A novel mechanism for isoprenaline-stimulated proliferation of rat parotid acinar cells involving the epidermal growth factor receptor and cell surface galactosyltransferase

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Chronic injections of epidermal growth factor (EGF) or the β -adrenergic receptor agonist isoprenaline resulted in rat parotid gland hypertrophy and hyperplasia. Introduction of a polyclonal antibody to EGF or the EGF-receptor (EGF-R) caused a specific retardation of acinar cell proliferation when injected along with the growth factor. Meanwhile, only the antibody to EGF-R caused a dose-dependent retardation of proliferation on co-administration with isoprenaline both *in vivo* and *in vitro*. The antibody injected alone had no effect on cell growth. When cells were incubated in the presence of EGF, plasma membranes from isoprenaline-treated and control animals showed phosphorylation of the EGF-R tyrosine moieties and transient increases in membrane-associated phospholipase C_γ . Isoprenaline did not stimulate phosphorylation of the EGF-R in isolated plasma membranes. However, activation of the phosphotyrosine-signalling pathway could be duplicated by incubating isoprenaline-treated acinar cells, but not control cells, with bovine galactosyltransferase. Immunopurified EGF-R demonstrated variations in reactivity with two different lectins after treatment of the cells with the β -agonist as well as increased galactosyltransferase substrate capacity *in vitro*. In addition, incubation of intact acinar cells and isolated plasma-membrane fractions from isoprenaline-treated rats with UDP-[^{14} C]galactose resulted in an increased incorporation of label into the EGF-R. The results suggest that the carbohydrate moiety of the EGF-R has been altered in isoprenaline-treated animals allowing galactosyltransferase now to recognize this receptor. This interaction may in part mediate proliferation of parotid acinar cells. Indeed, we have previously shown that an antibody to galactosyltransferase is capable of blocking isoprenaline-mediated acinar cell proliferation *in vivo* [Humphreys-Beher, Schneyer, Kidd & Marchase (1987) *J. Biol. Chem.* **262**, 11706–11713].

INTRODUCTION

Isoprenaline, a β -adrenergic receptor agonist, has a dramatic growth-stimulating effect on the salivary glands of rats and mice, with chronic administration leading to hyperplastic and hypertrophic gland enlargement [1–3]. At the molecular level, DNA, RNA and protein synthesis are all increased after isoprenaline administration, which is mediated in part by cyclic nucleotide second messengers [4–6]. The intracellular increases in cyclic AMP (cAMP) give rise to the activation of protein kinase A, Ca^{2+} mobilization and inositol lipid hydrolysis [7–9]. Acinar cell hyperplasia and gland hypertrophy can also be duplicated by dietary changes as well as by administration of growth hormones such as epidermal growth factor (EGF) [10–12].

The response of a cell to an extracellular signal is initiated by binding to specific plasma membrane receptors. This interaction leads to the generation of intracellular second messengers which subsequently mediate various aspects of cell proliferation. In the generally accepted pathway following activation of the EGF receptor (EGF-R) by growth factors, activated EGF-R stimulates the phosphorylation of one of the enzymes involved in the generation of intracellular second-messenger signals. The tyrosine phosphorylation of phospholipase C_γ by the EGF-R may take place by direct interaction in the plasma membrane [13–16]. Phospholipase C_γ is involved in phosphoinositide metabolism generating inositol 1,4,5-triphosphate and diacylglycerol which

cause the release of intracellular Ca^{2+} and activation of protein kinase C respectively [17,18].

In our examinations of the growth exhibited by rat acinar cells in response to isoprenaline, our attention has become focused on the role of cell surface galactosyltransferase (Gal Tase) [19]. The transition to a growth state in acinar cells is accompanied by an increased expression and topographic localization of Gal Tase to the plasma membrane [19,20]. A dramatic decrease in cell hypertrophy and hyperplasia can be elicited by introduction *in vivo* and *in vitro* of enzyme substrates, the specific modifier protein α -lactalbumin or antibody to Gal Tase at the time of β -agonist or growth hormone treatment [12,21]. Additional treatment with the wheat germ agglutinin lectin restores hyperplasia but is not mitogenic for untreated acinar cells [22]. This finding suggests that there is an increase in glycoconjugates in the plasma membrane which specifically terminate in the Gal Tase substrate saccharide *N*-acetylglucosamine.

The glycosyltransferases constitute a family of intracellular membrane-bound enzymes involved in the biosynthesis of the oligosaccharide moieties of glycoproteins, glycolipids and glycosaminoglycans [23,24]. One of the most studied of these enzymes, β 1-4 galactosyltransferase (EC 2.4.1.38), is primarily localized to the *trans*-Golgi network where the enzyme transfers Gal in a β 1-4 anomeric configuration to GlcNAc residues of glycoconjugates [25]. Although most activity is found in the Golgi apparatus, a portion of the enzyme activity has been localized to the plasma

Abbreviations used: ConA, concanavalin A; WGA, wheat-germ agglutinin; CorT, coral-tree lectin; PHA, *Phaseolus vulgaris* erythroagglutinin; SBA, soya-bean agglutinin; DBA, *Dolichos biflorus* agglutinin; EGF(-R), epidermal growth factor (receptor); Gal Tase, galactosyltransferase.

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membrane [25–29]. On the cell surface, Gal Tase has been shown to mediate various cell–cell and cell–matrix interactions (for a review see [30,31]). Plasma-membrane and soluble isoenzymes have been implicated in the growth control of normal cells [20,30,32], as an abnormality leads to uncontrolled growth in certain transformed cells [31,33–35]. Indeed, the concept of using Gal Tase as a potential tumour marker has been the subject of numerous reports [36–41]. It has firmly been established that the serum activity of a specific isoenzyme (GTII) is elevated in patients with certain solid tumours [41,42]. Alterations in the biosynthesis of the oligosaccharide moieties of membrane glycoproteins are an additional hallmark of transformed cells [43–47]. Transformations by chemical mutagens as well as by oncogenic viruses results in alteration of the size of *N*-asparagine and *O*-linked glycans [44,45]. Many studies have also shown direct correlation between metastatic potential and altered glycosylation of plasma-membrane glycoproteins [46–48].

In the present investigation, we present evidence showing that both isoprenaline- and EGF-stimulated proliferation of parotid gland acinar cells involves the activation of the EGF-R. This activation is consistent with tyrosine autophosphorylation of the EGF-R and movement of phospholipase C_y from a soluble to plasma-membrane association. However, the effects of isoprenaline are not mediated by circulating EGF since injection of antibody to EGF inhibited growth factor-mediated proliferation but not that induced by isoprenaline treatment. Subsequent isolation and characterization of the EGF-R from β -agonist-treated parotid glands revealed alterations in lectin binding and capacity to interact with endogenous and exogenous Gal Tase. This modification of the receptor may allow cell surface Gal Tase from treated animals to mimic the second-messenger signalling activity of EGF. This capacity is not demonstrated in control untreated acinar cells.

MATERIALS AND METHODS

Materials

UDP-Gal, ovalbumin, EGF, bovine Gal Tase, DL-isoprenaline, biotinylated lectins [soya-bean agglutinin (SBA), concanavalin A (ConA), *Phaseolus vulgaris* erythroagglutinin, (PHA), wheat-germ agglutinin (WGA) and *Dolichos biflorus* agglutinin (DBA)] and extravidin–peroxidase conjugate were purchased from Sigma Chemical Co. Coral Tree lectin (CorT) was a generous gift from Dr. C. West, Department of Anatomy and Cell Biology of the University of Florida. UDP-[^{14}C]galactose (specific radioactivity 300 $\mu\text{Ci}/\text{mmol}$) and [^3H]thymidine (specific radioactivity 6.7 Ci/mmol) were obtained from Amersham International.

Antibody preparation and characterization

The specificity of the antibodies is detailed in Fig. 1. Antibodies to mouse EGF and the human A431 carcinoma cell EGF-R [29(1)IgG] were obtained from Sigma Chemical Co. Immunoprecipitation of [γ - ^{32}P]ATP-labelled control plasma membranes with the monoclonal antibody to the EGF-R (Fig. 1a) detected radiolabelled proteins of molecular mass 170, 95 and 90 kDa after solubilization and separation on SDS/polyacrylamide gels. The proteins of 95 and 90 kDa are also present in the immunoprecipitation with preimmune serum and therefore represent non-specific contaminating proteins. Hydrolysis of the immunoprecipitate with NaOH did not alter the level of phosphorylation of the 170 kDa receptor, indicating that the label was most likely linked to tyrosine [49,50]. On Western blot analysis, the monoclonal antibody only detects the mature 170 kDa plasma-membrane form of the EGF-R [12].

Previously characterized polyclonal antibody to the rat EGF-

Fig. 1. Characterization of the monoclonal and polyclonal antibodies to the EGF-R

(a) Immunoprecipitation of ^{32}P -labelled plasma-membrane proteins using monoclonal antibody (mAb) 29(1) to the EGF-R from control acinar cells. The cell membranes were first incubated in the presence or absence of EGF and [γ - ^{32}P]ATP. The membranes were solubilized in detergent and immunoprecipitated with mAb 29(1) as described in the Materials and methods section. Lane 1, no added EGF, precipitation with preimmune serum; lane 2, no added EGF, precipitation with mAb 29(1); lane 3, addition of EGF and mAb 29(1); lane 4, addition of EGF and immunoprecipitation with mAb 29(1), followed by hydrolysis of non-tyrosine-associated phosphate by heating to 50 $^{\circ}\text{C}$ in 1 M-NaOH for 30 min [53]. (b) Identification of parotid gland EGF-R using the polyclonal antibody to immunoprecipitate [^{32}P]autophosphorylated receptor [55]. Lane 1, control plasma membrane preincubated with [^{32}P]ATP before immunoprecipitation. Lane 2, stimulation of control plasma membrane with EGF in the presence of [^{32}P]ATP before immunoprecipitation. (c) Immunoprecipitation of the EGF-R from control and isoprenaline-treated cell membranes (500 μg of protein) using the polyclonal antibody. After precipitation the samples were transferred to nitrocellulose and the blot was allowed to react with the polyclonal antibody to detect the receptor. Lane C, control membrane EGF-R. Lane I, isoprenaline-treated membrane EGF-R.

R was monospecific for the protein from parotid tissue as detailed by Dunn and co-workers [51]. The polyclonal antibody immunoprecipitated a 170 kDa phosphorylated form of the EGF-R from the plasma membrane of parotid gland acinar cells after EGF stimulation (Figs. 1b and 1c) [51]. This antibody also immunoprecipitated equal quantities of receptor from control and isoprenaline-treated membranes when an equal quantity of protein was used (Fig. 1c). Monoclonal antibody to phospholipase C_y was generously provided by Dr. S. G. Rhee, Laboratory of Biochemistry, NHLBI, Bethesda, MD, U.S.A. The characterization of this antibody has previously been described by Suh *et al.* [52] and Margolis *et al.* [15].

Western blot analysis

After separation of the samples on polyacrylamide gels, the proteins were electrophoretically transferred to nitrocellulose at 17 V for 12 h [53]. After incubation with the primary polyclonal antibody to the EGF-R, the nitrocellulose was incubated in goat anti-rabbit polyvalent IgG–peroxidase conjugate (1:1000) for 1 h, followed by staining for peroxidase activity as described elsewhere [12]. When appropriate, the stained proteins were then cut from the nitrocellulose and dissolved in 0.5 ml of 10 M-NaOH at 65 $^{\circ}\text{C}$ for 30 min. The slurry was then placed in aqueous

counting fluid, dark-adapted for 2 h and counted for ^{14}C or ^{32}P radioactivity for 10 min.

Animal procedures and thymidine incorporation

Female Sprague–Dawley rats were purchased from the University of Florida breeding colony. All animals weighed 180–250 g at the initiation of the experiments. One group of rats received twice daily intraperitoneal injections of isoprenaline in 0.5 ml at a concentration of 10 mg/ml for a period of 3 days. Other groups of animals receiving isoprenaline were also injected twice daily 20 min before the administration of β -agonist with 0.5 ml of the polyclonal antibody to EGF-R diluted as indicated. The hormone, mouse EGF, was injected at 20 $\mu\text{g}/\text{kg}$ body weight [11]. The dilution of EGF antibody to a final 1:1000 dilution is sufficient to precipitate a minimum of 225 ng of hormone as per manufacturer's description. Parotid gland hypertrophy and hyperplasia were determined by noting the parotid gland wet weight and the incorporation of [^3H]thymidine into DNA by the intraperitoneal injection of 100 μCi of radiolabel for 3 h before removal of the parotid gland [10,12]. Animals were anaesthetized with 0.3 ml of sodium pentobarbital and killed by exsanguination of the heart. Samples were corrected for background and expressed as mean c.p.m. $\pm$ s.d./mg of total protein.

Isolation of parotid acinar cells

For intact cell labelling, parotid gland acinar cells were freed of connective tissue and dissociated by incubation with collagenase and hyaluronidase [20]. The cells were subsequently washed in Hanks solution and incubated for 2.5 h at 37 °C to allow recovery of surface proteins affected by the dissociation process before the labelling experiments were continued. Radiolabelling of intact cells was accomplished by incubation with 1.0 μCi of UDP-[^{14}C]Gal with or without the addition of Gal Tase for 30 min at 37 °C. Acinar cells were resuspended in a final volume of 1 ml of Hanks balanced salts solution plus 0.2% sodium azide to prevent energy-dependent membrane rearrangements [20].

Membrane preparations and fractionation

A total membrane fraction was prepared from parotid glands as previously described [19]. The parotid gland from rats was identified by gross morphology, trimmed of connective tissue and lymph nodes and homogenized at 4 °C with a Dounce apparatus in 10 mM-Tris/HCl buffer, pH 8.0, containing the proteinase inhibitor phenylmethanesulphonyl fluoride (4 $\mu\text{g}/\text{ml}$) and phosphatase inhibitors: NaF (100 mM), sodium pyrophosphate (10 μM) and sodium orthovanadate (200 μM). Remaining connective tissue and unlysed cells were removed by low-speed centrifugation at 500 g for 15 min and the resulting slurry was centrifuged at 100000 g for 1 h to produce a total membrane pellet.

Plasma membranes were isolated by the method of Arvan & Castle [54]. The final membrane pellet was resuspended in 10 mM-Tris/HCl, pH 8.0. The purity of the membranes prepared in this manner is documented elsewhere [12,20]. The plasma-membrane fraction was shown to be enriched 10-fold over total membranes for γ -glutamyltranspeptidase, an enzyme marker appropriate for rat parotid acinar cells [54].

Immunopurification of the EGF-R

The monoclonal antibody to human A431 carcinoma cell EGF-R, purified by ammonium sulphate precipitation of ascites

fluid (Sigma Chemical Co.), was used for the purification of EGF-R. The immunoaffinity column for purification of the EGF-R was generated according to the manufacturer's (Bio-Rad) instructions. The plasma-membrane fraction from rat parotid gland, solubilized in PBS containing 0.5% NP-40, was applied to the immunoaffinity column. The unbound protein was removed by washing the column with PBS containing 0.5 M-NaCl. After further washing with PBS containing 0.2 M-NaCl, the bound antigen was eluted with a buffer of 0.2 M-glycine/HCl, pH 2.5. Protein recovery was determined by a modification of the method of Lowry [55]. Confirmation that the 170 kDa glycoprotein isolated by immunoaffinity column chromatography was the EGF-R was provided by an autophosphorylation reaction in the presence of EGF. ^{32}P from ATP was incorporated into the protein to a greater degree in the presence of growth factor than without (results not shown).

Immunoprecipitation of the EGF-R

Immunoprecipitation of either [^{14}C]Gal or ^{32}P -labelled solubilized receptor was performed using 500 μg of plasma-membrane preparations in 500 μl of Tris buffer containing protein and phosphatase inhibitors, 0.5% Triton X-100 and 0.1% SDS. Monoclonal antibody 29(1) or polyclonal antibody was added at 1:500 dilution and co-incubation performed for 12 h at 4 °C. Subsequently, 100 μg of protein A-agarose (Sigma) was added and incubation at 4 °C continued for an additional 30 min. The slurry was centrifuged at 12000 g for 5 min to recover the protein A-antibody-antigen complexes, washed three times in the same buffer and the receptor was eluted in SDS/gel sample buffer.

Lectin blotting

EGF-R glycosylation was measured as described by Heegaard & Halken [56]. Immunopurified receptor was electrophoresed on a 9% SDS/polyacrylamide gel, transferred to nitrocellulose, and incubated with biotinylated lectins as follows. The biotinylated lectins were first dialysed (0.1 M-NaCl, overnight stirring at 4 °C) to avoid background staining on the blots caused by free biotin. The standard buffer used for lectin incubations was 0.01 M-Tris/HCl (pH 7.2)/1 M-NaCl/0.05% polyvinylpyrrolidone/1.0 mM- CaCl_2 /1.0 mM- MgCl_2 /1.0 mM- MnCl_2 . The sample buffer containing 2% (v/v) polyvinylpyrrolidone served as blocking buffer. After electrophoretic transfer to nitrocellulose, the filter was blocked with the above buffer (2 h, 10 ml of buffer per strip) followed by three 5 min washes. The lectins (10 $\mu\text{g}/\text{ml}$) in 10 ml of buffer per lane were incubated with the filters for 1 h at room temperature by gentle shaking. After additional washes with buffer, the filter was incubated for 2 h with extravidin-horseradish peroxidase, and finally stained for peroxidase activity [53].

Gal Tase assays

The activity of Gal Tase was measured as described previously [19]. Plasma membranes were resuspended as described above at a final protein concentration of 100 $\mu\text{g}/\text{ml}$. The assay mixture (total volume 50 μl) used 10 mM-ovalbumin as acceptor to measure enzyme activity [19]. Enzyme specific activity was expressed as c.p.m. incorporated/h per mg of membrane protein (mean $\pm$ s.d.).

Autogalactosylation of Gal Tase

Bovine milk Gal Tase was allowed to undergo autogalactosylation in the presence of unlabelled UDP-galactose, after purification by α -lactalbumin-agarose affinity chromatography, as described by Torres & Hart [57], using the above assay buffer without Triton X-100. The autogalactosylated enzyme was concentrated in an Amicon ultrafiltration cell (model 8010) using

a PM-30 filter. The enzyme was stored at a final concentration of 50 units/ml at -20°C after dialysis against 10 mM-Tris/HCl buffer, pH 7.2, containing 10 mM-MnCl₂.

RESULTS

Inhibition of parotid gland hypertrophy by antiserum to EGF and the EGF-R

Injection of rats with EGF or isoprenaline resulted in hypertrophic and hyperplastic growth of the parotid gland as measured by gland wet weight and [³H]thymidine incorporation into DNA respectively (Figs. 2a and 2b). EGF administration resulted in a 1.5-fold increase in gland weight and [³H]thymidine concentration whereas isoprenaline treatment alone routinely caused a 2.5- to 3-fold increase. The growth-stimulating potential of EGF was completely inhibited by co-injection of a polyclonal antibody to either the growth factor or its specific cell surface receptor. Although isoprenaline treatment of rodents has been shown to cause the transient release of EGF from the submandibular gland [58], inclusion of anti-EGF antibodies with the β -agonist drug regimen failed to retard acinar cell proliferation,

suggesting the isoprenaline effects were not mediated directly by EGF (Figs. 2a and 2b).

To examine further the possible role of the EGF-R in mediating isoprenaline-stimulated growth, groups of animals were injected with a polyclonal antibody monospecific for the rat EGF-R. In addition to isoprenaline, three groups of animals were injected with antibody to the rat EGF-R. The antiserum, provided in twice daily injections over 3 days, had a moderate influence on gland hypertrophy. At final concentrations of 1:3000 and 1:6000 dilutions in the animal, gland wet weight was decreased by 54% and 25% ($P < 0.01$) respectively (Fig. 2c), whereas at higher concentrations of 1:500 no effect on gland size was observed. The antiserum had no growth-stimulating effect on its own when introduced into control animals. The most dramatic effect on gland hyperplasia was revealed by using [³H]thymidine to measure DNA synthesis stimulated by isoprenaline. A significant dose-dependent decrease ($P < 0.001$) was observed in DNA synthesis when the anti-EGF-R serum was introduced along with the isoprenaline drug regimen (Fig. 2d). Again the highest concentration of antibody (1:500) had no effect on retarding DNA synthesis. Preimmune serum at a final dilution of 1:3000 had no effect on acinar cell growth either when injected alone or in combination with isoprenaline (Fig. 2f). The inhibitory effects

Fig. 2. Effects of anti-EGF and anti-EGF-R antibodies on rat parotid gland hypertrophy and hyperplasia

(a) Acinar cell hypertrophy was determined by gland wet weight in control glands (■), chronic isoprenaline injection (▨), isoprenaline and anti-EGF antibody at 1:1000 dilution (▤), chronic EGF injections (■), EGF and antibody to EGF (□), EGF injection along with antibody to EGF-R (▩). (b) Acinar cell hyperplasia was determined in control and experimental animals treated as in (a) by measurement of [³H]thymidine incorporation into DNA. Key to histogram is the same as in (a). The results in (a) and (b) are from three animals per experimental condition. (c) Parotid gland wet weight was quantified after no treatment (■), anti-EGF-R antibody alone (▨), chronic isoprenaline injection (▨), isoprenaline and antiserum to the EGF-R (▩) at 1:500 (1), 1:3000 (2) and 1:6000 (3) dilution. The number of animals used for each experimental condition was four except the animals receiving isoprenaline and the 1:3000 dilution of antiserum where it was six. (d) Parotid gland hyperplasia was measured by [³H]thymidine incorporation into DNA treated as in (c). (e) Cell surface Gal Tase activity in control and experimental animals as detailed in (c). (f) Gland hypertrophy (left) and hyperplasia (right) after chronic isoprenaline and preimmune antiserum (1:3000 dilution) treatment of rat parotid glands. Control (■), preimmune serum alone (■), isoprenaline alone (▨), isoprenaline and preimmune serum (▨).

Table 1. Growth control of parotid acinar cells *in vitro* by antibody to the EGF-R

Acinar cells (approx. 2×10^6) were cultured as described by Kiser *et al.* [73]. To assay cell growth *in vitro* in the presence of the EGF-R antibodies, cultures were maintained for 48 h. The cell culture medium was modified to contain 5 ng of EGF and 10% rat serum, heat inactivated for 30 min at 56 °C. At 4 h of incubation, acinar cells were given 10 μ Ci of [^{3} H]thymidine, and incubation at 37 °C continued for an additional 4 h. Cell culturing was performed in 10 ml of media using 35 mm $\times$ 10 mm Costar plastic Petri dishes. Isoprenaline was added at a final concentration of 2 μ M. Antibody was added to the incubation mixture at a final dilution of 1:500 and 1:2000. EGF was added at a final concentration of 100 nM. All determinations of [^{3} H]thymidine incorporation are expressed as means $\pm$ S.D. for two experimental determinations performed in duplicate.

Condition	[^{3} H]Thymidine (c.p.m./mg of protein)
Control	830 $\pm$ 140
Isoprenaline	2050 $\pm$ 175
Isoprenaline + mAb to EGF-R (1:500)	1790 $\pm$ 150
Isoprenaline + mAb to EGF-R (1:2000)	1115 $\pm$ 130
Isoprenaline + anti-EGF-R (1:500)	1780 $\pm$ 130
Isoprenaline + anti-EGF-R (1:2000)	875 $\pm$ 140
EGF	1760 $\pm$ 150
EGF + anti-EGF-R (1:500)	1630 $\pm$ 200
EGF + anti-EGF-R (1:2000)	1105 $\pm$ 140

of anti EGF-R were not due to changes in the activity of cell surface Gal Tase, as shown in Fig. 2(e).

Inhibition of acinar cell proliferation by the polyclonal antibody was again confirmed *in vitro* using dilutions of the antibody at 1:500 and 1:2000. As shown in Table 1, the addition of isoprenaline or EGF to acinar cells caused approximately a 2.5- or 2.1-fold increase in [^{3} H]thymidine incorporation into DNA respectively ($P < 0.005$). Inclusion of antibody at the higher concentration of 1:500 again failed to cause a significant decrease in growth (13%), whereas the 1:2000 dilution inhibited growth by 54%. The same results were observed for the incorporation of the monoclonal antibody into the cell culture medium (Table 1). Finally, the use of the polyclonal antibody to the EGF-R in combination with EGF produced the same pattern as its inclusion with isoprenaline, namely the 1:500 dilution decreased growth by 7% whereas the 1:2000 dilution of the anti-EGF-R antibody inhibited thymidine incorporation by 37%.

Labelling of plasma-membrane glycoproteins *in vitro* and *in vivo*

When intact cells were incubated with UDP-[^{14} C]Gal, a protein of 170 kDa was the major protein labelled in the isoprenaline-treated acinar cells (Fig. 3). A second protein of 60 kDa was also faintly detected in the autoradiograph. Control membranes were unlabelled owing to the fact that cell surface Gal Tase is not normally present in quiescent cells (Figs. 3c and 3d). As an additional control, autogalactosylated exogenous Gal Tase was added to overcome any destruction of the cell surface enzyme which may have occurred after cell isolation as well as to compensate for differences in cell surface activities of Gal Tase caused by the isoprenaline treatment of the acinar cells. In both control and isoprenaline-treated cells the 170 kDa protein was labelled in the presence of exogenously added bovine Gal Tase and could be immunoprecipitated by polyclonal antibody to the EGF-R (Fig. 3c). Additional proteins of lower molecular mass (60–30 kDa) were also labelled to a greater extent in the isoprenaline-treated cells, indicating possible alterations in oligo-saccharide processing of other cell surface glycoproteins.

When exogenous bovine Gal Tase was incubated with Triton X-100-solubilized membranes (to increase possible substrate GlcNAc accessibility) and UDP-[^{14} C]Gal, several proteins from isoprenaline-treated membranes were uniquely labelled relative to control membranes (Fig. 3a). Of particular interest was a labelled protein that had a relative mobility similar to the EGF-R (170 kDa). This glycoprotein demonstrated an increase in [^{14} C]Gal labelling after isoprenaline treatment. Confirmation

Fig. 3. Identification of plasma-membrane acceptor macromolecules for Gal Tase from control (C) and isoprenaline-treated (I) rat parotid glands after electrophoresis on 10% SDS/polyacrylamide gels

(a) Gel fluorograph of plasma-membrane samples solubilized with 0.1% Triton X-100 and labelled with [^{14} C]Gal using exogenous bovine Gal Tase and 50 μ g of plasma-membrane protein in each sample. The membrane pellets (50 μ g) were resuspended in 100 μ l of Mes buffer containing 0.1% Triton X-100, 25 mM-MnCl₂, 0.25 units of Gal Tase stock solution and 1.0 μ Ci of UDP-[^{14} C]Gal. The mixture was then incubated at 37 °C for 30 min after which time the labelled protein was recovered by the addition of an equal volume of 10% trichloroacetic acid and centrifugation [22]. The pellet was washed with 500 μ l of 80% ethanol, dried by vacuum and resuspended in 20 μ l of SDS/polyacrylamide gel sample buffer for electrophoretic analysis. (b) Immunoprecipitation of the EGF-R using the rabbit polyclonal antibody. The [^{14} C]-labelled proteins from (a) were visualized by gel fluorography after immunoprecipitation of the parotid gland EGF-R. An equal quantity of protein was precipitated from each sample as indicated in Fig. 1(c). (c) Gel fluorograph of [^{14} C]Gal-labelled plasma-membrane proteins (50 μ g) from intact parotid gland acinar cells after incubation with exogenous bovine Gal Tase and UDP-[^{14} C]Gal(+) or UDP-[^{14} C]Gal alone (-). (d) Immunoprecipitation of [^{14} C]-labelled EGF-R from intact cells incubated with exogenous bovine Gal Tase (c, right side); 500 μ g of membrane protein was used for the immunoprecipitation to account for changes in cell size caused by isoprenaline treatment. Specific [^{14} C] radiolabelling of the plasma-membrane fraction glycoproteins was achieved by a modification of standard protocols [19,22,57].

Table 2. Levels of [¹⁴C]Gal incorporated into plasma membrane EGF-R glycoprotein

Immunoaffinity-purified EGF-R (see Fig. 4a) (0.5 µg) was incubated under conditions for galactosylation by bovine Gal Tase. The reaction was terminated by boiling the sample in SDS/gel sample buffer and the EGF-R cut from the polyacrylamide gel after electrophoresis and staining with Coomassie Brilliant Blue R-250. Plasma membranes from control and isoprenaline-treated parotid glands were radiolabelled with UDP-[¹⁴C]Gal and bovine Gal Tase, then solubilized with 0.1 M-Mes and 0.5% Triton X-100, and the EGF-R immunoprecipitated with the polyclonal antibody specific for the receptor following procedures described in the Materials and methods section. The immunoprecipitated proteins were further purified by SDS/polyacrylamide gel, and the 170 kDa band was cut from the polyacrylamide after X-ray fluorography, and the associated radiolabel determined by scintillation counting.

Detection/isolation	Control	Isoprenaline-treated
Anti-EGF-R-IgG Affigel	802 ± 125	3840 ± 175
Immunoprecipitation of EGF-R	107 ± 15	463 ± 30

that this protein was the EGF-R was obtained by specific immunoprecipitation of the ¹⁴C label in the 170 kDa protein by the polyclonal antibody to the EGF-R (Fig. 3b). After gel fluorography, the protein was cut from the gel and the radiolabel associated with the immunopurified receptor determined. As shown in Table 2, more [¹⁴C]Gal was incorporated into the EGF-R after isoprenaline treatment than into the control receptor.

Detection of carbohydrate alterations on the EGF-R after isoprenaline treatment

To characterize a possible alteration in the carbohydrate moieties contributing to the increase in the ability of this glycoprotein to interact with Gal Tase, the EGF-R was purified from NP-40-solubilized plasma membranes on an immunoaffinity column of mouse anti-EGF-R IgG. As shown in Fig. 4(a), a

protein with an apparent molecular mass of 170 kDa was isolated along with a minor contaminating protein migrating at approx. 75–80 kDa. The difference in mobility on Coomassie Blue-stained gels in Fig. 4(a) of the low-molecular-mass protein was not apparent in subsequent electrophoresis and transfer of proteins to nitrocellulose. The purified receptor preparation was also allowed to react with anti-(Gal Tase) antibody to determine if the transferase co-purified with the EGF-R. Under the conditions of purification on the immunoaffinity column, no Gal Tase was detected, although the purified protein reacted with the anti-EGF-R antibody. This observation is not unexpected, since Gal Tase has a low affinity for substrate sugars [59].

Alterations in the composition of the oligosaccharide structures for the EGF-R were determined by the reactivity of biotinylated lectins with various carbohydrate specificities. Equal amounts of immunopurified EGF-R were applied to SDS/polyacrylamide gels and transferred to nitrocellulose. The specificity of each lectin was verified by using known standard glycoproteins immobilized on nitrocellulose as well as blocking their binding by the inclusion of mono- or di-saccharide competitors (results not shown). Reaction with the lectins DBA, ConA and SBA demonstrated very similar levels of binding between control and isoprenaline-treated receptors (Fig. 4b). An increased lectin binding for the EGF-R after isoprenaline treatment was observed with WGA and CorT lectins. In contrast, PHA bound better to control EGF-R than to receptor isolated from experimental parotid glands. The changes in the lectin-binding profile of the EGF-R from isoprenaline-treated cells are consistent with the possible interaction of the receptor with Gal Tase in the plasma membrane during proliferation.

Autophosphorylation of the EGF-R

Activation of intrinsic protein tyrosine kinase activity of cell surface growth factor receptors is necessary for biological responses to EGF [13]. Preincubation of membranes with EGF stimulated incorporation of ³²P from γ-ATP into the EGF-R, 2- and 3.5-fold over the level of incorporation into receptor from unstimulated control and isoprenaline-treated acinar cell mem-

Fig. 4. Biotinylated lectin characterization of the EGF-R oligosaccharide composition from control (C) and isoprenaline-treated (I) animals

Equal amounts of immunopurified EGF-R (1 µg) was applied to a 9% SDS/polyacrylamide gel and either stained with Coomassie Brilliant Blue R-250 (a) or transferred to nitrocellulose and allowed to react with six different lectins as described in the Materials and methods section (b). Equal transfer of the purified receptor to nitrocellulose was determined by additional reaction of the blots to antibody to the receptor protein (results not shown). The figure is representative of results obtained from three separate experiments. The lectins used are identified in the Results section.

Table 3. Autophosphorylation *in vitro* of the EGF-R from plasma membranes

All values are expressed as mean d.p.m. $\pm$ s.d. for two experimental determinations performed in triplicate using 50 μ g of plasma-membrane protein. The EGF-R was isolated after autophosphorylation by immunoprecipitation with the polyclonal antibody to rat EGF-R.

Conditions	Control	Isoprenaline-treated
No additions	2216 $\pm$ 140	2168 $\pm$ 248
+ EGF	4424 $\pm$ 175	7564 $\pm$ 502
+ Gal Tase	2560 $\pm$ 330	7434 $\pm$ 898
+ Isoprenaline	2513 $\pm$ 131	2213 $\pm$ 183

Fig. 5. Effects of EGF and Gal Tase on the EGF-R

(a) Autophosphorylation of the EGF-R after incubation with EGF or Gal Tase. Plasma-membrane preparations were assessed for intrinsic EGF-R tyrosine kinase activity by the method of Defize *et al.* [68] using [^{32} P]ATP. Membrane protein (50 μ g) was incubated for 10 min at 20 $^{\circ}$ C in 10 mM-Tris, pH 7.4, buffer containing 10 mM-MnCl₂, under the following modifications: no additions, 100 ng of EGF, or 5 μ g of affinity-purified bovine Gal Tase. The sample volume was adjusted to 60 μ l with 10 mM-Tris buffer, pH 7.4, containing phosphatase inhibitors and 0.5 μ Ci of [γ - ^{32} P]ATP. The labelling reaction was continued for 10 min on ice followed by the addition of 440 μ l of solubilization buffer and polyclonal antibody to the rat EGF-R. The EGF-R was isolated by immunoprecipitation using the rat polyclonal antibody. The immunisolated proteins were further purified on an 8% SDS/polyacrylamide gel before autoradiography. Control membranes are indicated by C and isoprenaline-treated membranes are indicated by I. The data correspond to the results presented in Table 2. (b) Two-dimensional analysis of ^{32} P-labelled amino acids from the EGF-R after membrane stimulation with either EGF (1) or bovine Gal Tase (2). The EGF-R was isolated by immunoprecipitation using the polyclonal antiserum to the rat liver receptor [51] as described in (a). Phosphoamino acid analysis was performed by the method of Ottaviano and Gerace [71].

branes respectively (Table 3 and confirmed in Fig. 5). Although the same amount of receptor is present in both membranes (Fig. 1 and [12]), the ability of isoprenaline-treated membranes to incorporate more radiolabel elicited by the inclusion of EGF may be related to a receptor subpopulation with the carbohydrate modifications observed with the lectins (unpublished work). The substitution of bovine Gal Tase for EGF resulted in an increased incorporation of ^{32} P into the EGF-R from isoprenaline-treated membranes. The stimulation of autophosphorylation by bovine Gal Tase was comparable with that seen with EGF and occurred without the addition of UDP-Gal to the incubation mixture (Table 3). The phosphorylation induced by EGF and Gal Tase on the EGF-R was present as phosphotyrosine, a result consistent with the intrinsic tyrosine kinase activity of the receptor (Fig. 5b). Plasma membranes from control parotid glands did not show an increase in phosphorylation when incubated with Gal Tase. The inclusion of isoprenaline did not increase the level of ^{32} P incorporated into protein in either untreated or treated plasma-membrane samples.

Observation of increased membrane association of phospholipase C_γ in acinar cells

Further evidence for the ability of Gal Tase to stimulate the phosphotyrosine-signalling pathway was obtained by analysing the plasma-membrane fraction from a low-speed centrifugation for the association of phospholipase C_γ [16]. In parotid gland acinar cells, incubation of both control and isoprenaline-treated cells with EGF resulted in a transient increase in membrane-associated phospholipase C_γ with an apparent molecular mass of 140 kDa (Figs. 6a and 6b). The size detected for the protein from

Fig. 6. Detection of membrane-associated phospholipase C_γ after growth stimulation

Intact acinar cells were isolated as described previously [21]. After isolation, the cells were placed into RPMI 1060 containing 5% fetal bovine serum and incubated at 37 $^{\circ}$ C for 1 h. The cells were then incubated with EGF (100 ng/ml) or bovine Gal Tase (5 μ g/ml) for 10, 30 or 60 min. The cells were subsequently collected and disrupted by sonication. Low-speed membrane fractions were isolated as described by Todderud *et al.* [16] and Cohen and Fava [72]. In brief, the resulting cell lysate was subjected to centrifugation for 1 min at 8000 g in an Eppendorf microfuge. The supernatant was removed and re-centrifuged for 4 min at 8000 g with the resulting supernatant centrifuged a third time for 12 min. The pellet from these three centrifugations constituted the plasma-membrane fraction from low-speed centrifugations [16]. The proteins were then separated, after solubilization in SDS/gel buffer, on an 8% SDS/polyacrylamide gel followed by transfer to nitrocellulose. Western blotting was performed as described above using monoclonal antibody to phospholipase C_γ [52]. (a) Untreated control acinar cells co-cultured with 100 ng of EGF/ml; (b) isoprenaline-treated acinar cells co-cultured with 100 ng of EGF/ml; (c) control acinar cells co-cultured with 5 μ g of Gal Tase/ml; (d) isoprenaline-treated cells co-cultured with 5 μ g of Gal Tase/ml. Some 2 $\times$ 10⁶ cells were used for each time-sampling. The results presented are indicative of three repeated assays for phospholipase C_γ concentrations detected by Western blot.

parotid gland acinar cells is consistent with the size reported by others [14,15,60]. Only those cells pretreated with isoprenaline showed an ability to respond to incubation with bovine Gal Tase (Figs. 6c and 6d). The amount of membrane-associated phospholipase C_γ detected by the monoclonal antibody was elevated at 10 and 30 min. By 60 min, the concentrations of phospholipase C_γ had returned to unstimulated values (results not shown). The control cells treated with Gal Tase consistently showed higher amounts of phospholipase C_γ at zero time than at later times of incubation in the presence of Gal Tase (Fig. 6c). The meaning of this observation is unclear at the present time but may reflect the loss of the enzyme from the membrane with time in culture.

DISCUSSION

Isoprenaline or EGF administration to rats causes normally quiescent acinar cells of the parotid gland to undergo proliferation. Although the general pathway of EGF activation of DNA synthesis and cell division is beginning to become clear, how β -agonists bring about such changes in cell growth is not yet understood. In this regard, the present data suggest a novel mechanism for signal transduction at the plasma membrane in the transition from stasis to active cell growth.

The incorporation of anti-EGF-R antibody along with isoprenaline demonstrated the requirement for the receptor in stimulation of acinar cell growth. The inability of the high concentration of anti-EGF-R antibody to block growth may in part be related to the necessity of cross-linking receptors to mask a specific binding site or to initiate receptor internalization. The binding of antibodies to the EGF-R, however, did not cause growth stimulation by itself. At the same time, acinar cell proliferation in response to isoprenaline does not depend on increased binding of EGF, since the introduction of antibody to this growth factor did not decrease cell growth. Although it has been shown that β -agonists can cause the release of hormone from the submandibular gland [58], the results presented here suggest that EGF may not play a direct role in mediating isoprenaline-stimulated proliferation. This may be due to the fact that the maximum increase in gland hypertrophy and hyperplasia induced by isoprenaline treatment could eclipse any observed hormone effect. Indeed, co-injection of isoprenaline and EGF does not result in an additive increase in cell growth [11]. The limited growth produced by EGF injections alone may be due to the rapid clearance of the hormone from the blood [57]. This may again be true for β -agonist-induced increases in plasma hormone concentrations thereby limiting their growth-promoting potential.

To support further our concept of EGF-R-mediated hyperplasia, we have found an increase in phosphotyrosine in the 170 kDa EGF-R after isoprenaline treatment of acinar cells. The EGF-R contains a protein tyrosine kinase activity which is activated after EGF binding. This event leads to tyrosine phosphorylation of intracellular substrates and the EGF-R itself. The tyrosine phosphorylation is part of the signal-transduction pathway activated by the receptor [14,61]. The EGF-R from isoprenaline-treated cells appears to become autophosphorylated, as determined by immunoprecipitation of autophosphorylated receptor after isoprenaline treatment (Fig. 1b). The activation of the tyrosine kinase signalling pathway is not due to the direct action of isoprenaline, since activation of β -adrenergic receptor leads to stimulation of cAMP-dependent serine kinases [8]. However, activation of the cAMP-signalling pathway can also lead to the activation of phospholipase C and inositol phosphate metabolism [7]. The contribution of this second signalling pathway to cell proliferation could account for the partial

inhibition of growth observed with the anti-EGF-R antibodies. A subsequent substrate of the activated EGF-R appears to be phospholipase C, which acts in the plasma membrane to activate inositol phosphate metabolism [61,62]. Stimulation of the EGF-R by EGF leads to the rapid membrane association and phosphorylation of phospholipase C_γ in the signal transduction leading to DNA synthesis and cell division [13–16]. At present, we have shown the phosphorylation of the EGF-R as well as membrane association for phospholipase C_γ after EGF or Gal Tase stimulation of acinar cells *in vitro*. Further evidence for the ability of Gal Tase to mimic EGF-receptor interaction has been provided by showing both EGF and Gal Tase to induce adenylate cyclase activity and subsequent cAMP accumulation in isoprenaline-treated acinar cells in a β -adrenergic-receptor-independent fashion [63].

The introduction of α -lactalbumin and other Gal Tase-specific substrates [21,64] has been found to retard transferase-mediated hyperplasia, suggesting that an interaction of this enzyme with other cell surface proteins may be necessary. It is now a generally accepted fact that Gal Tase can be isolated as a constituent of the plasma membrane of various types of proliferating cells. Localization of the enzyme to the plasma membrane has subsequently been correlated with a number of cellular events [27,29]. More recently, attention has been focused on elucidating the role Gal Tase appears to play in mediating cell growth [12,20,21]. In the plasma membrane, Gal Tase has been proposed to interact with both glycolipid [65] and glycoprotein macromolecules [22,66]. In adrenal carcinoma cells, tumour metastasis correlates with the concentration of a 110 kDa membrane glycoprotein which is an apparent substrate for plasma-membrane Gal Tase [65]. In the rat parotid gland, Gal Tase also plays an intimate role in regulating acinar cell proliferation [12,20]. The addition of the enzyme modifier α -lactalbumin dramatically curtails isoprenaline-mediated cell growth [21]. Using this model, we suggest that the EGF-R is possibly the cellular macromolecule responsible for the signal transduction necessary for acinar cell proliferation. Immunopurified EGF-R, as well as being a receptor in both intact cells and isolated plasma membranes from isoprenaline-treated rats, is capable of accepting Gal in the presence of Gal Tase.

The EGF-R from A431 cells is a glycoprotein with 10–12 oligosaccharide chains, primarily composed of *N*-asparagine-linked high-mannose and complex structures [67,68]. Increased EGF-R activity has been demonstrated in A431 cells on the loss of blood group A⁺ antigens from the carbohydrate structures [67–69]. Variations in carbohydrate structure and their possible role in receptor regulation has been reported by others. Alterations in carbohydrate processing of the insulin receptor in CHO cell variants can lead to either increased or decreased biological activity [70]. The increased level of these biological properties can be extended in the parotid gland to a heightened ability of the receptor to interact with Gal Tase after the isoprenaline drug regimen, as revealed by the [¹⁴C]Gal labelling studies (Fig. 3, Table 2). At present, we have accumulated evidence suggesting that the EGF-R has undergone alterations (apparently different from A431 cells) in the carbohydrate structures after chronic isoprenaline treatment of rat parotid gland acinar cells. Western blot analysis of plasma-membrane preparations from control and isoprenaline-treated parotid glands with the lectins WGA and ConA show increased binding capacity for a large number of glycoproteins after β -agonist injection (unpublished work). From our results, this would appear to not only make the receptor a biological substrate capable of interaction with cell surface Gal Tase, but may additionally contribute to a receptor population with increased tyrosine kinase activity. Indeed, Gal Tase was able to substitute for EGF in activating this pathway after

Fig. 7. Schematic representation of the action of EGF or isoprenaline on the stimulation of acinar cell proliferation through the EGF-R

isoprenaline stimulation of proliferation, as reflected by the activation of phospholipase C_γ and induction of autophosphorylation of the receptor.

The incomplete retardation observed of both hypertrophy and hyperplasia of the parotid gland by treatment with antibody to the EGF-R and isoprenaline may be due to several factors. It is possible that a greater inhibition of growth could be obtained by a continuous application of the reagents rather than twice daily large dose injections. The rate of clearance from the circulation will place a limit on the time the receptor is blocked from interaction with cell surface Gal Tase, which is continuously present in the membrane [20]. Alternatively there may be more than one plasma-membrane protein capable of signal transduction stimulated by isoprenaline. Labelling of the plasma membrane by exogenous Gal Tase *in vitro* revealed several proteins in addition to the EGF-R that appear to undergo increased labelling after isoprenaline treatment (Fig. 3b).

In summary, we have demonstrated here that in the rat parotid gland acinar cells, the transition from stasis to active proliferation is accompanied by the appearance of cell surface Gal Tase (Fig. 7). At the same time, the EGF-R undergoes altered processing of carbohydrate structures and these changes promote the interaction of the EGF-R with Gal Tase (events 1 and 3). Changes *in vivo* in the carbohydrate structure being to appear as early as 2 h after the first isoprenaline injection (K. R. Purushotham, Y. Nakagawa & M. G. Humphreys-Beher, unpublished work). This is a similar time-course to that previously observed for the appearance of cell surface Gal Tase, although *in vitro* it is much sooner [20]. The juxtaponition of such membrane constituents may lead to activation of receptor tyrosine kinase activity and signal transduction leading to cell division. In this regard, our data suggest that Gal Tase can be thought of as acting in a fashion similar to receptor-ligand-type interactions. This interpretation is supported by the ability of Gal Tase to activate EGF-R autophosphorylation (Table 2), the ability of the EGF-R to act as enzyme substrate (Table 1 and Fig. 3) and the ability of the transferase to initiate a redistribution of

phospholipase C_γ (Fig. 6). The level of membrane incorporation of [^{14} C]Gal or binding of WGA is not affected by the inclusion of growth inhibitors such as antibody to Gal Tase (unpublished work). The addition of Gal Tase to isoprenaline-treated plasma membranes caused an induction of EGF-R autophosphorylation *in vitro* without the need for exogenous UDP-Gal. Alternatively, the transition to cell proliferation through the EGF-R, as indicated in Fig. 7, can occur by the unmodified receptor binding substrate hormone EGF. This again would lead to activation of receptor tyrosine kinase activity (events 1 and 2). The ability of the isolated EGF-R to continue to be a Gal acceptor macromolecule and the unavailability of UDP-Gal at the cell surface suggests that receptor activation does not require the enzymic transfer of the sugar moiety to the carbohydrate structures of the glycoprotein substrate.

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