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EPIDERMAL GROWTH FACTOR ACTIVATION OF RAT PAROTID GLAND ADENYLATE CYCLASE AND MEDIATION BY A GTP-BINDING REGULATORY PROTEIN

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Abstract—Injection of rats with a single dose of epidermal growth factor (EGF) or isoproterenol increased parotid gland acinar cell levels of cyclic AMP (cAMP) significantly above control basal concentrations (34, 177 and 11.5 pmol/g tissue/100 g body weight, respectively). Following a chronic regimen of isoproterenol (3 days), EGF, bovine galactosyltransferase (Gal Tase, EC 2.4.1.22) and isoproterenol increased cAMP levels, albeit to a lower level than observed for the single dose (21, 17 and 51 pmol, respectively). Using isolated parotid gland membranes, EGF and bovine galactosyltransferase also stimulated adenylate cyclase (EC 2.7.4.3) activity in a concentration-dependent manner. Introduction of the β-adrenergic receptor antagonist propranolol blocked isoproterenol-stimulated adenylate cyclase activity and cAMP accumulation, but not that observed with EGF or the transferase treatment. Growth factor-stimulated adenylate cyclase activity required the presence of the guanosine triphosphate (GTP) analogue, guanyl-5′-imidodiphosphate (p[NH]ppG), while cAMP accumulation could additionally be blocked by introducing the GDP analog, guanosine 5′[β-thio] diphosphate (GDP[S]). The ability of EGF to activate adenylate cyclase was not affected by pretreatment of acinar cell membranes with pertussis toxin, whereas pretreatment with cholera toxin eliminated EGF-stimulated cyclase activity. The experimental results presented here expand to the parotid gland our knowledge of the ability of EGF to stimulate the cAMP second messenger signalling pathway via a G-binding regulatory protein, by a mechanism independent of β-adrenergic receptor activation.

Administration of the catecholamine isoproterenol causes a number of morphological and biochemical changes in the parotid gland of rats. Chronic injection leads to gland hypertrophy and hyperplasia [1–3]. Changes in DNA, RNA and protein synthesis are mediated in part by agonist interaction with the β_1-adrenergic receptor on acinar cells and the subsequent accumulation of cyclic AMP (cAMP‖) as a consequence of activation of membrane adenylate cyclase (EC 2.7.4.3) [4–6]. In addition, changes in physiological conditions (such as dietary alterations leading to prolonged mastication) cause an increase in gland size [7, 8]. Growth factors such as nerve growth factor (NGF) and epidermal growth factor (EGF) have also been shown to cause a specific increase in cell division and gland size [9–11]. In all instances of acinar cell proliferation, indicated above, other biochemical alterations take place, e.g. the *de novo* biosynthesis and appearance of cell surface galactosyltransferase (Gal Tase; EC 2.4.1.22). This enzyme, normally a constituent of the Golgi complex, plays an intimate role in regulating acinar cell proliferation when present in the plasma membrane [11–14]. The introduction of the enzyme modifier α-lactalbumin has been shown to block cell proliferation following isoproterenol treatment as well as that induced by growth factors. Under conditions in which the cells become "primed" (e.g. isoproterenol and α-lactalbumin co-injection), proliferation can be re-established by introduction of soluble Gal Tase or lectins specific for terminal *N*-acetylglucosamine [15]. The mechanism by which Gal Tase perpetuates the signal for the transition from stasis to active acinar cell proliferation is not understood at the present time, but it may involve interaction with a cell surface glycoprotein in a fashion similar to a "receptor-ligand" type binding [15].

On the other hand, our understanding of cell growth potentiated by EGF and other growth factors has progressed rapidly. Upon interaction with its specific cell surface receptor, EGF causes the activation of intracellular second messenger signals. The initial coupling of receptor with ligand leads to activation of intrinsic receptor tyrosine kinase activity [16, 17]. The subsequent phosphorylation of substrate proteins activates inositol phospholipid metabolism

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‖ Abbreviations: cAMP, cyclic AMP; PROP, propranolol; Gal Tase, β1,4-galactosyltransferase; ISO, isoproterenol; EGF, epidermal growth factor; TCA, trichloroacetic acid; p[NH]ppG, guanyl-5′-yl-imidodiphosphate; G_s, stimulatory G-protein for adenylate cyclase; G_i, inhibitory G-protein for adenylate cyclase; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; IBMX, 3-isobutyl-1-methylxanthine; GDP[S], guanosine 5′[β-thiol]diphosphate; and GTA kinase, galactosyltransferase associated kinase.

[18] with the production of Ins-1,4,5-P_3 and diacylglycerol: two second messenger signals that upon accumulation trigger Ca^{2+} release from intracellular stores and activation of protein kinase C, respectively [19, 20]. Evidence has been provided more recently for activation of another second messenger signalling pathway. Both A431 epidermoid carcinoma and MDA-468 breast cell carcinoma (which overexpress the EGF-receptor) show EGF-mediated changes in cAMP levels and the involvement of G-binding proteins in the modulation of growth factor signal transduction [21, 22].

Rat cardiac tissue, like the parotid gland, will undergo hypertrophy and hyperplasia in responses to β-agonist treatment. Chronotropic effects of EGF on the rat heart are similar to those observed for β-agonist treatment of the heart [23, 24]. In studies by Nair and colleagues [25, 26], these physiological changes introduced by EGF were mediated by a G-binding regulatory protein interaction with, and subsequent stimulation of adenylase cyclase. In the present paper, we have examined the effects of EGF on rat parotid gland acinar cells and report a similar mechanism of adenylate cyclase activation and cAMP accumulation. This action of EGF appears to be dependent on interaction with a G-binding regulatory protein, most likely the stimulatory subunit of adenylate cyclase (G_s). The accumulation of cAMP by EGF is mechanistically similar to that observed in β-agonist stimulation of rat parotid glands in that both involve activation of adenylate cyclase. However, EGF activation of the enzyme is independent of β-adrenergic receptor interaction. To better understand how cell surface Gal Tase may be involved in the signal transduction mechanism leading to cell proliferation, acinar cells were also examined for the transferase's ability to alter cAMP production following "priming" of the acinar cells by prior isoproterenol treatment. Incubation of isoproterenol-treated parotid gland membranes with bovine Gal Tase of EGF was subsequently observed to cause a β-receptor independent activation of adenylate cyclase.

MATERIALS AND METHODS

Materials. Creatine phosphokinase, creatine phosphate, cholera toxin, pertussis toxin, adenosine triphosphate (ATP), guanosine triphosphate (GTP), guanosine 5′[β-thio]diphosphate (GDP[S]), nicotinamide adenine dinucleotide (NAD^+). guanyl-5′-yl-imidodiphosphate (p[NH]ppG), EGF, bovine Gal Tase, cAMP, 3-isobutyl-1-methylxanthine (IBMX), propranolol and isoproterenol were purchased from the Sigma Chemical Co. [α-^{32}P]ATP (sp. act. 3000 Ci/mmol), [α-^{32}P]NAD^+ (sp. act. 3000 Ci/mmol), and cAMP [^{3}H] Assay kit were obtained from Amersham. Female Sprague–Dawley strain rats, weighing 180–220 g, were purchased from the University of Florida breeding colony or Harlan Sprague–Dawley. Animals were allowed food and water *ad lib*. Reagents for sodium dodecyl sulfate (SDS)–polyacrylamide gels were obtained from Bio-Rad. All other reagents were of ultrapure quality and obtained from commercial sources.

Animal treatment and salivary gland isolation. Separate groups of animals were given the following drug regimen prior to assay of cAMP levels (also see Fig. 1): control animals, 0.5 mL saline injection; a single injection of *d,l*-isoproterenol (5 mg/200 g body weight); a single injection of propranolol (5 mg/200 g body weight); a single injection of isoproterenol and propranolol together; a single 10 μg/mL injection of EGF (0.5 mL injected); a single injection of EGF and propranolol; 3 days of isoproterenol injections; 3 days of isoproterenol injections followed 24 hr later by a single injection of EGF; and 3 days of isoproterenol injections followed 24 hr later by a single injection of 0.5 mg bovine Gal Tase. The animals were anesthetized with pentobarbital and the parotid gland was identified by gross morphology following killing by exsanguination. For assays of cAMP levels, the glands were quickly weighed and homogenized, with a hand-held tissue homogenizer, into 5 mL of 5% trichloroacetic acid (TCA). For assays of adenylate cyclase activity the tissue was homogenized in 10 mM Tris/HCl (pH 8.0) containing 1% aprotinin and 0.5 mM phenylmethylsulfonyl fluoride (PMSF).

Assay for cAMP levels. Following gland homogenization in 5% TCA, insoluble denatured protein was removed by centrifugation at 3500 rpm in a Sorvall RC-3B centrifuge. The aqueous material was subsequently extracted four times with an equal volume of water-saturated ether [27]. The remaining ether was allowed to evaporate at room temperature in a fume hood for 3–4 hr. The level of cAMP was measured using an Amersham assay kit based on the competition between unlabeled sample cAMP and a fixed quantiy of ^{3}H-labeled cAMP for binding to a high affinity binding protein [28]. The concentration of cAMP in the test samples was determined relative to a standard curve generated from the test kit standards. The values for parotid gland cAMP are expressed as picomoles per gram tissue per 100 grams of body weight. Experimental groups of animals were injected with the appropriate reagent or reagent combinations for 20 min prior to killing and removal of the parotid gland. Statistical significance for all experimental results for both cAMP levels and adenylate cyclase activity (see below) were determined by Student's *t*-test.

Total membrane preparation. For the preparation of membrane samples for adenylate cyclase assays, gland cell samples were homogenized in Tris buffer and were further lysed with a Dounce apparatus. Connective tissue and unlysed acinar cells were removed by low speed centrifugation at 1000 rpm in a Sorvall RC-5B centrifuge (SS 34 rotor) for 10 min at 4°. The remaining slurry was diluted into the Tris buffer containing protease inhibitors and centrifuged at 26,000 rpm for 1 hr (SW 28 rotor) at 4°. The supernatant was discarded and the membrane pellet was resuspended in 0.3 to 0.5 mL of 1.0 mM $NaHCO_3$ (pH 7.4) containing 0.7 mM EDTA and 0.5 mM $MgCl_2$. Protein assays were performed by a modification of the Lowry method with bovine serum albumin as the standard [29].

Assay of adenylate cyclase enzyme activity. Adenylate cyclase was assayed by the method of Salomon *et al.* [30]. In brief, a typical reaction

consisted of 75 μg of untreated or 72-hr isoproterenol-treated membrane protein in 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 20 mM creatine phosphate, 100 U/mL creatine phosphokinase, 1 mM IBMX, and 0.1 mM [α-^{32}P]ATP. EGF was added to most reactions at a concentration of 10 nM, while bovine Gal Tase was included at concentrations of 0.04 and 0.2 μM. The activity of [^{32}P]ATP per assay was such that each reaction consisted of 5×10^5 dpm. The total volume of the reaction was 250 μL. The enzyme activity was determined following a 10-min incubation period at 30°. The reactions were terminated by the addition of 100 μL of termination buffer consisting of 2.0% SDS, 1 mM ATP, 1.4 mM cAMP and 50 mM Tris/HCl (pH 7.4). Radiolabeled cAMP formed in the assay was subsequently separated by ion exchange column and $ZnSO_4$–$Ba(OH)_2$ treatment [30, 31]. The sample was passed through a 1.0-mL cation exchange volume consisting of Dowex AG-50W-X4. The column was washed with distilled deionized water and the final eluate collected in a volume of 3.0 mL of water. To this fraction 200 μL of saturated $BaSO_4$ was added and vortexed. The precipitate was collected following a 10-min centrifugation at 4500 rpm in a Sorvall RC-3B centrifuge and the supernatant decanted into scintillation vials containing 12 mL of Aquasol (Fisher Scientific) scintillation fluid. The recovery of cAMP was monitored in selective reactions by the copurification of ^{3}H-labeled cAMP added to the assay tubes following the incubation at 30°. All assays were performed a minimum of four times in duplicate.

Cholera toxin treatment of parotid gland membranes was performed by the method of Lotersztajn *et al.* [32]. Prior activation of the toxin was accomplished by incubation of the toxin with 20 mM dithiothreitol (DTT) for 30 min at 37°. After incubation, 150 μg of cholera toxin or buffer was incubated with 750 μg parotid acinar cell membrane protein in a final volume of 1.0 mL containing 50 mM Tris–HCl (pH 7.5), 1 mM ATP, 3 mM DTT, 0.1 mM GTP and 10 μM NAD^+ for 30 min at 30°. The membranes were then washed with 50 mM Tris/HCl and centrifuged in a microfuge at 15,000 rpm for 10 min. After washing two times, the membranes were resuspended in buffer containing 1.0 mM $NaHCO_3$ (pH 7.4), 0.7 mM EDTA and 0.5 mM $MgCl_2$.

ADP-ribosylation of parotid gland membrane proteins by pertussis toxin was also performed by the method of Lotersztajn *et al.* [32]. Activation of the toxin was accomplished as described above in DTT. All pretreatment of parotid gland membranes with pertussis toxin was performed as described for cholera toxin treatment. Typically, in the ADP-ribosylation reaction, 25 μg/mL pertussis toxin was incubated with 750 μg parotid gland membrane.

Radiolabeling of toxin-treated parotid gland membranes. ADP-ribosylation of membrane proteins was assessed by specific radiolabeling using [α-^{32}P] NAD^+ (2.2 μCi) in the above reactions with pertussis toxin to label the inhibitory subunit of adenylate cyclase ($G_{i\alpha}$). Labeling of proteins was performed as described by Pobiner *et al.* [33]. Following the washing of labeled membranes, 35 μg of protein was dissolved in 40 μL of Laemmli sample buffer [34] and heated to 100° for 5 min. SDS–polyacrylamide gel electrophoresis was performed as described by Pugsley and Schnaitman [34]. After staining and destaining [35], the gels were dried and the ^{32}P-labeled proteins located by autoradiography on Kodax XAR-5 film at −80°. The exposure times varied from 2 to 4 days for the visualization of radiolabeled protein.

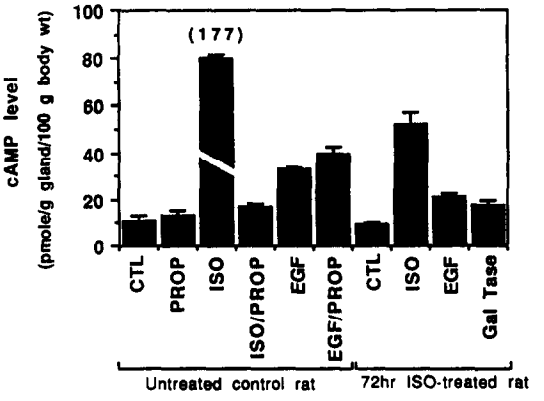

Fig. 1. Effects of EGF, isoproterenol (ISO), propranolol (PROP) and bovine Gal Tase on *in vivo* cAMP levels in rat parotid gland. Untreated or 72-hr chronic isoproterenol-treated rats received intraperitoneal injections of the above compounds. The glands were removed 20 min following the administration of the drug combinations indicated in Materials and Methods. Data are the means ± SEM of three observations, each performed in duplicate. The actual value (177 pmol) for ISO-stimulated cAMP levels in control animals is indicated above the histogram.

RESULTS

Parotid gland cAMP levels. To investigate the effect of EGF on the glandular levels of cAMP, rats were injected with growth factor of the β-adrenergic agonist isoproterenol (which elicits maximal cAMP production from parotid gland acinar cells). Under conditions of a single injection followed by removal of the parotid gland 20 min later, EGF caused an approximately 3-fold increase in cAMP over that observed in the control saline-injected animals ($P < 0.05$, Fig. 1). Isoproterenol alone caused an approximately 16-fold increase over control values ($P < 0.005$). Introduction of the β-receptor antagonist, propranolol, abolished the isoproterenol-mediated increase in cAMP but did not substantially alter EGF-induced changes in cAMP levels (34 pmol vs 40 pmol following propranolol, $P > 0.01$). Propranolol alone did not increase basal levels of cAMP. Cyclic AMP levels were also determined following a 3-day chronic regimen of isoproterenol. Under conditions of active hyperplasia, the basal level of cAMP in the acinar cells was similar to that in untreated control animals (24 hr following the final isoproterenol injection). Injection of isoproterenol or EGF under conditions of chronic β-agonist treatment, while causing a rise in cellular cAMP, did not result in the same level of stimulation. The increases in cyclic nucleotide were 5- and 2-fold

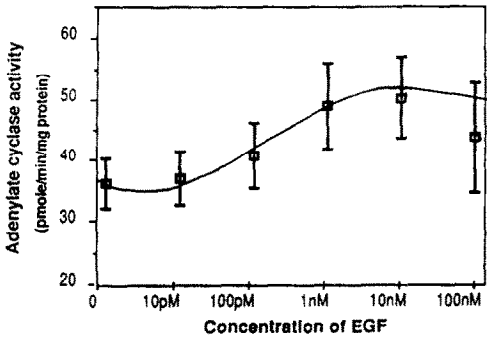

Fig. 2. Adenylate cyclase activity as a function of EGF concentration. Adenylate cyclase activity was measured in parotid gland acinar cell membranes in the presence of increasing concentrations of EGF. The reaction mixtures contained 10 μM p[NH]ppG as described by Nair *et al.* [25]. Each value is the mean ± SEM of four experimental observations. The value for adenylate cyclase activity in the absence of p[NH]ppG and EGF was 13.7 ± 0.4 pmol/min/mg protein.

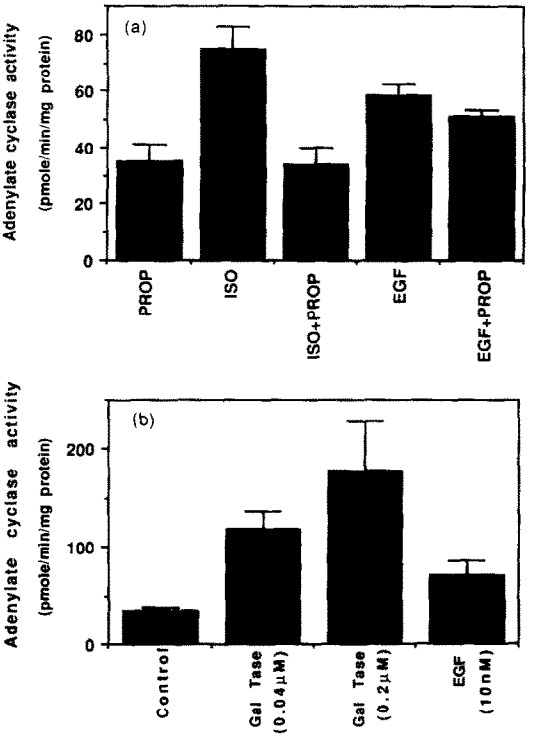

Fig. 3. Adenylate cyclase activity in rat parotid gland membranes stimulated with propranolol (PROP) EGF Gal Tase and isoproterenol (ISO). (a) Parotid gland membranes from untreated rats were incubated with either 100 nM propranolol, 100 nM ISO or 10 nM EGF. Propranolol (100 nM) was also included in combination with ISO or EGF in separate reactions to determine the involvement of β-adrenergic receptors in adenylate cyclase activation. Assay incubations were performed at 30° for 10 min. Values are means ± SEM of four experimental determinations. (b) ISO-treated membranes were incubated with 0.04 or 0.2 μM Gal Tase to determine the ability of the enzyme to directly effect adenylate cyclase stimulation. Stimulation with 10 nM EGF was included as a comparison. Values are means ± SEM of two experimental determinations performed in triplicate.

for isoproterenol and EGF, respectively ($P < 0.05$). The glycosyltransferase Gal Tase was injected into isoproterenol-treated rat in an effort to elucidate the possible mechanism responsible for the transferase-mediated role in acinar cell proliferation. Since cell surface Gal Tase is not normally present in untreated parotid acinar cells and does not demonstrate growth-stimulation under these circumstances [15], isoproterenol-treated animals were used to assess the possible transferase effects on cellular cAMP second messenger levels. The injection of Gal Tase 24 hr following the last injection of isoproterenol into rats resulted in a stimulation of cAMP accumulation to levels comparable to treatment with EGF ($P < 0.05$; Fig. 1). The administration of Gal Tase into untreated animals did not alter cAMP levels (data not shown).

Activation of adenylate cyclase by EGF. To demonstrate the specificity of EGF in the increase in cellular cAMP levels, total membranes were isolated and used in *in vitro* adenylate cyclase assays. As indicated in Fig. 2, EGF demonstrated a concentration-dependent ability to activate adenylate cyclase. Maximal stimulation occurred with 10 nM concentrations of EGF. Half-maximal stimulation was determined to be at approximately 110 pM EGF.

Evidence for a β-adrenergic receptor-independent modulation of cellular cAMP levels was obtained by assaying total membrane functions for adenylate cyclase activity following incubation with EGF, and a β-receptor antagonist. Again, isoproterenol was used as a positive control. As shown in Fig. 3a, isoproterenol treatment resulted in a 2-fold increase in adenylate cyclase activity which was sensitive to inhibition by co-treatment with the antagonist, propranolol ($P < 0.05$). The addition of EGF also increased enzyme activity approximately 1.7-fold. Incubation of membranes with EGF and propranolol did not decrease significantly EGF-stimulated adenylate cyclase ($P < 0.01$, Fig. 3a). When isoproterenol-treated membranes were substituted in the reaction, both Gal Tase and EGF ($P < 0.01$) increased the level of adenylate cyclase activity. As with control membranes, the stimulation was independent of the presence of a β-adrenoceptor antagonist (data not shown, Fig. 3b). As indicated in the figure, the ability of Gal Tase to stimulate adenylate cyclase activity was concentration dependent.

Involvement of G-proteins in the modulation of the EGF response. The incorporation of GTP or a non-hydrolyzable analogue such as p[NH]ppG or GTP[S] has been shown to be necessary for the stimulation of adenylate cyclase activity. These compounds facilitate adenylate cyclase activity and cAMP signal transduction by potentiating the activation of G-binding regulatory proteins [36]. The dependence of EGF-stimulated adenylate cyclase activity on GTP was therefore investigated by the incorporation of the analog p[NH]ppG into *in vitro* membrane assays in the presence (Fig. 4b) or absence

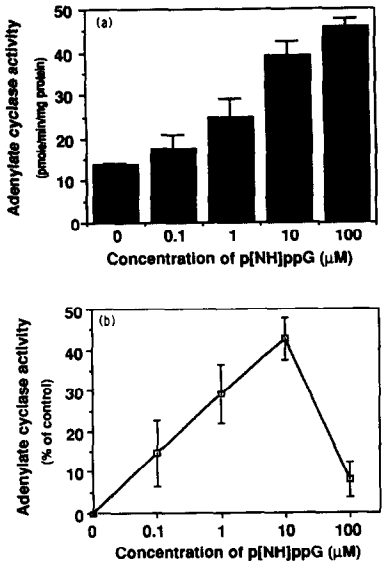

Fig. 4. Effect of various concentrations of p[NH]ppG on adenylate cyclase in the absence (a) and presence (b) of 10 nM EGF. Assay reactions were performed as described in Materials and Methods with concentrations of p[NH]ppG ranging from 0.1 to 100 μM in the absence of EGF (a). Separate parallel experiments were carried out in the presence of 10 nM EGF (b). Data are the means ± SEM of four separate experimental determinations performed in duplicate. Figure 4b represents the data expressed as the percentage stimulation of adenylate cyclase activity with 10 nM EGF in the reaction over the respective control (absence of EGF) values from panel (a). The actual enzyme activities for 0.1 μM p[NH]ppG in the absence and presence of EGF were, respectively, 17 and 20 pmol/min/mg protein.

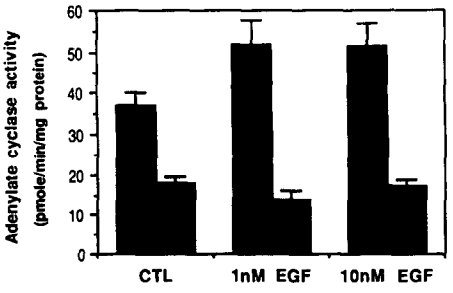

Fig. 5. Influence of GDP[S] on EGF-stimulated adenylate cyclase activity. Enzyme activity of parotid gland membranes was assayed in the presence and absence of EGF in the reaction mixtures. Separate reactions with (hatched bars) and without (solid bars) 10 μM GDP[S] were performed. All reactions contained 10 μM p[NH]ppG and are reported as the means ± SEM of four determinations performed in duplicate.

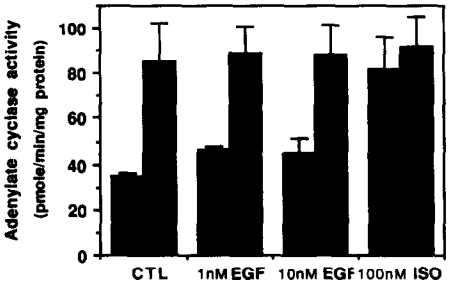

Fig. 6. Comparison of adenylate cyclase activity in control and cholera toxin-treated parotid gland acinar cell membranes. Control and EGF-containing membrane samples were assayed for adenylate cyclase activity following preincubation with cholera toxin (hatched bars) or activation buffer only (solid bars). All assays included 10 μM p[NH]ppG, and are the means ± SEM of four separate experiments performed in duplicate.

(Fig. 4a) of EGF. The ability of this analogue to stimulate adenylate cyclase in membrane preparations was concentration dependent. Maximal stimulation occurred between 10 and 100 μM p[NH]ppG, although the increase between these two concentrations was not substantially different. The inclusion of 10 nM EGF in the reaction (Fig. 4b) showed, however, that at the highest concentration of 100 μM p[NH]ppG, the ability of the growth factor to stimulate adenylate cyclase was reduced.

To further elucidate the requirement for G-proteins in the EGF stimulation of parotid membranes, the non-hydrolyzable GDP analogue GDP[S] was included in assays to block the activation of these regulatory proteins and subsequent participation of adenylate cyclase in second messenger signal transduction [37, 38]. The inclusion of this analogue along with EGF completely eliminated growth factor-stimulated adenylate cyclase from parotid acinar cell membranes. Enzyme activity was reduced at two different concentrations of EGF (1 and 10 nM) to basal levels of enzyme activity found in untreated control membranes (Fig. 5).

Action of cholera and pertussis toxins on EGF-stimulated adenylate cyclase activity. To investigate the mechanism by which G-proteins may regulate EGF-mediated accumulation of adenylate cyclase, modifiers of G_i or G_s activity were included in the *in vitro* enzyme assays. Modification of the regulatory subunits of G_i and G_s by pertussis toxin and cholera toxin, respectively, occurs by ADP-ribosylation. The results presented in Fig. 6 show that when parotid membranes were pretreated with cholera toxin, further stimulation of adenylate cyclase activity was not observed irrespective of the addition of either EGF or isoproterenol. Pretreatment of membranes with pertussis toxin and ADP-ribosylation of the G_i regulatory subunit enhanced the stimulation of parotid membrane adenylate cyclase by either EGF or isoproterenol (Fig. 7). The completeness of ADP-ribosylation of $G_{i\alpha}$ was assessed by autoradiography of ^{32}P label from [α-^{32}P]NAD^+ into proteins of a molecular mass of approximately 40 kDa. As shown in Fig. 8, preincubation of membranes with unlabeled NAD^+ along with pertussis toxin and subsequent re-exposure to toxin and [^{32}P]NAD^+ did not radiolabel the protein at ~40 kDa (lane C) as compared with preincubation of membranes with pertussis toxin

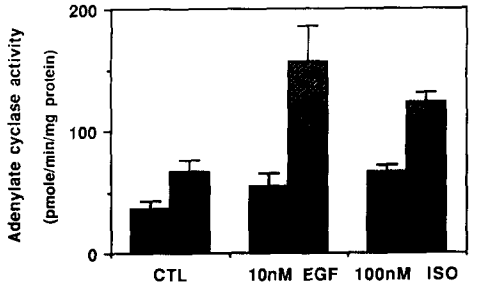

Fig. 7. EGF stimulation of adenylate cyclase in control and pertussis toxin-treated parotid membranes. Membranes were ADP-ribosylated as described in Materials and Methods. EGF (10 nM) or ISO (100 nM) was added to the reactions of untreated (solid bars) or pertussis-treated (hatched bars) membranes and assayed for adenylate cyclase activity. The incubation reactions contained 10 μM p[NH]ppG. Values are the means ± SEM of four separate experiments performed in duplicate.

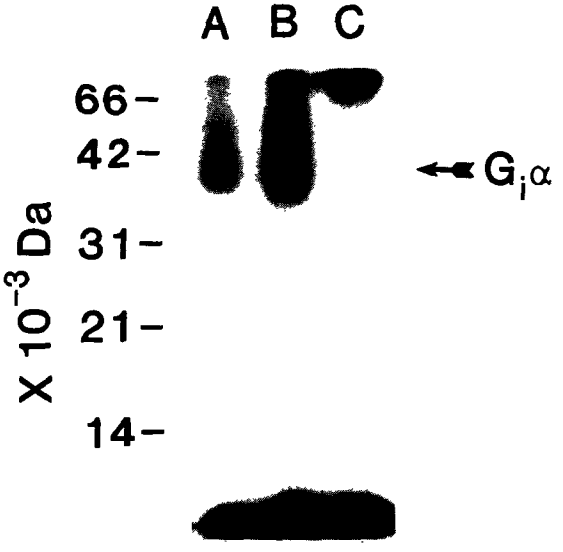

Fig. 8. ADP-ribosylation of $G_{i\alpha}$ in parotid membranes by pertussis toxin. The incubations were performed in two stages. In the primary incubation (ADP-ribosylation), the control membranes were incubated in the absence (lanes A and B) or presence (lane C) of unlabeled NAD^+ and pertussis toxin. Later (in the secondary incubation) the contents of the reactions were re-exposed to [α-^{32}P]NAD^+ in the presence (lanes B and C) or absence (lane A) of pertussis toxin. At the end of the incubation, the contents were washed with 50 mM Tris–HCl (pH 7.4) and the membrane pellet, isolated following centrifugation, was resuspended in SDS–polyacrylamide gel sample buffer. Thirty-five micrograms of protein was electrophoresed per well. Following separation, the gel was fixed and dried prior to exposure to X-ray film at −80°. Molecular weight standards are: bovine serum albumin, 66 kDa; ovalbumin, 42 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor soybean, 21 kDa; and lysozyme, 14 kDa.

and [^{32}P]NAD^+ (lanes A and B). The same experiment performed with cholera toxin again showed the labeling of G_s (~42 kDa) in the presence of [^{32}P]NAD^+ under the experimental conditions described in Materials and Methods (data not shown).

DISCUSSION

The results of experiments presented in this study demonstrate that EGF stimulates rat parotid gland adenylate cyclase and subsequently causes the accumulation of cAMP. While studies of hepatocytes and fibroblasts treated with EGF have not shown increased levels of cAMP in response to treatment with growth factor [39, 40], our finding with the parotid acinar cells more closely reflects the responses observed in the EGF-treated perfused rat heart. In the studies of Nair *et al.* [25, 26], EGF activation of adenylate cyclase and cAMP accumulation occurred in a concentration-dependent manner which was not inhibited by application of the β-adrenergic receptor antagonist propranolol. We have found the same to be true for our system as well. The inability of propranolol to reduce enzyme activity or cAMP accumulation suggests that EGF action was not a consequence of an indirect mechanism such as catecholamine release and is thus independent of β-adrenergic receptor activation [20, 21, 25]. As with the heart, *in vitro* stimulation of adenylate cyclase activity by EGF required the presence of p[NH]ppG. The requirement for this GTP analogue, as well as inhibition of cAMP synthesis and accumulation in the presence of the GDP-analogue GDP[S], would further imply that the mechanism of EGF action involves the use of a GTP-binding regulatory protein. More recent studies by several groups [21, 22, 25, 40] have indeed determined the requirement for a G-binding protein in both the stimulatory and inhibitory effects on EGF receptor signal transduction mediated by intracellular changes in cAMP metabolism.

Treatment of parotid acinar cell membranes with pertussis toxin resulted in the radiolabeling of a protein at M_r ~40 kDa. This protein is most likely the ADP-ribosylation substrate of adenylate cyclase, the α-subunit of G_i. The inactivation of the inhibitory subunit did not alter the ability of EGF to further stimulate adenylate cyclase, thus suggesting that the effects of the growth factor are not mediated by G_i down-regulation. Again, in agreement with the effects of EGF on the rat heart, treatment of membranes with cholera toxin abolished EGF stimulation of adenylate cyclase enzyme activity. The ADP-ribosylation of G_s and subsequent inability of acinar cells to increase cAMP levels in response to EGF are consistent with studies involving antibody to $G_{s\alpha}$ with heart membranes, which also was shown to inhibit EGF-stimulated enzyme activities [25].

Chronic treatment of rats with EGF or isoproterenol results in parotid gland hypertrophy and hyperplasia [1, 3]. This also is true to a limited extent in the rat heart [25]. The β-adrenergic agonist stimulation of cell surface Gal Tase has also been observed in the treatment of rats with EGF as well as the fact that introduction of α-lactalbumin will

inhibit either β-agonist or growth factor-stimulated cell proliferation [11]. The appearance of cell surface Gal Tase has been proposed to be mediated through activation of chromosomal gene expression for a novel Gal Tase associated (GTA) kinase [41]. The GTA kinase appears to play a role in the targeting of Golgi localized Gal Tase to the plasma membrane [11, 14]. The EGF-stimulated increase in cellular cAMP levels would be consistent with this mechanism of intracellular cAMP as a critical positive regulatory element necessary for the cellular expression genes involved in proliferation [21].

It is interesting to note in this regard that injection of isoproterenol-treated rats with exogenous Gal Tase was also capable of eliciting an increase in cAMP comparable to treatment with EGF. Gal Tase addition to isoproterenol-treated membranes in the adenylate cyclase assay *in vitro* caused an increase in cAMP as well. The mechanism by which Gal Tase generates the signal for proliferation in the plasma membrane is not known at the present time, but as with EGF, the co-administration of propranolol had no inhibitory action on the stimulation of adenylate cyclase activity. The similarity of β-adrenergic receptor-independent stimulation of adenylate cyclase by Gal Tase to that observed in EGF treatment may be indicative of a shared pathway for intracellular signal transduction. Our laboratory has shown previously the need of a cell surface glycoprotein (presumably the signal transducer) in Gal Tase-mediated acinar cell proliferation [15]. What this cell surface protein is or how it functions in the signal transduction has not been elucidated. The present findings suggest that part of this mechanism may involve intracellular cAMP accumulation similar to that observed in EGF-stimulated acinar cell proliferation. The mechanism by which Gal Tase signal transduction mediates acinar cell proliferation is being further investigated.

Interaction of EGF with its cell surface receptors produces a number of biological responses including cell proliferation through various protein phosphorylations [42, 43]. The action of EGF at the cell surface is initially mediated by the intrinsic tyrosine kinase activity of the receptor, with the accompanying phosphorylation of a number of cellular proteins at the cytoplasmic–plasma membrane interface [44]. The subsequent phosphorylation of phospholipase C_γ leads to the activation of intracellular second messengers Ins-1,4,5-P_3 and diacylglycerol which cause an increase in intracellular Ca^{2+} and activation of protein kinase C, respectively [18, 19]. Only recently has evidence accumulated from a number of cell lines as to the possible interaction of G-proteins in mediating specific growth responses of cells to exogenous EGF [22, 26]. It would thus appear that specific phosphorylation of protein components regulates various aspects of transmembrane signal transduction. Crosstalk among phosphorylation reactions mediated by protein kinase A [44, 45], protein kinase C [19, 21], and the protein tyrosine kinases [44], may be involved in the regulation of growth factor-dependent signal transduction. The results from our study would therefore further confirm the involvement of G-proteins in the EGF effects on adenylate cyclase activity in parotid gland acinar cells. Our observation suggests that the activated EGF–receptor complex is coupled to a cholera toxin sensitive G-binding regulatory protein which is capable of stimulating the cAMP-mediated second messenger signalling pathway. Experiments are underway to define the specific proteins involved in this process.

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