α1-Adrenergic and cholinergic agonists activate MAPK by separate mechanisms to inhibit secretion in lacrimal gland

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Ota, Isao, Driss Zoukhri, Robin R. Hodges, José D. Rios, Vanja Tepavcevic, Isabelle Raddassi, Li Li Chen, and Darlene A. Dartt. α1-Adrenergic and cholinergic agonists activate MAPK by separate mechanisms to inhibit secretion in lacrimal gland. Am J Physiol Cell Physiol 284: C168–C178, 2003. First published September 11, 2002; 10.1152/ajpcell.00151.2002.—The purpose of this study was to determine the role of p42/p44 mitogen-activated protein kinase (MAPK) in α1-adrenergically and cholinergically stimulated protein secretion in rat lacrimal gland acinar cells and the pathways used by these agonists to activate MAPK. Acini were isolated by collagenase digestion and incubated with the α1-adrenergic agonist phenylephrine or the cholinergic agonist carbachol, and activation of MAPK and protein secretion were then measured. Phenylephrine and carbachol activated MAPK in a time- and concentration-dependent manner. Inhibition of MAPK significantly increased phenylephrine- and carbachol-induced protein secretion. Whereas phenylephrine-induced activation of MAPK was completely inhibited by AG1478, activation of MAPK by carbachol was not. Phenylephrine stimulated tyrosine phosphorylation of the EGFR, whereas carbachol stimulated p60Src, and possibly Pyk2, to activate MAPK. We conclude that, in the lacrimal gland, activation of MAPK plays an inhibitory role in α1-adrenergically and cholinergically stimulated protein secretion and that these agonists use different signaling mechanisms to activate MAPK.

THE LACRIMAL GLAND is the major contributor to the aqueous portion of the tear film (2). Its function is to secrete protein, water, and electrolytes onto the ocular surface to maintain, nourish, and protect the cornea and conjunctiva. The lacrimal gland is composed of several cell types including acinar cells, the major cell type comprising ~80% of the gland, myoepithelial cells, and ductal cells. Acinar cells are highly polarized and joined by tight junctions at the luminal membrane, thus creating distinct basolateral and apical membranes (2). The lacrimal gland is highly innervated, and these nerves release their neurotransmitters, which interact with receptors on the basolateral membrane of the acinar cells. This initiates the signaling pathways necessary for secretion of proteins, electrolytes, and water across the apical membrane.

We have previously shown that lacrimal gland secretion is under neural control with cholinergic agonists, released from parasympathetic nerves, and α1-adrenergic agonists, released from sympathetic nerves, being the major regulators of lacrimal gland protein secretion (1, 7). We have also previously shown that these agonists activate separate signal transduction pathways to stimulate protein secretion in lacrimal gland acinar cells (8). Cholinergic agonists interact with muscarinic M3 receptors through a Gαq/11 G protein to activate phospholipase C (PLC) (19). Activation of PLC generates the Ca2+-mobilizing second messenger inositol 1,4,5-trisphosphate and the protein kinase C (PKC) activator diacylglycerol (DAG) (19). Ca2+ either alone or with Ca2+/calmodulin-dependent protein kinases phosphorylates specific substrates, which lead to protein secretion. DAG activates the PKC isoforms α, δ, and ε, which play a direct role in stimulating secretion (26). We have also shown that cholinergic agonists activate phospholipase D (PLD) to generate phosphatidic acid, which can be converted to DAG (25).

Unlike those in other tissues, lacrimal gland α1-adrenergic receptors are not coupled to activation of PLC or PLD (8, 25). There are, in fact, few details regarding the pathway these receptors activate in lacrimal gland acinar cells. The subtypes of α1-adrenergic receptors present in these cells and the effector enzyme(s) activated by these receptors are unidentified. It is known that PKCε plays a major role in protein secretion stimulated by phenylephrine [an α1-adrenergic agonist in the lacrimal gland (1)], whereas PKCa and -δ play inhibitory roles (26).

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases. These members include MAPKs (also known as extracellular sig...
nal-regulated kinases (ERKs) or p42/p44 MAPK), the p38 MAPKs, and the c-Jun NH2-terminal kinases (JNKs) (24). The p42/p44 MAPK (hereafter called MAPK) pathway is best known for its involvement in the signaling pathway of many growth factors, including epidermal growth factor (EGF) (23). This pathway involves autophosphorylation of the EGF receptor (EGFR), which then acts as a scaffold for the recruitment of the adaptor proteins Shc and Grb2. Shc is tyrosine phosphorylated, resulting in recruitment of the adapter protein Grb2 and the guanine nucleotide exchange factor Sos (20). Sos then activates Ras (15), which in turn initiates the activation of a cascade of protein kinases, namely Raf (MAPK kinase kinase), MEK (MAPK kinase), and MAPK. Traditionally, activation of MAPK is thought to regulate long-term cellular processes such as cell proliferation and differentiation (13).

G protein-coupled receptors (GPCRs) have been traditionally thought of as regulators of short-term processes such as protein secretion. It is now widely documented that GPCRs can also activate the MAPK pathway (14, 16), linking these receptors to cell proliferation and differentiation. The mechanisms by which GPCRs activate MAPK are not completely understood. One hypothesized mechanism involves the release of heparin-binding EGF (HB-EGF) through cleavage of its precursor molecule, proHB-EGF, by metalloproteinases (22). This process, termed shedding, occurs at or near the cell surface and converts the membrane-bound form (proHB-EGF) to the soluble HB-EGF (17). Pierce et al. (21) have shown that this shedding is also dependent on the Gβγ subunit and Src, implying that Gβγ subunit activates Src, which in turn activates one or more metalloproteinases, leading to cleavage of proHB-EGF. The released HB-EGF can then stimulate the EGFR in an autocrine or paracrine fashion, stimulating the MAPK cascade. In addition, GPCRs can activate MAPK without transactivating the EGFR. PKC has been shown to directly activate Raf, whereas p60Src has been shown to be activated by the Ca2+-dependent non-receptor tyrosine kinase Pyk2, leading directly to activation of Raf (13). Phosphoinositide 3-kinase, which can be a target of Gβγ, also activates Src (13).

In the present study we show that inhibition of MAPK increases α1-adrenergically and cholinergically stimulated protein secretion in the lacrimal gland. α1-Adrenergic agonists transactivate the EGFR, leading to the tyrosine phosphorylation of Shc and recruitment of Grb2, two early events mediating MAPK activation. Conversely, cholinergic agonists do not transactivate the EGFR to activate MAPK. They do however, activate p60Src and possibly Pyk2, to activate MAPK. We conclude that activation of MAPK in freshly isolated lacrimal gland epithelial cells by α1-adrenergic and cholinergic agonists occurs through different mechanisms to inhibit protein secretion.

**MATERIALS AND METHODS**

**Materials.** EGF and recombinant protein A-agarose were obtained from Upstate Biotechnology (Lake Placid, NY). Collagenase type III was from Worthington Biochemical (Freehold, NJ). AG1478 [4-[3-chloroanilino]-6,7-dimethoxyquinazoline], an inhibitor of the tyrosine phosphorylation activity of the EGFR, was from Calbiochem (La Jolla, CA). Amplex Red was from Molecular Probes (Eugene, OR). U0126 [1,4-diamo-2,3-dicyano-1,4-bis(α-aminophenylmercapto)buta diene], an inhibitor of MEK, was from Cell Signaling Technology (Beverly, MA). PP1 [4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine], a Src inhibitor, was purchased from Biomol (Plymouth Meeting, PA). Other chemicals were obtained from Sigma Chemical (St. Louis, MO).

**Antibodies.** Antibody against phosphorylated p42/44 MAPK that detects phosphorylated tyrosine 204 of p42/44 MAPK was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody against total p42 MAPK was from Santa Cruz Biotechnology. A polyclonal antibody against Shc [amino acids (aa) 359–473 in the SH2 domain] was purchased from Transduction Laboratories (Lexington, KY); and a monoclonal antibody against Shc (aa 366–473) was from Santa Cruz Biotechnology. A monoclonal anti-phosphotyrosine antibody (clone 4G10) was from Upstate Biotechnology. Polyclonal antibodies against Grb2, total Ras and the EGFR were from Santa Cruz Biotechnology. Antibody against total p60Src was purchased from Biosource International.

**Preparation of lacrimal gland acini.** All experiments conformed to the U.S. Department of Agriculture Animal Welfare Act (1985) and were approved by the Schepens Eye Research Institute Animal Care and Use Committee. Both exorbital lacrimal glands were removed from male Sprague-Dawley rats that had been anesthetized with CO2 for 1 min and then decapitated. Lacrimal glands were trimmed of fatty and connective tissue and fragmented into small pieces 2–3 mm in diameter. The pieces were then washed at 37°C in Krebs-Ringer bicarbonate (KRb) buffer (in mM: 119 NaCl, 4.8 KCl, 1 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, and 25 NaHCO3) supplemented with 10 mM HEPES, 5.5 mM glucose, and 0.5% BSA (KRb-HEPES), pH 7.4. Lacrimal gland acini were prepared by incubating tissue pieces with collagenase (CLS III; 150 U/ml) in 10 ml of KRb-HEPES buffer for 30 min at 37°C under a stream of 95% O2-5% CO2. The dispersed acini were allowed to recover for 30 min in 5 ml of fresh KRb-HEPES buffer containing 0.5% BSA. Cell viability was monitored with trypan blue (Sigma).

**Detection of MAPK activation by Western blotting.** Lacrimal gland acini were incubated for the indicated time period with phenylephrine (10–4 M), EGF (10–7 M), or carbachol (10–4 M). For inhibition experiments, acini were preincubated with either the MEK inhibitor U0126 (10–8–10–6 M) (4) or the p60Src inhibitor PP1 (10–5 M) (6) for 30 min or with the EGF inhibitor AG1478 (10–7 M) (12) for 15 min. To terminate incubation, the acini were centrifuged, the supernatant was discarded, 300 μl of ice-cold RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 100 μg/ml PMSF, 30 μg/ml aprotinin, and 1 mM Na3VO3), supplemented with protease and phosphatase inhibitors, were then added, and the acini were sonicated. The homogenate was centrifuged at 3,000 rpm for 30 min at 4°C, and proteins in the supernatant were separated by SDS-PAGE (10% acrylamide gels) and transferred to nitrocellulose membranes. Activated MAPK
was detected with antibodies that specifically recognize the phosphorylated (activated) pools of enzymes. Films were scanned, and data were analyzed using NIH Image. Values for phosphorylated enzymes (amounts for p42 and p44 MAPK were added together) were normalized to the amount of total enzyme by using antibodies to nonphosphorylated enzyme and were expressed as multiples of increase above the control value, which was set as 1.

**Immunoprecipitation experiments.** Lacrimal gland acini were incubated for the indicated time period with carbachol (10⁻⁴ M), phenylephrine (10⁻⁴ M), or EGF (10⁻⁷ M). To terminate incubation, the acini were centrifuged, the supernatant was discarded, and 1 ml of ice-cold RIPA buffer was added. The homogenate was centrifuged at 3,000 rpm for 30 min at 4°C. The supernatant (cell lysate) was incubated overnight at 4°C on a rocker platform in the presence of the specified immunoprecipitating antibody. After the addition of 100 μl of protein A-agarose for 2 h at 4°C, the immunoprecipitate was collected by brief centrifugation. After the pellet had been washed four times with RIPA buffer, the immunoprecipitate was resuspended in Laemmli sample buffer and boiled for 5 min. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoreactive bands were visualized using the enhanced chemiluminescence method. The films were scanned and analyzed using NIH Image. Western blotting was also performed, using the immunoprecipitating antibody to normalize for the amount of cells in each condition.

**Measurement of protein secretion.** Lacrimal gland acini were incubated for 20 min with the α₁-adrenergic agonists phenylephrine (10⁻⁴ M) or EGF (10⁻⁷ M) or the cholinergic agonist carbachol (10⁻⁷ M). Either U0126 (10⁻⁸–10⁻⁶ M) or PP1 (10⁻⁷ M) was added to acini 30 min before addition of the agonists. AG1478 (10⁻⁷ M) was added 15 min before addition of the agonists. To terminate the reaction, acini were centrifuged at 500 g for 30 s, and the supernatant was removed. The acini (pellet) were then homogenized in 10 mM Tris·HCl, pH 8.0. The amount of peroxidase, our marker for protein secretion, was measured in the supernatant and pellet spectrophotometrically using Amplex Red, according to the manufacturer’s protocol (Molecular Probes). In short, in the presence of peroxidase and hydrogen peroxide, Amplex Red reacts to form a highly fluorescent molecule. Fluorescence is read at an excitation wavelength of 560 nm and an emission wavelength of 590 nm on a fluorescent microplate reader (Bio-Tek, Winooski, VT). The amount of peroxidase in the supernatant (secreted) was expressed as a percentage of the total peroxidase (amount of peroxidase in the supernatant plus amount of peroxidase in the pellet).

**Data presentation.** Data are expressed as means ± SE and were analyzed using Student’s t-test with P < 0.05 considered statistically significant.

**RESULTS**

Effect of α₁-adrenergic and cholinergic agonists on activation of MAPK in freshly isolated lacrimal gland acinar cells. To determine whether MAPK is activated by α₁-adrenergic and cholinergic agonists, we incubated lacrimal acinar cells in the presence of phenylephrine (10⁻⁴ M), a selective α₁-adrenergic agonist in the lacrimal gland (1), or carbachol (10⁻⁴ M). Activation of MAPK was measured by Western blotting techniques using antibodies that selectively recognize the phosphorylated (active) form of MAPK as described in MATERIALS AND METHODS. As shown in Fig. 1A, phenylephrine induced a time-dependent activation of MAPK. Cirazoline, another α₁-adrenergic agonist, gave similar results (data not shown). Activation of cholinergic receptors with carbachol and EGF (10⁻⁷ M), used as a positive control, also induced a time-dependent activation of MAPK (Fig. 1A). When the blots from three independent experiments were analyzed by densitometry, the combined results showed that the kinetics of
phenylephrine-, carbachol-, and EGF-induced activation of MAPK were similar with a statistically significant effect reached after 5 min and returning to basal by 30 min (Fig. 1B).

Acini were then incubated in varying concentrations of phenylephrine, carbachol, and EGF for 5 min, and activation of MAPK was measured. Statistically significant increases of MAPK activation were obtained with $10^{-5}$–$10^{-3}$ M phenylephrine (Fig. 2A), $10^{-6}$–$10^{-4}$ M carbachol (Fig. 2B), and $10^{-8}$ and $10^{-7}$ M EGF (Fig. 2C).

Mauduit et al. (19) have shown that the lacrimal gland contains M$_3$ muscarinic receptors exclusively, whereas in the lacrimal gland, phenylephrine activates $\alpha_1$-adrenergic receptors (18). To determine whether the effects on MAPK activation were mediated through $\alpha_1$-adrenergic and cholinergic receptors, we incubated acini with the $\alpha_1$-adrenergic receptor antagonist prazosin ($10^{-4}$ M) or the M$_3$ muscarinic receptor antagonist 4-DAMP ($10^{-4}$ M) and stimulated with phenylephrine ($10^{-4}$ M) or carbachol ($10^{-4}$ M), respectively. MAPK activation was analyzed by Western blot as described earlier. Prazosin completely blocked phenylephrine-induced MAPK activation. Similarly, 4-DAMP completely inhibited carbachol-induced MAPK activation (data not shown). These results show that activation of two GPCRs and a growth factor receptor leads to a time- and concentration-dependent activation of MAPK in freshly isolated lacrimal gland acinar cells.

**Effect of U0126 on MAPK activation.** To determine whether the MEK inhibitor U0126 inhibited MAPK in the lacrimal gland, we preincubated acini with U0126 ($10^{-8}$–$10^{-6}$ M) for 30 min before stimulation with the cholinergic agonist carbachol ($10^{-4}$ M) for 10 min. The cells were homogenized and subjected to Western blot analysis with the antibodies for phosphorylated MAPK and total MAPK as described earlier. As shown in Fig. 3A, carbachol increased MAPK activity, which was...
blocked by U0126 in a concentration-dependent manner. U0126 had little effect on basal activity of MAPK. When the blots from three independent experiments were analyzed by densitometry, the combined results showed that carbachol-stimulated MAPK was inhibited 10, 15, 78, 91, and 96% with U0126 at $10^{-8}$, $10^{-7}$, $3 \times 10^{-7}$, $6 \times 10^{-7}$, and $10^{-6}$ M, respectively (data not shown). These results indicate that U0126 inhibits MAPK activation in the lacrimal gland.

Effect of inhibition of MAPK on $\alpha_1$-adrenergic and cholinergic-stimulated protein secretion. To determine the role of MAPK in phenylephrine- and carbachol-stimulated peroxidase secretion, we preincubated lacrimal gland acini for 30 min with the MEK inhibitor U0126 at concentrations of $10^{-7}$, $3 \times 10^{-7}$, and $6 \times 10^{-7}$ M, which we had previously shown to inhibit carbachol-stimulated MAPK activation (Fig. 3A), before stimulation with either phenylephrine ($10^{-4}$ M) or carbachol ($10^{-4}$ M). We did not use U0126 at $10^{-6}$ M because it increased basal secretion in these cells (data not shown). U0126 significantly increased phenylephrine-induced peroxidase secretion to 135 ± 11 and 134 ± 10% at $3 \times 10^{-7}$ and $6 \times 10^{-7}$ M, respectively (Fig. 3B). Carbachol-stimulated peroxidase secretion was significantly increased to 201 ± 40% at $3 \times 10^{-7}$ M U0126 and to 255 ± 50% at $6 \times 10^{-7}$ M U0126 (Fig. 3C). Secretion stimulated by either agonist was not significantly affected by U0126 at $10^{-7}$ M. The two concentrations of U0126 that were most effective at inhibiting carbachol-stimulated MAPK activation were also the most effective at increasing phenylephrine- and carbachol-induced protein secretion. These results indicate that, in the lacrimal gland, activation of MAPK by $\alpha_1$-adrenergic and cholinergic agonists plays an inhibitory role in stimulated peroxidase secretion.

Effect of inhibition of the EGFR on $\alpha_1$-adrenergic- and cholinergic-activation of MAPK. We next determined the ability of $\alpha_1$-adrenergic and cholinergic agonists to transactivate the EGFR. Thus we tested the effect of AG1478, a selective inhibitor of the EGFR intrinsic tyrosine kinase ([12], on agonist-induced activation of MAPK. As shown in Fig. 4A, AG1478 ($10^{-7}$ M) inhibited EGF- and phenylephrine-induced MAPK activation, whereas there was little effect on carbachol-induced MAPK activation. Because these experiments were performed separately, both the controls are shown. When the blots from independent experiments were analyzed by densitometry, the combined results showed that AG1478 inhibited EGF-induced MAPK activation by 81% and phenylephrine-induced MAPK activation by 93% (note that the control value was set to 1). In contrast, AG1478 did not inhibit carbachol-induced MAPK activation (Fig. 4B). To show that AG1478, at the concentration used, inhibited phosphorylation of the EGFR, we measured both basal and EGF-induced tyrosine phosphorylation of the EGFR by immunoprecipitation and Western blotting techniques. As shown in Fig. 4C, AG1478 ($10^{-7}$ M) completely inhibited EGF-stimulated phosphorylation of the EGFR. These results suggest that $\alpha_1$-adrenergic, but not cholinergic, agonists activate MAPK through transactivation of the EGFR in lacrimal gland acinar cells.

Effect of inhibition of the EGFR on $\alpha_1$-adrenergic- and cholinergic-induced protein secretion. To determine the role of EGFR in MAPK-mediated inhibition of protein secretion, we incubated acini with AG1478 ($10^{-7}$ M) and measured peroxidase secretion. As shown in Fig. 5, peroxidase secretion was statistically significantly stimulated above basal secretion in the presence of the each agonist alone, whereas AG1478 alone had no effect on basal protein secretion. Inhibition of the EGFR with AG1478 statistically significantly increased phenylephrine-induced peroxidase secretion in a concentration-dependent manner. In contrast, AG1478 had no significant effect on carbachol-stimulated peroxidase secretion (Fig. 5). These results again
suggest that α1-adrenergic, but not cholinergic, agonists transactivate the EGFR to activate MAPK, which in turn inhibits protein secretion in lacrimal gland acinar cells.

α-Adrenergic agonists stimulate tyrosine phosphorylation of Shc and recruitment of Grb2. To determine whether α1-adrenergic agonist-induced activation of the EGFR results in tyrosine phosphorylation of Shc and recruitment of Grb2, we performed immunoprecipitation experiments in which acinar cells were stimulated with phenylephrine, EGF, or carbachol and immunoprecipitated with an anti-Shc antibody and then performed Western blot analysis with antibodies against phosphotyrosine, Grb2, or Shc. As shown in Fig. 6A, when acinar cells were stimulated with phenylephrine (10^{-4} M) and the cell lysates were immunoprecipitated with an anti-Shc antibody, there was a time-dependent increase in the amount of tyrosine phosphorylated p52 Shc. EGF (10^{-7} M), used as a positive control, also increased the amount of tyrosine-phosphorylated p52 Shc (Fig. 6A). Importantly, when the Shc immunoprecipitates were blotted with an anti-Grb2 antibody, phenylephrine also increased the

Fig. 4. Effect of AG1478 on agonist-induced activation of MAPK. A: freshly isolated acinar cells were pre-treated with or without AG1478 (10^{-7} M) for 15 min and were then stimulated with EGF (10^{-7} M), Ph (10^{-4} M), or Cch (10^{-4} M) for 5 min. Acini were homogenized and processed for SDS-PAGE and Western blotting. Activated MAPK was detected with an antibody against phospho-MAPK (p42 and p44 MAPK). B: values for the amount of active MAPK were normalized to the amount of total MAPK using an antibody against p42 MAPK and expressed as fold increase above control, which has been set at 1. Data represent means ± SE from 3–5 independent experiments. C: acinar cells were pretreated with AG1478 (10^{-7} M) for 10 min before addition of EGF (10^{-7} M) for 5 min. Cell lysates were processed for immunoprecipitation (IP) with an anti-EGFR antibody and analyzed by Western blotting with anti-phosphotyrosine (PY) or anti-EGFR antibodies. Blots are representative of 2 independent experiments.

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Fig. 5. Effect of AG1478 on Ph- and Cch-induced protein secretion. Freshly isolated acinar cells were preincubated for 15 min with 10^{-8}–10^{-6} M AG1478 before stimulation for 20 min with no agonist (○), Ph (10^{-4} M, ■), or Cch (10^{-4} M, ▲), and peroxidase secretion was measured. Data represent means ± SE from 4–6 independent experiments. *Statistically significant increase in secretion over that for agonist alone.
amount of Grb2 associated with Shc in a time-dependent manner (Fig. 6A). EGF also increased the amount of Grb2 associated with Shc. When four independent experiments were analyzed densitometrically, results showed that phenylephrine caused a significant increase in the amount of tyrosine phosphorylation of Shc after 30 s (Fig. 6B) and also increased the association of Grb2 with Shc (Fig. 6C). EGF also significantly increased the tyrosine phosphorylation of Shc after 5 min as well as its association with Grb2 (n = 3). It is worth noting that, similar to MAPK, there is a substantial amount of tyrosine phosphorylated Shc under resting conditions. In contrast, when three independent experiments were analyzed, results showed that activation of cholinergic receptors did not increase the amount of tyrosine-phosphorylated p52 Shc (Fig. 6D).

These results show that α1-adrenergic agonists, but not cholinergic agonists, stimulate tyrosine phosphorylation of p52 Shc and its association with the adapter protein Grb2.

α-Adrenergic agonists transactivate the EGFR. To confirm that α1-adrenergic agonists transactivate the EGFR and confirm the results shown in Figs. 4–6, we stimulated acinar cells with EGF (10^{-7} M) as a positive control, phenylephrine (10^{-4} M), or carbachol (10^{-4} M). Immunoprecipitation experiments were performed either with an EGFR antibody and the amount of tyrosine phosphorylation quantitated by immunoblotting or with a phosphotyrosine antibody and the amount of EGFR quantitated by immunoblotting. Figure 7A is a representative blot showing that stimulation with EGF (10^{-7} M) and phenylephrine (10^{-4} M) increased the amount of tyrosine phosphorylation of the EGFR after 5 min, whereas carbachol did not. As shown in Fig. 7B, when the results of three to four experiments were summed, they showed that EGF induced a significant increase (3.8-fold) over basal in the amount of tyrosine phosphorylated EGFR. Importantly, phenylephrine also significantly increased the amount of tyrosine phosphorylated EGFR (2.2-fold) over basal. Carbachol did not increase the amount of tyrosine phosphorylation of the EGFR. It is worth noting that we often found a substantial amount of tyrosine phosphorylated EGFR under basal conditions, similar to phosphorylated Shc and MAPK (Fig. 7A). These results show that α1-adrenergic agonists transactivate the EGFR. They

![Fig. 6. Effect of Ph, EGF, and Cch on tyrosine phosphorylation of Shc and its association with Grb2. A: freshly isolated acinar cells were stimulated with Ph (10^{-4} M) or EGF (10^{-7} M) for 0–20 min. Cell lysates were processed for IP with an anti-Shc antibody and analyzed by Western blotting with an anti-PY, anti-Grb2, or anti-Shc antibody. Blots from 4 independent experiments were subjected to densitometric analyses. B: the amount of EGF (-) and Ph (●)-stimulated tyrosine phosphorylation of Shc is shown. C: the amount of EGF (-) and Ph (●)-stimulated Grb2 association with Shc is shown. D: freshly isolated acinar cells were stimulated with EGF (10^{-7} M) or Cch (10^{-4} M) for 5 min, and then cell lysates were immunoprecipitated with an anti-Shc antibody and subjected to Western blotting with an anti-PY antibody. C, control. Data represent means ± SE from 3 independent experiments. *Statistically significant increase over that for agonist alone.](http://ajp-cell.physiology.org/)

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suggest that transactivation of the EGFR mediates α1-adrenergic agonist-induced MAPK activation.

Cholinergic agonists stimulate tyrosine phosphorylation of Pyk2 and p60Src. Another possible pathway to activate MAPK, independent of the EGFR, involves activation of Pyk2, which in turn can activate p60Src. To determine whether α1-adrenergic and cholinergic agonists also activate Pyk2 and p60Src to activate MAPK, we stimulated acini with phenylephrine (10⁻⁴ M) or carbachol (10⁻⁴ M) for 5 min. Cell lysates were processed for IP with either an anti-EGFR antibody or anti-phosphotyrosine and were analyzed by Western blotting with anti-phosphotyrosine or anti-EGFR antibodies. Representative blots are shown. B: data represent means ± SE from 3–4 independent experiments. *Statistically significant increase in tyrosine phosphorylated EGFR above control (C).

also suggest that transactivation of the EGFR mediates α1-adrenergic agonist-induced MAPK activation.

Effect of inhibition of p60Src on cholinergic and α1-adrenergic agonist-stimulated MAPK activation and protein secretion. Because carbachol activates p60Src, we investigated the role of this enzyme in carbachol- and phenylephrine-stimulated MAPK activation and peroxidase secretion using the specific p60Src inhibitor PP1 (6). Acini were preincubated with PP1 (10⁻⁵ M) for 30 min and stimulated with carbachol (10⁻⁴ M) or phenylephrine (10⁻⁴ M) for either 5 min (MAPK activation) or 20 min (peroxidase secretion). A representative blot, shown in Fig. 9A, indicates that preincubation of acini with PP1 inhibited carbachol activation of MAPK. It is important to note that the amount of total MAPK in the cells stimulated with phenylephrine and phenylephrine plus PP1 is substantially less than the amount in the other conditions. These variations are a
result of the fact that the lacrimal gland acini used in these studies were freshly isolated for each experiment. When the blots from three independent experiments were densitometrically scanned, results showed that PP1 statistically significantly inhibited carbachol-stimulated MAPK activation by 43 ± 8% while having no effect on phenylephrine-induced activation of MAPK (Fig. 9B). Values for the amount of phosphorylated MAPK were corrected for the amount of total MAPK. Conversely, PP1 statistically significantly increased carbachol-stimulated peroxidase secretion over carbachol stimulation alone and had no effect on phenylephrine-induced protein secretion (Fig. 9C). PP1 had no effect on basal secretion (data not shown). These results imply that p60Src activation is required for carbachol-stimulated MAPK activation and inhibition of peroxidase secretion.

DISCUSSION

In the present study, we have shown that the MAPK pathway is involved in the negative regulation of protein secretion from the lacrimal gland as inhibition of MAPK increases α1-adrenergically and cholinergically induced protein secretion. α1-Adrenergic agonists activate MAPK through transactivation of the EGFR and recruitment of Shc and Grb2. These agonists do not use Pyk2 or p60Src to activate the EGFR. Cholinergically induced activation of MAPK does not occur through transactivation of the EGFR and involves activation of p60Src, and possibly Pyk2, which activates MAPK distal to the recruitment of Shc and Grb2. Classically, MAPK is involved in long-term events such as cell growth and differentiation. This study demonstrates the involvement of MAPK in the short-term process, protein secretion. Keely et al. (9, 10) have shown that carbachol activates MAPK, which is involved in the negative regulation of chloride secretion from T84 cells. This is similar to the lacrimal gland in that GPCR-induced MAPK activation also leads to a negative regulation of the secretory response. However, in contrast to the lacrimal gland, in which carbachol activates Pyk2 and p60Src but not the EGFR, in T84 cells carbachol transactivates the EGFR to activate MAPK, an effect mediated by intracellular Ca2+, Pyk2, and p60Src. Also in contrast to the lacrimal gland, MAPK in T84 cells mediates carbachol inhibition of secretion, whereas in the lacrimal gland MAPK inhibits carbachol stimulation of secretion. Thus, although similar signaling components are used in different cell types, the effects are cell specific. It is possible that different anchoring proteins could recruit the components in various configurations depending on the cell type and, thus, have varying effects.

The main function of the lacrimal gland is to synthesize proteins and secrete them onto the ocular surface, with cholinergic and α1-adrenergic agonists being potent stimuli of lacrimal gland secretion (2). Many of these proteins possess antimicrobial activities or are growth factors and are crucial for the homeostasis of the ocular surface. As a result, the regulation of protein secretion must be highly regulated, and the net secretion of α1-adrenergic and cholinergic agonists must be the result of a tight balance of stimulatory and inhibitory pathways. Therefore, it is of interest that cholinergic and α1-adrenergic agonists activate both stimulatory and inhibitory pathways. We have identified two...
such inhibitory pathways for α₁-adrenergic agonists. We have previously shown that whereas α₁-adrenergic agonists activate PKCα, -δ, and -ε, activation of PKCα and -δ inhibits α₁-adrenergically stimulated protein secretion and PKCε stimulates this secretion. Similarly, α₁-adrenergic agonists and cholinergic agonists activate MAPK, which also inhibits protein secretion in the lacrimal gland. It is tempting to speculate that PKCα and -δ are a part of the pathway involved in the activation of MAPK leading to inhibition of α₁-adrenergically stimulated protein secretion, although there is no evidence to corroborate this hypothesis. It is also possible that α₁-adrenergic agonists activate two independent pathways, both of which inhibit protein secretion. It is not known why these agonists would activate both stimulatory and inhibitory pathways. It is possible that this inhibition acts as a braking system to slow protein secretion, eventually bringing secretion back to basal levels.

In all other cells studied, cholinergic and α₁-adrenergic agonists activate a common pathway, namely the Ca²⁺/PKC pathway, which occurs through activation of PLC and/or PLD (5). Unique to the lacrimal gland acinar cells, we previously found (8, 25) that α₁-adrenergic receptors are not coupled to the activation of PLC or PLD, in contrast to cholinergic receptors. Moreover, we showed (26) that even though both cholinergic and α₁-adrenergic agonists activate PKC, they each activate distinct isoforms of PKC to stimulate secretion, further supporting the fact that cholinergic and α₁-adrenergic receptors operate through separate pathways to control lacrimal gland function. The results reported in the present study expand on this premise. Both the phosphorylation of the EGFR and the recruitment of Shc and Grb2 indicate that α₁-adrenergic agonists transactivate the EGFR to activate MAPK, whereas cholinergic agonists do not. Cholinergic, but not α₁-adrenergic, agonists activated p60Src, leading to MAPK activation and inhibition of protein secretion. Although we have shown that cholinergic agonists also activate Pyk2, we have no direct evidence for its role in activation of MAPK. The fact that the p60Src inhibitor PP1 only partially inhibited carbachol-stimulated activation of MAPK could imply that there are multiple pathways within the lacrimal gland used by cholinergic agonists to activate MAPK. It is interesting to note that even a partial inhibition of MAPK by PP1 still leads to a significant increase in carbachol-stimulated protein secretion.

The mechanisms involved in GPCR-induced transactivation of the EGFR are complicated, and many aspects are still not clearly understood. It is known that G proteins, metalloproteinases, and PKC play pivotal roles in activation of MAPK. Prenzel et al. (22) showed that transactivation of the EGFR by GPCR requires cleavage of proHB-EGF by metalloproteinases. Luttrell et al. (21) showed that the Gβγ subunits activate Sre, which subsequently activates metalloproteinases, leading to phosphorylation of the EGFR with subsequent recruitment of adapter proteins Shc and Grb2 and activation of MAPK. The EGFR can also be completely bypassed, because PKC can directly activate Raf and Pyk2 to activate MAPK (11). In addition, Pyk2 is known to be activated by Ca²⁺ as well (3). The involvement of metalloproteinases, PKC, or Ca²⁺ in α₁-adrenergic and cholinergic agonist-induced MAPK activation in the lacrimal gland and its regulation of protein secretion is not understood. It is unlikely that metalloproteinases are involved in cholinergic activation of MAPK because cholinergic agonists do not transactivate the EGFR. We hypothesize that cholinergic agonists activate Pyk2 and p60Src, which in turn directly activate Raf, leading to activation of MAPK, although the exact mechanisms by which Pyk2 and p60Src activate MAPK are not known. It is not known whether α₁-adrenergic agonists activate metalloproteinases, since these agonists do not activate Pyk2 or p60Src. Thus the exact mechanisms by which α₁-adrenergic agonists transactivate the EGFR to activate MAPK are not known.

During our investigations, we consistently found substantial amounts of tyrosine phosphorylated Shc associated with Grb2, tyrosine phosphorylated EGFR, and active MAPK under basal conditions. Protein secretion in the lacrimal gland occurs through two distinct pathways: a constitutive pathway in which proteins are constantly synthesized and secreted, and a regulated pathway in which synthesized proteins are stored in secretory granules awaiting external stimuli to be secreted. It is possible that the basal level of activated MAPK is necessary for regulation of protein synthesis for the constitutive secretory pathway in the lacrimal gland as well as the regulatory pathway. This is supported by the observations that inhibition of MAPK with PD-980059 and relatively high concentrations of U0126 significantly increased secretion in the absence of exogenous addition of stimuli.

It is worth noting that α₁-adrenergic agonists consistently gave a smaller response in the activation of the EGFR than EGF. This might occur if α₁-adrenergic agonists results in tyrosine phosphorylation of Shc and activation of MAPK in freshly isolated lacrimal gland epithelial cells. We have shown, using an inhibitor of the EGFR and measuring its phosphorylation, that α₁-adrenergic agonists transactivate the EGFR to activate MAPK. Transactivation of the EGFR by α₁-adrenergic agonists results in tyrosine phosphorylation of Shc and recruitment of Grb2, two early events in the biochemical cascade leading to activation of MAPK. In contrast, cholinergic agonists do not transactivate the EGFR but do activate Pyk2 and p60Src, though the role of Pyk2 in the activation of
MAPK is not completely clear. Activation of MAPK, by either mechanism, leads to inhibition of lacrimal gland protein secretion.

REFERENCES