EGF inhibits muscarinic receptor-mediated calcium signaling in a human salivary cell line

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Zhang, Bin-Xian, Chih-Ko Yeh, Tazuko K. Hymer, Meyer D. Lifschitz, and Michael S. Katz. EGF inhibits muscarinic receptor-mediated calcium signaling in a human salivary cell line. Am J Physiol Cell Physiol 279: C1024–C1033, 2000.—The effects of epidermal growth factor (EGF) on intracellular calcium ([Ca2+]i) responses to the muscarinic agonist carbachol were studied in a human salivary cell line (HSY). Carbachol (10–4 M)-stimulated [Ca2+]i mobilization was inhibited by 40% after 48-h treatment with 5 × 10–10 M EGF. EGF also reduced carbachol-induced [Ca2+]i in Ca2+ -free medium and Ca2+ influx following repletion of extracellular Ca2+. Under Ca2+ -free conditions, thapsigargin, an inhibitor of Ca2+ uptake to internal stores, induced similar [Ca2+]i signals in control and EGF-treated cells, indicating that internal Ca2+ stores were unaffected by EGF; however, in cells exposed to thapsigargin, Ca2+ influx following Ca2+ repletion was reduced by EGF. Muscarinic receptor density, assessed by binding of the muscarinic receptor antagonist L-[benzilic-4,4]-H(3)CN(quinuclidinyl benzilate (3H)QNB), was decreased by 20% after EGF treatment. Inhibition of the carbachol response by EGF was not altered by phorbol ester-induced downregulation of protein kinase C (PKC) but was enhanced upon PKC activation by a diacylglycerol analog. Phosphorylation of mitogen-activated protein kinase (MAP kinase) and inhibition of the carbachol response by EGF were both blocked by the MAP kinase pathway inhibitor PD-98059. The results suggest that EGF decreases carbachol-induced Ca2+ release from internal stores and also exerts a direct inhibitory action on Ca2+ influx. A decline in muscarinic receptor density may contribute to EGF inhibition of carbachol responsiveness. The inhibitory effect of EGF is mediated by the MAP kinase pathway and is potentiated by a distinct modulatory cascade involving activation of PKC. EGF may play a physiological role in regulating muscarinic receptor-stimulated salivary secretion.

carbachol; signal transduction; protein kinase C; mitogen-activated protein kinase; epidermal growth factor

FLUCTUATIONS of intracellular calcium ([Ca2+]i) mediate the cellular actions of many neurotransmitters, hormones, and growth factors. In salivary gland cells, activation of G protein-coupled muscarinic-cholinergic receptors results in hydrolysis of phosphatidylinositol 4,5-bisphosphate, yielding diacylglycerol and inositol 1,4,5-trisphosphate (IP3). Diacylglycerol is an endogenous activator of protein kinase C (PKC). IP3 induces calcium release from internal calcium stores, followed by the entry of extracellular Ca2+ and a sustained elevation of [Ca2+]i (2). The mobilization of [Ca2+]i in salivary cells is directly related to the level of fluid secretion (30). Currently, factors modulating the muscarinic receptor-coupled calcium signaling pathway in salivary cells have not been well characterized.

Epidermal growth factor (EGF), which is produced in abundance by salivary glands, is a multifunctional factor known to influence the proliferation, differentiation, and physiological function of a wide variety of cell types (reviewed in Ref. 5). The actions of EGF are mediated via a receptor tyrosine kinase cascade, leading to a number of signaling events including activation of PKC and the mitogen-activated protein kinase (MAP kinase) pathway (7, 21). Previous studies implicating, or “cross talk,” between EGF-induced signals and other signaling pathways. For example, EGF modulates protein-coupled receptor activation of adenyl cyclase in a number of tissues (10); purinergic receptor-induced calcium signaling also appears to be influenced by EGF (13). In salivary glands, EGF modifies muscarinic agonist-mediated amylase secretion (29). Although this finding suggests a role for EGF in the regulation of muscarinic receptor function in salivary cells, to our knowledge no previous investigations have demonstrated an interaction between EGF and muscarinic signaling pathways. In the present study, we have used HSY cells, a ductal cell line from human parotid (42), as a model system to determine whether the EGF receptor tyrosine kinase cascade modulates muscarinic receptor signaling in salivary cells. Our results indicate that EGF inhibits muscar-
rinic receptor-mediated \([\text{Ca}^{2+}]_i\) mobilization in HSY cells by activation of the MAP kinase pathway.

**MATERIALS AND METHODS**

**Materials.** Recombinant human EGF (rhEGF), insulin-like growth factor I (rhIGF-I), and transforming growth factor-\(\alpha\) (rhTGF-\(\alpha\)) were purchased from Promega (Madison, WI); platelet-derived growth factor (rhPDGF-AB) was from Pепrotech (Rocky Hill, NJ). Fura 2-AM was from Molecular Probes (Eugene, OR). Thapsigargin, 2-(2-amino-3-methoxyphenyl)-oxanaphthenal-4-one (PD-98059), and digitonin were purchased from RBI (Natick, MA). Monoclonal anti-PKC antibody (which recognizes \(\alpha\)- and \(\beta\)-isomers of PKC) was from Amersham (Arlington Heights, IL). Antibodies against p44/p42 MAP kinase and phospho-p44/p42 MAP kinase were from New England Biolabs (Beverly, MA). L-[Benzilic-4,4'-\text{HCN}]quinclidinyl benzilate ([\text{3H}]QNB; 30 Ci/nmol) was obtained from New England Nuclear (Boston, MA). Trypsin-EDTA and DMEM were from Life Technologies (Gaithersburg, MD). Aprotinin, leupeptin, phorbol 12-myristate 13-acetate (PMA), carbachol, atropine, 1,2-dioctanoyl-sn-glycero, and other chemicals were purchased from Sigma (St. Louis, MO).

**Cell culture.** The HSY cell line was originally established by Yanagawa et al. (42) and was kindly provided by Dr. James Turner (NIDCR/NIH, Bethesda, MD). Cells were plated at a density of about 2 \(\times\) 10\(^4\) cells/cm\(^2\) in 60- or 100-mm culture dishes and cultured in DMEM supplemented with 10% fetal calf serum and penicillin (100 U/ml)/streptomycin (100 \(\mu\)g/ml) at 37°C in a humidified 5% \(\text{CO}_2\) atmosphere incubator. Cells were grown to near confluence at 72 h and were then harvested with trypsin (0.05%)-EDTA (0.02%) for \([\text{Ca}^{2+}]_i\), and receptor binding measurements. Unless otherwise specified, EGF and other growth factors (TGF-\(\alpha\), PDGF, or IGF-I) were added 48 h prior to confluence, i.e., 24 h after plating. Addition of EGF caused a small but significant increase in cell number (\(P < 0.001\)) at 72 h.

**Measurement of intracellular calcium.** \([\text{Ca}^{2+}]_i\), was determined by spectrofluorometric measurements in cell suspensions. HSY cells were loaded with 1.2 \(\mu\)M fura 2-AM in a high-salt glucose (HNG) buffer containing 20 mM Tris, 10 mM KCl, 2 mM EDTA, 0.5 mM EGTA, 2.5 \(\mu\)g/ml leupeptin, 10 \(\mu\)g/ml aprotinin, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM sodium orthovanadate (Na\(_3\)VO\(_4\)), pH 7.5. The cells were homogenized in a 1.5-ml tissue grinder (Kontes Duall, from Fisher Scientific) with 30 strokes on ice. The homogenate was centrifuged (1,000 \(g\) for 10 min, and supernatant proteins (20 \(\mu\)g) were separated on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Schleicher & Schuell, Keene, NH). The membranes were immunoblotted with p44/p42 MAP kinase or phospho-p44/p42 MAP kinase primary antibody (1:1,000) and secondary antibody (1:1,000) with p44/p42 MAP kinase or phospho-p44/p42 MAP kinase primary antibody (1:1,000) and secondary antibody (1:1,000) conjugated to horseradish peroxidase (Amersham). MAP kinasers were visualized by an enhanced chemiluminescence system (ECL Plus, Amersham) and quantified by a phosphorimager system (Storm 860; Molecular Dynamics, Sunnyvale, CA).

**Data analysis.** Agonist-induced increases in \([\text{Ca}^{2+}]_i\) were calculated by subtracting unstimulated (basal) values from maximal agonist responses. Data from multiple experiments are means \(\pm\) SE. Statistical significance of single comparisons was determined using Student’s t-test. Multiple comparisons were performed by either ANOVA followed by the Dunnett multiple comparison test or two-factor ANOVA with the Sidak multiple comparison test. Scatchard analysis of \([\text{3H}]\text{QNB}\) saturation binding curves was used to determine muscarinic receptor density (\(B_{\text{max}}\)) and \(K_d\) (34).

**RESULTS**

**Attenuation of carbachol-induced \([\text{Ca}^{2+}]_i\) mobilization by EGF.** Figure 1A shows \([\text{Ca}^{2+}]_i\) responses to carbachol (10\(^{-4}\) M) in suspensions of EGF-treated and untreated HSY cells. In both groups of cells, carbachol stimulated a rapid increase in \([\text{Ca}^{2+}]_i\), followed by a sustained plateau phase. However, the \([\text{Ca}^{2+}]_i\) response to carbachol was markedly reduced by treatment of cells with EGF. EGF (5 \(\times\) 10\(^{-10}\) M, 48 h) caused a 40% decrease of carbachol-stimulated \([\text{Ca}^{2+}]_i\), mobilization (+ EGF, 381 \(\pm\) 30 nm vs. – EGF, 629 \(\pm\) 42 nm; \(P < 0.001\)) (Fig. 1B). Treatment with EGF had no
apparent effect on basal levels of $[\text{Ca}^{2+}]_i$ or the reversal of the carbachol-induced $[\text{Ca}^{2+}]_i$ signal following addition of atropine, a muscarinic receptor antagonist (Fig. 1A). Inhibition of carbachol-mediated $[\text{Ca}^{2+}]_i$ mobilization was dependent on EGF concentration and time of incubation with the growth factor (Fig. 2). The concentration of EGF producing half-maximal inhibition of carbachol response was $1.9 \pm 0.2 \times 10^{-10} \text{ M}$. Inhibition of the carbachol-induced $[\text{Ca}^{2+}]_i$ signal was observed within about 2 h of EGF treatment, whereas the time of incubation required for maximal inhibition was 4–8 h.

To determine whether growth factor inhibition of carbachol-induced $[\text{Ca}^{2+}]_i$ mobilization is specific to EGF, we compared carbachol responses in HSY cells treated with EGF and other growth factors (TGF-$\alpha$, PDGF, and IGF-I) known to act through receptor tyrosine kinase pathways. TGF-$\alpha$ binds and activates EGF receptors (5), whereas PDGF and IGF-I activate tyrosine kinase signaling via their own distinct receptors. Like EGF, TGF-$\alpha$ also decreased carbachol-induced $[\text{Ca}^{2+}]_i$ mobilization in HSY cells. In contrast, PDGF and IGF-I did not alter carbachol responsive-ness (Fig. 3). These data implicate a specific inhibitory effect of EGF receptor activation on carbachol-induced $[\text{Ca}^{2+}]_i$ mobilization in HSY cells.

Effects of EGF on carbachol-induced $[\text{Ca}^{2+}]_i$ signaling in the absence of extracellular Ca$^{2+}$ and during Ca$^{2+}$ repletion. In nonexcitable cells, G protein-coupled receptor-activated $[\text{Ca}^{2+}]_i$ mobilization consists of initial Ca$^{2+}$ release from IP$_3$-sensitive internal stores, followed by influx of extracellular Ca$^{2+}$ across the plasma membrane. The influx of Ca$^{2+}$ is thought to be activated by depletion of the internal Ca$^{2+}$ stores via a process of “capacitative calcium entry” (31). In a Ca$^{2+}$-free medium, Ca$^{2+}$ influx does not occur and $[\text{Ca}^{2+}]_i$ signals represent mainly release of internal Ca$^{2+}$ stores. To investigate the effect of EGF on carbachol-induced Ca$^{2+}$ release from internal stores, we examined $[\text{Ca}^{2+}]_i$ responsiveness of HSY cells to carbachol under Ca$^{2+}$-free conditions. Figure 4 shows that in the absence of extracellular Ca$^{2+}$, EGF reduced the carbachol-induced $[\text{Ca}^{2+}]_i$ signal by about one-half (+EGF,

![Fig. 1. Effect of EGF on carbachol-stimulated intracellular Ca$^{2+}$ ($[\text{Ca}^{2+}]_i$) mobilization. Mobilization of $[\text{Ca}^{2+}]_i$, in response to a maximally stimulatory concentration of carbachol ($10^{-4} \text{ M}$) was determined in HSY cells either treated with EGF (+EGF, $5 \times 10^{-10} \text{ M}$) or untreated (−EGF) for 48 h, as described in MATERIALS AND METHODS. A: representative experiment showing carbachol-responsive $[\text{Ca}^{2+}]_i$ signals in EGF-treated and untreated cells. Arrows indicate time of addition of carbachol followed by the muscarinic receptor antagonist atropine ($10^{-5} \text{ M}$). B: values are means ± SE for carbachol-induced increases in $[\text{Ca}^{2+}]_i$ from 12 experiments. *P < 0.001 vs. −EGF.]

![Fig. 2. Concentration dependence and time course of EGF effect on carbachol-induced $[\text{Ca}^{2+}]_i$ mobilization. A: HSY cells were treated with increasing concentrations of EGF for 48 h. Values are means ± SE for carbachol ($10^{-4} \text{ M}$)-induced increases in $[\text{Ca}^{2+}]_i$, from 6 experiments; the $[\text{Ca}^{2+}]_i$ response to carbachol in cells not treated with EGF is shown on the ordinate. B: HSY cells were incubated with $5 \times 10^{-10} \text{ M}$ EGF for 1–48 h as indicated. Means ± SE of carbachol ($10^{-4} \text{ M}$)-induced $[\text{Ca}^{2+}]_i$ signals are from 3–4 experiments.]

**Influence of EGF on muscarinic receptor binding.**

Because EGF had no apparent effect on internal Ca$^{2+}$ stores, EGF inhibition of carbachol-induced Ca$^{2+}$ release is likely to occur at one or more proximal steps of the muscarinic receptor-activated signaling pathway. In the present study, we examined the effect of EGF on muscarinic receptors assessed by binding of the muscarinic receptor antagonist [3H]QNB to HSY cell membrane preparations. Scatchard analysis of [3H]QNB saturation binding curves revealed a single class of high-affinity binding sites. EGF caused a 20% decrease of receptor density ($B_{\text{max}}$) without affecting receptor binding affinity ($K_d$) (Fig. 6). Thus EGF inhibition of carbachol-induced [Ca$^{2+}$]$_i$ mobilization could be mediated at least partly by a reduction in muscarinic receptor density.

146 ± 20 nM vs. -EGF, 286 ± 47 nM; $P < 0.05$). Subsequent repletion of extracellular Ca$^{2+}$ (1 mM) after carbachol stimulation induced a rapid rise of [Ca$^{2+}$]$_i$, presumably as a result of Ca$^{2+}$ influx (Fig. 4); this apparent Ca$^{2+}$ influx was also significantly reduced by EGF (+EGF, 586 ± 111 nM vs. -EGF, 885 ± 106 nM; $P < 0.05$) (Fig. 4). These findings suggest that EGF inhibition of carbachol-induced [Ca$^{2+}$]$_i$ mobilization in HSY cells (Fig. 1) involves reduction in both Ca$^{2+}$ release and Ca$^{2+}$ influx.

To explore further the action(s) of EGF on Ca$^{2+}$ release and Ca$^{2+}$ influx, we compared the [Ca$^{2+}$]$_i$ responses of EGF-treated and untreated HSY cells to thapsigargin, a specific inhibitor of endoplasmic reticulum Ca$^{2+}$-ATPase (36). Thapsigargin inhibits Ca$^{2+}$ uptake to the internal stores, resulting in depletion of the internal pool and elevation of [Ca$^{2+}$]$_i$. In the absence of extracellular Ca$^{2+}$, thapsigargin-induced elevation of [Ca$^{2+}$]$_i$ provides an indirect measure of Ca$^{2+}$ in the internal stores. Under Ca$^{2+}$-free conditions, thapsigargin produced equivalent increases in [Ca$^{2+}$]$_i$ in both EGF-treated and untreated HSY cells (Fig. 5), suggesting that the growth factor does not alter the internal pool. Interestingly, whereas subsequent addition of Ca$^{2+}$ to the medium increased [Ca$^{2+}$]$_i$, this response to extracellular Ca$^{2+}$ was significantly diminished in EGF-treated cells (+EGF, 702 ± 55 nM vs. -EGF, 908 ± 42 nM; $P < 0.03$) (Fig. 5). The observation that EGF decreases the [Ca$^{2+}$]$_i$ response to extracellular Ca$^{2+}$ repletion without affecting the internal stores suggests a direct inhibitory effect of EGF on Ca$^{2+}$ influx.

![Fig. 3. Effects of various growth factors on carbachol-induced [Ca$^{2+}$]$_i$ mobilization.](Image)

![Fig. 4. Effects of EGF on carbachol-induced [Ca$^{2+}$]$_i$, signaling in the absence of extracellular Ca$^{2+}$ and during Ca$^{2+}$ repletion.](Image)
EGF-responsive signals involved in modulating carbachol-induced \([\text{Ca}^{2+}]_i\) mobilization. The EGF receptor tyrosine kinase is coupled to activation of phospholipase \(C\gamma\) (PLC-\(C\gamma\)), which in turn generates \(IP_3\) and the endogenous PKC activator diacylglycerol (23). Activation of PKC has been linked to modulation of muscarinic agonist-sensitive \([\text{Ca}^{2+}]_i\) responses and also muscarinic receptor expression (3, 11, 26, 27, 32, 33). We therefore examined the role of PKC in EGF inhibition of carbachol-responsive \([\text{Ca}^{2+}]_i\) mobilization. PKC in HSY cells was downregulated by prolonged (18–20 h) exposure to the phorbol ester PMA (10^{-5} M) prior to EGF treatment (18). Complete downregulation of PKC \(\alpha\)- and \(\beta\)-isoforms in PMA-treated cells was confirmed by immunoblot analysis as described previously (Ref. 18, data not shown). EGF reduced the carbachol response by one-third with or without PMA pretreatment (Fig. 7). In contrast, pretreatment with 1,2-dioctanoyl-sn-glycerol (10^{-5} M), a cell-permeable diacylglycerol analog that causes initial stimulation without subsequent downregulation of PKC (Ref. 18, data not shown), markedly enhanced the inhibitory effect of EGF on carbachol-induced \([\text{Ca}^{2+}]_i\) responses.
EGF. As shown in Fig. 7, EGF completely abolished the carbachol-induced \([Ca^{2+}]_i\) signal in cells pretreated with the diacylglycerol analog. Taken together, these data suggest that activation of PKC, although not required for the inhibitory effect of EGF, does potentiate EGF inhibition of the carbachol response.

EGF stimulation of IP_3 formation causes a rapid increase of \([Ca^{2+}]_i\) in a number of tissues (14). In HSY cells we have found that EGF causes an acute elevation of \([Ca^{2+}]_i\) (i.e., within seconds of EGF exposure) in addition to the decrease in carbachol-responsive \([Ca^{2+}]_i\) mobilization occurring after more prolonged exposure to EGF. However, the acute effect of EGF was observed only at much higher concentrations than those required to inhibit the carbachol response (Fig. 8). At a concentration of \(5 \times 10^{-10}\) M, EGF produced near maximal inhibition of carbachol-induced \([Ca^{2+}]_i\) mobilization without any detectable acute change in \([Ca^{2+}]_i\) (cf. Figs. 2A and 8). Therefore, although EGF causes a rapid increase of \([Ca^{2+}]_i\) in HSY cells, this acute \([Ca^{2+}]_i\) signal is not likely to mediate inhibition of the carbachol response observed after prolonged EGF treatment.

Another important signaling cascade activated by the EGF receptor tyrosine kinase is the MAP kinase pathway, which plays an essential role in mediating cellular responses to EGF and a variety of other growth factors (21). In HSY cells, phosphorylation of MAP kinase by EGF was determined using immunoblot analysis. EGF induced the phosphorylation of the p44 isoform of MAP kinase without affecting the protein level. Treatment of HSY cells with PD-98059 (\(5 \times 10^{-5}\) M), a synthetic inhibitor of the MAP kinase pathway (1), blocked EGF-induced phosphorylation of p44 MAP kinase (Fig. 9A). As shown in Fig. 9B, inhibition of carbachol-stimulated \([Ca^{2+}]_i\) mobilization by EGF was prevented by PD-98059. These results suggest that the inhibitory effect of EGF on the carbachol response in HSY cells is mediated by activation of the MAP kinase pathway.

DISCUSSION
The results of the present study demonstrate a novel role for EGF in the regulation of muscarinic receptor-coupled \([Ca^{2+}]_i\), signaling in a human salivary cell line (HSY). We found that EGF inhibited carbachol-responsive \([Ca^{2+}]_i\) mobilization in a concentration- and time-dependent manner (Figs. 1 and 2). The \([Ca^{2+}]_i\) response to carbachol was decreased by both EGF and
TGF-α, which activate the EGF receptor, but not by growth factors (IGF-I and PDGF) acting on other receptor tyrosine kinases (Fig. 3).

Inhibition of the carbachol response by EGF was observed in the absence of extracellular Ca\(^{2+}\) (Fig. 4), suggesting a modulatory action of the growth factor on release of Ca\(^{2+}\) from carbachol-sensitive internal stores. The internal Ca\(^{2+}\) pool, as reflected by the [Ca\(^{2+}\)]i response to thapsigargin in cells incubated without external Ca\(^{2+}\), was intact after EGF treatment (Fig. 5). EGF has also been reported to have no effect on thapsigargin-sensitive Ca\(^{2+}\) stores in a rat pheochromocytoma cell line (PC12) (13). Therefore, inhibition of Ca\(^{2+}\) release by EGF is likely to be exerted on one or more elements of the signaling pathway coupling activation of the muscarinic receptor to mobilization of the internal Ca\(^{2+}\) store. Measurement of [\(^{3}H\)]QNB binding to HSY cell membranes revealed a small but significant decrease in muscarinic receptor density (Bmax) after EGF treatment (Fig. 6). The loss of muscarinic receptor binding sites could be the result of alterations in receptor expression, posttranslational modifications, or desensitization mechanisms. Our data do not exclude actions of EGF on other elements of muscarinic receptor signaling, including receptor/G protein coupling, PLC-mediated generation of IP3, or activation of IP3-sensitive Ca\(^{2+}\) release channels (IP3 receptors) in the endoplasmic reticulum. In a variety of cell types, IP3 receptor activity is regulated by tyrosine and serine/threonine phosphorylation (8, 15, 35, 44). Whether EGF exerts direct or indirect effects on the phosphorylation characteristics of IP3 receptors in HSY cells is not known.

Depletion of internal Ca\(^{2+}\) stores activates influx of extracellular Ca\(^{2+}\) across the plasma membrane in a process known as capacitative calcium entry (31). This process is thought to be mediated by a plasma membrane Ca\(^{2+}\)-channel termed the “Ca\(^{2+}\)-release-activated Ca\(^{2+}\)-channel” (CRAC channel) or “store-operated Ca\(^{2+}\)-channel” (SOC channel) (45). In HSY cells exposed to carbachol in the absence of extracellular Ca\(^{2+}\), we found that EGF decreased Ca\(^{2+}\) influx following Ca\(^{2+}\) repletion (Fig. 4). Inhibition of Ca\(^{2+}\) influx under these conditions could reflect either reduced Ca\(^{2+}\) release, leading to less depletion of internal stores and a secondary decline in capacitative Ca\(^{2+}\) entry, or a direct effect of EGF on Ca\(^{2+}\) influx. Growth factor inhibition of Ca\(^{2+}\) influx was also observed in cells treated with thapsigargin, even though EGF had no effect on thapsigargin-induced depletion of internal stores (Fig. 5). Our findings therefore suggest that EGF not only inhibits muscarinic receptor-coupled Ca\(^{2+}\) release but also modulates Ca\(^{2+}\) influx independently of internal store depletion.

An inhibitory effect of EGF on Ca\(^{2+}\) influx could be exerted directly on the Ca\(^{2+}\)-influx channel, although growth factor modification of as yet undefined signals coupling internal store depletion to Ca\(^{2+}\) influx cannot be excluded. The CRAC channel protein(s) has yet to be identified but may be a mammalian homolog of one or more products of the trp (transient receptor potential) gene family in Drosophila photoreceptors. Six trp homologs (trp1–trp6) have been identified in a variety of mammalian tissues (45), and a trp1 homologous sequence has been detected in rat submandibular gland RNA (40). Calcium influx mediated by a mouse Trp6 protein appears to be stimulated by G protein-coupled receptors independently of internal Ca\(^{2+}\) store depletion (4). Interestingly, a muscarinic receptor-activated Ca\(^{2+}\) influx pathway resembling Ca\(^{2+}\) entry through Trp6 has been described in a human submandibular gland cell line (HSG) (16). Whether one or more Trp homologs mediate Ca\(^{2+}\) influx in HSY cells has not yet been determined, and a role for EGF in regulating Trp channels in salivary or other cell types remains speculative.

Agonists binding to the EGF receptor modulate cellular functions by a complex series of signaling events, including activation of the MAP kinase and PKC pathways (7, 21). In the present study EGF was found to induce phosphorylation of MAP kinase in HSY cells. Phosphorylation of MAP kinase and inhibition of carbachol-induced [Ca\(^{2+}\)]i mobilization by EGF were both blocked by PD-98059, a selective inhibitor of the MAP kinase pathway (Fig. 9). In contrast, downregulation of PKC by prolonged exposure of HSY cells to phorbol ester had no effect on EGF inhibition of carbachol-sensitive [Ca\(^{2+}\)]i mobilization (Fig. 7). Thus activation of the MAP kinase pathway, but not PKC, appears to play an essential role in the inhibitory effect of EGF on muscarinic receptor-coupled [Ca\(^{2+}\)]i signaling.

Although our data do not support the involvement of PKC activation as a signaling intermediate in modulation of the muscarinic response by EGF, the level of PKC activity may influence carbachol-induced [Ca\(^{2+}\)]i signaling in HSY cells. Our results revealed an increase in the [Ca\(^{2+}\)]i response to carbachol following downregulation of PKC by phorbol ester, as well as a reduced [Ca\(^{2+}\)]i response after PKC activation by a diacylglycerol analog (Fig. 7). These changes in [Ca\(^{2+}\)]i mobilization, although relatively small in magnitude and not statistically significant, suggest that PKC may exert an inhibitory effect on [Ca\(^{2+}\)]i signaling independent of the modulatory action of EGF. Activation of PKC in a number of cell types, including human salivary cells (HSG and HSY), is known to exert differential effects, both stimulatory and inhibitory, on Ca\(^{2+}\) release responsive to muscarinic and other G protein-coupled receptors and also on capacitative Ca\(^{2+}\) entry (3, 11, 19, 26, 27, 32, 41). In addition, downregulation of several muscarinic receptor subtypes, occurring via phosphorylation-dependent receptor internalization or altered receptor gene transcription, is enhanced by PKC stimulation in various cell lines (33). A particularly striking result of our own studies was the finding that the inhibitory effect of EGF on carbachol-stimulated [Ca\(^{2+}\)]i mobilization was greatly accentuated in HSY cells pretreated with the diacylglycerol analog (Fig. 7). Whereas EGF alone inhibited the carbachol-responsive [Ca\(^{2+}\)]i signal by one-third, the carbachol response was completely blocked by the combination of growth factor and diacylglycerol analog (Fig. 7). This
marked synergy between the regulatory actions of an EGF receptor-activated, MAP kinase-dependent pathway and a distinct PKC activation cascade has not, to our knowledge, been described previously. Studies are currently underway in our laboratory to determine the cross talk mechanisms by which PKC potentiates EGF inhibition of carbachol-induced \([Ca^{2+}]_i\) signaling in HSY cells.

In this study EGF was found to exert two time-dependent actions on \([Ca^{2+}]_i\) levels in HSY cells. Exposure of cells to EGF for prolonged periods (i.e., \(\geq 2\) h) reduced carbachol-responsive \([Ca^{2+}]_i\) mobilization (Fig. 2B), whereas a rapid increase in \([Ca^{2+}]_i\) occurred within seconds of EGF exposure in the absence of other \([Ca^{2+}]_i\) mobilizing agonists (Fig. 8). Although both prolonged and rapid actions of EGF were concentration dependent, inhibition of carbachol responsiveness was observed at much lower concentrations of EGF than those required to cause rapid \([Ca^{2+}]_i\) mobilization (cf. Figs. 2A and 7). The two effects of EGF were further dissociated by treatment of cells with the MAP kinase inhibitor PD-98059, which totally blocked the modulatory action of EGF on carbachol-sensitive \([Ca^{2+}]_i\) signaling (Fig. 9) but did not decrease the acute \([Ca^{2+}]_i\) response to EGF (data not shown). Growth factor inhibition of carbachol-responsive Ca\(^{2+}\) release also could not be attributed to depletion of internal Ca\(^{2+}\) stores following the rapid \([Ca^{2+}]_i\) mobilizing action of EGF, because the internal stores were unaltered after prolonged EGF treatment. These observations indicate that the rapid and prolonged \([Ca^{2+}]_i\) responses to EGF represent distinct and independent actions of the growth factor.

Our results suggest a potential role for EGF in regulation of salivary gland function. EGF modulation of secretory response to muscarinic receptor activation has been reported in salivary gland and other gastrointestinal epithelial tissues. In a previous study examining the effect of EGF on rat parotid gland secretory function, EGF treatment in vivo caused an increase in muscarinic agonist-induced amylase secretion, although no change in salivary flow rate was detected (29). Other investigations have demonstrated acute inhibitory effects of EGF in vitro on carbachol-stimulated secretory functions in rabbit and canine gastric parietal cells and in human T84 colonic epithelial cells (6, 20, 38, 39). Whereas EGF inhibition of carbachol responsiveness in parietal cells may be mediated via activation of PKC, the inhibitory actions of EGF in both parietal and colonic cells do not appear to involve modulation of \([Ca^{2+}]_i\) transients (20, 38, 39). In contrast to the acute inhibitory effect of EGF on carbachol responsiveness, prolonged EGF treatment of rabbit parietal cells enhances carbachol-stimulated secretory activity. Like the modulatory action of EGF described in the present study of HSY cells, the prolonged effect of EGF in rabbit parietal cells appears to be mediated by the MAP kinase signaling pathway (6). EGF has also been found to produce acute inhibition, as well as prolonged enhancement, of G protein-coupled receptor-mediated secretory function in pancreatic acinar cells (28). Both inhibition and stimulation of G protein-coupled receptor-activated \([Ca^{2+}]_i\) mobilization have been reported in other cell types after prolonged EGF treatment (13, 22).

Insofar as EGF is produced and secreted by salivary cells, our findings also raise the possibility that EGF may regulate salivary secretory function through autocrine or paracrine mechanisms. An analogous regulatory role for EGF has been suggested in studies of kidney tubule cells, which produce and secrete EGF and at the same time exhibit a variety of differentiated functions subject to modulation by EGF. Although under baseline conditions renal EGF may be localized to the luminal membrane of tubular epithelial cells, the actions of EGF on sensitive tubule segments are exerted on receptors at the antiluminal, or basolateral, surface. Interestingly, autocrine or paracrine actions of renal EGF may, under some conditions, be facilitated by redistribution of intracellular EGF to the basolateral membrane (24). Whether the in vivo actions of EGF in salivary cells are subject to similar spatial considerations as exist in kidney epithelial cells is not known. Of note in this regard are recent studies demonstrating EGF-independent transactivation of EGF receptors by muscarinic agonists in human embryonic kidney 293 cells and T84 colonic epithelial cells (17, 37). In T84 cells, EGF receptor activation has been proposed to function as an inhibitory signal by which a muscarinic agonist negatively regulates its own secretory response (17). These findings, together with our own observations, suggest that HSY cells may be a useful model to explore the regulation of salivary secretion by spatial and functional interactions between EGF and muscarinic receptor signaling pathways.

In summary, the present study demonstrates that EGF inhibits carbachol-activated \([Ca^{2+}]_i\) mobilization in the HSY salivary cell line. EGF decreases muscarinic agonist-induced release of Ca\(^{2+}\) from internal stores and also exerts a direct inhibitory action on influx of Ca\(^{2+}\) across the plasma membrane. A decline in muscarinic receptor density may contribute to EGF inhibition of carbachol responsiveness. The inhibitory effect of EGF is mediated by the MAP kinase signaling pathway and is potentiated by a distinct modulatory cascade involving activation of PKC. These data provide the basis for further investigation of possible mechanisms by which cross talk between EGF and muscarinic receptor signaling pathways may function in the physiological regulation of salivary secretion.

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