0026-895X/00/040738-08$3.00/0

MOLECULAR PHARMACOLOGY, **57:**738–745 (2000).



# Differential Inhibition of Multiple cAMP Phosphodiesterase Isozymes by Isoflavones and Tyrphostins

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Received August 10, 1999; accepted December 20, 1999

This paper is available online at http://www.molpharm.org

## ABSTRACT

A series of isoflavone and tyrphostin compounds were found to inhibit the degradation of cAMP by several cyclic nucleotide phosphodiesterase (PDE) isozymes. Specific hydroxyl groups on the isoflavone structure were critical for PDE isozyme-selective inhibition. Replacement of the C-7 hydroxyl group of the isoflavone with a methoxy group raised the $IC_{50}$ for PDE1, PDE3, and PDE4. The absence of the C-5 hydroxyl group raised the $IC_{50}$ from 5 to >100 $\mu$M for PDE4, but actually lowered the $IC_{50}$ for PDE3 and PDE1. Replacement of the C-4′ hydroxyl group with a methoxy group raised the $IC_{50}$ for PDE3 and PDE1, yet only slightly changed the $IC_{50}$ for PDE4. Various tyrphostins were also potent inhibitors of PDE1, PDE3, and PDE4. The four-carbon side chained tyrphostins were much less potent; however, a very interesting pattern was observed in which removal of phenolic hydroxyls on the tyrphostin structure increased the potency for PDE1 and PDE3, but not PDE4. These results may help to explain some of the therapeutic and intracellular signaling effects of isoflavones and tyrphostins. Moreover, the isozyme selectivity demonstrated by the isoflavones and tyrphostins can serve as a pharmacophore for the design of specific PDE inhibitors.

Cyclic nucleotide phosphodiesterases (PDEs) are ubiquitously found in mammalian tissues and cells. These enzymes control cyclic nucleotide levels by catalyzing the hydrolysis of cGMP or cAMP, thereby rendering these critical signaling molecules inactive. There are currently ten gene families of PDE that possess unique substrate specificities, kinetic characteristics, regulatory properties, and inhibitor sensitivities (Beavo, 1995; Manganiello et al., 1995; Fisher et al., 1998; Soderling et al., 1998, 1999). PDEs are believed to play a role in many illnesses, disorders, and biological processes including diabetes (Wijkander et al., 1998; Ma et al., 1999), cardiovascular disease (Yu et al., 1996), inflammation (Teixeira et al., 1994), immune response (Michie et al., 1998), lymphocytic leukemia (Kim and Lerner, 1998), and erectile dysfunction (Moreland et al., 1999). Moreover, the involvement of PDE in numerous signaling pathways demonstrates the potential of PDE as a vital therapeutic target and reinforces the need for further understanding their regulation and the design of isozyme-specific PDE inhibitors.

The isoflavone family of compounds includes the well known tyrosine kinase inhibitor, genistein, as well as daidzein, biochanin A, and prunetin. These compounds are naturally occurring in the legume soybean. There have been numerous reports of the beneficial health effects of soy and isoflavones, such as chemoprevention (Messina et al., 1994), cardioprotection (Anthony et al., 1996), antioxidant (Giles and Wei, 1997), antiestrogenic (Martin et al., 1978), immune response (Middleton and Kandaswami, 1992), and inflammation (Middleton and Kandaswami, 1992).

Tyrphostins are a synthetic family of compounds that make up another class of tyrosine kinase inhibitors (Gazit et al., 1989). The tyrphostins also have demonstrated biological activity toward other targets. Some of these targets include GTP-using enzymes (Wolbring et al., 1994) and calcineurin (Martin, 1998).

Our earlier finding that genistein and tyrphostin 51 inhibited PDE4 in a tyrosine kinase-independent manner (Nichols and Morimoto, 1999), led us to investigate whether PDE inhibition by these compounds was specific for PDE4 or whether other PDE isozymes were sensitive as well. Using carefully chosen isoflavone and tyrphostin derivatives, we obtained information on specific molecular substituents on these compounds that altered the potency of PDE inhibition and conferred selectivity toward PDE isozymes.

This work was supported, in part, by the National Institutes of Health, NS33230; by a predoctoral fellowship to M.R.N. from the American Heart Association, Indiana Affiliate; and by funding to B.H.M. as a Cottrell Scholar of the Research Corporation.

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**ABBREVIATIONS:** PDE, cyclic nucleotide phosphodiesterase; EHNA, erythro-9-(2-hydroxy-3-nonyl)-adenine; ScAMP-TME, succinyl cAMP tyrosine methyl ester; RIA, radioimmunoassay; EGFR, epidermal growth factor receptor; TNF$\alpha$, tumor necrosis factor $\alpha$.

## Experimental Procedures

**Materials.** Na[$^{125}$I] (17 Ci/mg) was purchased from DuPont-NEN (Boston, MA). Iodination of 2′*O*-succinyl adenosine 3′, 5′ cyclic monophosphate tyrosyl methyl ester (ScAMP-TME) with Na[$^{125}$I] and chloramine T was as described previously (Brooker et al., 1979). Milrinone, calmodulin, succinyl cAMP tyrosine methyl ester (ScAMP-TME), cGMP, insoluble protein A, poly ($Glu_4Tyr_1$) copolymer, cyclic nucleotide phosphodiesterase (P-0134), genistein, daidzein, biochanin A, tyrphostins 51, 25, 23, 63, and fetal bovine serum were obtained from Sigma Chemical Co. (St. Louis, MO). Prunetin was obtained from Indofine (Somerville, NJ). Rolipram, tyrphostins 48 and 8 were purchased from Calbiochem (San Diego, CA). [$\gamma$-$^{32}$P]ATP (7000 Ci/mmol) was obtained from ICN Chemical Co. (Costa Mesa, CA), and mouse anti-calmodulin activated cyclic nucleotide phosphodiesterase monoclonal antibody (MAB1039) was purchased from Chemicon (Temecula, CA). Protein G-agarose and mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Dulbecco's modified Eagle's medium containing high glucose (4.5 g/liter) was obtained from Irvine Scientific (Santa Ana, CA). Erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) was purchased from Research Biochemicals International (Natick, MA).

**Cell Growth.** HT4 cells were obtained from Ronald McKay (Whittemore et al., 1991) and subcloned by limiting dilution. A single clone, HT4.7, was selected for all subsequent studies. HT4.7 cells were grown to confluence in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 $\mu$g/ml streptomycin for 3 to 5 days at 33°C in a 5% $CO_2$/95% air atmosphere.

**Generation of cAMP Antibodies.** Antibodies to cAMP were generated as described previously (Nichols and Morimoto, 1999). Antibody specificity was tested with AMP, ADP, ATP, cGMP, GMP, GDP, GTP, and cIMP. The anti-cAMP antibody was 10,000 times more selective for cAMP when compared with all other nucleotides except cIMP, in which the antibody had only a 100-fold greater affinity for cAMP.

**cAMP Radioimmunoassay.** cAMP was determined by competition binding with [$^{125}$I]-ScAMP-TME (Brooker et al., 1979). Radioactive tracer solution containing approximately 200,000 cpm/ml of [$^{125}$I]-ScAMP-TME was prepared in 50 mM sodium acetate, pH 4.75 containing 0.1% (w/v) $NaN_3$. The radioimmunoassay was performed in a 96-well filtration plate (Multiscreen HV; Millipore), containing 50 $\mu$l of neutralized sample, 50 $\mu$l of tracer, and 50 $\mu$l of 1:4000 diluted anti-cAMP antibody. The assay was incubated for 14 to 20 h at 4°C, and terminated with 0.2 ml of 0.1% (w/v) insoluble protein A. The amount of bound radioactivity was separated by vacuum filtration, and determined by gamma counting. The assay is able to detect 3 to 200 fmol when the samples are acetylated.

**HT4.7 Cell Extract Preparation.** Growth media was removed from confluent cells and the cells washed with LK buffer (125 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM $K_2HPO_4$, 10 mM glucose, and 20 mM HEPES, pH 7.4). Cells were removed into lysis buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM DTT, 10 $\mu$g/ml leupeptin, and 100 $\mu$M phenylmethylsulfonyl fluoride) and transferred to ice for 5 min. The cells were Dounce homogenized (30 strokes), and the cell lysate centrifuged at 10,000*g* for 10 min at 4°C.

**Preparation of Bovine Heart Phosphodiesterase Crude Complex.** Lyophilized cyclic nucleotide phosphodiesterase (2 U) from Sigma (bovine heart crude complex) was reconstituted in 20 ml of lysis buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM DTT, 10 $\mu$g/ml leupeptin, and 100 $\mu$M phenylmethylsulfonyl fluoride) to a concentration of 0.1 U/ml. The bovine heart PDE preparation was then applied to an ion-exchange column or used at this concentration to determine tyrosine kinase activity. One unit of bovine heart phosphodiesterase can hydrolyze 1.0 $\mu$mol of cAMP/min at pH 7.5 and 30°C.

**PDE Isozyme and Tyrosine Kinase Purification.** Phosphodiesterase and tyrosine kinase activities were partially purified by ion-exchange chromatography at 4°C. For PDE4, approximately 7 ml of an HT4.7 cell extract (2–3 mg/ml) was loaded on a Q2, strong anion exchange column (7 × 52 mm; Bio-Rad). PDE4 activity was eluted at a rate of 1 ml/min with a 30-ml linear gradient of NaCl (75 to 500 mM NaCl in 20 mM HEPES, pH 7.4) using a Bio-Rad Biologic system. Fractions 19 to 22 (1 ml each) at approximately 350 to 375 mM NaCl were pooled for PDE4 activity and assayed by the methods described previously. PDE activity in the bovine heart preparation was partially purified from 0.5 U of crude PDE complex in the same fashion as PDE4 although a more complex NaCl gradient was used. A 15-ml linear gradient from 0 to 250 mM NaCl was followed by a 15-ml plateau at 250 mM NaCl. The elution continued with a 30-ml linear gradient from 250 to 500 mM NaCl followed by an additional 10 ml at 500 mM NaCl. Fractions (1-ml) were collected and assayed for PDE activity in the presence of 1.5 mM calcium and 200 U/ml calmodulin.

**Phosphodiesterase Assays.** Preparations of partially purified PDE1, PDE3, and PDE4 were incubated at 30°C in a final concentration of 20 mM HEPES, pH 7.4 containing 90 mM KCl, 5 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 1 $\mu$M cAMP. Aliquots (100-$\mu$l) of the reaction were removed and terminated at various times by acidifying with $HClO_4$ to give a final concentration of 0.4 M. The acid extract was deproteinized by centrifugation and neutralized with $KHCO_3$. After a dilution in 50 mM sodium acetate, pH 4.75, containing 0.1% (w/v) $NaN_3$, 200 $\mu$l were acetylated with 10 $\mu$l of a 2:1 mixture of triethylamine and acetic anhydride for 10 to 20 min at room temperature. A final dilution was made before cAMP levels were measured by radioimmunoassay (RIA). In general, the phosphodiesterase assay consisted of measuring cAMP degradation during a timed incubation usually between 8 and 15 min. Phosphodiesterase activity is reported as the amount (picomoles per milliliter) of cAMP hydrolyzed by partially purified PDE during the assay incubation time. Data is presented as the mean ± S.E. $IC_{50}$ is defined as the concentration of inhibitor at 50% inhibition of the total enzyme activity.

**Tyrosine Kinase Assays.** The assay for tyrosine kinase activity was adapted from several methods (Corbin and Reimann, 1974; Braun et al., 1984; Ueki et al., 1997). Briefly, 25 $\mu$g of a HT4 cell extract was incubated with 1 mg/ml of the tyrosine kinase substrate poly($Glu_4Tyr_1$) in the presence of 20 mM HEPES pH 7.4, 5 mM $MnCl_2$, 10 mM $MgCl_2$, 10 $\mu$M ATP, and 5 $\mu$Ci [$^{32}$P]ATP in a final volume of 50 $\mu$l for 30 min at room temperature. The reaction was stopped with the addition of 1 mM ATP. After centrifugation at 10,000*g* for 3 min, 40 $\mu$l of the supernatant was spotted onto a Whatman 3 MM Chr filter paper square. The squares were washed four times with 10% TCA for 15 min, followed by 5 min each with ethanol and then ether. After drying, the squares were placed in scintillation fluid and counted for $^{32}$P incorporation.

**Immunoprecipitation.** Bovine heart PDE (0.1 U/ml) was precleared by incubation with 10 $\mu$l of mouse IgG (1:100) and 20 $\mu$l of protein G-agarose (1:200) for 30 min on an orbital rocker at 4°C. After centrifugation in a clinical centrifuge, 0.9 ml of supernatant was removed and incubated on ice with 10 $\mu$l of mouse anti-calmodulin activated cyclic nucleotide phosphodiesterase monoclonal antibody (MAB1039) for 1.5 h. Protein G-agarose (20 $\mu$l) was added followed by orbital rocking overnight at 4°C. The reaction mixture was centrifuged, the supernatant removed, and the agarose pellet was washed ten times with lysis buffer. The pellet was resuspended in 600 $\mu$l of lysis buffer and assayed for tyrosine kinase and phosphodiesterase activities.

## Results

**Characterization of Bovine Heart Phosphodiesterase.** We have previously shown that genistein and tyrphostin 51 inhibit PDE4 activity in the HT4.7 neural cell line by a tyrosine kinase-independent mechanism (Nichols and Morimoto, 1999). In light of this observation, it is important to

understand whether genistein was a selective inhibitor of PDE4 or whether it could inhibit other PDE isozymes. In the original report of genistein as a tyrosine kinase inhibitor, genistein was found to have no effect on bovine heart phosphodiesterase from Sigma (Akiyama et al., 1987). This result contradicted an earlier study in which genistein inhibited bovine heart PDE obtained from Boehringer with an $IC_{50}$ of 121 $\mu$M (Nikaido et al., 1982).

Three major forms of PDE have been isolated and characterized in bovine heart tissues. These are calcium/calmodulin-stimulated PDE1 (Ho et al., 1976), cGMP-stimulated PDE2 (Martins et al., 1982) and cGMP-inhibited PDE3 (Harrison et al., 1986). In earlier reports of the effect of genistein on bovine heart PDE activity, the PDE activity was tested without determining which PDE isozymes were present. To understand the effect of genistein and tyrphostin 51 on bovine heart PDE, a thorough characterization of the PDE activity in the Sigma bovine heart preparation was conducted.

Ion-exchange chromatography was used to separate 0.5 U of bovine heart phosphodiesterase. A series of two linear NaCl gradients were used to elute the protein from a strong anion exchange column. The fractions were assayed for PDE activity in the presence of 1.5 mM calcium and 200 U/ml calmodulin (Fig. 1). Two peaks of activity were clearly present in the bovine heart PDE preparation. The first peak of activity eluted at approximately 175 to 250 mM NaCl (fractions 10–20), whereas the second peak of activity eluted at 280 to 430 mM NaCl (fractions 35–50).

To characterize the two peaks of PDE activity and determine which PDE isozymes were present in the bovine heart preparation, we took advantage of several family-specific regulators and inhibitors. Fractions containing the highest PDE activity were collected into separate pools and tested for their sensitivity to the PDE1 regulator calcium and calmodulin, or to the PDE2 and PDE3 inhibitors, EHNA and milrinone, respectively (Fig. 2).

It was apparent that the first peak of activity was a calcium- and calmodulin-stimulated PDE1 isozyme because these regulators enhanced the activity almost 3-fold. EHNA and milrinone had no effect on peak 1 activity, indicating the absence of any contaminating PDE2 or PDE3 activity. In contrast, the PDE activity in peak 2 was not stimulated by calcium and calmodulin, and EHNA had no effect on PDE activity. However, 10 $\mu$M milrinone abolished 96% of peak 2 activity (Fig. 2).

The second peak of PDE activity was further probed with increasing concentrations (0.1–10 $\mu$M) of cGMP, milrinone, or 40 $\mu$M EHNA (Fig. 3). Milrinone, a PDE3-selective inhibitor, eliminated 92% of the activity with an $IC_{50}$ of approximately 400 nM, and cGMP inhibited 96% with an $IC_{50}$ of 1 $\mu$M. EHNA, a PDE2-selective inhibitor, had no effect on PDE activity in peak 2. This type of pharmacological profile is indicative of PDE3.

The presence of PDE4 activity was assessed on the crude PDE preparation before ion-exchange chromatography. The PDE4-selective inhibitor, rolipram, had no effect on bovine heart PDE activity even at concentrations of 3 $\mu$M. PDE activity was 3.29 nmol/min/mg in the absence of rolipram, 3.42 nmol/min/mg in the presence of 0.03 $\mu$M rolipram, 3.70 nmol/min/mg at 0.3 $\mu$M rolipram, and 3.29 nmol/min/mg at 3 $\mu$M rolipram. We have previously used rolipram to elucidate a PDE4 isoform in the HT4 cell line (Nichols and Morimoto, 1999). In that case, activity was significantly decreased at low rolipram concentrations in contrast to the bovine heart PDE crude complex. Collectively, this data demonstrates the Sigma bovine heart PDE preparation is composed of calcium and calmodulin-stimulated PDE1 (peak 1) and cGMP-inhibited PDE3 (peak 2).

**Inhibition of PDE1 and PDE3 by Genistein and Tyrphostin 51.** The effect of genistein and tyrphostin 51 was studied on the bovine heart PDE isozymes. The activity of partially purified PDE1 and PDE3 was measured in the

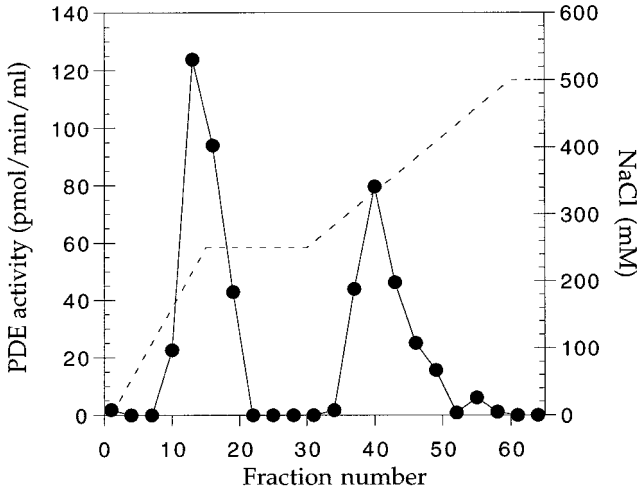



**Fig. 1.** Separation of bovine heart PDE activities. Bovine heart PDE crude complex (0.5 U, 1.67 mg) was loaded on, and PDE activity eluted from an anion exchange column as described in *Experimental Procedures*. Every third fraction (0.05 ml) was assayed for PDE activity in the presence of 1.5 mM calcium and 200 U calmodulin (●). The hydrolysis of 1 $\mu$M cAMP was measured over a 15-min period. The NaCl gradient is represented by the dashed line.

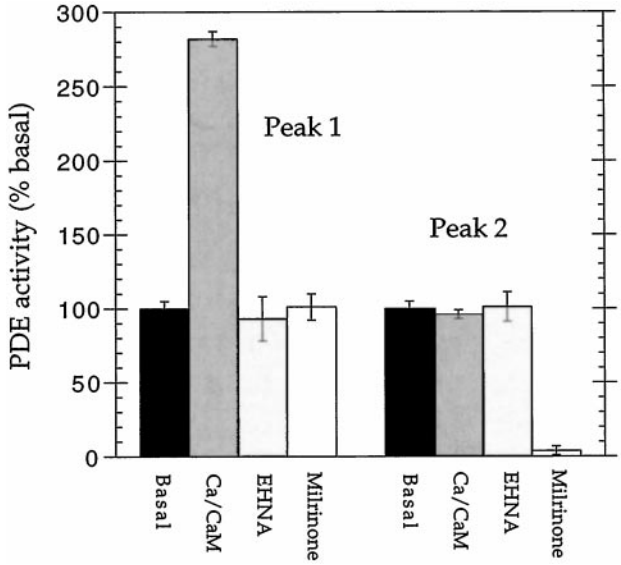



**Fig. 2.** Characterization of bovine heart PDE activity. After partial purification of the two peaks of PDE activity by ion-exchange chromatography, the effect of 1.5 mM calcium and 200 U/ml calmodulin, 40 $\mu$M EHNA and 10 $\mu$M milrinone was tested on both peaks of PDE activity. Fractions 13 to 16 were used for peak 1 and 39 to 42 for peak 2. The points are the average ± S.E.M. ($n$ = 6). PDE activity is reported as % basal activity in the presence of 1 mM EGTA and carrier dimethyl sulfoxide. Basal activity averaged 69 pmol/min/ml for peak 1 and 104 pmol/min/ml for peak 2.

presence of increasing concentrations of genistein (Fig. 4). Genistein inhibited up to 60% of the total PDE1 or PDE3 activity. The potency of genistein inhibition varied for the two PDE isozymes. The $IC_{50}$ value of genistein was 40 $\mu$M for PDE1 and 20 $\mu$M for PDE3, indicating that genistein was more potent at inhibiting PDE3 as compared with PDE1.

The ability of genistein to inhibit these two bovine heart PDE isozymes suggests that genistein can interact with multiple PDE isozymes, including the bovine heart forms. As we have shown previously (Nichols and Morimoto, 1999), genistein potently inhibited 92% of partially purified PDE4 activity with an $IC_{50}$ of 5 $\mu$M (Fig. 4). These differences in the inhibitory strength of genistein on different isozymes may provide important insight into the specificity of inhibition between PDE isozymes.

Tyrphostin 51 is a member of another class of tyrosine kinase inhibitor, and its ability to inhibit PDE1, PDE3, and PDE4 was studied. Increasing concentrations of tyrphostin 51 (1 to 100 $\mu$M), inhibited 80 to 95% of the total PDE activity (Fig. 5). Differences were noted in the potency of tyrphostin 51 on the various PDE isozymes (Fig. 5), with PDE1 being most sensitive, then PDE3 and PDE4. The $IC_{50}$ values for tyrphostin 51 were 7 $\mu$M for PDE1, 20 $\mu$M for PDE3, and 30 $\mu$M for PDE4.

**Tyrosine Kinase-Independent Inhibition of PDE1 and PDE3.** PDE3 has been shown to be regulated by tyrosine phosphorylation (Ueki et al., 1997). It was therefore possible that genistein and tyrphostin 51 were modulating PDE1 and PDE3 through inhibition of a tyrosine kinase. To address this possibility, the crude bovine heart phosphodiesterase preparation was assayed for tyrosine kinase activity, and 300 fmol/mg/min of activity was detected. This activity could be partially inhibited by both genistein (34% inhibition at 100 $\mu$M) and tyrphostin 51 (54% inhibition at 100 $\mu$M). Daidzein, an inactive analog of genistein, had no effect on bovine heart tyrosine kinase activity.

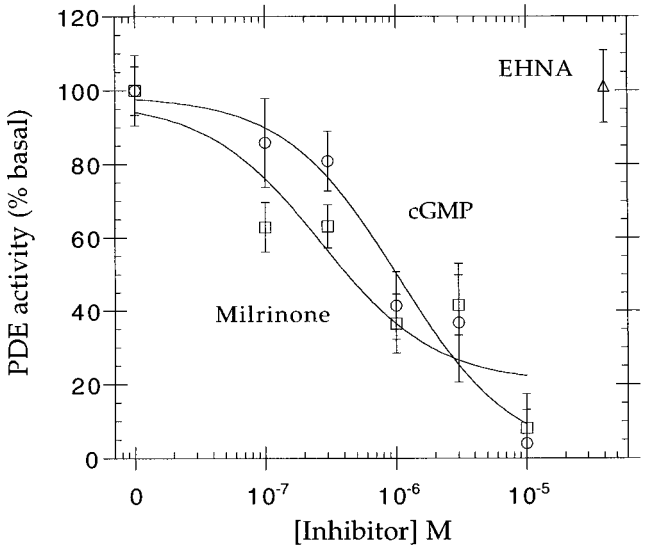



**Fig. 3.** Effect of various modulators on Peak 2 bovine heart PDE activity. After partial purification of the second peak of PDE activity by ion-exchange chromatography, the effect of increasing concentrations of milrinone (□), cGMP (○), or 40 $\mu$M EHNA (△) on PDE activity was determined using pooled fractions. The points are the average ± S.E.M. ($n$ = 6) and were fit to a nonlinear inhibition curve. PDE activity is reported as average % of basal activity (115 pmol/min/mg) in the presence of carrier dimethyl sulfoxide.

To determine whether inhibition of PDE1 and PDE3 by genistein and tyrphostin 51 was mediated by a protein tyrosine kinase, the bovine heart PDE isozymes were purified away from tyrosine kinase activity and tested for sensitivity to the inhibitors. Ion-exchange chromatography separated PDE3 away from any tyrosine kinase activity (Fig. 6, open circles, peak 2). However, bovine heart tyrosine kinase activ-

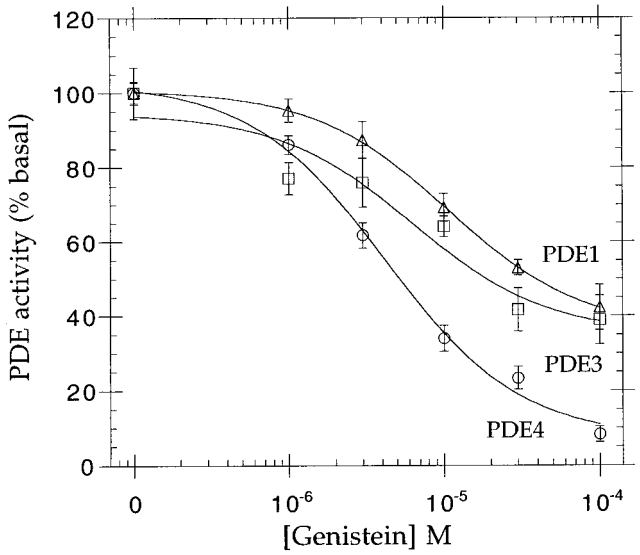



**Fig. 4.** Inhibition of PDE1, PDE3, and PDE4 by genistein. PDE activity was measured in partially purified preparations of PDE1 (△), PDE3 (□), or PDE4 (○) in the presence of increasing concentrations of genistein. The data points represent the average ± S.E.M. ($n$ = 6). Incubation times used to determine PDE activity and the basal activities were 6 min and 264 pmol/min/ml for PDE1, 8 min and 159 pmol/min/ml for PDE3, and 15 min and 148 pmol/min/ml for PDE4. A curve fit to the data points produced $IC_{50}$ values of 5 $\mu$M for PDE4, 20 $\mu$M for PDE3, and 40 $\mu$M for PDE1.

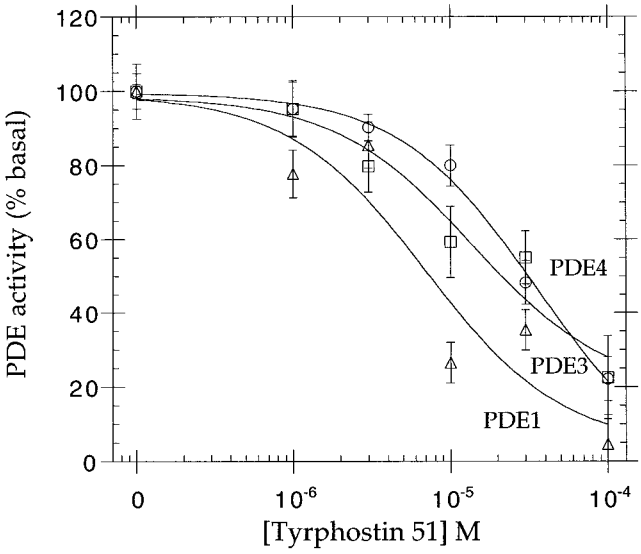



**Fig. 5.** Inhibition of PDE1, PDE3, and PDE4 by tyrphostin 51. PDE activity was measured in partially purified preparations of PDE1 (△), PDE3 (□), or PDE4 (○) in the presence of increasing concentrations of tyrphostin 51. The data points represent the average ± S.E.M. ($n$ = 6). Incubation times used to determine PDE activity and the basal activities were 10 min and 237 pmol/min/ml for PDE1, 10 min and 166 pmol/min/ml for PDE3, and 15 min and 76 pmol/min/ml for PDE4. A curve fit to the data points produced $IC_{50}$ values of 7 $\mu$M for PDE1, 20 $\mu$M for PDE3, and 30 $\mu$M for PDE4.

ity coeluted with PDE1 (Fig. 6). Bovine heart PDE1 was therefore separated from the tyrosine kinase activity by immunoprecipitation using an antibody to PDE1. Immunoprecipitation removed greater than 99.5% of the contaminating tyrosine kinase activity. Despite the removal of PDE1 from tyrosine kinases, PDE1 activity remained sensitive to genistein and tyrphostin 51. Genistein and tyrphostin 51 at 100 $\mu$M resulted in 43 $\pm$ 4 and 19 $\pm$ 3% ($n$ = 16) of the basal PDE1 activity, respectively. These data support the conclusion that the regulation of PDE1 and PD3 by genistein and tyrphostin 51 is independent of protein tyrosine kinases.

**Structure-Activity Relationship of PDE Inhibition by Isoflavones and Tyrphostins.** To gain insight into the important structural elements of isoflavone and tyrphostin compounds for PDE inhibition, a series of structural analogs or positional isomers of genistein and tyrphostin 51 were used (Fig. 7). Partially purified PDE1, PDE3, or PDE4 were studied to assess the inhibitory effect of the isoflavone or tyrphostin compounds. The $IC_{50}$ values were determined by locating the point on the curve at 50% inhibition of the PDE activity.

These studies produced striking results with respect to the differences in inhibition potency of the compounds. Daidzein, which is similar to genistein but lacks a C-5 hydroxyl group (Fig. 7), was a weak inhibitor of PDE4 ($IC_{50} > 100$ $\mu$M), yet inhibited PDE1 and PDE3 with an $IC_{50}$ of 30 and 12 $\mu$M, respectively (Table 1). This observation is interesting in that daidzein has been described as an inactive analog of genistein in the inhibition of tyrosine kinases. In fact, daidzein was the most potent inhibitor of PDE3 of all the compounds tested. It is apparent that the C-5 hydroxyl of the isoflavone is important for inhibition of PDE4 but not PDE1 or PDE3.

The importance of substitutions at the C-4′ position was investigated with biochanin A, which differs from genistein by a methoxy instead of a hydroxyl group. The absence of a hydroxyl group at this position lowered the inhibitory activity of the isoflavone for PDE1 and PDE3 ($IC_{50} > 100$ $\mu$M for both). However, PDE4 inhibition was not significantly affected, and biochanin A was as potent as genistein with an $IC_{50}$ of 9 $\mu$M (Table 1). This result indicates that the isoflavone C-4′ hydroxyl group is important for inhibition of PDE1 and PDE3, but not PDE4.

Replacement of the C-7 isoflavone hydroxyl group with a methoxy group significantly reduced the inhibition of all three PDE isozymes, with $IC_{50}$ values $> 100$ $\mu$M (Table 1, genistein compared with prunetin). Therefore, the C-7 hydroxyl probably interacts with a residue common to the three PDE isozymes.

A series of tyrphostin compounds also revealed important structure-activity information about PDE inhibition (Table 1). Tyrphostin 51 (trihydroxy) was a strong inhibitor of PDE1, PDE3, and PDE4 when compared with the monohydroxy tyrphostin 48. Both of these compounds were better inhibitors of PDE1 than any of the isoflavones.

Variation of the aliphatic chain at the Z position also affected activity. Tyrphostins 25, 23, and 8, which have a shorter 4 carbon (C4) aliphatic chain than tyrphostins 51 and 48, were considerably weaker inhibitors of all three PDE isozymes (Table 1). $IC_{50}$ values were near or greater than 100 $\mu$M for all of the short side chain tyrphostins.

Additional tyrphostin structure-activity information was obtained by measuring the extent of PDE inhibition by tyrphostins 25, 23, 8, and 63. The activity of partially purified PDE isozymes were measured in the presence of these tyrphostins at a concentration of 100 $\mu$M (Fig. 8). This series of tyrphostins were more potent inhibitors of PDE1 and PDE3 than of PDE4. For PDE4, modification of the tyrphostin pharmacophore had little effect on the inhibitory potential with the extent of inhibition ranging from 20% for tyrphostin 25 to 30% for tyrphostin 8.

The extent of inhibition by the tyrphostins was greater for PDE1 and PDE3. Tyrphostin 23 and tyrphostin 8 were able to inhibit approximately 50% of PDE3 activity and 50 to 60% of PDE1 activity. Interestingly, decreasing the number of hydroxyls on these short-chain tyrphostins increased the inhibitory potency for PDE1. Three phenolic hydroxyls of tyrphostin 25 resulted in inhibition of 27% of PDE1 activity, whereas tyrphostin 23 with two hydroxyls resulted in 48% inhibition, and tyrphostin 8 with only one hydroxyl inhibited 60%.

The tyrphostin trend of increasing inhibition by decreasing the number of hydroxyls was also observed for PDE3 (Fig. 8). Tyrphostin 25 with 3 hydroxyls inhibited only 10% of PDE3 activity, whereas tyrphostin 23 (two hydroxyls) and tyrphostin 8 (one hydroxyl) inhibited approximately 50% of the PDE3 activity. This pattern is quite different from the longer chain tyrphostins such as tyrphostin 51 and tyrphostin 48 (Fig. 7), in which removal of the hydroxyls decreased PDE inhibition potency for all PDE isozymes tested (Table 1).

The importance of the tyrphostin side chain is apparent from the tyrphostin 48, tyrphostin 8, and tyrphostin 63 series. Each of these tyrphostins has a single phenolic hydroxyl, but differ in the length and degree of saturation of the side chain (Fig. 7). Tyrphostin 48 has a C7 unsaturated side chain, and was effective at inhibiting all three PDE isozymes. The extent of inhibition was 90% for PDE1, and 75% of the PDE3 and PDE4 activity. Tyrphostin 8, a C4 unsaturated

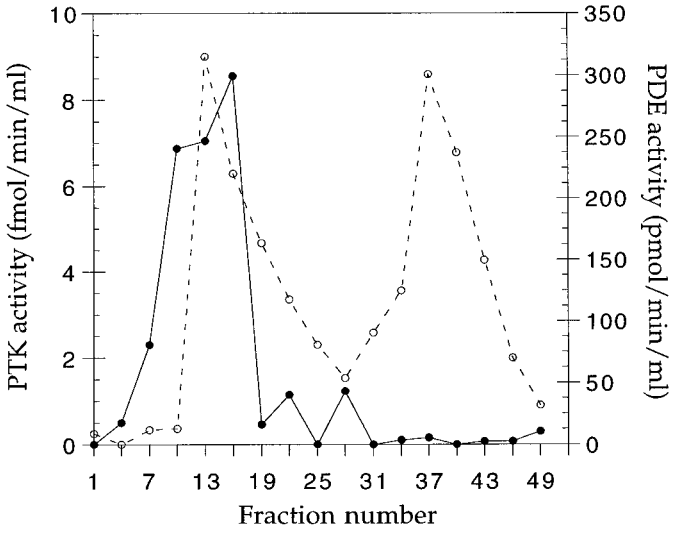



**Fig. 6.** Separation of bovine heart tyrosine kinase and PDE3 activities. Bovine heart PDE complex (1 U) was loaded on, and PDE activity eluted from an ion-exchange as described in *Experimental Procedures*. The fractions were then assayed for both PDE and tyrosine kinase activity. PDE activity was measured in the presence of 1.5 mM calcium and 200 U/ml calmodulin and the hydrolysis of 1 $\mu$M cAMP monitored over an 8-min period (○). Tyrosine kinase activity was determined in the same fractions (●).

tyrphostin, was moderately effective at inhibiting PDE, with the extent of inhibition being 50 to 60% of PDE1 and PDE3, and 30% for PDE4 (Fig. 8). Tyrphostin 63, which is identical with tyrphostin 8 except that the carbon side chain is saturated, was not effective at inhibiting any of the PDE isozymes. At most, 10% of any of the PDE isozyme activities were inhibited by 100 $\mu$M tyrphostin 63.

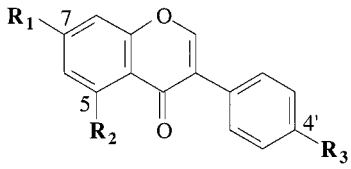
## Discussion

The ability of various isoflavones to inhibit PDE appears to be isozyme-selective. We demonstrated previously that genistein was a mixed-competitive inhibitor of PDE4, and that inhibition was independent of tyrosine kinase activity (Nichols and Morimoto, 1999). In investigating the order of potency of the isoflavones, it became apparent that inhibition of PDE1 and PDE3 was similar, but for PDE4 was different. This suggests that the isoflavone binding site on PDE1 and PDE3 are much more structurally similar than that of PDE4. It is interesting to note that PDE1 and PDE3 can hydrolyze both cAMP and cGMP, whereas PDE4 is selective for cAMP. This substrate selectivity may be related to isoflavone inhibition.

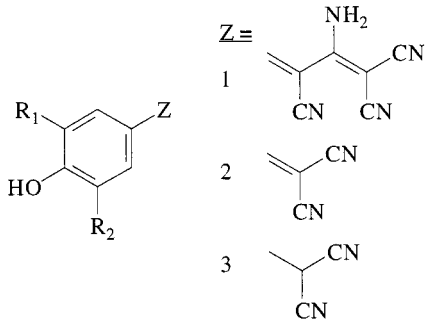
A summary of the structure-activity relationship of isoflavones is provided in Fig. 9. The hydroxyl groups on this structure are labeled for their importance in interacting with different PDE isozymes and subsequent inhibition of PDE activity. The overall picture of isoflavone interaction and inhibition of multiple PDE isozymes reflects differences in the active sites of PDE families and also demonstrates particular components of the isoflavone structure that recognize these active site differences.

The C-7 hydroxyl group of the isoflavone structure was important for inhibition of all three PDE isozymes tested (Fig. 9). Replacement of the C-7 hydroxyl of genistein with a methoxy group, as in prunetin, resulted in an $IC_{50}$ >100 $\mu$M for all PDE isozymes. This suggests that the isoflavone C-7 hydroxyl interacts with all three isozymes. The loss of activity seen with prunetin can be attributed to either a loss of hydrogen bonding or an increase in bulk.

From our data, the C-5 hydroxyl group of isoflavones appears to be important only for PDE4 inhibition (Fig. 9). A sharp loss of activity occurs with the removal of the hydroxyl group as evidenced in the shift in $IC_{50}$ for genistein ($IC_{50}$ = 5 $\mu$M) to daidzein ($IC_{50}$ > 100 $\mu$M). Daidzein is structurally identical with genistein, expect for the substitution of hydrogen for the C-5 hydroxyl. The inhibition of PDE1 or PDE3 was unaffected by the loss of the C-5 hydroxyl group. In fact, daidzein was a slightly stronger inhibitor of these two PDE isozymes than genistein.




| Isoflavone | $R_1$ (C-7) | $R_2$ (C-5) | $R_3$ (C-4') |
|---|---|---|---|
| Genistein | OH | OH | OH |
| Daidzein | OH | H | OH |
| Biochanin A | OH | OH | OMe |
| Prunetin | OMe | OH | OH |




| Tyrphostin | $R_1$ | $R_2$ | Z |
|---|---|---|---|
| 51 | -OH | -OH | 1 |
| 48 | -H | -H | 1 |
| 25 | -OH | -OH | 2 |
| 23 | -H | -OH | 2 |
| 8 | -H | -H | 2 |
| 63 | -H | -H | 3 |

**Fig. 7.** Isoflavone and Tyrphostin structures. Structure of various isoflavone and tyrphostin compounds.

For many other enzymes, daidzein is often used as an inactive analog of genistein. For example, genistein, but not daidzein, was able to activate cystic fibrosis transmembrane regulator chloride current (Chiang et al., 1997) and to inhibit both the epidermal growth factor receptor (EGFR) tyrosine kinase (Akiyama et al., 1987) and GLUT1, the type 1 isozyme of the hexose transporter (Vera et al., 1996).

The C-4′ hydroxyl of isoflavones appears to be important for inhibition of PDE1 and PDE3, but not to PDE4 (Fig. 9). Biochanin A selectively inhibited PDE4; however, the C-4′ methoxy group of biochanin A prevented an important interaction with PDE1 or PDE3, thereby reducing its ability to inhibit these enzymes. Our data demonstrates that both genistein and biochanin A are potent inhibitors of PDE4. A previous study investigating isoflavone inhibition of EGF receptor found biochanin A to be 30-fold less potent than genistein (Akiyama et al., 1987).

The tyrphostins were also potent inhibitors of PDE isozymes through a tyrosine kinase-independent mechanism. Similar to the isoflavones, the tyrphostins demonstrated PDE isozyme selectivity. The basic tyrphostin structure appears to be a good pharmacophore for PDE1 and PDE3 selective inhibitors. The absence of the C-3 and C-5 phenolic hydroxyl groups on the tyrphostin structure (tyrphostin 48) reduced the potency of PDE inhibition. The longer C7 side chain (Fig. 7, Z group) of tyrphostin 51 and 48 increased the potency of PDE inhibition dramatically over the shorter C4 side chained tyrphostins. Additionally, unsaturation of the side chain appears to be important for PDE inhibition. Tyrphostin 63 with an identical hydroxyl substitution as tyrphostin 8, but a saturated side chain is much less potent of an inhibitor. Although saturation would allow tyrphostin 63 to be more flexible, a dramatic decrease in inhibitory potency is observed. This may be related to the importance of electron delocalization and $\pi$-pi electron interactions with PDE or by providing a planar extension important in molecular recognition.

The pattern of PDE inhibition by the tyrphostins is quite different from inhibition of the EGFR tyrosine kinase (Gazit et al., 1989). Removal of phenolic hydroxyl groups on the shorter C4 side chain tyrphostins appeared to increase the extent of PDE inhibition. This was seen with PDE1 and to a smaller degree with PDE3 and PDE4. This result is in contrast to EGFR inhibition. Gazit and coworkers found that reducing the number of hydroxyls on the tyrphostin structure significantly weakened inhibition of EGFR activity (Gazit et al., 1989). Removal of the hydroxyls also decreased the inhibition of GTPase activity of transducin (Wolbring et al., 1994). We found that removal of the phenolic hydroxyls reduces the PDE inhibitory activity, but only for the longer, C7 chain tyrphostins (51 and 48). Thus additional factors must be involved in the ability of the short-chained tyrphostins to inhibit PDE, such as hydrophobic interactions, that compensate for the shorter side chain and increase the potency of inhibition with the removal of the hydroxyl groups.

The intracellular signaling events mediated by PDE and the biological activities of the isoflavones may converge in many yet to be determined diseases or signaling pathways. One example of convergence may be the signaling events

TABLE 1

$IC_{50}$ values of PDE isozymes by isoflavones and tyrphostins

The activity of partially purified preparations of PDE1, PDE3, and PDE4 was measured in the presence of increasing concentrations of isoflavones and tyrphostins. $IC_{50}$ values were determined by nonlinear curve fit to the PDE activity (average ± S.E.M. for $n = 6$) as a function of inhibitor concentration.

| Compound | $IC_{50}$ |
|---|---|
| | $\mu M$ |
| PDE1 | |
| Tyrphostin 51 | 7 |
| Tyrphostin 48 | 26 |
| Daidzein | 30 |
| Genistein | 40 |
| Biochanin A | >100 |
| Prunetin | >100 |
| PDE3 | |
| Daidzein | 12 |
| Genistein | 20 |
| Tyrphostin 51 | 20 |
| Tyrphostin 48 | 43 |
| Biochanin A | >100 |
| Prunetin | >100 |
| PDE4 | |
| Genistein | 5 |
| Biochanin A | 9 |
| Tyrphostin 51 | 30 |
| Tyrphostin 48 | 40 |
| Daidzein | >100 |
| Prunetin | >100 |

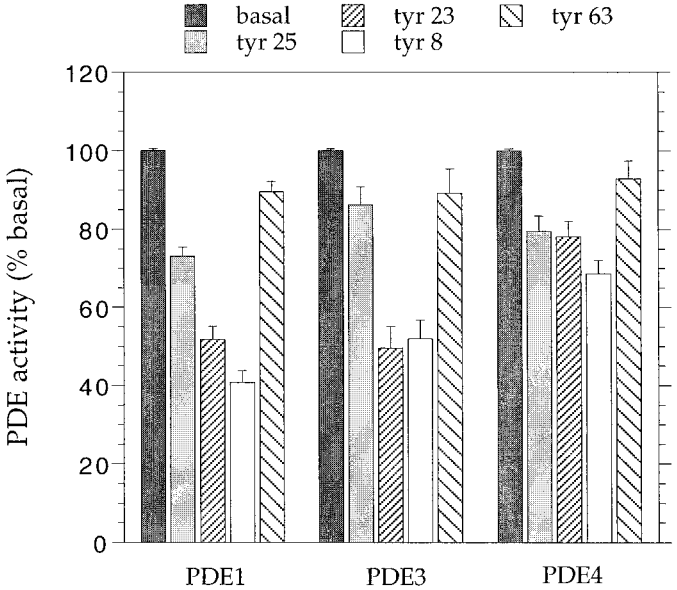



**Fig. 8.** Extent of PDE inhibition by short side chain tyrphostins. The activity of PDE1, PDE3, or PDE4 was measured in the absence and presence of 100 $\mu$M tyrphostin 25, tyrphostin 23, tyrphostin 8, or tyrphostin 63. PDE activity (average ± S.E.M., $n = 6$) is presented as % basal activity in the presence of carrier dimethyl sulfoxide. The basal activity averaged 111 pmol/min/ml for PDE1, 66 pmol/min/ml for PDE3, and 111 pmol/min/ml for PDE4.

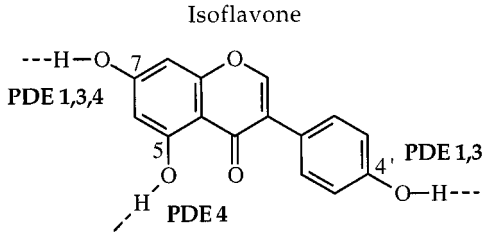



**Fig. 9.** PDE inhibition specificity of isoflavone hydroxyl groups. The C-7 hydroxyl group was important for inhibition of all three PDE isozymes. The C-5 hydroxyl was important for PDE4 inhibition and the C-4′ hydroxyl was important for PDE1 and PDE3 inhibition.

involved in allergic reactions (anaphylaxis) where both PDE inhibition (Teixeira et al., 1994) and flavonoids (Sloan et al., 1991) were important inhibitors of eosinophil accumulation and degranulation. One hypothesis is that the flavonoid-sensitive target is a PDE, which in turn mediates the activation of eosinophils.

Convergence can also occur with tumor necrosis factor $\alpha$ (TNF$\alpha$) production. Tyrphostins have been found to inhibit lipopolysaccharide-induced TNF$\alpha$ production in macrophages (Novogrodsky et al., 1994) and PDE inhibitors, in particular PDE4 inhibitors, have also been shown to inhibit lipopolysaccharide-induced TNF$\alpha$ production (Souness et al., 1996). The mechanism of the physiological consequence of tyrphostin could be mediated by PDE inhibition.

Our results on the structure-activity relationship of isoflavones and tyrphostins on various cAMP phosphodiesterase isozymes reveal structural information important for inhibition of PDE and provide a possible connection between PDE and numerous signaling processes that were heretofore thought to be mediated by tyrosine kinases.

**Acknowledgments**

We thank Drs. Patricia LiWang and Phil Low at Purdue University and Dr. Terrone Rosenberry at the Mayo Clinic Jacksonville for generously allowing us to use their laboratory facilities.

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