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REGULATION OF CARDIAC ADENYLYL CYCLASE BY EPIDERMAL GROWTH FACTOR (EGF)

ROLE OF EGF RECEPTOR PROTEIN TYROSINE KINASE ACTIVITY

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Abstract—We have shown previously that the α subunit of the stimulatory GTP binding regulatory component of adenylyl cyclase ($G_{s\alpha}$) mediates epidermal growth factor (EGF)-elicited stimulation of rat cardiac adenylyl cyclase (Nair *et al.*, *J Biol Chem* **265**: 21317–21322, 1990). Employing purified protein phosphotyrosine phosphatase, and benzylidene derivatives (tyrphostins: compounds 11 and 12) that selectively inhibit EGF receptor protein tyrosine kinase (EGFRK) activity, the role of EGFRK in EGF-mediated stimulation of cardiac adenylyl cyclase was investigated. The ability of the tyrphostins to inhibit the EGFRK activity in cardiac membranes was determined by monitoring tyrosine phosphorylation of either the 170 kDa protein or immunoprecipitated EGF receptor at 0° and room temperature, respectively. Compounds 11 and 12, in a concentration-dependent manner, inhibited EGF receptor tyrosine kinase activity. In assays of adenylyl cyclase activity neither compound 11 nor compound 12 altered Gpp(NH)p- or isoproterenol-stimulated activity. However, both compounds, in a concentration-dependent manner, attenuated the ability of EGF to stimulate adenylyl cyclase activity without altering specific binding of [^{125}I]EGF to cardiac membranes. Similarly, protein phosphotyrosine phosphatase obliterated the ability of EGF, but not isoproterenol, to stimulate adenylyl cyclase. Thus, we conclude that protein tyrosine kinase activity of the EGF receptor is essential for the stimulation of cardiac adenylyl cyclase by EGF.

Upon binding to its receptor, epidermal growth factor (EGF†) activates the intrinsic protein tyrosine kinase activity of the receptor with the resultant phosphorylation of a number of cellular proteins [1–3]. Activation of the EGF receptor protein kinase (EGFRK) has been shown to be important for a number of, but not all, biological events that are stimulated by EGF. For instance, studies performed in cells expressing a mutated EGF receptor devoid of kinase activity have demonstrated that the EGFRK activity is important for EGF-elicited receptor internalization [4, 5], DNA synthesis and phospholipase C activation [6]. Moreover, recent studies have demonstrated that the EGFRK can phosphorylate phospholipase Cγ on tyrosine residues and that this phosphorylation is important for stimulation of phospholipase Cγ activity in response to EGF [for review see Ref. 7]. On the other hand, a recent study has demonstrated that EGF-dependent tyrosine phosphorylation of mitogen-activated protein (MAP) kinase does not require EGFRK activity [8]. Therefore, it would appear that EGFRK activity is essential for some, but not all, biological actions of EGF.

Previous studies from our laboratory have shown that EGF produces inotropic and chronotropic actions in the rat heart by increasing cyclic AMP (cAMP) accumulation [9]. Moreover, despite the presence of functional EGF receptors in cardiac myocytes and non-myocytes derived from hearts, EGF stimulates cAMP accumulation only in cardiac myocytes [10]. EGF-elicited stimulation of cellular cAMP accumulation in the heart is the result of stimulation of adenylyl cyclase activity by a mechanism involving the participation of a G protein [11]. Employing an antibody directed against the carboxy-terminus of the α subunit of the stimulatory GTP binding regulatory component of adenylyl cyclase ($G_{s\alpha}$), we have also demonstrated that $G_{s\alpha}$ mediated the actions of EGF on adenylyl cyclase [12]. In the wake of reports which demonstrate that the protein tyrosine kinase activity of the EGF receptor is important in most, but not all, of EGF-elicited biological effects (see above), the purpose of the studies presented here was to investigate the role of the EGFRK activity in EGF-elicited stimulation of rat cardiac adenylyl cyclase. The protein tyrosine kinase inhibitors, "tyrphostins" [13], and purified protein phosphotyrosine phosphatase [14] were employed to attenuate the EGFRK. Tyrphostins have been shown to be highly selective in inhibiting EGFRK activity and in attenuating EGF-mediated cellular proliferation [13, 15, 16]. Likewise, protein phosphotyrosine phosphatase

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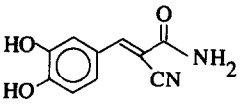
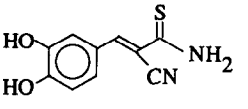
† Abbreviations: EGF, epidermal growth factor; EGFRK, EGF receptor protein tyrosine kinase; Gpp(NH)p, guanyl-5′-yl imidiphosphate; PTPase, protein phosphotyrosine phosphatase; G_s, stimulatory GTP binding regulatory component of adenylyl cyclase; and $G_{s\alpha}$, α subunit of G_s.



Compound 11



Compound 12

Fig. 1. Structural representation of the tyrphostins, compounds 11 and 12.

(PTPase) has been shown to effectively attenuate EGF-elicited tyrosine phosphorylations [14]. Employing purified PTPase [14] and the two most potent tyrphostins, compound 11 and compound 12 [13], data are presented to demonstrate that the EGFRK is essential for stimulation of the cardiac adenylyl cyclase by EGF.

MATERIALS AND METHODS

Materials. EGF was purchased from Biomedical Technologies, Inc. Phospocreatine, guanyl-5′-yl imidophosphate (Gpp(NH)p), cAMP, 3-isobutyl-1-methylxanthine and isoproterenol were obtained from the Sigma Chemical Co. [^{125}I]EGF, [^{3}H]cAMP, and [α-^{32}P]ATP were purchased from DuPont-New England Nuclear. Rabbit muscle creatine kinase was purchased from Boehringer Mannheim. Anti-phosphotyrosine antibody (PY20) was purchased from ICN Inc. Goat anti-mouse horseradish peroxidase conjugate was obtained from Bio-Rad Laboratories. An enhanced chemiluminescence (ECL) western blot detection system and anti-EGF receptor antibody (EGF-R1) were purchased from the Amersham Corp. Purified preparation of protein phosphotyrosine phosphatase was a gift from Dr. David L. Brautigan, Brown University, Providence, RI. All other chemicals were of the highest purity commercially available.

Isolation of cardiac membranes. Freshly excised hearts from rats of the Sprague–Dawley strain (200–240 g body weight) were homogenized in isolation medium containing 5 mM Tris–HCl, pH 7.4, 250 mM sucrose, and 1 mM ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). Cardiac membranes were isolated from this homogenate by the method of Bristow *et al.* [17]. Protein content of these members was determined by the method of Bradford [18], employing bovine serum albumin (BSA) as standard.

Synthesis of compound 11 *and compound* 12. The tyrphostins were synthesized according to the methods of Levitzki *et al.* [19], University of Jerusalem, Israel. Briefly, compound 11 (Fig. 1) was synthesized by adding 700 μL of piperidine to a mixture of 10 mM 3,4-dihydroxybenzaldehyde and 10.7 mM cyanoacetamide in 30 mL ethanol. The reaction was refluxed for 40 min and 100 mL of water was added. After cooling for 1 hr, the yellow crystalline powder was filtered, washed, and dried to give the final product. Similarly, for compound 12 (Fig. 1), 6 mM 3,4-dihydroxybenzaldehyde was mixed with 7 mM cyanothio-acetamide in 30 mL ethanol and 400 μL of piperidine was added. The reaction was refluxed for 1 hr and poured into ice water. After filtering, washing, and drying, compound 12 was obtained as an orange crystalline powder. As ascertained by their respective melting points (241°, compound 11; 217°, compound 12) and by proton NMR spectra, both compounds were approximately 98% pure.

EGFRK activity assays. The EGFRK activity in cardiac membranes was assessed employing two different approaches. In both of these methods, we monitored the ability of EGF to phosphorylate on tyrosine residues either the 170 kDa protein or the immunoprecipitated EGF receptor. The first approach involved incubation of cardiac membranes (150 μg protein) for 1 hr at 0° in a medium (pH 7.4) containing the following at the final indicated concentrations in 50 μL: β-glycerophosphate, 20 mM; glycerol, 2.5% (v/v); NaCl, 70 mM; Triton, 0.025% (v/v); sodium *ortho*-vanadate, 100μM; *p*-nitrophenyl phosphate, 20 μM; ATP, 200 μM; creatine kinase, 1 mg/mL; and phosphocreatine, 12 mM. EGF (50 nM) and the EGFRK inhibitors, compounds 11 and 12, were added at the desired final concentrations. The reactions were terminated by the addition of 50 μL of 2X Laemmli sample buffer [20] and heating at 100° for 3 min. Proteins in the samples were separated on 7.5% polyacrylamide gels according to the procedure of Laemmli [20] and transferred to nitrocellulose membranes for western blotting with the monoclonal anti-phosphotyrosine antibody (PY20) and the Amersham ECL detection system according to our previously published methods [10, 12]. The most prominent protein phosphorylated on tyrosine residues by EGF migrated at a position corresponding to a molecular mass of 170 kDa. Therefore, tyrphostin-mediated attenuation of EGF-stimulated tyrosine phosphorylation of the 170 kDa protein was monitored. Phosphorylation of the 170 kDa protein was analyzed by quantitative densitometric scanning using an automated Bio-image analyzer (BioScan, Milligen Corp.).

The second approach to monitor EGFRK activity in cardiac membranes involved incubations similar to those described above, except that the assays were performed at room temperature for a 15-sec period and the tyrosine phosphorylation of the immunoprecipitated EGF receptor was monitored. The purpose of these short incubations was to determine the rate of tyrosine phosphorylation of the EGF receptor (which migrates as a 170 kDa protein); EGF-elicited autophosphorylation of the immunoprecipitated EGF receptor from cardiac membranes was empirically determined to be linear for 20 sec at room temperature (not shown). The incubations, performed in the presence and absence of EGF (50 nM), with and without various concentrations of compounds 11 and 12, were

terminated by the addition of lysis buffer containing 1% (v/v) Triton X-100, hexokinase (2 U) and glucose (5 mM) in addition to the components of the incubation buffer. The hexokinase and glucose were included to rapidly convert ATP to ADP and thereby prevent any further phosphorylation of proteins. Following pre-clearing of the membrane lysates with goat anti-mouse IgG-agarose, the supernatants were incubated overnight at 4° with monoclonal anti-EGF receptor antibody (EGF-R1, Amersham). The immunocomplex was precipitated by centrifugation following the addition of goat anti-mouse IgG-agarose. After washing three times with the lysis buffer devoid of Triton, the immunoprecipitate was solubilized in Laemmli sample medium [20]. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [20], and western blots with anti-phosphotyrosine antibody were performed as described above.

Adenylyl cyclase activity. Adenylyl cyclase was assayed by the method of Salomon *et al.* [21] as described in our earlier reports [11, 12]. EGF, isoproterenol, protein phosphotyrosine phosphatase, and compounds 11 and 12 were added to the assay at the desired concentration. The GTP analog Gpp(NH)p was present in all activity assays unless otherwise indicated. Adenylyl cyclase activity was assayed in quadruplicates over a 30-min time period in a reaction volume of 250 μL. Activities were linear with time and protein under the various experimental conditions.

Binding of [^{125}I]EGF to cardiac membranes. To determine the effects of compounds 11 and 12 on the binding of EGF to its receptors on cardiac membranes, 50 μg of cardiac membrane protein was incubated at 30° in medium containing 50 mM Hepes, pH 7.4, 1.5 mM $MgCl_2$, 0.1% (w/v) BSA, 0.2% (w/v) bacitracin, and [^{125}I]EGF at a concentration of 5×10^{-11} M. Following equilibrium binding for 60 min (determined empirically from previous experiments), membrane-bound [^{125}I]EGF was separated from unbound [^{125}I]EGF by the addition of 3 mL of ice-cold 0.15 M NaCl and filtration through a Whatman GF/C filter soaked in polyethyleneimine [22]. The filters were washed two more times with 3-mL aliquots of NaCl prior to counting in a γ-counter. Non-specific binding was determined in parallel incubations performed in the presence of excess unlabeled EGF (1 μM) and subtracted from total binding to determine specifically bound [^{125}I]EGF.

RESULTS AND DISCUSSION

Initially, experiments were performed to evaluate the ability of EGF to phosphorylate on tyrosine residues the various proteins present in cardiac membranes. The most prominent augmentation in EGF-elicited tyrosine phosphorylation was associated with a 170 kDa protein. Therefore, to ascertain the concentration-dependent inhibition of EGFRK activity by compounds 11 and 12 in cardiac membranes, the ability of various concentrations of the inhibitors to attenuate EGF-stimulated phosphorylation on tyrosine residues of the 170 kDa protein was monitored by western blotting with the monoclonal anti-phosphotyrosine antibody PY20. In rat cardiac membranes, EGF stimulated phosphorylation of the 170 kDa protein by ~6 fold (Fig 2, A and B). Compounds 11 and 12 attenuated the ability of EGF to stimulate the phosphorylation of the 170 kDa protein in a concentration-dependent manner (Fig. 2). Hence, EGF-elicited tyrosine phosphorylation of the 170 kDa protein was inhibited by 70% at 100 and 20 μM concentrations of compounds 11 and 12, respectively (Fig. 2C). To determine the effects of tyrphostins on the EGFRK activity rather than the extent of phosphorylation of the 170 kDa protein, autophosphorylation of the immunoprecipitated EGF receptor was monitored at a time point (15 sec) that was within the linear portion of EGF-elicited augmentation of tyrosine phosphorylation of this protein (see Methods and Materials). EGF increased tyrosine phosphorylation of the immunoprecipitated EGF receptor by 4-fold and the tyrphostins, in a concentration-dependent manner, decreased EGF-mediated autophosphorylation of the immunoprecipitated EGF receptor (Fig. 2D). Most interestingly, in both experimental approaches, the concentration dependence of the inhibition and the extent of attenuation of either the 170 kDa protein phosphorylation (Fig. 2, A–C) or the rate of EGF receptor autophosphorylation (Fig. 2D) by tyrphostins were similar, i.e. the maximum inhibition attained was ~70–80% at concentrations of 100 and 20 μM of compound 11 and 12, respectively (cf. Fig. 2, C and D). In addition, consistent with the findings of Yaish *et al.* [13] in A431 cells, compound 12 was approximately five times more potent than compound 11 in inhibiting the EGFRK reactivity of cardiac membranes. Moreover, the concentrations at which cardiac membrane EGFRK was inhibited by compounds 11 and 12 were similar to those reported by Yaish *et al.* [13].

To address the possibility that the EGFRK is important in the stimulation of cardiac adenylyl cyclase by EGF, experiments were performed to determine the effects of compounds 11 and 12 on basal and Gpp(NH)p-stimulated adenylyl cyclase activity. The data in Table 1 show that, at concentrations which inhibited by ~80% the EGFRK activity (Fig. 2), neither compound 11 nor compound 12 altered basal or Gpp(NH)p-stimulated adenylyl cyclase activity. These data, therefore, indicate that the EGFRK inhibitors do not alter the activity of adenylyl cyclase in a non-selective manner. Likewise, at all concentrations tested, the EGFRK inhibitors, compounds 11 and 12, did not alter the ability of the β-adrenergic receptor agonist, isoproterenol to stimulate cardiac adenylyl cyclase activity (Figs. 3 and 4). However, the ability of EGF to stimulate adenylyl cyclase activity was attenuated by compound 11 (Fig. 3) and compound 12 (Fig. 4) in a concentration-dependent manner. The inability of EGFRK inhibitors to alter significantly isoproterenol-stimulated adenylyl cyclase activity demonstrates that compounds 11 and 12 do not, non-selectively, interfere with β-adrenergic receptor-G_s and G_s-effector (adenylyl cyclase) interactions. Hence, it would appear that compounds 11 and 12 abolish the ability of EGF to stimulate the cardiac adenylyl

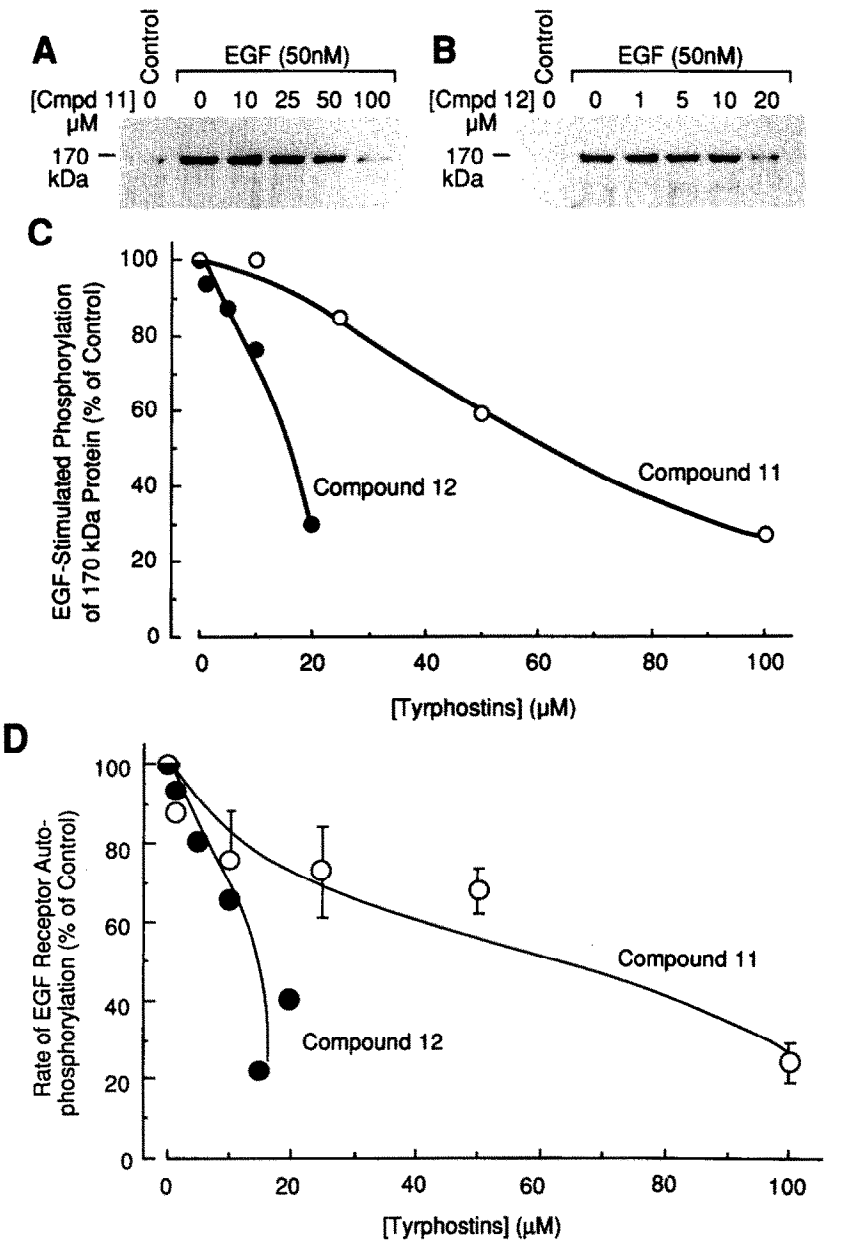


Fig. 2. Effect of various concentrations of tyrphostins on EGF-stimulated phosphorylation of (i) the 170 kDa protein (panels A–C) and (ii) immunoprecipitated EGF receptor (panel D) from cardiac membranes. (A and B): Cardiac membranes (150 μg protein) were incubated in the absence or presence of EGF (50 nM) with increasing concentrations of compound 11 (0–100 μM; panel A) or compound 12 (0–20 μM; panel B). Following incubation and after separating proteins on 7.5% polyacrylamide gels by SDS–PAGE, the proteins were electrophoretically transferred onto nitrocellulose. The blots were developed with the monoclonal anti-phosphotyrosine antibody as described in Materials and Methods. (C): Quantitation by densitometric scanning of EGF-stimulated tyrosine phosphorylation of the 170 kDa protein presented in panels A and B. Data represented as percent of control EGF-stimulated phosphorylation in the absence of the inhibitors; from the densitometric scans, EGF-elicited increase in tyrosine phosphorylation was assigned the value of 100%. Data in panels A–C are from one of three similar experiments. (D): Cardiac membranes (90 μg protein) were incubated at room temperature in the presence or absence of EGF and the indicated concentrations of tyrphostins for a period that was within the linear region of EGF-elicited autophosphorylation of its receptor (15 sec, see Materials and Methods). The membranes were lysed and the EGF receptor was immunoprecipitated employing the monoclonal EGF-R1 antibody. Proteins in the immunoprecipitate were separated and detected with anti-phosphotyrosine antibody. Autophosphorylation of the EGF receptor, which migrated as a 170 kDa protein, was quantitated by densitometric scanning of the entire band, and rates of phosphorylation were calculated by dividing the densities of bands by 15 sec. Following densitometric scanning, data are presented as percent of EGF-mediated augmentation of autophosphorylation of the EGF receptor under control conditions (assigned as 100%) and are the means ± SEM of three experiments.

Table 1. Effects of compounds 11 and 12 on basal and Gpp(NH)p-stimulated adenylyl cyclase activity

Condition	Adenylyl cyclase activity (pmol/min/mg protein)	
	−Gpp(NH)p	+Gpp(NH)p
Control	12.0 ± 1.3	17.5 ± 0.8
+ Cmpd 11 (100 μM)	10.2 ± 1.6	17.3 ± 2.2
+ Cmpd 12 (20 μM)	12.1 ± 0.8	21.9 ± 1.2

Adenylyl cyclase activity was measured in the absence and presence of 10 μM Gpp(NH)p, and the indicated concentrations of compounds 11 and 12 were added. Details of the adenylyl cyclase assay are provided in Materials and Methods. The means ± SEM of three separate experiments are presented.

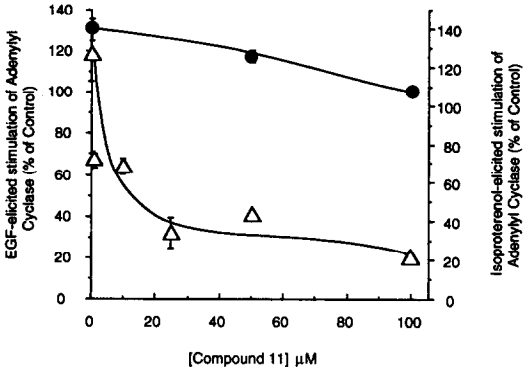


Fig. 3. Effect of various concentrations of the EGFRK inhibitor compound 11 on the abilities of isoproterenol and EGF to stimulate cardiac adenylyl cyclase activity. Cardiac membranes (50 μg protein) were assayed for adenylyl cyclase activity in the presence of 100 nM isoproterenol (●) and 10 nM EGF (△) with various concentrations (10–100 μM) of compound 11 for 30 min. All assays were performed in a total volume of 250 μL and contained 10 μM Gpp(NH)p. Data are the means ± SEM (N = 4 experiments) of percent agonist (EGF/isoproterenol) mediated stimulation over corresponding controls performed in the presence of the various indicated concentrations of inhibitor. Unstimulated control adenylyl cyclase activity, in the presence of Gpp(NH)p (10 μM), which remained unaffected by compound 11 was 16.9 ± 1.1 pmol/min mg protein. Wherever not seen, error bars are within the symbol size.

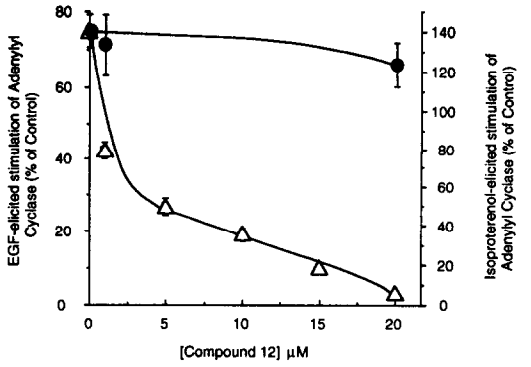


Fig. 4. Effect of various concentrations of the EGFRK inhibitor, compound 12 on the ability of 100 nM isoproterenol (●) and 10 mM EGF, (△) to stimulate cardiac adenylyl cyclase activity. The experimental protocol and conditions were the same as those described in the legend to Fig. 3, except that compound 11 was replaced with various concentrations (1–20 μM) of compound 12. Data are the means ± SEM (N = 4 experiments) of percent agonist (EGF/isoproterenol) mediated stimulation over corresponding controls performed in the presence of the various indicated concentrations of inhibitor. Unstimulated control adenylyl cyclase activity, in the presence of Gpp(NH)p (10 μM), which remained unaffected by compound 12 was 19.6 ± 0.6 pmol/min mg protein. Wherever not seen, error bars are within the symbol size.

cyclase by selectively inhibiting the activity of the cardiac EGFRK.

The role of EGFRK activity in EGF-elicited stimulation of cardiac adenylyl cyclase was evaluated further by employing PTPase purified from human placenta [14]. The rationale in these experiments was that by increasing the PTPase activity, the effective activity of the EGFRK would be diminished and, therefore, if the EGFRK was important in mediating the actions of EGF on cardiac adenylyl cyclase, then the phosphotyrosine phosphatase would attenuate the ability of EGF to simulate adenylyl cyclase. As demonstrated by the data in Table 2, phosphotyrosine phosphatase did not alter either the control (Gpp(NH)p-stimulated) or isoproterenol-stimulated adenylyl cyclase activity. However, phosphotyrosine phosphatase addition to the adenylyl cyclase system, in a concentration-dependent manner, attenuated the ability of EGF to stimulate adenylyl cyclase such that in the presence of 5.7 nmol/min and 22.8 nmol/min of phosphotyrosine phosphatase, the effects of EGF on adenylyl cyclase were inhibited by 33% and 100%, respectively. The data in Table 2 along with the experiments depicted in Figs. 2–4 indicate that attenuation of the EGFRK diminishes the ability of EGF to stimulate adenylyl cyclase.

The role of the EGFRK in EGF-elicited stimulation of cardiac adenylyl cyclase was also investigated with another structurally different protein tyrosine kinase inhibitor, genistein [23]. However, concentrations (400 μM) of genistein which inhibited the cardiac EGFRK activity also attenuated by 45% the ability of isoproterenol to stimulate cardiac adenylyl cyclase (data not shown). This effect of genistein on isoproterenol-stimulated cyclase activity demonstrated that unlike compounds 11 and 12, genistein is non-selective in its actions on cardiac adenylyl cyclase. The non-selective effects of genistein may be related to the mechanism by which it inhibits protein tyrosine kinases. In contrast to compounds 11 and 12 which inhibit the EGFRK by competing for the substrate protein(s) [13], genistein inhibits EGFRK by competing for ATP [23]. Thus, it is possible that genistein also inhibits adenylyl cyclase by competing for ATP. Although genistein inhibited by 65% the ability of EGF to stimulate cardiac

Table 2. Attenuation by protein phosphotyrosine phosphatase of the ability of EGF, but not isoproterenol, to stimulate cardiac adenylyl cyclase

Condition	Adenylyl cyclase activity (pmol/min/mg protein)		
	No PTPase	+ PTPase (5.7 nmol/min)	+ PTPase (22.8 nmol/min)
Control	34.3 ± 4.4	34.6 ± 2.5	39.7 ± 2.8
+ EGF (10 nm)	54.9 ± 2.4 (60.0%)	48.3 ± 4.4 (39.6%)	33.8 ± 3.9 (0.0%)
+ Iso (100 nM)	85.6 ± 4.1 (149.6%)	83.1 ± 3.1 (140.0%)	91.7 ± 1.1 (131.9%)

Adenylyl cyclase assays were performed in the presence and absence of the indicated activities of the purified protein phosphotyrosine phosphatase (PTPase) from human placenta. Values are the means ± SEM of three separate experiments. Numbers in parentheses represent percent stimulation over the corresponding control values.

adenylyl cyclase, the non-selective effects of genistein on isoproterenol-stimulated adenylyl cyclase activity detracted from the further use of this compound to manipulate the activity of the cardiac EGFRK.

One possible explanation for the selective inhibition of EGF-stimulated adenylyl cyclase activity by compounds 11 and 12 could be that the inhibitors alter the ability of EGF to bind to its receptors. In this context, it should be noted that Yaish *et al.* [13] have reported previously that compounds 11 and 12 do not interfere with the binding of EGF to its receptors. Nonetheless, we investigated the effects of maximally effective concentrations of compounds 11 and 12 to alter specific binding of [^{125}I]EGF to cardiac membranes. For this purpose specific binding of K_d equivalent concentration of EGF (50 pM; [24, 25]) to membranes was examined because any modulation of EGF binding to its receptors would be most prominent at this concentration of the ligand. Despite the performance of these experiments under the most optimal conditions (30°) to observe alterations in [^{125}I]EGF binding, the data (not shown) demonstrated that neither compound 11 nor compound 12 altered the specific binding of [^{125}I]EGF to cardiac membranes. Therefore, we conclude that compounds 11 and 12 do not modulate EGFRK activity or EGF-mediated stimulation of adenylyl cyclase by altering the binding of EGF to its receptor.

It is noteworthy, that to ensure that the tyrphostins were indeed inhibiting the EGFRK activity, the kinase activity was monitored both at 0° as well as at room temperature, the latter representing the temperature at which adenylyl cyclase was studied. Thus, from the data presented above, it may be concluded that, as described previously in A431 cells [13], compounds 11 and 12 are potent inhibitors of the cardiac EGFRK. Additionally, unlike genistein, the tyrphostins do not non-selectively alter the ability of agonists whose receptors do not possess protein tyrosine kinase activity (e.g. the β-adrenergic receptors) to stimulate adenylyl cyclase. However, compounds 11 and 12 selectively attenuated the ability of EGF to stimulate cardiac adenylyl cyclase activity and, as evident from experiments involving binding of [^{125}I]EGF to cardiac membranes, this inhibition is not the result of alterations in the interactions between the EGF receptor and its ligand. Similarly, the protein phosphotyrosine phosphatase purified from human placenta also attenuated the ability of EGF, but not Gpp(NH)p or isoproterenol, to simulate adenylyl cyclase. Hence, we conclude that the EGFRK activity is important in EGF-elicited stimulation of cardiac adenylyl cyclase. We have also demonstrated that $G_{s\alpha}$ mediates the actions of EGF on cardiac adenylyl cyclase [12]. This coupled with our findings described here would suggest an intimate interaction between the receptor protein tyrosine kinase activity and activation of G_s. In view of the growing body of experimental evidence which demonstrates that low molecular weight G proteins and G protein components are phosphorylated by EGF and insulin receptor tyrosine kinases and protein kinase C [26–30], and that phosphorylation of the α-subunit of G_i by protein kinase C decreases the functional activity of G_i [30], it is tempting to speculate that the EGFRK either directly or via some phosphorylation cascade phosphorylates a component(s) of G_s and modulates its activity. In this respect, it is noteworthy that in a recent report [31], in *in vitro* experiments, pp60^{src} has been demonstrated to phosphorylate on tyrosine residues the α subunits of G_s and other G proteins. Although the kinase activities of pp60^{src} and EGF receptor are related, because of the non-selective phosphorylation of various α subunits of G proteins by pp60^{src} [31], the precise sequence of events that lead to G_s activation following stimulation of the activity remains to be elucidated and forms the focus of our future studies.

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