Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 318 (2004) 247–252
BBRC
www.elsevier.com/locate/ybbrc

Salivary phospholipid secretion in response to β-adrenergic stimulation is mediated by Src kinase-dependent epidermal growth factor receptor transactivation

Bronislaw L. Slomiany* and Amalia Slomiany

Research Center, University of Medicine and Dentistry of New Jersey, Newark, NJ 07103-2400, USA

Received 2 April 2004

Abstract

Communication between receptor tyrosine kinase and G protein-coupled receptor (GPCR)-mediated signaling is recognized as a common integrator linking diverse aspects of intracellular signaling systems. Here, we report that G protein-coupled β-adrenergic receptor activation leading to stimulation of salivary phospholipid release occurs with the involvement of epidermal growth factor receptor (EGFR). Using sublingual gland acinar cells, we show that prosecretory effect of isoproterenol on phospholipid release was subjected to suppression by EGFR kinase inhibitor, PD153035, and wortmannin, an inhibitor of PI3K, but not by PD98059, an inhibitor of extracellular signal regulated kinase (ERK). Furthermore, wortmannin, but not the ERK inhibitor, caused the reduction in the acinar cell secretory responses to β-adrenergic agonist-generated cAMP as well as adenyl cyclase activator, forskolin. The acinar cell phospholipid secretory responses to isoproterenol, moreover, were inhibited by PP2, a selective inhibitor of tyrosine kinase Src responsible for ligand-independent EGFR phosphorylation. Taken together, our data are the first to demonstrate the requirement for Src kinase-dependent EGFR transactivation in regulation of salivary phospholipid secretion in response to β-adrenergic GPCR activation.

Keywords: β-Adrenergic GPCR activation; Src kinase; EGFR transactivation; Salivary phospholipid secretion

Among the components of salivary gland secretions recognized of importance to a variety of functional properties of saliva, including the maintenance of soft and hard oral tissue integrity, are lipids [1]. The elevated levels of lipids in saliva are associated with the high incidence of caries, and the development of plaque, calculus, and periodontal disease [2]. The lipids of saliva are known to affect the penetration of oral mucosa by lipophilic substances, alter the interaction of salivary proteins with calcium, and influence the extent of glycosyltransferase activity associated with cariogenic potential of bacteria [1,2]. Moreover, salivary lipids, and phospholipids in particular, readily enter into heterotypic interaction with proteins and glycoproteins of saliva to form a dynamic continuum that determines many physicochemical and biological properties of saliva [1,3]. Included among these are viscoelastic and lubricative properties, proteolytic susceptibility, and the formation of the protective coatings covering tooth enamel and oral mucosa [3,4].

Considerably less is known about the factors regulating the level of lipids in saliva, although the control of salivary secretion is mainly derived through neural, paracrine, and endocrine systems [5,6]. The parasympathetic nerve stimulation leads mainly to increase in salivary fluid secretion, while sympathetic nerve stimulation affects the secretion of macromolecular constituents of saliva [6]. Indeed, studies show that acinar cells of salivary glands respond to cAMP-mediated β-adrenergic agonists with increase in protein and mucin secretion [6], and previous work from our laboratory revealed that sublingual gland cells in culture respond to isoproterenol stimulation by the increase in phospholipid release [7].

While it is well recognized that the cellular effects of G protein-coupled receptors (GPCRs) that respond to

* Corresponding author. Fax: 1-973-972-7020.
E-mail address: slomiabr@umdnj.edu (B.L. Slomiany).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.04.016

β-adrenergic stimulation are mediated by the generation of cAMP and subsequent protein kinase A (PKA) activation, more recent data indicate that GPCR agonists, such as isoproterenol and vasoactive intestinal peptide (VIP), also have the ability to induce the activation of receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) [8–10]. This ligand-independent EGFR activation, referred to as EGFR transactivation, involves selective phosphorylation of the tyrosine residues that are not autophosphorylation sites associated with EGF stimulation [9]. Moreover, studies reporting on GPCR activation of EGFR phosphorylation point to the involvement of Src kinase in this signaling process [8,11,12]. Thus, EGFR transactivation is emerging as a central integrator in regulation of cellular responses induced by GPCR agonists in epithelial cells.

In the present study, using acinar cells of rat sublingual gland, we have investigated the role of EGFR transactivation in regulation of salivary phospholipid secretion in response to β-adrenergic agonist, isoproterenol. The data we present identify Src kinase-dependent EGFR transactivation as a key factor in salivary phospholipid elaboration.

Materials and methods

Sublingual gland acinar cell isolation and phospholipid labeling. Freshly dissected rat sublingual salivary gland was trimmed of fat and connective tissue, cut into small dices, and minced by passage through a 50 mesh metal grid [7]. The minced tissue was suspended in 5 volumes Dulbecco's modified (Gibco) Eagle's minimal essential medium (DMEM), supplemented with fungizone (50 μg/ml), penicillin (50 U/ml), streptomycin (50 μg/ml), and 2% albumin, and dispersed into single cells by trituration with a glass homogenizer, and settled by centrifugation [7]. Following rinsing, the cells were resuspended in the medium to a concentration of 5×10^5 cell/ml, transferred in 1 ml aliquots to DMEM in culture dishes containing [^{14}C]choline (20 μCi/ml), and incubated for 3 h under 95% O_2/5% CO_2 atmosphere at 37 °C [13]. The cells were then centrifuged at 300*g* for 5 min, washed three times with DMEM containing 5% albumin to remove free radiolabel, and resuspended in a fresh DMEM free of albumin.

Cell incubation. To assess the effect of β-adrenergic agonist on the acinar cells phospholipid secretory responses, the cells were preincubated for 20 min either with saline diluent or indicated concentrations of β-adrenergic antagonist, alprenolol (Sigma), followed by 30 min incubation with 0–15 μM isoproterenol (Sigma). In the experiments on the effect of EGFR inhibitor, PD153035 (Calbiochem), ERK1/2 inhibitor, PD98059 (Calbiochem), PI3K inhibitor, wortmannin (Sigma), and Src kinase selective inhibitor, PP2 and its inactive analog, PP3 (Calbiochem), the cells prior to addition of the isoproterenol were first incubated for 20 min with the indicated concentration of the agent. The effects of dibutyryl-cAMP (Sigma) and forskolin (Sigma) were assessed following 20 min preincubation with wortmannin or saline diluent. At the end of incubation period, the cells were centrifuged at 300*g* for 5 min and washed three times with fresh medium. The medium and washes were combined and used for the isolation of [^{14}C]choline labeled phospholipids.

Cell viability. Cell preparations before and during the experimentation were assessed for viability and cellular integrity using trypan blue uptake assay and the determination of lactate dehydrogenase released into the medium [13].

Phospholipid analysis. Extraction of lipids from the combined cell wash and incubation medium was performed with chloroform/methanol (2:1, v/v) [7]. The extracts were filtered through a grade F sintered glass funnel, and the lipids contained in the filtrates were dried under stream of nitrogen. The lipids contained in the residue were dissolved in a small volume of chloroform, applied to silicic acid column, and separated into neutral lipid, glycolipid, and phospholipid fractions [7]. Aliquots of phospholipid fraction, eluted from the columns with methanol, were subjected to analysis for [^{14}C]choline labeled phospholipids by scintillation spectrometry, while the remainder was used for individual phospholipid analysis by thin-layer chromatography. Following two consecutive developments in chloroform/methanol/water (80:35:5, by vol.), the plates were scanned for individual [^{14}C]choline-containing phospholipids using Berthold Digital Autoradiograph Analyzer.

Data analysis. All experiments were carried out using duplicate sampling and the results are expressed as means ± SD. Analysis of variance (ANOVA) was used to determine significance and the significance level was set at $P > 0.05$.

Results

The mechanism underlying β-adrenergic agonist activated release of salivary phospholipids was investigated using [^{14}C]choline-labeled acinar cells of rat sublingual salivary glands exposed to isoproterenol. Under the employed incubation conditions, the cell viability remained over 98% with little if any (0.5–0.7%) cellular damage, and the label incorporated into phosphatidylcholine (PC), lysophosphatidylcholine (LPC), and sphingomyelin (SM). The analyses indicated that at the end of 3 h labeling period, 94.2% of choline-containing phospholipids were represented by PC, 2.3% by LPC, and 3.1% by SM (Fig. 1). The effect of β-adrenergic agonist, isoproterenol, on the choline-containing phospholipid release is presented in Fig. 2A.

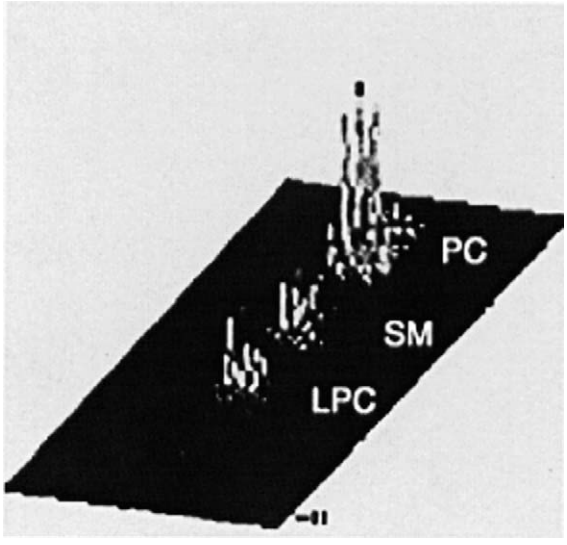

Fig. 1. Autoradiograph scan of thin-layer chromatography plate of choline-labeled phospholipids from rat sublingual salivary gland incubated in the presence of [^{14}C]choline. PC, phosphatidylcholine; SM, sphingomyelin; and LPC, lysophosphatidylcholine.

The phospholipid secretion showed a dose-dependent increase with the isoproterenol concentration up to 10 μM, at which point a 3.1-fold increase in phospholipids release was observed. Introduction to the incubation medium of a specific β-adrenergic antagonist, alprenolol, led to the impedance of the isoproterenol-induced increase in phospholipid release. The effect of alprenolol was dose-dependent and the optimal concentration of 20 μM produced an 86% impedance of the isoproterenol stimulatory effect (Fig. 2B).

The prosecretory effect of isoproterenol on choline-containing phospholipid release by salivary gland acinar cells was subjected to suppression by the pretreatment with a specific inhibitor of EGFR kinase,

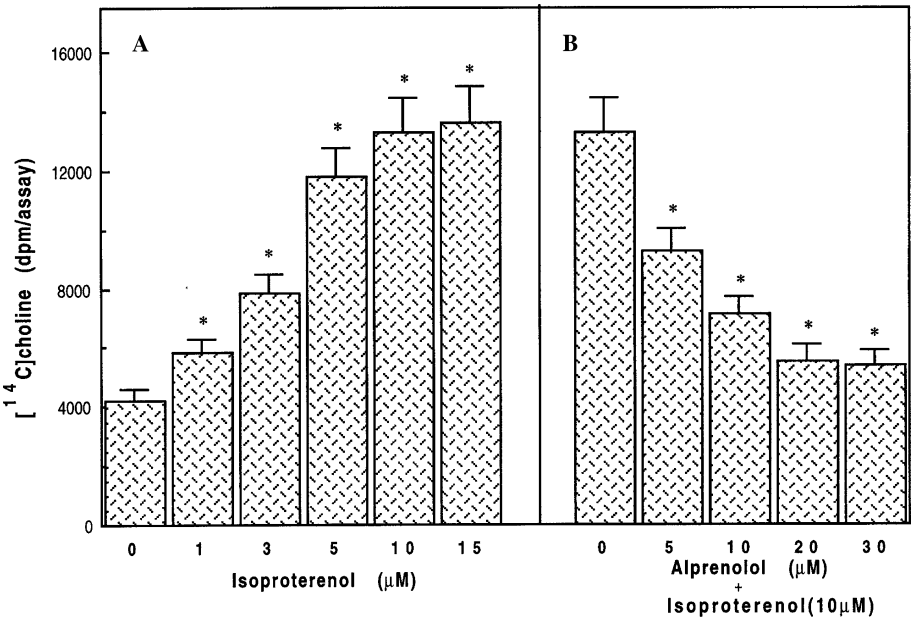

Fig. 2. Effect of β-adrenergic agonist, isoproterenol, on the secretion of [^{14}C]choline-containing phospholipids by sublingual salivary gland cells in culture. The [^{14}C]choline-labeled cells, pretreated with saline diluent (A) or β-adrenergic antagonist, alprenolol (B), were stimulated for 30 min with the indicated concentrations of isoproterenol and the medium was analyzed for choline-labeled phospholipids. Values represent means ± SD of five experiments. *$P < 0.05$ compared with that of control.

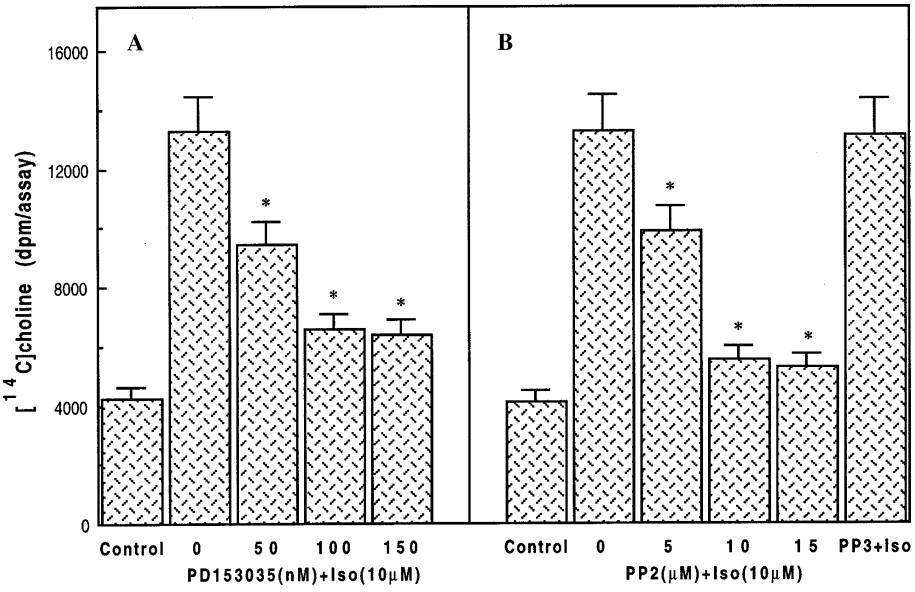

Fig. 3. Effect of EGFR inhibitor, PD153035, and Src kinase inhibitor, PP2, on sublingual salivary gland cells, phospholipid secretion in response to isoproterenol. The [^{14}C]choline-labeled cells, pretreated with the indicated concentrations of PD153035 (A) or PP2, or 10 μM of its inactive analog PP3 (B), were stimulated for 30 min with 10 μM isoproterenol (Iso) and the medium was analyzed for choline-labeled phospholipids. Values represent means ± SD of five experiments. *$P < 0.05$ compared with that of Iso.

PD153035, which at its optimal concentration of 100 nM reduced the isoproterenol-stimulated phospholipid release by 72.5% (Fig. 3A). Moreover, the secretory responses to isoproterenol were also inhibited by PP2, a selective inhibitor of tyrosine kinase Src responsible for ligand-independent EGFR transactivation, whereas PP3 a pharmacologically inactive analog of PP2 produced no effect (Fig. 3B). The effect of PP2 was concentration-dependent and at 10 μM produced a 69.1% reduction of the isoproterenol-stimulated phospholipid release.

As prosecretory actions of isoproterenol are exerted through cAMP-dependent activation of PKA, and the downstream effects of EGFR are mediated by PI3K, we next examined the acinar cell phospholipid secretory responses in the presence of PI3K inhibitor, wortmannin. The results revealed that pretreatment with wortmannin at 100 nM led to a 78.4% reduction in the acinar cell phospholipid secretory responses to isoproterenol, the phospholipid secretory responses to a cell permeable analog of cAMP, dibutyryl-cAMP, decreased by a 72.8%, while the prosecretory effects of adenyl cyclase activator, forskolin, were reduced by 81.3% (Fig. 4). In contrast, pretreatment of the acinar cells with the inhibitor of ERK pathway, PD98059, caused only marginal if any subsequent alterations in phospholipid

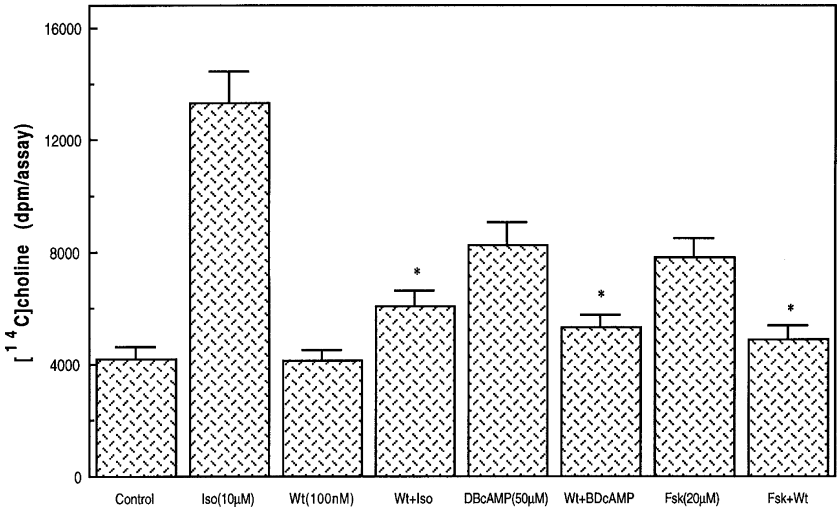

Fig. 4. Effect of PI3K inhibitor, wortmannin (Wt), on sublingual salivary gland cells, phospholipid secretion in response to isoproterenol (Iso), dibutyryl-cMAP (DBcAMP), and forskolin (Fsk). The [^{14}C]choline-labeled cells, pretreated with 100 nM Wt, were stimulated for 30 min with 10 μM Iso or 50 μM DBcAMP, or 20 μM Fsk and the medium was analyzed for choline-labeled phospholipids. Values represent means ± SD of five experiments. *$P < 0.05$ compared with that of agonist alone.

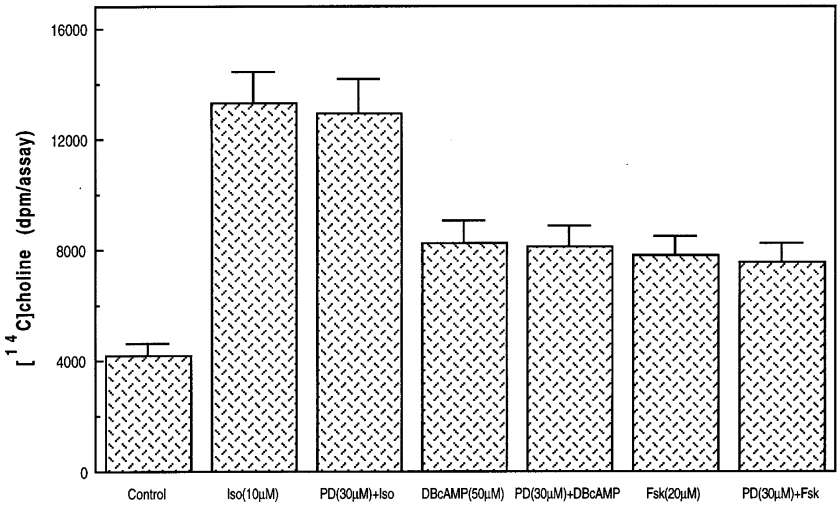

Fig. 5. Effect of ERK inhibitor, PD98059, on sublingual salivary gland cells, phospholipid secretion in response to isoproterenol (Iso), dibutyryl-cAMP (DBcAMP), and forskolin (Fsk). The [^{14}C]choline-labeled cells, pretreated with 30 μM PD98059 (PD), were stimulated for 30 min with 10 μM Iso or 50 μM DBcAMP, or 20 μM Fsk and the medium was analyzed for choline-labeled phospholipids. Values represent means ± SD of five experiments. *$P < 0.05$ compared with that of agonist alone.

secretory responses to isoproterenol, dibutyryl-cAMP or forskolin (Fig. 5).

Discussion

The principal regulatory factors controlling the extent and nature of salivary secretions are autonomic neurotransmitters released by innervating sympathetic and parasympathetic nerves, and the acinar cells of salivary glands possess receptors capable of responding to signal from either system [5,6]. While the secretion of salivary fluid is promoted by activation of cholinergic and α-adrenergic receptors coupled to GPCRs that regulate the generation of Ca^{2+} and PKC second messengers, the β-adrenergic agonists couple to GPCRs that regulate the generation of cAMP and PKA activation, and stimulate the secretion of macromolecular constituents of saliva [6,7]. Moreover, recent studies with several different cell systems into the nature of cellular responses mediated by GPCR signaling systems provided well-documented evidence that cAMP-dependent agonists have the capability to induce EGFR transactivation [8–12]. Indeed, activation of GPCR by β-adrenergic receptor agonist, isoproterenol, has been reported to lead to EGFR transactivation with the involvement of PKA-induced stimulation of ERK and Src kinases in transformed kidney cells [8], and colonic epithelial cells respond to stimulation by VIP with GPCR-mediated signaling pathway through cAMP- and PKA-dependent EGFR transactivation that involves PI3K [10]. Thus, communication between receptor tyrosine kinase activation and GPCR-mediated signaling is emerging as a common integrator linking diverse aspects of intracellular signaling pathways.

Hence, in the study presented herein, using secretory cells of sublingual salivary gland, we examined the involvement of EGFR transactivation in regulation of salivary phospholipid secretion in response to stimulation with β-adrenergic agonist, isoproterenol. The results revealed that exposure of the acinar cells to isoproterenol led to a dose-dependent stimulation in phospholipid release, and that this effect of isoproterenol was sensitive to inhibition by β-adrenergic antagonist, alprenolol. Moreover, the prosecretory effect of isoproterenol on phospholipid release was subjected to suppression by a specific inhibitor of EGFR kinase activity, PD153035, as well as wortmannin, a specific inhibitor of PI3K. Wortmannin, furthermore, caused the reduction in the acinar cell phospholipid secretory responses to β-adrenergic agonist generated second messenger, cAMP as well as adenyl cyclase activator, forskolin. Together, these data provide evidence for the role of EGFR in mediation of β-adrenergic agonist prosecretory action on salivary phospholipid release, and point to PI3K as a critical regulator of cAMP-dependent secretory responses.

Our findings are supported by the results obtained recently with intestinal epithelial cells where stimulation with VIP or dibutyryl-cAMP led to a rapid recruitment of a p85 regulatory subunit of PI3K to the EGFR [10]. This process is associated with autophosphorylation of tyrosine residue within the regulatory subunit and leads to an increase in catalytic activity of p110 subunit of PI3K towards membrane inositol lipids that trigger protein kinaseB/Akt activation [14,15]. Interestingly, Akt activation and PI3K-dependent generation of phosphatidylinositol phosphates have been shown to be sensitive to an inhibitor of Src kinase [14], and the inhibition of PI3K with wortmannin attenuated secretory responses not only to VIP and dibutyryl-cAMP, but also to the adenyl cyclase activator, forskolin [10].

As the events of EGFR transactivation are also associated with the activation of ERK MAPK and the non-receptor tyrosine kinase Src [9,12,16,17], we focused further in our studies on the role these kinases play in mediation of the acinar cell phospholipid secretory responses to β-adrenergic agonist stimulation. The results revealed that while the inhibition of Src kinase with a selective pharmacological inhibitor, PP2, evoked a significant reduction in the acinar cell phospholipid secretory responses to isoproterenol, the ERK inhibitor, PD98059, caused only marginal if any alterations in subsequent phospholipid secretory responses to the agonist. The inhibition of ERK, furthermore, did not attenuate the acinar cell secretory responses to dibutyryl-cAMP and the adenyl cyclase activator, forskolin. These findings are consistent with the data gathered with intestinal epithelial cells and demonstrating that, although cMAP-dependent agonist-induced EGFR transactivation is accompanied by ERK phosphorylation, ERK MAPK does not appear to be involved in regulation of cAMP-induced secretory responses [10]. Moreover, our finding that the acinar cell phospholipid secretory responses to isoproterenol stimulation were blocked by PP2, and the reports indicating that β-adrenergic receptor-mediated Src activation precedes EGFR transactivation [8,12,17] attest to a critical requirement for Src in the regulation GPCR-mediated secretory events that require EGFR phosphorylation.

References

[1] B.L. Slomiany, V.L.N. Murty, A. Slomiany, Salivary lipids in health and disease, Prog. Lipid Res. 24 (1985) 311–324.

[2] B.L. Slomiany, A. Slomiany, I.D. Mandel, Lipids of saliva and salivary concretions, in: J. Tenovue (Ed.), Clinical Chemistry of Human Saliva, vol. II, CRC Press, Boca Raton, 1989, pp. 121–145.

[3] B.L. Slomiany, V.L.N. Murty, J. Sarosiek, J. Piotrowski, A. Slomiany, Role of associated and covalently bound lipids in

salivary mucin hydrophobicity, Biochem. Biophys. Res. Commun. 151 (1988) 1046–1053.
[4] B.L. Slomiany, V.L.N. Murty, I.D. Mandel, G. Zalesna, A. Slomiany, Physicochemical characteristics of mucus glycoproteins and lipids of the human oral mucosal mucus coat in relation to caries status, Arch. Oral Biol. 34 (1989) 229–237.
[5] B.J. Baum, Neurotransmitter control of secretion, J. Dent. Res. 66 (1987) 628–632.
[6] D.I. Cook, E.W. Van Lennep, M.L. Roberts, J.A. Young, Secretion by the major salivary glands, in: L.R. Johnson (Ed.), Physiology of the Gastrointestinal Tract, vol. 2, Raven Press, New York, 1994, pp. 1061–1117.
[7] B.L. Slomiany, S. Sengupta, Z. Fekete, V.L.N. Murty, A. Slomiany, Adrenergic and cholinergic regulation of phospholipids release in sublingual salivary gland in vitro, Gen. Pharmacol. 22 (1991) 969–973.
[8] S. Maudsley, K.L. Pierce, A.M. Zamah, W.E. Miller, S. Ahn, Y. Daaka, R.J. Lefkowitz, L.M. Luttrell, The β2-adrenergic receptor mediates extracellular signal-regulated kinase activation via assembly of a multi-receptor complex with epidermal growth factor receptor, J. Biol. Chem. 275 (2000) 9572–9580.
[9] A. Piiper, M.P. Lutz, H. Cramer, R. Elez, B. Cornbergger, I. Dikic, W. Muller-Esterl, S. Zeuzem, Protein kinase A mediates cAMP-induced tyrosine phosphorylation of the epidermal growth factor receptor, Biochem. Biophys. Res. Commun. 301 (2003) 848–854.
[10] L.S. Bertelsen, K.E. Barrett, S.J. Keely, Gs protein-coupled receptor agonists induce transactivation of the epidermal growth factor receptor in T_{84} cells, J. Biol. Chem. 279 (2004) 6271–6279.
[11] L.M. Luttrell, Y. Daaka, R.J. Lefkowitz, Regulation of tyrosine kinase cascades by G-protein coupled receptors, Curr. Opin. Cell Biol. 11 (1999) 177–183.
[12] E.M. Hur, Y.S. Park, B.D. Lee, I.H. Ho, H.S. Kim, T.D. Kim, P.G. Suh, S.H. Ryu, K.T. Kim, Sensitization of epidermal growth factor-induced signaling by bradykinin is mediated by c-Src, J. Biol. Chem. 279 (2004) 5852–5860.
[13] B.L. Slomiany, V.L.N. Murty, E. Piotrowski, M. Morita, J. Piotrowski, A. Slomiany, Activation of arachidonoyl phospholipase A_2 in prostaglandin-mediated action of sucralfate, Gen. Pharmacol. 25 (1994) 261–266.
[14] M.P. Haynes, L. Li, D. Sinha, K.S. Russell, K. Hisamoto, R. Baron, M. Collinge, W.C. Sessa, J.R. Bender, Src kinase mediates phosphatidylinositol 3-kinase/Akt-dependent rapid endothelial nitric-oxide synthase activation by estrogen, J. Biol. Chem. 278 (2003) 2118–2123.
[15] H.Q. Wag, T. Quan, T. He, T.F. Franke, J.J. Voorhees, G.J. Fisher, Epidermal growth factor receptor-dependent, NF-κB-independent activation of the phosphatidylinositol 3-kinase/Akt pathway inhibits ultra violet irradiation-induced caspase-3, -8, and -9 in human keratinocytes, J. Biol. Chem. 278 (2003) 45737–45745.
[16] M. Leserer, A. Gschwind, A. Ullrich, Epidermal growth factor receptor signal transactivation, IUBMB Life 49 (2000) 405–409.
[17] Y. Ren, S. Meng, L. Mei, Z.J. Zhao, R. Jove, J. Wu, Roles of Gab1 and SHP2 in paxillin tyrosine dephosphorylation and Src activation in response to epidermal growth factor, J. Biol. Chem. 279 (2004) 8497–8505.