Epidermal growth factor upregulates β-adrenergic receptor signaling in a human salivary cell line

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Am J Physiol Cell Physiol 284: C1164–C1175, 2003. First published January 22, 2003; 10.1152/ajpcell.00343.2002.—The effects of epidermal growth factor (EGF) on the β-adrenergic receptor-coupled adenylyl cyclase system were studied in a human salivary cell line (HSY). The β-adrenergic agonist isoproterenol (10−5 M) stimulated adenylyl cyclase activity by ~2-fold, and the isoproterenol response was increased 1.8-fold after prolonged (48 h) exposure to EGF (5 × 10−10 M). In contrast, enzyme activation via stimulatory prostaglandin receptors and by agents acting on nonreceptor components of the adenylyl cyclase system was not enhanced by EGF. β-Adrenergic receptor density, assessed by binding of the β-adrenergic receptor antagonist (−)[125I]iodopindolol, was increased threefold after EGF treatment. Competition binding studies with unlabeled antagonists selective for β1- and β2-adrenergic receptor subtypes indicated that the increase in (−)[125I]iodopindolol binding sites induced by EGF reflected an increased number of β2-adrenergic receptors. Likewise, Northern blot analysis of RNA from EGF-treated cells revealed selective induction of β2-adrenergic receptor mRNA, which was blocked by the RNA synthesis inhibitor actinomycin D. The increase in β-adrenergic receptor density produced by EGF was unaltered by phorbol ester-induced downregulation of protein kinase C (PKC). Enhancement of isoproterenol-responsive adenylyl cyclase activity and phosphorylation of mitogen-activated protein kinase (MAPK) by EGF were both blocked by the MAPK pathway inhibitor PD-98059. The results suggest that in HSY cells EGF enhances β-adrenergic responsiveness by upregulating β2-adrenergic receptor expression at the transcriptional level. Moreover, the stimulatory effect of EGF on β2-adrenergic receptor signaling appears to be mediated by the MAPK pathway and independent of PKC activation.

Adenylyl cyclase; G protein-coupled receptor; signal transduction; mitogen-activated protein kinase; protein kinase C pathways (3). In general, cholinergic-muscarinic and α-adrenergic receptor agonists induce secretion of salivary fluid and electrolytes via activation of the intracellular calcium ([Ca2+]i) cascade (3). On the other hand, β-adrenergic receptor agonists stimulate the secretion of salivary proteins by activation of adenylyl cyclase and the generation of intracellular cAMP (3). Stimulation of the β-adrenergic receptor-coupled adenylyl cyclase pathway also causes transcriptional and posttranslational modifications of salivary gland proteins (52). In vivo, the β-adrenergic agonist isoproterenol has been demonstrated to induce hypertrophy and hyperplasia of salivary glands (10, 50). In a number of cell types, β-adrenergic receptor activation of adenylyl cyclase is modulated by systemic hormones as well as local growth factors and cytokines (21, 29, 51, 54). Whether similar modulation of β-adrenergic receptor signaling might play a regulatory role in salivary cell secretion and growth has not been well characterized.

Epidermal growth factor (EGF) is a multifunctional factor produced in abundance by rodent salivary glands; lesser amounts of EGF have also been identified in human salivary cells (33). The actions of EGF are mediated by a receptor tyrosine kinase linked to multiple intracellular signaling events such as activation of protein kinase C (PKC) and the extracellular signal-regulated kinase (ERK/mitogen-activated protein kinase (MAPK) cascade (14, 37). Interactions, or “cross talk,” between EGF-induced signals and G protein-coupled receptor signaling pathways are recognized in a variety of tissues (18, 19, 44, 47). Earlier studies showed that EGF administration to rats modulates muscarinic receptor activation of salivary secretion (46). Using a ductal cell line (HSY) from human parotid (57), we recently demonstrated (60) inhibition of muscarinic receptor-mediated [Ca2+]i mobilization by EGF. In contrast, EGF regulation of signaling events mediating β-adrenergic receptor-linked functions in salivary cells has not been described previously. In the present study we have examined the effect of EGF on the β-adrenergic receptor-stimulated

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adenylyl cyclase system in HSY cells. Our results indicate that EGF enhances β-adrenergic receptor signaling via an increase in β-adrenergic receptor expression at the transcriptional level. Moreover, the stimulatory action of EGF on β-adrenergic responsiveness appears to be mediated by activation of ERK/MAPK but not PKC.

MATERIALS AND METHODS

Materials. Recombinant human (rh) EGF, insulin-like growth factor-I (rhIGF-I), and transforming growth factor-α (rhTGF-α) were purchased from Promega (Madison, WI); platelet-derived growth factor (rhPDGF-AB) was from Peprotech (Rocky Hill, NJ). (-)-[125I]-iodopindolol (2,200 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA). Dulbecco’s modified Eagle’s medium (DMEM) was from Life Technologies (Gaithersburg, MD). (-)-Isoprotorenol (+) bitartrate salt, phorbol 12-myristate 13-acetate (PMA), 3-isobutyl-1-methylxantine (IBMX), prostaglandin E2 (PGE2), chola toxin, actinomycin D, and other chemicals were purchased from Sigma (St. Louis, MO). CGP-20712A methanesulfonate was obtained from Research Biochemicals International (RBI; Natick, MA), and forskolin was from Calbiochem (San Diego, CA). Rabbit antibodies against p44/p42MAPK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling Technology (Beverly, MA), respectively; goat anti-rabbit IgG Fc conjugated to horseradish peroxidase was from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell culture. The HSY cell line, which was originally established by Yanagawa et al. (57), was kindly provided by Dr. James Turner [National Institute of Dental and Craniofacial Research (NIDCR), National Institutes of Health, Bethesda, MD]. Cells were plated at a density of ~2 × 104 cells/cm2 in 48-well culture plates (for adenyl cyclase assay) or 100-mm culture dishes (for β-adrenergic receptor binding assay and Northern and immunoblot analyses) and cultured in DMEM supplemented with 10% fetal calf serum (FCS) and penicillin (100 U/ml)-streptomycin (100 μg/ml) at 37°C in a humidified 5% CO2 atmosphere incubator. Unless otherwise specified, cells were grown to near confluency at 72 h. EGF and other growth factors (TGF-α, PDGF, or IGF-I) were generally added to the culture medium containing 10% FCS 48 h before confluence, i.e., 24 h after plating. Under these conditions EGF caused a small but significant increase in cell number (~16% increase; P < 0.005) at 72 h. Adenyl cyclase assay. Adenyl cyclase activity was measured in intact HSY cells cultured for 72 h. Enzyme activity was determined as the conversion of [3H]ATP to [3H]cAMP after cellular incorporation of [3H]adenine, as described previously (20). Briefly, cells were incubated with [3H]adenine (1 μCi/well) in DMEM for 2 h at 37°C and then exposed to adenyl cyclase-stimulating agents (isoprotorenol, PGE2, or forskolin) for 10 min in the presence of the phosphodiesterase inhibitor IBMX (0.5 mM). For measurements of cholera toxin-stimulated adenyl cyclase activity, cholera toxin (1 μg/ml) was present during the 2-h incubation with [3H]adenine. The reaction was terminated with ice-cold trichloroace- tic acid (0.12 mM), and the [3H]cAMP product was isolated by two-column chromatography (19). Cell numbers were determined in separate cultures grown under conditions identical to those used for cells undergoing enzyme assay. The ade- 

nyl cyclase activity is expressed as counts per minute (cpm) of [3H]cAMP per 104 cells per 10-min incubation period.

β-Adrenergic receptor binding assay. β-Adrenergic receptor binding in membrane preparations from HSY cells cultured for 72 h was measured by an equilibrium binding assay using the radiolabeled β-adrenergic receptor antagonist (-)-[125I]-iodopindolol (28). Membranes (43,000 g pellets) were prepared as described previously (60), and 10–100 μg of membrane protein were incubated with (-)[125I]iodopindolol in 125–250 μl of reaction buffer (15.2 mM Na-HEPES (pH 7.5), 115 mM NaCl, 0.66 mM l-ascorbic acid) for 30 min at 30°C. Reactions were terminated by adding 4 ml of wash buffer [10 mM Tris (pH 7.5), 154 mM NaCl] at room temperature, and membrane-bound radioligand was collected on Whatman glass fiber filters (GF/F) with a Brandel Cell Harvester (Biomedical Research and Development Laboratories, Gaithersburg, MD). Nonspecific binding of (-)-[125I]iodopindolol was defined as the amount of radioligand bound in the presence of an excess (10−3 M) of the β-adrenergic agonist (-)-isoprotorenol. Specific binding was 70–90% of the total binding at radioligand concentrations in the range of 10−11 to 10−4 M.

Saturation binding curves were constructed by measuring specific binding of (-)-[125I]iodopindolol at eight concentrations of radioligand in the range of 0.01–0.5 nM. Competition binding studies were performed by measuring the binding of (-)-[125I]iodopindolol (at a concentration approximating the KD) in the presence of 17–19 concentrations of nonlabeled (-)-isoprotorenol (a nonselective β-adrenergic receptor ago- 

nist; Ref. 36), ICI-118,551 (a selective β2-adrenergic receptor antagonist; Ref. 6), or CGP-20712A (a selective β1 adrenergic receptor antagonist; Ref. 15) in the range of 10−11 to 10−4 M. Northern blot analysis. Total RNA was isolated from HSY cells by the guanidinium thiocyanate-phenol-chloroform extraction method with TRI Reagent (Molecular Research Center, Cincinnati, OH; Refs. 12, 13). RNA samples (20 μg) were electrophoresed on 1.2% agarose-formaldehyde gels and transferred to nitrocellulose membranes (Micron Separations, Westborough, MA). The membranes were presoaked, hybridized with 32P-labeled β1 or β2-adrenergic receptor cDNA insert, and washed as described previously (59). Each membrane was then exposed to a PhosphorImager screen for 1–10 days, and hybridization signals were quantified with a Molecular Dynamics Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). As a control for RNA loading and transfer, the membranes were stripped and reprobed with a 5′-end labeled antisense 18S ribosomal RNA oligonucleotide (5′-GCCGTGCCTACATTAGACATGCATG), followed by washing and exposure to PhosphorImager screens for 30 min.

The β1-adrenergic receptor cDNA, a Petl fragment of the rat β1-adrenergic receptor gene in pGEM3zf (+), was a generous gift from Dr. C. A. Machida (Oregon Regional Primate Research Center, Beaverton, OR; Ref. 49). The β2-adrenergic receptor probe, a NcoI/SalI fragment of human β2-adrenergic receptor cDNA in the expression vector pBC12MI, was kindly provided by Dr. R. J. Lefkowitz (Duke University, Durham, NC). RNA samples from rat hippocampus and COS-7 cells (American Type Culture Collection, Manassas, VA) were used as positive controls for β1- and β2-adrenergic receptor mRNAs, respectively.

Immunoblot analysis of ERK/MAPK. Immunoblot analysis was performed as described previously, with minor modification (60). HSY cells cultured for 24 h were washed three
times with cold PBS and lysed in a buffer containing 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 10% NP-40, 2 mM EDTA, 0.5 mM EGTA, 2.5 μg/ml leupeptin, 10 μg/ml aprotinin, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate (Na3VO4). After centrifugation of cell lysates at 8,000 g for 2 min at 4°C, supernatant proteins (50 μg) were separated on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Schleicher & Schuell, Keene, NH). The membranes were immunoblotted with p44/p42MAPK or phospho-p44/p42MAPK primary antibody (1:1,000) and a secondary horseradish peroxidase-conjugated antibody (1:1,000). MAPKs were visualized by an enhanced chemiluminescence system (ECL Plus, Amersham Pharmacia Biotech) and quantified with ImageQuant computer software (version 5; Molecular Dynamics, Sunnyvale, CA).

Data analysis. Data from multiple experiments are expressed as means ± SE. Statistical significance of single comparisons was determined with Student’s t-test. Multiple comparisons were performed by analysis of variance (ANOVA) followed by Sidak’s multiple-comparison test. Scattered data were analyzed with a weighted, nonlinear least-squares curve-fitting program (28).

RESULTS

Enhancement of isoproterenol-stimulated adenylyl cyclase activity by EGF. The effect of EGF on β-adrenergic-responsive adenylyl cyclase activity in HSY cells is shown in Fig. 1. The β-adrenergic agonist isoproterenol (10⁻⁵ M) stimulated adenylyl cyclase activity by about twofold relative to unstimulated (basal) enzyme activity. The isoproterenol response was markedly increased (1.8 ± 0.1-fold) by treatment of cells with EGF (5 × 10⁻¹⁰ M, 48 h), even though the basal level of adenylyl cyclase activity was slightly reduced (~12%) by EGF treatment. Enhancement of isoproterenol-stimulated adenylyl cyclase activity was dependent on EGF concentration and time of incubation with the growth factor (Fig. 2). The stimulatory effect of EGF was maximal at ~5 × 10⁻¹⁰ M (3 ng/ml), with half-maximal effect at 1 × 10⁻¹⁰ M (Fig. 2A). The response to isoproterenol increased within 24 h of EGF treatment, with progressive enhancement observed through 48 h (Fig. 2B).

To examine the specificity of EGF action on isoproterenol-stimulated adenylyl cyclase, we compared isoproterenol responses in HSY cells treated with EGF and other growth factors (TGF-α, PDGF, and IGF-I). TGF-α acts by binding to EGF receptors (11), whereas PDGF and IGF-I activate distinct receptor tyrosine kinases. TGF-α (3 ng/ml), like EGF, enhanced isoproterenol responsiveness in HSY cells (1.4 ± 0.1-fold, n = 17; P < 0.001). In contrast, PDGF (10 ng/ml) and IGF-I (50 ng/ml) had no effect on isoproterenol-stimulated adenylyl cyclase activity (data not shown). The concentrations of PDGF and IGF-I used in these experiments have been reported to elicit biological responses in a variety of cell types. The results implicate a specific
stimulatory effect of EGF receptor activation on isoproterenol-responsive adenylyl cyclase in HSY cells.

Adenylyl cyclase activation via stimulatory receptors other than β-adrenergic receptors was not enhanced by EGF. In the current study we found that adenylyl cyclase in HSY cells is linked not only to β-adrenergic receptors but also to stimulatory receptors for prostaglandins. However, unlike the β-adrenergic response, stimulation of adenylyl cyclase by PGE2 was unchanged after EGF treatment (Fig. 3). We also determined whether EGF modified non-receptor-mediated activation of adenylyl cyclase in response to cholera toxin, which stimulates enzyme activity by catalyzing ADP-ribosylation of the stimulatory Gs protein, and forskolin, which exerts a direct stimulatory effect on adenylyl cyclase (48). EGF had no effect on cholera toxin-stimulated enzyme activity. In contrast, a significant reduction (~20%, P < 0.002) in the forskolin response was observed after EGF treatment, suggesting an inhibitory effect of the growth factor at the level of the adenylyl cyclase enzyme (Fig. 3).

**Effects of EGF on β-adrenergic receptors.** Because EGF preferentially increased β-adrenergic responsive adenylyl cyclase activity, we examined the effects of EGF on β-adrenergic receptors assessed by binding of the β-adrenergic receptor antagonist (-)[125I]iodopindolol to HSY cell membrane preparations. Scatchard analysis of (-)[125I]iodopindolol saturation binding curves revealed a single class of binding sites with high affinity (Kd = 9.6 ± 1.7 × 10^{-11} M) for the radioligand. In untreated HSY cells the density (Bmax) of β-adrenergic receptors was 4.4 ± 0.8 fmol/mg protein. EGF caused a threefold increase in Bmax (12.9 ± 2.5 fmol/mg protein; P < 0.001 vs. untreated cells) without affecting receptor binding affinity (Kd = 11.1 ± 3.2 × 10^{-11} M; Fig. 4).

Modulation of β-adrenergic receptor binding by EGF was further characterized in studies measuring competition for (-)[125I]iodopindolol binding sites by unlabeled isoproterenol and antagonists selective for β2- and β1-adrenergic receptor subtypes. The results of the competition binding studies and curve-fitting analysis of binding data are presented in Fig. 5 and Table 1, respectively. As in a variety of other tissues, competition studies with HSY cell membranes demonstrated binding of the nonselective β-adrenergic agonist isoproterenol to both high- and low-affinity binding sites. Treatment of HSY cells with EGF resulted in a threefold increase (P < 0.02) in the number of receptor sites binding isoproterenol with high affinity; an apparent increase in the number of low-affinity receptors was not statistically significant (Fig. 5A; Table 1). Both high- and low-affinity binding sites for the β2-selective adrenergic receptor antagonist ICI-118,551 were also identified in HSY cell membranes (Fig. 5B). In contrast, only a single class of low-affinity binding sites for the β1-selective adrenergic receptor antagonist CGP-20712A was detected (Fig. 5C). The sites binding ICI-118,551 with high affinity, presumably receptors of the β2-adrenergic subtype, were increased about threefold (P < 0.01) in number by EGF treatment. Growth factor-induced increases in low-affinity binding for both β1- and β2-selective antagonists were not statistically significant. The dissociation constants of high- and low-affinity binding sites for isoproterenol, ICI-118,551, and CGP-20712A were unaltered by EGF (Table 1). Together with the (-)[125I]iodopindolol saturation binding data, the results of the competition studies suggest that increased high-affinity binding of (-)[125I]iodopindolol and isoproterenol in EGF-treated HSY cells reflects at least in part an increased number of receptors of the β2-adrenergic subtype.

The β1- and β2-adrenergic receptor subtypes, like many G protein-coupled receptors, are subject to well-recognized regulatory processes in which the number of cell surface receptors decreases progressively over time of exposure to agonist (53). In view of the stimulatory action of EGF on β-adrenergic receptor number in HSY cells, we performed studies to determine whether agonist-induced reduction of membrane receptor content might be altered by growth factor treatment. Figure 6 shows that exposure of HSY cells to isoproterenol (10^{-5} M) caused a time-dependent decrease in (-)[125I]iodopindolol binding to cell membranes, with a >40% decline in radioligand binding occurring by 30 min and >80% loss of binding by 24 h.

![Fig. 3. Effects of EGF on prostaglandin receptor and non-receptor-mediated activation of adenylyl cyclase. HSY cells were treated with EGF (+EGF; 5 × 10^{-10} M) or left untreated (-EGF) for 48 h and then assayed for adenylyl cyclase responses to prostaglandin E2 (PGE2, 10^{-6} M; n = 5 experiments), cholera toxin (1 μg/ml; n = 4), and forskolin (10^{-7} M; n = 21) as described in MATERIALS AND METHODS. Values represent mean ± SE enzyme activities; *P < 0.002 vs. -EGF. Unstimulated, or basal, activities with and without EGF treatment were also measured in each experiment; the effect of EGF on basal activity was similar to that shown for a larger number of experiments in Fig. 1.](http://ajpcell.physiology.org/doi/fig/10.1152/ajpcell.00021.2003)
Treatment of cells with EGF had no significant effect on isoproterenol-induced decreases in (125I)iodopindolol binding. Thus EGF increases β2-adrenergic receptor binding in HSY cells by regulatory processes that appear to be unrelated to those induced by β1-adrenergic agonist.

We next examined the effect of EGF on expression of β1- and β2-adrenergic receptor subtypes at the mRNA level. Treatment of HSY cells with EGF for periods of 1–24 h produced a biphasic increase in the levels of β2-adrenergic receptor mRNA assessed by Northern blot analysis. As shown in Fig. 7, β2-adrenergic receptor mRNA levels increased transiently by threefold at 1 h of EGF treatment; after a return to the basal level of expression at 4 h, receptor mRNA levels rose again by twofold at 8 h and remained elevated through 24 h of EGF exposure. In contrast, treatment of HSY cells with EGF for periods up to 24 h had no effect on β1-adrenergic receptor mRNA expression, with the exception of a relatively small increase (40%) in mRNA.

Table 1. Effects of EGF on high- and low-affinity binding sites for isoproterenol, ICI-118,551, and CGP-20712A

<table>
<thead>
<tr>
<th>Ligand Competing for (125I)Iodopindolol Binding</th>
<th>Isoproterenol</th>
<th>ICI-118,551</th>
<th>CGP-20712A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 6)</td>
<td>(n = 7)</td>
<td>(n = 4)</td>
</tr>
<tr>
<td></td>
<td>RH</td>
<td>RL</td>
<td>RH</td>
</tr>
<tr>
<td>−EGF</td>
<td>2.57 ± 0.63</td>
<td>2.62 ± 0.48</td>
<td>2.91 ± 0.36</td>
</tr>
<tr>
<td>+EGF</td>
<td>7.37 ± 1.52*</td>
<td>9.99 ± 3.45</td>
<td>9.79 ± 2.02**</td>
</tr>
<tr>
<td></td>
<td>KH</td>
<td>KL</td>
<td>KH</td>
</tr>
<tr>
<td>−EGF</td>
<td>1.57 ± 0.71</td>
<td>239 ± 81</td>
<td>3.14 ± 1.48</td>
</tr>
<tr>
<td>+EGF</td>
<td>3.73 ± 1.76</td>
<td>219 ± 48</td>
<td>1.41 ± 0.48</td>
</tr>
</tbody>
</table>

Values are means ± SE from n experiments. Competition for (125I)iodopindolol binding by isoproterenol, ICI-118,551 and CGP-20712A was measured in cell membranes from EGF (+EGF, 5 × 10−10 M)-treated and untreated (−EGF) HSY cells. Competition binding curves from the experiments presented in Fig. 5 were subjected to curve-fitting analysis as described in MATERIALS AND METHODS. Isoproterenol and ICI-118,551 competition curves were best fit by a 2-site model describing high- and low-affinity binding sites; CGP-20712A binding data were best fit by a 1-site model of low-affinity binding. RH and RL (fmol/mg protein), numbers of sites binding isoproterenol, ICI-118,551, or CGP-20712A with high and low affinity; KH and KL, dissociation constants (nM) of high- and low-affinity binding sites. * P < 0.02 vs. −EGF; ** P < 0.01 vs. −EGF.
levels observed at 1 h (Fig. 7). The increase in \( \beta_2 \)-adrenergic receptor mRNA induced by 1 h of EGF treatment was blocked by actinomycin D, an inhibitor of RNA synthesis (Fig. 8). These findings suggest that although both \( \beta_1 \)- and \( \beta_2 \)-adrenergic receptor subtypes are expressed in HSY cells, EGF selectively stimulates \( \beta_2 \)-adrenergic receptor expression at the level of transcription.

Effects of EGF-induced signals on \( \beta_2 \)-adrenergic receptor-coupled adenylyl cyclase. The EGF receptor tyrosine kinase modulates cellular function by activating a variety of intracellular signaling cascades including the PKC and ERK/MAPK pathways (14, 37, 38). Previous studies indicate that activation of PKC regulates \( \beta \)-adrenergic-responsive adenylyl cyclase activity in several cell types through effects on \( \beta \)-adrenergic receptor expression, receptor coupling to G protein, and activity of the adenylyl cyclase enzyme (7, 34, 42). We therefore examined whether EGF-induced enhancement of \( \beta \)-adrenergic receptor signaling in HSY cells may be dependent on activation of PKC. PKC was downregulated by pretreatment of cells with the phorbol ester PMA (10\(^{-5}\) M) for 4–6 h before addition of EGF. Exposure of HSY cells and other cell types to high concentrations (\( \geq 10^{-5}\) M) of PMA for periods of >1 h results in nearly complete loss of PKC activity and/or immunoreactive PKC isoforms (30, 58, 60). As shown in Fig. 9, EGF caused a twofold increase in isoproterenol-responsive adenylyl cyclase activity irrespective of whether cells were pretreated with PMA; PMA also had no effect on the threefold increase in \( \beta \)-adrenergic receptor density induced by EGF. These findings suggest that activation of PKC is not required for the stimulatory effect of EGF on \( \beta \)-adrenergic receptor signaling. Interestingly, PMA itself produced twofold increases in both isoproterenol responsiveness and \( \beta \)-adrenergic receptor density with or without addition of EGF. Moreover, PMA and EGF in combination augmented the isoproterenol response and \( \beta \)-adrenergic receptor density to a much greater extent (4- and >6-fold increases, respectively) than did either

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Fig. 5. Competition for \((-)\)\(^{125}\)I-iodopindolol binding sites by isoproterenol, ICI-118,551, and CGP-20712A: effects of EGF. Cell membranes were obtained from HSY cells treated with EGF (+EGF; 5×10\(^{-10}\) M) or left untreated (−EGF) for 48 h. Binding of \((-)\)\(^{125}\)I-iodopindolol to cell membranes was assayed in the presence of increasing concentrations of unlabeled isoproterenol (A), ICI-118,551 (B), and CGP-20712A (C) as described in MATERIALS AND METHODS. Composite curves are presented with mean ± SE values from 4–7 experiments.

Fig. 6. Isoproterenol-induced loss of \((-)\)\(^{125}\)I-iodopindolol binding to membranes from EGF-treated and untreated HSY cells. HSY cells cultured for a total of 72 h were treated with EGF (+EGF; 5×10\(^{-10}\) M) or left untreated (−EGF) for 48 h as described in MATERIALS AND METHODS; cells were preincubated with isoproterenol (10\(^{-5}\) M) for 0–24 h before the end of the 72-h culture period. Specific binding of \((-)\)\(^{125}\)I-iodopindolol to cell membrane preparations was measured at a saturating concentration (0.25 nM) of radioligand. Binding data are expressed as % of control binding measured in the absence of isoproterenol preincubation [100% binding at time 0 = 4.9 ± 0.4 fmol/mg protein (−EGF), 14.4 ± 2.1 fmol/mg protein (+EGF); n = 10 experiments]. Values are means ± SE from 3–10 experiments. No significant effect (\(P > 0.05\)) of EGF on % binding was observed at any time of isoproterenol preincubation.
agent alone (Fig. 9). Thus PMA and EGF appear to exert independent modulatory influences on the \( \beta_2 \)-adrenergic receptor-coupled adenylyl cyclase system in HSY cells.

Other experiments were performed to examine whether the ERK/MAPK pathway may be involved in regulation of \( \beta_2 \)-adrenergic receptor signaling by EGF. The effect of EGF on isoproterenol-stimulated adenylyl cyclase activity was determined in HSY cells pretreated with PD-98059, a synthetic inhibitor of the ERK/MAPK pathway. The inhibitor abolished the increase in isoproterenol-responsive enzyme activity produced by EGF. In control experiments EGF was observed to induce phosphorylation of ERK in HSY cells, and this action of EGF was inhibited by PD-98059 (Fig. 10). The results suggest that EGF-induced enhancement of \( \beta_2 \)-adrenergic receptor signaling is mediated by activation of the ERK/MAPK pathway.

**DISCUSSION**

The results of this study demonstrate that EGF enhances \( \beta_2 \)-adrenergic receptor-mediated activation of adenylyl cyclase in a human salivary gland cell line (HSY) (Fig. 1). The stimulatory action of EGF is directed specifically at the \( \beta_2 \)-adrenergic receptor signaling pathway, because prostaglandin receptor-responsive adenylyl cyclase activity is unaffected by growth factor treatment. Moreover, EGF has no stimulatory effect on enzyme activation by agents acting directly on \( G_{\alpha} \) or adenylyl cyclase (cholera toxin and forskolin, respectively; Fig. 3). Receptor binding experiments, including competition studies using antagonists selective for \( \beta_2 \) and \( \beta_1 \)-adrenergic receptor subtypes, confirm that increased \( \beta_2 \)-adrenergic responsiveness in EGF-treated HSY cells reflects a threefold increase in the number of membrane receptors of the \( \beta_2 \)-adrenergic subtype. In our studies no high-affinity binding sites of the \( \beta_1 \)-adrenergic subtype were detected in either control or EGF-treated cells (Figs. 4 and 5; Table 1).

Our work is the first to report that in HSY cells \( \beta_2 \)-adrenergic receptors are predominantly of the \( \beta_2 \)-type.
have observed in EGF-treated HSY cells is not related to altered mechanisms of agonist-induced receptor regulation, because losses of receptor binding following isoproterenol exposure were unaffected by EGF pretreatment (Fig. 6). In contrast to this finding, EGF treatment of vascular smooth muscle cells has been shown to increase the cell surface content of the multifunctional receptor low-density lipoprotein receptor-related protein by altering receptor distribution and recycling (56).

EGF-induced upregulation of β-adrenergic receptor content in HSY cells is likely to result, at least in part, from an increase in β2-adrenergic receptor mRNA expression. Northern blot analyses revealed a bimodal increase in levels of β2-adrenergic receptor subtype.

Agonist-induced reduction of membrane receptor content is characteristic of β-adrenergic and other G protein-coupled receptors. Interestingly, the two subtypes of the β-adrenergic receptor undergo distinct patterns of agonist-induced regulation. Losses of receptor binding are of greater magnitude, and occur earlier after agonist exposure, for the β2- than the β1-adrenergic receptor subtype (53). In HSY cells the marked decline in membrane β-adrenergic receptor binding following agonist exposure closely resembles that previously reported for the β2-adrenergic receptor subtype (53). The upregulation of β2-adrenergic receptors was

Fig. 9. Enhancement of β-AR signaling by EGF: effects of pretreatment with phorbol 12-myristate 13-acetate (PMA). HSY cells cultured for 24 h were incubated with or without PMA (10^{-5} M) for 4–6 h, followed by addition of EGF (5 \times 10^{-9} M) or vehicle for 44 h in the continued presence of PMA. Cells were then assayed for isoproterenol (10^{-5} M)-stimulated and unstimulated (basal) adenylyl cyclase activities or used to obtain membranes for determination of β-AR density (B_{max}) by Scatchard analysis of [(-)-[125]iodopindolol saturation binding curves (see MATERIALS AND METHODS). Open bars, means ± SE of isoproterenol-stimulated adenylyl cyclase activities from 11 experiments. Hatched bars, means ± SE of β-AR density (B_{max}) from 5 experiments. EGF and PMA individually increased values of isoproterenol-stimulated adenylyl cyclase activity and B_{max} relative to values in untreated cells (P < 0.01); isoproterenol responsiveness and B_{max} in cells treated with both EGF and PMA were greater than in cells treated with either agent alone (P < 0.01). PMA had no effect on basal adenylyl cyclase activity (data not shown).

Fig. 10. Effects of PD-98059 on EGF-induced enhancement of isoproterenol-stimulated adenylyl cyclase activity and phosphorylation of extracellular signal-regulated kinase (ERK). A: HSY cells cultured for 24 h were treated with PD-98059 (+ PD-98059; 2.5 \times 10^{-5} M) or left untreated (−PD-98059) for 30 min, followed by addition of EGF (+EGF, 5 \times 10^{-10} M) or vehicle (−EGF) for 48 h. Cells were then assayed for isoproterenol (10^{-5} M)-stimulated and unstimulated (basal) adenylyl cyclase activities as described in MATERIALS AND METHODS. Values represent means ± SE of isoproterenol-stimulated adenylyl cyclase activity from 15 experiments. *P < 0.0001 vs. −EGF. PD-98059 had no effect on basal enzyme activity (data not shown). B: Cells cultured for 24 h were treated with PD-98059 (2.5 \times 10^{-5} M) or left untreated as in A. Phosphorylation of ERK (p44/p42MAPK) was determined after 10-min incubation with or without EGF (5 \times 10^{-10} M) by immunoblot analysis as described in MATERIALS AND METHODS. In this representative experiment PD-98059 inhibited EGF-induced phosphorylation of p44/p42MAPK by 41%; when added at a higher concentration (10^{-4} M), PD-98059 caused 84% inhibition of ERK phosphorylation (data not shown).
mRNA after addition of EGF, with two- to threefold elevations of receptor mRNA levels occurring at 1 h and again at 8–24 h of EGF exposure (Fig. 7). The time required for expression and membrane targeting of increased numbers of β2-adrenergic receptors presumably accounts for the finding that EGF enhances isoproterenol-responsive adenyl cyclase activity only after prolonged incubation periods (≥24 h) (Fig. 2). The increase in β2-adrenergic receptor mRNA observed at 1 h of EGF treatment was found to be blocked by the RNA synthesis inhibitor actinomycin D; in the absence of growth factor, receptor transcript levels were unaffected by actinomycin D added for 1 h (Fig. 8). These results suggest that EGF upregulates expression of the β2-adrenergic receptor subtype by a mechanism involving increased transcription of the receptor gene. Our experiments do not exclude the possibility of an effect of EGF on β2-adrenergic receptor mRNA stability over longer periods of growth factor exposure. In earlier studies EGF was shown to reduce the expression of other G protein-coupled receptors, i.e., luteinizing hormone/chorionic gonadotropin receptors in Leydig tumor cells and angiotensin II receptors in vascular smooth muscle cells, through inhibitory effects on receptor gene transcription (40, 41). Interestingly, the 5′-flanking region of the human and/or rat β2-adrenergic receptor gene contains recognition sites for several transcription factors (nuclear factor-κB, Sp1, cAMP response element-binding protein) implicated in the cellular actions of EGF (26). However, additional studies will be required to clarify whether EGF regulates expression of the β2-adrenergic receptor gene by transcriptional or posttranscriptional mechanisms.

Regulation of β-adrenergic receptor expression by EGF had not been described before the current study in HSY cells. Moreover, only limited observations relating to long-term effects of EGF on adenyl cyclase activation via β-adrenergic or other stimulatory G protein-coupled receptors have been reported in other cell types. In an earlier study, prolonged (24–48 h) incubation of rat granulosa cells with EGF was found to reduce follicle-stimulating hormone activation of adenyl cyclase (31). Work from our laboratory (19) showed that exposure of a rat clonal osteoblast-like cell line (UMR-106) to EGF for 48 h decreased adenyl cyclase stimulation by isoproterenol and parathyroid hormone (PTH) but not PGE2. Although we did not determine the effect of EGF on β-adrenergic receptor binding in UMR-106 cells, a subsequent study by others demonstrated reduced numbers of PTH receptors in UMR-106 cells treated for 22 h with EGF (5). In this study PTH receptor binding was found to be distributed heterogeneously among morphologically distinct populations of UMR-106 cells. Of note, EGF appeared to amplify a proliferating pool of UMR-106 cells containing reduced numbers of PTH receptors and at the same time deplete a quiescent population of receptor-enriched cells (5). The distribution of β-adrenergic receptors among UMR-106 or HSY cell populations has not been analyzed. Nonetheless, the contrasting effects of EGF on isoproterenol-responsive adenyl cyclase activities in the two cell types suggest at least some degree of tissue specificity in the stimulatory action of EGF on β-adrenergic receptor expression observed in HSY cells. Decreased forskolin stimulation of adenyl cyclase activity in both HSY and UMR-106 cells, and reduced basal enzyme activity in HSY cells, after long-term treatment with EGF may reflect an inhibitory effect of the growth factor on one or more adenyl cyclase isozymes common to a number of tissues (Figs. 1 and 3; Refs. 19, 22). It should be emphasized that EGF also causes rapid (i.e., occurring within minutes) changes of adenyl cyclase activities in a variety of in vitro systems, including rat parotid cell membranes (39, 47). These short-term effects of EGF, which are generally thought to be mediated by Gs and/or the inhibitory G, protein, are distinct from the changes in G protein-coupled receptor expression and adenyl cyclase activation occurring in cells after prolonged exposure to EGF.

A number of observations confirm that the stimulatory effect of EGF on β-adrenergic receptor expression in HSY cells is mediated by signaling events of the EGF receptor tyrosine kinase pathway. The concentration range over which EGF increases isoproterenol-stimulated adenyl cyclase activity (Fig. 2) is characteristic of growth factor actions at the EGF receptor (19). The finding that isoproterenol responsiveness was increased by both EGF and TGF-α, which act by binding to the EGF receptor, but not by agonists (PDGF, IGF-1) binding to other receptor tyrosine kinases further implicates involvement of the EGF receptor tyrosine kinase in growth factor regulation of β-adrenergic receptor function in HSY cells. In preliminary experiments (not shown) we have also detected the expression of immunoreactive EGF receptors in HSY cells by immunoblot analysis. Because the EGF receptor tyrosine kinase activates the ERK/MAPK signaling cascade in many tissue types including the HSY cell line (60), we examined whether ERK/MAPK may play a role in the modulation of β-adrenergic responsiveness by EGF. The ERK/MAPK pathway inhibitor PD-98059 abolished the increase in isoproterenol-stimulated adenyl cyclase activity caused by EGF; EGF-induced phosphorylation of ERK/MAPK was also inhibited by PD-98059 (Fig. 10). Thus the regulatory action of EGF on the expression of functional β-adrenergic receptors appears to be exerted at least partly via EGF receptor tyrosine kinase activation of the ERK/MAPK signal transduction pathway. Recent studies have suggested that the EGF receptor may also translocate to the nucleus and act as a transcription factor (35). It is not known whether this alternate signaling pathway might occur in salivary cells and play a modulatory role in β-adrenergic receptor expression.

The EGF receptor tyrosine kinase is also coupled to activation of PKC in numerous tissues. In our studies EGF-induced increases in isoproterenol-responsive adenyl cyclase activity and β-adrenergic receptor number were unchanged after downregulation of PKC by prolonged (4–6 h) exposure of HSY cells to phorbol ester (Fig. 9). This finding suggests that activation of
PKC does not play any significant role in the modulatory action of EGF on β-adrenergic receptor expression. Treatment with phorbol ester alone was found to increase the expression of functional β-adrenergic receptors (Fig. 9), although under the experimental conditions used it could not be determined whether this effect of phorbol ester was attributable to initial activation or subsequent downregulation of PKC. Phorbol ester-induced activation of PKC was reported previously to decrease expression of β-adrenergic receptor mRNA, but not β1- and β2-adrenergic receptor transcripts, in murine 3T3-F442A adipocytes and also β1-adrenergic receptor gene transcription in rat C6 glioma cells (16, 34). Whereas β2-adrenergic receptor binding was said to be downregulated in phorbol ester-treated C6 cells, supportive receptor binding data were not presented (34). PKC exerts a complex array of regulatory effects on the functions and distribution of the EGF receptor (2). As indicated above, however, any possible changes in EGF receptor action induced by either activation or downregulation of PKC were no longer apparent in HSY cells after 4- to 6-h exposure to phorbol ester. Because PMA in combination with EGF produced such a marked increase (>6-fold) in β-adrenergic receptor content (Fig. 9), additional studies are warranted to define further the signaling processes by which PKC and EGF receptor tyrosine kinase may function as independent yet interactive determinants of β-adrenergic receptor expression in HSY cells.

In recent years it has become increasingly clear that activation of G protein-coupled receptors regulates not only classic effectors of signal transduction (e.g., adenyl cyclase and phospholipase C) but also mitogenic signaling cascades (e.g., ERK and other MAPK pathways) involved in cell growth and differentiation. Many G protein-coupled receptors, including β2-adrenergic receptors, exert proliferative effects via “transactivation” of EGF and other tyrosine kinase receptors coupled to the ERK/MAPK pathway (18, 44). The growth-promoting effects of isoproterenol on rat parotid acinar cells have long been thought to involve activation of the EGF receptor (45). Interestingly, growth of rat parotid glands after chronic administration of isoproterenol in vivo is accompanied by an increase in the number of parotid membrane β2-adrenergic receptors (with a corresponding decline in β1-adrenergic receptor content) (25). This observation is consistent with possible involvement of the β2-adrenergic receptor subtype in the control of salivary cell growth (24); a similar growth-promoting role for the β2-adrenergic receptor has been demonstrated in rat FRTL5 thyroid cells (23). Together with previous data, then, our studies of HSY cells suggest a reciprocal relationship between β2-adrenergic and EGF receptors, in which upregulation of β2-adrenergic receptors induced by the EGF receptor tyrosine kinase signaling pathway could in turn promote salivary cell growth via EGF receptor transactivation. In this regard it should be emphasized that activation of the EGF receptor plays a critical role in salivary gland morphogenesis (9, 55). Also, the HSY cell line used in our experiments is thought to originate from salivary intercalated duct cells, which may act as progenitors in the replenishment of both acinar and duct cells. Thus EGF-induced upregulation of β-adrenergic receptor signaling, as observed in HSY cells, could conceivably contribute to EGF-dependent processes involved in salivary gland development and regeneration.

In summary, the present study demonstrates for the first time that in a human salivary cell line (HSY) EGF enhances β-adrenergic-responsive adenyl cyclase activation via an increase in β2-adrenergic receptor expression at the transcriptional level. We previously showed (60) that, in contrast to β-adrenergic receptor signaling, the muscarinic receptor-mediated calcium mobilization pathway in HSY cells is inhibited by EGF. The effects of EGF on both β-adrenergic and muscarinic receptor signaling pathways appear to be mediated by the ERK/MAPK cascade and potentiated by distinct modulatory actions of PKC. Our findings provide the basis for further investigation of mechanisms by which cross talk between EGF receptor tyrosine kinase and G protein-coupled receptor signaling pathways may function in the regulation of salivary cell secretion and growth.

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