corroborate previous studies by Nakano (13) who showed that staurosporine inhibited in a non-selective manner the activity of a variety of protein kinases *in vitro* but, since the isoquinolinefulfonamides do not compete with [^{3}H]-staurosporine, raise the question of the presence of different binding sites for inhibitors already known to act on the catalytic domain of these various protein kinases.

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