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Inhibition of Calcineurin by the Tyrphostin Class of Tyrosine Kinase Inhibitors

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ABSTRACT. Because of their similarity to tyrosine, members of the tyrphostin family of tyrosine kinase inhibitors were tested as possible inhibitors of the protein serine/threonine phosphatase calcineurin. Calcineurin was inhibited by tyrphostins A8 (also designated AG10), A23 (AG18), and A48 (AG112) with *p*-nitrophenyl phosphate as substrate. The IC_{50} values estimated with this substrate were 21, 62, and 30 μM for A8, A23, and A48, respectively. Two other tyrphostins, A46 (AG99) and A63 (AG13), did not inhibit calcineurin at concentrations up to 200 μM. Similar inhibition was observed with tyrphostins A8 and A23 using a phosphopeptide substrate (1.0 mM). Tyrphostin A8 showed competitive inhibition against *p*-nitrophenyl phosphate as the substrate, with an inhibition constant of 18 μM, comparable to the IC_{50} value. Possible chemical and structural features influencing inhibition are discussed based on a comparison of the structures of the tyrphostins tested. BIOCHEM PHARMACOL **56**;4:483–488, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. calcineurin; calmodulin-activated phosphatase; dual specificity phosphatase; tyrphostins; tyrosine kinase inhibitors

Protein phosphorylation and dephosphorylation reactions are broadly classified by target specificity into serine/threonine specific, tyrosine specific, and dual specific kinases and phosphatases. Commonly, kinase specificity is highly influenced by the primary structure near the target site to be phosphorylated [1]. Protein phosphatases show less dependency on the primary structure and seem to require aspects of secondary structure for substrate recognition [2]. The basis for the differences in target site specificities is not entirely clear. Resolution of the crystal structure of tyrosine phosphatases has revealed a deep substrate binding crevice [3–6] that likely limits accessibility of active site residues for phosphoseryl or phosphothreonyl residues. Resolved structures [7–10] of serine phosphatases do not show a similar feature to explain specificity.

There are four major protein serine/threonine phosphatases [11] distinguished primarily by substrate specificities and susceptibility to specific phosphatase inhibitors; the phosphatases are designated type-1, -2A, -2B, and -2C. Phosphatase-1 is the traditional phosphorylase phosphatase. Phosphatase-2B is activated by calmodulin and is known as calcineurin. Phosphatase-1, -2A, and -2B share extensive sequence identity and predictions of secondary structure; predictions of essential amino acid residues can be made.

Calcineurin is an informative model system to study specificity of protein serine/threonine phosphatases. First, calcineurin has activity with selected low molecular weight phosphate esters [12–14] enabling the systematic evaluation of substrate specificity using chemically well-defined structures. Application of related substrates has provided some insight into the mechanism of the enzyme. Second, calcineurin has activity with phosphopeptides, providing a means to characterize target site recognition [15, 16]. Finally, calcineurin has activity *in vitro* with phosphoseryl, phosphothreonyl, and phosphotyrosyl residues in various proteins [17, 18]. This property technically makes calcineurin a member of the dual specificity class of protein phosphatases, but it is premature to consider all of these activities physiologically relevant. These observations do indicate that the active site of calcineurin can tolerate and interact with a phosphotyrosyl residue, larger than the phosphoseryl or phosphothreonyl residues.

Similar in size and topology to tyrosine, the family of tyrphostin molecules has been developed as inhibitors of tyrosine kinases [19, 20]. Specifically, these compounds have been shown to inhibit the epidermal growth factor receptor/kinase with half-maximal inhibition requiring micromolar concentrations [19, 20]. Because of the potential for recognition of a tyrosine structural mimic, members of the tyrphostin family were tested as inhibitors of calcineurin.

MATERIALS AND METHODS

Materials

The substrate pNPP† (Sigma 104 substrate) was purchased from Sigma as were EGTA, MOPS, and phenyl-Sepharose.

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† *Abbreviations:* IC_{50}, concentration resulting in inhibition of 50% phosphatase activity; MOPS, 3-(*N*-morpholino)propanesulfonic acid; pNP, *p*-nitrophenol; and pNPP, *p*-nitrophenyl phosphate.

Tyrphostins (A8, A23, A46, A48, and A63) were obtained from Calbiochem. The tyrphostins have also been designated AG10, AG18, AG99, AG112, and AG13, respectively [20]. A protein phosphatase assay kit was purchased from the Promega Corp. Other chemicals (metal salts, etc.) were obtained from Aldrich Chemical or Fisher Scientific. Chelex-100 was purchased from Bio-Rad Laboratories and was used to remove metal from water and buffers used in these experiments.

Proteins

Calcineurin was isolated from bovine brain by the method of Sharma *et al.* [21] except that MOPS buffers were used in place of Tris buffers. Calcineurin purified with this protocol requires Mn^{2+} or Ni^{2+} for full activity [12–14] and is not further activated by the addition of $CaCl_2$. Calmodulin was purified by the procedure of Sharma and Wang [22] with a slight modification. Following DE-52 chromatography, the calmodulin was loaded onto phenyl-Sepharose and eluted with EGTA [23]. Protein concentrations were determined by the method of Bradford [24].

Calcineurin Assay with pNPP

Calcineurin was assayed by measuring the release of pNP spectrophotometrically at 410 nm in a Spectramax 250 microtiter plate reader (Molecular Devices) with a total volume of 200 μL. The standard assay for characterization of activity was performed at 30° in 25 mM MOPS, pH 7.0; 1.0 mM $MnCl_2$; 20 μg/mL of calmodulin; and 20 μg/mL of calcineurin with 10 mM pNPP as the substrate. Inhibition assays with the tyrphostins were done with 2–6 μg/mL of calmodulin and calcineurin. Tyrphostins were prepared as 10 mM stocks in DMSO. Working solutions were prepared daily at concentrations of 1 mM in 10% DMSO (v/v) and 1 mM MOPS, pH 7.0. Samples of the tyrphostins were added to the reaction mix, with final concentrations indicated in the figures. In all sample and control reactions, DMSO was maintained at 5% (v/v). Kinetic inhibition analysis with tyrphostin A8 was performed using 6 μg/mL of calmodulin and 6 μg/mL of calcineurin and substrate concentrations ranging from 5.0 to 50.0 mM. Tyrphostin A8 was included at 0.0, 15.0, and 30.0 μM. The initial rate data (converted to units of sec^{-1}) were fit to equations for the different modes of inhibition, using the programs Enzyme Kinetics (Trinity Software) and Psi-Plot (PsiPlot, Inc.).

$$v = \frac{k_{cat} \cdot [pNPP]}{K_m(pNPP) \cdot (1 + [I]/K_I) + [pNPP]} \quad \text{Competitive inhibition} \quad (1)$$

$$v = \frac{k_{cat} \cdot [pNPP]}{K_m(pNPP) \cdot (1 + [I]/K_I) + [pNPP] \cdot (1 + [I]/K_I)} \quad \text{Noncompetitive inhibition} \quad (2)$$

$$v = \frac{k_{cat} \cdot [pNPP]}{K_m(pNPP) + [pNPP] \cdot (1 + [I]/K_I)} \quad \text{Uncompetitive inhibition} \quad (3)$$

Calcineurin Assay with Phosphopeptide

Calcineurin activity was also measured with a phosphopeptide supplied with the phosphatase assay kit (Promega). Activity was monitored by measuring the release of inorganic phosphate, using a malachite green dye method. Briefly, the assay mixture was 25 mM MOPS, pH 7.0; 1.0 mM $MnCl_2$; 20 μg/mL of calmodulin; and 20 μg/mL of calcineurin with 1.0 mM phosphopeptide of sequence Arg-Arg-Ala-Thr(P)-Val-Ala. This peptide was hydrolyzed by calcineurin with rates higher than analogous serine phosphopeptides, which have values of K_m in the range between 50 and 100 μM [25]. The total volume was 50 μL and was incubated at 30° in the Spectramax 250 plate reader after initiation of the reaction by the addition of phosphopeptide (12.5 μL of 4.0 mM stock solution). After 15 min, the plate was removed, and 50 μL of the dye reagent was added. The mixture was incubated for 10 min for color development, and then the absorbance of each mixture was measured at 630 nm. According to the kit protocol, any wavelength between 600 and 660 nm is suitable, but the best results in this study were obtained at 630 nm. For the negative control, calcineurin was added after the dye reagent. Inhibition by tyrphostins was tested by including 0.1 mM tyrphostin during the 15-min reaction.

RESULTS AND DISCUSSION

Inasmuch as calcineurin can hydrolyze tyrosine phosphate and dephosphorylate phosphotyrosyl-containing proteins, it seemed likely that calcineurin would recognize the basic structure of the tyrphostin family of tyrosine kinase inhibitors. Five different tyrphostins were checked as possible inhibitors. The structures are shown in Fig. 1, with tyrosine shown for comparison.

Inhibition of Calcineurin

Tyrphostins were first characterized as possible inhibitors of calcineurin using pNPP as the substrate. Using pNPP as the substrate, only tyrphostins A8, A23, and A48 were found to inhibit calcineurin activity. These data are shown in Fig. 2A. The IC_{50} values were determined as 21 μM for tyrphostin A8, 30 μM for tyrphostin A48, and 62 μM for tyrphostin A23. As shown, tyrphostins A46 and A63 did not inhibit the activity of calcineurin. Increasing the concentration of these compounds up to 0.2 mM did not result in any inhibition. Concentrations above 0.2 mM provided greater difficulty for the measurement of enzyme activity because of background absorbance, and they have less significance for physiological concerns. Figure 2B shows

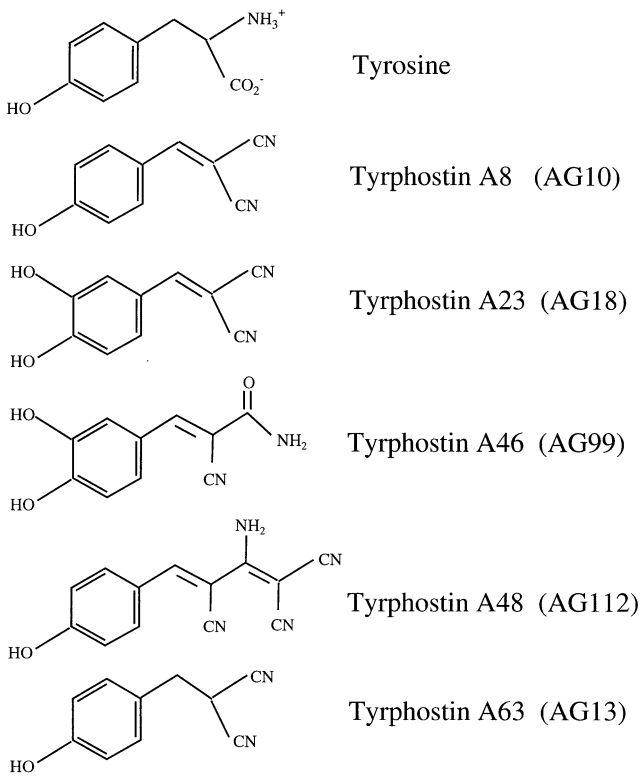

FIG. 1. Structures of the tyrphostin inhibitors. The structures of the different tyrphostins tested are shown with the structure of tyrosine for comparison. The nomenclature A8, A23, A46, A48, and A63 was taken from the commercial supplier. The designation from the AG nomenclature system of Levitzki and Gazit [20] is also provided.

a comparison of the inhibition observed using a 50 μM concentration of each tyrphostin. The pattern in this figure more clearly shows the relative abilities of the tyrphostins to inhibit calcineurin.

Inhibition Pattern by Tyrphostins

The type of inhibition for tyrphostin A8 was determined using pNPP as the substrate. The results are shown in Fig. 3 as double-reciprocal plots of the initial rate data. The pattern indicated that tyrphostin A8 was a competitive inhibitor of calcineurin against substrate. An inhibition constant (K_I) was evaluated from a non-linear fit to the Michaelis–Menten form of the equation and was estimated as 18 μM, comparable to the estimated IC_{50} value (Fig. 2). The data fit was also consistent with competitive inhibition and not consistent with a noncompetitive model. Although not proving the site of binding, it was likely that these inhibitors bound directly at the active site based on the similarity of structure to the substrate and product of the reaction, particularly considering the *in vitro* tyrosine phosphatase activity of calcineurin.

Inhibition of Calcineurin with Peptide Substrate

Inhibition by the tyrphostin molecules (0.1 mM) was also evaluated using a peptide substrate for calcineurin. Enzyme activity was monitored by detection of released inorganic phosphate with the malachite green dye method, using a phosphatase assay kit obtained from the Promega Corp. The substrate was a phosphopeptide with the sequence

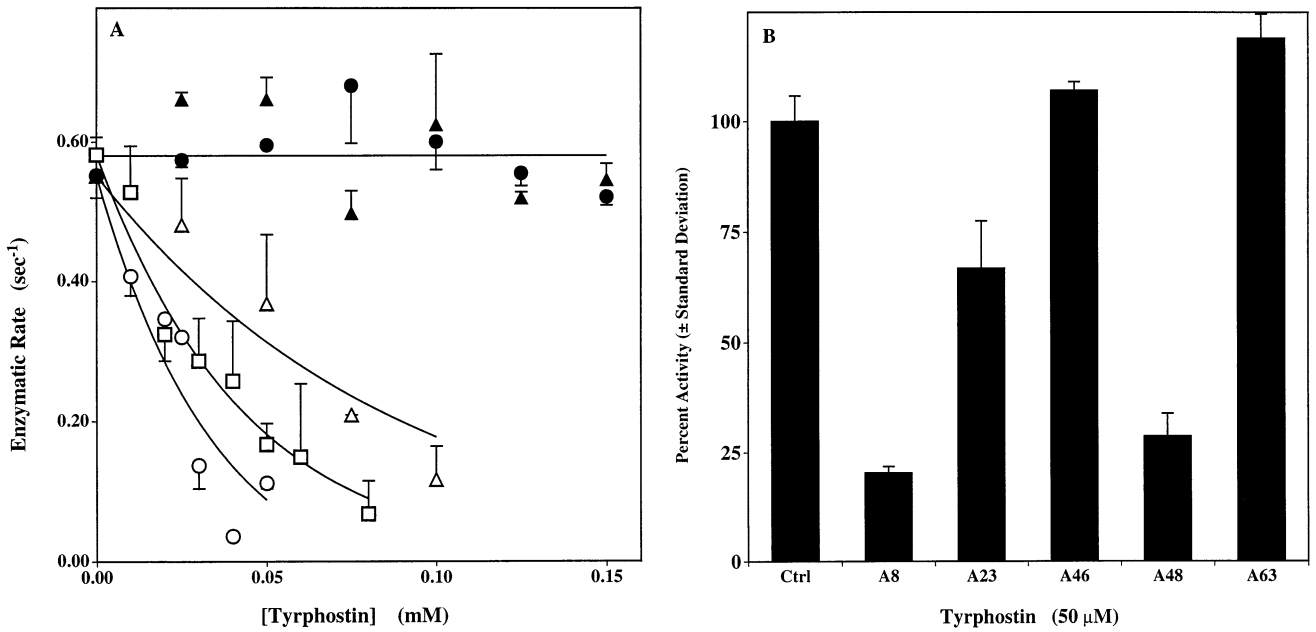

FIG. 2. Tyrphostin inhibition of calcineurin. In panel A, each tyrphostin was added to the indicated concentration, and calcineurin (2–6 μg/mL) activity was assayed using 10.0 mM pNPP in 5% (v/v) DMSO as described. The tyrphostins evaluated as inhibitors were A8 (○), A23 (△), A46 (●), A48 (□), and A63 (▲). Each point represents the average of at least triplicate assays, and error bars shown represent standard deviations. Error bars are shown in one direction only to enhance the clarity of the figure. In panel B, the inhibition observed with a 50 μM concentration of each tyrphostin is compared. Error bars show the standard deviation of an N of at least 3.

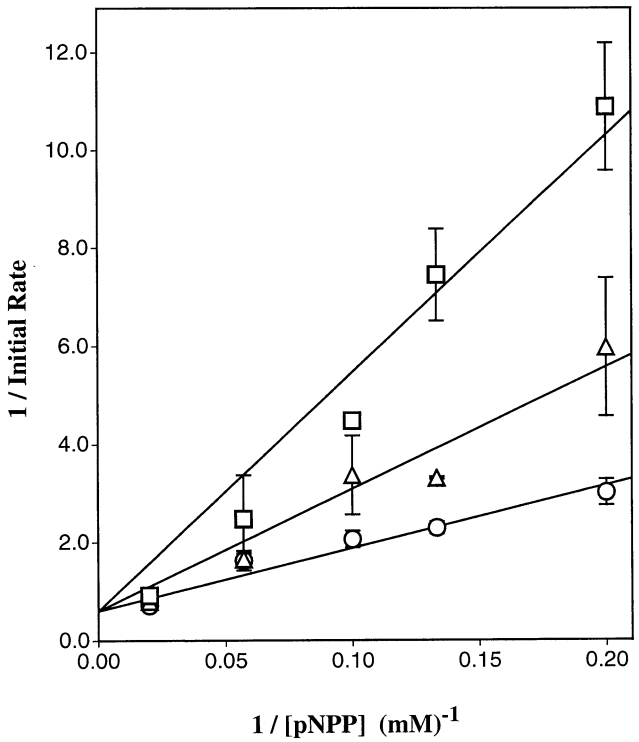

FIG. 3. Inhibition pattern for tyrphostin A8 inhibition of calcineurin. Initial velocities were measured as described with 5.0, 7.5, 10.0, 17.5, and 50.0 mM pNPP in 5% (v/v) DMSO with 6 μg/mL of calcineurin and 6 μg/mL of calmodulin. Tyrphostin was omitted (○) or added to the assays at a concentration of 15.0 μM (△) or 30.0 μM (□). Each substrate–velocity pair was determined as duplicate activity measurements in two separate experiments: four estimates for each substrate concentration plotted. The two experiments were averaged, and each activity measurement was plotted as the reduced unit, sec^{-1}. Error bars show the standard error between the two experiments.

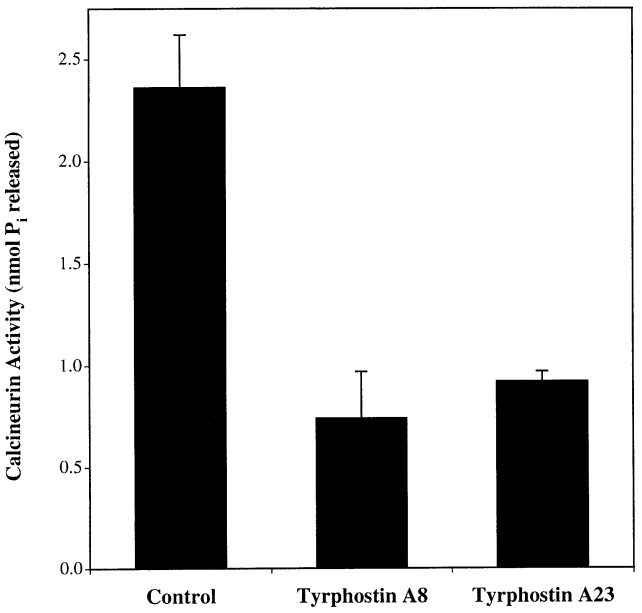

FIG. 4. Tyrphostin inhibition of calcineurin with a phosphopeptide substrate. Each tyrphostin was added at 0.1 mM, and calcineurin activity was assayed using 1.0 mM peptide as the substrate according to the phosphate release assay described in Materials and Methods. The tyrphostins evaluated as inhibitors were A8 and A23, and their activities were compared with control activity in the presence of 5% (v/v) DMSO. Activity is shown as nanomoles of phosphate released during the 5-min reaction time and represents the average of four to seven measurements. The error bars shown represent standard deviations of the calculated values.

Arg-Arg-Ala-Thr(P)-Val-Ala, known to be hydrolyzed by calcineurin [3, 25]. As with pNPP, inhibition of peptide phosphatase activity was found with tyrphostins A8 and A23 (Fig. 4). Tyrphostin A48 was not tested as it was similar to A8 for the inhibition of pNPP hydrolysis. There was less discrimination between A8 and A23 with the peptide as substrate. These data did verify that the tyrphostin molecules inhibited calcineurin independently of the substrate.

Influence of Structure on Inhibition

The observed differences in inhibitory potency may be related to differences in structures of the inhibitors. Tyrphostin A23 contains an additional hydroxyl group compared with A8 and was a weaker inhibitor of calcineurin. A slight amount of steric interference or perturbation of some enzyme–inhibitor interaction was likely responsible for the difference between these two inhibitors. Like A23, tyrphostin A46 also has an additional hydroxyl group, but the aliphatic portion of A46 is significantly different. Tyrphostin A46 has a carboxamido group in place of one of the cyano groups present in A8 and A23. This group is sterically larger and may have prevented a critical interaction of A46 with calcineurin. Tyrphostin A48 is similar to A8 with a single aromatic hydroxyl substituent; the aliphatic portion contains an additional carbon—carbon double bond unit and an additional cyano substituent, but has an amino substituent. The larger aliphatic chain did not seem to cause significant steric problems for inhibition, with tyrphostin A48 only slightly weaker than A8 in blocking activity.

The additional cyano group of A8 and A23, compared with A46, may also change the nature of conjugation of unsaturated bonds in these inhibitors. The carboxamido group of tyrphostin A46 may support some conjugation, but not as effectively as the cyano group in A8 and A23. The effect of bond conjugation was likely apparent in the difference between A8 and A63. Tyrphostins A8 and A63 are identical except for the double bond between C_α and C_β of the aliphatic chain. Tyrphostin A8 (as well as A23) retains the double bond, whereas the aliphatic portion of tyrphostin A63 is saturated. The unsaturated aliphatic group in A8 and A23 enables conjugation along the entire backbone with electron density delocalized throughout the entire structure. In A63, such delocalization is not possible. As noted, the structure of tyrphostin A48 is similar to that of A8, with the aliphatic portion containing an additional

carbon—carbon double bond unit and an additional cyano substituent. These would likely enhance electron withdrawal from the ring and provide for additional conjugation, but the amino substituent would reduce the electron withdrawing potential of the aliphatic chain. The inhibition caused by tyrphostin A48 resembled A8 more so than A23, a result consistent with the structures of the inhibitors. The additional cyano group and the additional conjugation in A48 likely provided the basis for greater inhibition than found with A23, but the amino substituent caused slightly reduced inhibition compared with tyrphostin A8.

There are likely two possible explanations for a role of electron delocalization in enhancing inhibitor potential. First, significant electron delocalization may enable better interaction with protein residues such as tyrosine that also have delocalized character. Akin to aromatic–aromatic stacking interactions, centers of delocalized electron density may form tight interactions. A second possible explanation is that the delocalized electron density may interact with the metal ions known to be involved in calcineurin activity [26–30]. Such an interaction between delocalized π-electrons and metals is commonplace in organometallic compounds [31]. If the metal was the target, it was not likely related to simple preference for the substituents present in the different tyrphostins. Tyrphostins A8 and A63 are identical with the same spatial orientation of the phenol ring and the cyano groups in the aliphatic chain. The only difference between these structures is the double bond present in tyrphostin A8 and missing in tyrphostin A63. Both intrinsic metals and the required exogenous metal are candidates for such interactions. The positive character of the metal centers would strongly interact with the electron dense compounds, and any catalytic role of the metal(s) might be disrupted.

Comparison to Other Inhibitors of Calcineurin

The inhibition observed with tyrphostins A8 and A23 was more potent than has been observed with other simple tyrosine analogs tested as inhibitors. Simple phenols and tyrosine derivatives failed to inhibit at concentrations of 1.0 mM or lower [32], consistent with the effects of tyrphostin A63. 4-(Fluoromethyl)phenyl phosphate [33] was reported to be a mechanism-based inhibitor of calcineurin with a K_i of approximately 40 mM, a value higher than the K_m for low molecular weight phosphate esters. Inhibition by cyclosporin A [34, 35] was characterized by a $K_i \approx 33$ nM, but required the complex of cyclosporin A and one of the cyclophilin proteins [34]. Pyrethroid compounds [36] are multi-ring compounds and have been reported to inhibit calcineurin more potently than the inhibition reported herein, but the effect of pyrethroids has been questioned [37]. For simple, single-ring structures, the tyrphostins may be the most potent inhibitors of calcineurin. It may be possible to use the tyrphostin structure as a starting basis for the development of novel, potent inhibitors of calcineurin. Any inhibitors developed would have to be tested as inhibitors of type-1 and type-2A protein phosphatases, other members of the same gene family.

Significance for the Use of Tyrphostins

Inhibition of calcineurin was found in the range of 5–100 μM tyrphostins (A8 or A23), similar to the range for inhibition of protein tyrosine kinases [19, 20] and transducin GTPase activity [38]. Another similarity between calcineurin and tyrosine kinases was the relative failure of tyrphostin A63 to inhibit any activity. The saturated, tyrphostin A63 has an IC_{50} in the millimolar range for tyrosine kinases [19, 20] and is used as a negative control for inhibition by other tyrphostins. These studies provide further evidence that tyrphostins must be used with caution in studies of protein phosphorylation in cellular processes. Besides inhibition of tyrosine kinase activity, there is the potential for the inhibition of phosphatase activity and other enzymes including the GTPase activity of transducin [38] and DNA topoisomerase I [39]. These results would predict that protein tyrosine phosphatases also would be inhibited by tyrphostins.

These data have no current physiological significance, but it is intriguing that tyrphostins and cyclosporin A have been tested in at least one clinical situation, the treatment of psoriasis and psoriatic arthritis [40–42]. Tyrphostins (A23 was one example) were shown to block cell growth of psoriatic keratinocytes [40]. It may be possible that calcineurin is a common link in the efficacy of tyrphostins and cyclosporin in the treatment of psoriasis and psoriatic arthritis. Still, the data from these trials are difficult to interpret because of the pleiotropic effects of tyrphostins [19, 20, 38, 39].

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