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Effect of protein kinase C and Ca^{2+} on p42/p44 MAPK, Pyk2, and Src activation in rat conjunctival goblet cells

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Abstract

Conjunctival goblet cells synthesize and secrete mucins onto the ocular surface to lubricate it and protect it from bacterial infections. Mucin secretion is under neural control, and cholinergic agonists released from parasympathetic nerves are major stimuli of this secretion. The signal transduction pathways these agonists use to stimulate secretion involve activating protein kinase C (PKC) and increasing intracellular [Ca^{2+}] to activate the non-receptor kinases Pyk2 and p60Src (Src) to transactivate the EGF receptor. Transactivation of the EGF receptor activates a kinase cascade culminating in the activation of p42/p44 MAPK (MAPK) and ultimately that leads to secretion of high molecular weight glycoconjugates (HMWGC), including mucins. To further examine the roles of PKC and Ca^{2+} in the activation of MAPK, Pyk2, and Src in mucin secretion, rat conjunctival pieces and cultured goblet cells were incubated with the PKC activator phorbol myristate acid (PMA), the cholinergic agonist carbachol, or the calcium ionophore, ionomycin for varying times. Conjunctival pieces were preincubated with PKC inhibitors 10 min prior to addition of carbachol (10^{-4} M) for 10 min. The amount of phosphorylated (activated) MAPK, Pyk2 and Src was determined by Western blotting techniques using antibodies specific to the phosphorylated forms of each kinase. PMA significantly increased the activation of MAPK, Pyk2, and Src in a time and concentration-dependent manner. PMA-stimulated MAPK activity was completely inhibited by the EGF receptor inhibitor AG1478 (10^{-7} M). Carbachol-stimulated MAPK activity was inhibited by three PKC inhibitors, calphostin C, chelethyrine, and staurosporine. Ionomycin (10^{-6} M)-stimulated MAPK activity was inhibited 66% by AG1478 (10^{-7} M). Ionomycin also significantly increased Pyk2 and Src in time dependent manner. PKC and ionomycin also activated p42/p44 MAPK, Pyk2, and Src in cultured conjunctival goblet cells. We conclude that PKC and intracellular Ca^{2+} activate Pyk2 and Src and phosphorylate the EGF receptor leading to stimulation of MAPK in conjunctival goblet cells.

Keywords: goblet cells; signal transduction; MAPK; mucin secretion

1. Introduction

Goblet cells of the conjunctiva are responsible for synthesis, storage, and secretion of mucins, which make up the mucous layer of the tear film (Dartt, 2004; Gipson and Argueso, 2003). Mucins serve to lubricate the ocular surface, protect from bacterial infections and provide for a smooth refractive surface. These cells are highly specialized epithelial cells that are interspersed throughout the stratified squamous cells of the conjunctiva either singly or in clusters, depending on the species. A decrease in the quantity of goblet cells or their ability to secrete mucins is deleterious to the ocular surface.

Conjunctival goblet cell mucin secretion, similar to secretion from other tissues, is under neural control. We have shown that parasympathetic and sympathetic nerves surround conjunctival goblet cells (Dartt et al., 1995). Neurotransmitters released from parasympathetic nerves, namely the cholinergic agonist acetylcholine and vasoactive intestinal peptide (VIP), caused secretion of high molecular weight glycoconjugates (HMWGC), including mucins, from these cells (Dartt et al., 1996; Rios et al., 1999). In addition, activating of sensory

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nerves in the cornea caused goblet cell mucin secretion by activation the efferent parasympathetic and sympathetic nerves (Dartt et al., 1995; Kessler et al., 1995).

In the conjunctiva, cholinergic agonists transmit their extracellular signal by binding to the M_2 and M_3 muscarinic receptors on the conjunctival goblet cells (Kanno et al., 2003; Rios et al., 1999). These receptors are G-protein coupled receptors (GPCR) that are present on the plasma membrane of the goblet cells. Upon agonist binding, the receptor is activated which in turn stimulates the hydrolysis of phosphatidylinositolbisphosphate (PIP_2) by phospholipase C. Hydrolysis of PIP_2 increases the intracellular concentrations of diacylglycerol (DAG) and 1,4,5 inositol trisphsphate (IP_3). DAG activates the classical and novel isoforms of protein kinase C (PKC). IP_3 releases Ca^{2+} from intracellular stores to increase intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$). Both of these events, PKC activation and the increase in $[Ca^{2+}]_i$, lead to phosphorylation of additional proteins and ultimately to HMWGC secretion.

It is now well established that G-protein coupled receptors, such as muscarinic receptors, can interact with receptor tyrosine kinases such as the EGF receptor (Gschwind et al., 2001). Activation of the EGF receptor involves phosphorylation of the receptor on specific tyrosine residues resulting in recruitment of adaptor molecules. These adaptor molecules cause the EGF receptor to dimerize and autophosphorylate (Bazley and Gullick, 2005) leading to downstream effects. In conjunctival goblet cells, we previously showed that cholinergic agonists activate the focal adhesion kinase Pyk2 through PKC and Ca^{2+}. Pyk2 binds to and activates the non-receptor tyrosine kinase p60src (Src) (Kanno et al., 2003). This complex can then transactive the EGF receptor recruiting the adaptor proteins Shc, Grb2, and the Ras guanine nucleotide exchange factor Sos. Sos binds to the low molecular weight GTPase, Ras, causing the exchange of GDP for GTP. Ras then activates a cascade of protein kinases, Raf (MAPK kinase kinase), MEK (MAPK kinase) and p42/p44 MAPK (also known as Erk). p42/p44 MAPK has been implicated in a variety of cellular processes, both long term processes such as gene expression, differentiation, and cell proliferation, and short term processes such as secretion of HMWGC secretion from conjunctival goblet cells (Dartt et al., 1996; Kanno et al., 2003; Rios et al., 1999).

In the current study, we examined the roles of PKC and $[Ca^{2+}]_i$ in cholinergic agonist-stimulated p42/p44 MAPK, Pyk2, and p60Src activation that ultimately leads to HMWGC secretion from goblet cells.

2. Materials and methods

2.1. Materials

Monoclonal antibodies to phosphorylated (active) p42/p44 MAPK, total p42 MAPK, and total Pyk2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to Pyk2 phosphorylated at tyrosine 881 and total Src were from Biosource International (Camarillo, CA). The antibody directed against Src phosphorylated on tyrosine 416 was purchased from Cell Signaling Technology (Beverly, MA). Anti-rabbit and anti-mouse IgG secondary antibodies conjugated to horseradish peroxidase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Ionomycin, phorbol 12-myristate 13-acetate (PMA), staurosporine, calphostin C, and chelerythrine chloride were from LC Labs (Waltham, MA). Keratinocyte basal medium (KBM) was from Clonetics (San Diego, CA). Chemiluminescence reagents were from Pierce (Rockville, IL). All other reagents were from Sigma (St. Louis, MO).

2.2. Methods

All experiments were approved by the Schepens Eye Research Institute Animal Care and Use Committee. Male Sprague–Dawley rats (250–300 g) were obtained from Taconic Farms (Germantown, NY). Rats were anesthetized for 1 min in CO_2 and decapitated. The entire conjunctiva was dissected around the limbus of the cornea, placed on filter paper and cut into four pieces per eye. The pieces were preincubated for 60 min in KBM.

2.2.1. Culture of rat conjunctival goblet cells

Rat conjunctival goblet cells were grown in organ culture as described previously (Shatos et al., 2001). In brief, the conjunctival pieces were placed in RPMI 1640 media supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C. Goblet cells were identified by the presence of numerous secretory granules and contaminating non-goblet cells were removed with a rubber policeman (Shatos et al., 2001). Goblet cells were allowed to grow, trypsinized and placed in 6-well culture plates and grown to confluency.

2.2.2. Measurement of MAPK, Pyk2, and c-Src activity

The activation of p42/44 MAPK, Pyk2, and Src were examined using Western blot techniques. Conjunctival pieces or cultured rat goblet cells were incubated with the cholinergic agonist carbachol (10^{-4} M), ionomycin (10^{-6} M) or PMA (10^{-7} M) for 10 min. Inhibitors were added 10 min prior to stimulation. The pieces or cells were removed and homogenized in RIPA buffer (10 mM Tris–HCl, pH7.4, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 100 μg/ml phenylmethylsulfonyl fluoride, 30 μl/ml aprotinin, 1 mM Na_3VO_3). The homogenate was centrifuged at 10,000 × *g* for 30 min at 4 °C. Proteins in the supernatant were separated by SDS-PAGE on a 10% gel, transferred onto nitrocellulose membranes, which were blocked overnight at 4 °C in 5% non-fat dried milk in buffer containing 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween-20 (TBST). The blots were then probed with antibodies directed against the non-phosphorylated form of the enzyme (total) or the phosphorylated form of the enzyme (activated) followed by HRP-conjugated secondary antibody. Immunoreactive bands were digitally scanned and analyzed using NIH Image. The amount of phosphorylated enzyme in each