

Lipoxin A₄ activates ALX/FPR2 receptor to regulate conjunctival goblet cell secretion

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Conjunctival goblet cells play a major role in maintaining the mucus 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 proresolution agonists such as lipoxin A₄ (LXA₄) that can also function under physiological conditions. The purpose of this study was to determine the actions of LXA₄ on cultured rat conjunctival goblet cell mucin secretion, intracellular [Ca²⁺]_i ([Ca²⁺]_i), and identify signaling pathways activated by LXA₄. ALX/FPR2 (formyl peptide receptor2) was localized to goblet cells in rat conjunctiva and in cultured goblet cells. LXA₄ significantly increased mucin secretion, [Ca²⁺]_i, and extracellular regulated kinase 1/2 (ERK 1/2) activation. These functions were inhibited by ALX/FPR2 inhibitors. Stable analogs of LXA₄ increased [Ca²⁺]_i to the same extent as LXA₄. Sequential addition of either LXA₄ or resolvin D1 followed by the second compound decreased [Ca²⁺]_i of the second compound compared with its initial response. LXA₄ activated phospholipases C, D, and A₂ and downstream molecules protein kinase C, ERK 1/2, and Ca²⁺/calmodulin-dependent kinase to increase mucin secretion and [Ca²⁺]_i. We conclude that conjunctival goblet cells respond to LXA₄ 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 for 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 from the external environment, preventing desiccation 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 proinflammatory

mediators such as leukotrienes and prostaglandins to the generation of proresolution mediators such as the resolvins (Rv), lipoxins (LX), protectins, and maresins.⁵ These compounds are known, collectively, as specialized proresolving lipid mediators (SPMs).⁵ Lipoxins are biosynthesized from arachidonic acid during the resolution of inflammation. Two main types of lipoxins are produced *in vivo*: lipoxin A₄ (LXA₄) and lipoxin B₄ (LXB₄). In the presence of aspirin, aspirin-triggered epimers of LXA₄ (ATL) and LXB₄ are formed.⁶ In the conjunctiva, pretreatment of goblet cells with the proresolution 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 LTD₄ 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 LXA₄, ALX/FPR2, is endogenously expressed in numerous tissues.^{9,10} In the eye, LXA₄ and the enzymes responsible for its synthesis are expressed in a healthy, noninflamed mouse cornea.^{11,12}

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The role of LXA₄ under normal, physiological conditions in the conjunctiva is not known. Therefore, we explored the role of LXA₄ on conjunctival goblet cell glycoconjugate secretion and intracellular [Ca²⁺]_i and the effects of several stable analogs of LXA₄ on [Ca²⁺]_i in the absence of proinflammatory mediators. We demonstrate that LXA₄ responses are mediated via its receptor ALX/FPR2 (formyl peptide receptor 2) to activate phospholipases C, D, and A₂ that in turn stimulates the downstream signaling molecules protein kinase C (PKC), extracellular regulated kinase 1/2 (ERK 1/2), and calcium/calmodulin-dependent protein kinase (Ca²⁺/CaMK) to increase [Ca²⁺]_i and glycoconjugate secretion.

RESULTS

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

To determine whether ALX/FPR2 receptor is present in goblet cells, reverse transcriptase-PCR was performed using complementary DNA (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 ~75 kDa that 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 *Ulex europaeus* agglutinin-1 (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 reverse transcriptase-PCR, western blot analysis, and immunofluorescence microscopy.

LXA₄ increases glycoconjugate secretion and [Ca²⁺]_i and activation of ERK 1/2

Cholinergic 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 [Ca²⁺]_i in rat and human cultured goblet cells.⁷ Therefore, we determined whether activation of the ALX/FPR2 receptor by LXA₄ also increases secretion and [Ca²⁺]_i. Goblet cells were stimulated for 2 h with LXA₄ at 10⁻¹⁰, 3 × 10⁻¹⁰, and 10⁻⁹ M and glycoconjugate secretion measured. LXA₄ significantly increased glycoconjugate secretion 1.2 ± 0.05-fold (*P* = 0.03) and 1.3 ± 0.04-fold (*P* = 0.002) above basal at 3 × 10⁻¹⁰ and 10⁻⁹ M, respectively (**Figure 2a**).

As an increase in [Ca²⁺]_i is a major mechanism by which several agonists, including cholinergic agonists and histamine, cause glycoconjugate secretion,^{7,14} [Ca²⁺]_i was measured in response to LXA₄. LXA₄ (10⁻¹¹–10⁻⁷ M) increased [Ca²⁺]_i in cultured goblet cells in a concentration-dependent manner. [Ca²⁺]_i was significantly increased at 10⁻⁹ M (*P* = 0.003) and

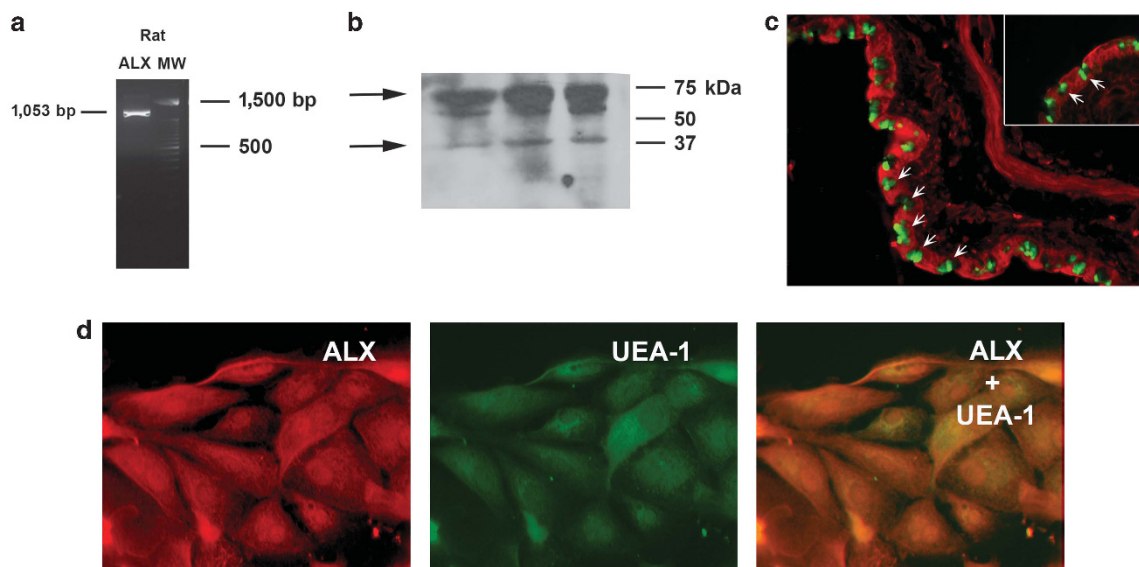


Figure 1 Presence and localization of ALX/FPR2 in rat conjunctiva and cultured rat goblet cells. Presence of ALX/FPR2 was determined in rat goblet cells by (a) reverse transcriptase-PCR (RT-PCR) and (b) western blot analysis where each lane represents a different animal. (c) Localization of ALX/FPR2 in rat conjunctiva is shown by immunofluorescent microscopy. ALX/FPR2 is shown in red. Green staining indicates binding of the lectin *Ulex europaeus* agglutinin (UEA-1) that specifically binds to secretory products of conjunctival goblet cells. Arrows indicate goblet cells. Original magnification 200 ×, inset 400 ×. (d) Presence and localization of ALX/FPR2 in cultured rat goblet cells. Red staining indicates presence of ALX/FPR2. Green staining indicates binding of UEA-1. Micrographs are representative of three separate experiments. Original magnification 200 ×. FPR2, formyl peptide receptor 2.

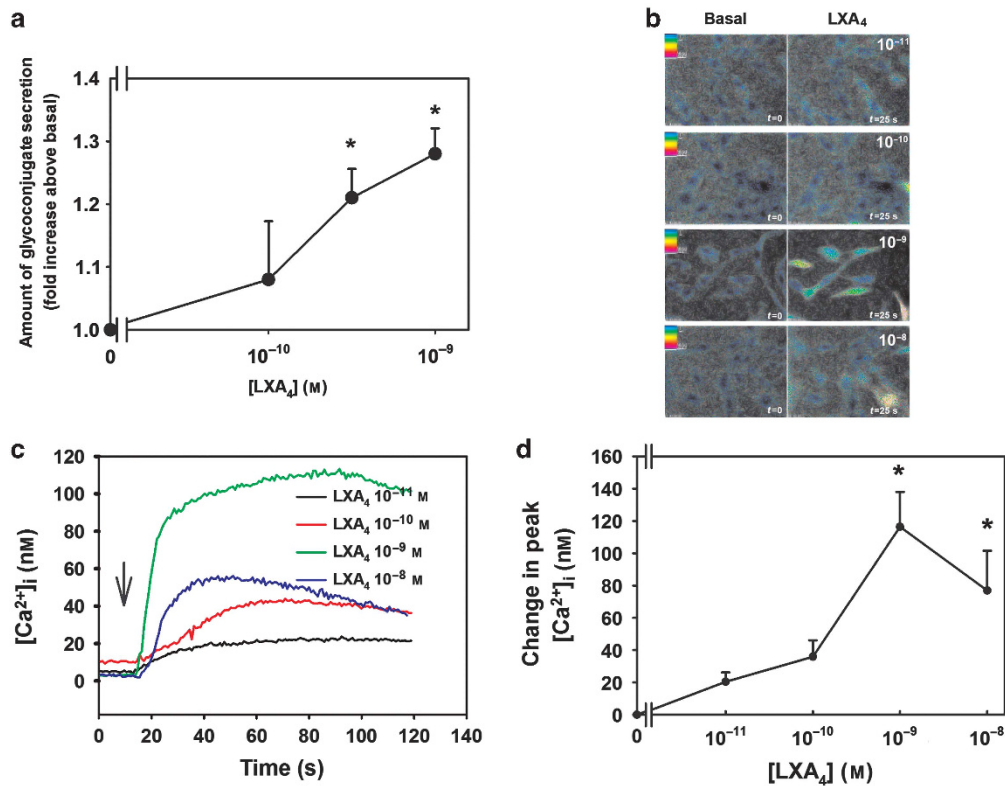


Figure 2 Lipoxin A₄ (LXA₄) increased glycoconjugate secretion and intracellular [Ca²⁺]_i ([Ca²⁺]_i) in rat conjunctival goblet cells. **(a)** Glycoconjugate secretion is shown. Data are mean ± s.e.m. from three independent experiments. **(b)** Pseudo-colored images of increase in [Ca²⁺]_i in single goblet cells over time after no additions (left panel) or addition of LXA₄ 10⁻¹¹–10⁻⁸ M are shown. **(c)** [Ca²⁺]_i response over time in response to increasing concentrations of LXA₄ (10⁻¹¹–10⁻⁸ M) is shown. Data are mean from three independent experiments. Arrow indicates addition of LXA₄. **(d)** Peak [Ca²⁺]_i in response to LXA₄ is shown. Data are mean ± s.e.m. of three independent experiments. *Significantly different from vehicle.

10⁻¹⁰ M ($P=0.04$) (**Figure 2b,c**) with a value maximum of 116.4 ± 21.5 nM at LXA₄ 10⁻⁹ M (**Figure 2d**).

To demonstrate that the rise in [Ca²⁺]_i stimulated by LXA₄ leads to glycoconjugate secretion, goblet cells were incubated with the calcium chelator acetoxyethyl ester of 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (BAPTA/AM) and glycoconjugate secretion measured. To determine the concentration of BAPTA/AM necessary to chelate Ca²⁺_i, cells were preincubated with BAPTA/AM from 10⁻⁶ to 10⁻⁴ M for 30 min and fura-2 for 1 h. The increase in [Ca²⁺]_i was then measured in response to LXA₄ (10⁻⁹ M). LXA₄ significantly increased [Ca²⁺]_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 [Ca²⁺]_i and secretion in these cells.^{14,15} In these experiments, histamine significantly increased [Ca²⁺]_i to 265 ± 39 nM ($P=0.0005$). The increases in peak [Ca²⁺]_i in response to both LXA₄ 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 [Ca²⁺]_i with 10⁻⁵ M BAPTA/AM on LXA₄-stimulated mucin secretion was then examined. Chelation of Ca²⁺_i with 10⁻⁵ M BAPTA/AM did not alter basal secretion (**Figure 3b**). LXA₄ (10⁻⁹ M)

and histamine (a positive control, 10⁻⁵ M) both significantly stimulated mucin secretion 2.2 ± 0.4-fold ($P=0.02$) and 1.8 ± 0.2-fold (0.009) above basal, respectively (**Figure 3b**). LXA₄-stimulated secretion was significantly inhibited ($P=0.05$) by 80.8 ± 6.8% to 1.2 ± 0.2-fold above basal, although histamine-stimulated secretion was completely inhibited. Thus, the increase in [Ca²⁺]_i by LXA₄ is necessary for mucin secretion to occur.

To determine whether LXA₄ binds to and activates the ALX/FPR2 receptor to increase [Ca²⁺]_i and glycoconjugate secretion, the effects of two different types of ALX/FPR2 inhibitors were examined. First, BOC-2, a peptide inhibitor of ALX/FPR2, was used. In these experiments, LXA₄ (10⁻⁹ M) significantly increased [Ca²⁺]_i by 125.5 ± 44.2 nM ($P=0.01$; **Figure 4a,b**). 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,b**). Similar results were obtained using a second type of inhibitor, small interfering RNA (siRNA) directed against the ALX/FPR2 receptor. LXA₄ (10⁻⁹ M) significantly increased [Ca²⁺]_i by 411.6 ± 119.6 nM ($P=0.02$; **Figure 4c,d**). Incubation with a nonspecific, scrambled siRNA did not significantly alter the LXA₄ response. In contrast, incubation with ALX/FPR2 siRNA significantly decreased LXA₄-stimulated [Ca²⁺]_i 95% to 14.9 ± 3.0 nM ($P=0.03$). These data indicate that LXA₄ exerts its actions via through the ALX/FPR2 receptor.

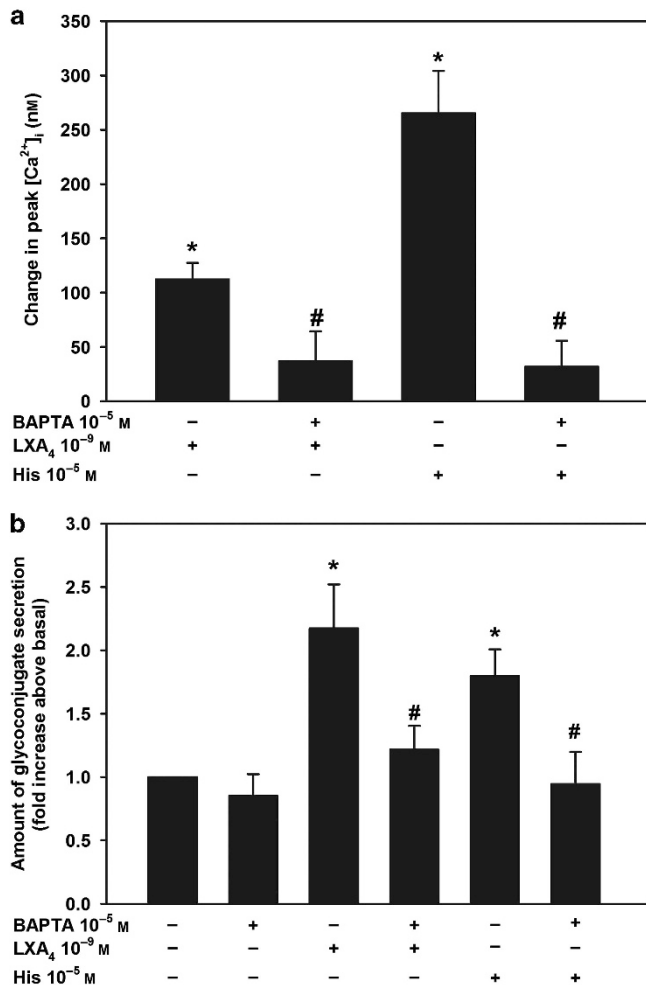


Figure 3 Chelation of intracellular $[Ca^{2+}]_i$ inhibits lipoxin A₄ (LXA₄)-stimulated increase in $[Ca^{2+}]_i$ and glycoconjugate secretion. (a) Effect of chelation of $[Ca^{2+}]_i$ with BAPTA (10^{-5} M) on LXA₄-stimulated and histamine (His)-stimulated increase in $[Ca^{2+}]_i$ and (b) basal, LXA₄- and histamine-stimulated glycoconjugate secretion. Mean \pm s.e.m. of four independent experiments. *Significantly different from vehicle, #significantly different from LXA₄ or histamine.

As cholinergic agonists, histamine, and other proresolution mediators, namely RvD1 and AT-RvD1, stimulate the phosphorylation and activation of ERK 1/2, we determined whether LXA₄ also increased ERK 1/2 activity. Goblet cells were stimulated with LXA₄ 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**, LXA₄ increased ERK 1/2 activity. When six independent experiments were analyzed, LXA₄ increased ERK 1/2 activity in a concentration-dependent manner with a maximum activity of 1.6 ± 0.1 -fold ($P=0.025$) 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, LXA₄ alone stimulates secretion, an increase in $[Ca^{2+}]_i$, and ERK 1/2 activation in cultured conjunctival goblet cells through the ALX receptor.

LXA₄ analogs on $[Ca^{2+}]_i$ and their effects on LXA₄-stimulated increase in $[Ca^{2+}]_i$

Because LXA₄ undergoes rapid inactivation, several, more stable, LXA₄ 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 LXA₄-stimulated increase in $[Ca^{2+}]_i$. 15-epi-16-phenoxy-LXA₄ and 15-epi-16-parafluorophenoxy-LXA₄, which have each been shown to prevent neutrophil recruitment¹⁶ and inflammation,¹⁷ were compared with LXA₄, ATL, and LTB₄. All compounds (10^{-9} M) increased $[Ca^{2+}]_i$ to a similar extent as LXA₄ and with a similar time frame to reach peak $[Ca^{2+}]_i$ (**Table 1**). If the analogs were given 2 min before LXA₄, the LXA₄-stimulated increase in $[Ca^{2+}]_i$ was significantly decreased when compared with the LXA₄ response when added first (**Table 1**). These results indicate that LXA₄ analogs could bind to the same or overlapping binding sites on ALX, causing a desensitization of the receptor to a subsequent addition of LXA₄.

Interaction of LXA₄, RvD1, and annexin A1 binding to ALX/FPR2

The ALX/FPR2 receptor has multiple agonists that can bind to it. These agonists include the resolvin RvD1, the protein annexin A1 (AnxA1), as well as LXA₄.¹⁸ We explored the interaction between the ligands of ALX/FPR2 in goblet cells using the following experimental paradigm: addition of first agonist followed 2 min 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,b**). There was no additional increase in $[Ca^{2+}]_i$ after a second addition of RvD1. The same result was obtained if LXA₄ is added first followed by a subsequent addition of RvD1 (**Figure 6a,b**). The second RvD1 response after initial addition of either RvD1 or LXA₄ was significantly decreased ($P=0.005$ and $P=0.02$, respectively) from the result obtained when RvD1 is added first.

If LXA₄ (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 LXA₄ did not result in any increase in $[Ca^{2+}]_i$ (**Figure 6a,b**). This is also true if RvD1 is given before LXA₄. Therefore, the second LXA₄ response after initial addition of either LXA₄ or RvD1 was significantly decreased ($P=0.01$ and $P=0.01$, respectively) from the result obtained when LXA₄ is added first.

These results indicate that, in rat goblet cells, RvD1 and LXA₄ 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,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, and this was significantly decreased ($P=0.004$) from the response when AnxA1 was added first. Similar results were obtained when AnxA1 was added after RvD1 (10^{-8} M) or LXA₄ (10^{-9} M) as the change in peak

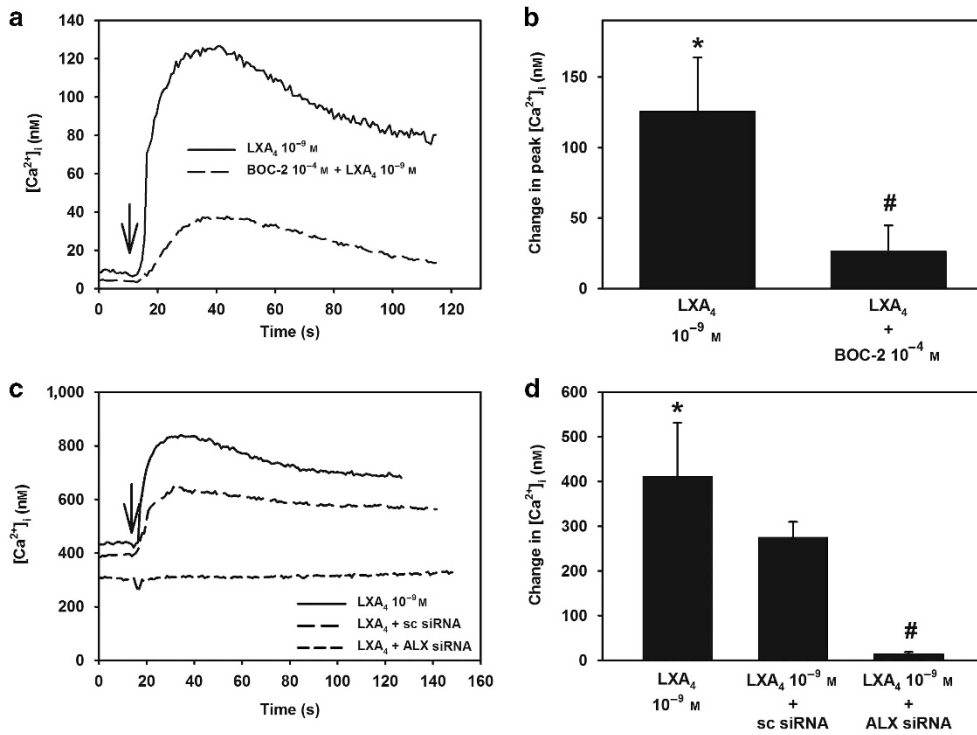


Figure 4 Inhibition of ALX/FPR2 inhibits lipoxin A₄ (LXA₄)-stimulated increase in [Ca²⁺]_i. Effect of preincubation with the ALX/FPR2 inhibitor BOC-2 (a, b) or scrambled (sc) or small interfering RNA (siRNA) to ALX/FPR2 (c, d) on LXA₄-stimulated increase in [Ca²⁺]_i. FPR2, formyl peptide receptor 2. Mean ± s.e.m. of four independent experiments (a, b) and three independent experiments (c, d). *Significantly different from vehicle, #significantly different from LXA₄. Arrow indicates addition of LXA₄.

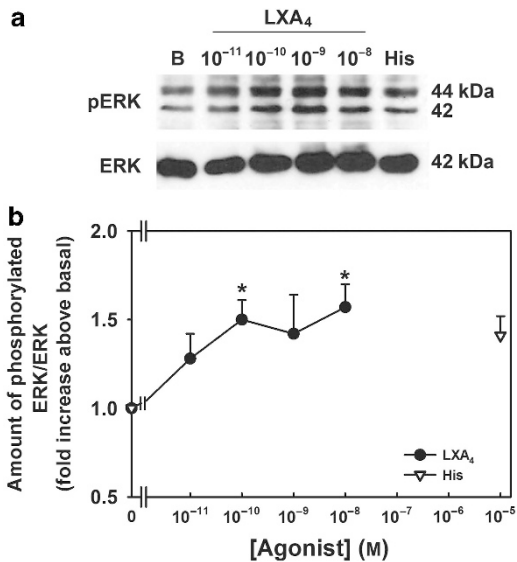


Figure 5 Lipoxin A₄ (LXA₄) stimulates activation of extracellular regulated kinase 1/2 (ERK 1/2). Goblet cells were incubated with LXA₄ 10⁻¹¹–10⁻⁸ M or histamine (His) 10⁻⁵ M for 5 min. (a) Representative blot is shown. (b) Mean ± s.e.m. of six independent experiments with increasing concentrations of LXA₄ (closed circles) or histamine at 10⁻⁵ M (open triangle). *Significantly different from vehicle.

[Ca²⁺]_i significantly decreased (*P* = 0.02 and *P* = 0.05, respectively) to 89.7 ± 16.8 and 80.7 ± 70.8 nM, respectively (Figure 6e,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 [Ca²⁺]_i was significantly increased by 161.6 ± 60.7 nM (*P* = 0.02; Figure 6e,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,f). If LXA₄ is added first, the change in peak [Ca²⁺]_i was significantly increased to 377.7 ± 78.8 nM (*P* = 0.0007). After AnxA1 addition, the LXA₄ response was significantly decreased and was 174.5 ± 44.4 nM (*P* = 0.04; Figure 6e,f).

These data indicate that AnxA1 alters the interactions of LXA₄ and RvD1 to the ALX/FPR2 receptor.

Signaling pathways activated by LXA₄ to increase [Ca²⁺]_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 LXA₄ in goblet cells, cells were first preincubated for 30 min with inhibitors to proteins in the phospholipase C (PLC) pathway. The increase in [Ca²⁺]_i and glycoconjugate secretion were measured after addition of LXA₄. Inhibition of PLC with U73122 (10⁻⁷ M) significantly inhibited LXA₄-stimulated increase in [Ca²⁺]_i by 80.8 ± 10.2% (*P* = 0.04) and completely abolished LXA₄-stimulated mucin secretion (Figure 7a,b). It is well established that PLC stimulates the release of [Ca²⁺]_i from the endoplasmic reticulum through production of inositol trisphosphate (IP₃) that binds to its receptor. Using 2-aminoethyl diphenylborinate (2-APB; 10⁻⁵ M), an inhibitor

Table 1 LXA₄ analogs stimulate [Ca²⁺]_i and alter LXA₄-stimulated increase in [Ca²⁺]_i response

Analog	Change in peak [Ca ²⁺] _i (nM)	Time to peak increase (s)	LXA ₄ response ^a ; change in peak [Ca ²⁺] _i (nM)
LXA ₄	347.4 ± 58.3 (<i>P</i> = 0.00002)	67.4 ± 5.8	48.1 ± 27.7 ^b (<i>P</i> = 0.005)
ATL	356.6 ± 82.3 (<i>P</i> = 0.0005)	61.3 ± 7.7	54.4 ± 64.0 ^b (<i>P</i> = 0.01)
15-epi-16-phenoxy-LXA ₄	403.3 ± 106.3 (<i>P</i> = 0.002)	52.7 ± 9.1	107.5 ± 62.9 ^b (<i>P</i> = 0.03)
15-epi-16-parafluorophenoxy-LXA ₄	398.4 ± 126.3 (<i>P</i> = 0.006)	66.9 ± 8.5	78.3 ± 51.0 ^b (<i>P</i> = 0.01)
LXB ₄	379.4 ± 79.7 (<i>P</i> = 0.0002)	53.2 ± 7.6	-19.8 ± 34.0 ^b (<i>P</i> = 0.002)

Abbreviations: ATL, aspirin-triggered LXA₄; LXA₄, lipoxin A₄; LXB₄, lipoxin B₄.

^aLXA₄ response 2 min after addition of analogs.

^bIndicates significantly different from LXA₄.

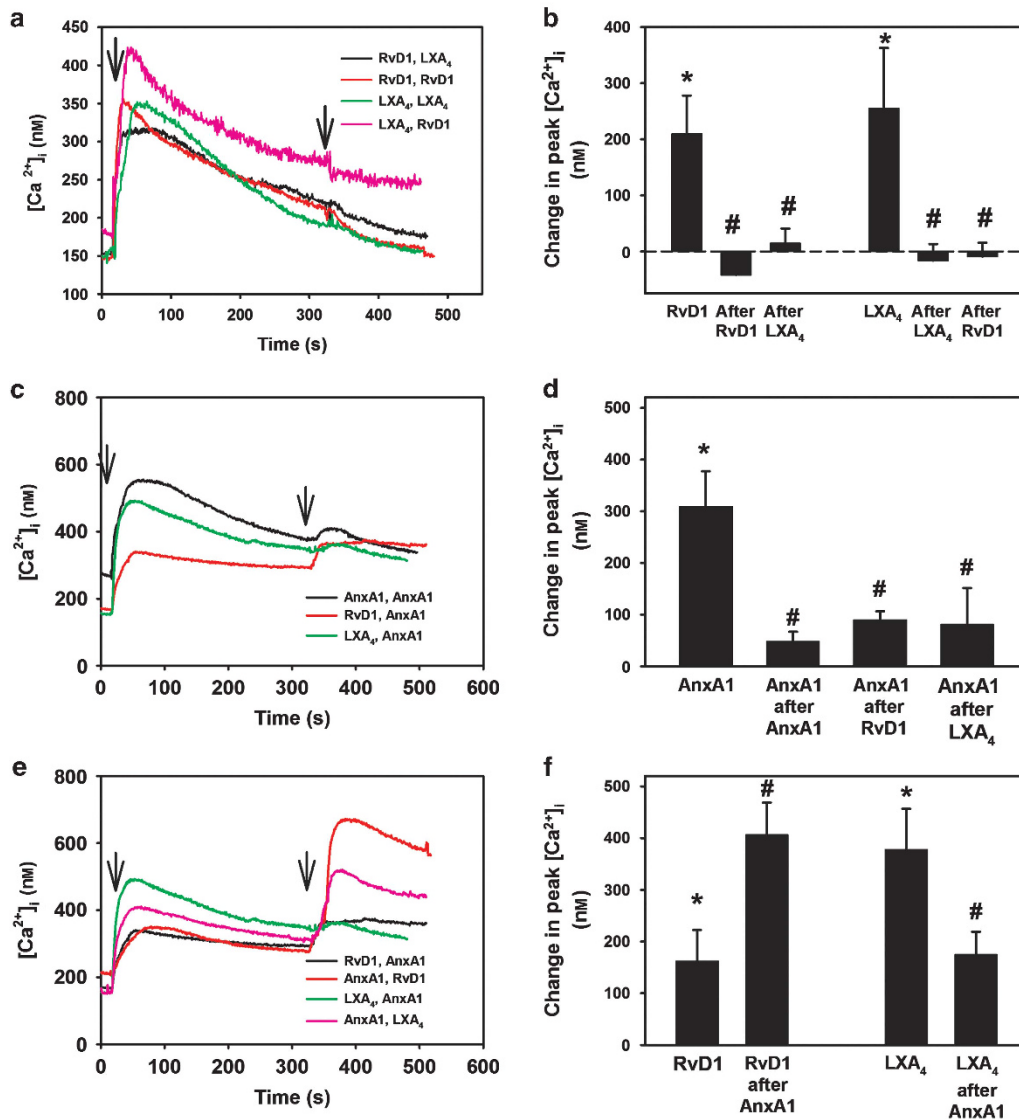


Figure 6 Receptor interaction of resolvin D1 (RvD1), lipoxin A₄ (LXA₄), and annexin A1 (AnxA1). Addition of RvD1 followed by LXA₄ 2 min later (a, b) or LXA₄ followed by RvD1 2 min later (a, b). Addition of AnxA1 followed by either RvD1 or LXA₄ 2 min later (c, d) or RvD1 or LXA₄ followed by AnxA1 2 min later (e, f). (a, c, e) Rise of [Ca²⁺]_i over time. Arrows indicate addition of either AnxA1, RvD1, or LXA₄. (b, d, f) Peak [Ca²⁺]_i from four (b) or six (d, f) independent experiments. *Significantly different from vehicle, #significantly different from first addition of compound.

of the IP₃ receptor, LXA₄-stimulated increase in [Ca²⁺]_i was significantly blocked (*P* = 0.01) by 68.4 ± 20.2% and secretion was significantly blocked by 63.3 ± 4.5% (Figure 7a,b). As a

positive control, goblet cells were pretreated with APB 10⁻⁵ M and stimulated with the cholinergic agonist carbachol (Cch) that is also known to increase [Ca²⁺]_i.^{19,20} 2-APB significantly

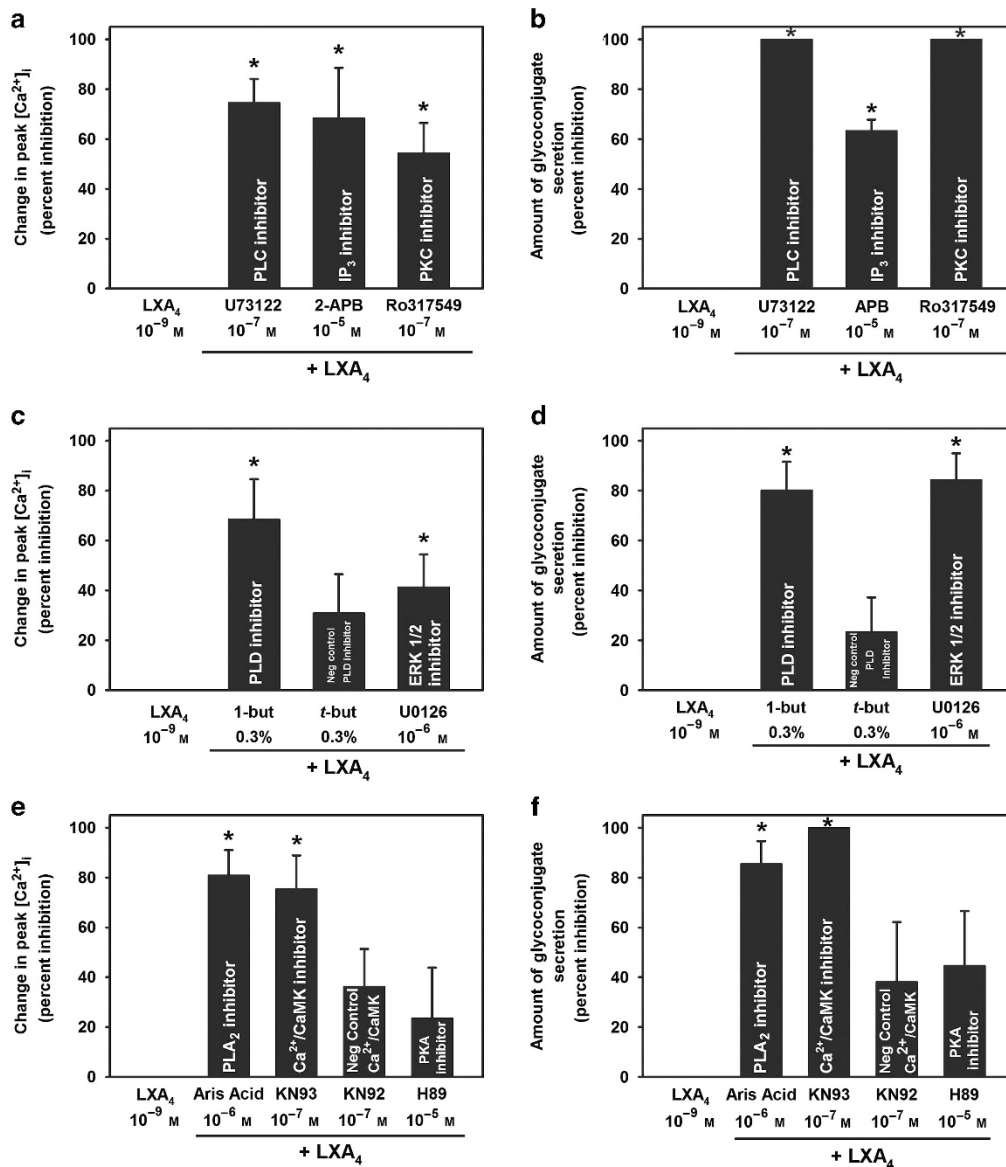


Figure 7 Signal transduction pathways used by lipoxin A₄ (LXA₄) to increase [Ca²⁺]_i and glycoconjugate secretion. Goblet cells were preincubated with inhibitors (**a, b**) U73122 (10⁻⁷ M), 2-aminoethyl diphenylborinate (APB; 10⁻⁹ M), and Ro317549 (10⁻⁷ M); (**c, d**) 1-butanol (1-but, 0.3%), *t*-butanol (*t*-but, 0.3%), and U0126 (10⁻⁶ M); (**e, f**) aristolochic acid (Aris Acid, 10⁻⁶ M), KN93 (10⁻⁷ M), KN92 (10⁻⁷ M), and H89 (10⁻⁵ M). The increase in [Ca²⁺]_i (**a, c, e**) and glycoconjugate secretion was measured (**b, d, f**). Mean ± s.e.m. of three to five independent experiments. *Significantly different from LXA₄ alone. Ca²⁺/CaMK, calcium/calmodulin-dependent protein kinase; ERK 1/2, extracellular regulated kinase 1/2; IP₃, inositol trisphosphate; Neg, negative; PKA, protein kinase A; PKC, protein kinase C; PLA₂, phospholipase A₂; PLC, phospholipase C; PLD, phospholipase D.

blocked Cch-stimulated [Ca²⁺]_i response by 69.2 ± 17.2% ($P = 8 \times 10^{-6}$, data not shown).

In addition to generation of IP₃, activation of PLC also leads to activation of PKC through the release of diacylglycerol (DAG). When goblet cells were preincubated with PKC inhibitor Ro317649 (10⁻⁷ M), LXA₄-stimulated increase in [Ca²⁺]_i was significantly blocked by 54.4 ± 12.1% ($P = 0.02$). Glycoconjugate secretion was completely blocked by Ro317649 (**Figure 7a,b**).

To investigate the phospholipase D (PLD) pathway, goblet cells were incubated with the PLD inhibitor 1-butanol (0.3%) and its negative control *t*-butanol (0.3%). 1-Butanol

significantly reduced LXA₄-stimulated increase in [Ca²⁺]_i by 68.5 ± 16.1% ($P = 0.01$), whereas *t*-butanol had no effect (**Figure 7c**). The same inhibitors were used before measurement of mucin secretion. Inhibition of PLD with 1-butanol significantly blocked LXA₄-stimulated secretion by 80.2 ± 11.4% ($P = 0.0004$), whereas *t*-butanol 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-Butanol, but not *t*-butanol, completely blocked Cch-stimulated glycoconjugate secretion (data not shown).

As LXA₄ activates ERK 1/2 (**Figure 5**), we determined whether this activation was downstream of the increase in [Ca²⁺]_i and whether 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). LXA₄-stimulated increase in [Ca²⁺]_i was significantly inhibited by 41.3 ± 13.1% (*P* = 0.03; **Figure 7c**). Mucin secretion was also significantly inhibited by 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 [Ca²⁺]_i and secretion stimulated by Cch were both significantly inhibited by U0126 (data not shown).

An increase in [Ca²⁺]_i can activate cytosolic phospholipase A₂ (PLA₂) through binding of Ca²⁺. To determine whether PLA₂ is activated by LXA₄, goblet cells were incubated with the PLA₂ inhibitor aristolochic acid (10⁻⁶ M). Aristolochic acid significantly blocked LXA₄-stimulated increase in [Ca²⁺]_i by 80.8 ± 10.2% (*P* = 1 × 10⁻⁵) and glycoconjugate secretion by 85.4 ± 9.2% (*P* = 1 × 10⁻⁶; **Figure 7e,f**).

The involvement of Ca²⁺/CaMK in LXA₄-stimulated increase in [Ca²⁺]_i was investigated as Ca²⁺/CaMK is also a major calcium binding protein.²⁷ The Ca²⁺/CaMK inhibitor KN93 (10⁻⁷ M) significantly inhibited LXA₄ stimulated increase in [Ca²⁺]_i by 75.4 ± 13.5% (*P* = 7 × 10⁻⁴) and completely blocked glycoconjugate secretion (**Figure 7e,f**). The inactive analog KN92 had no effect on LXA₄-stimulated [Ca²⁺]_i or secretion.

To determine whether LXA₄ increases cyclic adenosine monophosphate levels, goblet cells were incubated with the protein kinase A (PKA) inhibitor H89 (10⁻⁵ M). H89 did not alter either LXA₄-stimulated increase in [Ca²⁺]_i or glycoconjugate secretion (**Figure 7e,f**). However, in the same experiments, vasoactive intestinal peptide (which is known to activate PKA to generate cyclic adenosine monophosphate) stimulated increase in [Ca²⁺]_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 [Ca²⁺]_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 IP₃, and activate ERK 1/2, PKC, and Ca²⁺/CaMK, but not PKA, to increase [Ca²⁺]_i and glycoconjugate secretion in goblet cells.

DISCUSSION

In the present study, we showed that in goblet cells activation of ALX/FPR2 by LXA₄ induces a number of signaling components involved in cellular pathways leading to an increase in [Ca²⁺]_i and mucin secretion. These signaling components include PLD, PLC, and PLA₂ and a number of downstream molecules, ERK 1/2, PKC, and Ca²⁺/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 LXA₄.^{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 that include PLA₂, PLD, ERK 1/2, c-Jun N-terminal kinase, and p38.²⁹

The signaling pathways activated by LXA₄ are cell specific. In human monocytes, LXA₄ stimulates a robust increase in [Ca²⁺]_i, whereas LXA₄ stimulates a small increase in [Ca²⁺]_i in polymorphonuclear neutrophils³⁰⁻³² and activates PLD in a PKC-dependent manner.³³ However, neither the increase in [Ca²⁺]_i nor PLD activation have been linked to a physiological process³¹⁻³³ (**Figure 8b**). LXA₄ also causes lipid remodeling and a release of arachidonic acid in polymorphonuclear neutrophils.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, LXA₄ elicits a robust increase in [Ca²⁺]_i in a concentration-dependent manner. The increase in [Ca²⁺]_i in goblet cells is directly linked to glycoconjugate secretion as chelation of Ca²⁺ abolishes LXA₄-stimulated secretion.

One pathway activated in conjunctival goblet cells is the PLC pathway. Activation of this pathway induced release of [Ca²⁺]_i by activating the IP₃ receptor and PKC. The increase in [Ca²⁺]_i subsequently activated Ca²⁺/CaMK. As PLD and PLA₂ also increased [Ca²⁺]_i, these enzymes could have also activated Ca²⁺/CaMK. In support of this suggestion is our finding that inhibition of Ca²⁺/CaMK completely blocked LXA₄-stimulated increase in [Ca²⁺]_i and secretion. In contrast to Ca²⁺/CaMK, blockage of the IP₃ receptor, although it significantly inhibited secretion, is not as effective a blockade of secretion as is the Ca²⁺/CaMK inhibitor. This finding is consistent with only PLC, not PLD or PLA₂, activating the IP₃ receptor.

Inhibition of PKC or ERK 1/2 caused a different effect than inhibition of Ca²⁺/CaMK. Inhibition of PKC or ERK 1/2 did not completely suppress LXA₄-induced increase in [Ca²⁺]_i, but completely impaired LXA₄ stimulation of secretion. These results suggest that PKC and ERK 1/2, when activated, use both Ca²⁺-dependent and Ca²⁺-independent pathways to induce secretion. PKC consists of a family of multiple isoforms that are divided into three types: (i) DAG- and Ca²⁺-dependent isoforms, (ii) DAG-dependent but Ca²⁺-independent isoforms, and (iii) DAG- and Ca²⁺-independent isoforms.³⁴ ERK 1/2 can also be activated in a Ca²⁺-independent manner.³⁵⁻³⁷ Our findings suggest that LXA₄ activates multiple isoforms of PKC with at least one being a Ca²⁺-dependent isoform and one Ca²⁺-independent isoform. These data also indicate that ERK 1/2 activation by LXA₄ occurs via both Ca²⁺-dependent and Ca²⁺-independent mechanisms.

LXA₄ in our studies does not activate PKA to increase [Ca²⁺]_i nor to stimulate secretion. In conjunctival goblet cells, vasoactive intestinal peptide uses cyclic adenosine monophosphate and PKA to stimulate mucin secretion and does so in a Ca²⁺-dependent manner.³⁸ This is consistent with activation of Ca²⁺-dependent isoforms of adenylyl cyclase. In spite of the fact that LXA₄ in the present study increases [Ca²⁺]_i, this agonist appears not to be coupled to G_{αs} and adenylyl cyclase.

Interestingly, LXA₄ has an effect on goblet cells alone in the absence of any proinflammatory molecules as these

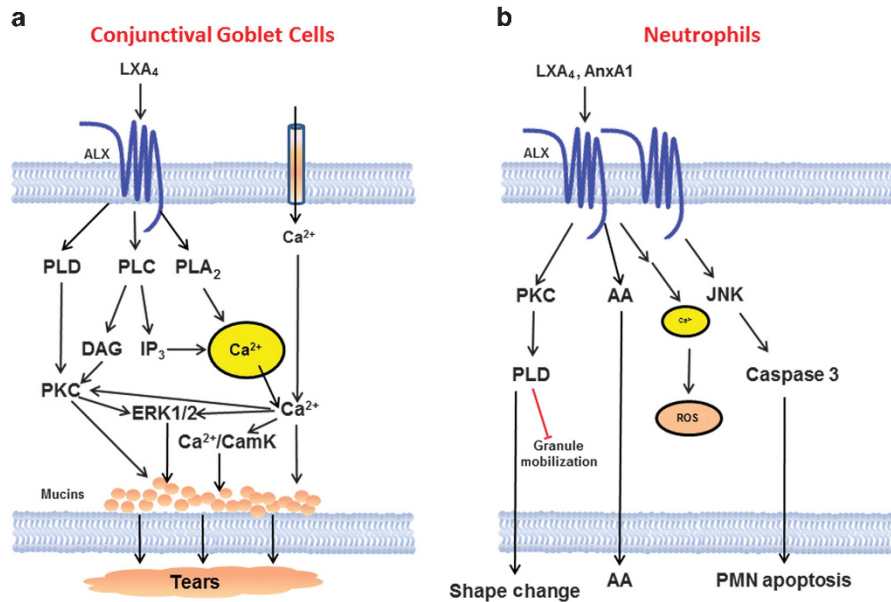


Figure 8 Schematic diagram of signaling pathways of ALX/FPR2 receptor in goblet cells and neutrophils. **(a)** Pathways in goblet cells are shown. lipoxin A₄ (LXA₄), resolvin D1 (RvD1), or annexin A1 (AnxA1) bind to ALX/FPR2 to activate phospholipases D, C, and A₂ (PLD, PLC, and PLA₂). PLD activates protein kinase C (PKC) whereas PLC causes the generation of diacylglycerol (DAG) and inositol trisphosphate (IP₃). DAG activates PKC whereas IP₃ causes the release of [Ca²⁺]_i from intracellular stores. Extracellular regulated kinase 1/2 (ERK 1/2) is also activated. PLA₂ causes the release of Ca²⁺. Intracellular Ca²⁺ activates calcium/calmodulin-dependent protein kinase (Ca²⁺/CamK). All pathways lead to mucin secretion. **(b)** Pathways in neutrophils are shown. LXA₄ 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 Ca²⁺ is also released leading to an increase in reactive oxygen species (ROS). Arachidonic acid (AA) is secreted. LXA₄ also activates c-Jun N-terminal kinase (JNK) that in turn activates caspase 3, leading to polymorphonuclear neutrophil (PMN) apoptosis.

experiments in this study were performed *in vitro*. Similar results were obtained with RvD1 and AT-RvD1.⁷ These resolvins increased [Ca²⁺]_i, glycoconjugate secretion, and short-term activation of ERK 1/2 (<30 min) but blocked histamine-stimulated actions⁷ over longer times. We hypothesize that this activation by SPMs including LXA₄ is vital to maintaining the normal homeostasis of the ocular surface. This would imply that regulation of SPMs biosynthesis with goblet cells must be tightly controlled.

The role of LXA₄ in the goblet cells is very complicated. Under normal, physiological conditions, endogenous LXA₄ 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, LXA₄ likely plays an anti-inflammatory function to reduce mucin secretion. Similarly, LXA₄ is endogenously expressed in the cornea without inflammation present and the amount of LXA₄ was increased after corneal wounding.¹² Exogenous addition of LXA₄ promoted wound healing¹² and increased goblet cell number and mucin area in a desiccating stress model of dry eye.³⁹ It is not known whether the conjunctiva produces LXA₄ but it is possible that LXA₄ produced by the cornea could diffuse via tears to activate goblet cells.

Activation of ALX/FPR2 in goblet cells with either LXA₄ or RvD1 blocked subsequent addition of either of these SPMs. This could be because of the fact that the receptor has desensitized. Maderna *et al.*⁴⁰ have shown that ALX/FPR2 is internalized after treatment with LXA₄, although the maximum

internalization did not occur until 30 min after the addition. It is also possible that LXA₄ and RvD1 bind to the same or overlapping binding sites in the receptor. LXA₄ was shown to bind to the extracellular loop III and the related transmembrane area.⁴¹ The location to which RvD1 binds to ALX/FPR2 has not yet been determined.

In goblet cells, addition of AnxA1 or LXA₄ blocks the increase in [Ca²⁺]_i stimulated by a second addition of either agonist. This would be consistent with AnxA1 and LXA₄ binding to the same or overlapping sites. Interestingly, although an initial addition of RvD1 blocked the increase in [Ca²⁺]_i to a subsequent addition of AnxA1, the peak [Ca²⁺]_i stimulated by RvD1 after addition of AnxA1 was significantly increased compared with the response obtained when RvD1 was added first. Binding of ligands to ALX is necessarily complicated as the receptor can bind proteins, peptides, and lipids²⁸ and can form homo- and heterodimers.¹³

LXA₄ 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 with LXA₄, ATL, and LTB₄. All analogs behaved similarly to LXA₄ in that intracellular [Ca²⁺]_i was mobilized to similar extents. In addition, as all compounds blocked a second addition of LXA₄, it is likely that all bind to ALX, similar to LXA₄.

Several SPMs including LXA₄, RvD1, and RvE1 stimulate conjunctival goblet cell secretion. These three SPMs are biosynthesized by three different pathways. LXA₄ is derived from arachidonic acid, RvD1 from docosahexaenoic 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 LXA₄ and other SPMs to maintain the homeostasis of the ocular surface. As LXA₄ directly affects goblet cells, lipoxins and other SPMs could be a novel treatment for dry eye diseases.

METHODS

Synthetic LXA₄ 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 before 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 CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 10 mM HEPES, and 5.5 mM glucose (pH 7.45)) to the desired concentrations and added to the cells. The LXA₄ 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. AnxA1 was purchased from MyBiosource (San Diego, CA). Aristolochic acid, 2-APB, 1-butanol and *t*-butanol were from Sigma-Aldrich (St Louis, MO), and U0126 was purchased from R&D Systems (Minneapolis, MN). H89 and R0-31317549 were obtained from EMD Millipore.

Animals. Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing between 125 and 150 g were anesthetized with CO₂ for 1 min, decapitated, and the bulbar and forniceal conjunctival epithelia removed from both eyes. All experiments were in accordance with the US 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.^{7,8,14,42-44} 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 UEA-1 (detects goblet cell secretory product) to ensure that goblet cells predominated.

Reverse transcriptase-PCR. Cultured goblet cells were homogenized in TRIzol (Sigma-Aldrich, St Louis, MO) and total RNA isolated according to manufacturer's instructions. Total RNA at $1\ \mu\text{g}$ was used for cDNA synthesis using the Superscript First-Strand Synthesis system for reverse transcriptase PCR (Invitrogen, Carlsbad, CA). The cDNA was amplified by the PCR using primers specific to ALX/FPR2 receptor using the Jumpstart REDTaq Readymix Reaction Mix (Sigma-Aldrich) in a thermal cycler (Master Cycler, Eppendorf, Hauppauge, NY). The primers were from previously published sequences.⁹ For rat goblet cells, the forward primer sequence was 5'-ATGGAAGCCAACTATTCCATC-3' and the reverse primer sequence was 5'-TCATATTGCTTTTATATCAATGTT-3'. These primers generated 1,053 bp fragments. The conditions were as follows: 5 min at 95°C followed by 35 cycles of 1 min at 94°C , 30 s at annealing temperature for 1 min at 72°C , with a final hold at 72°C 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 ($10^{-5}\ \text{M}$) 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). The homogenate was centrifuged at $2,000\ g$ for 30 min at 4°C . Proteins were separated by SDS-polyacrylamide gel electrophoresis and processed for western blotting. The antibody against the ALX/FPR2 receptor (Novus Biologics, Littleton, CO) was diluted 1:1,000. Immunoreactive bands were visualized by the enhanced chemiluminescence method. The films were analyzed with ImageJ software (<http://rsbweb.nih.gov/ij/>).

Immunofluorescence microscopy. First-passage cells grown on glass coverslips and dissected rat conjunctiva were fixed in 4% formaldehyde diluted in phosphate-buffered saline (145 mM NaCl, 7.3 mM Na₂HPO₄, and 2.7 mM NaH₂PO₄ (pH 7.2)) for 4 h at 4°C . Conjunctival tissue was preserved in 30% sucrose in phosphate-buffered saline at 4°C overnight and embedded in optimal cutting temperature embedding compound. Then, $6\ \mu\text{m}$ sections were cut, placed on slides, and air dried for 2 h. The sections or coverslips were rinsed for 5 min in phosphate-buffered saline, and nonspecific sites were blocked by incubation with 1% bovine serum albumin, and 0.2% Triton X-100 in phosphate-buffered saline for 45 min at room temperature. ALX/FPR2 receptor antibody (Novus Biologics) was used at 1:100 dilution overnight at 4°C . Secondary antibodies were conjugated to Cy3 (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, 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 LXA₄, histamine, or the cholinergic agonist Cch in serum-free RPMI-1640 supplemented with 0.5% bovine serum albumin for 2 h. Goblet cell secretion was measured using an enzyme-linked lectin assay 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.⁸ Glycoconjugate secretion was expressed as fold increase over basal that was set to 1.

Measurement of $[\text{Ca}^{2+}]_i$. Goblet cells were incubated for 1 h at 37°C with KRB-HEPES plus 0.5% bovine serum albumin containing $0.5\ \mu\text{M}$ fura-2/AM (Invitrogen, Grand Island, NY), $8\ \mu\text{M}$ pluronic acid F127, and $250\ \mu\text{M}$ 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 $[\text{Ca}^{2+}]_i$ with time or as the change in peak $[\text{Ca}^{2+}]_i$. Change in peak $[\text{Ca}^{2+}]_i$ was calculated by subtracting the average of the basal value (no added agonist) from the peak $[\text{Ca}^{2+}]_i$. Although data are not shown, the plateau $[\text{Ca}^{2+}]_i$ was affected similarly to the peak $[\text{Ca}^{2+}]_i$.

Knockdown of ALX/FPR2 receptor. Goblet cells were incubated with $100\ \mu\text{M}$ siRNA against a scrambled sequence or ALX/FPR2 receptor for

72 h (Thermo Scientific, Waltham, MA) in Accell delivery media according to the manufacturer's instructions (Thermo Scientific). The siRNA was a pool of four molecules whose sequences were: (i) 5'-CCAUCAGGUUCGUUAUUGG-3', (ii) 5'-CCUGCAGACAUUGAGAUAA-3', (iii) 5'-GUUAAUACUCGUUACGGA-3', and (iv) 5'-GUACAAACACUUGUGAAA-3'.

Statistical analysis. Results were expressed as the fold increase above basal. Results are presented as mean \pm s.e.m. Data were analyzed by one-way analysis of variance followed by Tukey's *post hoc* test or Student's *t*-test. $P < 0.05$ was considered statistically significant.

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DISCLOSURE

C.N.S. is an inventor on patents (resolvins) assigned to Brigham and Women's Hospital and licensed to Resolvix Pharmaceuticals. He was a scientific founder of Resolvix Pharmaceuticals and owns equity in the company. His interests were reviewed and are managed by the Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policies.

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