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Epidermal growth factor stimulates rat cardiac adenylate cyclase through a GTP-binding regulatory protein

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In isolated perfused rat hearts, epidermal growth factor (EGF; 15 nM) increased cellular cyclic AMP (cAMP) content by 9.5-fold. In rat cardiac membranes, EGF also stimulated adenylate cyclase activity in a dose-dependent manner, with maximal stimulation (35% above control) being observed at 10 nM-EGF. Half-maximal stimulation of adenylate cyclase was observed at 40 pM-EGF. Although the β-adrenergic-receptor antagonist propranolol markedly attenuated the isoprenaline-mediated increase in cAMP content of perfused hearts and stimulation of adenylate cyclase activity, it did not alter the ability of EGF to elevate tissue cAMP content and stimulate adenylate cyclase. The involvement of a guanine-nucleotide-binding protein (G-protein) in the activation of adenylate cyclase by EGF was indicated by the following evidence. First, the EGF-mediated stimulation of adenylate cyclase required the presence of the non-hydrolysable GTP analogue, guanyl-5′-yl-imododiphosphate (p[NH]ppG). Maximal stimulation was observed in the presence of 10 μM-p[NH]ppG. Secondly, in the presence of 10 μM-p[NH]ppG, the stable GDP analogue guanosine 5′-[β-thio]diphosphate at a concentration of 10 μM blocked the stimulation of the adenylate cyclase by 1 nM- and 10 nM-EGF. Third, $NaF + AlCl_3$-stimulated adenylate cyclase activity was not altered by EGF. The ability of EGF to stimulate adenylate cyclase was not affected by pertussis-toxin treatment of cardiac membranes. However, in cholera-toxin-treated cardiac membranes, when the adenylate cyclase activity was stimulated by 2-fold, EGF was ineffective. Finally, PMA by itself did not alter the activity of cardiac adenylate cyclase, but abolished the EGF-mediated stimulation of this enzyme activity. The experimental evidence in the present paper demonstrates, for the first time, that EGF stimulates adenylate cyclase in rat cardiac membranes through a stimulatory GTP-binding regulatory protein, and this effect is manifested in elevated cellular cAMP levels in perfused hearts exposed to EGF.

INTRODUCTION

Apart from the well-established effects of EGF on cellular hypertrophy and hyperplasia (Bucher *et al.*, 1978), several studies have demonstrated that EGF regulates metabolic processes in the cell by altering the activities of several enzymes (e.g. phosphorylase), metabolite (e.g. amino-acid)-transport processes, and ion fluxes across the plasma membrane (Carpenter, 1979; Bosch *et al.*, 1986; Moule & McGivan, 1987). These early events after cellular activation by EGF appear to be mediated by either activation or modulation of different second-messenger systems by EGF. Hence it is well established that, after binding of EGF to its receptor, the intrinsic tyrosine kinase activity of the receptor is stimulated (Ushiro & Cohen, 1980). This activation of the EGF-receptor tyrosine kinase activity results in the phosphorylation of a number of cellular and plasma-membrane-bound proteins (Carpenter *et al.*, 1979; also see Hunter & Cooper, 1985, for review). Additionally, in rat hepatocytes and A431 cells, EGF has been demonstrated to increase cytosolic free Ca^{2+} after generation of the second messenger, inositol 1,4,5-trisphosphate ($InsP_3$) (Johnson & Garrison, 1987; Tilly *et al.*, 1988). The increase in PtdIns(4,5)P_2 hydrolysis, which results in elevated cellular $InsP_3$ levels and consequent increase in cytosolic free Ca^{2+}, has been reported to result from a direct activation of phospholipase C after binding of EGF to the receptor. In rat hepatocytes, studies by Johnson & Garrison (1987) suggest that EGF-elicited activation of phospholipase C is mediated by a pertussis-toxin-sensitive GTP-binding protein (G-protein). However, in A431 epidermoid-carcinoma cells, EGF-mediated activation of phospholipase C is not affected by pertussis-toxin treatment of cells (Tilly *et al.*, 1988; Pike & Eakes, 1987), and recent studies (Wahl *et al.*, 1988, 1989; Nishibe *et al.*, 1989) suggest that EGF-elicited activation of phospholipase C in A431 epidermoid-carcinoma cells involves phosphorylation on tyrosine residues of phospholipase C. Although these latter findings indicate that in A431 cells the EGF receptor is not coupled to phospholipase C via G-protein(s), it is noteworthy that in A431 cells EGF has been demonstrated to stimulate a phosphatidylinositol kinase activity which increases cellular phosphatidylinositol 4-monophosphate content via a cholera-toxin-sensitive, but cyclic-AMP-independent, process (Pike & Eakes, 1987). Hence the studies described above would indicate

Abbreviations used: G-protein, GTP-binding regulatory protein; G_s, stimulatory G-protein of adenylate cyclase; G_i, inhibitory G-protein of adenylate cyclase; $G_i\alpha$, α subunit of G_i; IBMX, 3-isobutyl-1-methylxanthine; GDP[S], guanosine 5′-[β-thio]diphosphate; p[NH]ppG, guanyl-5′-yl imidodiphosphate; GTP[S], guanosine 5′-[γ-thio]triphosphate; EGF, epidermal growth factor; cAMP, cyclic AMP; PMA, 4β-phorbol 12-myristate 13-acetate; 4α-PDD, 4α-phorbol 12,13-didecanoate.

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that EGF-elicited activation of phospholipase C in some (e.g. hepatocytes), but not all (e.g. A431 cells), tissues is mediated by a G-protein. Similarly, in the same cell type (e.g. A431 cells), certain (e.g. phosphatidylinositol kinase stimulation), but not all (e.g. phospholipase C activation), biological effects of EGF may be mediated via activation of G-proteins.

With regard to the cAMP second-messenger system, studies by Bosch *et al.* (1986) have demonstrated that in rat hepatocytes EGF decreases the glucagon-elicited increase in cellular cAMP, and therefore attenuates activation of metabolic processes in response to glucagon (e.g. phosphorylase activation). It is noteworthy that EGF by itself does not alter the basal cAMP level in hepatocytes. Similarly, EGF has been reported to decrease prostaglandin E_1-stimulated cAMP content of fibroblastic cells without affecting the basal unstimulated cAMP content (Anderson *et al.*, 1979). Whether the effects of EGF in these two cell systems are the result of inhibition of adenylate cyclase or activation of cAMP phosphodiesterase activities remains to be elucidated.

In chicken heart mesenchymal cells, EGF has been demonstrated to stimulate cellular proliferation (Balk *et al.*, 1985). Additionally, EGF has also been demonstrated to produce chronotropic effects in chick-embryonic ventricular cells in culture (Rabkin *et al.*, 1987). Although EGF has been demonstrated to produce these effects in cardiac tissue, the second-messenger system(s) and mechanism(s) involved in EGF-mediated responses in the heart remain unknown. Since agonists such as isoprenaline (isoproterenol) elevate cellular cAMP content and produce inotropic and chronotropic effects in the heart, studies in the present paper were designed to address the hypothesis that EGF stimulates cardiac adenylate cyclase and increases the accumulation of cAMP in the heart.

MATERIALS AND METHODS

Male rats of the Sprague–Dawley strain (200–240 g body wt.) were used in these studies. Animals were allowed free access to food and water. Heart perfusions were carried out essentially by the technique described by Patel & Olson (1984). The hearts were excised from animals anaesthetized with pentobarbital sodium, and retrograde Langendorff perfusion was established after aortic cannulation. The hearts were perfused at 37 °C, with Krebs–Henseleit bicarbonate buffer, pH 7.4, modified to contain 1.3 mM-$CaCl_2$ and 5.5 mM-glucose. Perfusion was maintained at a constant flow rate of 10 ml/min. After a 20 min equilibration period, hearts were perfused for a 10 min control period with medium alone, or for the same control period followed by an additional 10 min of EGF or isoprenaline. In experiments where the effects of propranolol were investigated, an identical protocol was employed, except that the β-adrenergic antagonist was present in the perfusion medium throughout the experimental period. EGF, isoprenaline and propranolol were infused at a point proximal to the aortic cannula by means of syringe pumps. At appropriate times in the perfusion period, the hearts were freeze-clamped with a pair of aluminium tongs pre-chilled in liquid N_2. The frozen hearts were pulverized and stored at −70 °C.

Extraction of cAMP from the frozen heart samples was carried out as described previously for liver tissue (Rashed & Patel, 1987). Radioimmunoassay for determination of cAMP in the extracts was performed by the method of Brooker *et al.* (1979).

Freshly excised rat hearts, homogenized in isolation medium containing 5 mM-Tris/HCl, pH 7.4, 250 mM-sucrose and 1 mM-EGTA were employed for isolation of cardiac membranes by the method of Bristow *et al.* (1982). Protein content of these membranes was determined by the method of Bradford (1976), with bovine serum albumin as standard. Portions (2 mg) of the membranes were stored at −70 °C until further use.

Adenylate cyclase was assayed by the method of Salomon *et al.* (1974). Membrane protein (20–30 μg) was added to a reaction mixture consisting of 50 mM-Tris/HCl (pH 7.4), 5.0 mM-$MgCl_2$, 12.0 mM-phosphocreatine, 1 mg of creatine kinase/ml, 1 mM-IBMX and 0.1 mM-[α-^{32}P]ATP (200 d.p.m./pmol). EGF, p[NH]ppG, GDP[S], isoprenaline, propranolol, NaF and $AlCl_3$ were added to the assay at the desired concentration. ^{3}H-labelled cAMP (15000–18000 d.p.m./assay) was also added to the assay mixture for determination of recovery of [^{32}P]cAMP after column chromatography. The final reaction volume was 250 μl, and activities were determined over a 30 min period. The reactions were terminated by transferring 100 μl of incubation mixture to tubes containing 100 μl of 2% SDS, 1 mM-ATP, 1.4 mM-cAMP and 50 mM-Tris/HCl, pH 7.4, and freezing the mixture in solid CO_2. [^{32}P]cAMP was then separated by the method of Salomon *et al.* (1974). Recovery of cAMP ranged from 70 to 80%. All assays were performed in quadruplicate, and activity was linear with respect to membrane protein concentration and time.

Cholera toxin treatment of cardiac membranes was carried out by the method of Lotersztajn *et al.* (1987). Before use, cholera toxin was activated by incubation with 20 mM-dithiothreitol for 30 min at 37 °C. Cardiac membranes (1.5–2.0 mg of protein) were incubated with 300 μg of cholera toxin/ml or vehicle in a final volume of 1.2 ml containing 50 mM-Tris/HCl, pH 7.4, 1 mM-ATP, 3 mM-dithiothreitol, 0.1 mM-GTP and 10 μM-NAD^+ for 30 min at 30 °C. Membranes were then washed and separated from the incubation medium by 10-fold dilution in ice-cold 50 mM-Tris/HCl (pH 7.4) and centrifugation. The final washed membrane pellets were resuspended in medium containing 250 mM-sucrose, 1 mM-EGTA and 5 mM-Tris/HCl, pH 7.4.

ADP-ribosylation of cardiac membrane proteins by pertussis toxin was also performed by the method of Lotersztajn *et al.* (1987). Membranes (1.5–2.0 mg of protein) were incubated with 50 μg of pertussis toxin/ml. The toxin was activated by incubation with 20 mM-dithiothreitol for 30 min at 37 °C. For adenylate cyclase activity measurements, membranes which had been ADP-ribosylated with pertussis toxin were washed and resuspended by procedures identical with those described above for cholera-toxin treatment. To ensure that pertussis-toxin-catalysed ADP-ribosylation of G_i was complete, an approach similar to that described by Pobiner *et al.* (1985) was employed. Essentially, the incorporation of ^{32}P label from [α-^{32}P]NAD^+ (1 μM; 30 μCi/nmol) into the 40 kDa protein (presumably $G_i\alpha$) was assessed in membranes which had been ADP-ribosylated with pertussis toxin and unlabelled NAD^+ (1 μM) in a preliminary incubation. Control membranes were similarly treated, except that pertussis toxin and NAD^+ (1 μM) were present only in the secondary incubation. In these experiments,

Table 1. Effect of EGF (15 nM), isoprenaline (10 nM) and propranolol (10 nM) on cAMP accumulation in perfused rat hearts

Isolated rat hearts were perfused with Krebs–Henseleit buffer containing 5.5 mM-glucose, as described in the Materials and methods section. After a 20 min equilibration period, hearts were perfused for a further 10 min under control conditions or for the same control period followed by an additional 10 min in the presence of agonists. When the effects of propranolol were studied, the β-adrenoreceptor antagonist was present throughout the experimental period. Hearts were freeze-clamped at the end of the respective infusion periods, and frozen cardiac tissue was analysed for cAMP content. Data are presented as means ± S.E.M. from at least seven hearts perfused under each condition.

Perfusion condition	Tissue cAMP content (pmol/g of tissue)
Control	3.50 ± 1.65
EGF (15 nM)	33.25 ± 4.65
Isoprenaline (10 nM)	118.19 ± 33.5
Propranolol (10 nM)	3.02 ± 0.47
EGF (15 nM) + propranolol (10 nM)	37.35 ± 6.20
Isoprenaline (10 nM) + propranolol (10 nM)	10.60 ± 2.22

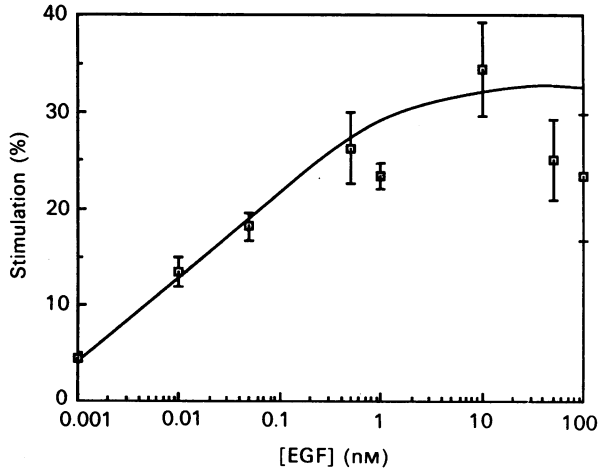

Fig. 1. Effect of different EGF concentrations on the activity of adenylate cyclase in membranes isolated from rat hearts

Adenylate cyclase activity in cardiac membranes was assayed in the presence of p[NH]ppG (10 μM) as described in the Materials and methods section. Assays were performed either in the absence of EGF or in the presence of the various concentrations of EGF. Data are presented as percentage stimulation of adenylate cyclase over controls performed in the absence of EGF. Values represented are means ± S.E.M. of four separate experiments, in which each assay was performed in quadruplicate. In this series of experiments control values in the absence of EGF were 48.6 ± 2.6 pmol/min per mg of protein.

$G_i\alpha$ was identified by pertussis-toxin-specific labelling of the 40 kDa protein. After incubation for 60 min at 30 °C, the reactions were terminated by 10-fold dilution in ice-cold potassium phosphate buffer (50 mM, pH 7.4) and centrifuged. The membrane proteins were dissolved in 250 μl of Laemmli (1970) sample buffer at 100 °C for 5 min. After SDS/polyacrylamide-gel electrophoresis, by the method of Laemmli (1970), ^{32}P-labelled proteins were located by autoradiography on Kodak XAR film.

EGF, p[NH]ppG, GDP[S], cAMP, IBMX, isoprenaline, PMA, 4α-PDD, cholera toxin and phosphocreatine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{3}H]cAMP was obtained from New England Nuclear Corp. [α-^{32}P]ATP was purchased from ICN Radiochemicals, Irvine, CA, U.S.A. Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA, U.S.A.). Rabbit skeletal-muscle creatine kinase was obtained from Boehringer Mannheim. All other chemicals were of the highest purity commercially available.

RESULTS

To investigate the effect of EGF on cellular cAMP content and to compare it with isoprenaline-elicited elevation of tissue cAMP levels, hearts were perfused in the absence and presence of either EGF (15 nM) or isoprenaline (10 nM). The perfused hearts were freeze-clamped just before or 10 min after initiation of agonist infusion. The data in Table 1 demonstrate that infusion of EGF (15 nM) into hearts increased the tissue cAMP content by 9.5-fold over control values. Similarly, isoprenaline (10 nM) stimulated cAMP accumulation by 33.7-fold over control values (Table 1). Although the β-adrenergic antagonist propranolol (10 nM) by itself did not alter the cAMP content of hearts, it inhibited by 91% the action of isoprenaline without attenuating the EGF-mediated increase in cAMP content of hearts (Table 1). Therefore these data indicate that EGF-induced cellular cAMP accumulation in the heart is independent of β-adrenoreceptor activation. Since the EGF-elicited elevation of tissue cAMP content may result from either stimulation of adenylate cyclase activity or inhibition of cAMP phosphodiesterase activity in the heart, experiments were performed to investigate if EGF stimulated cardiac adenylate cyclase. The data in Fig. 1 demonstrate that in isolated cardiac membranes EGF stimulated the adenylate cyclase activity in a dose-dependent manner. Maximal stimulation of adenylate cyclase was observed at 10 nM-EGF, with no further increase being observed at higher concentrations of EGF (Fig. 1). Half-maximal stimulation of adenylate cyclase was observed at approx. 40 pM-EGF. It is noteworthy that, in the experiments depicted in Fig. 1, the GTP analogue p[NH]ppG was present in the adenylate cyclase assay at a concentration of 10 μM. To confirm further that the effects of EGF on cardiac adenylate cyclase were independent of β-adrenoreceptor activation, the experiments depicted in Fig. 2 were performed. Essentially, adenylate cyclase activity in cardiac membranes was measured in the absence and presence of EGF or isoprenaline with and without the addition of propranolol (100 nM) to the incubation. In support of our findings with perfused hearts (Table 1), these experiments demonstrated that propranolol, which by itself did not alter adenylate cyclase activity, almost completely abolished the actions of isoprenaline without altering the ability of EGF to stimulate adenylate cyclase (Fig. 2).

In hormone-receptor-mediated regulation of adenylate

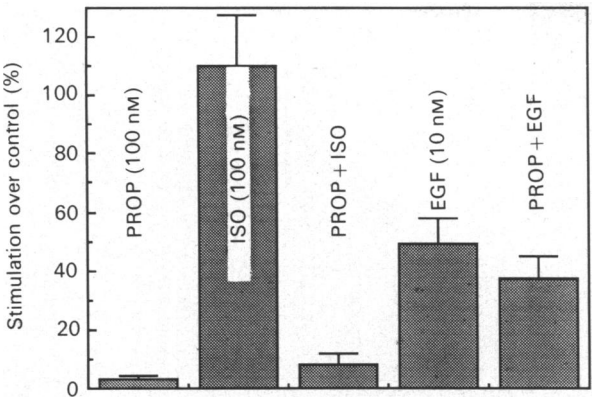

Fig. 2. Effect of propranolol on EGF- and isoprenaline-stimulated adenylate cyclase activity in membranes isolated from rat hearts

Adenylate cyclase activity in cardiac membranes was assayed in the presence of p[NH]ppG (10 μM). Propranolol (PROP) (100 nM) was added to the assay reaction in the absence and presence of EGF (10 nM) or isoprenaline (ISO) (100 nM). Data are presented as means $\pm$ S.E.M. of four separate experiments. In this series of experiments, control values in the absence of EGF, isoprenaline or propranolol were 32.12 $\pm$ 1.07 pmol/min per mg of protein.

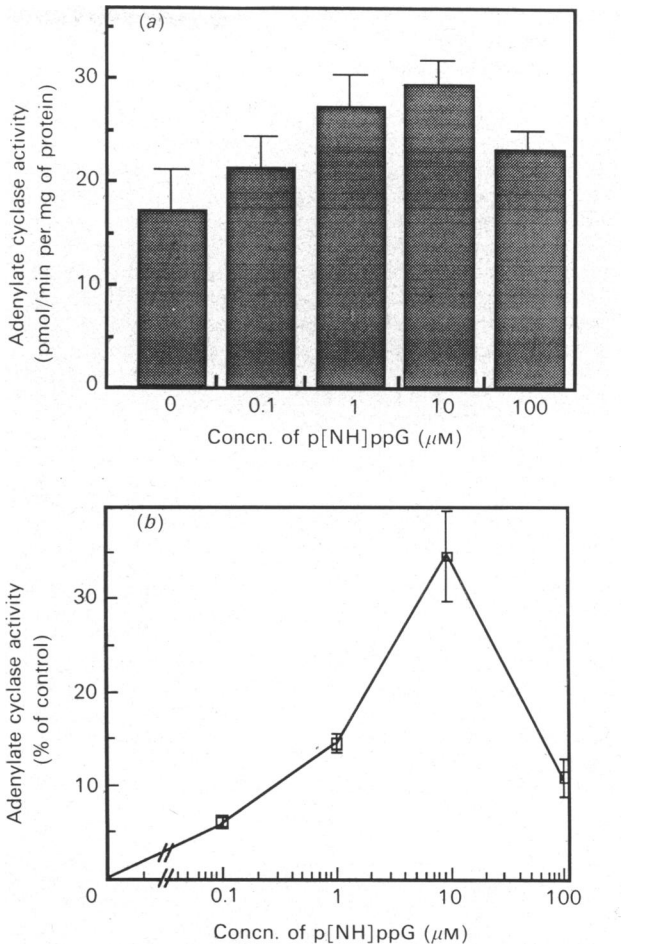

Fig. 3. Effect of different p[NH]ppG concentrations on stimulation of cardiac adenylate cyclase activity in the absence (*a*) and presence (*b*) of EGF (10 nM)

In these experiments, one set of control assay incubations (*a*) were performed with cardiac membranes in the absence of EGF, and the effect of various p[NH]ppG concentrations (0–100 μM) on adenylate cyclase activity was determined. In an identical set of assays performed in parallel to the control set of incubations (*b*), EGF (10 nM) was added. Data in (*a*) are presented as means $\pm$ S.E.M. ($n = 4$). Data in (*b*) are presented as percentage stimulation of adenylate cyclase observed with EGF over the respective controls (i.e. same p[NH]ppG concentration) in the absence of EGF. Values represented are means $\pm$ S.E.M. of four separate experiments in which each condition was assayed in quadruplicate

cyclase, GTP or its non-hydrolysable analogues p[NH]-ppG and GTP[S] have been demonstrated to facilitate signal transduction, presumably by activating G-proteins (Gilman, 1987). To investigate if EGF-elicited stimulation of adenylate cyclase was dependent on the presence of GTP or its analogue p[NH]ppG, the effect of various concentrations of p[NH]ppG on the stimulation of adenylate cyclase in the absence and presence of EGF was studied. As shown in Fig. 3(*a*), p[NH]ppG stimulated the adenylate cyclase activity in a concentration-dependent manner. Maximal stimulation of adenylate cyclase was observed in the presence of 10 μM-p[NH]ppG (Fig. 3*a*). At higher concentrations (e.g. 100 μM), p[NH]-ppG stimulated adenylate cyclase activity to a lesser extent as compared with the stimulation observed in the presence of 10 μM-p[NH]ppG. Such an observation may be related to the differential activation of G_s and G_i elements at different p[NH]ppG concentrations (Cooper *et al.*, 1979). For instance, if G_s was activated at lower concentrations of p[NH]ppG, and G_i activation required higher concentrations of the nucleotide, the concentration–response relationship observed in Fig. 3(*a*) would be expected. In any event, in the absence of p[NH]ppG, EGF at 10 nM did not stimulate adenylate cyclase activity (Fig. 3*b*). However, when p[NH]ppG concentration in the assay mixture was varied over the range 0.1–100 μM, EGF stimulated adenylate cyclase activity over and beyond the increase in activity observed with p[NH]ppG alone (Fig. 3*b*). The optimal concentration of p[NH]ppG required to observe the maximal EGF-elicited stimulation of adenylate cyclase was 10 μM. At higher concentrations of p[NH]ppG (e.g. 100 μM), the ability of EGF to stimulate adenylate cyclase was attenuated (Fig. 3*b*). The decreased stimulation of adenylate cyclase by EGF in the presence of 100 μM-p[NH]ppG as compared with that observed in the presence of 10 μM-p[NH]ppG (Fig. 3*b*) is similar to the attenuation of histamine-stimulated adenylate cyclase at higher concentration of p[NH]ppG reported by Bristow *et al.* (1982). This concentration-dependent requirement for p[NH]ppG to observe the stimulation of adenylate cyclase by EGF suggested that a G-protein mediated the effects of EGF on adenylate cyclase. Therefore, to confirm further the involvement of a G-protein in the stimulation of adenylate cyclase by EGF, the non-hydrolysable GDP analogue GDP[S] was employed to block the activation of G-proteins (Codina *et al.*, 1984). GDP or its non-hydrolysable analogue GDP[S], by stabilizing the α, β and γ subunits of G-proteins into their heterotrimeric conformation, eliminates the participation of G-proteins in signal-transduction mechanisms (Codina *et al.*, 1984). As described

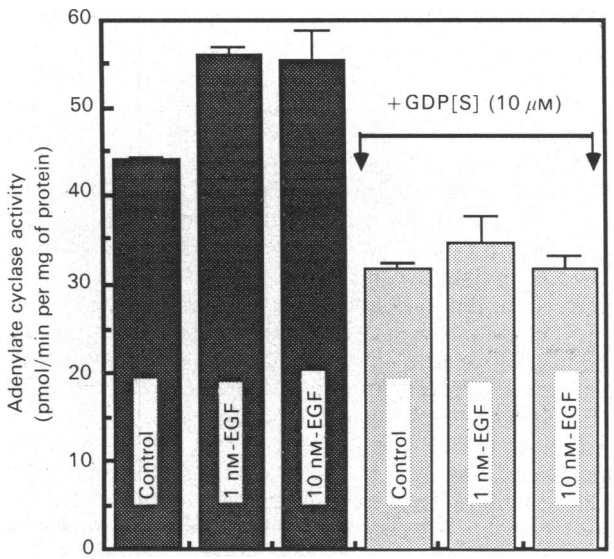

Fig. 4. Effect of GDP[S] on EGF-elicited stimulation of adenylate cyclase activity in isolated cardiac membranes

In a control set of incubations, adenylate cyclase activity was measured in the presence of p[NH]ppG (10 μM) with and without the addition of either 1 nM- or 10 nM-EGF. In parallel assay incubations identical with the controls, GDP[S] (10 μM) was added. Data are presented as means ± S.E.M. of four separate experiments in which each experimental condition was assayed in quadruplicate.

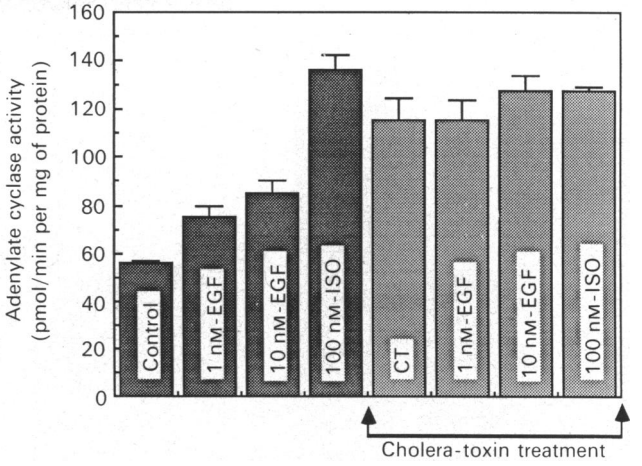

Fig. 6. Comparison of EGF- and isoprenaline-elicited stimulation of adenylate cyclase between control membranes and membranes treated with cholera toxin

ADP-ribosylation of cardiac membrane protein was performed as described in the Materials and methods section. Control membranes were treated in an identical manner, except that cholera toxin was not added. Adenylate cyclase activity was measured in the presence of p[NH]ppG (10 μM). Data are presented as means ± S.E.M. of four separate experiments. All assays were performed in quadruplicate. Abbreviations used: CT, activity in cholera-toxin-treated membranes under control conditions; ISO, isoprenaline.

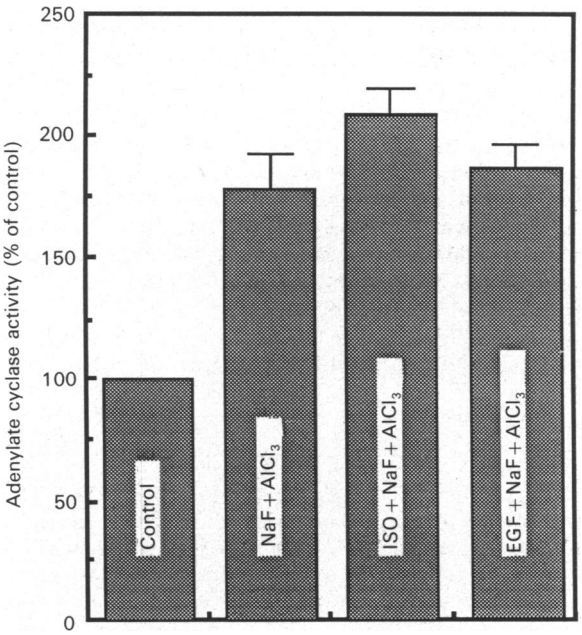

Fig. 5. Effect of NaF plus $AlCl_3$ on the ability of EGF to stimulate cardiac adenylate cyclase activity

NaF (2 mM) and $AlCl_3$ (10 μM) were added to the assay reaction in the absence and presence of EGF (10 nM) or isoprenaline (ISO) (100 nM). Data are presented as percentages of control activity monitored in the absence of NaF + $AlCl_3$, and are means ± S.E.M. of four separate experiments.

above (Fig. 1), in control experiments, in the absence of GDP[S], EGF at concentrations of 1 nM and 10 nM stimulated the cardiac adenylate cyclase activity (Fig. 4). Furthermore, as demonstrated previously for other G-protein-mediated effects (Ullrich & Wollheim, 1988; Vallar *et al.*, 1987), GDP[S] (10 μM) inhibited by 30% the control adenylate cyclase activity measured in the presence of p[NH]ppG (Fig. 4). Additionally, the data presented in Fig. 4 indicate that the stimulation of adenylate cyclase by 1 nM- and 10 nM-EGF was completely blocked by 10 μM-GDP[S]. It is noteworthy that a number of studies dealing with G-proteins have employed 100 μM-GDP[S]. However, as demonstrated by Vallar *et al.* (1987), lower concentrations of GDP[S] (e.g. 10 μM) can effectively block G-protein-mediated responses if the effector system is not stimulated to its maximal capacity (as appears to be the case with EGF and cardiac adenylate cyclase). Furthermore, since preliminary studies indicated that GDP[S] at 10 μM and 100 μM was equally effective at blocking EGF-elicited stimulation of adenylate cyclase, and because commercially available GDP[S] is only 85–90% pure, to minimize interference from contaminants the present studies were performed at the lowest effective concentration of GDP[S] (i.e. 10 μM). The findings from experiments employing GDP[S] also suggest that a G-protein is involved in the stimulatory actions of EGF on rat cardiac adenylate cyclase. The involvement of G-proteins in the EGF-mediated activation of cardiac adenylate cyclase is also borne out by experiments performed with NaF (3 mM) and $AlCl_3$ (10 μM). AlF_3 (derived from the mixture of NaF and $AlCl_3$) has been demonstrated to activate both G_i and G_s regulatory proteins of adenylate cyclase (Harwood & Rodbell, 1973; Bokoch *et al.*, 1984; Katada *et al.*, 1984),

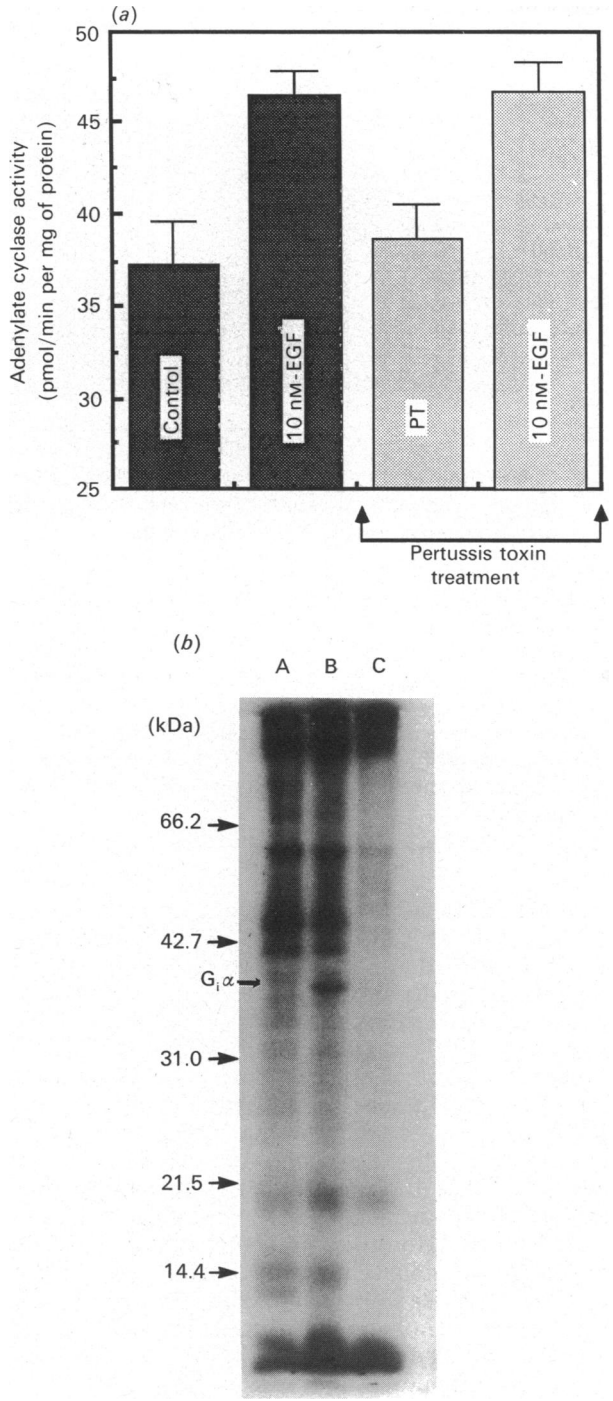

Fig. 7. Effect of pertussis-toxin treatment on EGF-elicited stimulation of cardiac adenylate cyclase activity

(*a*) EGF (10 nM)-mediated stimulation of adenylate cyclase in control membranes and membranes treated with pertussis toxin. ADP-ribosylation of cardiac membranes was performed as described in the Materials and methods section. Control membranes were treated in the same manner, except that pertussis toxin was not added. Adenylate cyclase activity was measured in the presence of p[NH]ppG (10 μM). Data are presented as pmol/min per mg of protein. Values are means ± S.E.M. of four separate experiments. PT represents activity in pertussis-toxin-treated membranes under control conditions. (*b*) Assessment of the extent of pertussis-toxin-catalysed ADP-ribosylation of $G_i\alpha$ in cardiac membranes. Membranes were preincubated with the ADP-ribosylation reaction mixture in the presence and absence of unlabelled NAD^+ and pertussis toxin. In a subsequent incubation the membranes were exposed to [α-^{32}P]NAD^+ either in the presence (lanes B and C) or in the absence (lane A) of pertussis toxin. Lanes: A, membranes incubated without pertussis toxin and NAD^+ in the primary incubation and exposed to [α-^{32}P]NAD^+ in the secondary incubation; B, membranes treated as described for lane A, but exposed to pertussis toxin and [α-^{32}P]NAD^+ in the secondary incubation only; C, membranes exposed to pertussis toxin and unlabelled NAD^+ in the primary incubation and to pertussis toxin and [α-^{32}P]NAD^+ in the secondary incubation. Autoradiograph of the membrane proteins resolved on SDS/polyacrylamide-gel electrophoresis is shown. For details, see the Materials and methods section.

and the effects of such an activation of G-proteins are manifested as a stimulation of the basal enzyme activity. The data in Fig. 5 demonstrate that NaF-stimulated adenylate cyclase activity of cardiac membranes was not further augmented by the addition of either isoprenaline or EGF, again implying the involvement of a G-protein in the signal-transduction mechanism of the EGF-evoked effects. Taken together, the data in Figs. 3–5 strongly suggest that a G-protein mediates the effects of EGF on cardiac adenylate cyclase.

Since stimulation of adenylate cyclase in cardiac membranes in response to EGF may result from either inhibition of G_i activity or stimulation of G_s, additional experiments were performed to elucidate the effects of EGF on this enzyme activity after covalent modification of G_s and G_i by cholera toxin and pertussis toxin, respectively. The data in Fig. 6 demonstrate that, when cardiac membranes were treated with cholera toxin to ADP-ribosylate the stimulatory GTP-binding protein of adenylate cyclase, G_s, and activate the enzyme, EGF at concentrations of 1 nM and 10 nM was ineffective in modulating adenylate cyclase. As shown in Fig. 6, when membranes were treated with cholera toxin, adenylate cyclase activity was not further stimulated by the β-adrenergic agonist isoprenaline. This latter finding demonstrates that, under the conditions employed in our experiments, cholera-toxin-mediated ADP-ribosylation of G_s was complete. On the other hand, ADP-ribosylation of G_i by pertussis-toxin treatment of cardiac membranes did not alter the activity of cardiac adenylate cyclase, suggesting that the G_i activity under basal conditions in these preparations is minimal (Fig. 7*a*). Similar results, i.e. no change of basal activity of adenylate cyclase by pertussis-toxin treatment of membranes despite ADP-ribosylation of G_i, have previously been reported (Anand-Srivastava *et al.*, 1987). Additionally, pertussis-toxin-mediated ADP-ribosylation did not alter the ability of EGF to stimulate adenylate cyclase activity (Fig. 7*a*). In these experiments the absence of the 40 kDa band in lane A (Fig. 7*b*), where pertussis toxin was not added, as compared with lane B, with pertussis toxin, demonstrates that the 40 kDa protein, presumably $G_i\alpha$, was indeed the substrate for ADP-ribosylation by pertussis toxin. To ensure that under our experimental conditions pertussis-toxin-mediated ADP-ribosylation of G_i was complete, the approach described by Pobiner *et al.* (1985) was employed. Essentially, the ability of pertussis toxin to ADP-ribosylate $G_i\alpha$ (40 kDa protein) of membranes

Table 2. Effect of active (PMA) and inactive (4α-PDD) phorbol esters on the ability of EGF to stimulate adenylate cyclase activity in rat cardiac membranes

Adenylate cyclase activity was assayed as described in the Materials and methods section. PMA (100 nM) or 4α-PDD (100 nM) was added to the assay reaction either alone or in the presence of EGF (10 mM). All incubations without PMA or 4α-PDD were performed in the presence of equivalent amount of the solvent (dimethyl sulphoxide) for PMA and 4α-PDD. Data are presented as means ± S.E.M. ($n = 4$).

Assay condition	Adenylate cyclase activity (pmol/min per mg of protein)
Control	34.2 ± 2.7
EGF (10 nM)	48.2 ± 1.3
PMA (100 nM)	37.2 ± 1.3
PMA (100 nM) + EGF (10 nM)	33.0 ± 2.0
4α-PDD (100 nM)	33.1 ± 1.5
4α-PDD (100 nM) + EGF (10nM)	42.5 ± 3.5

previously exposed to the ADP-ribosylation procedure in the presence or absence of pertussis toxin was tested. Fig. 7(*b*) shows that, in contrast with control membranes which had been exposed to pertussis toxin and NAD^+ in the secondary incubation only (lane B), when cardiac membranes were preincubated with the complete ADP-ribosylation reaction mixture (lane C), pertussis toxin did not catalyse incorporation of ^{32}P label in the 40 kDa band in the second incubation period (see the Materials and methods section for details). This observation indicates that ADP-ribosylation of G_i with pertussis toxin in the first incubation was indeed complete. Additionally, in membranes which had been preincubated with unlabelled NAD^+ (lane C, Fig. 7*b*), the incorporation of ^{32}P label from [α-^{32}P]NAD^+ in proteins of molecular mass > 40 kDa during the second incubation was also lower than that observed in membranes which were exposed to NAD^+ only during the second incubation (lanes A and B, Fig. 7*b*). Hence it would appear that the non-specific (i.e. pertussis-toxin-independent) labelling of proteins (see, e.g., lane A, Fig. 7*b*) is the result of endogenous ADP-ribosyltransferase(s) activity, and that in the preincubation with unlabelled NAD^+ these substrate proteins are nearly completely ADP-ribosylated (see lane C, Fig. 7*b*).

Finally, since EGF has been reported to increase the activity of phospholipase C and elevate cellular accumulation of diacylglycerol in several tissues (Johnson & Garrison, 1987; Pike & Eakes, 1987; Wahl *et al.*, 1988), and because activation of protein kinase C by diacylglycerol or tumour-promoting phorbol esters has been demonstrated to activate adenylate cyclase activity in fibroblasts and other cell types (Bell *et al.*, 1985; Magnaldo *et al.*, 1988; Simantov & Sachs, 1982), the possibility that EGF-mediated stimulation of adenylate cyclase is secondary to EGF-elicited activation of protein kinase C was considered. Stimulation of membrane-associated protein kinase C by 4β-phorbol 12-myristate 13-acetate (PMA) (100 nM) did not alter the basal activity of cardiac adenylate cyclase (Table 2). However, in the presence of PMA (100 nM), EGF (10 nM) did not stimulate the adenylate cyclase activity (Table 2). On the other hand, the inactive phorbol ester 4α-phorbol 12,13-didecanoate (4α-PDD) (100 nM) by itself did not alter adenylate cyclase activity, and, unlike PMA, 4α-PDD did not significantly alter the ability of EGF to stimulate adenylate cyclase activity (Table 2). To establish further the role of protein kinase C activation in attenuation of EGF-elicited stimulation of adenylate cyclase activity, experiments with inhibitors of protein kinase C, tamoxifen and sphingosine, were considered. However, tamoxifen and sphingosine by themselves stimulated (18%) and inhibited (12%) cardiac adenylate cyclase activity, respectively. In view of the non-specific effects of protein kinase C inhibitors, therefore, further studies with these compounds were not pursued.

DISCUSSION

The studies presented here demonstrate that EGF stimulates the cardiac adenylate cyclase activity and augments accumulation of cAMP in perfused rat hearts. This finding is different from the observations that, in hepatocytes and in fibroblastic cells, EGF attenuates agonist-elevated cellular cAMP levels without altering the basal cAMP content of cells (Bosch *et al.*, 1986; Anderson *et al.*, 1979). Clearly the mechanism(s) or coupling systems involved in modulation of the cAMP second-messenger system in response to EGF in hepatocytes or fibroblastic cells and in cardiac tissue must be different. EGF stimulated cardiac adenylate cyclase activity in a concentration-dependent manner, and half-maximal stimulation was observed at 40 pM concentration of EGF, a value similar to the K_D values reported for the high-affinity binding sites of EGF in various tissues (Carpenter, 1987; Faucher *et al.*, 1988). Interestingly, the β-adrenoreceptor inhibitor propanolol did not alter the ability of EGF to either increase cAMP content of perfused hearts or stimulate adenylate cyclase activity in cardiac membranes, suggesting that EGF did not mediate its actions by some indirect mechanism, such as increasing the release of catecholamines from nerve terminals. The stimulation of cardiac adenylate cyclase activity by EGF required the presence of p[NH]ppG in the assay mixture, and was maximal at 10 μM-p[NH]ppG (Fig. 3). The absolute requirement for the GTP analogue to observe stimulation of adenylate cyclase in response to EGF, coupled with the ability of the GDP analogue GDP[S] to abolish stimulation of adenylate cyclase by EGF, indicates that EGF effects on adenylate cyclase are mediated through a GTP-binding regulatory protein. This contention is further supported by our observations that, when G-proteins are activated by the addition of NaF plus $AlCl_3$, EGF, like isoprenaline, did not further increase the enzyme activity. From our studies with pertussis toxin, it is apparent that, although pertussis toxin catalysed ADP-ribosylation of the 40 kDa protein, presumably the α-subunit of G_i, the ability of EGF to stimulate adenylate cyclase was not altered. These findings suggest that EGF does not mediate its effects on adenylate cyclase via inhibition of the G_i function. On the other hand, when membranes were treated to ADP-ribosylate the G_s regulatory element and stimulate adenylate cyclase activity, EGF did not further enhance the enzyme activity. In our studies complete ADP-ribosylation of G_s by cholera toxin was indicated by the

inability of isoprenaline to alter enzyme activity. In view of the finding that cholera-toxin treatment of cardiac membranes abolished the effect of EGF on adenylate cyclase, it would be tempting to suggest that the G-protein mediating the actions of EGF in the heart is G_s. However, since cholera toxin, through ADP-ribosylation of G_s, maximally stimulates adenylate cyclase activity, it may be argued that any further activation of adenylate cyclase by EGF would be precluded even if the G-protein mediating EGF action was not G_s. Therefore, at present the conclusions that we can draw are that G_i is not involved in mediating the actions of EGF and that activation of either G_s or G_s-like G-protein by EGF stimulates cardiac adenylate cyclase activity. Additionally, since PMA did not alter the cardiac adenylate cyclase activity, it would appear that EGF-elicited stimulation of this enzyme activity is not mediated via protein kinase C. However, PMA abolished the ability of EGF to stimulate adenylate cyclase. This latter observation would suggest that, as reported previously for a variety of EGF-elicited responses (Macara, 1986; Johnson *et al.*, 1986; Moolenaar *et al.*, 1986), protein kinase C, perhaps via phosphorylation of the EGF receptor and subsequent attenuation of receptor function (Hepler *et al.*, 1988; Wahl & Carpenter, 1988), also modulates the actions of EGF on adenylate cyclase.

Since the chronotropic effects of EGF (Rabkin *et al.*, 1987) and stimulation of adenylate cyclase activity in cardiac tissue are analogous to the effects produced by β-adrenoreceptor agonists in the heart (Hofmann *et al.*, 1987), it is likely that stimulation of cardiac adenylate cyclase and cellular accumulation of cAMP in response to EGF is the mechanism by which EGF stimulates automaticity of cardiac cells in culture (Rabkin *et al.*, 1987). In this respect our preliminary studies demonstrate that infusion of a bolus dose of EGF (1.6 nmol) into the perfusion medium, at a point proximal to the aortic cannula, resulted in decrease of perfusion pressure (-16 mmHg), increase in heart rate ($+35$ beats/min), increase in left-ventricular pressure (3.33-fold) and a 3.75-fold elevation in the rate of left-ventricular pressure development (LV dp/dt) (results not shown). These effects of EGF (1.6 nmol) on mechanical function of the heart were very similar to those observed with the bolus administration of the β-adrenergic-receptor agonist isoprenaline (0.4 nmol) (results not shown). It should be noted that in the heart, apart from elevating cellular cAMP content, EGF has been demonstrated to phosphorylate and inactivate the inhibitory protein of the cAMP-dependent protein kinase (Van Patten *et al.*, 1987). The resultant increase in cAMP-dependent protein kinase activity may reinforce and perhaps also amplify the biological responses elicited by the cAMP which accumulates in response to EGF. Additionally, it is possible that the growth-inducing effects of EGF in mesenchymal cells of the heart (Balk *et al.*, 1985) are also related to the ability of EGF to stimulate cardiac adenylate cyclase and increase cellular cAMP content. Although the role of cAMP as a positive or negative regulator of cell growth has been a controversial issue for some time (Friedman, 1976), several studies have provided considerable evidence for cAMP as a critical positive regulatory element in growth (see Boynton & Whitfield, 1983, for review). It is likely that the effects of cAMP on cell growth are tissue- or cell-type-specific, and that in the heart cAMP is a positive regulator of growth.

Apart from stimulation of adenylate cyclase, the participation of G_s or G_s-like protein in EGF-activated processes in the heart may regulate other biological responses. Hence recent studies from Birnbaumer's laboratory have demonstrated that in the heart G_s activation can stimulate the dihydropyridine-sensitive Ca^{2+} channels (Yatani *et al.*, 1987). In view of this finding, it is conceivable that EGF, via G_s or G_s-like protein, may also alter Ca^{2+} fluxes across the myocardial plasma membrane, and thereby alter cardiac function and metabolism.

The structural motif considered to be common to all G-protein-linked receptors is the presence of seven hydrophobic putative transmembrane domains linked by hydrophilic loops (Gilman, 1987). However, the EGF receptor, like several other growth-factor receptors (Carpenter, 1987), consists of a single transmembrane region which links the extracellular glycosylated ligand-binding domain with the cytosolic protein-tyrosine-kinase-containing domain. Therefore, at present it is not clear how the EGF receptor may be coupled to a G-protein. In this respect, although our findings, as well as those of others (Johnson & Garrison, 1987; Pike & Eakes, 1987), indicate the involvement of G-proteins in mediating the biological effects of EGF, the possibility that these effects are secondary to the activation of the intrinsic tyrosine kinase activity of the EGF receptor, and not due to a direct interaction of the receptor with G-proteins, cannot be ruled out. Indeed, a previous study (Valentine-Braun *et al.*, 1986) has demonstrated that in human placenta the tyrosine kinase activity of the EGF receptor can phosphorylate tyrosine residues on a 35 kDa protein which is thought to be the β-subunit of the GTP-binding regulatory proteins of adenylate cyclase. However, to date phosphorylation of the β-subunit has not been demonstrated to alter the function of either the G_s or G_i regulatory components of adenylate cyclase, and therefore the significance of the findings of Valentine-Braun *et al.* (1986) remains to be evaluated. Clearly, further studies are required to elucidate the nature of the coupling between the EGF receptor and G-proteins.

In conclusion, the evidence presented in this paper demonstrates, for the first time, that in the heart EGF stimulates adenylate cyclase activity via a stimulatory GTP-binding regulatory protein. Whether the G-protein which mediates the actions of EGF is G_s or a G_s-like protein is at present not known. Although activation of adenylate cyclase can certainly account for the observed elevation in tissue cAMP content of hearts exposed to EGF, at present the possibility that EGF also modulates cAMP phosphodiesterase activity cannot be discarded.

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