Signal Transduction Pathways Used by EGF to Stimulate Protein Secretion in Rat Lacrimal Gland

Vanja Tepavcevic, Robin R. Hodges, Driss Zoukhri, and Darlene A. Dartt

PURPOSE. To determine the effects of epidermal growth factor (EGF) on lacrimal gland secretion of proteins and characterize its signal-transducing components.

METHODS. Both exorbital lacrimal glands were removed from male Sprague-Dawley rats. Dispersed acini were isolated by collagenase digestion in Krebs-Ringer bicarbonate (KRB) buffer at 37°C. Acini were incubated with EGF (10−7 M), the cholinergic agonist carbachol (10−5 M), or the α1-adrenergic agonist phenylephrine (10−4 M), and peroxidase secretion was measured by a fluorescence assay. To measure intracellular calcium ([Ca2+]i), acini were incubated in fura-2 tetra-acetoxymethyl ester for 60 minutes at 22°C, and fluorescence was measured at 340 and 380 nm with an emission wavelength of 505 nm. Extracellular Ca2+ was chelated with KRB-BSA without CaCl2 and with 2 mM EGTA before measurement of peroxidase secretion. Protein kinase C (PKC) was downregulated by incubating acini overnight, with or without the phorbol ester, phorbol 12-myristate 13-acetate (PMA; 10−6 M), and peroxidase secretion was measured.

RESULTS. EGF-stimulated peroxidase secretion in a concentration-dependent manner with a significant increase at 10−7 M. EGF-stimulated secretion was inhibited by the EGF receptor (EGFR) inhibitor AG1478, but not by the phosphoinositide-3 kinase inhibitor LY292004 or the mitogen-activated kinase kinase inhibitor U0126. EGF increased [Ca2+]i, whereas chelation of extracellular Ca2+ inhibited EGF-induced peroxidase secretion by 90%. Downregulation of PKC also inhibited EGF-stimulated peroxidase secretion.

CONCLUSIONS. EGF stimulates lacrimal gland secretion of protein by activating the EGFR to increase [Ca2+]i, and activate PKC. (Invest Ophthalmol Vis Sci. 2003;44:1075-1081) DOI:10.1167/iovs.0240794

The lacrimal gland is an exocrine gland that secretes water, ions, and proteins onto the ocular surface in response to neural stimulation1 and is the main source of the aqueous layer of the tear film.1 Nerves tightly control the amount and makeup of lacrimal gland fluid, and any alteration can be detrimental to the stability of the tear film. Tear film disorders can lead to irritation and inflammation of the ocular surface that, in the worst case, can induce corneal ulcerations and an increased risk of bacterial infections, possibly leading to visual impairment. Such alterations in the tear film are known collectively as dry-eye syndromes2 and can be classified into two groups: aqueous-deficient and evaporative dry eye. Aqueous-deficient dry-eye syndromes are a direct result of the absence of output from the lacrimal gland.2 It is imperative to understand how a normal lacrimal gland functions to develop treatments for dry-eye syndromes.

Protein secretion by the mammalian lacrimal gland is primarily a function of the acinar cells, which are highly polarized cells constituting the main cell type in the lacrimal gland,3 and is regulated by neurotransmitters released from sympathetic and parasympathetic nerves. Receptors for these neurotransmitters are located on the basolateral membranes of the acinar cells.3 Activation of these receptors results in stimulation of signal-transduction pathways culminating in release of water, electrolytes, and proteins across the apical membranes and into the glandular excretory duct and onto the ocular surface.

Lacrimal gland secretion of protein is stimulated by α1-adrenergic and cholinergic agonists, using separate signal-transduction pathways.4 α1-Adrenergic agonists, such as phenylephrine, stimulate secretion by binding to α1-adrenergic receptors and activating protein kinase C (PKC).e Cholinergic agonists, such as acetylcholine and carbachol, bind to muscarinic receptors and activate phospholipase Cβ (PLCβ).6 PLCβ hydrolyzes phosphatidylinositolbisphosphate (PIP2) into 1,4,5-inositoltrisphosphate (IP3) and diacylglycerol (DAG). DAG activates PKC. The lacrimal gland contains at least five PKC isoforms: PKCo, δ, ε, η, /, and μ. Although the roles of PKCs/ and μ in protein secretion in the lacrimal gland are unknown, activation of PKC, δ, ε, η leads to secretion.8 IP3 interacts with receptors on the endoplasmic reticulum to release stored Ca2+ into the cytosol. Emptying of these intracellular Ca2+ stores stimulates the influx of extracellular Ca2+ across the plasma membrane.7 This increase in intracellular Ca2+ concentration, in conjunction with Ca2+-calmodulin-dependent protein kinases, activates protein secretion.

Epidermal growth factor (EGF) is a much-studied growth factor and is part of a family of growth factors consisting of approximately 11 members.9,10 EGF is synthesized as a precursor molecule with a molecular mass of 170 kDa that spans the plasma membrane.9,10 Pro-EGF, with a molecular mass of 150 kDa, is produced by cleavage of the precursor molecule and is released from the membrane (called ectodomain shedding). The 6-kDa form of EGF can be released by further proteolytic cleavage of the pro-EGF. All three forms of EGF, the membrane-bound precursor, the soluble pro-EGF, and the 6-kDa form, are biologically active. Thus, EGF can act as a growth factor when it is associated with the membrane (juxtacrine interaction) or as a soluble molecule (paracrine or autocrine interaction).

It is well established that the lacrimal gland produces EGF, which has a presumed biological activity on the ocular surface.11,12 It is also possible that EGF has juxtacrine, paracrine, or autocrine effects within the lacrimal gland itself. However, the effects of growth factors on lacrimal gland functions remain to be determined. EGF is known to have long-term effects, such as activation of cell proliferation, differentiation, and migration. It also can have short-term effects on secretory functions that are tissue- and cell-type dependent. EGF is known to stimulate amylase release in pancreatic acini,13 secretion of mucus glycoproteins in human gastric mucosa.14

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thyrotropin secretion in the rat,\textsuperscript{15} and prolactin synthesis and secretion in rat pituitary cells in culture.\textsuperscript{16} Conversely, EGF inhibits secretagogue-induced CAMP production and amylase secretion by G-protein-coupled receptors in pancreatic acini,\textsuperscript{17} chloride secretion in T84 cells,\textsuperscript{18} and gastric acid secretion.\textsuperscript{19}

The classic signal-transduction pathway used by EGF involves activation of the p42/p44 mitogen-activated protein kinase (MAPK) cascade.\textsuperscript{20} EGF binds to its receptors (ErBB) inducing their homo- and heterodimerization, which activates the intrinsic tyrosine kinase activity of these receptors. Activated receptors recruit the adapter proteins Shc and Grb2. This leads to activation of the protein kinase Raf (MAPK kinase kinase) and MEK (MAPK kinase) that are part of the cascade of protein kinases ultimately leading to activation of MAPK.\textsuperscript{20} In addition, EGF can also recruit and activate PLC, leading to an increase in intracellular Ca\textsuperscript{2+} and activation of PKC.\textsuperscript{21} It is also known that EGF can stimulate phosphoinositide-3 kinase (PI-3K).\textsuperscript{22}

The purpose of this study was to determine whether EGF stimulates lacrimal gland secretion of protein and if so, what signal-transduction pathways are used by EGF. We show that EGF stimulates lacrimal gland secretion of protein through the EGFR by possibly activating PLC\textsubscript{γ} and leading to an increase in intracellular Ca\textsuperscript{2+} and activation of PKC. EGF does not activate either PI-3K or MAPK to stimulate protein secretion.

**Materials and Methods**

Carbachol and phenylephrine were purchased from Sigma Chemical Co. (St. Louis, MO). EGF was from Upstate Biotechnology (Lake Placid, NY). U0126 and tyrphostin AG1478 were from Biomol Research Laboratories (Plymouth Meeting, PA). LY292004 was purchased from Alexis Biochemicals (San Diego, CA). PMA was from L. C. Laboratories (Waltham, MA). RPMI media was from BioWhittaker (Walkersville, MD). Unless otherwise specified, all other reagents were purchased from Sigma Chemical Co. Antibodies to PKC\textsubscript{α}, -δ, and -ε were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Isolation and Preparation of Acini and Measurement of Protein Secretion**

All experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Schepps Eye Research Institute Animal Care and Use Committee. Both exorbital lacrimal glands were removed from male Sprague-Dawley rats (Taconic, Germantown, NY) that had been anesthetized with CO\textsubscript{2} for 1 minute and then decapitated. Dispersed acini were isolated by collagenase digestion, as previously described.\textsuperscript{4} After recovery in Krebs-Ringer bicarbonate (KRB) buffer (in mM: 119 NaCl, 4.8 KCl, 1 CaCl\textsubscript{2}, 1.2 MgSO\textsubscript{4}, 1.2 KH\textsubscript{2}PO\textsubscript{4}, and 25 NaHCO\textsubscript{3}) supplemented with 10 mM HEPES, 5.5 mM glucose, and 0.5% bovine serum albumin (BSA; pH 7.4) at 37\textdegree C, acini were resuspended in RPMI 1640 medium supplemented with penicillin (100 U/mL) and streptomycin (100 \mu g/mL) and incubated at 37\textdegree C in a humidified atmosphere containing 5% CO\textsubscript{2}, with or without the phorbol ester PMA (10 \textmu M). Long-term treatment of cells with phorbol esters downregulates PKC, resulting in a loss of PKC activity. Acini were collected, centrifuged, and washed twice with KRB buffer containing 0.5% BSA. They were then incubated for 20 minutes with or without phenylephrine (10 \textmu M), carbachol (10\textsuperscript{-4} M), PMA (10\textsuperscript{-6} M), and EGF (10\textsuperscript{-7} M). Secretion was measured as previously described.

To chelate extracellular Ca\textsuperscript{2+}, acini were incubated either in KRB-BSA without CaCl\textsubscript{2} and containing 2 mM EGTA. The cells were then incubated for 20 minutes, with or without carbachol (10\textsuperscript{-4} M) or EGF (10\textsuperscript{-7} M), and secretion was measured.

**Measurement of [Ca\textsuperscript{2+}]\textsubscript{i}**

Acini were incubated in KRB-HEPES buffer containing 0.5% BSA, 0.5 \mu M fura-2-tetra-acetoxyethyl ester, 10% phoronic F127, and 250 \mu M sulfinpyrazone for 60 minutes at 22°C. The cells were then washed with KRB-HEPES buffer containing 250 \mu M sulfinpyrazone, and fluorescence was measured at 22°C. Fluorescence was measured at excitation wavelengths of 340 and 380 nm and an emission wavelength of 505 nm using a luminescence spectrophotometer (model LS-5B; Perkin Elmer, Wellesley, MA). To calculate [Ca\textsuperscript{2+}]\textsubscript{i}, 5.6 mM EGTA, 7.5 mM Tris-HCl (pH 7.5), and 1% Triton X-100 were added at the end of the reaction to obtain the minimum level of fluorescence. The maximum fluorescence was determined by the addition of 14.5 mM CaCl\textsubscript{2}. The dissociation constant of 135 nM for fura-2 at 22°C was used to calculate [Ca\textsuperscript{2+}]\textsubscript{i}, by the ratio method.\textsuperscript{23}

**Detection of PKC by Western Blot Analysis**

Acini, incubated overnight in RPMI 1640 medium supplemented with penicillin (100 U/mL) and streptomycin (100 \mu g/mL), with or without PMA (10\textsuperscript{-7} M) to downregulate PKC, were centrifuged and washed twice with fresh KRB buffer containing 0.5% BSA. The pellet was suspended in radioimmunoprecipitation assay (RIPA) buffer (in mM: 10 Tris-HCl [pH 7.4], 150 NaCl, 1 Na\textsubscript{3}VO\textsubscript{3}, 1 EDTA, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 100 \mu g/mL phenylmethylsulfonyl fluoride, and 30 \mu g/mL aprotonin). The acini were sonicated, and the homogenate was centrifuged at 5000 rpm for 30 minutes at 4°C. To the supernatant (cell lysate), Laemmli sample buffer was added, and the samples were boiled for 5 minutes. Proteins were separated by SDS-PAGE (12% gels) and transferred to nitrocellulose membrane. Antibodies against PKCo and PKCe were used at 1:1000 dilution, and the antibody against PKCo δ was used at 1:50 dilution. Secondary antibodies were conjugated to horseradish peroxidase. Immunoreactive bands were visualized using the enhanced chemiluminescence method.
peroxidase secretion from rat lacrimal gland acini.

These data suggest that EGF stimulates 50.6% fold over basal (Fig. 1). These data suggest that EGF is acting through the EGFR to stimulate EGF-stimulated protein secretion by 50.6%.

It is well known that EGF activates a cascade of protein kinases, including Raf and MEK, that culminates in the activation of MAPK. We have shown that inhibition of MEK with U0126 inhibits cholinergic agonist-induced activation of MAPK in the lacrimal gland at $3 \times 10^{-7}$, $6 \times 10^{-7}$, and $10^{-6}$ M. In addition, we have shown that inhibition of MEK with U0126 significantly increases carbachol- and phenylephrine-induced lacrimal gland secretion of protein, implying that MAPK is involved in the negative regulation of stimulated peroxidase secretion. To determine whether MAPK is activated by EGF to alter EGF-stimulated lacrimal gland peroxidase secretion, lacrimal gland acini were preincubated with U0126 (10^{-7}, 3 \times 10^{-7}, and 6 \times 10^{-7} M) for 30 minutes before stimulation with EGF (10^{-7} M) for 20 minutes. As shown in Figure 3, U0126 did not have a significant effect on EGF-induced protein secretion at any concentration tested. At the highest concentration used ($6 \times 10^{-7}$ M), which we had shown caused a significant increase in carbachol-stimulated peroxidase secretion, U0126 had no effect on EGF-induced peroxidase secretion (115% ± 28.0% of total). U0126 alone did not have an effect on basal secretion (data not shown).

**FIGURE 1.** Effect of EGF on lacrimal gland peroxidase secretion. Lacrimal gland acini were stimulated with EGF (10^{-11}–10^{-6} M) for 20 minutes, and peroxidase secretion was measured. Data are the mean ± SE of results in five independent experiments. *Significantly different from $10^{-11}$ M.

**Data Presentation and Statistical Analysis**

Data are expressed as the mean ± SEM. Where indicated, the data were statistically analyzed using Student’s t-test for paired values. $P < 0.05$ was considered to be significant.

**RESULTS**

**Effect of EGF on Lacrimal Gland Secretion of Protein**

Because EGF has been shown to be present in the lacrimal gland by PCR and is known to act in either an autocrine or paracrine manner, we determined the effects of EGF on lacrimal gland peroxidase secretion. Freshly isolated acinar cells were incubated with increasing concentrations of EGF (10^{-11}–10^{-6} M) for 20 minutes, and peroxidase secretion was measured. Basal peroxidase secretion was measured to be 0.86% ± 0.36% total peroxidase. EGF at $10^{-7}$ and $10^{-6}$ M significantly increased peroxidase secretion with a net secretion over basal of 0.65% ± 0.25% and 1.02% ± 0.48% of total peroxidase, respectively. This represents an increase of 1.8-fold and 2.2-fold over basal (Fig. 1). These data suggest that EGF stimulates peroxidase secretion from rat lacrimal gland acini.

**Effect of Inhibition of EGFR Phosphorylation on Secretion**

To determine whether the effects of EGF on peroxidase secretion are mediated through activation of the EGFR, acini were incubated with AG1478 (10^{-7}–10^{-6} M), an inhibitor of the intrinsic tyrosine kinase activity of the EGFR, for 15 minutes before stimulation with $10^{-7}$ M EGF for 20 minutes. We have shown that preincubation with AG1478 (10^{-7} M) significantly inhibited EGF-stimulated tyrosine phosphorylation of the EGFR. Preincubation with AG1478 significantly inhibited EGF-stimulated protein secretion by 50.6% ± 13.1%, 78.2% ± 14.8%, and 89.9% ± 10.1%, at $10^{-7}$, $10^{-6}$, and $10^{-5}$ M, respectively (Fig. 2). AG1478 had no effect on basal secretion. These data suggest that EGF is acting through the EGFR to stimulate peroxidase secretion.

**Effect of Inhibition of MEK on EGF-Induced Protein Secretion**

It is well known that EGF activates a cascade of protein kinases, including Raf and MEK, that culminates in the activation of MAPK. We have shown that inhibition of MEK with U0126 inhibits cholinergic agonist-induced activation of MAPK in the lacrimal gland at $3 \times 10^{-7}$, $6 \times 10^{-7}$, and $10^{-6}$ M. In addition, we have shown that inhibition of MEK with U0126 significantly increases carbachol- and phenylephrine-induced lacrimal gland secretion of protein, implying that MAPK is involved in the negative regulation of stimulated peroxidase secretion. To determine whether MAPK is activated by EGF to alter EGF-stimulated lacrimal gland peroxidase secretion, lacrimal gland acini were preincubated with U0126 (10^{-7}, 3 \times 10^{-7}, and 6 \times 10^{-7} M) for 30 minutes before stimulation with EGF (10^{-7} M) for 20 minutes. As shown in Figure 3, U0126 did not have a significant effect on EGF-induced protein secretion at any concentration tested. At the highest concentration used ($6 \times 10^{-7}$ M), which we had shown caused a significant increase in carbachol-stimulated peroxidase secretion, U0126 had no effect on EGF-induced peroxidase secretion (115% ± 28.0% of total). U0126 alone did not have an effect on basal secretion (data not shown).

**FIGURE 2.** Effect of AG1478 (10^{-7}–10^{-6}) on EGF-stimulated peroxidase secretion. Lacrimal gland acini were preincubated with increasing concentrations of the EGFR tyrosine kinase inhibitor AG1478 for 15 minutes before stimulation of EGF (10^{-7} M) for 20 minutes. Data are the mean ± SE of results in three independent experiments. *Significantly different from EGF alone.

**FIGURE 3.** Effect of U0126 on EGF-stimulated peroxidase secretion. Lacrimal gland acini were preincubated with increasing concentrations of the MEK inhibitor for 30 minutes before stimulation of EGF (10^{-7} M) for 20 minutes. Data are the mean ± SE of results in four independent experiments.
before stimulation with 10^{-6} M EGF, acini were preincubated for 1 hour with fura 2 and stimulated with EGF (10^{-5} M). All three concentrations of EGF significantly increased intracellular Ca^{2+} (Fig. 6A). EGF 10^{-6} M increased [Ca^{2+}]_{i} to 56.9 ± 10.9 nM from a basal value of 24.3 ± 2.5 nM. EGF 10^{-7} M and 10^{-6} M increased [Ca^{2+}]_{i} to 56.9 ± 14.1 and 66.3 ± 18.9 nM, respectively. This response is in comparison to 145.9 ± 37.9 nM obtained in the presence of carbachol, a cholinergic agonist known to increase [Ca^{2+}]_{i}.

We then examined whether chelation of extracellular Ca^{2+} with 2 mM EGTA had an effect on EGF-stimulated lacrimal gland secretion of protein. Chelation of extracellular Ca^{2+} with EGTA significantly inhibited both carbachol- (a positive control) and EGF-induced protein secretion. Carbachol-induced secretion was inhibited 65.4% ± 2.7%, whereas EGF-induced protein secretion was inhibited 90.3% ± 9.7% (Fig. 6B). These data imply that EGF increases [Ca^{2+}]_{i}, and utilizes Ca^{2+} to stimulate peroxidase secretion.

Effect of Simultaneous Addition of Cholinergic or \( \alpha_{1} \)-Adrenergic Agonists and EGF

Cholinergic and \( \alpha_{1} \)-adrenergic agonists are major stimuli of lacrimal gland secretion of protein. We have shown that these two agonists use PKC and Ca^{2+} to various degrees to stimulate peroxidase secretion. To determine whether EGF used the same intracellular signaling pathways as cholinergic or \( \alpha_{1} \)-adrenergic agonists, acini were incubated in the presence of each agonist alone or with combination of agonists. EGF (10^{-7} M) significantly stimulated peroxidase secretion to 2.9% ± 0.9% from a basal value of 1.8% ± 0.6%. Carbachol and phenylephrine also significantly stimulated peroxidase secretion to 2.9% ± 0.7% of total peroxidase and 6.5% ± 1.1%, respectively (Fig. 5). Simultaneous addition of the cholinergic agonist carbachol (10^{-4} M) and EGF (10^{-7} M) resulted in secretion of 1.9% ± 0.2% (Fig. 5). This secretion was significantly less than the calculated theoretical secretion of 4.0% ± 1.0%. Simultaneous addition of the phenylephrine (10^{-4} M) and EGF (10^{-7} M) resulted in secretion of 4.7% ± 0.6%. This secretion was also significantly less than the calculated theoretical secretion of 7.4% ± 1.5% (Fig. 5). These data imply that EGF uses the same pathways as cholinergic and \( \alpha_{1} \)-adrenergic agonists to stimulate peroxidase secretion.

Effect of Ca^{2+} on EGF-Stimulated Lacrimal Gland Secretion of Protein

Because EGF seems to use the same signal transduction pathways as cholinergic and \( \alpha_{1} \)-adrenergic agonists, we examined the role of Ca^{2+} in EGF-stimulated secretion of peroxidase. To determine whether EGF stimulates lacrimal gland secretion of protein by increasing intracellular [Ca^{2+}]_{i}, acini were preincubated for 1 hour with fura 2 and stimulated with EGF (10^{-8} – 10^{-6} M). All three concentrations of EGF significantly increased intracellular Ca^{2+} (Fig. 6A). EGF 10^{-6} M increased [Ca^{2+}]_{i} to 56.9 ± 10.9 nM from a basal value of 24.3 ± 2.5 nM. EGF 10^{-7} M and 10^{-6} M increased [Ca^{2+}]_{i} to 56.9 ± 14.1 and 66.3 ± 18.9 nM, respectively. This response is in comparison to 145.9 ± 37.9 nM obtained in the presence of carbachol, a cholinergic agonist known to increase [Ca^{2+}]_{i}.

To determine whether PKC was involved in the signaling pathways leading to EGF-stimulated protein secretion in the lacrimal gland, we incubated lacrimal gland acini with the PKC activator PMA (10^{-6} M) overnight to downregulate PKC, as described in the Methods section. After overnight treatment with PMA, either PKC activation or peroxidase secretion was measured. Western blot analysis was performed with antibodies against PKCa, -\( \delta \), and -\( \epsilon \) (Fig. 7A). PKCa was downregulated by 79%, PKCe by 53%, and PKCd by 10%. Secretion experiments were also performed in which acini were stimulated

![FIGURE 4. Effect of LY292004 on EGF-stimulated secretion of peroxidase. Lacrimal gland acini were preincubated with increasing concentrations of the PI-3K inhibitor LY292004 for 30 minutes before stimulation with EGF (10^{-7} M) for 20 minutes. Data are the mean ± SE of results in six independent experiments.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932918/)

![FIGURE 5. Effect of simultaneous addition of cholinergic or \( \alpha_{1} \)-adrenergic agonists and EGF. Lacrimal gland acini were incubated for 20 minutes with carbachol (Cch, 10^{-4} M), phenylephrine (Ph, 10^{-4} M), EGF (10^{-7} M), Cch+EGF, or Ph+EGF, and peroxidase secretion was measured. (■) Actual secretion measured. (□) Calculated theoretical secretion. Data are the mean ± SE of results in four independent experiments. *Significantly different from basal secretion; †significantly different from calculated secretion.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932918/)
Lacrimal gland acini were incubated for 1 hour with the secretion. Which also involves activation of PLC-

In the lacrimal gland, EGF binds to the EGFR and may activate types but is known to stimulate secretion in only a few tissues. Because EGF has also been detected in the tear film, it is hypothesized that the lacrimal gland synthesizes and secretes EGF onto the ocular surface for the health and maintenance of the cells of the cornea and conjunctiva. Secretion of EGF occurs through the process of ectodomain shedding rather than regulated protein secretion, as is the case for peroxidase, which occurs through exocytosis. Precursor EGF is synthesized in the cell and inserted into the plasma membrane. It is then cleaved by specific enzymes to generate its soluble factors, pro-EGF and EGF. EGF is active when attached to the cell membrane or as both types of soluble molecules. Because of this shedding process, EGF synthesized by the lacrimal gland may also affect lacrimal gland cells directly. However, only precursor EGF located on the basal lateral membranes would affect the lacrimal gland directly, whereas precursor EGF on apical membranes would be released into the tears and affect the ocular surface.

EGF and other growth factors are most commonly involved in long-term events such as cell proliferation, differentiation, and migration. Indeed, Zieske et al. have shown that the mRNA for EGR and the EGF receptor increases during corneal epithelial wound repair. In addition, Wilson et al. showed that mRNA levels of EGF in the lacrimal gland increase after a corneal wound. Taken together, these results imply that the lacrimal gland is a primary source of growth factors to promote healing of the ocular surface. Although some of the effects of EGF on the ocular surface are known, its effects on the lacrimal gland itself are not completely understood. In this study, EGF stimulated protein secretion from lacrimal gland acini. In addition to the lacrimal gland, EGF is also known to stimulate protein secretion from pancreatic acini, gastric mucosa, and rat pituitary cells. It is also possible that EGF plays a role in maintenance and survival of the lacrimal gland. It has been

with carbachol (10^{-4} M) and PMA (10^{-6} M) as positive controls or EGF (10^{-7} M). Overnight treatment of cells with PMA completely abolished PMA-induced protein secretion and significantly inhibited carbachol- and EGF-induced protein secretion by 93\% \pm 7\% and 79\% \pm 21\%, respectively (Fig. 7B). These data imply that EGF uses PKC\(\alpha\) and \(\delta\) to stimulate peroxidase secretion.

**DISCUSSION**

EGF is a well-established growth factor that has been shown to be involved in cell growth and differentiation in many cell types but is known to stimulate secretion in only a few tissues. In the lacrimal gland, EGF binds to the EGFR and may activate PLC\(\gamma\). This, in turn, activates PKC\(\alpha\) and \(\delta\) and increases \([Ca^{2+}]_{i}\), but does not stimulate either PI-3K or MAPK to lead to protein secretion. This is similar to EGF-stimulated amylase secretion, which also involves activation of PLC-\(\gamma\) and EGF-stimulated prolactin secretion, which occurs through an increase in \([Ca^{2+}]_{i}\). EGF is also known to stimulate mucus secretion by activation of MAPK. In contrast to the lacrimal gland, EGF activates PI-3K to inhibit Cl\(^{-}\) secretion in T84 cells.

It has been shown that EGF is synthesized in the lacrimal glands of humans and rats. Because EGF has also been detected in the tear film, it is hypothesized that the lacrimal gland synthesizes and secretes EGF onto the ocular surface for the health and maintenance of the cells of the cornea and conjunctiva. Secretion of EGF occurs through the process of ectodomain shedding rather than regulated protein secretion, as is the case for peroxidase, which occurs through exocytosis. Precursor EGF is synthesized in the cell and inserted into the plasma membrane. It is then cleaved by specific enzymes to generate its soluble factors, pro-EGF and EGF. EGF is active when attached to the cell membrane or as both types of soluble molecules. Because of this shedding process, EGF synthesized by the lacrimal gland may also affect lacrimal gland cells directly. However, only precursor EGF located on the basal lateral membranes would affect the lacrimal gland directly, whereas precursor EGF on apical membranes would be released into the tears and affect the ocular surface.

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**FIGURE 6.** Role of Ca\(^{2+}\) in EGF-stimulated peroxidase secretion. (A) Lacrimal gland acini were incubated for 1 hour with the fluorescent Ca\(^{2+}\) indicator fura 2. Acini were then stimulated with increasing concentrations of EGF ( ), or carbachol ( ), and fluorescence was measured and [Ca\(^{2+}\)] calculated. Data are the mean ± SE of results in four independent experiments. Significantly different from basal value. (B) Lacrimal gland acini were stimulated for 20 minutes with EGF (10\(^{-7}\) M) or Cch (10\(^{-4}\) M) in buffer containing normal calcium (1 mm) or KRB buffer with no Ca\(^{2+}\) added and with 2 mM EGTA. Data are the mean ± SE of results in four independent experiments. Significantly different from secretion obtained in the presence of agonist alone.
shown that androgens play a critical role in preservation of the characteristics and function of the lacrimal gland.34 Of interest, EGF has been shown to play a role in the regulation of androgen expression through phosphorylation of the androgen receptor and alteration of its sensitivity to low levels of androgens.35 36 Thus, EGF appears to play an important role in the function (secretion) and maintenance of the lacrimal gland.

Of note, Marechal et al.37 showed that the mature 6-kDa form of EGF is not present in the rat lacrimal gland. The only form of EGF that they detected was the approximately 152-kDa pro-EGF, although they did not establish the location of the pro-EGF. They hypothesized that pro-EGF is released from the lacrimal gland onto the ocular surface where it is then cleaved by other enzymes present in the tears to produce the 6-kDa form. It is also possible that the 6-kDa form of EGF is formed only after stimulation of lacrimal gland acinar cells. The pro-EGF form of the molecule could stimulate lacrimal gland secretion if it is on the basal lateral membrane, because it is known that pro-EGF binds to and activates the EGFR.37,38

Other members of the EGF family of proteins—namely, transforming growth factor (TGF)—have also been shown to be present in the lacrimal glands of the mouse and human.39 In addition, other growth factors, such as fibroblast growth factor, hepatocyte growth factor, TGFβ, and keratinocyte growth factor have been detected in the lacrimal gland.35 40 The effects of these growth factors on lacrimal gland secretion of protein have not been investigated.

Peroxidase secretion in the presence of both cholinergic agonists and EGF or α1-adrenergic agonists and EGF is less than additive, indicating that portions of the pathway used by EGF to stimulate secretion are shared with those used by cholinergic and α1-adrenergic agonists. We have shown that cholinergic agonists activate PKCα, β, and ε and increase [Ca2+]i. Similar to cholinergic agonists, EGF also activates PKCa and -δ and increases [Ca2+]i. However, it is not clear at what point the pathways used by EGF and α1-adrenergic agonists overlap. It is known that α1-adrenergic agonists activate PKCe to stimulate protein secretion and increase [Ca2+]i, to a small extent.4 It is possible that the overlap between the two pathways occurs with the increase in [Ca2+]i, although the increase in [Ca2+]i, obtained in the presence of α1-adrenergic agonists is approximately half of that obtained in the presence of EGF.4 It is also possible that the overlap occurs at the level of the EGFR, because α1-adrenergic agonists have been shown to transactivate it.37 This transactivation, however, has so far been limited only to activation of MAPK, which inhibits α1-adrenergic agonist-induced protein secretion and does not appear to be involved in EGF-induced protein secretion.

Western blot analysis of PKC showed that overnight treatment with PMA downregulated PKCa and -δ, but not -ε. EGF-stimulated peroxidase secretion was inhibited by 79% by this treatment, which implies that EGF does not use PKCe, but rather uses PKCa and/or -δ to stimulate protein secretion. This is in comparison with results obtained with cholinergic agonists, which have shown uses PKCa and -ε primarily and PKCδ to a lesser extent, and α1-adrenergic agonists, which use only PKCe to stimulate protein secretion.37 Because the other PKC isoforms present in the lacrimal gland—PKCγ/λ and -μ—are not affected by treatment with phorbol ester, their role in EGF-stimulated protein secretion is not known.

It is interesting to note that inhibition of either PKC or chelation of Ca2+ resulted in significant inhibition of 79% and 90% of EGF-stimulated protein secretion. This implies that activation of either pathway can mediate EGF-induced secretion of protein. It is possible that the presence of both Ca2+ and PKC is necessary for exocytosis (i.e., fusion of the secretory granules with the plasma membrane) to occur. It has been shown in pancreatic acini that both PKC and Ca2+ trigger exocytosis.32 It is also possible that there are redundancies in the secretory pathways of the lacrimal gland, so that it contains multiple pathways to stimulate protein secretion. Because tears are absolutely required for the health and maintenance of the cells on the ocular surface, it follows that the lacrimal gland would have multiple pathways leading to secretion in the event that one pathway is defective.

In conclusion, in our study EGF stimulated protein secretion from rat lacrimal gland. EGF activated the EGFR, stimulating PLC to activate PKC and increase [Ca2+]i, thereby inducing protein secretion. Identification of this new regulatory pathway could lead to the development of new treatments for stimulation of lacrimal gland secretion to treat dry-eye syndromes.

References


