β-Adrenergic-responsive activation of extracellular signal-regulated protein kinases in salivary cells: role of epidermal growth factor receptor and cAMP

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1Geriatric Research, Education and Clinical Center and 2Research Service, Audie L. Murphy Division, South Texas Veterans Health Care System; and Departments of 3Dental Diagnostic Science, 4Surgery, 5Community Dentistry, and 6Medicine, University of Texas Health Science Center at San Antonio, San Antonio, Texas

Submitted 29 July 2004; accepted in final form 27 January 2005

Yeh, Chih-Ko, Paramita M. Ghosh, Howard Dang, Qun Liu, Alan L. Lin, Bin-Xian Zhang, and Michael S. Katz. β-Adrenergic responsive activation of extracellular signal-regulated protein kinases in salivary cells: role of epidermal growth factor receptor and cAMP. Am J Physiol Cell Physiol 288: C1357–C1366, 2005.—The β-adrenergic receptor agonist isoproterenol exerts growth-promoting effects on salivary glands. In this study, activation of ERKs, members of the mitogen-activated protein kinase family, by isoproterenol was examined in a human salivary gland cell line (HSY). Immunoblot analysis indicated that isoproterenol (10−5 M) induced transient activation of ERK1/2 (4.4-fold relative to basal at 10 min) similar to that caused by EGF (6.7 fold). Isoproterenol, like EGF, also induced phosphorylation of the EGF receptor. However, inhibition of EGF receptor phosphorylation by the tyrphostin AG-1478 only partially attenuated isoproterenol-induced ERK phosphorylation, whereas EGF-responsive ERK activation was completely blocked. The Gi, inhibitor pertussis toxin also caused partial inhibition of isoproterenol-stimulated ERK activation. The cAMP analog 8-(4-chlorophenylthio)adenosine 3’,5’-cyclic monophosphate (CPT-cAMP) and the CAMP-elevating agents IBMX and cholera toxin produced transient ERK1/2 activation, similar to the effect of isoproterenol, in HSY cells. The stimulatory effects of isoproterenol and cAMP on ERK phosphorylation were not reduced by the PKA inhibitor H-89, whereas the Src family inhibitor 4-amino-5-(4-chlorophenyl)-1-[(butyl)pyrazolo[3,4-d]pyrimidase (PP2) and transfection of a dominant-negative Src construct diminished isoproterenol-induced ERK activation. Isoproterenol induced marked overexpression of the cell growth-related adhesion molecule CD44, and this effect of isoproterenol was abolished by the ERK pathway inhibitor PD-98059. In summary, we show a dual mechanism of isoproterenol-induced ERK phosphorylation in HSY cells—one pathway mediated by EGF receptor transactivation and the other by an EGF receptor-independent pathway possibly mediated by cAMP. Our results also suggest that isoproterenol-induced growth of salivary tissue may involve ERK-mediated CD44 expression.

THE β-ADRENERGIC RECEPTOR is a member of the G protein-coupled receptor (GPCR) family, a large family of heptahelical or serpentine receptors that consist of seven transmembrane-spanning regions and are involved in the regulation of diverse cellular functions through trimeric guanine nucleotide-binding proteins (G proteins) (17). In the classic paradigm of β-adrenergic signaling, activation of the receptor causes the exchange of GTP for GDP on the α-subunit of Gαβγ, leading to the dissociation of the α-subunit from the βγ-subunits. β-Adrenergic receptors act through Gαs to stimulate adenyl cyclase, resulting in cAMP production and activation of the cAMP-dependent protein kinase, or PKA, cascade. β-Adrenergic receptors may also be coupled to Gαq, which classically inhibits adenyl cyclase. Recent studies have demonstrated that activation of β-adrenergic receptors and other GPCRs also stimulates MAPK cascades that mediate cell proliferation and/or cell differentiation (13, 19).

MAPK pathways constitute an evolutionarily conserved series of serine/threonine kinases traditionally linked to receptor tyrosine kinases such as receptors for EGF and PDGF. The ERKs are one class of MAPKs that are regulated by a three-tiered cascade composed of a MAPK kinase kinase (MAPKKK; e.g., c-Raf1 or B-Raf), a MAPK kinase (e.g., MEK1 and MEK2) and a MAPK (e.g., ERK1 and ERK2) (23). In the past several years, it has been demonstrated that numerous GPCR-stimulated cellular functions are mediated through the MAPK cascades. In a number of systems, β-adrenergic receptor-induced activation of ERKs proceeds through PKA-, Gαs-, and Gαq-, mediated signaling events and may be attributable to the transactivation of the EGF receptor (19). Transactivation of receptor tyrosine kinases after GPCR stimulation has been documented for EGF, PDGF, and insulin-like growth factor receptors and is the subject of several recent reviews (7, 13, 22). However, the mechanisms by which β-adrenergic receptors and other GPCRs activate the ERK/MAPK cascade are cell type-, ligand-, and G protein-specific and may be dependent on the physiological conditions of the cell (18).

In salivary gland tissues, activation of β-adrenergic receptors plays an important role in protein secretion, secretory protein expression, and postnatal development and growth (2). Chronic treatment of rodents with the β-adrenergic receptor agonist isoproterenol has long been known to cause salivary gland enlargement without the development of neoplasm (4, 28). There is some evidence suggesting that MAPKs are activated during isoproterenol treatment. One study showed that Ras guanine nucleotide exchange activity, which triggers the MAPKKK-MEK-MAPK cascade, was increased in parotid cell homogenates from isoproterenol-treated rats compared with untreated rats (31). The same group of investigators reported that in parotid gland lysates from isoproterenol-treated...
rats, levels of phosphorylated ERK2 increased for up to 12 h of treatment, whereas phosphorylated ERK1 levels decreased gradually over time; the activation of ERKs by isoproterenol was apparently not affected by cAMP accumulation or PKA activity (5). Other studies suggested that EGF receptor signaling cascades may be involved in mediating isoproterenol effects on the salivary gland in rodents (14, 26). Despite these earlier studies, the involvement of ERK signaling pathway(s) and downstream targets as mediators of salivary cell responses to β-adrenergic receptor activation has yet to be defined clearly. Among potential targets downstream of ERK signaling in salivary cells is CD44, an adhesion molecule thought to regulate salivary gland growth and development (8, 24).

In the present study we examined the cellular signals linking β-adrenergic receptors with the activation of ERKs in a human salivary cell line (HSY). Our results suggest that isoproterenol activates ERKs via two different signaling pathways: on the one hand, activation of ERKs is induced by transactivation of the EGF receptor, whereas an additional activation pathway possibly mediated by cAMP signaling appears to be at least partly independent of receptor tyrosine kinase. Furthermore, we have identified CD44 as a possible downstream effector of β-adrenergic receptor-induced ERK activation in salivary gland cells.

MATERIALS AND METHODS

Materials. Chemicals including (−)-isoproterenol (+)-bathitrate salt, IBMX, cholera toxin, 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (CPT-cAMP), aprotinin, leupeptin, phenanthroline, pepstatin A, benzamidine HCl, 4-amino-7-phenylpyrazolo[3,4-d]pyrimidine (PP2), 4-amino-7-phenylpyrazolo[3,4-d]pyrimidine (PP3), and sodium orthovanadate (Na3VO4) were obtained from Calbiochem (San Diego, CA). Antibodies against p44/p42MAPK and EGF receptor were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against phospho-p44/p42MAPK (Thr202/Tyr204), phospho-p44/p42MAPK (Tyr1068), and phosphotyrosine (pY) were purchased from Cell Signaling Technology (Beverly, MA). Goat anti-rabbit IgG Fc conjugated antibody (clone G44.26) and phycoerythrin-conjugated rat anti-mouse kappa-chain antibody (clone 187.1) were purchased from BD Biosciences Pharmingen (San Diego, CA).

Cell culture. The HSY cell line, which was originally established by Yanagawa et al. (33), 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 × 10^4 cells/cm² in 100-mm culture plates and cultured in DMEM supplemented with 10% FBS 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 confluence before use. The cells were treated with reagents such as isoproterenol or EGF for various time periods and then prepared for immunoblot or immunoprecipitation analysis.

Transfection. A dominant-negative c-Src (K295R/Y527F) construct (DnSrc) was kindly provided by Dr. Joan S. Brugge (Harvard University, Cambridge, MA). HSY cells were transiently transfected with 1 μg of DnSrc plasmid or empty vector (control) with LipofectAMINE PLUS reagent (Invitrogen, Carlsbad, CA) and cotransfected with 100 ng of green fluorescent protein (GFP) gene vector (pHGF-N1) in each 100-mm culture dish as described previously (10). Cells were incubated with the DNA-LipofectAMINE mix for 3–4 h, after which the cells were washed with PBS and allowed to recover in culture medium for 24 h. Cells were then trypsinized, and GFP-expressing cells were sorted and collected using flow cytometry with FACSCalibur (BD Biosciences Immunocytometry Systems, San Jose, CA). Sorted cells were continued in culture and used for experiments at 48 h posttransfection.

Western blot analysis. Immunoblot (Western blot) analysis was performed as described previously, with minor modification (35). HSY cells were washed three times with cold PBS, scraped, and lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% NP-40, 3 μg/ml leupeptin, 3 μg/ml aprotinin, 3 μg/ml pepstatin A, 3 μg/ml phenanthroline, 30 μM benzamidane HCl, 0.3 mM PMSF, 20 mM β-glycerol phosphate, 10 mM sodium fluoride, and 1 mM Na3VO4 at 4°C for 30 min. After centrifugation of the cell lysates at 10,000 g for 2 min at 4°C, supernatant protein samples (50 μg) were added to 15 μl of 4× sample buffer (150 mM Tris-HCl, pH 8.8, 1%
SDS, 40% glycerol) and β-mercaptoethanol and then diluted with lysis buffer (without protease and phosphatase inhibitors) to a total volume of 60 μl. The protein samples were separated on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Schleicher & Schuell, Keene, NH). The membranes were immunoblotted with primary antibody [1:500 dilution for p44/p42MAPK, phospho-p44/p42MAPK, and EGF receptor; 1:250 dilution for phospho-EGF receptor (Y1068)] and a secondary horseradish peroxidase-conjugated antibody (1:10,000). MAPKs/ERKs and EGF receptor were visualized using an enhanced chemiluminescence system (SuperSignal West Pico Chemiluminescent Substrate; Pierce Biotechnology, Rockford, IL), and immunoblots were quantified with ImageQuant computer software (version 5; Molecular Dynamics, Sunnyvale, CA).

**Immunoprecipitation assay of EGF receptor.** Phosphorylation of the EGF receptor was detected by immunoprecipitating of the protein from cell lysates, followed by immunoblotting with anti-phosphotyrosine antibodies as described elsewhere (11). Briefly, whole cell lysates (500 μg protein) were precleared by mixing them with 25 μl of 50% protein A-Sepharose beads in 400 μl of lysis buffer containing 1 μg/ml BSA for 1 h (11). The supernatants were incubated with EGF receptor antibody (1 μg/sample) overnight. Next, 20 μl of protein A-Sepharose was added for 1 h, and the immunocomplexes were washed three times with lysis buffer. Samples were separated using SDS-PAGE, and proteins were detected by performing Western blot analysis using anti-phosphotyrosine monoclonal antibody (pY; 1:500).

**Flow cytometric analyses.** Cells subjected to various treatments were harvested from culture plates with trypsin-EDTA and then washed with cold PBS. One million cells in balanced salt solution (pH 7.2) containing 0.02% NaN3 were incubated with 0.5 μg/ml of mouse anti-human CD44 antibody for 1 h at 4°C. After three washes with balanced salt solution, cells were incubated with 0.2 μg/ml of phycoerythrin-conjugated rat anti-mouse κ-chain antibody for 1 h at 4°C. Cells were again washed and fixed in 2% formaldehyde, and fluorescence was measured using flow cytometry with FACS Calibur. Cells incubated with the secondary antibody alone were used as negative controls.

**Data analysis.** The densities of Western blots were quantified with ImageQuant. Phosphorylation of p44/p42 MAPK, EGF receptor, or tyrosine induced by isoproterenol, EGF, or other reagents was normalized to the total immunoreactive protein of interest for each

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**Fig. 2.** Transactivation of the EGF receptor (EGFR) by Iso. A: immunoprecipitation (IP): HSY cells were cultured to near confluence in DMEM containing 10% FBS and treated with Iso (10^-5 M), EGF (5 × 10^-10 M), or vehicle (Con) for 10 min. Cell lysates were incubated with antibody to EGFR and precipitated with protein A-Sepharose as described in MATERIALS AND METHODS. The immunocomplex was subjected to SDS-PAGE, and proteins were detected by performing immunoblot (IB) analysis (left) using antibody against phosphotyrosine (pY) or EGFR. EGFR was used to monitor the protein loading in each lane. Quantitation of band densities, as described in MATERIALS AND METHODS, is shown on right. B: effect of 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG-1478) on Iso-induced phosphorylation of EGFR and ERKs: HSY cells were pretreated with (+) or without (−) AG-1478 (5 × 10^-6 M) for 30 min before addition of Iso (10^-5 M), EGF (5 × 10^-10 M), or vehicle (−) for 10 min. Western blot analysis was performed as described in MATERIALS AND METHODS with primary antibodies against phospho-Y1068 of EGFR, EGFR, phospho-p44/p42MAPK, and p44/p42MAPK. Representative blots are shown on left, and quantitative results (means ± SE) from 3–5 independent experiments are shown on right. *P < 0.01 vs. untreated level of phospho-EGFR; **P < 0.02 vs. level of phospho-p44/p42MAPK after AG-1478 alone.

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SDS, 40% glycerol, and β-mercaptoethanol, and then diluted with lysis buffer (without protease and phosphatase inhibitors) to a total volume of 60 μl. The protein samples were separated on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Schleicher & Schuell, Keene, NH). The membranes were immunoblotted with primary antibody [1:500 dilution for p44/p42MAPK, phospho-p44/p42MAPK, and EGF receptor; 1:250 dilution for phospho-EGF receptor (Y1068)] and a secondary horseradish peroxidase-conjugated antibody (1:10,000). MAPKs/ERKs and EGF receptor were visualized using an enhanced chemiluminescence system (SuperSignal West Pico Chemiluminescent Substrate; Pierce Biotechnology, Rockford, IL), and immunoblots were quantified with ImageQuant computer software (version 5; Molecular Dynamics, Sunnyvale, CA).

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sample and expressed as the fold increase relative to the normalized value in untreated (control) cells. Bar graphs show the untransformed mean values ± SE or range (if n = 2). For statistical analysis, single and multiple comparisons were conducted using Student’s t-test and Bonferroni analysis, respectively, with logarithmically transformed values.

RESULTS

Isoproterenol stimulates the activation of ERK1/2 (p44/p42MAPK) in HSY cells. To determine whether isoproterenol can induce the activation of ERK1/2 in HSY cells, we measured the effect of isoproterenol on the phosphorylation of p44/p42MAPK with immunoblot analysis. Because we previously showed (34, 35) that EGF induces ERK signaling in HSY cells, EGF was used as a positive control in these experiments. Isoproterenol, like EGF, induced the phosphorylation of ERK1/2 without affecting total ERK1/2 protein levels. Both isoproterenol and EGF induced a transient activation of ERK1/2. The activation of ERK1/2 by isoproterenol peaked at 5–15 min (Fig. 1A), with a return to basal level after 1–2 h. A similar time course of ERK1/2 activation was observed in EGF-treated cells (Fig. 1A). At 10 min, isoproterenol increased phosphorylation of ERK1/2 4.4 ± 0.8-fold (mean ± SE; n = 9) relative to basal levels, whereas EGF activated ERK phosphorylation 6.7 ± 2.2-fold (n = 5; P > 0.05 compared with isoproterenol stimulation) (Fig. 1B).

Isoproterenol-induced ERK activation is mediated partially by EGF receptor transactivation. Activation of ERKs by GPCRs in several cell types was previously shown to occur via transactivation of the EGF receptor (13, 19). In the present study of HSY cells, isoproterenol induced EGF receptor phosphorylation comparable to that induced by EGF (Fig. 2). To examine the role of the EGF receptor in isoproterenol-induced ERK activation, we pretreated HSY cells with the EGF receptor inhibitor AG-1478 for 30 min before stimulation with isoproterenol or EGF. Activation of the EGF receptor was detected by increased phosphorylation of the tyrosine residue Y1068 with a phosphorylation-specific antibody (Fig. 2B). AG-1478 completely inhibited the phosphorylation of the EGF receptor at Y1068 induced by both isoproterenol and EGF. However, AG-1478 eliminated EGF-stimulated ERK phosphorylation but had only a partial inhibitory effect on isoproterenol-induced ERK activation, indicating the presence of an alternate pathway for isoproterenol stimulation of ERKs (Fig. 2B).

Previous studies have indicated that the Gαi subunit may mediate GPCR stimulation of ERKs via transactivation of the EGF receptor (16, 18). We examined the involvement of Gαi in isoproterenol-induced ERK activation by treating HSY cells with the Gαi inhibitor pertussis toxin for 16 h before stimulation with isoproterenol. Like the EGF receptor inhibitor AG-1478 (see above), pertussis toxin caused partial inhibition of isoproterenol-stimulated ERK1/2 phosphorylation (Fig. 3). Although this finding suggests a role for Gαi in ERK activation by isoproterenol, a portion of the β-adrenergic response in HSY cells appears to involve stimulatory signals other than the Gαi-dependent pathway of EGF receptor transactivation described in some other tissues.

Isoproterenol-induced ERK1/2 activation may also be mediated by cAMP signaling. Stimulation of β-adrenergic receptors by isoproterenol results in Gαs-mediated activation of adenyl cyclase and increased production of cAMP. We previously demonstrated (34) the activation of adenyl cyclase activity by isoproterenol in HSY cells. To investigate whether activation of cAMP signaling stimulates ERKs in HSY cells, we tested the effects of the membrane-permeant cAMP analog CPT-cAMP and cAMP-elevating agents on phosphorylation of ERK1/2. Treatment of HSY cells with CPT-cAMP resulted in ERK activation with a time course similar to that observed in isoproterenol-treated cells, i.e., ERK phosphorylation induced by CPT-cAMP treatment peaked at 5–15 min and returned to basal level within 1 h (Fig. 4A). Interestingly, activation of ERKs by CPT-cAMP was observed even after pretreatment of cells with AG-1478 (5 × 10⁻⁶ M; data not shown); in this respect, CAMP, like isoproterenol (Fig. 2B), appears to stimulate ERK phosphorylation via signaling intermediate(s) at least partially distinct from the EGF receptor.

Cholera toxin activates adenyl cyclase by catalyzing ADP-ribosylation of the stimulatory Gαs protein. Our previous studies (34) demonstrated that cholera toxin causes substantial activation of adenyl cyclase in HSY cells. In the present study HSY cells were preincubated with cholera toxin, followed by measurement of ERK activation with or without isoproterenol. Phosphorylation of ERKs was increased by incubation with cholera toxin for 30 min but returned to basal level by 2 h and also at 24 h with toxin treatment. ERKs were further activated by isoproterenol after 30-min but not 2- and 24-h pretreatment with cholera toxin (Fig. 4B, left). Because
adenylyl cyclase of HSY cells remains activated after 2-h treatment with cholera toxin (34), the reduction in ERK activation after toxin treatment most likely reflects the transient stimulatory effect of cAMP on phosphorylation of ERKs (Fig. 4A). In a number of cell types, $G_{os}$ is either redistributed to cytosol or downregulated after prolonged activation by cholera toxin (5, 21, 29). Thus, in our experiments, the failure to observe stimulation of ERKs by isoproterenol after 24 h of cholera toxin exposure may at least partly reflect loss of $G_{os}$ coupled to $\beta$-adrenergic receptors in the plasma membrane. In contrast, prolonged (24 h) treatment with cholera toxin had no apparent effect on EGF-induced activation of ERKs (Fig. 4B, right). The results of these studies suggest that $G_{os}$ plays a critical role in isoproterenol-induced activation of ERKs.

PKA is thought to be involved in $\beta$-adrenergic receptor-mediated activation of ERKs in a variety of cell types (19). However, in HSY cells, the stimulatory effects of isoproterenol and cholera toxin on phosphorylation of ERK1/2 were not affected by pretreatment (2 h) with the PKA inhibitor H-89 (5 $\times$ 10^{-6} M; Fig. 5). Likewise, prevention of cAMP degradation with the phosphodiesterase inhibitor IBMX also increased ERK phosphorylation, whereas the stimulatory effect of IBMX was not influenced by pretreatment with H-89 (Fig. 5). Activation of ERKs by cAMP-elevating agents was not reduced by increasing concentrations of H-89 up to 2.5 $\times$ 10^{-5} M (data not shown). On the basis of these results, activation of PKA has no apparent role in mediating $\beta$-adrenergic receptor-, $G_{os}$-, or cAMP-responsive ERK activation in HSY cells.

Isoproterenol-induced ERK activation is Src dependent. Activation of nonreceptor protein tyrosine kinases of the Src family may be involved in GPCR-induced activation of ERKs by both EGF receptor transactivation and cAMP-dependent pathways (19). To determine whether Src mediates isoproterenol-induced activation of ERKs in HSY cells, we measured isoproterenol responsiveness in cells pretreated for 2 h with PP2 (5 $\times$ 10^{-6} M), a Src family-selective tyrosine kinase inhibitor. Incubation with PP2 reduced isoproterenol-stimulated EGF receptor phosphorylation and ERK activation. Pretreatment of cells with PP3,
an inactive PP2 analog, had no effect on ERK activation by isoproterenol (Fig. 6A). Because PP2 is known to inhibit all Src family kinases, we also used a DnSrc to inhibit Src specifically. The phosphorylation of ERK1/2 induced by isoproterenol was inhibited in DnSrc-transfected HSY cells (Fig. 6B). These results indicate that in HSY cells, Src plays a regulatory role in isoproterenol-induced ERK activation. Basal phosphorylation of ERK1/2 appeared to be increased in DnSrc-transfected cells; whether phosphorylation of nonmutated sites in the DnSrc construct might result in increased basal ERK activities is unknown (25).

Isoproterenol increases expression of CD44 via ERK activation. The cell adhesion molecule CD44 may play a role in regulating growth and differentiation of several tissues, including the salivary gland (8, 24). In addition, CD44 expression in some tissues appears to be ERK dependent (32). In the present study, flow cytometric analysis revealed that isoproterenol treatment of HSY cells produced a marked elevation in surface CD44 expression. Expression of surface CD44 was also increased by treatment of cells with either EGF or IBMX. The increase in CD44 expression induced by isoproterenol and EGF was eliminated by the ERK pathway inhibitor PD-98059, which also blocked isoproterenol stimulation of ERK phosphorylation. Interestingly, the EGF receptor inhibitor AG-1478 also blocked the CD44 response to isoproterenol (Fig. 7). Together, these results implicate CD44 as a downstream effector of isoproterenol-induced ERK activation in HSY cells. The findings are also generally consistent with a role for both cAMP- and EGF receptor-dependent signaling in the CD44 response to isoproterenol.

**DISCUSSION**

A major goal of current salivary gland research is to develop strategies to maintain or regenerate functional salivary tissue after glandular destruction by disease processes, e.g., Sjögren syndrome, or therapeutic maneuvers such as head and neck irradiation. Chronic treatment of rats with isoproterenol is known to induce salivary gland enlargement, suggesting that salivary epithelial cell proliferation is regulated by β-adrenergic receptor-mediated signaling. Using the HSY human salivary epithelial cell line, we have demonstrated in this study that isoproterenol induces activation/phosphorylation of ERK1/2 through two distinct pathways, one involving the transactivation of the EGF receptor and the other possibly dependent on cAMP-generated signals.
Activation of MAPKs by GPCRs such as β-adrenergic receptors has been demonstrated to be mediated by the transactivation of EGF receptors in other cell types, e.g., cardiac fibroblasts and COS-7 cells (12, 15, 20). Our results show that isoproterenol activates ERK1/2 in HSY cells by a signal transduction mechanism involving the transactivation of EGF receptors. Isoproterenol treatment was found to induce phosphorylation of the EGF receptor at Y1068, a major site for receptor autophosphorylation. Furthermore, blockade of EGF receptor phosphorylation with the specific inhibitor AG-1478 diminished isoproterenol-induced activation of ERK1/2 (Fig. 2). Together, these data demonstrate interreceptor signaling “cross-talk” between β-adrenergic and EGF receptors in HSY cells.

In this study, we performed experiments to elucidate the molecular mechanism by which isoproterenol induces EGF receptor transactivation in HSY cells. Previous work suggests that in a number of tissues, β-adrenergic receptor-induced activation of ERKs is sensitive to the Gs inhibitor pertussis toxin. In these systems, Gi-dependent ERK activation appears to be mediated through the Gi subunit and may involve EGF receptor transactivation (16, 18). Moreover, Gi-dependent activation of ERKs by the β-adrenergic receptor is thought to result from a PKA-induced phosphorylation of the receptor, which causes a “switch” in the coupling of the receptor from Gi to Gs (18). We found that pretreatment of HSY cells with pertussis toxin attenuated isoproterenol-induced ERK activation (Fig. 3); in preliminary work, isoproterenol-responsive phosphorylation of the EGF receptor was also reduced by pertussis toxin (data not shown). Short-term activation of Gαs with cholera toxin was observed to increase phosphorylation of ERK1/2, whereas long-term toxin treatment, which downregulates Gαs in several tissues (5, 21, 29), blocked ERK activation by isoproterenol (Fig. 4B). These findings, implicating the involvement of both Gi and Gs in the isoproterenol response, are consistent with a switch in G protein coupling by the β-adrenergic receptor in HSY cells. Interestingly, treatment of HSY cells with the PKA inhibitor H-89 had no effect on isoproterenol-induced ERK activation (Fig. 5A). Thus, in marked contrast to other cell types in which pertussis-sensitive isoproterenol responses were profoundly inhibited by H-89 (6), HSY cells appear to undergo a switch in G protein coupling without obvious involvement of PKA. It should be noted that the interpretation of data from experiments using H-89 could be limited by direct PKA inhibition of c-Raf1, an activator of ERKs downstream of the EGF receptor and the GTPase Ras (18). Indeed, basal levels of ERK phosphorylation in HSY cells tended to increase in the presence of...
higher concentrations (>5 × 10⁻⁶ M) of H-89, even though the isoproterenol response remained unaffected (data not shown). Although our studies do not entirely exclude a possible role for PKA in Gₛ-to-Gι switching in HSY cells, in preliminary experiments isoproterenol-induced phosphorylation of the EGF receptor (Y1068) was unaffected by H-89 (not shown).

Earlier reports have proposed that, in contrast to the Gi-dependent pathway, a distinct, pertussis toxin-insensitive pathway involving cAMP signaling may also mediate β-adrenergic receptor-induced activation of ERKs (9, 18). In HSY cells pertussis toxin and AG-1478 produced only partial inhibition of ERK activation by the β-adrenergic agonist (Figs. 2 and 3). These findings suggested that isoproterenol activates ERK1/2 in HSY cells in part by a signaling mechanism other than the Gi-dependent pathway involving transactivation of the EGF receptor. Like isoproterenol, the cAMP analog CPT-cAMP and cAMP-elevating agents (cholera toxin and IBMX) also activated ERK1/2 in HSY cells (Figs. 4 and 5). In preliminary experiments (not shown) the activating effect of CPT-cAMP on ERK phosphorylation was not blocked by the EGF receptor inhibitor AG-1478 (Fig. 5). Thus we found no evidence in HSY cells for PKA involvement in ERK activation by cAMP, although as noted above experiments using H-89 may be difficult to interpret, given the complex actions of PKA on ERK activation in any given cell type. We have detected immunoreactive EPAC and B-Raf in HSY cells (data not shown), yet it remains to be determined whether isoproterenol stimulation of ERKs in these cells might occur via an EPAC-Rap1-B-Raf signaling cascade.

Src tyrosine kinases are thought to act as early signaling intermediates in both Gₛₐ-mediated EGF receptor transactivation and PKA-dependent ERK activation induced by GPCR activation of ERKs by β-adrenergic receptor-stimulated cAMP production is thought to be mediated by PKA and/or the cAMP-regulated guanine nucleotide exchange factor EPAC (exchange protein directly activated by cAMP), which in turn cause sequential activation of the downstream intermediates Rap1 and B-Raf (18, 19, 30). In HSY cells stimulation of ERK1/2 by the cAMP-elevating agents IBMX and cholera toxin was unaffected by pretreatment of cells with H-89 (Fig. 5). Thus we found no evidence in HSY cells for PKA involvement in ERK activation by cAMP, although as noted above experiments using H-89 may be difficult to interpret, given the complex actions of PKA on ERK activation in any given cell type. We have detected immunoreactive EPAC and B-Raf in HSY cells (data not shown), yet it remains to be determined whether isoproterenol stimulation of ERKs in these cells might occur via an EPAC-Rap1-B-Raf signaling cascade.
stimulation (18, 19, 30). In our studies of HSY cells, isoproterenol-responsive phosphorylation of the EGF receptor was attenuated by the Src inhibitor PP2, whereas Src inhibition by either PP2 or transfection of a DnSrc blocked the ERK response to β-adrenergic agonist (Fig. 6). These findings suggest that Src is required for EGF receptor transactivation and possibly additional signaling events essential to ERK activation by isoproterenol. However, the precise target(s) for Src regulation in G<sub>i</sub>- and/or cAMP-mediated pathways of isoproterenol action remains to be identified.

Although β-adrenergic receptors are linked to the ERK cascade in multiple cell types, functional responses to the ERK-mediated actions of isoproterenol may be cell type specific. Of particular note in this regard, the growth-promoting effects of isoproterenol appear to be specific for the salivary gland and a few other tissues (i.e., pancreas and heart) (3, 27). The specificity of target cell responsiveness to β-adrenergic receptor signaling through the ERK pathway may reside, at least in part, in the downstream effectors generated in any given cell type. The CD44 proteins are a family of transmembrane glycoproteins thought to regulate cell proliferation, survival, and differentiation through transduction of signals originating in the extracellular matrix (24). CD44 is present in normal human salivary tissue and has been proposed to play a role in adult salivary gland regeneration and/or regrowth (8, 24). Moreover, in some tissues, alternative splicing of the CD44 gene is regulated by activation of the ERK pathway (32). In the current study, isoproterenol treatment of HSY cells caused a profound increase in surface CD44 expression, which was completely blocked by the ERK pathway inhibitor PD-98059 (Fig. 7). Thus CD44 could function as a downstream target mediating salivary cell growth responsive to β-adrenergic receptor-induced activation of the ERK pathway. EGF and IBMX were each found to increase CD44 expression, suggesting the involvement of both EGF receptor and cAMP signaling pathways in isoproterenol-induced regulation of CD44. Although treatment of cells over 24 h with the EGF receptor inhibitor AG-1478 completely blocked isoproterenol-induced CD44 expression, whether prolonged exposure to AG-1478 might have adverse effects on both pathways implicated in isoproterenol signaling requires further elucidation.

In summary, we propose that activation of β-adrenergic receptors in HSY cells induces the mitogenic signaling pathways depicted schematically in Fig. 8. Distinct pathways of ERK activation mediated by G<sub>i</sub>-dependent transactivation of EGF receptors or cAMP signaling processes have been individually demonstrated in several cell types (18, 19). To our knowledge, however, the coexistence of both pathways operating simultaneously in a single cell line has not been described previously. In HSY cells, both pathways appear to be G<sub>i</sub>-dependent because isoproterenol-induced phosphorylation of ERKs was abolished under conditions (i.e., prolonged incubation with cholera toxin) found to downregulate G<sub>ai</sub> in other systems (5, 21, 29). Hence, in Fig. 8, the two signaling pathways are shown to diverge at a step involving either G<sub>ai</sub> activation of adenylyl cyclase or switching of β-adrenergic receptor coupling from G<sub>ai</sub> to G<sub>os</sub> (18). Although the process of G protein switching is generally thought to require PKA-mediated phosphorylation of the β-adrenergic receptor, we found no evidence to indicate that isoproterenol-responsive ERK activation in HSY cells is PKA dependent; nonetheless, a possible role for PKA in G<sub>i</sub>- and/or cAMP-mediated signaling pathways has not been fully excluded. Finally, the identification of CD44 as a likely effector downstream of ERK1/2 is also summarized in Fig. 8. In a related study (34), we recently reported that EGF receptor-mediated stimulation of ERKs in HSY cells increases expression of β-adrenergic receptors coupled to activation of adenylyl cyclase. Additional investigations are required to clarify the mechanisms by which cross-talk between β-adrenergic receptor- and receptor tyrosine kinase-linked pathways elicits mitogenic signals promoting tissue-specific growth of salivary gland cells.

Fig. 8. Schematic illustration of coexisting signaling pathways involved in β-adrenergic receptor (β-AR)-responsive ERK1/2 activation in HSY cells. Agonist binding to the β-AR in HSY cells is proposed to activate ERK1/2 via 2 coexisting signaling cascades: 1) G<sub>os</sub>-mediated transactivation of EGFR, with resultant activation of the Ras/c-Raf1 pathway (right), and 2) generation of cAMP via stimulation of adenylyl cyclase (AC) and activation of Rap1/B-Raf signaling (left). Signaling intermediates tested in this study are shown in bold type. The sequence of individual signaling intermediates involved in the 2 pathways is adapted from recent consensus views of G protein-coupled receptor-mediated ERK activation (18, 19, 30). The precise involvement of Src in isoproterenol-induced ERK activation remains to be identified.
REFERENCES


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