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Lipoxin A_4 activates ALX/FPR2 Receptor to Regulate Conjunctival Goblet Cell Secretion

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Abstract

Conjunctival goblet cells play a major role in maintaining the mucous layer of the tear film under physiological conditions as well as in inflammatory diseases like dry eye and allergic conjunctivitis.. Resolution of inflammation is mediated by pro-resolution agonists such as lipoxin A_4 (LXA_4) that can also function under physiological conditions. The purpose of this study was to determine the actions of LXA_4 on cultured rat conjunctival goblet cell mucin secretion, intracellular [Ca^{2+}] ([Ca^{2+}]$_i$) and identify signaling pathways activated by LXA_4. ALX/FPR was localized to goblet cells in rat conjunctiva and in cultured goblet cells. LXA_4 significantly increased mucin secretion, [Ca^{2+}]$_i$, and ERK 1/2 activation. These functions were inhibited by ALX/FPR2 inhibitors. Stable analogs of LXA_4 increased [Ca^{2+}]$_i$ to the same extent as LXA_4. Sequential addition of either LXA_4 or resolvin D1 followed by the second compound decreased [Ca^{2+}]$_i$ of the second compound compared to its initial response. LXA_4 activated phospholipase C, -D, and A2 and downstream molecules protein kinase C, ERK 1/2, and Ca^{2+}/calmodulin dependent kinase to increase mucin secretion and [Ca^{2+}]$_i$. We conclude that conjunctival goblet cells respond to LXA_4 to maintain the homeostasis of the ocular surface and could be a novel treatment for dry eye diseases.

INTRODUCTION

The cornea and conjunctiva comprise the ocular surface of the eye. Within the conjunctiva, goblet cells are interspersed throughout the stratified squamous cells. Goblet cells are responsible the synthesis and secretion of mucins into the tears and onto the ocular surface. Mucins, along with other components of the tear film, protect the cornea and conjunctiva

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from the external environment preventing dessication as well as adherence of bacteria and allergens. Recent studies have demonstrated that goblet cells play an active role in the innate immune response of the conjunctiva and are directly affected by cytokines produced during inflammation [1–3]. In the context of the ocular surface, the types of inflammation observed include seasonal allergic conjunctivitis, vernal keratoconjunctivitis, atopic keratoconjunctivitis, giant papillary conjunctivitis, chemical and thermal burns and dry eye syndrome [4]. Uncontrolled inflammation is a hallmark of these diseases causing redness, itching, and discomfort.

Resolution of inflammation is an active process and occurs with the switch from the generation of pro-inflammatory mediators such as leukotrienes and prostaglandins to the generation of pro-resolution mediators such as the resolvins (Rv), lipoxins (LX), protectins, and maresins [5]. These compounds are known, collectively, as specialized proresolving lipid mediators (SPM)[5]. Lipoxins are biosynthesized from arachidonic acid during the resolution of inflammation. Two main types of lipoxins are produced in vivo: LXA_4 and LXB_4. In the presence of aspirin, aspirin-triggered epimers of LXA_4 (ATL) and LXB_4 are formed [6]. In the conjunctiva, pretreatment of goblet cells with the pro-resolution compounds RvD1 and aspirin-triggered RvD1 (AT-RvD1) significantly decreased histamine-stimulated mucin secretion [7]. In addition, RvD1 and RvE1 were both effective in inhibiting LTD_4-stimulation of glycoconjugate secretion [8]. In addition to their well-established role in resolution of acute and chronic inflammation, it is likely that SPMs also have a role in the normal, physiological state. The expression of the receptor for LXA_4, ALX, is endogenously expressed in numerous tissues [9, 10]. In the eye, LXA_4 and the enzymes responsible for its synthesis are expressed in a healthy, non-inflamed mouse cornea [11, 12].

The role of LXA_4 under normal, physiological conditions in the conjunctiva is not known. Therefore, we explored the role of LXA_4 has an effect on conjunctival goblet cell glycoconjugate secretion and $[Ca^{2+}]_i$ and the effects of several stable analogs of LXA_4 on $[Ca^{2+}]_i$ in the absence of pro-inflammatory mediators. We demonstrate that LXA_4 responses are mediated via its receptor ALX/FPR2 to activate phospholipases -C, -D, and -A2 which in turn stimulates the downstream signaling molecules protein kinase C (PKC), extracellular regulated kinase (ERK 1/2), and calcium/calmodulin-dependent protein kinase (Ca^{2+}/CaMK) to increase $[Ca^{2+}]_i$ and glycoconjugate secretion.

Results

ALX/FPR2 receptor is present in cultured rat conjunctival goblet cells

To determine if ALX/FPR2 receptor is present in goblet cells, RT-PCR was performed using cDNA isolated from rat conjunctival goblet cells. A single band at the expected number of base pairs was observed (Figure 1A). ALX/FPR2 was also detected in cultured goblet cells by western blot analysis using an antibody directed against ALX/FPR2 (Figure 1B). A band at the expected molecular weight of 38 kDa was seen as well as a band at approximately 75 kDa which is consistent with ALX dimers [13]. To determine the location of ALX/FPR2 within the conjunctiva, immunofluorescence microscopy experiments were performed. In rat conjunctiva, ALX/FPR2 (shown in red, Figure 1C) was present in goblet cells and stratified squamous cells. The lectin UEA-1 (shown in green, Figure 1C), which binds to the secretory

products of goblet cells, was used to identify the goblet cells. In cultured rat goblet cells, ALX/FPR2 (shown in red) was present throughout the cytosol (Figure 1D). UEA-1, shown in green was used to confirm that the cultured cells were indeed goblet cells (Figure 1D). There was significant overlap in the localization of ALX/FPR2 and UEA-1. Thus the presence of ALX/FPR2 was demonstrated in rat conjunctiva and goblet cells by RT-PCR, western blot analysis, and immunofluorescence microscopy.

LXA_4 Increases Glycoconjugate Secretion and $[Ca^{2+}]_i$ and Activation of ERK 1/2

Cholingeric agonists can be released from parasympathetic nerves, as well as histamine, and the resolvins RvD1 and AT-RvD1 were shown to alter mucin secretion and $[Ca^{2+}]_i$, in rat and human cultured goblet cells [7]. Therefore, we determined if activation of the ALX/FPR2 receptor by LXA_4 also increases secretion and $[Ca^{2+}]_i$. Goblet cells were stimulated for 2 h with LXA_4 at 10^{-10}, 3×10^{-10}, and 10^{-9} M and glycoconjugate secretion measured. LXA_4 significantly increased glycoconjugate secretion 1.2 ± 0.05 (p=0.03) and 1.3 ± 0.04 (p=0.002) fold above basal at 3×10^{-10} and 10^{-9} M, respectively (Figure 2A).

As an increase in $[Ca^{2+}]_i$ is a major mechanism by which several agonists, including cholinergic agonists and histamine, cause glycoconjugate secretion [7, 14], $[Ca^{2+}]_i$ was measured in response to LXA_4. LXA_4 (10^{-11}–10^{-7} M) increased $[Ca^{2+}]_i$ in cultured goblet cells in a concentration-dependent manner. $[Ca^{2+}]_i$ was significantly increased at 10^{-9} M (p=0.003) and 10^{-10} M (p=0.04) (Figure 2B and C) with a value maximum of 116.4 ± 21.5 nM at LXA_4 10^{-9} M (Figure 2D).

To demonstrate that the rise in $[Ca^{2+}]_i$ stimulated by LXA_4 leads to glycoconjugate secretion goblet cells were incubated with the calcium chelator BAPTA/AM and glycoconjugate secretion measured. To determine the concentration of BAPTA/AM necessary to chelate $[Ca^{2+}]_i$, cells were preincubated with BAPTA/AM from 10^{-6} to 10^{-4} M for 30 min and fura2 for 1h. The increase in $[Ca^{2+}]_i$ was then measured in response to LXA_4 (10^{-9} M). LXA_4 significantly increased $Ca^{2+}{}_i$ to 112 ± 15 nM (p=0.0003). Histamine (10^{-5} M) was also used as a positive control as histamine has also been shown to stimulate an increase in $[Ca^{2+}]_i$ and secretion in these cells [14, 15]. In these experiments, histamine significantly increased $[Ca^{2+}]_i$ to 265 ± 39 nM (p=0.0005). The increases in peak $[Ca^{2+}]_i$ in response to both LXA_4 and histamine response were significantly inhibited (p=0.05 and p=0.002, respectively) at 10^{-5} M BAPTA/AM (Figure 3A).

Based on these experiments, the effect of chelation of $Ca^{2+}{}_i$ with 10^{-5} M BAPTA/AM on LXA_4-stimulated mucin secretion was then examined. Chelation of $Ca^{2+}{}_i$ with 10^{-5} M BAPTA/AM did not alter basal secretion (Figure 3B). LXA_4 (10^{-9} M) and histamine (a positive control, 10^{-5} M) both significantly stimulated mucin secretion 2.2 ± 0.4 (p=0.02) and 1.8 ± 0.2 (0.009) fold above basal, respectively (Figure 3B). LXA_4 stimulated secretion was significantly inhibited (p=0.05) by 80.8 ± 6.8% to 1.2 ± 0.2 fold above basal and histamine-stimulated secretion was completely inhibited. Thus, the increase in $[Ca^{2+}]_i$ by LXA_4 is necessary for to mucin secretion to occur.

To determine if LXA_4 binds to and activates the ALX/FPR2 receptor to increase $[Ca^{2+}]_i$ and glycoconjugate secretion, the effects of two different types of ALX/FPR2 inhibitors were

examined. Firstly, BOC-2, a peptide inhibitor of ALX/FPR2 was used. In these experiments LXA_4 (10^{-9} M) significantly increased $[Ca^{2+}]_i$ by 125.5 ± 44.2 nM (p=0.01) (Figure 4A and 4B). Preincubation for 30 min with BOC-2 (10^{-4} M) significantly decreased this response by 77% to 26.4 ± 21.3 nM (p= 0.05) (Figure 4A and B). Similar results were obtained using a second type of inhibitor, siRNA directed against the ALX/FPR2 receptor. LXA_4 (10^{-9} M) significantly increased $[Ca^{2+}]_i$ by 411.6 ± 119.6 nM (p=0.02) (Figure 4C and D). Incubation with a non-specific, scrambled siRNA did not significantly alter the LXA_4 response. In contrast, incubation with ALX/FPR2 siRNA significantly decreased LXA_4 stimulated $[Ca^{2+}]_i$ 95% to 14.9 ± 3.0 nM (p=0.03). These data indicate that LXA_4 exerts its actions via through the ALX/FPR2 receptor.

As cholinergic agonists, histamine and other proresolution mediators, namely RvD1 and AT-RvD1, stimulate the phosphorylation and activation of ERK 1/2, we determined if LXA_4 also increased ERK 1/2 activity. Goblet cells were stimulated with LXA_4 10^{-11}–10^{-8} M or histamine (10^{-5} M), as a positive control, for 5 min. Cells were lysed and activation was determined by western blot analysis. As shown in Figure 5A, LXA_4 increased ERK 1/2 activity. When six independent experiments were analyzed, LXA_4 increased ERK 1/2 activity in a concentration-dependent manner with a maximum activity of 1.6 ± 0.1 (p=0.025) fold above basal occurring at 10^{-8} M (Figure 5B). As positive control histamine (10^{-5} M) increased ERK1/2 activity by 1.5 ± 0.2 fold.

The result of these experiments indicates that similar to cholinergic agonists, histamine and RvD1 and AT-RvD1, LXA_4 alone stimulates secretion, an increase in $[Ca^{2+}]_i$, and ERK 1/2 activation in cultured conjunctival goblet cells through the ALX receptor.

LXA_4 Analogs on $[Ca^{2+}]_i$ and Their Effects on LXA_4-stimualted Increase in $[Ca^{2+}]_i$

Because LXA_4 undergoes rapid inactivation, several, more stable, LXA_4 analogs have been synthesized. We tested the effects of several analogs on their ability to increase $[Ca^{2+}]_i$ in goblet cells and their effects on LXA_4-stimulated increase in $[Ca^{2+}]_i$. 15-epi-16-phenoxy-LXA_4 and 15-epi-16-parafluorophenoxy-LXA_4, which have each been shown to prevent neutrophil recruitment [16] and inflammation [17], were compared to LXA_4, ATL and LTB_4. All compounds (10^{-9} M) increased $[Ca^{2+}]_i$ to a similar extent as LXA_4 and with a similar time frame to reach peak $[Ca^{2+}]_i$ (Table I). If the analogs were given 2 min prior to LXA_4, the LXA_4-stimulated increase in $[Ca^{2+}]_i$ was significantly decreased when compared to the LXA_4 response when added first (Table I). These results indicate that LXA_4 analogs could bind to the same or overlapping binding sites on ALX causing a desensitization of the receptor to a subsequent addition of LXA_4.

Interaction of LXA_4, RvD1, and Annexin A1 Binding to ALX/FPR2

The ALX/FPR2 receptor has multiple agonists which can bind to it. These agonists include the resolvin RvD1, the protein annexin A1 (AnxA1), as well as LXA_4 [18]. We explored the interaction between the ligands of ALX/FPR2 in goblet cells using the following experimental paradigm: addition of first agonist followed 2 minutes later by addition of second agonist. Increase in $[Ca^{2+}]_i$ was measured after addition of each agonist. In rat goblet cells, an initial addition of RvD1 (10^{-8} M) resulted in an significant increase in peak $[Ca^{2+}]_i$

of 209.6 ± 64.0 nM (p=0.01) (Figure 6A and B). There was no additional increase in $[Ca^{2+}]_i$ after a second addition of RvD1. The same result was obtained if LXA_4 is added first followed by a subsequent addition of RvD1 (Figure 6A and B). The second RvD1 response after initial addition of either RvD1 or LXA_4 was significantly decreased (p= 0.005and p=0.02, respectively) from the result obtained when RvD1 is added first.

If LXA_4 (10^{-9} M) is added first, the initial response resulted in a significant change (p=0.02) in peak $[Ca^{2+}]_i$ of 255.2 ± 107.9 nM. A second addition of LXA_4 did not result in any increase in $[Ca^{2+}]_i$ (Figure 6A and B). This is also true if RvD1 is given before LXA_4. Therefore the second LXA_4 response after initial addition of either LXA_4 or RvD1 was significantly decreased (p= 0.01 and p= 0.01 respectively) from the result obtained when LXA_4 is added first.

These results indicate that, in rat goblet cells, RvD1 and LXA_4 could bind to the same or to overlapping sites causing a desensitization of the receptor to a subsequent addition of either of these two SPMs.

When AnxA1 (10^{-8} M) was added first, the change in peak $[Ca^{2+}]_i$ was significantly increased over baseline by 309.2 ± 68.2 nM (p=0.001) (Figure 6C and D). When AnxA1 was added again, 2 min after the first addition, the change in peak $[Ca^{2+}]_i$ was 47.8 ± 19.0 nM, which was significantly decreased (p=0.004) from the response when AnxA1 added first. Similar results were obtained when AnxA1 was added after RvD1 (10^{-8} M) or LXA_4 (10^{-9} M) as the change in peak $[Ca^{2+}]_i$ significantly decreased (p= 0.02 and p=0.05, respectively) to 89.7 ± 16.8 and 80.7 ± 70.8 nM, respectively (Figures 6E and F). These decreases were significantly different from the response obtained when AnxA1 was added first.

If RvD1 is added before AnxA1 the change in peak $[Ca^{2+}]_i$ was significantly increased by 161.6 ± 60.7 nM (p=0.02) (Figure 6E and F). Interestingly, the RvD1-stimulated response was significantly increased when RvD1 was added 2 min after AnxA1 to 405.8 ± 62.8 nM (p= 0.05) (Figure 6E and F). If LXA_4 is added first, the change in peak $[Ca^{2+}]_i$ was significantly increased to 377.7 ± 78.8 nM (p=0.0007). After AnxA1 addition, the LXA_4 response was significantly decreased and was 174.5 ± 44.4 nM (p=0.04) (Figure 6E and F).

These data indicate that AnxA1 alters the interactions of LXA_4 and RvD1 to the ALX/FPR2 receptor.

Signaling Pathways Activated by LXA_4 to Increase $[Ca^{2+}]_i$ and Glycoconjugate Secretion

ALX/FPR2 is known to bind multiple ligands implying that this receptor is coupled to a diverse array of signaling pathways. To explore which signaling pathways are activated by ALX/FPR2 and LXA_4 in goblet cells, cells were first preincubated for 30 min with inhibitors to proteins in the phospholipase C (PLC) pathway. The increase in $[Ca^{2+}]_i$ and glycoconjugate secretion were measured after addition of LXA_4. Inhibition of PLC with U73122 (10^{-7} M) significantly inhibited LXA_4 stimulated increase in $[Ca^{2+}]_i$ by 80.8 ± 10.2% (p=0.04) and completely abolished LXA_4-stimulated mucin secretion (Figure 7A and B). It is well established that PLC stimulates the release of $Ca^{2+}{}_i$ from the endoplasmic

reticulum through production of inositol trisphosphate (IP_3) which binds to its receptor. Using 2-APB (10^{-5} M), an inhibitor of the IP_3 receptor, LXA_4-stimulated increase in $[Ca^{2+}]_i$ was significantly blocked (p=0.01) by 68.4 ± 20.2% and secretion was significantly blocked by 63.3 ± 4.5% (Figures 7A and B). As a positive control, goblet cells were pretreated with APB 10^{-5} M and stimulated with the cholinergic agonist carbachol (Cch) which is also known to increase $[Ca^{2+}]_i$ [19, 20]. 2-APB significantly blocked Cch-stimulated $[Ca^{2+}]_i$ response by 69.2 ± 17.2% ($p=8\times10^{-6}$) (data not shown).

In addition to generation of IP_3, activation of PLC also leads to activation of protein kinase C (PKC) through the release of diacylglycerol. When goblet cells were preincubated with PKC inhibitor Ro317649 (10^{-7} M), LXA_4-stimulated increase in $[Ca^{2+}]_i$ was significantly blocked by 54.4 ± 12.1% (p=0.02). Glycoconjugate secretion was completely blocked by Ro317649 (Figure 7A and B).

To investigate the phospholipase D (PLD) pathway, goblet cells were incubated with the PLD inhibitor 1-butanol (0.3% 1-but) and its negative control *t*-butanol (0.3%, *t*-but). 1-But significantly reduced LXA_4-stimulated increase in $[Ca^{2+}]_i$ by 68.5 ± 16.1% (p=0.01) while *t*-but had no effect (Figure 7C). The same inhibitors were used prior to measurement of mucin secretion. Inhibition of PLD with 1-but significantly blocked LXA_4-stimulated secretion 80.2 ± 11.4% (p=0.0004) while *t*-but did not have a significant effect (Figure 7D). As Cch has been shown to activate PLD in other tissues [21–24], we used Cch stimulation of glycoconjugate secretion as a control for the effectiveness of PLD inhibitor. 1-But, but not *t*-but, completely blocked Cch-stimulated glycoconjugate secretion (data not shown).

As LXA_4 activates ERK 1/2 (Figure 5), we determined if this activation was downstream of the increase in $[Ca^{2+}]_i$ and if activation of ERK 1/2 led to mucin secretion. To this end, goblet cells were incubated with an inhibitor of the ERK1/2 pathway, U0126 (10^{-6} M). LXA_4-stimulated increase in $[Ca^{2+}]_i$ was significantly inhibited 41.3 ± 13.1% (p=0.03) (Figure 7C). Mucin secretion was also significantly inhibited 84.4 ± 10.6% (p=0.0002) (Figure 7D). Cch, which has been shown to activate ERK 1/2 in goblet cells [25, 26], was used as a control. The increase in $[Ca^{2+}]_i$ and secretion stimulated by Cch were both significantly inhibited by U0126 (data not shown).

An increase in $[Ca^{2+}]_i$ can activate cytosolic phospholipase A_2 ($cPLA_2$) through binding of Ca^{2+}. To determine if PLA_2 is activated by LXA_4, goblet cells were incubated with the PLA2 inhibitor aristolochic acid (aris acid, 10^{-6} M). Aris acid significantly blocked LXA_4-stimulated increase in $[Ca^{2+}]_i$ 80.8 ± 10.2% ($p=1\times10^{-5}$) and glycoconjugate secretion by 85.4 ± 9.2% ($p=1\times10^{-6}$) (Figure 7E and F).

The involvement of calcium/calmodulin-dependent protein kinase II (Ca^{2+}/CaMK) in LXA_4-stimulated increase in $[Ca^{2+}]_i$ was investigated as Ca^{2+}/CamK is also a major calcium binding protein [27]. The Ca^{2+}/CaMK inhibitor KN93 (10^{-7} M) significantly inhibited LXA_4 stimulated increase in $[Ca^{2+}]_i$ by 75.4 ± 13.5% ($p=7\times10^{-4}$) and completely blocked glycoconjugate secretion (Figure 7E and F). The inactive analog KN92 had no effect on LXA_4-stimulated $[Ca^{2+}]_i$ or secretion.

To determine if LXA_4 increases cAMP levels, goblet cells were incubated with the PKA inhibitor H89 (10^{-5} M). H89 did not alter either LXA_4-stimulated increase in $[Ca^{2+}]_i$ or glycoconjugate secretion (Figure 7E and F). However, in the same experiments, vasoactive intestinal peptide (which is known to activate PKA to generate cAMP) stimulated increase in $[Ca^{2+}]_i$ and glycoconjugate secretion that was significantly inhibited by H89 by 50.3 ± 11.5% (p=0.009) and 56.6 ± 26.0%, (p=0.05) respectively (data not shown).

None of the inhibitors alone altered basal levels of either $[Ca^{2+}]_i$ or mucin secretion (data not shown). These data indicate that ALX/FPR2 is coupled to PLD, PLC, and PLA2 in goblet cells. Activation of these phospholipases generate IP_3, and activate ERK 1/2, PKC, and Ca^{2+}/CaMK but not PKA to increase $[Ca^{2+}]_i$ and glycoconjugate secretion in goblet cells.

Discussion

In the present study, we showed that in goblet cells activation of ALX/FPR2 by LXA_4 induces a number of signaling components involved in cellular pathways leading to an increase in $[Ca^{2+}]_i$ and mucin secretion. These signaling components include the phospholipases -C, -D, and –A2 and a number of downstream molecules, ERK 1/2, PKC, and Ca^{2+}/CaMK but not PKA (Figure 8A). ALX/FPR2 is known to bind a wide variety of ligand types including peptides, lipids, and small molecules including LXA_4 [18, 28]. This receptor has been shown to have interactions with multiple G proteins after binding the peptide *N*-formyl-methionyl-leucyl-phenylalanine [29]. This allows for the coupling of a variety of signaling molecules which include phospholipase A2 and D, ERK 1/2, JNK, and p38 [29].

The signaling pathways activated by LXA_4 are cell-specific. In human monocytes, LXA_4 stimulates a robust increase in $[Ca^{2+}]_i$ while LXA_4 stimulates a small increase in $[Ca^{2+}]_i$ in polymorphonuclear neutrophils (PMN) [30–32] and activates PLD in a PKC dependent manner [33]. However, neither the increase in $[Ca^{2+}]_i$ nor PLD activation have been linked to a physiological process [31–33] (Figure 8B). LXA_4 also causes lipid remodeling and a release of arachidonic acid in PMNs [32]. However this release of arachidonic acid did not lead to the oxidation of arachidonic acid or neutrophil aggregation and adhesion to endothelial cells [32]. In goblet cells, LXA_4 elicits a robust increase in $[Ca^{2+}]_i$ in a concentration dependent manner. The increase in $[Ca^{2+}]_i$ in goblet cells is directly linked to glycoconjugate secretion as chelation of $Ca^{2+}{}_i$ abolishes LXA_4-stimulated secretion.

One pathway activated in conjunctival goblet cells is the PLC pathway. Activation of this pathway induced release of $Ca^{2+}{}_i$ by activating the IP_3 receptor and PKC. The increase in $[Ca^{2+}]_i$ subsequently activated Ca^{2+}/CaMK. As PLD and PLA2 also increased the $[Ca^{2+}]_i$, these enzymes could have also activated Ca^{2+}/CaMK. In support of this suggestion is our finding that inhibition of Ca^{2+}/CaMK completely blocked LXA_4-stimulated increase in $[Ca^{2+}]_i$ and secretion. In contrast to Ca^{2+}/CaMK, blockage of the IP_3 receptor, although it significantly inhibited secretion, is not as effective a blockade of secretion as is the Ca^{2+}/CaMK inhibitor. This finding is consistent with only PLC, not PLD or PLA2, activating the IP_3 receptor.

Inhibition of PKC or ERK1/2 caused a different effect than inhibition of Ca^{2+}/CaMK. Inhibition of PKC or ERK1/2 did not completely suppress LXA_4-induced increase in $[Ca^{2+}]_{i,}$ but completely impaired LXA_4 stimulation of secretion. These results suggest that PKC and ERK1/2, when activated, use both Ca^{2+}- dependent and Ca^{2+} independent pathways to induce secretion. PKC consists of a family of multiple isoforms that are divided into three types: 1) DAG and Ca^{2+}- dependent isoforms 2) DAG dependent but Ca^{2+}-independent isoforms, and 3) DAG and Ca^{2+}-independent isoforms [34]. ERK 1/2 can also be activated in a Ca^{2+} independent manner [35–37]. Our findings suggest that LXA_4 activates multiple isoforms of PKC with at least one being a Ca^{2+}-dependent isoform and one Ca^{2+}-independent isoform. These data also indicate that ERK 1/2 activation by LXA_4 occurs via both Ca^{2+}- dependent and Ca^{2+}-independent mechanisms.

LXA_4 in our studies does not activate PKA to increase $[Ca^{2+}]_i$ nor to stimulate secretion. In conjunctival goblet cells VIP uses cAMP and PKA to stimulate mucin secretion and does so in a Ca^{2+}-dependent manner [38]. This is consistent with activation of Ca^{2+}-dependent isoforms of adenylyl cyclase. In spite of the fact the LXA_4 in the present study increases $[Ca^{2+}]_{i,}$ this agonist appears not to be coupled to Gαs and adenylyl cyclase.

Interestingly, LXA_4 has an effect on goblet cells alone in the absence of any pro-inflammatory molecules as these experiments in this study were performed *in vitro*. Similar results were obtained with RvD1 and AT-RvD1 [7]. These resolvins increased $[Ca^{2+}]_i$, glycoconjugate secretion, and short term activation of ERK 1/2 (less than 30 min) but blocked histamine-stimulated actions [7] over longer times. We hypothesize that this activation by SPMs including LXA_4 is vital to maintaining the normal homeostasis of the ocular surface. This would imply that regulation of SPM biosynthesis within goblet cells must be tightly controlled.

The role of LXA_4 in the goblet cells is very complicated. Under normal, physiological conditions, endogenous LXA_4 likely stimulates mucin secretion to maintain the barrier functions and antimicrobial activities necessary to protect the cornea. Under inflammatory conditions in which there is excess mucin secretion LXA_4 likely plays an anti-inflammatory function to reduce mucin secretion. Similarly, LXA_4 is endogenously expressed in the cornea without inflammation present and the amount of LXA_4 was increased after corneal wounding [12]. Exogenous addition of LXA_4 promoted wound healing [12] and increased goblet cell number and mucin area in a desiccating stress model of dry eye [39] It is not known if the conjunctiva produces LXA_4 but it is possible that LXA_4 produced by the cornea could diffuse via tears to activate goblet cells.

Activation of ALX/FPR2 in goblet cells with either LXA_4 or RvD1 blocked a subsequent addition of either of these SPMs. This could be due to the fact that the receptor has desensitized. Maderna *et al* has shown that ALX/FPR2 is internalized after treatment with LXA_4 though the maximum internalization did not occur until 30 min after the addition [41]. It is also possible that LXA_4 and RvD1 bind to the same or overlapping binding sites in the receptor. LXA_4 was shown to bind to the extracellular loop III and the related transmembrane area [42]. The location to which RvD1 binds to ALX/FPR2 has not yet been determined.

In goblet cells, addition of AnxA1 or LXA_4 blocks the increase in $[Ca^{2+}]_i$ stimulated by a second addition of either agonist. This would be consistent with AnxA1 and LXA_4 binding to the same or overlapping sites. Interestingly, while an initial addition of RvD1 blocked the increase in $[Ca^{2+}]_i$ to a subsequent addition of AnxA1, the peak $[Ca^{2+}]_i$ stimulated by RvD1 after addition of AnxA1 was significantly increased compared to the response obtained when RvD1 was added first. Binding of ligands to ALX is necessarily complicated as the receptor can bind proteins, peptides and lipids [28] and can form homo- and heterodimers [13].

LXA_4 and ATL are rapidly synthesized in response to stimuli and are also rapidly degraded [16]. Stable analogs of these compounds would be advantageous for long term treatments. We tested two such analogs and compared them to LXA_4, ATL, and LTB_4. All analogs behaved similarly to LXA_4 in that intracellular $[Ca^{2+}]$ was mobilized to similar extents. In addition, as all compounds blocked a second addition of LXA_4, it is likely that all bind to ALX, similar to LXA_4.

Several SPMs including LXA_4, RvD1, and RvE1 stimulate conjunctival goblet cell secretion. These three SPMs are biosynthesized by three different pathways. LXA_4 is derived from arachidonic acid, RvD1 from docsahexaenoic acid, and RvE1 from eicosapentaenoic acid. As conjunctival goblet cell secretion is critical for the health of the ocular surface, multiple different pathways are available to produce goblet cell secretion. If one pathway is blocked or depleted, others can potentially take over. Multiple, redundant pathways protect the cornea and ensure clear vision.

We conclude that goblet cells in the conjunctiva are able to respond to LXA_4 and other SPMS to maintain the homeostasis of the ocular surface. As LXA_4 directly affects goblet cells, lipoxins and other SPMs could be a novel treatment for dry eye diseases.

MATERIALS AND METHODS

Synthetic LXA_4 was purchased from EMD Millipore (Billerica, MA) and RvD1 was purchased from Cayman Chemical, Ann Arbor, MI). Both compounds were dissolved in ethanol as supplied by the manufacturer and were stored at −80 °C with minimal exposure to light. Immediately prior to use, each SPM was diluted in with Krebs-Ringer bicarbonate buffer with HEPES (KRB-HEPES, 119 mM NaCl, 4.8 mM KCl, 1.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES, and 5.5 mM glucose [pH 7.45]) to the desired concentrations and added to the cells. The LXA_4 analogs were prepared as described previously [16, 17]. The cells were incubated at 37 °C in the dark. Daily working stock dilutions were discarded following each experiment. Annexin A1 (AnxA1) was purchased from MyBiosource (San Diego, CA). Aristolochic acid, 2-aminoethyl diphenylborinate (2-APB), 1 butanol and *t*-butanol were from Sigma Aldrich (St Louis, MO) while U0126 was purchased from R&D Systems (Minneapolis, MN). H89 and R0-31317549 were obtained from EMD Millipore (Billerica, MA).

Animals

Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing between 125 and 150 g were anesthetized with CO_2 for 1 min, decapitated, and the bulbar and forniceal

conjunctival epithelia removed from both eyes. All experiments were in accordance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and were approved by the Schepens Eye Research Institute Animal Care and Use Committee.

Cell Culture

Goblet cells from rat conjunctiva were grown in organ culture as described and extensively characterized previously [7, 8, 14, 43–45]. The tissue plug was removed after nodules of cells were observed. First passage goblet cells were used in all experiments. The identity of cultured cells was periodically checked by evaluating staining with antibody to cytokeratin 7 (detects goblet cell bodies) and the lectin Ulex europaeus agglutinin (UEA)-1 (detects goblet cell secretory product) to ensure that goblet cells predominated.

Reverse Transcriptase (RT)-PCR

Cultured goblet cells were homogenized in TRIzol and total RNA isolated according to manufacturer's instructions. One microgram of total RNA was used for complementary DNA (cDNA) synthesis using the Superscript First-Strand Synthesis system for RT-PCR (Invitrogen, Carlsbad, CA). The cDNA was amplified by the polymerase chain reaction (PCR) using primers specific to ALX/FPR2 receptor using the Jumpstart REDTaq Readymix Reaction Mix (Sigma-Aldrich, St. Louis, MO) in a thermal cycler (Master Cycler, Eppendorf, Hauppauge, NY). The primers for were from previously published sequences [9]. For rat goblet cells, the forward primer sequence was ATG GAA GCC AAC TAT TCC ATC, and the reverse primer sequence TCA TAT TGC TTT TAT ATC AAT GTT. These primers generated 1053 bp fragments. The conditions were as follows: 5 min at 95 ºC followed by 35 cycles of 1min at 94ºC, 30 s at annealing temperature for 1 min at 72 ºC with a final hold at 72 ºC for 10 min. Samples with no cDNA served as the negative control. Amplification products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

Western blotting analyses

Cultured goblet cells were stimulated with increasing concentration of LXA4 and histamine (10–5 M) for 5 min. The reaction was stopped with the addition of excess ice cold KRB-HEPES and homogenized in RIPA buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, and 1 mM EDTA) in the presence of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The homogenate was centrifuged at 2000g for 30 min at 4°C. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and processed for western blotting. The antibody against the ALX/FPR2 receptor (Novus Biologics, Littleton, CO) was diluted 1:1000. Immunoreactive bands were visualized by the enhanced chemiluminescence method. The films were analyzed with Image J software (http://rsbweb.nih.gov/ij/).

Immunofluorescence Microscopy

First passage cells grown on glass cover slips and dissected rat conjunctiva were fixed in 4% formaldehyde diluted in phosphate buffered saline (PBS, 145 mM NaCl, 7.3 mM Na_2HPO_4,

and 2.7 mM NaH_2PO_4 (pH 7.2)) for 4 hours at 4ºC. Conjunctival tissue was preserved in 30% sucrose in PBS at 4ºC overnight and embedded in optimal cutting temperature embedding compound. Six micron sections were cut, placed on slides, and air dried for 2 hours. The sections or coverslips were rinsed for 5 minutes in PBS, and nonspecific sites were blocked by incubation with 1% bovine serum albumin, and 0.2% Triton X-100 in PBS for 45 minutes at room temperature. ALX/FPR2 receptor antibody (Novus Biologics) was used at 1:100 dilution overnight at 4 °C. Secondary antibodies were conjugated to Cy 3 (Jackson ImmunoResearch Laboratories, West Grove, PA) and were used at a dilution of 1:150 for 1 h at room temperature. Negative control experiments included incubation with the isotype control antibody. The cells were viewed by fluorescence microscopy (Eclipse E80i; Nikon, Tokyo, Japan) and micrographs were taken with a digital camera (Spot; Diagnostic Instruments, Inc, Sterling Heights, MI).

Measurement of Glycoconjugate Secretion

Cultured goblet cells were serum starved for 2 h before use, preincubated with inhibitors for 30 min, and then stimulated with either LXA_4, histamine, or the cholinergic agonist carbachol (Cch) in serum-free RPMI 1640 supplemented with 0.5% BSA for 2 h. Goblet cell secretion was measured using an enzyme-linked lectin assay (ELLA) with the lectin UEA-I. UEA-1 detects high molecular weight glycoconjugates including mucins produced by rat goblet cells. The media were collected and analyzed for the amount of lectin-detectable glycoconjugates, which quantifies the amount of goblet cell secretion as described earlier[46]. Glycoconjugate secretion was expressed as fold increase over basal that was set to 1.

Measurement of $[Ca^{2+}]_i$

Goblet cells were incubated for 1 h at 37 °C with KRB-HEPES plus 0.5% BSA containing 0.5 μM fura-2/AM (Invitrogen, Grand Island, NY), 8 μM pluronic acid F127, and 250 μM sulfinpyrazone followed by washing in KRB-HEPES containing sulfinpyrazone. Calcium measurements were made with a ratio imaging system (InCyt Im2; Intracellular Imaging, Cincinnati, OH) using wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. At least 10 cells were selected in each experimental condition. After addition of agonists, data were collected in real time. Data are presented as the actual $[Ca^{2+}]_i$ with time or as the change in peak $[Ca^{2+}]_i$. Change in peak $[Ca^{2+}]_i$ was calculated by subtracting the average of the basal value (no added agonist) from the peak $[Ca^{2+}]_i$. Although data is not shown, the plateau $[Ca^{2+}]_i$ was affected similarly to the peak $[Ca^{2+}]_i$.

Knockdown of ALX/FPR2 Receptor

Goblet cells were incubated with 100 μM siRNA against a scrambled sequence or ALX/FPR2 receptor for 72 h (Thermo Scientific, Waltham, MA) in Accell delivery media according to manufacturer's instructions (Thermo Scientific, Waltham, MA). The siRNA was a pool of 4 molecules whose sequences were: 1) CCAUCAGGUUCGUUAUUGG, 2) CCUGCAGACAUUGAGAUAA, 3) GUUUAAUACUCGUUACGGA, and 4) GUACAAACACUUGUGAAA.

Statistical analysis

Results were expressed as the fold-increase above basal. Results are presented as mean ± SEM. Data were analyzed by One-way ANOVA followed by tukey post hoc test or Student's *t*-test. $P<0.05$ was considered statistically significant.

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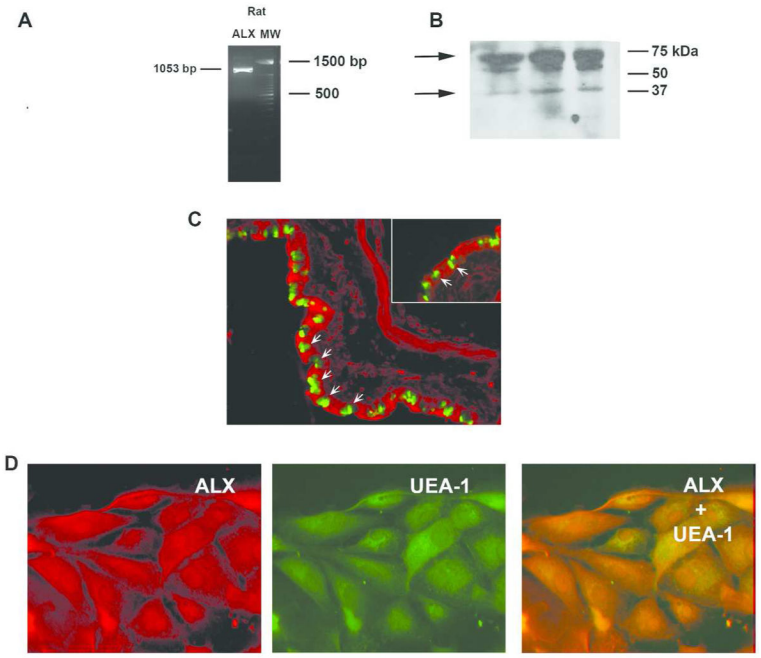

Figure 1.

Presence of ALX/FPR2 was determined in rat goblet cells by RT-PCR (**A**) and western blot analysis where each lane represents a different animal (**B**). Localization of ALX/FRP2 in rat conjunctiva is shown in by immunofluorescent microscopy (**C**). ALX/FPR2 is shown in red. Green staining indicates binding of the lectin UEA-1 which specifically binds to secretory products of conjunctival goblet cells. Arrows indicate goblet cells. Magnification 200x, inset 400x. Presence and localization of ALX/FPR2 in cultured rat goblet cells (**D**). Red staining indicates presence of ALX/FPR2. Green staining indicates binding of UEA-1. Micrographs are representative of three separate experiments. Magnification 200x.

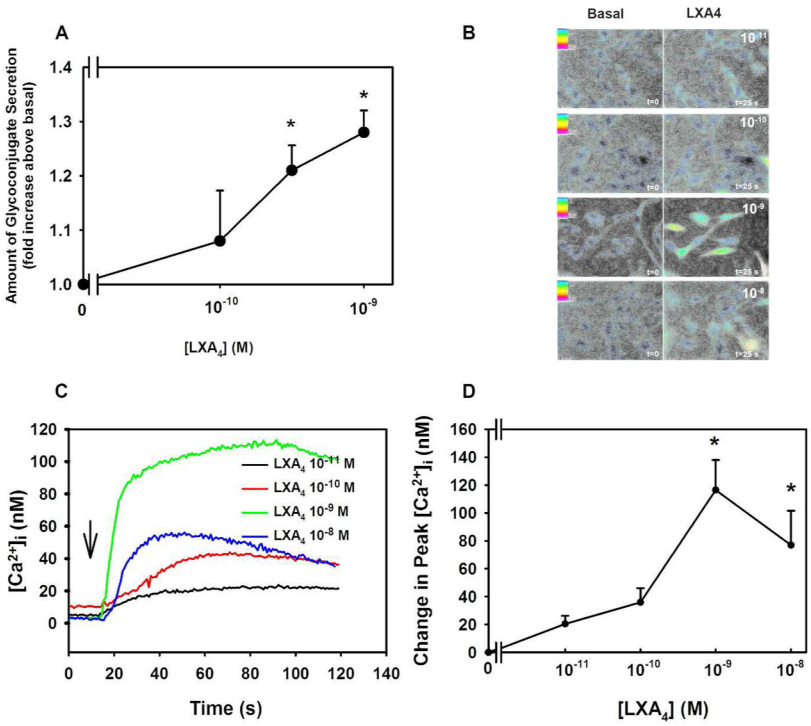

Figure 2.

Lipoxin A_4 (LXA_4) increased glycoconjugate secretion and intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) in rat conjunctival goblet cells. Glycoconjugate secretion is shown in **A**. Data are mean ± SEM from three independent experiments. Pseudo-colored images of increase in $[Ca^{2+}]_i$ in single goblet cells over time after no additions (left panel) or addition of LXA_4 10^{-11} – 10^{-8} M are shown in **B**. $[Ca^{2+}]_i$ response over time in response to increasing concentrations of LXA_4 (10^{-11}– 10^{-8}) is shown in **C**. Data are mean from three independent experiments. Arrow indicates addition of LXA_4. Peak $[Ca^{2+}]_i$ in response to LXA_4 is shown in **D**. Data are mean ± SEM of three independent experiments. * Significantly different from vehicle.

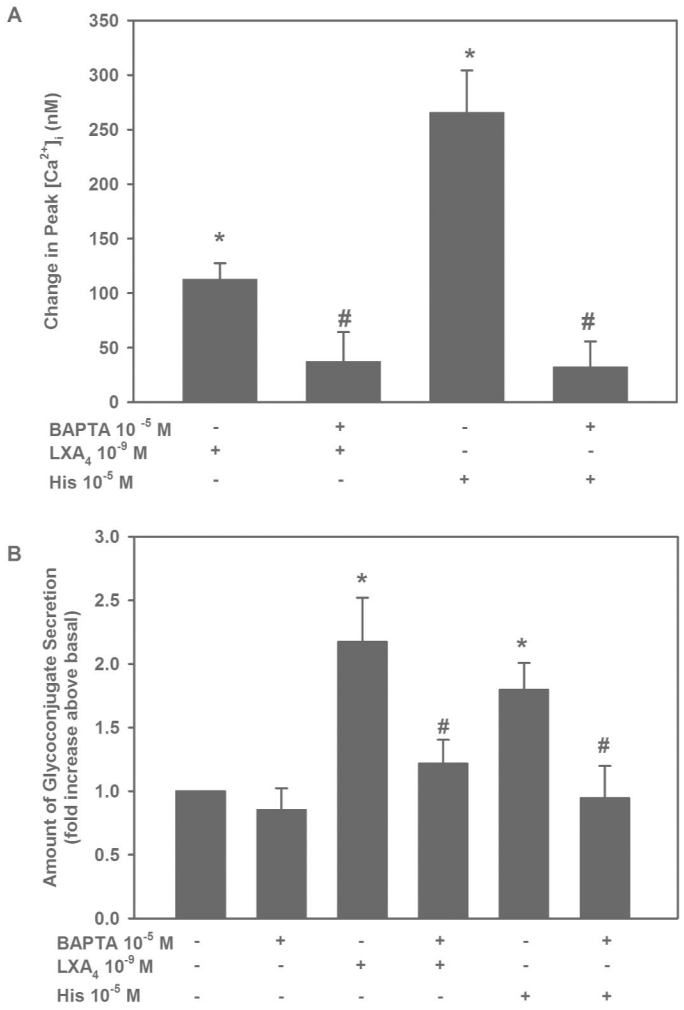

Figure 3.

Chelation of Intracellular $[Ca^{2+}]_i$ inhibits lipoxin A_4 (LXA_4) stimulated increase in $[Ca^{2+}]_i$ and glycoconjugate secretion. Effect of chelation of $[Ca^{2+}]_i$ with BAPTA (10^{-5} M) on LXA_4- and histamine- (His) stimulated increase in $[Ca^{2+}]_{i.}$ (**A**) and basal, LXA_4 and histamine-stimulated glycoconjugate secretion (**B**). Mean ± SEM of four independent experiments. * significantly different from vehicle, # significantly different from LXA_4 or histamine.

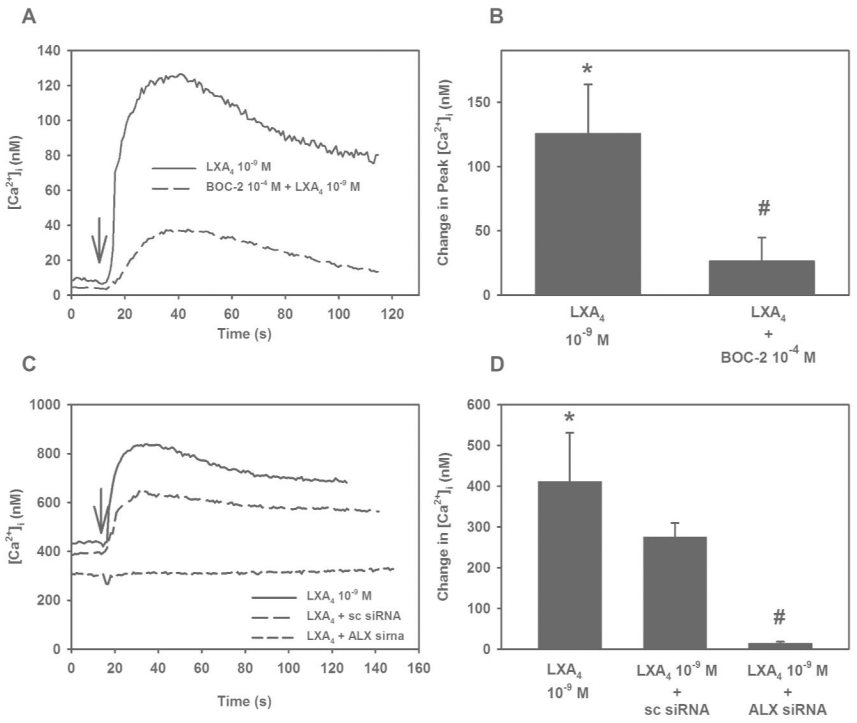

Figure 4.

Inhibition of ALX/FPR2 inhibits lipoxin A_4 (LXA_4) stimulated increase in $[Ca^{2+}]_i$. Effect of preincubation with the ALX/FPR2 inhibitor BOC-2 (**A** and **B**) or scrambled (sc) or siRNA to ALX/FPR2 (**C** and **D**) on LXA_4-stimulated increase in $[Ca^{2+}]_i$. Mean ± SEM of four independent experiments (**A** and **B**) and three independent experiments (**C** and **D**). * Significantly different from vehicle, # significantly different from LXA_4. Arrow indicates addition of LXA_4.

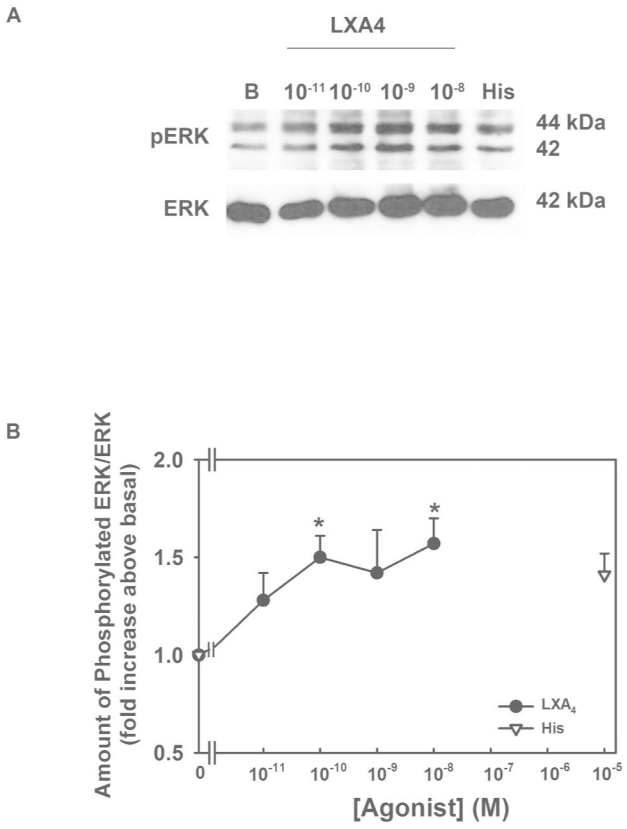

Figure 5.

Lipoxin A_4 (LXA_4) stimulates activation of ERK 1/2. Goblet cells were incubated with LXA_4 10^{-11}–10^{-8} M or histamine 10^{-5} M (his) for 5 min. Representative blot is shown in **A**. Mean ± SEM of six independent experiments with increasing concentrations of LXA_4 (closed circles) or histamine at 10^{-5} M (his, open triangle) is in **B**. * significantly different from vehicle.

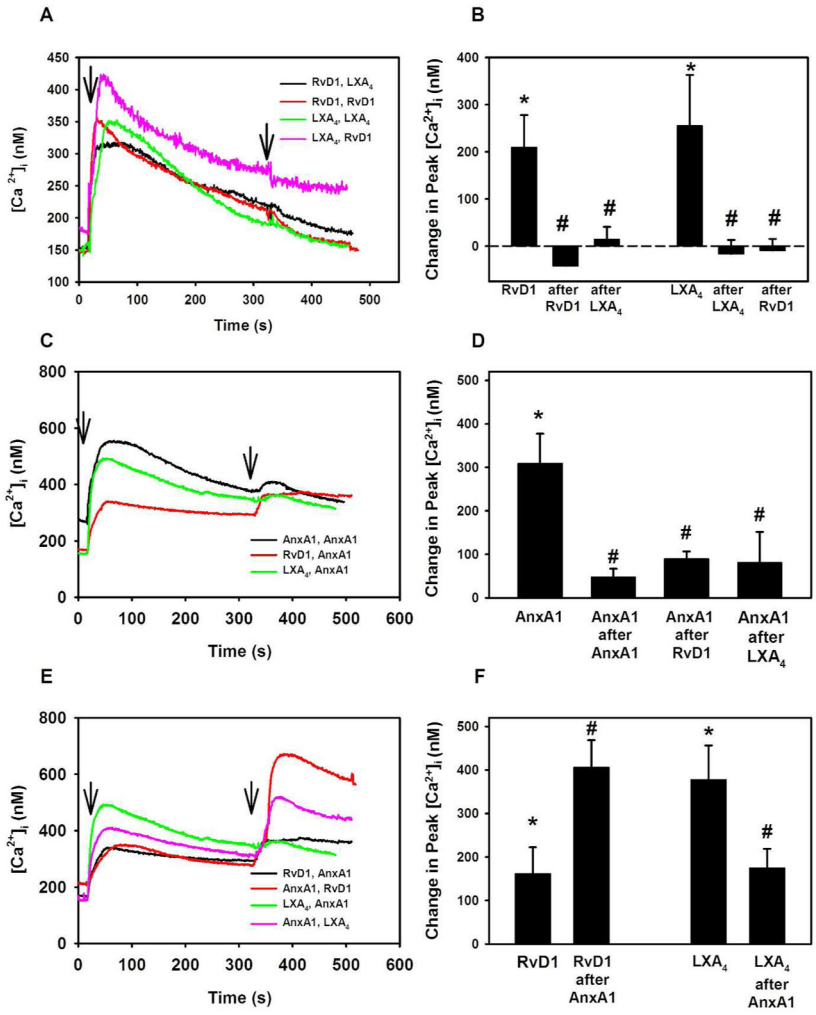

Figure 6.

Receptor interaction of Resolvin D1 (RvD1), Lipoxin A_4 (LXA_4), and Annexin A1 (AnxA1). Addition of RvD1 followed by LXA_4 2 minutes later (**A** and **B**) or LXA_4 followed by RvD1 2 minutes later (**A** and **B**). Addition of annexin A1 (AnxA1) followed by either RvD1 or LXA_4 2 minutes later (**C** and **D**) or RvD1 or LXA_4 followed by AnxA1 2 minutes later (**E** and **F**). Panels **A**, **C**, and **E** are rise of $[Ca^{2+}]$ over time. Arrows indicate addition of either AnxA1, RvD1 or LXA_4. Panels **B**, **D** and **F** are peak $[Ca^{2+}]_i$ from four (**B**) or six (**D** and **F**) independent experiments. * significantly different from vehicle, # significantly different from first addition of compound.

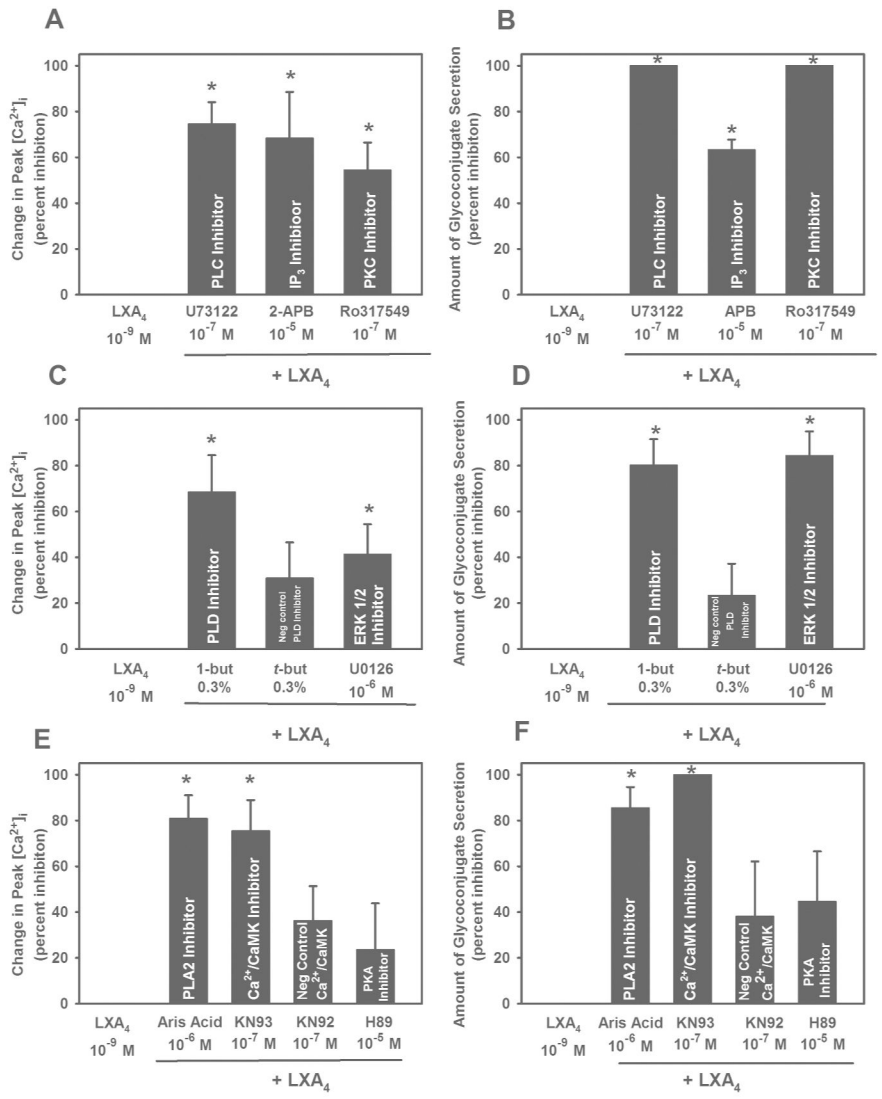

Figure 7.

Signal transduction pathways used by LXA_4 to increase $[Ca^{2+}]_i$ and glycoconjugate secretion. Goblet cells were preincubated with inhibitors U73122 (10^{-7} M), APB (10^{-5} M), and Ro317549 (10^{-7} M) (**A** and **B**); 1-butanol (0.3%), t-butanol (0.3%), and U0126 (10^{-6} M) (**C** and **D**); aristolochic acid (aris acid, 10^{-6} M), KN93 (10^{-7} M), KN92 (10^{-7} M), and H89 (10^{-5} M) (**E** and **F**). The increase in $[Ca^{2+}]_i$ (**A,C,** and **E**) and glycoconjugate secretion was measured (**B, D**, and **F**). Mean ± SEM of three-five independent experiments. * Significantly different from LXA_4 alone.

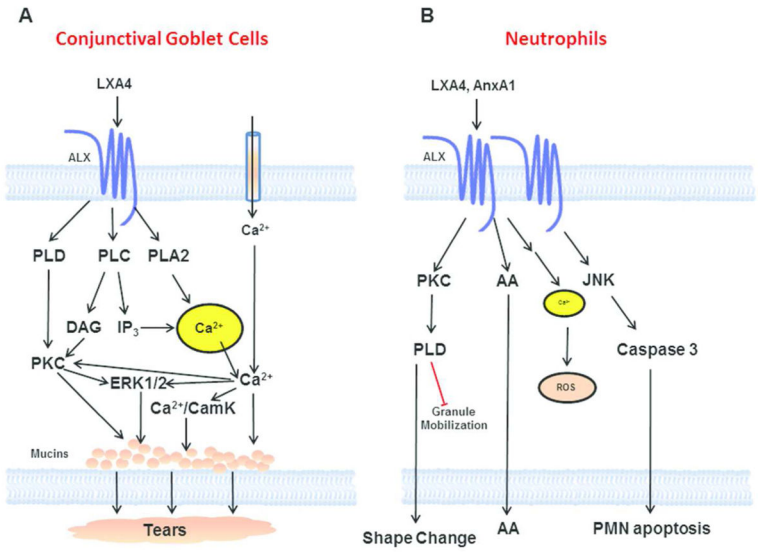

Figure 8.

Schematic Diagram of Signaling Pathways of ALX Receptor in Goblet Cells and Neutrophils. Pathways in goblet cells are shown in **A**. LXA_4, RvD1 or annexin A1 (AnxA1) bind to ALX to activate phospholipases (PL) –D, -C, and -A2. PLD activates protein kinase C (PKC) while PLC causes the generation diacylglycerol (DAG) and inositol trisphosphate (IP_3). DAG activates PKC while IP_3 causes the release of Ca^{2+} from intracellular stores. Extracellular regulated kinase 1/2 (ERK 1/2) is also activated. PLA2 causes the release of Ca^{2+}. Intracellular Ca^{2+} activates Ca^{2+}/calmodulin kinase (Ca^{2+}/CamK). All pathways lead to mucin secretion. Pathways in neutrophils are shown in **B**. LXA_4 or AnxA1 bind to ALX to activate PLD in a PKC dependent manner leading to shape changes and inhibition of granule mobilization A small amount of Ca^{2+} is also released leading to increase in reactive oxygen species (ROS). Arachadonic acid (AA) is secreted. LXA_4 also activates c-Jun N-terminal kinase (JNK) which in turn activates caspase 3 leading to polymorphonuclear neutrophils (PMN) apoptosis.

Table I

LXA_4 analogs stimulate $[Ca^{2+}]_i$ and alter LXA_4-stimulated increase in $[Ca^{2+}]_i$ response

Analogs	Change in Peak $[Ca^{2+}]_i$ (nM)	Time to Peak Increase (s)	LXA_4 Response [§]; Change in Peak $[Ca^{2+}]_i$
LXA_4	347.4 ± 58.3 (P=0.00002)	67.4 ± 5.8	(nM) 48.1 ± 27.7 * (P=0.005)
ATL	356.6 ± 82.3 (P=0.0005)	61.3 ± 7.7	54.4 ± 64.0 * (P=0.01)
15-epi-16-phenoxy-LXA_4	403.3 ± 106.3 (P=0.002)	52.7 ± 9.1	107.5 ± 62.9 * (P=0.03)
15-epi-16-parafluorophenoxy-LXA_4	398.4 ± 126.3 (P=0.006)	66.9 ± 8.5	78.3 ± 51.0 * (P=0.01)
LXB_4	379.4 ± 79.7 (P=0.0002)	53.2 ± 7.6	−19.8 ± 34.0 * (P=0.002)

* Indicates significantly different from LXA_4

§ LXA_4 response 2 min after addition of analogs

ATL, aspirin-triggered LXA_4; LXB_4, lipoxin B_4