**ABSTRACT**

Muscarinic type 3 receptor (M3R) plays a pivotal role in the induction of glandular fluid secretions. Although M3R is often the target of autoantibodies in Sjögren’s syndrome (SjS), chemical agonists for M3R are clinically used to stimulate saliva secretion in patients with SjS. Aside from its activity in promoting glandular fluid secretion, however, it is unclear whether activation of M3R is related to other biological events in SjS. This study aimed to investigate the cytoprotective effect of chemical agonist-mediated M3R activation on apoptosis induced in human salivary gland (HSG) cells. Carbachol (CCh), a muscarinic receptor-specific agonist, abrogated tumor necrosis factor α/interferon γ-induced apoptosis through pathways involving caspase 3/7, but its cytoprotective effect was decreased by a M3R antagonist, a mitogen-activated protein kinase kinase inhibitor, a phosphatidylinositol 3-kinase/Akt inhibitor, or an epidermal growth factor receptor (EGFR) inhibitor. Ligation of M3R with CCh transactivated EGFR and phosphorylated ERK and Akt, the downstream targets of EGFR. Inhibition of intracellular calcium release or protein kinase C δ, both of which are involved in the cell signaling of M3R-mediated fluid secretion, did not affect CCh-induced ERK or Akt phosphorylation. CCh stimulated Src phosphorylation and binding to EGFR. A Src inhibitor attenuated the CCh/M3R-induced cytoprotective effect and EGFR transactivation cascades. Overall, these results indicated that CCh/M3R induced transactivation of EGFR through Src activation leading to ERK and Akt phosphorylation, which in turn suppressed caspase 3/7-mediated apoptotic signals in HSG cells. This study, for the first time, proposes that CCh-mediated M3R activation can promote not only fluid secretion but also survival of salivary gland cells in the inflammatory context of SjS.

**Introduction**

Sjögren’s syndrome (SjS) is a chronic autoimmune disease characterized by lymphocytic infiltration, gland destruction, and eye and mouth dryness (Fox and Kang, 1992; Kroneld et al., 1997; Fox and Stern, 2002). Although the cause of SjS remains unclear, many studies have suggested that T and B lymphocytes that infiltrate the affected glands are involved in the pathogenesis of SjS, because of their production of tissue-destructive proinflammatory cytokines and autoantibodies, respectively (Lee et al., 2009). It was reported that levels of proinflammatory cytokines, such as tumor necrosis factor α (TNFα) and interferon γ (IFNγ), are elevated in the affected glands in SjS (Fox et al., 1994; and 1997; Fox and Stern, 2002).

**ABBREVIATIONS:** SJS, Sjögren’s syndrome; HSG, human salivary gland; M3R, muscarinic type 3 receptor; CCh, carbachol; ERK, extracellular signal-regulated kinase; EGFR, epidermal growth factor receptor; GPCR, G protein-coupled receptor; NC, nitrocellulose; TNFα, tumor necrosis factor α; IFNγ, interferon γ; AMPK, AMP-activated protein kinase; HB, heparin-binding; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; GPCR, G protein-coupled receptor; PTK, protein kinase C; PI3K, phosphatidylinositol 3-kinase; 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine methiodide; BAPTA-AM, 1,2-bis(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid, tetraacetoxyethyl ester; LY294002, 2-morpholin-4-yl-8-phenylchromen-4-one; AG1478, N-(3-chlorophenyl)-6,7-dimethoxy-4-quinazolinamine; PP2, 4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine; GF109203X, 2-[1-(3-dimethylamino-propyl)-1H-indol-3-yl]-3-[1H-indol-3-yl]maleimide; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; U0126, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene.
Kolkowski et al., 1999). Those proinflammatory cytokines can induce apoptosis of salivary gland cells through caspase 3 signaling (Kamachi et al., 2002; Kulkarni et al., 2006). In contrast, it is thought that hypofunction of fluid secretion from affected glands is caused by autoantibodies against muscarinic type 3 receptor (M3R) (Li et al., 2004; Koo et al., 2008). M3R is the major muscarinic acetylcholine receptor in the salivary glands, and it plays a pivotal role in the induction of salivary fluid secretion (Baum, 1993). More specifically, acetylcholine released from the parasympathetic nerves activates M3R to induce intracellular Ca2+ influx, which initiates the cell signaling required for fluid secretion from acinar cells (Ambudkar et al., 1993; Ambudkar, 2000; Park et al., 2001). Chemical agonists for M3R, such as pilocarpine and cevimeline, are often used clinically to stimulate saliva secretion among patients with SjS (Mavragani and Moutsopoulos, 2007).

M3R, a G protein-coupled receptor (GPCR), belongs to the largest transmembrane receptor superfamily in humans and mice, and it is characterized by a seven-transmembrane α-helix structure (Pierce et al., 2002). In general, the binding of an agonistic ligand to its cognate GPCR elicits Ca2+ and/or PKC signaling cascades that induce the expression of genes required for multiple fundamental functions, including exocrine and endocrine secretion, smooth muscle and cardiac muscle contraction, pain transmission, fluid homeostasis, blood pressure regulation, and immune responses (Pierce et al., 2002). GPCRs, such as endothelin receptors and protease-activated receptor I, also activate mitogenic signaling networks, such as PKC/protein kinase D, MEK/ERK, and the PI3K/Akt cascade, which leads to the induction of a variety of biological responses, including cell proliferation, differentiation, migration, and survival (Rozengurt, 1998, 2007).

Many studies have shown the relevance of Ca2+ signaling in M3R-induced fluid secretion. However, mitogenic signaling pathways such as those involving PKC, ERK, and Akt, as well as their M3R-mediated downstream events, are still poorly understood in the context of salivary gland cells. Only recently has a muscarinic receptor agonist, namely, carbachol (CCh), been reported to stimulate in vitro ERK phosphorylation in human immortalized salivary gland cells or rat submandibular acinar cells (Soltoff and Hedden, 2010). Because SjS is a chronic degenerative disease characterized by the gradual progression of cell apoptosis and resulting tissue destruction, it is plausible that mitogenic signaling, which can down-regulate apoptosis induced through the caspase pathway (Steelman et al., 2008), may play a role in sustaining the configuration and functions of affected glands. Although it was shown that M3R promotes neuroblastoma cell survival through activation of the ERK signaling pathway (Greenwood and Dragunow, 2010), it remains unclear whether M3R signaling in salivary gland cells is similarly cytoprotective against proinflammatory cytokine-induced apoptosis.

In the present study, we hypothesized that chemical agonist-mediated M3R activation could induce cell signaling in salivary gland cells, thereby contributing to the protection of cells against apoptosis caused by inflammatory insult. To test this hypothesis, apoptosis was induced in human salivary gland (HSG) cells through inflammatory stimulation with TNFα/IFNγ. CCh was used as a chemical agonist for M3Rs, on the basis of studies that showed that CCh could efficiently activate M3Rs expressed on cultured HSG cells (Cha et al., 2006; Pauley et al., 2011). We then investigated the cytotoxic effect of CCh-mediated M3R ligation, as well as the molecular mechanism underlying such M3R-mediated cytoprotective effects.
nonfat milk for 1 h, followed by overnight reaction at 4°C with rabbit anti-human phosphorylated ERK antibody (1:2000; Cell Signaling Technology), rabbit anti-human total ERK antibody (1:2000; Cell Signaling Technology), rabbit anti-human phosphorylated Akt antibody (1:2000; Cell Signaling Technology), rabbit anti-human total Akt antibody (1:2000; Cell Signaling Technology), rabbit anti-human phosphorylated PKCδ antibody (1:500; Cell Signaling Technology), rabbit anti-human total PKCδ antibody (1:1000; Cell Signaling Technology), rabbit anti-human phosphorylated EGFR antibody (1:500; Cell Signaling Technology), rabbit anti-human total EGFR antibody (1:5000; Cell Signaling Technology), rabbit anti-human phosphorylated Src family (Tyr416) antibody (1:500; Cell Signaling Technology), rabbit monoclonal anti-human total c-Src antibody (clone 3D610, 1:4000; Cell Signaling Technology), and horseradish peroxidase-conjugated mouse anti-β-actin antibody (1:5000; AbCam Inc., Cambridge, MA). After extensive washes, the NC membranes were incubated with peroxidase-conjugated donkey anti-rabbit IgG antibody (1:5000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 1 h at room temperature. The localization of specific antibody deposited to the molecule of interest on the NC membranes was detected by developing color with the Immobilon Western chemistry (Millipore Corp., Billerica, MA).

**Immunoprecipitation.** Equal amounts of cell lysates (500 µg of total protein in 1 ml of lysis buffer) were incubated overnight at 4°C with rabbit anti-human total EGFR antibody (1:50). GammaBind Plus Sepharose beads (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) were then washed three times with lysis buffer, and then incubated for 30 min at room temperature. HSG cells were lysed, and immunoblotting assays were performed as described above. Total c-Src protein levels were determined with rabbit anti-human total c-Src monoclonal antibody (clone 32G6, 1:500; Cell Signaling Technology).

**[Ca^{2+}], Measurements.** [Ca^{2+}], was measured by using a fluo-8 no-wash calcium assay kit (AAT Bioquest, Sunnyvale, CA), as described previously (Ohta et al., 2011). HSG cells were incubated for 30 min at 37°C in phenol red-free Hank’s buffer with 20 mM HEPES containing fluo-8 no-wash dye-loading solution, in the presence or absence of 4-DAMP (1 µM) or BAPTA-AM (10 µM), and then incubated for 30 min at room temperature. HSG cells were exposed to 100 µM CCh for 1 min, and the fluorescence intensity (excitation wavelength, 490 nm; emission wavelength, 525 nm) was detected with a fluorometric imaging plate reader. The fluorescence intensities were quantified from three independent cell culture experiments.

**Transfection of siRNA.** Validated PKCδ siRNA, EGFR siRNA, and negative-control siRNA were obtained from Invitrogen (identification nos. PRKCDVHS41574 for PKCδ siRNA, EGFRVHS41680 for EGFR siRNA, and 12935-300 for negative-control siRNA). HSG cells in medium A were seeded at a density of 1.25 × 10^4 cells per well in 24-well plastic culture plates and were cultured for 24 h at 37°C. Then, 20 nM levels of PKCδ siRNA, EGFR siRNA, or negative-control siRNA were transfected into the cells by using RNAiMAX reagent (Invitrogen), according to the manufacturer’s instructions. After 48 h of incubation, the cells treated with or without 100 µM CCh were lysed, and immunoblotting assays were performed as described above.

**Statistical Analysis.** Differences between two groups of interest were analyzed with Student’s t tests.

**Results**

**CCh Protects HSG Cells from Apoptosis Induced through TNFα/IFNγ Stimulation.** To examine the cytotoxic protective effects of M3R in HSG cells, the muscarinic receptor agonist CCh was tested with respect to TNFα/IFNγ-induced apoptosis, which was reported previously to activate apoptosis signaling in HSG cells (Kamachi et al., 2002; Kulkarni et al., 2006). Consistent with a previous report, the combined TNFα/IFNγ treatment reduced cell viability among HSG cells (Fig. 1A). However, CCh protected against cell death induced by TNFα/IFNγ in a dose-dependent manner (Fig. 1A). TUNEL staining showed that TNFα/IFNγ stimulation increased the number of apoptotic cells, whereas CCh treatment significantly abrogated the increase (Fig. 1, B and C). We monitored the effect of CCh on the caspase 3/7 death signal activity induced by the TNFα/IFNγ challenge in HSG cells. As shown in Supplemental Fig. 1A, caspase 3/7 activity reached a significantly higher level than control values at 24 h after stimulation with inflammatory cytokines. Therefore, in the following experiments, measurements of caspase 3/7 activity were performed 24 h after TNFα/IFNγ challenge. It should be noted that the protocol for incubating HSG cells for 24 h with proinflammatory cytokines to induce caspase activity was published previously (Kulkarni et al., 2006).
The increased caspase 3/7 activity induced by TNFα/IFNγ (Fig. 1D) was significantly attenuated by the addition of CCh (Fig. 1D). It is noteworthy that neither U0126 nor CCh was markedly inhibited by LY294002 but not by contrast, the up-regulation of Akt phosphorylation caused by M3R signaling was inhibited by U0126 (MEK/ERK1/2 inhibitor), (Fig. 2, A and B). Whereas CCh-induced ERK phosphorylation was inhibited by U0126 (MEK/ERK1/2 inhibitor), LY294002 (PI3K/Akt inhibitor) had no effect (Fig. 2C). In contrast, the up-regulation of Akt phosphorylation caused by CCh was remarkably inhibited by LY294002 but not by U0126 (Fig. 2C). It is noteworthy that neither U0126 nor LY294002 affected TNFα/IFNγ-induced caspase 3/7 activity, whereas those inhibitors clearly attenuated the CCh-mediated protective effect in HSG cells (Fig. 2, D and E). TNFα/IFNγ stimulation transiently increased the phosphorylation of ERK and Akt but phosphorylation decreased below basal levels 20 min after the challenge with inflammatory cytokines (Supplemental Fig. 2), which suggests that the net impact of TNFα/IFNγ on ERK and Akt phosphorylation levels in HSG cells was suppressive. These findings indicate that the up-regulation of both ERK and Akt signaling induced by CCh is responsible for its cytoprotective effect in HSG cells.

M3R Activation Is Responsible for CCh-Mediated Cell Survival Signaling in HSG Cells. To examine the relationship between M3R activation and CCh-induced cell survival signaling, the M3R antagonist 4-DAMP was used. Pretreatment with 4-DAMP abrogated the phosphorylation of both ERK and Akt induced by CCh in HSG cells (Fig. 3A). Furthermore, the CCh-mediated protective effect on TNFα/IFNγ-induced caspase 3/7 activity was significantly inhibited by 4-DAMP. These findings suggest that CCh activates the ERK and Akt mitogenic survival signaling cascade through M3R in HSG cells.

Neither Ca2+ nor PKCδ Signaling Is Associated with CCh-Induced Cytoprotective ERK and Akt Signaling Cascades. It is well known that Ca2+ signaling, which is caused by M3R activation, plays a pivotal role in fluid secretion in salivary gland cells (Li et al., 2004; Koo et al., 2008); therefore, we investigated whether Ca2+ signaling, in addition to the activation of ERK and Akt signaling described above, might be involved in CCh-induced cell survival signaling in HSG cells. As expected, CCh did increase [Ca2+]i, whereas both a M3R inhibitor (4-DAMP) and a Ca2+-chelator (BAPTA-AM) suppressed CCh-induced increases in [Ca2+]i, in HSG cells (Fig. 4A). BAPTA-AM, which completely blocked calcium mobility in HSG cells, failed to inhibit CCh-induced phosphorylation of ERK and Akt (Fig. 4B). Moreover, the CCh-mediated suppressive effect on TNFα/IFNγ-induced caspase 3/7 activity was not affected by BAPTA-AM treatment (Fig. 4C). These findings indicate that CCh causes cell survival mitogenic signaling in a Ca2+-independent manner.

Next, we investigated the possible engagement of PKCδ in M3R signaling, because activation of PKC has been linked to muscarinic receptor-induced ERK activation in several cell types (Keely et al., 1998; Jiménez and Montiel, 2005). On the basis of the following lines of evidence, we focused on PKCδ expressed in HSG cells. First, CCh caused cell survival mi-
Fig. 3. M3R is involved in CCh-induced cell survival signaling in HSG cells. A, HSG cells were pretreated with or without 4-DAMP (1 μM) for 30 min and then were exposed to CCh (100 μM) for 10 min. The phosphorylated (p) and total (t) ERK and Akt levels were analyzed through immunoblotting. Quantification of the band density was performed through densitometric scanning of each band by using National Institutes of Health Image software. B, HSG cells were pretreated with or without 4-DAMP (1 μM) for 30 min. The cells then were treated with or without 100 μM CCh, in the absence or presence of TNFα (50 ng/ml)/IFNγ (10 ng/ml), and were incubated for 24 h. Caspase 3/7 activity was indicated by luminescence activity, as described under Materials and Methods. Values represent the mean ± S.D. of three cultures. *, p < 0.05; **, p < 0.01, values differ significantly (t test).

togenic signaling in a Ca²⁺-independent manner (Fig. 4). Second, in contrast to the major isoforms of PKC (α, βI, βII, and γ), which require Ca²⁺⁺ for full activation, activation of a second class of PKC isoforms, including PKCδ, occurs in a Ca²⁺-independent manner (Parker and Murray-Rust, 2004). Third, B lymphocytes infiltrating the salivary glands of patients with SS could cause epithelial cell apoptosis through activation of PKCδ (Varin et al., 2012). We found that CCh increased PKCδ phosphorylation levels in a time-dependent manner (Fig. 4D) and the CCh-induced PKCδ up-regulation was blocked by 4-DAMP (Fig. 4E), which indicates that PKCδ is activated through M3R in HSG cells. Transfection of PKCδ siRNA decreased PKCδ expression (Fig. 4F). Contrary to our expectation, PKCδ knockdown did not affect the CCh-induced phosphorylation of ERK and Akt in HSG cells (Fig. 4G), whereas phosphorylated PKCδ expression was undetectable (data not shown). Furthermore, PKCδ siRNA failed to attenuate the CCh-mediated protective effect against TNFα/ IFNγ-induced caspase 3/7 activity in HSG cells (Fig. 4H). These findings demonstrate that CCh-induced cell survival signaling is independent of PKCδ signaling in HSG cells.

EGFR Transactivation Is Involved in CCh-Induced Cell Survival Signaling. It was reported that some muscarinic receptors could induce ERK activation through transactivation of EGFR in various cell types (Keely et al., 2000; Kanno et al., 2003). Therefore, we tested whether CCh-induced cell survival signaling is mediated by EGFR transactivation. CCh increased the phosphorylation of EGFR in a time-dependent manner (Fig. 5A) but the increase in phosphorylated EGFR levels was blocked by the M3R inhibitor 4-DAMP (Fig. 5B), which indicates that EGFR is transactivated through M3R in HSG cells. To test whether EGFR phosphorylation is involved in CCh-induced cell survival signaling, we next performed an inhibition assay using AG1478, an EGFR kinase-specific inhibitor. Pretreatment of HSG cells with AG1478 abrogated the CCh-induced phosphorylation of EGFR, ERK, and Akt (Fig. 5C). The protective effect of CCh on TNFα/IFNγ-induced caspase 3/7 activity was also significantly decreased by AG1478 (Fig. 5D). To confirm these findings, we performed an RNA interference-based, gene-silencing assay with EGFR siRNA. Transfection of EGFR siRNA decreased EGFR expression levels in HSG cells (Fig. 5E). EGFR siRNA transfection attenuated CCh-induced ERK and Akt phosphorylation (Fig. 5F). These findings suggest that M3R ligation with CCh leads to EGFR transactivation, which in turn initiates cytoprotective survival signaling in HSG cells.

The Intracellular Signal Adaptor c-Src Intervenes between M3R and EGFR in the CCh-Induced EGFR Transactivation Cascade. It is known that EGFR transactivation induced by GPCRs (such as muscarinic receptors) is dependent on activation of the cell signal adaptor c-Src, a nonreceptor tyrosine kinase, in various cell types (Rosenblum et al., 2000; Yeh et al., 2005). Therefore, we investigated whether c-Src might be associated with CCh-induced transactivation of EGFR signaling in HSG cells. CCh caused rapid transient phosphorylation of Src at Tyr416, indicating the active state of the kinase, which began to be expressed as early as 1 min after stimulation with CCh (Fig. 6A). The immunoprecipitation assay also showed that CCh stimulation enhanced the association between c-Src and EGFR in a time-dependent manner, which was detectable after 1 min (Fig. 6B). Moreover, 4-DAMP treatment blocked both CCh-induced phosphorylation of c-Src at Tyr416 (Fig. 6C) and the association of c-Src with EGFR (Fig. 6D). It is noteworthy that the Src tyrosine kinase inhibitor PP2 reduced CCh-induced phosphorylation of EGFR, ERK, and Akt in HSG cells (Fig. 6E). Finally, the protective effect of CCh against TNFα/IFNγ-induced caspase 3/7 activity was significantly attenuated by PP2 (Fig. 6F). Taken together, these findings demonstrate that CCh-induced Src activation through M3R causes EGFR transactivation, leading to cell survival signaling mediated by ERK and Akt phosphorylation in HSG cells.

Exogenously Applied EGF Emulates CCh-Induced Cytoprotective Cell Signaling. If it is true that transactivation of EGFR through M3R can induce survival mitogenic signaling, then direct (i.e., exogenous) activation of EGFR should induce mitogenic survival signaling in HSG cells. To test this supposition, we examined the effect of exogenously applied EGF on EGFR, ERK, and Akt phosphorylation and TNFα/IFNγ-induced caspase 3/7 activity in HSG cells (Fig. 7A). EGF treatment attenuated TNFα/IFNγ-induced caspase 3/7 activity in HSG cells (Fig. 7B). Taken together, these findings clearly suggest that EGFR activation plays a critical role in the protection of salivary gland cells from apoptotic challenge.

Discussion

The present study revealed that ligation of M3R, through transactivation of EGFR, could up-regulate not only ERK but also Akt, which suppresses the caspase 3/7 death signal, as well as apoptosis of HSG cells induced by an inflammatory insult (Fig. 8). Although Akt and ERK signaling are major intracellular signaling pathways for cell survival (Steelman et al., 2008; Kajiya et al., 2009;
Chappell et al., 2011), many previous studies on the pathophysiologic features of HSG cells focused on understanding M3R-induced Ca2⁺ signaling in the fluid secretion system (Li et al., 2004; Koo et al., 2008; Tobin et al., 2009) and not the underlying cytoprotective mechanism. Soltloff and Hedden (2010) demonstrated that activation of M3R promoted ERK phosphorylation in salivary gland cells, which supports our finding of CCh-mediated activation of ERK and Akt phosphorylation. However, the finding in the present study (i.e., that activation of M3R elicited cytoprotective signals through transactivation of EGFR) provides a novel scientific foundation for understanding the pathophysiologic characteristics of SjS and offers new molecular targets for SjS drug discovery.

The fact that some GPCR agonists can transactivate EGFR was originally reported in 1996 (Daub et al., 1996), and this theory is now applied to a wide variety of GPCR ligands (Gschwind et al., 2001). This paradigm requires the intervention of the intracellular signal adaptor molecule c-Src, which activates the intracellular tyrosine kinase domain of EGFR (Amorino et al., 2007; Rozengurt, 2007), as shown in this study (Fig. 6). However, studies demonstrated that transactivation of EGFR by GPCR ligands could occur through both intracellular c-Src intervention and extracellular EGFR-ligand transfer (Ohtsu et al., 2006; Bhola and Grandis, 2008). More specifically, activation of GPCR induces the expression of extracellular enzymes that cleave the ectodomain of hep-arin-binding (HB) EGF (cell membrane-bound form of EGFR ligands). Subsequently, the released (HB) EGF binds and activates EGFR (Ohtsu et al., 2006; Bhola and Grandis, 2008). To test whether such an alternative mechanism of extracellular M3R-EGFR transactivation is involved in CCh-stimulated HSG cells, we explored the effect of the HB EGF inhibitor CRM197 on CCh-induced EGFR, ERK, and Akt
phosphorylation (Supplemental Fig. 3). However, because the HB EGF inhibitor blocked neither CCh-induced EGFR transactivation nor ERK/Akt phosphorylation (Supplemental Fig. 3), M3R-induced EGFR transactivation in HSG cells seemed to be solely regulated by intracellular c-Src intervention between M3R and EGFR.

In addition to CCh-induced EGFR transactivation involving Src activation, the present study demonstrated that EGFR activation induced by exogenously applied EGF is involved in cytoprotective signaling in HSG cells. Although exogenously applied EGF elicited full inhibition of caspase 3/7 activity induced by TNFα/IFNγ (Fig. 7D), it is noteworthy that the CCh-mediated suppression of caspase 3/7 activity was rather modest (−50–60% inhibition) (Fig. 2, D and E). CCh also induced differences in the time course and signal intensity of EGFR, ERK, and Akt phosphorylation (Figs. 2, A and B, and 5A), compared with that induced by EGF (Fig. 7, A–C). More specifically, EGF induced EGFR phosphorylation and mitogenic signaling activity more rapidly and dramatically than did CCh. These findings indicated that the intensity of CCh-evoked EGFR transactivation was attenuated through the intervention of the M3R-Src signaling process, compared with direct activation of EGFR with EGF.

Ligation of M3R with its cognate agonist induces classic Gq protein-mediated phospholipase C β activation leading to the production of the second messengers inositol tris-phosphate and diacylglycerol, which causes increases in [Ca2+]i and activation of PKCs (Singer et al., 1997). In accordance with this classic theory, CCh stimulated intracellular Ca2+ mobilization and PKCδ phosphorylation through M3R in HSG cells (Fig. 4). Although these cell signaling cascades were demonstrated to regulate saliva formation and secretion, CCh mediation of these classic pathways was not associated with cell survival signaling in HSG cells (Fig. 4) (Ambudkar et al., 1993; Soltoff and Toker, 1995; Soltoff et al., 1998).

Because the PKC family is composed of more than 15 isozymes, we tested, in addition to PKCδ siRNA, the effects of the pan-PKC inhibitor GF109203X on CCh-induced cytoprotective signaling in HSG cells. Consistent with the results from the PKCδ siRNA experiments (Fig. 4), the PKC inhibitor failed to attenuate CCh-induced ERK and Akt phosphorylation or its cytoprotective effect against TNFα/IFNγ-induced caspase 3/7 activation (Supplemental Fig. 4). It is still possible that other PKCs are associated with the CCh-induced mitogenic signaling, because GF109203X inhibits PKCα, PKCβI, PKCδ, and PKCε but has little or no effect on other PKC isoforms, such as PKCβII and PKCγ. A total absence of PKCβII in the acinar epithelial cells of patients with SjS has been reported (Törnwall et al., 1997), which suggests that PKCβII activation may not be associated with inflammation-induced caspase 3/7 activation in the context of SjS lesions. However, it is possible that other GF109203X-resistant PKC isoforms, such as PKCγ, may participate in CCh-mediated cytoprotective signaling. For example, when the effects of the EGFR inhibitor AG1478 (Fig. 5C) on the CCh-elicted phosphorylation of EGFR, ERK, and Akt were compared with those of the Src inhibitor PP2 (Fig. 6E), discrepancies in the levels of suppression of phosphorylation by these two drugs were evident, which suggests the possible inter-
vention of other signaling pathways between Src and EGFR. Additional comprehensive studies will be required to elucidate the signaling networks that are involved in CCh-induced cytoprotective events.

Lin et al. (2008) reported that CCh stimulates ERK phosphorylation through PKC activity without EGFR transactivation, whereas another muscarinic agonist, pilocarpine, could up-regulate ERK phosphorylation through c-Src-dependent EGFR transactivation in the human salivary gland cell line HSY. Unlike the present study, which addressed the effects of CCh on cytoprotection, the study by Lin et al. (2008) focused only on CCh-induced ERK signaling, without considering the downstream outcomes of ERK activation. The HSG cells used in the present study predominantly express M3R among all muscarinic receptor isoforms (Nagy et al., 2007), whereas HSY cells express M1 and M3 receptors to equal degrees (Lin et al., 2008). To explain the discrepancy in the findings on the actions of CCh between the study by Lin et al. (2008) and the present study, it is thought that CCh binding to M1 receptors in HSY cells might have induced ERK phosphorylation without EGFR transactivation, whereas CCh-mediated M3R activation resulted in EGFR transactivation-dependent ERK activation in the present study. The study by Lin et al. (2008) supports our key finding (i.e., that activation of M3R can lead to cytoprotective ERK activation signaling through EGFR transactivation in HSG cells).

In native salivary gland tissue, CCh-induced elevations of \([\text{Ca}^{2+}]_i\), stimulate fluid secretion in salivary acinar cells by activating apically located Cl\(^-\) channels and basolaterally located K\(^+\) channels (Romanenko et al., 2006). In this context, CCh seems to increase the turnover of Na\(^+\)/K\(^+\)-ATPase and reduce...
intracellular ATP levels, which promotes the activation of AMP-activated protein kinase (AMPK) (Soltoff, 2004). It was reported that adiponectin could prevent IFNγ-induced apoptosis of salivary gland cells through AMPK activation (Katsiougiannis et al., 2010), which suggests that signaling that can activate AMPK, including CCh-evoked M3R stimulation, may also protect salivary gland cells from apoptosis. In contrast, immunohistochemical analysis of diseased gland tissue from a patient with SjS demonstrated that the surviving salivary gland cells strongly exhibited phosphorylated EGFR expression, although the number of apoptotic cells was increased (Nakamura et al., 2010), which suggests that signaling that can activate EGFRs of salivary gland cells are among patients with SjS.

Although M3R chemical agonists, such as pilocarpine and cevimeline, are often used clinically to stimulate salivary secretion in patients with SjS (Mavragani and Moutsopoulos, 2007), no studies have addressed whether such treatment can also protect against the progression of gland destruction. On the basis of our results, it is plausible that clinical treatment using M3R chemical agonists could yield cytoprotective effects against proinflammatory cytokine-induced apoptosis in salivary gland cells, particularly in the context of the tissue destruction that occurs in patients with SjS.

In summary, CCh-stimulated M3R transactivates EGFR through a signal intervention mediated by c-Src, which results in phosphorylation of both ERK and Akt. The culmination of these signaling events attenuates TNFα/IFNγ-induced caspase 3/7 activity and protects the salivary gland cells from apoptosis (Fig. 8). This study sheds light on the EGFR transactivation system and introduces novel molecular targets to the search for a therapeutic chemical compound that can protect HSG cells from inflammation-induced apoptosis, potentially leading to the development of novel therapeutic interventions against SjS.
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Authorship Contributions

Participated in research design: Kajiya, Jin, Yu, Cha, and Kawai.

Conducted experiments: Kajiya, Ichimonji, Min, Zhu, and Almazroa.

Contributed new reagents or analytic tools: Kajiya and Cha.

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Wrote or contributed to the writing of the manuscript: Kajiya, Jin, Yu, Cha, and Kawai.

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