

Inhibition of GTP-utilizing Enzymes by Tyrphostins*

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Tyrphostins are a group of organic compounds which are widely used as a tool to specifically inhibit protein tyrosine kinases (Yaish, P., Gazit, A., Gilon, C., and Levitzki A. (1988) *Science* 242, 933–935; Gazit, A., Yaish, P., Gilon, C., and Levitzki A. (1989) *J. Med. Chem.* 32, 2344–2352; Lyall, R. M., Zilberstein, A., Gazit, A., Gilon, C., Levitzki, A., and Schlessinger J. (1989) *J. Biol. Chem.* 264, 14503–14509; Osherov, N., Gazit, A., Gilon, C., and Levitzki, A. (1993) *J. Biol. Chem.* 268, 11134–11142). We report here that members of the tyrphostin family inhibit the GTPase activity of transducin and the enzymatic activities of other GTP-utilizing proteins in retinal rod outer segments, such as guanylyl cyclase or fructose-6-phosphate kinase. In contrast, ATP-utilizing enzymes such as hexokinase or rhodopsin kinase were not effected.

Since the discovery in the late 1970s that the transforming gene of the tumorigenic Rous sarcoma virus (p60^{src}) encodes a protein tyrosine kinase (Collett and Erikson, 1978; Hunter and Sefton, 1980), there has been an intense focus on the role of both oncogenic and protooncogenic tyrosine kinases in processes related to normal and tumorigenic cell growth. It has been hypothesized that diseases such as psoriasis, certain cancers, chronic myeloid leukemia, atherosclerosis, myelofibrosis, and pulmonary fibrosis may be associated with the increase in activity of specific protein tyrosine kinases (Levitzki, 1992). These diseases, therefore, are thought to be candidates for therapy by protein tyrosine kinase inhibitors such as the tyrphostins (Levitzki, 1992). To date, well over 100 tyrphostin derivatives have been synthesized and tested for their inhibitory activity in various tyrosine kinase assay systems (Yaish *et al.*, 1988; Gazit *et al.*, 1989, 1991; Lyall *et al.*, 1989; Osherov *et al.*, 1993). Tyrphostins are widely used (10–100 μ M) in studies done both *in vitro* (Yaish *et al.*, 1988; Gazit *et al.*, 1989, 1991) and *in vivo* (Lyall *et al.*, 1989; Osherov *et al.*, 1993) to evaluate the potential metabolic roles of protein tyrosine kinase activi-

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ties and their potential link to pathological conditions such as cancer and psoriasis. We report here that the widely used tyrphostins 23, 25, and 47 (also designated AG-18, -82, and -217) are also potent inhibitors of bovine retinal rod outer segment (ROS)¹ guanylyl cyclase, ROS fructose-6-phosphate kinase, and the GTPase activity of transducin, with IC₅₀ values (2–30 μ M) in the same range as the IC₅₀ values observed previously for the inhibition of tyrosine kinase activity of the epidermal growth factor receptor (Gazit *et al.*, 1989).

MATERIALS AND METHODS

Pyrophosphate reagent, phosphoenolpyruvate, GTP, GDP, ATP, ADP, NADH, lactate dehydrogenase (EC 1.1.1.27), and pyruvate kinase (EC 2.1.1.740) were obtained from Sigma; GTP γ S was obtained from Boehringer Mannheim; tyrphostin 23 (AG-18), tyrphostin 25 (AG-82), tyrphostin 47 (AG-217), and tyrphostin 9 (AG-17) were obtained from Calbiochem; tyrphostin 8 (AG-10) was synthesized according to Gazit *et al.* (1989).

Preparation of Bovine ROS—Bovine eyeballs were purchased from a local slaughterhouse, and the retinas were dissected as soon as possible. Ca²⁺-depleted intact bovine ROS were purified from freshly dissected retinas as described previously (Schnetkamp *et al.*, 1979; Schnetkamp, 1986) with an additional sucrose gradient purification step (20–50% (w/v) sucrose, 20 mM Hepes, 10 mM D-glucose, 500 μ M EDTA). Intact bovine ROS were stored at 4 °C as a concentrated suspension containing 200–300 μ M rhodopsin in 600 mM sucrose, 5% (w/v) Ficoll 400, 100 μ M EDTA, and 20 mM Hepes (the pH of this and all other solutions was adjusted to pH 7.4 with arginine).

GTPase and ATPase Assay—GTPase and ATPase activities were measured with an enzyme-coupled assay in which pyruvate kinase (EC 2.1.1.740) and lactate dehydrogenase (EC 1.1.1.27) couple GDP or ADP formation to the oxidation of 1 mol of β -NADH/mol of GDP or ADP formed. Intact ROS were bleached and diluted in 1 ml containing 50 mM Hepes (pH 7.4), 10 mM magnesium acetate, 100 mM potassium acetate, and 0.01% saponin (final opsin concentration 2–4 μ M). ROS membranes were sedimented in a table-top centrifuge at 10,000 rpm for 30 s. ROS membranes were resuspended, and the GTPase or ATPase assay was performed in a volume of 2 ml in: 50 mM Hepes (pH 7.4), 10 mM magnesium acetate, 500 μ M phosphoenolpyruvate, 100 mM potassium acetate, 0.01% saponin, 5.0 units of lactate dehydrogenase, 3.5 units of pyruvate kinase, and 70 μ M NADH.

Assay of Guanylyl Cyclase and Fructose-6-phosphate Kinase—The enzyme guanylyl cyclase (EC 4.6.1.2) catalyzes the reaction GTP \rightarrow cGMP + PP_i. The key enzyme for the enzyme-coupled assay, the PP_i-dependent bacterial fructose-6-phosphate kinase (EC 2.7.1.90), uses fructose-6-phosphate as a substrate and couples PP_i formation to an enzymatic cascade, which leads to the oxidation of 2 mol of β -NADH/mol of PP_i formed. The bacterial PP_i-dependent fructose-6-phosphate kinase depends on PP_i rather than GTP or ATP, whereas the mammalian ROS fructose-6-phosphate kinase uses GTP or ATP but not PP_i. By using GTP or ATP as phosphate donors and ROS as a source of mammalian fructose-6-phosphate kinase, this assay could therefore also be used to measure the activity of the ROS fructose-6-phosphate kinase. Intact ROS were diluted to a final rhodopsin concentration of 1 μ M in a volume of 2 ml in: 400 μ M EGTA, 15 mM sodium fluoride, 75 mM sodium chloride, 1 mM citrate, 0.01% saponin, and 0.6 ml of the SIGMA PP_i detection kit. A more detailed description and validation of this assay will be presented elsewhere.²

Optical Recordings—The optical recordings of the β -NADH oxidation for both GTPase and guanylyl cyclase assays were performed in an

¹ The abbreviations used are: ROS, rod outer segment(s); GTP γ S, guanosine 5'-O-(thiotriphosphate); GC, guanylyl cyclase; tyrphostin 8, [(4-monohydroxybenzylidene)malononitrile]; tyrphostin 23, [(3,4-dihydroxybenzylidene)malononitrile]; tyrphostin 25, [(3,4,5-trihydroxyphenyl)methylene]propanedinitrile; tyrphostin 47, [(3,4-dihydroxybenzylidene)thiocyanacetamide]; tyrphostin 9, [(3,5-di-*t*-butyl)-4-dihydroxy- α -cyanocinnamide].

² G. Wolbring and P. P. M. Schnetkamp, submitted for publication.

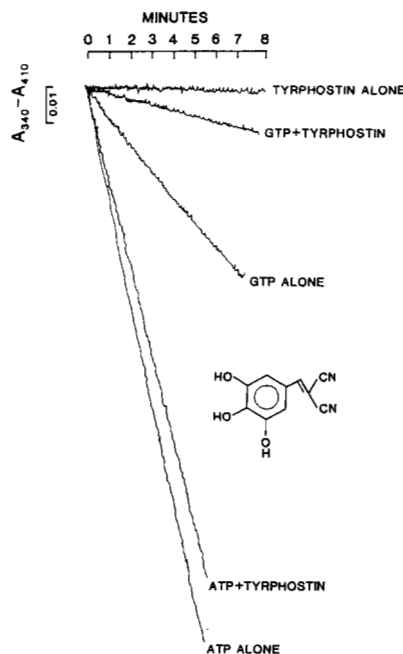


FIG. 1. Effect of tyrphostin 25 on ROS GTPase and ATPase activities. Real-time traces of ROS GTPase and ATPase activities are illustrated in the presence or absence of 40 μM tyrphostin 25. The real-time traces represent GTP- or ATP-induced production of GDP or ADP, respectively. Production of GDP or ADP was coupled to oxidation of NADH as described under "Materials and Methods," and was measured in a dual-wavelength spectrophotometer as a decrease in light absorption at 340 nm (with 410 nm as reference wavelength). Saponin-permeabilized ROS were used at a final opsin concentration of 2.8 μM . GTPase or ATPase activities were initiated by the addition of 100 μM GTP or 100 μM ATP, respectively.

SLM-Aminco DW2C dual-wavelength spectrophotometer in dual-wavelength mode with a wavelength pair of 340/410 nm and slit width of 6 nm. The temperature (25 $^{\circ}\text{C}$) was controlled with a circulating water bath, and the suspension was mixed with a magnetic spin bar.

Phosphorylation of rhodopsin by rhodopsin kinase was determined as described elsewhere (Schnetkamp *et al.*, 1979). Phosphorylation of glucose by hexokinase was determined as described previously (Schnetkamp and Daemen, 1981). Neither the solvent Me_2SO nor the tyrphostins affected the optical calibration signal of the assays.

RESULTS AND DISCUSSION

In previous work we have developed two optical assays to measure GC activity in retinal rod outer segments and observed that ATP could modulate GC activity.² In order to establish whether tyrosine phosphorylation might be involved in the effect of ATP on ROS GC, we decided to use, among other tools, the tyrphostins, a group of organic compounds that are widely used to inhibit specifically protein tyrosine kinases (Yaish *et al.*, 1988; Gazit *et al.*, 1989; Lyall *et al.*, 1989; Oshero *et al.*, 1993). However, in our initial experiments, we found that tyrphostin 23 instantaneously inhibited ROS GC with an IC_{50} of 26 μM (S.D. — 5, 3 experiments). This result indicated that tyrphostin 23 was acting directly on GC and not via the inhibition of a protein tyrosine kinase. Since GC is a GTP-utilizing enzyme, we tested another GTP-utilizing enzyme (ROS fructose-6-phosphate kinase) that could be measured with the GC assay as described under "Materials and Methods."

ROS Fructose-6-phosphate Kinase—Because ROS fructose-6-phosphate kinase can utilize ATP or GTP as a phosphate donor, we were able to evaluate the action of tyrphostins in the presence of either of these two phosphate donors. We found that if GTP was used as a phosphate donor, tyrphostin 23 inhibited ROS fructose-6-phosphate kinase with an IC_{50} of 6.9 μM (S.D.—1.9, 3 experiments) and, in one experiment, tyrphostin 25 in-

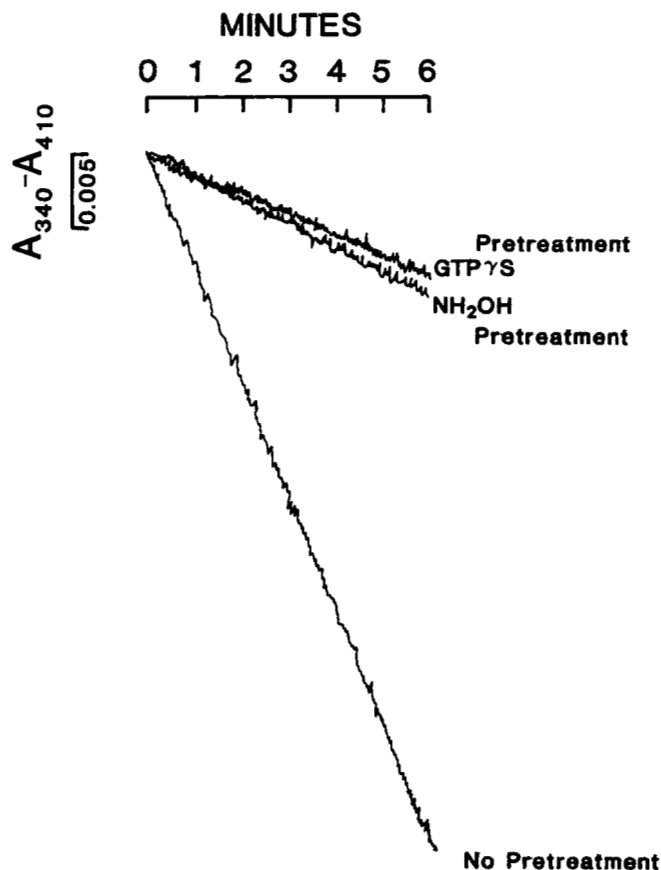


FIG. 2. The GTPase rate of transducin. Real-time traces of GTPase activity of saponin-permeabilized ROS were measured as a decrease in light absorption at 340 nm as described in the legend for Fig. 1. Saponin-permeabilized ROS were used at a final opsin concentration of 2.8 μM . GTPase activity was initiated by the addition of 25 μM GTP to bleached and saponin-permeabilized ROS that (a) received no special pretreatment, (b) were bleached in the presence of 2 mM hydroxylamine prior to addition of saponin, or (c) were treated with 10 μM GTP γS for 5 min prior to addition of GTP. The GTPase reaction was initiated by the addition of 25 μM GTP.

hibited ROS fructose-6-phosphate kinase with an IC_{50} of 2.2 μM . In contrast, if ATP was used as a phosphate donor, neither tyrphostin 23 nor 25 inhibited ROS fructose-6-phosphate kinase at concentrations up to 100 μM . The above results suggested that tyrphostins 23 and 25 may inhibit proteins that use GTP but not ATP or PP_i as a phosphate donor. Therefore, we tested ATP-utilizing enzymes present in ROS such as hexokinase or rhodopsin kinase, as well as the PP_i -dependent bacterial fructose-6-phosphate kinase, an enzyme that cannot use ATP or GTP, but uses PP_i instead as a phosphate donor. We found that none of these enzymes were inhibited by tyrphostins 23 and 25 at concentrations up to 100 μM (data not shown).

Inhibition of the GTPase Activity of Transducin by Tyrphostins—GTP hydrolysis represents another GTP-utilizing process. Using an enzyme-coupled real-time assay to measure ROS GTPase and ATPase activities as described under "Materials and Methods," we found that 40 μM tyrphostin 25 inhibited the GTPase activity of saponin-permeabilized ROS by 90% when 100 μM GTP was used. In contrast, the ATPase activity measured in a separate cuvette was only inhibited by 10% or less when 100 μM ATP was used (Fig. 1).

In a last step, we wished to establish the contribution of transducin-associated GTPase to the overall GTPase activity in ROS preparations. Transducin, a peripheral ROS protein and a member of the G-protein family, mediates the signal coupling between rhodopsin and the cGMP-dependent phosphodiester-

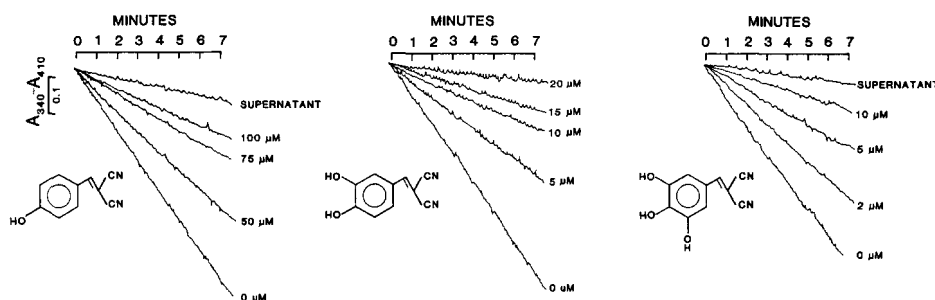


FIG. 3. Inhibition of the GTPase activity of transducin by tyrphostins 8, 23, and 25. Transducin-associated GTPase activity was measured in saponin-permeabilized ROS (final opsin concentration of 1–3 μM) as described in the legend of Fig. 1 and as defined in Fig. 2. The GTPase reaction was initiated by the addition of 25 μM GTP in the presence of the indicated concentrations of tyrphostin (left, tyrphostin 8; middle, tyrphostin 23; right, tyrphostin 25). Supernatant indicates the GTPase activity observed in the clear supernatant after sedimentation of ROS membranes and in the presence of the highest tyrphostin concentration applied.

ase. Activation of transducin involves interaction of the G-protein with light-activated rhodopsin and the use of GTP as a cofactor. Therefore, the transducin-associated GTPase activity was selectively inhibited in two ways (Fig. 2); 1) bleached ROS were treated with 2 mM hydroxylamine to remove the chromophore from opsin and to prevent binding to and activation of transducin by opsin (Hofmann *et al.*, 1983), and 2) 10 μM GTP γS was added to the assay medium, which led to the formation of a stable complex between GTP γS and transducin preventing binding of GTP to transducin and subsequent hydrolysis of GTP (Yamanaka *et al.*, 1985). At 25 μM GTP, transducin proved to be the predominant GTPase activity, as both hydroxylamine and GTP γS treatments reduced the GTPase activity by at least 5-fold when bleached saponin-permeabilized and washed ROS membranes were used (Fig. 2).

Next, the effect of tyrphostins on the GTPase rate of transducin was tested. We found that the potency to inhibit GTPase activity of transducin increased from the monohydroxy (tyrphostin 8) via the dihydroxy (tyrphostin 23) to the trihydroxy (tyrphostin 25) derivative of tyrphostin (Fig. 3). The observed IC_{50} values for tyrphostin 8, tyrphostin 23, and tyrphostin 25 were 45 μM (1 experiment), 10 μM (S.D. — 2, 12 experiments), and 7 μM (S.D. — 3, 7 experiments), respectively. In one experiment, we determined that another widely used derivative, tyrphostin 47, inhibited GTPase activity with an IC_{50} of 5 μM . The derivative tyrphostin 9, where the 3' and 5' hydroxy groups in tyrphostin 25 were substituted with *t*-butyl groups, did not inhibit transducin GTPase (not illustrated). Genistein, another protein tyrosine kinase inhibitor that, like tyrphostin, possesses a *p*-OH-phenyl moiety in its structure (Akiyama *et al.*, 1987), did not inhibit either the GTPase activity of transducin or the activities of ROS GC and ROS fructose-6-phosphate kinase. Increasing the GTP concentration in the assay from 25 to 250 μM did not affect the IC_{50} values of tyrphostin 23 and 25 toward transducin (data not shown), indicating that the inhibition by the tyrphostins was non competitive with GTP.

Taken together, our results suggest that tyrphostins possessing unsubstituted hydroxyl groups at the 3-, 4-, and 5-positions of the phenyl ring are effective inhibitors of three markedly different GTP-utilizing proteins (*i.e.* transducin, GC, and fructose-6-phosphate kinase) at concentrations at which ATP-utilizing enzymes were unaffected. We believe that the observed effects of tyrphostins 23, 25, and 47 on GTP-utilizing enzymes are direct effects on these enzymes and are not due to a secondary effect, reflecting the inhibition of protein tyrosine kinases by tyrphostin 23, 25, and 47 because: (a) the effects of tyrphostins 23, 25, and 47 on the activities of transducin, GC, and fructose-6-phosphate kinase were immediate, and (b) genistein, another protein tyrosine kinase inhibitor, had no effect on the activity of transducin, GC, and fructose-6-phosphate kinase at concentrations up to 100 μM .

The tyrphostins we have studied are widely used in a range between 10 and 100 μM in studies done *in vitro* (Yaish *et al.*, 1988; Gazit *et al.*, 1989, 1991) and *in vivo* (Lyall *et al.*, 1989; Oshero *et al.*, 1993; Fujihara *et al.*, 1993; Glembotski *et al.*, 1993; Yamazaki *et al.*, 1993; Lee *et al.*, 1993; Seckl *et al.*, 1993; Campbell *et al.*, 1993; Roifman *et al.*, 1991; Rendu *et al.*, 1992) in order to evaluate the potential metabolic effects of protein tyrosine kinases. We believe it is of importance that the IC_{50} values (2–26 μM) we measured for the inhibition of ROS guanylyl cyclase and transducin GTPase were close to the IC_{50} values observed for the inhibition of the epidermal growth factor receptor kinase (Gazit *et al.*, 1989). In view of our results, studies employing tyrphostins 23, 25, and 47 in cell culture systems must also consider the ability of these compounds to inhibit GTP-utilizing enzymes. Our results indicate that the different tyrphostins can inhibit GTP-utilizing enzymes with different potencies, raising the possibility of synthesizing tyrphostin derivatives that may specifically inhibit GTP-utilizing enzymes such as Ras, without affecting tyrosine kinases.

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