The Pro-resolving Mediators Maresin 1, Maresin 2 and Annexin A1 in Maintenance of Ocular Surface Health

Doctor of Philosophy (Ph.D.) thesis by
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2022
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Nature is the source of all true knowledge. She has her own logic, her own laws, she has no effect without cause nor invention without necessity.

Leonardo Da Vinci (1452 – 1519)
1 Foreword and Acknowledgments

This thesis is dedicated to our lab manager Robin R. Hodges, you will be deeply missed.

Financial support

The Ph.D. project was supported by the Norwegian Research Council and Department of Medical Biochemistry, Oslo University Hospital to Markus V. Olsen, the National Institute of Health R01 EY019470 to Darlene A. Dartt and R01 GM038765 to Charles N. Serhan.

Execution of the project

All experiments were performed at Vecellio Dry Eye Laboratory, Schepens Eye Research Institute/ Mass Eye and Ear and Department of Ophthalmology, Harvard Medical School, Harvard University, Boston, MA, USA.

Personal Acknowledgments

I want to thank my supervisor Darlene A. Dartt. Being a part of a lab with an experienced professor like Professor Dartt has been of significant importance throughout the project. In addition of having plenty of knowledge, Professor Dartt is an extremely creative and inspiring person. Most importantly, Professor Dartt is a warm-hearted person, contributing to a great lab environment. I will bring everything I have learnt from Professor Dartt with me in the future.

Due to an interest in ophthalmology and eye physiology in medical School I was lucky to meet my supervisor Tor P. Utheim. Professor Utheim presented me a research project for the medical school research program “Forskerlinjen”. After a troubling start, with a lot of trying and failing, Tor had an ability to motivate me to continue the hard work. Without his positivity and support this project would not have been possible to complete. In addition to his motivating character, Professor Utheim
is intelligent, creative, supportive, and has an admirable work ethic. Professor Utheim has been a huge inspiration, both academically and personally, and I really appreciate our friendship.

Anne Viktoria Lyngstadaas is both my wife and my research partner. Due to a common interest in research we decided to apply for research projects at Forskerlinjen together. We helped each other throughout the projects, finally leading to two Ph.D.-theses. I am very lucky to have her, first of all as a wife, but also as a partner in research. Our cooperation has been essential for our projects, and I appreciate that we are able to both have a professional and a non-professional relationship.

Robin R. Hodges, our beloved lab manager, sadly passed away in March 2021. Robin was a wonderful human being, who always spread positivity. Robin always had a solution when there were any problems at the lab. With both her theoretical and practical expertise, her work and support have been crucial during my time at the lab. She is irreplaceable, both as a lab manager and as a person. My heart goes out to Robin’s family.

I will thank my other lab co-workers Jeffrey Bair, Marie Shatos, Dayu Li and Laura Garcia-Posadas for all your help during my time at the Dart Lab. I want to thank Dr. Charles N. Serhan for valuable discussions about mediators and intracellular pathways. I also want to thank Kaisa Filtvedt for making wonderful illustrations.

Finally, I want to thank my family, my mother, father and sister who have been supportive in all aspects of life. My father has always helped me with schoolwork, in addition to being an assistant coach in our football team. My mother is really warm hearted, and helps me keep on working, always without any pressure. I will also thank my “new family”, Petter, Anita and Ole Nikolai for always being there.
2 Abbreviations

1-but – 1-butanol
2APB – 2-aminoethyl diphenylborinate
AA – Aristolochic acid
AnxA1 – Annexin A1
ARDS – Acute respiratory distress syndrome
BAPTA/AM – 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester)
BOC2 – N-BOC-Phe-Leu-Phe-Leu-Phe
BSA – Bovine serum album
Ca²⁺/CaMKII – Ca²⁺/calmodulin-dependent protein kinase II
[Ca²⁺]i – Intracellular calcium concentration
CALT – Conjunctiva-associated lymphoid tissue
CSF – cerebrospinal fluid
Cch – Carbachol
CK7 – Cytokeratin 7
COPI – Coat protein I
DAG – Diacylglycerol
DHA – Docosahexaenoic acid
EGFR – Epidermal growth factor receptor
ELLA – Enzyme linked lectin assay
EPA – Eicosapentaenoic acid
EPAC – Exchange proteins directly activated by cyclic AMP
ERK 1/2 – Extracellular-regulated kinase 1/2
Fura 2/AM – Fura 2 acetoxymethyl ester
GDP – Guanosine diphosphate
GPCR – G-protein coupled receptor
GTP – Guanosine triphosphate
IP₃ – Inositol 1,4,5-trisphosphate
LGR6 – Leucin-rich repeat containing G protein-coupled receptor 6
LOX – Lipoxygenase
LTB₄ – Leukotriene B₄
LXA₄ – Lipoxin A₄
МΦ – Macrophage
MaR1 – Maresin 1
MaR2 – Maresin 2
MAPK – Mitogen-activated protein kinase
MUC5AC – Mucin 5AC
PKC – Protein kinase C
PGE₄ – Prostaglandin E₄
PIP₂ – Phosphatidylinositol 4,5-bisphosphate
PLC – Phospholipase C
PMN – Polymorphonuclear neutrophil
PUFA – Polyunsaturated fatty acids
RVD1 – Resolvin D1
RVD2 – Resolvin D2
RVE1 – Resolvin E1
SERCA – Sarco-/endoplasmic reticulum Ca²⁺-ATPase
SOC-3 – Suppressor of cytokine signaling 3
SPM – Specialized pro-resolving mediator
t-but – tertiary-butanol
TRP – Transient receptor potential
TRPV1 – Transient receptor potential vanilloid 1
List of papers

**Paper 1:**
**Markus V. Olsen**, Anne V. Lyngstadaas, Jeffrey A. Bair, Robin R. Hodges, Tor P. Utheim, Charles N. Serhan, Darlene A. Dartt.

Maresin 1, a specialized proresolving mediator, stimulates intracellular $[\text{Ca}^{2+}]_{i}$ and secretion in conjunctival goblet cells. *Journal of Cellular Physiology*. 2021 Jan;236(1):340-353.

**Paper 2:**
**Markus V. Olsen**, Anne V. Lyngstadaas, Jeffrey A. Bair, Robin R. Hodges, Tor P. Utheim, Charles N. Serhan, Darlene A. Dartt. Comparison of Signaling Pathways Used by the Specialized Pro-Resolving Mediators Maresin 1 and Maresin 2 to Regulate Goblet Cell Function. Submitted March 2022.

**Paper 3:**
**Anne V. Lyngstadaas**, Markus V. Olsen, Jeffrey A. Bair, Robin R. Hodges, Tor P. Utheim, Charles N. Serhan, Darlene A. Dartt.

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5 Background

5.1 Inflammatory eye disease and specialized pro-resolving mediators

Inflammatory eye diseases, like allergic conjunctivitis, are prevalent diseases affecting up to 40% of the North American population, causing reduced quality of life and productivity (1). The disease is a type 1 IgE-mediated hypersensitivity reaction and typically leads to signs and symptoms like itching, redness, burning, eyelid edema, conjunctival edema (chemosis) and watery discharge (alterations in the tear film) (2).

Treatment options, in addition to basic eye care, include antihistamines, alpha adrenergic vasoconstrictors, mast cell stabilizers and glucocorticoids in various combinations (3). The treatment options are limited, in many cases ineffective and associated with side effects (4). For instance, glucocorticoids may increase intraocular pressure resulting in glaucoma (5).

To develop new and effective treatment alternatives for inflammatory eye diseases a better understanding of cellular physiology of the ocular surface and resolution of inflammation is required.

In the late stages of inflammation, leukocytes switch from generating pro-inflammatory chemical mediators to pro-resolving mediators, including maresins, resolvins, and protectins (6-8). The specialized pro-resolving mediators (SPMs) Maresin 1 (MaR1) and Maresin 2 (MaR2) are derived from the ω-3 fatty acid docosahexaenoic acid (DHA) (9, 10). DHA has been detected in the tear film of humans diagnosed with chronic inflammatory eye disease, indicating that SPMs may play a role in the immunological response in inflammatory ocular disease (11).

Conjunctival goblet cells have an important role in maintaining a balanced secretion of the high molecular weight glycoproteins including mucins. Inflammatory diseases such as Sjögren’s syndrome, allergic conjunctivitis and viral- and bacterial-induced conjunctivitis are associated with alterations in mucin secretion from conjunctival goblet cells, contributing to symptoms of disease (12). Goblet cell activation is
primarily caused by an increase in intracellular calcium concentration ([Ca^{2+}]_i) leading to mucin secretion. Our group previously showed that the specialized pro-resolving mediators (SPMs) Lipoxin (Lx) A_4, Resolvin (RV) D1, RVD2 and RVE1, play a role in Ca^{2+}-signaling and high molecular weight glycoprotein secretion from cultured rat conjunctival goblet cells (13-16).

The pro-resolving mediator, Annexin A1 (AnxA1), is a protein released from leukocytes that mediates a portion of the anti-inflammatory effect of glucocorticoids (17). As mentioned earlier, glucocorticoids are associated with serious side effects and can only be used for a limited time period (3). By utilizing the anti-inflammatory effect of glucocorticoids through Annexin A1, fewer side effects occur, but an effective treatment of inflammatory disease remains.

The present thesis describes the effect of MaR1, MaR2 and AnxA1 on rat conjunctival goblet cell function including an effect on [Ca^{2+}]_i and high molecular weight glycoprotein secretion and identification of which receptors and intracellular pathways are utilized by the SPMs. This work will hopefully contribute to a better understanding of resolution of inflammation and how conjunctival goblet cells are modulated by SPMs; possibly having potential as a future treatment of inflammatory disease, especially ocular allergy.

5.2 The ocular surface

5.2.1 Anatomy of the ocular surface

The ocular surface system consists of the cornea, conjunctiva, lacrimal gland, accessory lacrimal glands, Meibomian glands, glands of Moll and Zeis, and the nasolacrimal duct (18) (Fig. 1). The apical most layer of the cornea and conjunctiva are stratified, non-keratinized epithelia that both function to protect against the external environment. The corneal and conjunctival epithelia are continuous and interface with the epithelia of the nasolacrimal duct, the lacrimal gland ducts, and the
Meibomian gland ducts (18). The organization of the epithelium is complex and varies due to differential exposure to the external environment (19).

The apical surface of the conjunctiva is directly exposed to potential threats from the environment and is covered by a glycocalyx containing transmembrane mucins called MUC1, MUC4 and MUC16. The mucin rich environment provides protection against infection, and the O-glycans in the mucins prevent apical surface adhesion (20). MUC1 and MUC16 interacts with a carbohydrate associated protein called galectin-3, in order to enhance the integrity of the epithelial barrier (21, 22). The basolateral epithelium face underlying and neighboring cells and promotes a barrier function due to inter-cellular junctions (19).

Figure 1: An illustration of the components of the ocular surface. The conjunctiva has a palpebral- and a bulbar part, with an intermediate fornix. Illustrated by Kaisa Filtvedt.
5.2.2 The cornea – Avascular and extensively innervated

The cornea is transparent and avascular, with an overlaying tear film which provides a refractive surface essential for the optical function of the eye, as well as protection against threats from the external environment (23). The cornea consists of cellular components including epithelial cells, keratocytes and endothelial cells, as well as the acellular components consisting of collagen and glycosaminoglycans (24). The cornea consists of five layers; 1) The innermost layer covering the aqueous humour, the endothelium which is a single layer of cells that maintains clarity by removing H₂O from the stroma, 2) Apically to the endothelium, Descemet’s membrane that is an extracellular matrix consisting of different types of IV collagen and laminin, 3) Next is the corneal stroma consisting of keratocytes and specifically layered collagen fibrils that maintain transparency and have mechanical and refractive functions, 4) Next is Bowman’s layer that is an acellular extracellular matrix consisting of collagen fibrils in random directions, contributing to the shape of the cornea, and 5) Apical most is the epithelium that consists of seven layers of epithelia cells and which is a tight barrier to chemicals, microbes and other types of external hazards (24-26). The cornea is one of the most densely innervated and sensitive tissues in the body containing predominantly sensory and limited sympathetic nerves. The sensory nerves are protective to the eye as they react to any stimulus that could be a sign of potential danger for this transparent, anterior part of the eye (23).

5.2.3 The conjunctiva - Conjunctival goblet cells and mucins

The conjunctiva surrounds the cornea and lines the eyelids. It is divided into a superior and inferior tarsal (palpebral) part that line the lids, a superior and inferior bulbar part that surround the cornea, and an intermediate fornix the connect the bulbar and tarsal areas (27). Furthermore, the conjunctiva is composed of two layers, the stratified, non-keratinized epithelium and the underlying stroma. There are three types of functional cells in the conjunctival epithelium; the goblet cells, stratified squamous cells, and undifferentiated cells (12). The epithelium is well innervated with parasympathetic and sympathetic nerves, but not as well innervated as the cornea by
afferent sensory nerves (28). In contrast to the cornea, the conjunctiva is richly vascularized, with blood vessels both in the stroma and epithelium (28). In the underlying loose connective tissue there is a mucosal immune system called conjunctiva-associated lymphoid tissue (CALT) (29). The conjunctiva is much more permeable than the cornea.

The conjunctival goblet cells are either interspersed among stratified squamous epithelial cells or clustered (30). The goblet cells produce and secrete mucins, including MUC5AC (31). The gel-forming MUC5AC forms oligomeric structures by disulfide-bond binding between monomers. The mucin is stored in cytoplasmic vesicles within conjunctival goblet cells prior to secretion (32). When stimulated by a secretory signal the goblet cells release secretory granules with the whole content of the secretory vesicle (27). The mucins are an important component of the tear film, which will be described in the next section.

5.2.4 The tear film

The tear film covers the ocular surface (cornea and conjunctiva). A balanced and consistent composition of tears is essential for protection against external stress, both in health and disease. There is controversy over how many layers are in the tear film, two or three (Fig. 2). In the three-layer model, the innermost layer of the tear film consists of secreted mucins, as MUC5AC, and other proteins produced by the conjunctival goblet cells, as well as electrolytes and water secreted by the goblet cells and stratified squamous cells. The functionality of the innermost layer is dependent on the amount of mucin produced and secreted, in addition to the quality (12).

The middle aqueous layer consists of proteins, electrolytes and water produced by the lacrimal glands and by a lesser extent the conjunctiva. Neural stimuli initiate lacrimal gland secretion resulting in fluid essential for both transparency and nutrition. The fluid contains proteins, some of them antibacterial, preventing infectious diseases (33).
The outer lipid layer is produced by the Meibomian glands (27). The lipid layer functions to prevent evaporation of liquid from the tear film and contribute to stabilization by lowering surface tension (34).

In the two-layer model, there is a gradient of aqueous and mucous in one layer and a superficial lipid layer produced by the Meibomian glands. The mucin concentration decreases towards the surface (35).

The tear film with its exceedingly complex composition is accordingly essential for refractive function of the eye by contribution of transparency, as well as protection against threats and danger from the external environment due to a mechanical barrier and chemical quality (23).

**Figure 2:**
The three-layer model of the tear film consisting of the mucin layer (secreted by the conjunctival goblet cells and partly by stratified squamous epithelial cells), the aqueous layer and the lipid layer protecting the ocular surface. Illustrated by Kaisa Filtvedt.
5.2.5 Ca$^{2+}$ and mucin secretion

The intracellular [Ca$^{2+}$], compared to bound intracellular Ca, is an important intracellular signaling messenger in various cellular processes. The cells maintain a resting level of [Ca$^{2+}$] at approximately 100 nM to survive and function properly. There are multiple mechanisms for stabilizing the level of [Ca$^{2+}$]. Sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase (SERCA) is responsible for pumping Ca$^{2+}$ into the ER, while plasma-membrane Ca$^{2+}$-ATPase (PMCA) and Na$^+$/Ca$^{2+}$ exchanger (NCX) remove Ca$^{2+}$ from the cell. Furthermore, the mitochondrial uniporter transports Ca$^{2+}$ at a wide dynamic range (36).

The Ca$^{2+}$-signaling systems are complex, but have some similar characteristics. They mainly function by generating a brief pulse of intracellular Ca$^{2+}$ initiating cellular activity. The increase in cytoplasmic [Ca$^{2+}$] is derived from intracellular calcium stores, primarily in the endoplasmic reticulum (ER) or by influx of extracellular Ca$^{2+}$ (36). An increase in intracellular Ca$^{2+}$ ([Ca$^{2+}$]$\text{i}$) stimulates mucin secretion in goblet cells, a mechanism confirmed in several species (37, 38). When an agonist binds to its specific receptor, intracellular signaling pathways are activated. The signaling cascade causes an increase in [Ca$^{2+}$], leading to fusion of the cytoplasmic vesicle containing mucins with the apical plasma membrane and releasing all of the granules from a given cell. One of the main pathways generating intracellular Ca$^{2+}$ is the phospholipase C (PLC) pathway, which is important in cellular processes as exocytosis, muscle contraction, proliferation and ion channel opening (39). When an agonist binds to a G-protein coupled receptor (GPCR), PLC hydrolys phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to generate diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP$_3$). Cytosolic IP$_3$ binds to IP$_3$-receptors that are cation channels located on the ER and release Ca$^{2+}$ from the ER causing a rapid increase in ([Ca$^{2+}$]$\text{i}$) (39).

Store-operated Ca$^{2+}$ release activated Ca$^{2+}$ (CRAC) channels are involved in regulation of [Ca$^{2+}$]$\text{i}$. When intracellular Ca$^{2+}$ stores (ER) are depleted, STIM1, which is located along the microtubules in the ER, senses a drop in the Ca$^{2+}$ level inside of the ER. The reduction in Ca$^{2+}$ level leads to STIM1 oligomerization and translocation,
which cause activation of ORAI1, a Ca\(^{2+}\) specific ion channel in the plasma membrane that causes Ca\(^{2+}\) influx into the cell (40). To bring back [Ca\(^{2+}\)] to homeostasis by refilling the intracellular Ca\(^{2+}\) stores, SERCA pumps Ca\(^{2+}\) back into ER. This process is known as the capacitative calcium entry.

### 5.3 Inflammation

Inflammation is a non-specific, beneficial host response to tissue injury or infection. The purpose of inflammation is bringing the tissue back to a normal structural and functional state known as homeostasis (41). Within the host, the immune system is a central actor in both the triggering and avoidance of inflammation. The immune system is divided into the innate and adaptive system. The innate immune system consists of the natural defending barriers in the organism, both physically and chemically, as well as neutrophils, monocytes, macrophages, complement, cytokines and acute phase proteins. The adaptive immune system consists of T- and B-lymphocytes acting in an antigen-specific reaction (42).

Furthermore, inflammation can be divided to acute and chronic inflammation. Acute inflammation typically lasts for minutes to days, while chronic inflammation may last from days to years (43). Infectious agents, trauma, tissue necrosis, foreign bodies and hypersensitivity reactions are typical triggers of inflammation (43). Triggers of acute inflammation cause secretion of inflammatory mediators, such as histamine, prostaglandins, leukotrienes and interleukins, leading to vasodilation, increased vasopermeability, leukocyte migration, chemotaxis and potential tissue damage. These events are related to the cardinal signs of inflammation: calor (fever), rubor (vasodilation or redness), tumor (swelling from increased vasopermeability and cell infiltration), dolor (pain), and loss of function (from release of chemical substances and tissue damage) (44).

Leukocytes are recruited from the blood to the site that triggered inflammation by rolling, adhesion, transmigration and migration towards the chemotactic stimulus (43). In the first phase of acute inflammation, polymorphonuclear neutrophils (PMNs)
arrive to the local site of inflammation. After 2-3 days, monocytes appear and differentiate to macrophages (45). PMNs and macrophages arise from common precursors and are both phagocytes and express similar antigens (46). The leukocytes cooperate in action, however, possess distinct abilities. Neutrophils regulate the level of chemoattractants, stimulating recruitment of macrophages. Furthermore, neutrophils enhance transmigration of monocytes by altering vascular permeability (46). While neutrophils are stored in the bone marrow and released rapidly during acute inflammation, macrophages arise from monocytes produced in the bone marrow and circulating in the blood vessels (47).

Macrophages are activated in two phenotypes, termed “M1” or “M2”. “M1”-macrophages are activated by the classical pathway, induced by IFN-γ and foreign substances, stimulating inflammation by increased cytokine secretion and termination of ingested organisms (43). Interestingly, neutrophils are able to induce M1 phenotype increasing pro-inflammatory activity (46). The alternative pathway activates “M2”-macrophages induces by cytokines as IL-4 and IL-13 stimulating tissue repair (43).

If the acute inflammatory response is successful the cause of tissue injury is eliminated and the pro-inflammatory phase is followed by a phase of resolution (48). For centuries, it was thought that resolution of inflammation was a passive process, caused by dilution of pro-inflammatory stimulators. However, resolution of inflammation is now known to be an active process, initiated by chemical mediators and cellular programs during acute inflammation (49). A more detailed explanation of this process is described in section “5.5 – Resolution of inflammation – Pro-resolving mediators”.

If the inflammatory response fails to resolve the acute inflammation, a state of chronic inflammation develops, associated with tissue destruction and fibrosis. A continual activation of the adaptive immune system is thought to be one of the drivers of chronic inflammation. Acute and chronic inflammation may coexist, as in diseases as asthma, rheumatoid arthritis, ulcerative colitis and chronic obstructive pulmonary disease (50). Being a central pathological mechanism in a numerous of diseases, for
instance the diseases mentioned above, inflammation is one of the most common causes of disease burden worldwide.

5.4 Ocular surface inflammation – role of the tear film and its mucin in ocular allergy, an ocular surface inflammatory disease

For the tear film to function properly the secretion of mucin has to be tightly regulated, as both mucin deficiency and over secretion are associated with ocular surface disease (28). Inflammation is one of the underlying disease mechanisms in multiple diseases on the ocular surface, for instance in allergic conjunctivitis, a disease associated with an increased mucin secretion (28, 51). In predisposed individuals, the mast cells display IgE antibodies specific for antigens on specific allergens. Antigen-antibody binding is followed by degranulation of the mast cells. The degranulation causes release of mediators like histamine which plays a key role in allergic inflammatory disease (51). An increase in vascular permeability causes infiltration of mast cells, neutrophils, eosinophils and T-cells. Production and secretion of cytokines amplifies and prolongs the allergic response (51).

The increase in mucin secretion during allergic conjunctivitis contributes to a destabilization of the tear film, causing reduced protection against external danger. In addition, a non-homeostatic tear film contributes to the symptoms of the disease. In other words, inflammation affects mucin secretion, and mucin secretion needs to function optimally to avoid and protect against inflammation.

5.5 Resolution of inflammation – Pro-resolving mediators

As previously mentioned, PMNs play a key role in the initial process of acute inflammation (45). Inflammatory mediators such as leukotrienes and prostaglandins are released from leukocytes, including PMNs, enhancing the inflammatory response. Leukotriene B₄ amplifies chemotaxis of PMNs, while Prostaglandin E₂
(PGE$_2$) induces acute inflammation and contributes to the symptoms of the inflammatory disease (52, 53).

Emerging findings suggest that resolution of inflammation is an active process, and the pro-inflammatory cells, like PMNs and pro-inflammatory mediators, including prostaglandins, play a key role in initiating resolution of inflammation (49, 54). For instance, the pro-inflammatory mediator PGE$_2$ amplifies the synthesis of the pro-resolving mediator lipoxin (LX)A$_4$ during acute inflammation (49, 54). The process of switching pro-inflammatory lipid mediators, like leukotrienes and prostaglandins, into pro-resolving lipid mediators is called “lipid mediator class switching” (54).

Serhan and colleagues have over the last decades systematically identified novel lipid mediators called specialized pro-resolving mediators (SPMs) (7, 55). The SPMs are a family of locally acting resolution mediators, further subdivided in groups based on the biosynthesis, structure, and origin. These mediators have been conserved in living organisms, thus they are suggested as evolutionally conserved molecules essential for host protection (56). The SPMs are biochemically derived from polyunsaturated fatty acids (PUFA) and include the lipoxins, resolvins, protectins and maresins (8, 57).

In mammals, the precursors of the SPMs, such as EPA and DHA, are rapidly present at the local site of inflammation, diffusing directly from the circulation to the tissue (58). As an illustration to demonstrate their actions, the SPMs activate immune cells to clear apoptotic cells (59, 60) and limit and regulate PMN invasion (9, 60, 61), thereby contributing to resolution of inflammation. In other words, the SPMs have various functions and actions, but generally lead to resolution of inflammation and restoration of tissue homeostasis. The resolution mediators, the SPMs, are derived from $\omega$-6 and $\omega$-3 fatty acids. Oxygenation of arachidonic acid and $\omega$-6 fatty acids contributes to both pro-inflammatory and pro-resolving mediators, whereas $\omega$-3 fatty acids mainly lead to formation of anti-inflammatory and pro-resolving mediators (62). Lipoxins are biosynthesized from the $\omega$-6 fatty acid arachidonic acid. The E-series resolvins (63) are derived from the $\omega$-3 fatty acid eicosapentaenoic acid (EPA), while the D-series resolvins (64), protectins (65) and maresins (9) are derived from the $\omega$-3 fatty acid docosahexaenoic acid (DHA).
To clarify, there is a distinction between anti-inflammatory and pro-resolving actions. Anti-inflammatory mediators block pro-inflammatory mediators and reduce the cardinal signs of inflammation. The anti-inflammatory effect can both prolong and reduce the time it takes to achieve tissue homeostasis. Some of these anti-inflammatory mediators work in a resolution-toxic manner, meaning that they increase extend tissue inflammation and prolong the resolution phase. In order to be called a pro-resolving, the mediator must contribute to the restoration of tissue homeostasis (55). By enhancing uptake of cellular debris and apoptotic cells by macrophages and limit neutrophil recruitment and microbial invasion to the local inflammation, the SPMs contribute to homeostasis (66).

One of the major advantages of SPMs is their ability to resolve inflammation without suppressing the immune system, thereby helping the host to defeat the underlying disease of inflammation. Illustratively, the SPM RVD2 contributes to an increased survival in microbial sepsis in mice without immunosuppression (67).

The most common way for a mediator to function is by binding as a ligand to a receptor causing activation of a cascade of intracellular signaling pathways. The SPMs mainly activate specific GPCRs. So far six different GPCRs for SPMs have been identified (68, 69). Because these receptors are detected on distinctive cell types, binding of ligands to these receptors can cause tissue selectivity (68).

5.6 Maresin 1

In 2009, Serhan and collaborators detected a novel group of lipid mediators and labeled them maresins (9). The name “Maresin” is based on the origin and actions of the mediator; macrophage mediator (Ma) in resolving (res) inflammation (in) (9). By using unbiased, targeted mediator lipidomics in mice with peritonitis, Serhan et al found that Maresin 1 originated from the ω-3 fatty acid DHA in macrophages (MΦs). 12-lipoxygenase (LOX) converts DHA to 14-hydroperoxydocosahexaenoic acid. Further series of reactions, including steps of reduction, dioxygenation and
hydrolyzation generate 14-dihydroxydocosa-4Z, 8, 10, 12, 16Z, 19Z-hexaenoic acid, Maresin 1 (MaR1) (9). Interestingly, an isomer of MaR1 was identified, 7S,14S-diHDNA, with a lower potency, indicating stereoselective actions (9). The total stereochemistry of MaR1 was detected by Serhan and colleagues in 2012 by using an organic synthesis and matching approach. The complete stereochemistry of MaR1 is 7R, 14S-dihydroxydocosa-4Z,8E,10E,12Z,16Z,19Z-hexaenoic acid (70).

The key coupling reactions in the synthesis of MaR1 are chiral titanium complex-promoted enantioselective alkyne addition to the aldehyde, Julia-Kocienski olefination and BF3-mediated alkyne attack on the epoxide (71). Similar to the SPMs RvE1 and Protectin D1, MaR1 contains 1,8-dihydroxy groups and a conjugated triene, suggesting a bioactive importance of these parts of the molecule (71).

An intermediate in the synthesis of MaR1 called 13S,14S-epoxydocosa-4Z,7Z,9E,11E,16Z,19Z-hexaenoic acid (13S,14S-epoxy-DHA) was found to inhibit formation of LTB₄, in addition to reducing expression of CD54 and CD80 expression in M1 macrophages and to upregulate CD163 and CD206 in M2 macrophages that would contribute to resolving inflammation (72). Furthermore, incubation of 13S,14S-DHA resulted in a higher level of MaR1 in M2 macrophages compared with M1 macrophages consistent with a pro-resolution effect of MaR1.

MaR1 blocks both PMN infiltration and stimulates macrophage phagocytosis thus contributing to resolution of inflammation (9). In addition, MaR1 has tissue regenerative and anti-nociceptive actions in capsaicin induced pain (70).

In a newly published study (69), MaR1 was shown to be an activator of human leucin-rich repeat containing G protein-coupled receptor 6 (LGR6) expressed on phagocytes. LGR6 is well known to play a role in stem cell and tissue regeneration. Chiang et al showed that MaR1-LGR6 interaction in mice and human phagocytes contribute to resolution (69).
Figure 3: The chemical structure of Maresin 1. The molecule has one carboxyl group in the end, two hydroxyl groups at position 7 and 14 and double bonds at 4, 8, 10, 12, 16 and 19. The carbon chain of the fatty acid is 22 carbons long, thereby a docosahexaenoic acid. The complete stereochemistry is 7R, 14S-dihydroxydocosa-4Z,8E,10E,12Z,16Z,19Z-hexaenoic acid. E/Z is a stereo description defining the attached groups or atoms on each side of a double bond. If the selected groups are on the same side, the capital “Z” is used, when the selected groups are on the opposite side, the capital “E” is used (73). S/R is a stereo description which describes if the attached group in a counterclockwise (“S”=Sinister) or a clockwise direction (“R”=Rectus) (74).

5.7 Maresin 2

In addition to MaR1, there are other similar end products in the biosynthesis of DHA with pro-resolving actions. In 2014, Deng et al discovered Maresin2 (MaR2) (10). By an enzymatic epoxidation by 12-LOX that produces 13S, 14S-epoxy-maresin and a series of other steps that result in the product 13R,14S-dihydroxy-4Z,7Z,9E,11E,16Z,19Z-hexaenoic acid, MaR2 (10). Recently, the total stereoselective synthesis of MaR2 was identified as (7Z,9E,11E,13R,14S,16Z,19Z)-13,14-dihydroxydocosa-7,9,11,16,19-pentaenoic acid (75). The central intermediate 13S,14S-epoxy-maresin inhibits conversion of AA by 12-LOX, without altering the
production of end products of DHA, indicating a feed-forward mechanism in maresin synthesis (10, 76). MaR2 inhibits PMN infiltration in mouse peritonitis and enhanced phagocytosis of apoptotic PMNs by macrophages, thus MaR2 is a pro-resolving mediator by definition (10).

**Figure 4:** The chemical structure of Maresin 2. MaR2 has two hydroxyl groups in positions 13 and 14, and double bonds in position 4, 7, 9, 11, 16 and 19. The total stereoselectivity of MaR2 is (7Z,9E,11E,13R,14S,16Z,19Z)-13,14-dihydroxydocosa-7,9,11,16,19-pentaenoic acid (75). S=sinister. R=rectus. E=An isomer with the atoms on the opposite side. Z=An isomer with the atoms on the same side.

5.8 Annexin A1

In addition to pro-resolving mediators derived from ω-3 fatty and ω-6 fatty acids, leukocytes release a 37 kDa protein called AnxA1, previously known as lipocortin 1 (17). AnxA1 consists of a highly conserved core domain extending from the C-terminal including four repeated sequences with 70 amino acids, and a N-terminal region with specific biological actions (77). AnxA1 is a potent inhibitor of phospholipase A₂, an enzyme essential for eicosanoid biosynthesis, thereby limiting inflammatory activity (78). By injecting recombinant protein of AnxA1 an inhibition of PMN migration is initiated, an action similar to that of steroids like dexamethasone (79). To confirm the importance of AnxA1 in inflammatory disease, an exacerbation
of the chronic inflammatory disease adjuvant-induced arthritis occurred when anti-annexin antibodies neutralized the intracellular Annexin A1 (80).

The relationship between AnxA1 and glucocorticoids has been well-known for decades. Goulding et al were the first to show that administration of hydrocortisone increases the amount of AnxA1 in human organisms (81). AnxA1 was mainly detected in PMNs and monocytes (81). AnxA1 inhibits leukocyte activation and trafficking by working through specific amino acids in the protein structure (82). In other words, the protein contains distinct functional peptides; Ac2-26, Ac9-25 and Ac2-12. Anx1 predominantly works by Ac2-26 or Ac9-25 binding to the formyl peptide receptor to modulate neutrophil function (83). AnxA1 contains a membrane-binding domain – the annexin core – that has a shape of a curved disc. Studies showed a substantially increased effect of AnxA1 when the protein is in a correctly refolded state (79). Ca\(^{2+}\) has multiple functions in the action of Annexins. The Ca\(^{2+}\) forms the membrane contact with AnxA1 by influencing its chemical groups of carboxyl, carbonyl and phosphoryl, as well as expose the buried N-terminal domain using conformational switching.

5.9 Receptors and intracellular pathways

There are three main classes of cell surface receptors;

1) G-protein-coupled receptors (GPCRs) consist of a polypeptide chain that crosses the plasma membrane lipid layer seven times. The receptors activate guanosine triphosphate (GTP)-binding proteins (G proteins) which activate/inactivate enzymes or ion channels in the plasma membrane, activating an intracellular signaling pathways (84). There are approximately 800 GPCRs. Examples of the receptor type are the extensively studied \(\beta_2\)-adrenergic receptor and the opioid receptor (85).

2) Ion-channel coupled receptors which change the permeability through the plasma membrane to ions, altering the membrane potential (84). The ionototropic glutamate receptors AMPA and NMDA are examples of ion-channel coupled receptors (86).
3) Enzyme-coupled receptors working as an enzyme or associated with enzymes inside the cells causing activation of intracellular signaling pathways (84). Examples of enzyme-coupled receptors are the receptor tyrosine kinases including Epidermal growth factor receptor (EGFR) and the platelet derived growth factor receptor (PDGF) (87).

The specialized pro-resolving mediators and the mediators with pro-resolving actions mainly act as ligands to GPCRs (13-16, 88-90). About 1/3 of all drugs work by binding to a GPCR and the ligand to the receptor can be lipids, fatty acids, amino acids, peptides and proteins (84).

The GPCRs initiate their signaling cascade through heterotrimeric G proteins, moreover the receptors may activate G-protein-independent pathways by G-protein-coupled receptor kinase-mediated (GRK) phosphorylation and arrestin coupling (91). The G-proteins consist of three subunits, α, β, and γ (α subunit and βγ-complex). The α subunit has a guanosine diphosphate (GDP) bound in inactive state. When the receptor is activated the GDP dissociates and is exchanged by GTP. The α subunit of the G-protein is one of three variants, Gs, Gi and Gq, each activating/inactivating different intracellular signaling pathways. Gs stimulates adenylyl cyclase (AC), Gi inhibits AC and Gq activates phospholipase C (PLC). The βγ-complex may bind to ion channels altering their permeability (84).

When Gs activates AC, cyclic AMP is produced from ATP. cAMP is a messenger that is mainly known to activate the enzyme cyclic-AMP-dependent protein kinase (PKA). When Gi is activated, an inhibition of AC occurs. When Gq is activated, PLC cleaves an inositol phospholipid attached to the internal part of the plasma membrane. The cleavages cause activation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 binds to the IP3-receptor located on the surface of endoplasmic reticulum (ER) causing a release of Ca2+ to the cytosol (39). DAG activates protein kinase C (PKC) (84).
6 Aims

6.1 Hypothesis:

The pro-resolving mediators MaR1, MaR2 and AnxA1 activate rat conjunctival goblet cells by increasing the intracellular Ca\(^{2+}\) concentration and stimulating mucin secretion.

6.2 Primary goal:

The primary goal is to gain knowledge about the physiology of MaR1, MaR2 and AnxA1 in cultured rat conjunctival goblet cells by 1) identifying the receptors utilized, 2) mapping the essential intracellular signaling pathways activated and 3) studying the effect on mucin secretion.

6.3 Secondary goal:

The secondary goal is to develop new treatments for inflammatory diseases, such as allergic conjunctivitis.

7 Materials and methods

7.1 Materials

7.1.1 MaR1, MaR2 and AnAX1

Maresin 1 (14-dihydroxydocosa-4Z, 8, 10, 12, 16Z, 19Z-hexaenoic acid, MaR1) and Maresin 2 (13R,14S-dihydroxy-4Z,7Z,9E,11E,16Z,19Z-hexaenoic acid, MaR2) were purchased from Cayman Chemical (Ann Arbor, MI, USA), dissolved in an ethanol solution as supplied by the manufacturer. The solutions were stored at -80°C with
minimal exposure to light. Annexin A1 was obtained from MyBiosource (San Diego, CA, USA), stored at -20°C with minimal exposure to light. The solutions were diluted immediately before use in 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 7.40-7.45)) to the desired concentrations (10⁻⁸ M) and stored on ice during the experiments. Daily working stock dilutions were discarded following each experiment.

7.1.2 Inhibitors, positive controls and negative controls

VIP, KN92, KN93 U73122 and U73343 were purchased from Tocris Bioscience (Ellisville, MO, USA). Histamine, Carbachol (Cch), 2-aminoethyl diphenylborinate (2APB), Aristolochic acid (AA), RO317549, 1-butanol (1-but) and tertiary-butanol (t-but) were all obtained from Sigma-Aldrich (St. Louis, MO, USA). N-BOC-Phe-Leu-Phe-Leu-Phe (BOC2) was ordered from GenScript (Piscataway, NJ, USA). BLT-1 (U-75302) and LTB₄ were ordered from Cayman Chemical (Ann Arbor, MI, USA). BAPTA/AM was purchased from Life Technologies (Grand Island, Ny, USA). Lipoxin A₄, H89, Thapsigargin and RO-317549 were ordered from EMD Millipore (Billerica, MA, USA). UO126 was obtained from R&D Systems (Minneapolis, MN, USA).

7.2 Animals

4-6 week old male albino Sprague-Dawley rats (Taconic Farms, Germantown, NY, USA), weighing between 125-150 g were anesthetized with CO₂ for 5 min. As a secondary confirmation of death, the rats were decapitated with a guillotine. Immediately after decapitation, the bulbar and fornical conjunctival epithelia were 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 of Committee.
7.3 Cell culture

Rat conjunctival goblet cells were cultured from male albino Sprague-Dawley rats. The technique of isolation, characterization and propagation of rat conjunctival goblet cells was developed by Shatos et al (92) and the technique has been used extensively since (13, 14, 16, 88, 93-100). The conjunctival tissue was cut and tissue plugs were placed in 6 well plates with 0.5 ml RPMI 1640 medium (from Lonza (Walkerville, IL, USA)) supplemented with 10% Fetal bovine serum ((FBS) from Atlanta Biologicals (Norcross, GA, USA)), 2mM L-glutamine (from Lonza) and 100 mcg/ml penicillin-streptomycin. The cells prepared for glycoconjugate secretion experiments were plated in 24 well plates. RPMI media was changed every second day, and 2 ml media was used in each well. The cells proliferated in culture for a maximum of 7-10 days before preparation for experiments. Goblet cells grew from the tissue plug in a circular pattern, as evenly spaced nodules. Fibroblasts that grew outside the circular structure were removed by mechanical scraping (92). Cultured goblet cells were identified periodically by staining with anti-cytokeratin 7 antibody, anti-MUC5AC antibody and the lectin UEA-1 directly conjugated to fluorophore, a procedure further described below. The cells were trypsinized and transferred to calcium dishes 24 hours before the experiments were performed.

7.4 Immunofluorescence Microscopy

First passage rat conjunctival goblet cells were grown on glass coverslips and fixed in 100% methanol for 30 minutes at room temperature. The coverslips were rinsed in 1 x phosphate-buffered saline (137 mM NaCl, 2.7 KCl, 10 mM Na₂HPO₄ and 1.8 mM KaH₂PO₄, 10 x PBS) (pH 7.2-7.4)). Nonspecific sites were blocked by incubation with 1% bovine serum albumin, and 0.2% Triton X-100 in 1 x PBS for 45 minutes at room temperature. An antibody to cytokeratin 7 was used at a dilution of 1:100 in order to identify the cytoskeleton of conjunctival goblet cells, and to identify goblet cell secretory product UEA-1 (Sigma-Aldrich) was used at a dilution of 1:300 and MUC5AC was used at a dilution of 1:100. 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, USA).
Figure 5: Immunofluorescence microscopy images at 40 x magnification of rat conjunctival goblet cell. Anti-cytokeratin 7 (CK7) antibody fluoresces green and detects the cytoskeleton in goblet cells. Directly labeled UEA1 fluoresces red and indicates secretory product. 4’,6-diamidino-2-phenylindole (DAPI) fluoresces blue and detects the cellular nucleus.

Figure 6: Immunofluorescence microscopy images at 40 x magnification of rat conjunctival goblet cell. MUC5AC is stained in red and detects secretory mucins. UEA1
is stained in green and also discovers secretory products. 4’,6-diamidino-2-phenylindole (DAPI) detects the cellular nucleus.

7.5 Measurement of changes in intracellular calcium concentration [Ca^{2+}].

Cultured rat conjunctival goblet cells were transferred after trypsinization to 35-mm glass bottom dishes and incubated in 37.0°C overnight. The cells were then incubated at 37.0°C for 1 hour with Krebs Ringer bicarbonate buffer with HEPES (KRB-HEPES) containing 0.5% BSA, 0.5 mM of fura2/AM (Life Technologies), 250 mM Sulfinpyrazone (Sigma-Aldrich) and 8 mM Pluronic acid F127 (Sigma-Aldrich).

[Ca^{2+}] was measured with a ratio imaging system (InCytIm2; intracellular imaging, Cincinnati, OH, USA) using wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. A minimum of 10 goblet cells were selected and the calcium response was followed for approximately 2 minutes. The agonist, the positive control or the mediator of interest (MaR1, MaR2 or AnXA1), was either added alone or treated with inhibitors before addition, and data were collected in real time. The inhibitors or inactive controls H89, BOC2, BAPTA/AM, Kn92, Kn93, AA, UO126, U75302, RO317549, 2APB, U73122, or U73343 were added 30 minutes prior to the agonist. 1-butanol, t-butanol and thapsigargin were added 15 minutes prior to the agonist. Changes in peak [Ca^{2+}] were calculated by subtracting the average basal [Ca^{2+}], from the peak [Ca^{2+}]. Responses from cells with a basal value above 500nM or any value above 2000 nM Ca^{2+} were not included in the analyses. The former value indicated a dysfunctional cell. The later value was above the highest value used to calibrate the fluorescent microscope.
Figure 7: Pseudocolor images showing changes in $[Ca^{2+}]_i$ in rat conjunctival goblet cells stimulated before at $t=0$ or after histamine $10^{-5}$ M at $t=30$, 50 and 80 s. Changes in $[Ca^{2+}]_i$ were detected using the ratio imaging system InCytIm2 (intracellular imaging, Cincinnati, OH, USA). Calibration bar is in upper left corner.

7.6 Measurement of high molecular weight glycoprotein secretion

Cultured rat conjunctival goblet cells were trypsinized and transferred to 24 well plates. The cells were serum starved in serum free RPMI 1640 media containing 0.5% bovine serum albumin (BSA) for 120 minutes. An agonist at a specific concentration was then added alone or the cells were incubated with an inhibitor for 30 minutes, and then stimulated with an agonist at a specific concentration for 2 hours for SPMs or 4 hours for carbachol or histamine. The amount of goblet cell mucin secretion was measured using the lectin UEA-1, that detects high molecular-weight glycoproteins in an enzyme linked lectin assay (ELLA). The media were collected and analyzed for lectin-detectable glycoproteins including MUC5AC. The UEA-1 was detected by Amplex Red (Invitrogen, Carlsbad, CA, USA). Glycoprotein secretion is shown as fold increase above basal (which was set to 1). The protocol used is described in detail by Dartt et al in a paper about the effect of leukotrienes and resolvins on conjunctival goblet cells.
7.7 Reverse transcriptase-PCR

Reverse transcriptase-PCR was performed in the third paper to investigate if AnXA1 was present in goblet cells. The procedure was described by Hodges et al in a paper investigating LXA4 and the ALX/FPR2-receptor (15). Cultured goblet cells were homogenized in TRIzol (Sigma-Aldrich) and total RNA isolated according to manufacturer's instructions. 1 µg of total RNA was used for complementary DNA (cDNA) synthesis using the Superscript First-Strand Synthesis system for Reverse transcriptase PCR (RT-PCR) (Invitrogen). The cDNA was amplified by the polymerase chain reaction (PCR) using primers specific to Annexin A1 using the Jumpstart REDTaq Readymix Reaction Mix (Sigma-Aldrich) in a thermal cycler (Master Cycler, Eppendorf, Hauppauge, NY, USA). The forward primer was GTGATCGCTGTGAGGATATGAG, and the reverse primer was TACAGAGCAGTTGGGATGTTTAG. These primers generated 504 BP fragments. The conditions were as follows: 5 min at 95°C followed by 35 cycles of 1 min at 94°C, 30 seconds 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.

7.8 Measurement of cAMP

Goblet cells seeded in 24-well plates were grown to 75% confluence. All cells were incubated with IBMX 10⁻³ M for 40 minutes total. MaR1 (10⁻⁸ M ) was added for 40 minutes. VIP (10⁻⁸ M) added for 5 min was the positive control. Cells were lysed in 0.1 M HCl. Total cell cAMP was assayed by direct cAMP ELISA kit following the manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY, USA). The acetylation protocol was used to increase sensitivity. Total cell protein was determined by Bradford assay, and cellular cAMP was normalized to total protein. cAMP levels are presented in real numbers.
7.9 Statistical Analysis

Data are expressed as mean ± SEM. Data were analyzed by either Student’s *t*-test or one-way ANOVA followed by Tukey test. *p* < 0.05 was considered significant.

8 Ethical considerations

All experiments were in accordance with the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and the National Institutes of Health guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). The animal protocols were approved by the Schepens Institutional Animal Care and Use of Committee (IACUC). Animal courses were taken and approval obtained before animal contact.

9 Summary of results

**Paper 1 - Maresin 1, a specialized pro-resolving mediator, stimulates intracellular [Ca$^{2+}$], and secretion in conjunctival goblet cells**

In paper 1, we determined the intracellular pathways used by MaR1 and the effect on mucin secretion. We also examined the effect of MaR1 on histamine stimulated cells. Rat conjunctival goblet cells were cultured and measurements of intracellular [Ca$^{2+}$] changes and enzyme linked lectin assay (ELLA) to measure glycoprotein secretion were performed. MaR1 stimulates mucin secretion by increasing the [Ca$^{2+}$] in rat conjunctival goblet cells. When inhibitors of phospholipase C, protein kinase C, Ca$^{2+}$/calmodulin-dependent protein kinase II and extracellular-regulated kinase 1/2 were added, MaR1-stimulated [Ca$^{2+}$] increase and secretion were blocked. An
inhibitor of PLD only blocked secretion. Histamine-stimulated increase in $[\text{Ca}^{2+}]_{i}$ and secretion were blocked by preincubation of MaR1 (89).

**Figure 8:** Schematic diagram of signaling pathways activated by Maresin 1 (MaR1). The diagram is from the paper “Maresin 1, a specialized pro-resolving mediator, stimulates intracellular $[\text{Ca}^{2+}]_{i}$ and secretion in conjunctival goblet cells” (89). The figure is used with the permission of Journal of Cellular Physiology.

**Paper 2 - Comparison of signaling pathways used by the specialized pro-resolving mediators Maresin 1 and Maresin 2 to regulate goblet cell function**

In paper 2, we examined the effect of MaR2 on rat conjunctival goblet cells and identified the intracellular pathways used to stimulate mucin secretion. We compared the effect of MaR2 to that of MaR1. Lastly, we established the effect of MaR2 on histamine-stimulation of goblet cell function. Rat conjunctival goblet cells were cultured and measurements of intracellular calcium changes by Fura2 and secretion by enzyme linked lectin assay (ELLA) were performed. MaR2 increases $[\text{Ca}^{2+}]_{i}$ and stimulates mucin secretion an in rat conjunctival goblet cells. In contrast to MaR1, MaR2-stimulated $[\text{Ca}^{2+}]_{i}$ increase was blocked by an inhibitor of cAMP-dependent
protein kinases known as protein kinase A (PKA), but not by PLC. Similar to MaR1, MaR2 increase in $[\text{Ca}^{2+}]_i$ and secretion were blocked by an inhibitor of PLD (101).

Table 1. The receptors and intracellular pathways used by MaR1 and MaR2 to increase $[\text{Ca}^{2+}]_i$.

<table>
<thead>
<tr>
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<th>Pathway/Process</th>
<th>Maresin 1</th>
<th>Maresin 2</th>
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<tbody>
<tr>
<td>U-73122</td>
<td>PLC activation</td>
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<tr>
<td>2-APB</td>
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<td>Thapsigargin</td>
<td>SERCA</td>
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<td>Removing CaCl$_2$</td>
<td>Extracellular Ca$^{2+}$</td>
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<tr>
<td>RO-317549</td>
<td>PKC activation</td>
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<td>+</td>
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<td>Ca$^{2+}$/CamKII</td>
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<td></td>
</tr>
<tr>
<td>1-Butanol</td>
<td>PLD activation</td>
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<tr>
<td>Aristolochic acid</td>
<td>PLA$_2$ activation</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>H89</td>
<td>cAMP-dep. PKA</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>U-75302</td>
<td>BLT-1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>BOC2</td>
<td>ALX/FPR2</td>
<td>+</td>
<td>-</td>
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<tr>
<td>UO126</td>
<td>ERK 1/2</td>
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+=dependent on and -=independent of.

Table 2. The receptors and intracellular pathways used by MaR1 and MaR2 to stimulate glycoprotein secretion.

<table>
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<th>Inhibitor</th>
<th>Pathway/Process</th>
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<th>Maresin 2</th>
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</thead>
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<td>BAPTA/AM</td>
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<td>RO-317549</td>
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<td>ERK 1/2</td>
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+=dependent on and -=independent of.
Paper 3 - The pro-resolving mediator Annexin A1 regulates MUC5AC secretion in cultured goblet cells suggesting a new use in inflammatory conjunctival diseases

In paper 3, the effect of the protein AnXA1 on rat conjunctival goblet cells was examined. The intracellular pathways used by AnXA1 and its effect on mucin secretion were explored. Rat conjunctival goblet cells were cultured and measurements of intracellular calcium changes by Fura2 and secretion by enzyme linked lectin assay (ELLA) were performed. AnXA1 stimulates mucin secretion and an increase in the [Ca\(^{2+}\)]\(_{i}\) in rat conjunctival goblet cells. AnXA1 works through the ALX/FPR2-receptor. AnXA1-stimulated [Ca\(^{2+}\)]\(_{i}\) increase was blocked by inhibitors of IP\(_3\), sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) and PKC (90). Inhibitors of PLD, ERK1/2, and PLA2 only blocked secretion.

![Figure 9: Schematic diagram of signaling pathways activated by Annexin A1 (AnXA1). From the paper “Pro-Resolving Mediator Annexin A1 Regulates Intracellular Ca\(^{2+}\) and Mucin Secretion in Cultured Goblet Cells Suggesting a New Use in Inflammatory Conjunctival Diseases” (90). The figure is used with the permission of Frontiers in Immunology.](image-url)
10 Discussion

With the discovery of mediators with pro-resolving characteristics biosynthesized from ω-3 fatty acids, the understanding of the pathophysiology of resolution of inflammation changed from being a passive process to an active, programmed process (102). In this thesis we have described the effect of the specialized pro-resolving lipids (SPMs) MaR1 and MaR2 and the pro-resolving protein AnxA1 on rat conjunctival goblet cells. By using Fura2 and a fluorescence microscopic ratio imaging system and ELLA on cultured rat conjunctival goblet cells, we found that all the SPMs used increased [Ca^{2+}]_{i} and stimulated glycoprotein secretion (89, 90, 101). Furthermore, we found that MaR1 and AnxA1 were dependent on the PLC pathway to increase [Ca^{2+}]_{i} and stimulate glycoprotein secretion (89, 90). Surprisingly, MaR2 was dependent on cAMP-dependent PKA to increase [Ca^{2+}]_{i} and stimulate secretion and not on the PLC pathway (101). AnxA1, was as presumed, a ligand of the ALX/FPR2 receptor in rat conjunctival goblet cells (90). MaR1 was found to use the LTB_{4} receptor BLT-1 and the ALX/PFR2 receptor to increase [Ca^{2+}]_{i}, but only the BLT-1 to stimulate glycoconjugate secretion. Furthermore, MaR2 only utilized the BLT-1 receptor to increase [Ca^{2+}]_{i} (101). Interestingly, we found that MaR1, MaR2 and AnxA1 blocked the function of the inflammatory mediator histamine (89, 90, 101). Collectively, our results support the conclusion that these SPMs may play a role both in health and disease through regulation of conjunctival goblet cell function, thus contributing to tissue homeostasis.

In the section below, the methods utilized, the receptors and intracellular pathways of AnxA1, MaR1 and MaR2, the function of the mediators in other organs and a comparison to other SPMs will be discussed.

10.1 Discussion of methods

10.1.1 Discussion of cell culture methods

Only male, albino Sprague-Dawley rats 4-6 weeks old were used in these studies to avoid age- and gender-related dissimilarities. The rats were anesthetized for 3-5
minutes and decapitated, then the inferior and superior tarsal parts of the conjunctiva were surgically removed from both eyes. The tissue was rinsed under a microscope and all potential fibroblasts were removed. We underwent a practice period of several months to master the technique of isolating the right cell type before we started with the experiments included in the studies. Furthermore, immunohistochemical staining with anti-CK7 and anti-MUC5AC antibodies was performed regularly to confirm culturing of the correct cell type, goblet cells. The RPMI medium was changed every two days and the same amount of medium was consistently put in each well. The protocol developed by Shatos et al. describing isolation and culturing of rat conjunctival goblet cells was strictly followed (92). In addition to following the protocol, we always tried to use the same instruments, incubators, rooms and microscopes during the procedure, to be as consistent as possible. We inspected the 6 well-plates for contamination regularly.

In our projects, we used an in vitro model, culture of primary cells (not cell lines or immortalized cells) to measure $[\text{Ca}^{2+}]_i$ and secretion. Our group has performed several studies with the in vitro method, validating the method (13-16, 88-90). A major advantage of the in vitro cell culture model compared to in vivo models is the possibility of examining goblet cells isolated from other cell types, for instance stratified squamous cells and fibroblasts (27). Fukagawa et al. found that corneal and conjunctival fibroblasts produce chemokines attracting eosinophils that play a role in allergic conjunctivitis (103). This finding confirms that other cell types may cause alterations in glycoprotein secretion. SPMs are important mediators in both health and disease. Thus, SPMs and SPM-precursors already present in the conjunctiva in living organisms may influence the response of the mediator which is studied. This may cause an advantage of in vitro models compared to in vivo models.

A problem faced working with primary cell lines is contamination with stromal cells, which causes a high number of failed experiments (104). In order to control that goblet cells were the main cell type in the conjunctival tissue after tissue rinsing, we performed immunohistochemistry regularly with markers of goblet cells (anti-CK7 and anti-MUC5AC). Another weakness of the method of cell culturing of primary cell lines is the risk of contamination. For instance, minor changes in room temperature increase the risk of the fungus mold. When contamination was suspected or
confirmed, the cells were not included in the experiments, and all instruments, incubators and rooms were rinsed.

In addition of being a practical advantage, we think that the in vitro model is more ethical than the in vivo model, knowing that the animal does not suffer during the experiments and more experiments can be performed per animal.

To measure the effect of SPMs on ocular surface disease an in vivo disease model applying topical SPM therapy is an applicatory option to the in vitro model performed in our studies. For instance, topical applied RVD1 decreased clinical symptoms, the level of MUC5AC in tears, the number of immune cells recruited to the ocular surface and modulated cytokines in the conjunctiva in an allergic eye disease mouse model (105). A major advantage of the in vivo model is that it is more convertible to normal physiology and pathophysiology compared to the in vitro model. In living organisms multiple cell types and mediators work together to function properly, both in health and disease. Most likely, SPMs influence different cell types to perform their actions.

However, a problem the in vivo model may face is the risk of alterations in the secretory process due to other cell types than goblet cells. For instance, sensory stimulation of the cornea and conjunctiva induce goblet cell secretion by a reflex arc and parasympathetic and sympathetic nerves directly stimulate glycoprotein secretion (106). Another problem of the in vivo model is the risk of pain and suffering of the animals involved in the experiments. The number of animals used in research should be reduced and the animals involved should not suffer. This subject is more thoroughly described below in the section “ethical consideration”.

Another potential way to stimulate goblet cells is by intravenous stimulation in vivo. In a study by Norris et al, a cluster of SPMs in human plasma and serum were detected after ω-3 supplementation combined with a low dose intravenous LPS challenge causing an inflammatory reaction (107). RvE1, RvD1, LXB₄, 18-HEPE, 17-HDHA and AT-LXA₄ were detected in human plasma and serum (107). The conjunctiva is richly vascularized which increases the probability that the SPM-precursors and SPMs migrate to the ocular surface cells during inflammation. An in vivo model combining ocular surface inflammation, for instance allergic keratoconjunctivitis, with ω-3
supplementation prior to measuring the level of SPMs and SPM-precursors would be interesting. However, in this model it is not possible to rule out effects of other mediators that migrate through the circulation during an inflammation. For instance, ω-3 supplementation with a high proportion of EPA and DHA increased the effect of topical tacrolimus therapy in dogs (108).

Another method exploring conjunctival goblet cells is cultured immortalized cell lines. A major advantage of immortalized cell lines is that the cells can be grown indefinitely. Another advantage of this method is the reduction of animals utilized in research. There are several disadvantages, such as change in phenotype when immortality is established and epigenetic changes that affect the cell line during cellular differentiation (109). In addition, to avoid genetic changes and contamination and to maintain exponential growth, high authenticated and characterized cells must be used and transferred properly from flask to flask with fresh nutrient (110). Unfortunately, there are no conjunctival goblet cell lines.

10.1.2 Ethical consideration

The experiments were performed in accordance with the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and the National Institutes of Health guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). The animal protocols were approved by the Schepens Institutional Animal Care and Use of Committee (IACUC). The “3R principle” of animal research was introduced by Russell and Burch in 1959 and has ever since been a valuable tool in the discussion of animal research ethics (111).

1) Replacement: According to the “3R principle”, we should consider replacing rat conjunctiva with other alternatives. A possible solution could be to rat conjunctiva with human conjunctiva from donors. Our institute was not offered human conjunctiva from donors as regularly as rats, human cells take longer to grow, and there are age, gender, disease, and medication differences between human donors. Thus, the studies would be difficult to perform.
2) Reduction: As few animals as possible were included in our experiments. By mastering the technique of cell culturing, we could use one rat to perform several different experiments. Moreover, when the control was significantly different from the control + inhibitor, our experiments were terminated.

3) Refinement: We performed animal courses focusing on animal handling and early signs of distress/suffering to make sure that the rats were treated correctly. The rats were never put in cages alone, to reduce distress and increase social contact with other rats. The professional employees at the animal facility made sure that the rats had all their essential needs and reduced all factors that could cause distress. To sacrifice the rats as humanely as possible the rats were anesthetized with CO₂, one of the most used and recommended methods for euthanasia of animals in research.

10.1.3 Discussion of ratio imaging - calcium

Measurements of intracellular calcium ([Ca^{2+}]_i) using the ratio imaging system InCytIm 2 was the basis of the studies included in this thesis. Tsien et al. developed the highly fluorescent indicator Fura-2, an indicator of [Ca^{2+}] that is based on the structure of EGTA. This family of dyes has been widely used since its discovery and Tsien was awarded the Noble Prize in Chemistry in 2008 for his discovery (112). Fura-2 acetoxyethyl ester (AM) is a non-Ca^{2+} sensitive but a lipid soluble compound, that makes it possible for the compound to pass through cell membranes (99). Once inside non-specific esterases cleave Fura-2 AM to Fura-2. A major advantage with use of these fluorescent dyes compared to other invasive experimental methods is avoidance of disruption of the plasma membrane (113). Compared to previously used fluorescent dyes, one of the major advantages of Fura 2 is increased brightness and allowing it to bind less Ca^{2+} for a strong signal. Thus Fura-2 does not itself decrease the [Ca^{2+}] as an earlier dye Quin-2 did. Other important advantages of Fura 2 are its ability to bind more selectively to Ca^{2+} (and less to Mg^{2+}) and that the excitation wavelengths of Fura 2 depend on the [Ca^{2+}]_i. Another advantage is the total dye concentration is less significant compared with other compounds (112). When [Ca^{2+}]_i is elevated the 340-nm excitation wavelength increases, while the 380-nm wavelength decreases (113). The excitation wavelength
of 340-nm is directly proportional and the excitation wavelength of 380-nm is inversely proportional of the free [Ca$^{2+}$] (114). The shifting of excitation absorbance of the fluorescent depending on the binding to Ca$^{2+}$, comparing one specific wavelength to another wavelength originating from the same volume (ratio imaging), makes the measurement obtained independent of intracellular dye concentration and of the number of cells being recorded from (114).

To be able to respond to altered levels of [Ca$^{2+}$], the Fura 2/AM must be completely de-esterified to its free acid. If not, the partially de-esterified compound is still highly fluorescent but insensitive to Ca$^{2+}$, which can lead to problems during experiments. Another potential problem with Fura 2 is accumulation of the compound in intracellular organelles like endoplasmic reticulum, mitochondria and secretory granules, especially during longer loading times (113). Loading is improved using the amphiphilic agent Pluronic F-127 (115, 116). To avoid efflux of Fura 2 from the cell by anion transporters, the uric acid transport inhibitor sulfipyrazone is used (117). We used both Pluronic F-127 and sulfipyrazone in all our experiments.

Another potential weakness of the method is dependency on user. To reduce the influence of this factor, we underwent several months of practice using mediators with known effects on rat conjunctival goblet cells, for example histamine (95). Furthermore, all the [Ca$^{2+}$] experiments with the same mediator or inhibitor were performed by the same person, to avoid inter-scientist measurement variability.

10.1.4 Discussion of Enzyme-linked Lectin Assay – glycoprotein secretion

Enzyme-linked lectin assay (ELLA) was performed using the lectin UEA-1. UEA-1 detects rat conjunctival goblet cell secretory proteins containing the carbohydrate moiety $\alpha$(1,2)-linked fucose that includes the mucin MUC5AC. The ELLA was performed according to the Pierce Protocol. The same protocol and procedure have been thoroughly described in previous studies performed at Dartt Laboratory at the Schepens Eye Research Institute (13-16, 89, 94, 99, 100). In addition, the ELLA method has been shown to have a high sensitivity and does not require hazardous reagents (118). In vivo, mucin production is regulated by goblet cell proliferation, mucin synthesis and mucin secretion. By performing ELLA on cultured rat
conjunctival goblet cells (in vitro), we were able to measure isolated mucin secretion (100). To avoid inter-scientist measurement variability, all ELLA procedures were performed by the same scientist.

10.1.5 Reverse transcriptase-PCR

The development of polymerase chain reaction (PCR) late in the last century has revolutionized research, medicine and forensic medicine (119). Being a widely used method in research makes it highly validated. Some of the advantages of the method is that it is simple to use and to understand, as well as being low-priced and highly sensitive (120). Limitations of the method is that a known sequence is needed to design primers to detect the DNA sequence of interest and the primer may bind non-specifically to a similar sequence (120). Another limitation is that PCR is only able to detect proteins/peptides and not lipids, which would have been valuable to detect the lipid mediators. In order to analyze lipids a more expensive and advanced method of lipidomics by tandem mass spectroscopy (LC-MS-MS) is required.

10.1.6 Discussion of statistical analysis

A minimum of three (3-8) rats were utilized in our experiments. For each condition two experiments were performed, and for each condition approximately 20 rat conjunctival goblet cells were included. Controls with known effect in rat conjunctival goblet cells were involved in all experiments. 120-320 individual cell responses for each condition were measured and the analyses were conducted. Each individual value was treated as random, independent variable. Thus, the data was analyzed with a Student’s t-test. To exclude dead cells and unusual cell responses for the [Ca^{2+}] analysis all cells starting above a value of 500 nM and all cells above 2000 nM during the experiment were removed from the analyses.

10.2 Discussion of results
Table 3. The receptors and intracellular pathways used by MaR1, MaR2 and AnXA1 to increase \([\text{Ca}^{2+}]\).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Pathway/Process</th>
<th>Maresin 1</th>
<th>Maresin 2</th>
<th>Annexin A1</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAPTA/AM</td>
<td>([\text{Ca}^{2+}])</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>U-73122</td>
<td>PLC activation</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2-APB</td>
<td>IP3 receptor</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>SERCA</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Removing CaCl2</td>
<td>Extracellular Ca(^{2+})</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td>RO-317549</td>
<td>PKC activation</td>
<td>+</td>
<td>+</td>
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<tr>
<td>KN92</td>
<td>Ca(^{2+})/CamKII</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>1-Butanol</td>
<td>PLD activation</td>
<td>-</td>
<td>+</td>
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<tr>
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<td>PLA2 activation</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>H89</td>
<td>cAMP-dep. PKA</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>U-75302</td>
<td>BLT-1</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>BOC2</td>
<td>ALX/FPR2</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>UO126</td>
<td>ERK 1/2</td>
<td>+</td>
<td>-</td>
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</tr>
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</table>

+=dependent on and -=independent of.

Table 4. The receptors and intracellular pathways used by MaR1, MaR2 and AnXA1 to stimulate glycoprotein secretion.

<table>
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<th>Inhibitor</th>
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10.2.1 Receptors
We found that MaR1 uses both the BLT-1 receptor and the ALX/FPR2 receptor to increase \([\text{Ca}^{2+}]\), but only the BLT-1 receptor to stimulate glycoprotein secretion in rat conjunctival goblet cells (101).

Recently, multiple SPMs, including MaR1, have been detected in human peripheral blood and lymphoid organs (121). In a study by Chiurchiù et al RVD1, RVD2 and MaR1 were each shown to both modulate and prevent the generation of \(T_H1\) and \(T_H17\) cells, which play a central role in chronic inflammation (122). The GPR32 receptor and the ALX/FPR2 receptor were detected on the T cells, and preincubation with specific neutralizing antibodies directed toward these receptors suppressed the action of the specialized pro-resolving mediators on these cells (122). This might indicate that MaR1 plays a central role in resolution of chronic inflammation in human cells by binding to the ALX/FPR2 receptor. In addition, this finding suggests that AnxA1, which is also known to bind to ALX/FPR2 and to show pro-resolving actions, may bind to T cells to cause pro-resolving actions. Hodges et al. identified the ALX/FPR2 receptor in rat conjunctival goblet cells by RT-PCR (15). Furthermore, the receptor has been found to be synthetized in corneal epithelial cells in mice, and expressed in human cornea epithelial cells, suggesting a central role in the functioning of the eye surface (123, 124). Thus, both the ALX/FPR2 receptor, MaR1 and AnxA1 have all been found to play a role in human lymphoid cells and rat conjunctival goblet cells. Hence, we hypothesize that MaR1 and AnxA1 dampen chronic inflammatory diseases, like allergic conjunctivitis, possibly by binding or by modulating the ALX/FPR2 receptor by activating protein kinases to phosphorylate and block the activity of the receptor (15).

The BLT-1 receptor is a G-protein-coupled receptor known as the specific receptor of the pro-inflammatory chemoattractant Leukotriene B\(_4\) (LTB\(_4\)) (125). The receptor is expressed in cells that play a central role in inflammation, including granulocytes, eosinophils, macrophages, several T-cell subclasses (\(T_H1\), \(T_H2\) and \(T_H17\)) and dendritic cells (126). Recently, the BLT-1 receptor was detected in both human and rat conjunctival goblet cells, and RvE1 was found to use the BLT1 receptor to increase \([\text{Ca}^{2+}]\) and to stimulate glycoprotein secretion (127).
We found that MaR1 activates BLT-1 to increase $[\text{Ca}^{2+}]_i$ and to stimulate secretion, while MaR2 used the receptor to increase $[\text{Ca}^{2+}]_i$, but not to stimulate secretion (101). Since MaR1 and MaR2 are pro-resolving mediators, in contrast to the pro-inflammatory LTB₄, our findings indicate that the function of the BLT-1 receptor depends on the agonist bound to the receptor.

When the BLT-1 receptor is “turned off”, inflammation is reduced. For instance, in bronchial asthma, eosinophil migration was suppressed and airway hyperresponsiveness was reduced in BLT-1 knock out (KO) mice (128). Because MaR1 and MaR2 are agonists, and not antagonists, we hypothesize that the BLT-1 receptor has an active pro-resolving function when MaR1 or MaR2 are bound to the receptor. Illustratively, MaR1 and its metabolite 22-OH MaR1 have been found to suppress LTB₄ signaling by acting on the BLT-1 receptor as partial agonists in a model of *E. Coli* peritonitis in mice (129). This finding indicated that both antagonists and pro-resolving agonists could be beneficial in treatment of inflammatory diseases.

In addition of being a receptor on the surface of immune cells, the BLT-1 receptor plays a role in other cell types. For instance, bone loss due to osteoclast activity was reduced in BLT-1 KO mice. LTB₄ is produced in osteoclasts and stimulates bone resorption by binding to BLT-1 on these cells (130). Furthermore, Terawaki et al. showed that lung goblet cell hyperplasia was reduced in BLT-1 KO mice with bronchial asthma (128). Thus, we suggest that MaR1 may play an important role in modulating conjunctival goblet cell secretion when bound to the BLT-1 receptor, contributing to tissue homeostasis. An interesting finding was that MaR1 when given 2 min after MaR2 attenuated the effect of MaR2 on $[\text{Ca}^{2+}]_i$, while MaR2 added 2 min after MaR1 did not alter the MaR1 response (101). In addition, we found that MaR1 given 30 min before LTB₄ decreased the LTB₄ induced increase in $[\text{Ca}^{2+}]_i$, while MaR2 given similarly to MaR1 did not affect the $[\text{Ca}^{2+}]_i$ response to LTB₄. MaR1 may attenuate both LTB₄- and MaR2 induced increase in $[\text{Ca}^{2+}]_i$ by binding or modulating a specific region of the BLT-1 receptor, a direct action (101). MaR1 may interact with a receptor that activate specific protein kinases that phosphorylate specific sites on the BLT-1 receptor, thus counter-regulating the receptor and
blocking its stimulation of cellular signaling pathways leading to an increase in 
$[\text{Ca}^{2+}]_i$, and/or secretion.

Another possible way of blocking LTB$_4$ and MaR2 by MaR1 is by indirectly blocking 
the BLT-1 receptor by binding to other receptors, such as the LRG6 receptor, 
described below. Another possible mechanism of desensitization of MaR2 by MaR1 
is through activation of the ALX/FPR2 receptor. Interestingly, AnxA1 was found to 
modulate inflammatory pain by desensitizing the TRPV1-receptor by activating the 
ALX/FPR2 receptor, through activation of PLC and calmodulin (77). We found MaR1 
to act on the ALX/FPR2 receptor to activate PLC and CaMKII, supporting this as a 
possible hypothesis (89).

Recently, a specific receptor of MaR1 was detected on the surface of phagocytes, 
the LRG6 receptor (69). The LRG6 receptor is known to play a role in tissue 
regeneration and repair (131). The receptor was detected on macrophages, PMNs 
and monocytes in mice and humans. In these species MaR1 stimulated macrophage 
phagocytosis in an LRG6-dependent manner. Furthermore, MaR1 did not use other 
known SPM receptors, including the ALX/FPR2 receptor. In addition, stimulation of 
phagocytosis by MaR1 was not dependent on the BLT-1 receptor (69). As described 
in the MaR2 paper, there could be multiple reasons for the differences detected, 
including cell- and species specificity, especially as LRG6 has yet to be detected in 
rats (101). The latter possibility occurs for the RvD1 specific GPR32 receptor. This 
receptor is found in humans, but not rats. In rats RvD1 activates the ALX/FPR2 
receptor instead (14). Future studies examining the role of the LRG6 receptor in 
conjunctival goblet cells in different species especially humans and mice would be of 
value.

As presented in the introduction, several SPMs contain 1,8-dihydroxy groups and 
conjugated trienes in their chemical structure, suggesting a biological importance of 
these structures in ligand-receptor binding (71). LTB$_4$, MaR1 and PD1 contain these 
chemical patterns in their structure. The potency of these three mediators on the 
human BLT-1 receptor varies, suggesting that the overall structure of the mediator is 
more important than separate chemical patterns (69). Future studies examining 
fragments of lipid mediators would be of interest.
To summarize, we hypothesize that MaR1 uses the ALX/FPR2 and the BLT-1 receptor to maintain tissue homeostasis in rat conjunctival goblet cells by contributing to a balanced glycoprotein and mucin secretion. Presumably, there exist several other receptors involved in the function of MaR1 that are not yet identified. The complete chemical structure, rather than parts of the mediator is so far found to be the most central factor in ligand-receptor binding. As in other organ systems and cells, AnxA1 uses the ALX/FPR2 receptor to perform its actions to contribute to tissue homeostasis by modulating goblet cell secretion. MaR2 might use the BLT-1 receptor to perform some of its actions, but this agonist is working through other undetected receptors to balance goblet cell secretion.

10.2.2 Comparison of Intracellular pathways used by MaR1, MaR2, and AnxA1 to increase $[\text{Ca}^{2+}]_i$ and stimulate mucin secretion

We compared the different receptors and intracellular pathways used by MaR1, MaR2, and AnxA1 to stimulate conjunctival goblet cell function. MaR1 uses intracellular calcium stores, PLC, IP$_3$, PKC, Ca$^{2+}$/CamKII and ERK 1/2 to increase $[\text{Ca}^{2+}]_i$ and stimulate glycoprotein secretion. On the other hand, it does not use PKA or PLA$_2$ to increase $[\text{Ca}^{2+}]_i$ nor to stimulate secretion. Furthermore, MaR1 uses PLD to stimulate secretion, but not to increase $[\text{Ca}^{2+}]_i$ (89).

In contrast to MarR1, MaR2 uses intracellular calcium stores, PKC, PLA$_2$, as well as cAMP-dependent PKA, but not PLC, IP$_3$ or extracellular Ca$^{2+}$ to increase $[\text{Ca}^{2+}]_i$. However, MaR2 is dependent on PLC, IP$_3$, PKC, PLD and cAMP-dependent PKA, but not PLA$_2$ to stimulate secretion (101).

Finally, AnxA1 uses intracellular calcium stores, PLC, IP$_3$, extracellular calcium, PKC and Ca$^{2+}$/CamKII to increase $[\text{Ca}^{2+}]_i$ and to stimulate secretion. Interestingly, AnxA1 uses PLA$_2$, PLD and ERK 1/2 to stimulate secretion, but not to increase $[\text{Ca}^{2+}]_i$ (90).

To sum up, the different mediators with pro-resolving qualities function through different intracellular pathways independently of origin. While MaR1 and AnxA1 use
the PLC pathway to increase \([Ca^{2+}]_i\), and stimulate glycoprotein secretion, MaR2 uses cAMP-dependent PKA to increase \([Ca^{2+}]_i\), and PLC to stimulate glycoprotein secretion. The intracellular pathways used by MaR1, MaR2 and AnxA1 will be described and discussed further in the discussion below.

10.2.2.1 PLC and \(IP_3\)

Vesicular exocytosis of mucins in goblet cells can be caused directly by \(Ca^{2+}\). One of the most studied cellular mechanisms known to cause an increase in \([Ca^{2+}]_i\) is the PLC pathway. There are several isoforms of PLC, for instance PLC\(\beta\) activated by GPCRs, PLC\(\gamma\) activated by tyrosine-kinase coupled receptors, PLC\(\delta\) activated by an increase in \([Ca^{2+}]_i\) and PLC\(c\) activated by Ras (36). Mucin secretion from goblet cells is known to be stimulated by cholinergic agonists transmitting signals through muscarine receptors, activating G\(\alpha_q\), which in turn activates PLC\(\beta\), stimulating an increase in \([Ca^{2+}]_i\) (27). We found that the pro-resolving mediators we studied interfere with the ALX/FPR2 and/or BLT-1 receptor that are receptors known to activate G\(\alpha_q\), which causes PLC to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP2) to \(IP_3\) and DAG (15, 39, 132). We suggest that MaR1 is uses both receptors to perform its actions, while AnxA1 is primarily dependent on ALX/FPR2. As already described, MaR2 is dependent on BLT-1 to increase \([Ca^{2+}]_i\), but not to stimulate secretion. MaR2 may activate another GPCR to stimulate secretion. However, MaR2 is dependent on PLC and the intracellular \(IP_3\) receptors to stimulate secretion, but not to increase \([Ca^{2+}]_i\) (101). Another possible explanation of the dependency of PLC to stimulate secretion, but not to increase \([Ca^{2+}]_i\) is production DAG, which is independent of \(Ca^{2+}\), and then activates PKC (37). Furthermore, we used the PLC inhibitor U73122, which predominantly inhibits the PLC\(\beta2\) isoform, but not PLC\(\beta1\), PLC\(\beta3\) and PLC\(\beta4\) (133). The mediators may work through several other PLC isoforms than PLC\(\beta2\). Accordingly, the actions of MaR2 may be caused by activation of other PLC isoforms to cause an increase in \([Ca^{2+}]_i\).

MaR2 is dependent on activation of the intracellular \(IP_3\) receptors to cause secretion, but not to increase \([Ca^{2+}]_i\) (101). We used the \(IP_3\) receptor inhibitor 2-
Aminoethoxydiphenyl Borate (2-APB). In addition of being an inhibitor of the IP$_3$ receptor, 2-APB has been found to interact with transient receptor potential (TRP) receptors. For instance, 2-APB was found to inhibit TRPC and TRPM, and activate TRPV1 which is known to play a central role in pain sensation (134). Another maresin, MaR1, inhibits TRPV1 in trigeminal nociceptive neurons, a finding that supports that 2-APB may have unspecific actions in the presence of the maresins (135). Thus, the inhibitory effect 2-APB on MaR2 may be caused by binding to TRP. Future studies exploring the role of TRP in ocular surface disease would be of value. In dry eye disease, corneal afferent nerves mediate responses through TRPM8 in response of drying of the cornea, indicating a central role of the TRP receptor in inflammatory dry eye disease (136).

To confirm a dependency of Ca$^{2+}$ released from intracellular stores for MaR1, MaR2, and AnxA1, we used the calcium chelator, Bapta/AM and the SERCA inhibitor, thapsigargin. The three mediators were all found to be dependent on release of Ca$^{2+}$ from intracellular calcium stores to increase [Ca$^{2+}$]$_i$ (89, 90, 101). MaR1 and AnxA1 are likely using the PLC – IP$_3$ pathway to stimulate release of Ca$^{2+}$ from the ER. In contrast to MaR1 and AnxA1, MaR2 uses both PLD and cAMP-dependent PKA to increase [Ca$^{2+}$]. A possible mechanism used by MaR2 to increase [Ca$^{2+}$]$_i$ by release from ER is potentiation of the IP$_3$-receptor by cAMP-dependent PKA, a mechanism described by Burgess et al (137). Moreover, MaR2 may use messengers in the PLC pathway, but is not dependent on these messengers to cause the actions measured in the present thesis.

10.2.2.2 Extracellular Ca$^{2+}$

Interestingly, both the MaR1 and MaR2 increase in [Ca$^{2+}$]$_i$ were independent of extracellular Ca$^{2+}$ to (89, 101). To refill the intracellular calcium stores with Ca$^{2+}$, extracellular Ca$^{2+}$ entering across the plasma membrane is crucial (39). Thus, to explain this finding, we have to take a more detailed look at the time interval for the different cellular events. Goblet cells secrete various amounts of mucin depending on the environment to keep a stable tear film (homeostasis), and an increase in [Ca$^{2+}$]$_i$ has a central role in exocytosis of mucins. Glycoprotein secretion is measured two
hours after addition of a mediator, while $[\text{Ca}^{2+}]_i$ is measured the first seconds after addition. Secretion is the end result of many cellular processes, for instance protein synthesis in the ER, modulation in the Golgi compartment and fusion with the cellular membrane (14). Thus, our experiments do not exclude that extracellular Ca$^{2+}$ might play a role in secretion or in $[\text{Ca}^{2+}]_i$ after the first two minutes, especially during the plateau phase that is particularly dependent upon the influx of extracellular Ca$^{2+}$.

In addition to playing a role in exocytosis of mucins, Ca$^{2+}$ has other important functions in goblet cells. For instance, Ca$^{2+}$ and H$^+$ shield the negative charges in oligosaccharide chains of mucins. The mucin oligomers/multimers are stored in mucin granules. Furthermore, in the mucin granules, Ca$^{2+}$ contributes to organization of a condensed polyanionic matrix (138). Future studies investigating the role of extracellular Ca$^{2+}$ in other pro-resolving events would be interesting.

### 10.2.2.3 CaMKII

CaMKII is a highly conserved, multi-functional protein encoded by four genes ($\alpha$, $\beta$, $\gamma$ and $\delta$) (139). The protein is well-known for playing a role in neuronal activity, like long-term potentiation (LTP) and is highly expressed in hippocampus and neocortex (140). In addition, this kinase plays a central role in exocytosis in different organ systems. For instance, pancreatic islet cells are dependent on CaMKII in the secretion of insulin (141). Exocytosis of mucins from goblet cells can be induced by activation of CaM kinases, like CaMKII (27). The underlying working mechanism of CaMKII in the exocytotic process has not been fully established, but the kinase has been found to regulate the vesicular trafficking of the molecule synataxin (142).

AnXA1 and MaR1 are dependent on CaMKII both to increase $[\text{Ca}^{2+}]_i$ and to stimulate secretion (89, 90). CaMKII is able to work in both a Ca$^{2+}$ dependent and Ca$^{2+}$ independent manner. The autoinhibitory domain of the kinase can be phosphorylated, which inhibits the inhibitory part of the kinase. Autophosphorylation of threonine (Th$^{286}$) increases the affinity between the kinase and calmodulin and the kinase is activated independently of the $[\text{Ca}^{2+}]_i$ (143). Additionally, high frequency Ca$^{2+}$ pulses are known to cause rapid CaMKII autophosphorylation, while lower frequencies fail to initiate autophosphorylation (144). Because AnxA1 and MaR1
cause an increase in $[Ca^{2+}]_i$, after activation of CaMKII, CaMKII can both be activated in a $Ca^{2+}$ dependent and $Ca^{2+}$ independent manner. AnxA1 and MaR1 may activate other intracellular signaling molecules causing an increase in $[Ca^{2+}]_i$ which rapidly activates CaMKII. The mediators may also directly activate CaMKII causing autophosphorylation and a $Ca^{2+}$ independent activation of the kinase.

10.2.2.4 PKC

PKC has multiple isoforms divided into classical, novel, atypical and PKCµ. Classical PKC isoforms are α, βI, βII, and γ. They are activated by $Ca^{2+}$, phosphatidylinerine, and DAG. Novel PKC isoforms are δ, ε, η, and θ; unlike the classical PKC isoforms, are $Ca^{2+}$ independent. Atypical PKC isoforms are ζ and i/l and are not activated by $Ca^{2+}$, phosphatidylinerine, or DAG. In rat conjunctival goblet cells, seven PKC isoforms have been detected by western blotting analyses and immunofluorescence microscopy (37). A well-known mechanism of PKC activation is through the PLC pathway. As for $Ca^{2+}$, PKC has been shown to directly stimulate secretion in rat conjunctival goblet cells (37). PLC hydrolyzes PIP$_2$ that activates DAG, ultimately activating PKC. This mechanism of action would activate the classical PKC isoforms. The novel PKC isoforms could be activated by different non-$Ca^{2+}$-dependent pathways.

MaR1 and AnxA1 activate PLC and PKC to increase $[Ca^{2+}]_i$, thus PKC is likely to be activated by DAG (89, 90). MaR2 was not dependent on PLC to increase $[Ca^{2+}]_i$ (101). However, MaR2 used PLC to stimulate secretion. This action could be explained by PKC-dependent secretion that is activated by other ways in addition to PLC. For instance, in retinal inflammatory bacterial disease, PLD generates phosphatic acid which is dephosphorylated to generate DAG, which in turn activates PKC (145). Thus, MaR2 may activate PKC through PLD.

Another possible mechanism of PKC activation is through cAMP. The SOC-3 (suppressor of cytokine signaling 3) limits pro-inflammatory signaling and is a target gene for combating chronic inflammatory disease. SOC-3 increases cAMP levels that
induce EPAC (exchange proteins directly activated by cyclic AMP), which in turn activates PKC. Activation of PKC causes an ERK-dependent mobilization of transcription factors and induction of the SOC-3 gene (146). As of now, it is not known if there is a connection between cAMP and PKC in exocytosis of mucins in goblet cells. Future studies exploring this would be of value.

10.2.2.5 PLD

We found that both MaR1 and AnxA1 were dependent on PLD to stimulate secretion, but not to increase [Ca\(^{2+}\)]. (89, 90). The mucin protein cores are synthesized in the ER and transported to the Golgi apparatus (27). Coat protein I (COPI) components function in vesicle formation. The late stages of vesicle fission are dependent on PLD2, as PLD2 generates phosphatic acid (147). This important step in exocytosis of mucins may explain why MaR1 and AnxA1 are dependent on PLD for secretion, but not through changes in [Ca\(^{2+}\)]. MaR2 may be dependent on PLD both through activation of [Ca\(^{2+}\)] to cause mucin secretion or by modulating the vesicle fission stage (101). Another possible mechanism of protein secretion is Ca\(^{2+}\)-independent activation of PLD through cholinergic agonist-stimulation, a mechanism found in lacrimal gland acinar cells. In a study by Hodges et al, a cholinergic agonist activated PLD1 to stimulate Rho/ROCK and ERK1/2, but did not increase [Ca\(^{2+}\)] or activate PKC, to stimulate protein secretion (148, 149). MaR1 and AnxA1 could use this type of pathway to stimulate protein secretion from conjunctival goblet cells.

10.2.2.6 ERK 1/2

Hypersecretion of mucins is a central pathological feature in multiple diseases involving goblet cells. For instance, cigarette smoke in pulmonary goblet cells causes hypersecretion and has a positive correlation with the expression of ERK 1/2. UO126 inhibits MEK to cause a reduction in activated ERK 1/2 and MUC5AC expression (150). We found that MaR1 is dependent on ERK 1/2 to increase [Ca\(^{2+}\)] and stimulate glycoprotein secretion (89). In a study exploring the effect of the SPM LXA\(_4\) on rat conjunctival goblet cells, ERK 1/2 inhibition partially suppressed LXA\(_4\) stimulated increase in [Ca\(^{2+}\)], but completely blocked LXA\(_4\) stimulated secretion (15). AnxA1 was dependent on ERK 1/2 activity for secretion, but, in contrast to LXA4, not
for increasing $[\text{Ca}^{2+}]_i$ (90). As for LXA₄, AnxA1 activates the ALX/FPR2 receptor, suggesting a shared mechanism of action. AnxA1 and LXA₄ are known to have several shared mechanisms of actions, for instance activation of PKC and PLC. Thus, ERK 1/2 may be activated in a PKC-dependent manner to cause glycoconjugate secretion (146). Their dissimilarities in signaling pathways used could be due to the binding of a lipid (LXA₄) and a protein (AnxA1) to different parts of the ALX/FPR2 receptor known to be promiscuous in its agonists.

Activation of ERK1/2 is also a component of the EGFR pathway stimulated by release of EGF from pro-EGF. RvD1, but not RvE1 activate a matrix metalloproteinase that releases EGF from pro-EGF (88). Activation of EGFR induces a cascade of protein kinase activation ending in ERK1/2. It is possible that AnxA1 activates the EGFR-dependent pathway to induce ERK1/2 and stimulate secretion.

### 10.2.2.7 cAMP

$G_{s\alpha}$ that causes catalyzation of ATP to cAMP is known to activate diverse downstream molecules in addition to PKA, including Epac (151). MaR1 and AnxA1 did not elevate cAMP nor were dependent on cAMP-dependent PKA to increase $[\text{Ca}^{2+}]_i$ or to stimulate secretion (89, 90). Chatterjee et al found that MaR1 elevates cAMP in human endothelial cells and smooth muscle cells (152). Furthermore, Chiang et al found that MaR1 increased cAMP by binding to the LRG6 receptor in human embryonic kidney cells (69). Gu et al found that MaR1 upregulated the cAMP concentration, which decreased lung injury in a mouse sepsis model (153). Our finding that the actions of MaR1 and AnxA1 were independent of cAMP in in rat conjunctival goblet cells may indicate both a cell- and species-specific mechanism of action. In fact, the LGR6 receptor that is cAMP dependent has yet to be found in rat cells. In addition, as suggested by Lyngstadaas et al, the PLC-IP₃ activation may serve as a mechanism maintaining a high $[\text{Ca}^{2+}]_i$, preventing inhibitors, like H89, from significantly blocking mediator-stimulated increase in $[\text{Ca}^{2+}]_i$ (90). Both MaR1 and AnxA1 are dependent on the PLC pathway to stimulate increase in $[\text{Ca}^{2+}]_i$, supporting this hypothesis.
In contrast to AnxA1 and MaR1, MaR2 is dependent on cAMP to increase $[\text{Ca}^{2+}]_i$ and stimulate secretion (101). Thus, it is unlikely that MaR2 works through the BLT-1 receptor to cause it actions, due to activation of Gi by BLT-1 (101). The SPM, RvD2 also utilizes cAMP-dependent PKA to stimulate an increase in $[\text{Ca}^{2+}]_i$ and secretion, and is known to activate the DRV2/GPR18 receptor (16). Thus, MaR2 may work through the DRV2/GPR18 receptor or through other as yet unidentified receptors.

10.2.2.8 PLA$_2$

PLA$_2$ is mainly known to cause hydrolysis of membrane phospholipids, leading to the release of arachidonic acid (154). So far, 19 enzymes with PLA$_2$ activity have been detected, which can further be divided into two main groups; secretory and cellular PLA$_2$ (154). The function of PLA$_2$ may both be a result of hydrolysis of membrane phospholipids and by an indirect action through the release of lipid signaling precursors/molecules (155). While MaR1 was found to be completely independent of PLA$_2$ to increase $[\text{Ca}^{2+}]_i$ and to stimulate glycoconjugate secretion, MaR2 was dependent on the lipase to cause an increase in $[\text{Ca}^{2+}]_i$ (89, 101). AnxA1 was found to be dependent on the lipase to stimulate glycoconjugate secretion (90). Aristolochic acid was shown to be anti-inflammatory in edema in mice. Inhibition of arachidonate mobilization by aristolochic acid is partly due to inhibition of cellular PLA$_2$. However, the inhibitor may have other, unspecific effects by influencing additional steps in eicosanoid synthesis (156). The unspecific actions of aristolochic acid may explain the differing results between the dependency of PLA$_2$ on $[\text{Ca}^{2+}]_i$ and glycoconjugate secretion in the mediators with pro-resolving effects used in the present study.

10.2.3 The role of MaR1, MaR2 and AnxA1 in resolution of inflammation.

MaR1, Mar2 and AnxA1 were all shown to decrease histamine-stimulated increase in $[\text{Ca}^{2+}]_i$ and glycoconjugate secretion, supporting their specialized pro-resolving actions in disease (89, 90, 101). Fujimoto et al found that the histamine H$_1$ receptor is desensitized by PKC activation which causes phosphorylation of the receptor and blockage of its action (157). Thus, the inhibitory effect of MaR1, MaR2 and AnxA1 on
histamine in goblet cells may be caused by phosphorylation by PKC of the H1 receptor subtype of histamine, which should be explored further in future studies. This is particularly important in conjunctival goblet cells as all four histamine receptor subtypes are present and each of these subtypes is phosphorylated by different protein kinases (95). In ocular allergy, the pro-inflammatory mediator histamine stimulates mucin overproduction, contributing to the disease and symptoms of allergic conjunctivitis. In addition to playing a role in decreasing the negative effect of histamine on the ocular surface, we propose that the pro-resolving mediators may have an essential role in goblet cell activation and secretion in normal physiology. We suggest that the mediators with pro-resolving actions contribute to tissue homeostasis by stimulating goblet cells to produce a balanced mucin secretion. Further studies exploring the effect of MaR1, MaR2 and AnxA1 in physiological and pathological models in different species and cell types are important in the future.

To illustrate the potential of SPMs in the role of clinical medicine as a treatment option in the future, we will describe some of the significant discoveries of MaR1, MaR2 and AnxA1.

10.2.3.1 A role of MaR1 in resolution of inflammation

MaR1 is present in human serum and secondary lymphoid tissues, including the spleen and lymph nodes (121). In human peripheral blood lymphocytes, MaR1 reduces the production of the pro-inflammatory cytokines TNF-α, IFN-γ in CD8⁺ T cells and CD4⁺ T cells, thereby regulating the adaptive immune system. In addition, MaR1 prevents generation of T_H1 and T_H17 cells (cells associated with chronic inflammation) and enhances differentiation of T_reg cells. Interestingly, both the GPR32 and ALX/FPR2 receptor are expressed on all T-cell subclasses (122).

MaR1 not only plays a role in the complex adaptive immune system, but also in the innate immune system. Serhan et al found that MaR1 reduces PMN infiltration and chemotaxis and enhances efferocytosis of apoptotic PMNs (70). In the flatworm planaria, MaR1 is biosynthesized after injury, supporting MaR1 as a central contributor to tissue homeostasis. Interestingly, MaR1 also stimulates tissue regeneration when the anterior section of planaria is surgically removed.
Furthermore, by finding a role of MaR1 in a large spectrum of species suggests a conserved role of the SPM throughout evolution.

Another function of MaR1 is to reduce pain in mice by regulating Transient Receptor Potential Vanilloid 1 (TRPV1) channel function (70). Thus, MaR1 might also play a role in pain control during an inflammatory state. Additionally, Park et al found that MaR1 regulates TRPV1 modulating synaptic plasticity causing analgesic actions in chronic temporomandibular joint pain in mice, a disease caused by somatic inflammatory pain (135). Tibia bone fractures are associated with postoperative pain. Treatment with \(\omega-3\) fatty acid DHA prior to surgery relieve pain, and peri-operative treatment with MaR1 reduces development of postoperative pain, being more effective than the SPM, RVD1 (158).

Bacterial infections are some of the most common inflammatory diseases in humans and may lead to potentially life-threatening diseases like sepsis. In *E. coli*-infected mice, MaR1 was found to bind to and modulate the BLT-1 receptor causing increased bacterial clearance by stimulating phagocytosis of *E. coli* and by regulating the chemoattractant LTB4 (129). In cecal ligation and puncture induced sepsis in mice, MaR1 improved survival rate by acting on the ALX/FPR2 receptor. MaR1 inhibited inflammatory cytokines including TNF-\(\alpha\), decreased sepsis associated mitochondrial dysfunction and ROS production, suppressed bacterial growth and protected lung tissue by decreasing the number of neutrophils (153). In the same disease model, Li et al found that MaR1 was protective by blocking the pro-inflammatory NF-\(\kappa\)B pathway (159). Thus, MaR1 decreases bacterial number and resolves inflammation in life-threatening sepsis by acting on multiple mechanisms to reduce disease burden.

Mucous membranes cover the surface of the body in cavities and organs in the body that contact the external environment, and form a protective barrier against external dangers. In addition to the cornea and conjunctiva, both the gastrointestinal tract, the respiratory tract, the oral cavity, and the genito-urinary tract are covered by a mucous membrane. In dust-induced lung inflammation, MaR1 regulates PMN influx to the airways by reducing pro-inflammatory cytokines like TNF-\(\alpha\) and IL-6 (160). In acid-
induced acute lung injury in mice MaR1 decreases cytokines, as well as inhibits neutrophil activation and influx to the lung interstitium. In addition, MaR1 is produced in platelets and inhibits LTBA₄ hydrolase, reducing LTB₄, a central mediator linked to neutrophil activation in acute respiratory distress syndrome (ARDS) (161). In asthma, MaR1 is produced in the lung tissue and limits inflammation by stimulating T_{reg} cells, and regulates the central pro-allergic type 2 innate lymphoid cells (162). In induced colitis in mice, MaR1 reduces disease activity by decreasing proinflammatory mediators, reducing neutrophil migration, inhibiting ROS production, inhibiting colonic cell infiltration, reducing NF-κB activity and stimulating M1- to M2 class switching in macrophages (163). In human localized aggressive periodontitis, MaR1 restores neutrophil- and macrophage function and stimulates clearance of periodontal pathogens (164). These findings support the hypothesis that MaR1 plays a role in resolution of inflammation in other mucous membrane covered tissues, including the ocular surface.

In addition to infectious diseases, inflammation is known to play a central role in diseases such as cardiovascular disease and rheumatological disease, for example rheumatoid arthritis (RA). By attenuating inflammatory mediators, reducing TNF-α associated ROS production and inhibiting adhesion of monocytes in human vascular smooth muscles cells and endothelial cells, MaR1 may play a role as a therapeutic option in vascular inflammatory diseases in the future (152). In RA, the serum levels of MaR1 are decreased in patients with active RA, and increased in patients with inactive RA. In RA, MaR1 may work by regulating the balance between T_{Reg} cells and Th17 cells (165). In addition to being a potential treatment in RA, MaR1 may play another role. In RA patients, MaR1 was detected in the synovial fluid (166). The level of MaR1 in the synovial fluid may reflect the level of inflammation in the joint and can thus be used to follow the progression of the disease (166).

Diseases and injuries of the nervous system may lead to a major loss of function and reduced quality of life. In addition, the treatment options are limited in progressive diseases such as amyotrophic lateral sclerosis (ALS). MaR1 shows protective effects in stress-induced motor neuron cell death, by inhibiting ROS production and NF-κB activation, and may thus be a potential treatment option in diseases affecting the
motor neuron cells (167). Following spinal cord injury (SCI), a dysregulated inflammatory response caused by an incomplete clearance of immune cells from the damaged area leads to limited ability to repair nerves and may contribute to irreversible functional disabilities (168). By selectively downregulating proinflammatory cytokines, such as IL-6, and by increasing neutrophil phagocytosis, MaR1 enhances resolution of inflammation (168).

A common postoperative complication of surgery in elderly patients is perioperative neurocognitive disorder, which leads to decreased cognitive function. Prophylactic treatment with MaR1 in mice prevents surgery-induced glial activation, reduced infiltration of macrophages to the hippocampus and improves memory function (169). In the same study, Yang et al explored the concentration of MaR1 in human cerebrospinal fluid (CSF) prior to and after surgery. No statistical significance was found between the groups. However, a weakness of the study was the sample size (11 patients), out of which only two patients showed clear changes in MaR1 levels before and after surgery (169). Furthermore, the level of MaR1 was only measured in the CSF, but not hippocampal tissue, which would be interesting. Moreover, there may be a difference in the role of MaR1 as a prophylactic mediator against cognitive declining and as a SPM in acute inflammation. By preventing chronic inflammation by long term macrophage regulation, MaR1 may play a role in maintaining cognitive function.

Due to the obesity epidemic, the prevalence of diseases including liver steatosis and non-alcoholic steatohepatitis (NASH) is increasing dramatically. In liver steatosis in mice caused by obesity, MaR1 decreased accumulation of fat, induced fatty acid oxidation and stimulated autophagy (170). Thus, MaR1 has a protective function and decreases symptoms in the inflammatory disease NASH by inducing M2 liver macrophages that are anti-inflammatory and repair injured tissues (171).

To summarize, MaR1 plays a role in in a numerous, diverse species and performs its actions in a number of distinct organ systems. In accordance with our findings, the ALX/FPR2 receptor and the BLT-1 receptor were found on cells central in the actions of MaR1. Furthermore, MaR1 plays a role in cells in the gastrointestinal tract and the lungs; organs covered by a mucous membrane where goblet cells have an essential
role. MaR1 blocks pro-inflammatory cytokines, reduces migration of pro-inflammatory leukocytes, reduces NF-κB activity, stimulates M1- to M2 class switching, stimulates tissue regeneration and causes analgesic actions by regulating pain receptors. We detected the main receptors and intracellular pathways used by MaR1 in rat conjunctival goblet cells in physiology and a role for MaR1 in disease by blocking the action of the pro-inflammatory autacoid histamine. Future studies exploring the effect of MaR1 in ocular surface disease and the underlying mechanisms of its actions will be of value in the future.

10.2.3.2 A role of MaR2 in resolution of inflammation

MaR2 was recently discovered and added to the SPM family. Deng et al. showed that MaR2 inhibited neutrophils in mouse peritonitis and that MaR2 enhanced phagocytosis of apoptotic PMNs by macrophages (10). Studies exploring the role of MaR2 are limited due to its recent discovery. Interestingly, we found that MaR2 uses different intracellular pathways in rat conjunctival goblet cells than other SPMs investigated. However, MaR2 also inhibits the effect of histamine, in accordance with the SPM family. Future studies of the role of MaR2 in ocular surface disease and the comparison with other SPMs will be interesting.

10.2.3.3 A role of Annexin A1 in resolution of inflammation

Similar to other pro-resolving mediators, AnxA1 plays a central role in regulating PMNs. In peritonitis in mice, AnxA1 inhibited PMN migration contributing to a reduced inflammatory response and tissue homeostasis. Furthermore, in the absence of AnxA1 PMN diapedesis continues (172). When mice are pre-treated with AnxA1, the pro-inflammatory IL-1β-induced PMN migration is inhibited (82). In PMNs in humans, AnxA1 reduced cell adhesion which inhibited PMN extravasation, thus contributing to resolution of inflammation (173). Moreover, AnxA1 caused controlled PMN activation and accelerated PMN apoptosis, contributing to resolution of inflammation (174). For instance, in pleural cavity inflammation in mice, AnxA1 resolved inflammation by inducing apoptosis of inflammatory cells like neutrophils
Thus, AnXA1 may have a central role in regulating PMNs contributing to resolution of inflammation.

Glucocorticoids are frequently used to suppress inflammatory diseases, for instance in inflammatory bowel disease, allergy and sinusitis. Their action is mostly known to be anti-inflammatory. However, by accelerating the uptake of apoptotic PMNs, glucocorticoids also contribute to resolution (176). When rat peritoneal cells were treated with dexamethasone the concentration of AnxA1 increased dramatically (78). The anti-inflammatory effect of glucocorticoids on the innate immune system might be caused by release of AnxA1 and its binding to ALX/FPR2 receptors in PMNs and macrophages. In contrast, the immunosuppressive effect of glucocorticoids on the adaptive immune system may be due to inhibition of AnxA1 expression by T cells (17). In induced arthritis in rats, anti-AnxA1 monoclonal antibodies reversed the effect of dexamethasone causing an exacerbation of the disease. AnxA1 acts as anti-inflammatory by inhibiting pro-inflammatory cytokine production and acts as a pro-resolving mediator by blocking PMN recruitment (80).

As for MaR1, AnxA1 has pro-resolving features in organs covered by a mucous membrane. In induced colitis in mice, AnxA1 was overexpressed and secreted during the cellular stage of the inflammatory process (177). In Streptococcus pneumoniae pneumonia in mice, the absence of AnxA1 and the ALX/FPR2 receptor caused increased bacterial dissemination and uncontrolled inflammation (178). In contrast, the level of AnxA1 was negatively correlated with lung function in human chronic obstructive pulmonary disease (COPD). AnxA1 also caused fibroblast activation, indicating a potential role in lung fibrosis (179). Thus, in lung disease the role of AnxA1 and if it is a protective pro-resolving mediator or pro-inflammatory, is not known. However, in another chronic inflammatory disease, endometriosis, the levels of both AnxA1 and ALX/FPR2 are reduced and IL-6 levels are increased, indicating that a low level of the pro-resolving mediator may not be able to resolve the chronic inflammation (180). In allergic conjunctivitis in mice, AnxA1 decreases clinical symptoms through several mechanisms, including inhibition of leukocyte recruitment and release of cytokines and chemokines. Deficiency of AnxA1 leads to an increased inflammatory response (181). By binding to the ALX/FPR2 receptor, AnxA1 contributes to the termination of allergy, both by anti-inflammatory and pro-resolving
actions. For instance, AnxA1-ALX/FPR2-binding has a pro-resolving effect by inhibition of eosinophil migration in allergic conjunctivitis. AnxA1 and ALX/FPR2 are co-localized in mast cells, neutrophils and eosinophils in allergic conjunctivitis, suggesting a role of the mediator and the receptor in resolution of allergic conjunctivitis (182).

In addition to its function in ocular surface diseases, AnxA1 plays a role in other ocular diseases. In uveitis, treatment with AnxA1 causes decreased neutrophil invasion and in AnxA1 KO-mice the inflammatory response is exacerbated. The AnxA1 effect on ocular inflammation may be caused both by reducing COX-2 expression and by binding to the ALX/FPR2 receptor, inhibiting release of pro-inflammatory mediators (183). The neurodegenerative disease glaucoma causes axonal damage to the optic nerve and retinal ganglion cell death. A decrease in AnxA1 secretion is associated with increased retinal inflammation and retinal ganglion cell apoptosis which exacerbate glaucoma (184).

Diseases involving ischemia are associated with inflammation. Reperfusion of ischemic cardiac cells is correlated with myocardial damage due to release of soluble mediators. In myocardial ischemia-reperfusion damage in rat, AnxA1 reduces PMN extravasation in a dose-dependent manner, thereby reducing leukocyte-induced damage, preserving cell integrity and reducing tissue necrosis (185). By activating the ALX/FPR2 receptor, AnxA1 provides cardioprotective effects (186). In mesenteric arteries damaged by ischemia/reperfusion, AnxA1 has an anti-adhesive effect blocking neutrophil extravasation due to binding to the ALX/FPR2 receptor (187). Cerebral artery occlusion causes ischemia and neuronal death. Microglia, cells with an immunological function in the central nervous system, express a high level AnxA1. AnxA1 is cerebroprotective in middle cerebral artery occlusion and the inflammatory response is increased by AnxA1 deficiency (188).

In addition to ischemic diseases in the central nervous system, spinal cord injuries are followed by an inflammatory reaction that contributes to tissue damage. After a spinal cord injury, the expression of annexins (I, II and V) is elevated in neurons and glial cells, and may have a protective role (189).
AnxA1 inhibits inflammatory pain by regulating the TRPV1 receptor in the dorsal horn neurons. AnxA1 being a ligand to the FPR2/ALX receptor activates PLC, increases \( \text{[Ca}^{2+}\text{]}_i \), which activates calmodulin. The activation of this intracellular pathway causes desensitization of TRPV1 and decreased pain. (77).

As for other mediators with pro-resolving features, AnxA1 may play a role in tissue regeneration. AnxA1 induces a pro-reparative phenotype of macrophages by binding to the ALX/FPR2 receptor. The activated macrophages then stimulate regeneration of muscle fibers in induced muscle injuries in mice (190).

Comparison of MaR1, MaR2 and AnxA1 and their action on histamine

The discovery of pro-resolving and SPMs uncovered a new potential target for development of treatments for inflammatory diseases (7, 49). As mentioned in the introduction, the SPMs clear apoptotic cells (59, 60) and limit and regulate PMN invasion, thereby contributing to resolution of inflammation (9, 60, 61). Our findings in rat conjunctival goblet cells on the actions of MaR1, MaR2 and AnxA1 to increase \( \text{[Ca}^{2+}\text{]}_i \), and stimulate glycoconjugate secretion that represents health, but inhibiting the histamine-induced \( \text{[Ca}^{2+}\text{]}_i \) and secretory responses that represents inflammatory disease support the hypothesis that these mediators may play a role in conjunctival tissue homeostasis.

AnxA1 mediates its actions at lower concentrations than other pro-resolving mediators used in the present study. While MaR1 and MaR2 caused a maximum \( \text{[Ca}^{2+}\text{]}_i \) and secretory response at \( 10^{-8} \text{ M} \), AnxA1 had a maximum response at \( 10^{-9} \text{ M} \) for \( \text{[Ca}^{2+}\text{]}_i \) and at \( 10^{-10} \text{ M} \) for secretion (89, 90, 101). The basis for the high potency of AnxA1 compared to the other SPMs is not yet understood, but could indicate that AnxA1 could have effect at a low concentration if applied pharmaceutically.

Surprisingly, MaR1 and MaR2 exerted their actions through different receptors and intracellular pathways. Both MaR1 and MaR2 contain a conjugated triene, however, only MaR1 contains a 1,8 dihydroxy group between the conjugated triene (71). The hydroxy group contributes to increased intermolecular binding by hydrogen bonding. Moreover, the dihydroxy group may convert adjacent molecules to alcohols which
increases water solubility. These chemical qualities may contribute to differences in receptor binding.

Unexpectedly, we found that MaR1 and AnxA1 use similar receptors and intracellular pathways. In addition to the shared pro-resolving functions of the two mediators, MaR1 and AnXA1 both decrease inflammatory pain by regulating TRPV1 (77, 135).

**Figure 10:** *Receptors and intracellular pathways used by MaR1, MaR2 and AnxA1 in rat conjunctival goblet cells.* (The last figure is a first draft of how the figure will look like. The signaling pathways of the first figure will be included in the last figure when the illustrator is done).

10.2.4 Comparison of pro-resolving mediators/SPMs

10.2.4.1 RvD1
As for MaR1 and MaR2, RvD1 is biosynthesized from the ω-3 fatty acid DHA (14). This mediator is known to bind to the DRV1/GPR32 receptor in humans and the ALX/FPR2 receptor in humans and rats (191). RvD1 increases [Ca²⁺]i and stimulates glycoconjugate secretion in rat conjunctival goblet cells. Furthermore, it is a ligand for ALX/FPR2 receptor activating PLC, PLD, PLA2, PKC, IP3, ERK 1/2 and Ca²⁺/CaMK (14). AnxA1 and MaR1 are ligands of the ALX/FPR2 receptor as well. However, AnxA1 and RvD1 activate different intracellular pathways, while MaR1 and RvD1 use almost identical intracellular pathways. MaR1 does not activate PLD to increase [Ca²⁺]i and RvD1 does not use PKC to stimulate glycoconjugate secretion, which is the only difference between these two mediators (14). In a study of murine peritonitis, the resolving effect of MaR1 was compared to that of RvD1. By stimulating an increased efferocytosis by macrophages of apoptotic PMNs, the resolving actions of MaR1 was found to be more potent than that of RvD1 (70). This finding suggests that similar SPMs with the same origin, the same receptor binding and intracellular pathway activation may have different resolving effects. Moreover, the ALX/FPR2-receptor is very promiscuous and has multiple agonists that bind to different parts of the molecule. For instance, a protein like AnxA1 and a lipid like MaR1 will probably bind to different sites of the receptor. This could account for the differences in signaling pathways activated by the ligands of ALX/FPR2 studied herein.

10.2.4.2 RvD2

RvD2 is biosynthesized from the ω-3 fatty acid DHA and activates the DRV2/GPR18 receptor which is expressed in leukocytes (192). RvD2 increases the [Ca²⁺]i and stimulates glycoconjugate secretion in rat conjunctival goblet cells (16). In contrast to RvD1, MaR1 and AnxA1, RvD2 activates adenylyl cyclase that increases [cAMP], that in turn activates cAMP-dependent PKA. In contrast to RvD1, MaR1, and AnxA1, RvD2 and MaR2 activate cAMP and PKA. In addition, RvD2 activates PLC and IP₃ to increase [Ca²⁺]i and stimulate secretion (16), but MaR2 was dependent on PLC and PLD, but not PLA₂ to stimulate secretion. MaR2 and may, like RvD2, use PKA to increase IP₃ dependent Ca²⁺ release from the ER to mediate its actions on Ca²⁺ and secretion. As IP₃ is produced from PLC activation, the action of PKA on IP₃-induced Ca²⁺ release is an interaction with the PLC pathway.
10.2.4.3 RvE1

In contrast to the maresins and the D-series resolvins, RvE1 is biosynthesized from the ω-3 fatty acid eicosapentanoic acid (EPA) (13). RvE1 activates PLC, IP3, PKC, PLD and PLA₂, but not Ca²⁺/CaMK to increase [Ca²⁺]i and to stimulate glycoconjugate secretion (13). Thus, the actions of RvE1 are similar to that of MaR1 and AnxA1, despite their different origins. RVE1 binds to Chem23 and like MaR1, RvE1 binds to the BLT-1 receptor (193, 194). By promoting neutrophil apoptosis through BLT-1 receptor binding, RvE1 contributes to tissue homeostasis, for instance in pulmonary diseases like Acute Respiratory Distress Syndrome (ARDS) (195). Thus, the various SPMs stimulate receptors and cellular signaling pathways in both similar and different manners regardless of their chemical structure and biosynthetic pathways.

10.2.4.4 LxA₄

Lipoxins are biosynthesized from the ω-6 fatty acid arachidonic acid (15). LXA₄ increases [Ca²⁺]i and stimulates glycoconjugate secretion in rat conjunctival goblet cells by binding to the ALX/FPR2 receptor activating PLD, PLC, PLA₂, ERK 1/2, Ca²⁺/CaMK and not PKA. Compared to other SPMs studied in rat conjunctival goblet cells, LXA₄ works similarly to MaR1 and RvD1, which are also known to activate ALX/FPR2 and matching intracellular pathways. This finding further supports the hypothesis that in conjunctival goblet cells SPMs may work in similar manner despite being of different biosynthetic origins.

10.3 Future perspectives

10.3.1 ω-3 Fatty acids

There has been an increasing interest in ω-3 fatty acids over the last decades due to their potential health benefits (196). Fish (body lipids and liver) and plants (flax, chia...
and canola) oils are the main dietary sources of ω-3 fatty acids as they are not synthesized by humans. The potential advantages of treatment with ω-3 fatty acids has been thoroughly explored in several diseases including cardiovascular disease, diabetes, cancer, depression, dementia and rheumatoid arthritis (196). Randomized trials have found multiple clinical effects of ω-3 fatty acids in rheumatoid arthritis (RA) (197). Furthermore, American Heart Association (AHA) recommends 1 g of EPA and DHA (combined) per day in patients with documented coronary heart disease due to a reduction in cardiovascular events and by lowering the level of triglyceride in the circulation (198, 199). However, novel systematic reviews and meta-analyses have questioned the protective effect of ω-3 fatty acids in diseases like cardiac disease and cancer, with findings of no significant difference between ω-3 fatty acid supplements and placebo (196, 200, 201).

In the inflammatory disease, dry eye disease, ω-3 fatty acid supplements (EPA and DHA) had a beneficial effect on tear production and tear osmolarity, but only a variable effect on symptoms when utilized alone without other dry eye disease treatments (202). Thus, both in other organ systems and in ocular surface diseases, more studies are needed to explore if ω-3 fatty acids should play a role as a treatment option or if their biosynthetic products, the lipoxins, resolvins, maresins and protectins should be used as these are the bioactive compounds.

There are several indications of an important function of ω-3 fatty acids in maintenance of tissue homeostasis. For instance, several SPMs and their precursors have been detected in human tears in healthy eyes in concentrations corresponding to their bioactive function (203). Moreover, both DHA and EPA have been detected in human tears and their levels corelate with the symptoms of dry eye disease (204). Furthermore, several studies have found an advantage of ω-3 fatty acids in disease prevention. Airway inflammation pre-treated with ω-3 fatty acids led to a reduced neutrophil invasion (205). In the gastrointestinal tract, MaR1 prevented chronic colitis (163). Moreover, there is an indication of an evolutionary preservation of ω-3 fatty acids, further supporting an important role for them in survival and health. Hamberg et. al. found a protective role of the ω-3 fatty acid oxylipin in bacterial infection in tobacco leaves, supporting the importance of lipid mediators in an evolutionary...
perspective (206). In addition, rainbow trout produce resolvins and protectins that are present in both neural and hemopoietic tissue (207, 208).

Thus, future studies are needed to investigate if SPMs like MaR1 and MaR2 could be a treatment option in inflammatory eye disease. The administration method, the concentration and the treatment duration should be investigated for different ω-3 fatty acids, both precursors like EPA and DHA and end products like MaR1 and MaR2.

10.3.2 AnxA1 and its peptides

AnxA1 is present in a large variety of cell types, for instance granulocytes, monocytes, stromal cells and kidney mesangial cells and is involved in resolution of inflammation *in vivo* (209). By imitating the anti-inflammatory and pro-resolving system in the body, AnxA1 is a major pharmaceutical candidate in multiple diseases. AnxA1 is a large protein (37 kDa), however, studies have found several functional peptides within the protein (209). Ac2-26 blocks leukocyte migration, Ac2-12 inhibits PMN infiltration and Ac9-25 inhibits PMN release of superoxide anions (82, 210, 211). Thus, distinct parts of the protein are likely to possess different functions in resolution of inflammation. Future studies of the different AnxA1 peptides will be interesting.

Chronic inflammatory diseases like inflammatory bowel disease and rheumatoid arthritis cause a large disease burden worldwide. Immunosuppressive treatment may cause major side effects, for instance leukopenia that increases the risk of dangerous infections. By causing resolution of inflammation as opposed to blockage of inflammation by inhibiting the SPM synthetic enzymes COX1 or COX2, we hope that SPMs will be useful as a treatment option for chronic inflammatory diseases in the future. In fact, RvE1, MaR1 and NPD1/PD1 are all in clinical development programs (102). In a phase 2 clinical trial, RVE1 was found to improve symptoms of dry eye disease (212).

10.3.3 Antibiotic resistance
One of the major global challenges in medicine is the increase in antibiotic resistance, which is associated with a higher risk of mortality and treatment failure (213). Studies found specific SPMs that shorten the resolution interval and increase host survival in bacterial E. coli infections (214). Furthermore, when an SPM is combined with antibiotics, the use of antibiotics can potentially be reduced (214). In addition to fighting bacterial infections, SPMs may play a role in reducing excessive inflammatory response. For instance, RVD2 has been shown to both reduce bacterial burden and excessive cytokine production (67). Thus, SPMs may be a future treatment option in infectious diseases, alone or in combination, both as an enhancer of treatment, but also as an “antibiotic resistance reducer”.

10.3.4 What is yet not known?

We investigated receptors and intracellular pathways used by MaR1, MaR2 and AnxA1 in rat conjunctival goblet cells. We found that these mediators, which are known to play a role in resolution of inflammation, increase $[\text{Ca}^{2+}]_i$ and stimulate glycoconjugate secretion. Future studies are needed to explore how this mucin secretion stimulation affects the tear film and if the stimulation is beneficial in health, but dysregulated in disease.

Due to cell- and species specificity, future studies investigating the role of these mediators in other cell types in the conjunctiva, including stratified squamous cells, fibroblasts, and immune cells and determining how these mediators work in human cells, would be interesting.

11 Conclusion

The goal of this thesis was to explore the specialized pro-resolving lipid mediators MaR1 and MaR2 and the protein mediator with specialized pro-resolving actions AnxA1. We found that MaR1, MaR2 and AnxA1 increased $[\text{Ca}^{2+}]_i$, stimulated glycoconjugate secretion and reduced the actions of the pro-inflammatory mediator
histamine in rat conjunctival goblet cells (89, 90, 101). Overall, our results suggest a potential role for MaR1, MaR2 and AnxA1 in resolution of ocular surface inflammation in health and disease.

12 Norwegian summary - Sammendrag

12.1 Introduksjon
Inflammatoriske øyesykdommer, slik som allergisk konjunktivitt, rammer opptil 40% av den nord-amerikanske populasjonen og medfører redusert livskvalitet og produktivitet (1). Ubalanse i mucinsekresjonen fra konjunktivale begerceller er en viktig bidragsgivende faktor ved ulike inflammatoriske øyesykdommer (12). I de seneste stadiene av en inflammasjon skifter leukocyter produksjonen fra pro-inflammatoriske til pro-resolverende mediatører, inkludert maresiner, resolviner og protectiner (6-8). Endringen fra leukotriener og prostaglandiner, til pro-resolverende lipid-mediatorer kalles «lipid mediator class switching» (54). Spesialiserte pro-resolverende mediatører (SPM-er) slik som LxA₄, RVD1, RVD2 and RVE1 har vist seg å spille en rolle i Ca²⁺-signalering og mucinproduksjon i konjunktivale begerceller hos rotter (13-16). Maresin 1 (MaR1) og Maresin 2 (MaR2) er SPM-er og dannes fra ω-3 fettsyren dokosaheksaensyre (DHA) (9, 10). Hos pattedyr, diffunderer fettsyrer som DHA raskt fra sirkulasjonen til vevet hvor inflammasjonen pågår (58). SPM-er bidrar til resolusjon ved eliminering av apopotiske celler (59, 60) og ved å begrense og regulere invasjonen av nøytrofile granulocyter (9, 60, 61). I tillegg til de pro-resolverende fettsyrene antas det pro-resolverende og anti-inflammatoriske proteinet Annexin A1 (AnxA1) å spille en viktig rolle i resolusjon av inflammasjon (17). Målet med denne avhandlingen er å utforske rollen til de pro-resolverende mediatorene MaR1, MaR2 og AnxA1 i konjunktivale begerceller og undersøke hvordan de påvirker mucinproduksjon.
12.2 Metode
Det ble dannet cellerkulturer av konjunktivale begerceller fra albinorotter (Sprague-Dawley). Konjunktiva ble dissekert løs og konjunktivale vevsbiter ble lagt i cellerkulturplater for proliferasjon i 6-10 dager. \([\text{Ca}^{2+}]\), ble målt ved hjelp av et «ratio imaging system» (InCytIm2). Mengden mucin ble målt ved hjelp av lektinet UEA-1 i et «enzyme linked lectin assay» (ELLA). Deteksjon av proteinet AnxA1 ble utført ved hjelp av revers transkriptase-PCR. cAMP ble målt ved hjelp av ELISA.

12.3 Resultat
MaR1, MaR2 og AnxA1 stimulerer mucinsekresjon gjennom økning av \([\text{Ca}^{2+}]\).
MaR1-stimulert \([\text{Ca}^{2+}]\)-økning og mucinsekresjon ble blokkert av inhibitorer av phospholipase C, proteinkinase C, \([\text{Ca}^{2+}]\)/calmodulin-avhengig proteinkinase II og extracellular-regulated kinase 1/2. PLD-inhibisjon hemmet sekresjon, men ikke \([\text{Ca}^{2+}]\)-økning. Histamin-stimulert \([\text{Ca}^{2+}]\)-økning og mucinsekresjon ble blokkert av MaR1-preinkubasjon (89). I motsetning til MaR1, ble MaR2-stimulert \([\text{Ca}^{2+}]\)-økning blokkert av en inhibitor av cAMP-avhengig proteinkinase A (PKA), men ikke av PLC. I likhet med MaR1, ble MaR2-stimulert \([\text{Ca}^{2+}]\)-økning og mucinsekresjon blokkert av PLD-inhibisjon (101). MaR1 bruker både BLT-1- og ALX/FPR2-receptoren for å øke \([\text{Ca}^{2+}]\), mens MaR2 bruker ukjente receptorer. AnxA1 virker via receptoren ALX/FPR2 og AnxA1-stimulert \([\text{Ca}^{2+}]\)-økning ble hemmet av inhibitorer av IP_{3}, sarco/endoplasmatiskt reticulum \([\text{Ca}^{2+}]\)-ATPase (SERCA) og PKC (90). Inhibiterer av PLD, ERK1/2 og PLA_{2} blokkerte kun mucinsekresjon.

12.4 Diskusjon/konklusjon
MaR1 bruker ALX/FPR2- og BLT-1-receptorene for å opprettholde vevshomeostase i konjunktivale begerceller hos rotter ved å bidra til en balansert mucinsekresjon. AnxA1 bruker ALX/FPR2-receptoren for å utøve sin virkning, mens MaR2 bruker ukjente receptorer for å påvirke mucinsekresjon. Både MaR1, MaR2 og AnxA1 reduserer histamin-stimulert \([\text{Ca}^{2+}]\)-økning og mucinsekresjon, som understøtter deres viktige rolle i resolusjon av inflammasjon (89, 90, 101). \(\omega\)-3-tilskudd (EPA og DHA) har vist seg å ha en positiv effekt på tåreproduksjon og tåreosmolaritet (202). I tillegg har flere SPM-er og deres forløpere blitt påvist i mennesketårer i konsentrasjoner som korresponderer med deres bioaktive funksjon (203). SPM-ene RvE1, MaR1 og NPD1/PD1 er alle i kliniske studier (102). RVE1 bedret symptomene på tørre øyne i en fase-2 klinisk studie (212). Våre funn indikerer at MaR1, MaR2 og AnxA1 spiller en viktig rolle i vevshomeostasen på øyets overflate og at disse
mediatorene kan ha en potensiell rolle som behandling mot inflammatoriske sykdommer.

13 English Summary

13.1 Introduction
Ocular surface inflammatory diseases, such as allergic conjunctivitis, harm up to 40% of the North American population. These diseases lead to reduced quality of life and productivity (1). Alterations in the amount of mucin secreted from conjunctival goblet cells contribute to the symptoms of inflammatory eye diseases (12). In the late stages of inflammation, leukocytes switch from producing pro-inflammatory mediators to pro-resolving mediators, such as maresins, resolvins and protectins (6-8). The switch of leukotriens and prostaglandins to pro-resolving lipid mediators is called “lipid mediator class switching” (54). Specialized pro-resolving mediators (SPMs) such as LxA₄, RVD1, RVD2 and RVE1 have been found to play a role in Ca²⁺-signaling and mucin secretion in conjunctival goblet cells in rats (13-16). Maresin 1 (MaR1) and Maresin 2 (MaR2) are SPMs generated from the ω-3 fatty acid docosahexaenoic acid (DHA) (9, 10). In mammals, fatty acids like DHA have been found to move rapidly from the circulation to inflammatory tissue through diffusion (58). SPMs contribute to resolution of inflammation by activating immune cells, eliminating apoptotic cells (59, 60) and by limiting and regulating invasion of neutrophils (9, 60, 61). In addition to the pro-resolving fatty acids, the pro-resolving and anti-inflammatory protein Annexin A1 (AnxA1) plays a role in resolution of inflammation (17). The main goal of this thesis is to explore the roles of the pro-resolving mediators MaR1, MaR2 and AnxA1 in conjunctival goblet cells and to investigate how the mediators influence mucin secretion.

13.2 Method
Experiments were performed on cell cultures of conjunctival goblet cells from albino Sprague-Dawley rats. Conjunctiva was dissected and cells proliferated for 6-10 days. [Ca²⁺] was measured using a ratio imaging system (InCytIm2). An “enzyme linked lectin assay” (ELLA) using UEA-1 was performed to measure the amount of mucins. AnxA1 was detected through reverse transcriptase-PCR. ELISA was used to measure the amount of cAMP.
13.3 Results
MaR1, MaR2 and AnxA1 stimulate mucin secretion by increasing $[\text{Ca}^{2+}]_{i}$. MaR1-stimulated $[\text{Ca}^{2+}]_{i}$-increase and mucin secretion were blocked by inhibitors of phospholipase C, protein kinase C, $\text{Ca}^{2+}$/calmodulin-dependent protein kinase II and extracellular-regulated kinase 1/2. PLD-inhibition blocked secretion, but not $[\text{Ca}^{2+}]_{i}$-increase. Histamine-stimulated $[\text{Ca}^{2+}]_{i}$-increase and secretion were blocked by MaR1 preincubation (89). Unlike MaR1, MaR2-stimulated $[\text{Ca}^{2+}]_{i}$-increase was blocked by an inhibitor of cAMP-dependent protein kinase A (PKA), but not by PLC. Like MaR1, MaR2-stimulated $[\text{Ca}^{2+}]_{i}$-increase and mucin secretion were blocked by PLD-inhibition (101). MaR1 uses both the BLT-1 receptor and the ALX/FPR2 receptor to increase $[\text{Ca}^{2+}]_{i}$, but only the BLT-1 receptor to stimulate mucin secretion. AnxA1 works by binding to the ALX/FPR2 receptor and AnxA1-stimulated $[\text{Ca}^{2+}]_{i}$-increase was blocked by inhibitors of IP$_3$, sarco/endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) and PKC (90). Inhibitors of PLD, ERK1/2 and PLA$_2$ only blocked secretion.

13.4 Discussion and conclusion
MaR1 uses the ALX/FPR2 receptor and the BLT-1 receptor to maintain tissue homeostasis in conjunctival goblet cells by balancing the mucin secretion. AnxA1 uses the ALX/FPR2 receptor, while MaR2 uses unknown receptors to influence mucin secretion. Both MaR1, MaR2 and AnxA1 reduce histamine-stimulated $[\text{Ca}^{2+}]_{i}$-increase and mucin secretion, which supports their potential important role in resolution of inflammation (89, 90, 101). $\omega$-3 supplements (EPA and DHA) have been shown to be beneficial for tear production and tear osmolarity (202). In addition, multiple SPMs and their precursors have been found in human tears in concentrations corresponding to their bioactive functions (203). The SPM RvE1, MaR1 and NPD1/PD1 are all in clinical trial programs (102). RVE1 decreased symptoms in dry eye disease in a phase 2 clinical trial study (212). Our findings indicate that MaR1, MaR2 and AnxA1 play an important role in ocular tissue homeostasis and that they have a potential future role in the treatment of ocular surface disease.
References


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**Errata**

Name of the candidate: Markus Vicente Tørud Olsen

Title of thesis: The Pro-resolving Mediators Maresin 1, Maresin 2 and Annexin A1 in Maintenance of Ocular Surface Health

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Paper 1
Maresin 1, a specialized proresolving mediator, stimulates intracellular \([\text{Ca}^{2+}]\) and secretion in conjunctival goblet cells

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Funding information
Norwegian Research Council; National Eye Institute, Grant/Award Number: R01 EY019470; National Institute of General Medical Sciences, Grant/Award Number: R01GM038765

Abstract
Mucin secretion from conjunctival goblet cells forms the tear film mucin layer and requires regulation to function properly. Maresin 1 (MaR1) is a specialized proresolving mediator produced during the resolution of inflammation. We determined if MaR1 stimulates mucin secretion and signaling pathways used. Cultured rat conjunctival goblet cells were used to measure the increase in intracellular \([\text{Ca}^{2+}]\) concentration and mucin secretion. MaR1 increased \([\text{Ca}^{2+}]\) and secretion were blocked by inhibitors of phospholipase C, protein kinase C, \text{Ca}^{2+}/\text{calmodulin-dependent} protein kinase II, and extracellular-regulated kinase 1/2. MaR1 added before addition of histamine counterregulated histamine-stimulated increase in \([\text{Ca}^{2+}]\), and secretion. We conclude that MaR1 likely has two actions in conjunctival goblet cells: first, maintaining optimal tear film mucin levels by increasing \([\text{Ca}^{2+}]\), and stimulating mucin secretion in health and, second, attenuating the increase in \([\text{Ca}^{2+}]\), and overproduction of mucin secretion by counterregulating the effect of histamine as occurs in ocular allergy.

KEYWORDS
conjunctiva, goblet cell, signal transduction, specialized proresolving mediator (SPM)

1 | INTRODUCTION

The ocular surface consists of the cornea and conjunctiva, the apical layer of which is stratified nonkeratinized epithelium. Present within the conjunctival epithelium are mucin-producing goblet cells that are essential for the protection of the ocular surface (Hodges & Dartt, 2013). The mucin secreted from the goblet cells is an essential component of the tear film and functions to clear allergens, pathogens, and other potentially damaging substances from the ocular surface and to lubricate the surface (Mantelli & Argueso, 2008). The goblet cells have an important role in maintaining the health of the ocular surface by ensuring a balanced amount of mucin secretion under normal, physiologic conditions. In disease, both mucin overproduction and deficiency can lead to diseases of the ocular surface (Dartt, 2004). Inflammatory diseases such as allergic conjunctivitis, keratoconjunctivitis sicca (dry eye), Sjögren’s syndrome, and bacterial and viral conjunctivitis can cause alterations in mucin homeostasis. These alterations in the normally balanced mucin secretion due to inflammatory disease can result in reduced quality of life and impairment of vision (Barabino, Labetoulle, Rolando, & Messmer, 2016; Mantelli & Argueso, 2008; Palmares et al., 2010; Uchino & Schaumberg, 2013). Treatment options for inflammatory diseases are limited and often ineffective for many patients.

The resolution of disease-induced inflammation is an active process with a complex time- and cell-dependent interaction between epithelial cells and inflammatory cells with proinflammatory and proresolving...
mediators (Serhan & Petasis, 2011). During resolution of the inflammatory process, a complex program of biochemical mediator production and leukocyte influx work to actively terminate the inflammatory process (Serhan & Savill, 2005). Immune and epithelial cells biosynthesize lipid compounds called specialized proresolving mediators (SPMs), which have both an anti-inflammatory and novel proresolving actions (Dalli & Serhan, 2016; Serhan, Dalli, Colas, Winkler, & Chiang, 2015). Macrophages are important immune cells in the inflammatory regulation process and biosynthesize specific family of SPMs, termed maresins (macrophage mediators in the resolution of inflammation, MaR; Serhan et al., 2009) Maresin 1 (MaR1) is the first MaR discovered and is produced by macrophages when stimulated by efferocytosis of polymorphonuclear neutrophils (PMNs) or PMN microparticles (Dalli & Serhan, 2016). MaR are biosynthesized from the precursor ω-3 fatty acid docosahexaenoic acid (DHA) known to be important in ocular physiology (Dartt & Masli, 2014; Hodges, Li, Shatos, Serhan, & Dartt, 2016; Li et al., 2013; Lipsettad, Hodges, Utheim, Serhan, & Dartt, 2017; Saban et al., 2019) and their production is increased as M1 macrophages are converted to M2 macrophages (Serhan et al., 2015). The complete structure of MaR1 is established and confirmed by total organic synthesis, and its structure portends proresolving actions (Serhan et al., 2012). MaR1 can then interact with PMNs and macrophages via specific receptors including the recently discovered leucine-rich-repeat-containing G protein-coupled receptor 6 (LRG6) that is responsible for MaR1 proresolving actions (Chiang, Liberos, Norris, de la Rosa, & Serhan, 2019; Colas et al., 2016; Gu et al., 2018). MaR1 limits PMN tissue infiltration during the inflammatory response, which helps to prevent and reduce local tissue damage, contributes to the resolution of inflammation, increases regeneration, and is nociective (Serhan et al., 2009). Many of the target tissues of MaR1 in which MaR1 functions have yet to be identified and are of interest.

The purpose of this study was to determine if MaR1 interacts with rat conjunctival goblet cells and regulates the secretory function of these cells under physiological regulation and during inflammatory disease. One of the main stimulatory signals for high molecular weight glycoprotein secretion that includes mucins is an increase in the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]). We used inhibitors of receptors, enzymes, and downstream molecules of different intracellular pathways to determine if MaR1 increases [Ca$^{2+}$], and stimulation mucin secretion and to identify the signaling pathways used. In addition, because MaR1 is thought to be a central proresolving mediator during inflammatory diseases, we investigated the actions of MaR1 on increase in [Ca$^{2+}$], and secretion stimulated by the al­lergic, proinflammatory mediator, histamine.

2 MATERIALS AND METHODS

2.1 Materials

Roswell Park Memorial Institute (RPMI) 1640 cell culture medium, penicillin/streptomycin, and l-glutamine were purchased from Lonza (Walkerville, IL). Fetal bovine serum (FBS) was from Atlanta Biologicals (Norcross, GA). Ulex europaeus agglutinin I (UEA-1), histamine, carbachol (Cch), aristolochic acid (AA), 2-aminoethoxydiphenyl borate (2-APB), 1-butanol (1-but), and t-butanol (t-but) were obtained from Sigma-Aldrich (St. Louis, MO). MaR1 (7R,14S-dihydroxy-4Z,8E,10E,12Z,16Z,19Z-docosahexaenoic acid) was from Cayman Chemical (Ann Arbor, MI). MaR1 was stored in an ethanol solution at -80°C as supplied by the manufacturer. The solution was diluted immediately before use in Krebs-Ringer bicarbonate buffer with 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid (KR-BEPEHS, 119 mM NaCl, 4.8 mM KCl, 1.0 mM CaCl$_2$, 1.2 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$, 10 mM HEPES, and 5.5 mM glucose [pH 7.40–7.45]) to the desired concentrations and added to the cells.

VIP, U73122, U73343, KN92, and KN93 were purchased from Tocris Bioscience (Ellisville, MO). Fura-2-acetoxyethyl ester (Fura-2/AM) and 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA/AM) were purchased from Life Technologies (Grand Island, NY). UO126 was ordered from R&D Systems. Lipoxin A$_4$ (LXA$_4$), H89, thapsigargin, and RO-317549 were ordered from EMD Millipore (Billerica, MA). All inhibitors were initially diluted and stored in dimethyl sulfoxide at -20°C. Immediately before use, the inhibitors were diluted to desired concentrations in KR-BEPEHS and added to the cells.

2.2 Animals

Four-to-eight-week-old male albino Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing between 125 and 150 g were anesthetized with CO$_2$ for 5 min, decapitated, and the bulbar and fornical conjunctival epithelia removed from both eyes. All experiments were in accordance with the US National Research Council’s Guide for the Care and Use of Laboratory Animals, the US Public Health Service’s Policy on Humane Care and Use of Laboratory Animals, and Guide for the Care and Use of Laboratory Animals and were approved by the Scheckens Eye Research Institute Animal Care and Use of Committee.

2.3 Cell culture

The conjunctival tissue was dissected and placed in six-well plates with 0.5 ml RPMI 1640 medium supplemented with 10% FBS, 2 mM l-glutamine, and 100 mg/ml penicillin-streptomycin. RPMI medium was changed every second day. Cultured goblet cells were identified periodically by staining with anti-cytokeratin 7, anti-MUC5AC, and the lectin UEA-1 directly conjugated to a fluorophore. The cells were trypsinized and transferred to glass bottom dishes for Ca$^{2+}$ experiments or 24-well plates for secretion experiments as described previously (Hayashi et al., 2012; Hodges et al., 2017, 2016; Li, Carozza, Shatos, Hodges, & Dartt, 2012; Li et al., 2013; Li, Jiao, Shatos, Hodges, & Dartt, 2013; Lipsettad et al., 2017).
2.4 Measurement of high molecular weight glycoprotein secretion

Cultured rat conjunctival goblet cells were trypsinized and transferred to 24-well plates. The cells were serum-starved in RPMI 1640 media containing 0.35% bovine serum album (BSA) for 2 hr. MaR1 (10^{-10}–10^{-7} M) was then added alone or the cells were incubated with inhibitors for 30 min, and then stimulated with MaR1 (10^{-9} M), histamine (10^{-5} M) or the cholinergic agonist Cch (10^{-4} M) for 2 hr. As an index of mucin secretion, the amount of goblet cell high molecular weight glycoprotein (HMWG) secretion was measured using the lectin UEA-1 in an enzyme-linked lectin assay (Hayashi et al., 2012; Hodges et al., 2017, 2016; Li et al., 2012; Li, Hodges, et al., 2013; Li, Jiao, et al., 2013; Lippestad et al., 2017). Cells were homogenized and the amount of protein was determined using the Bradford assay. Glycoprotein secretion was standardized to amount of protein in each condition and is shown as fold increase above basal (which was set to 1).

2.5 Measurement of [Ca^{2+}]

Cultured rat conjunctival goblet cells were trypsinized and transferred to 35-mm glass bottom dishes and incubated at 37°C overnight. The cells were then incubated at 37°C for 1 hr with KRB-HEPES containing 0.5% BSA, 0.5 μM of Fura-2/AM, 250 μM sulfinpyrazone, and 8 μM pluronic acid F127. [Ca^{2+}] was measured with a ratio imaging system (InCyt; Intracellular Imaging, Cincinnati, OH) using wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. A minimum of 10 goblet cells were selected and the Ca^{2+} response was followed for ~2 min. MaR1 was either added alone or cells were pretreated with inhibitors before addition of either MaR1 or a positive control. The inhibitors were added 30 min before MaR1 except I-but, t-but, and thapsigargin were added 15 min before MaR1. Change in peak [Ca^{2+}] was calculated by subtracting the average basal [Ca^{2+}] from the peak [Ca^{2+}].

2.6 Measurement of cyclic adenosine monophosphate level

Goblet cells seeded in 24-well plates were grown to 75% confluence. All cells were incubated with 3-isobutyl-1-methylxanthine 10^{-6} M for 40 min total. MaR1 (10^{-6}) M was added for 40 min. VIP (10^{-8} M) added for 5 min was the positive control. Cells were lysed in 0.1 M HCl. Total cell cyclic adenosine monophosphate (cAMP) was assayed by direct cAMP enzyme-linked immunosorbent assay kit following the manufacturer’s instructions (Enzo Life Sciences, Farmingdale, NY). The acetylation protocol was used to increase sensitivity. Total cell protein was determined by the Bradford assay, and the cellular cAMP was normalized to total protein. cAMP levels are presented in real numbers.

2.7 Statistical analysis

Data are expressed as mean ± standard error of the mean. Data were analyzed by either Student’s t-test or one-way analysis of variance followed by the Tukey test. A p < 0.05 was considered significant.

3 RESULTS

3.1 MaR1 increases high molecular weight glycoprotein secretion in rat conjunctival goblet cells

Other SPMs including LXAs, resolvin D1 (RvD1), and resolvin E1 (RvE1) stimulate HMWG secretion, our index of mucin secretion, in rat conjunctival goblet cells (Hodges et al., 2017; Li et al., 2013; Lippestad et al., 2017; Lippestad, Hodges, Utheim, Serhan, & Dartt, 2018). To determine if MaR1 increases HMWG secretion, goblet cells were incubated with MaR1 (10^{-10}–10^{-7} M) and secretion measured. The cholinergic agonist Cch 10^{-4} M was used as a positive control (Hayashi et al., 2012; Rios et al., 1999). MaR1 at 10^{-10} and 10^{-9} M increased HMWG secretion significantly above basal by 2.0 ± 0.1 fold and 2.4 ± 0.1 fold above basal (Figure 1; n = 3), respectively. The two highest concentrations of MaR1 (10^{-8} and 10^{-7} M) did not stimulate secretion. Cch significantly increased mucin secretion 2.2 ± 0.2 fold above basal (n = 3). Thus, MaR1 stimulates HMGC secretion with a peak at 10^{-9} M.

3.2 MaR1 increases [Ca^{2+}] in rat conjunctival goblet cells

In addition to stimulating secretion, the SPMs LXAs, RvD1, and RvE1 also increase [Ca^{2+}] in rat conjunctival goblet cells (Hodges et al., 2017;
Li et al., 2013; Lippes et al., 2018, 2017). To determine if MaR1 also increases \([Ca^{2+}]_i\), cultured goblet cells were incubated in fura-2/AM for 1 hr and stimulated with MaR1 (\(10^{-10} \text{--} 10^{-7} \text{M}\)). Each concentration of MaR1 statistically significantly increased \([Ca^{2+}]_i\) (Figure 2a-c; n = 6). MaR1-stimulated increase in \([Ca^{2+}]_i\) was 178.2 ± 68.4 nM at \(10^{-10} \text{M}\), 120.5 ± 29.0 nM at \(10^{-9} \text{M}\), 220.3 ± 37.8 at \(10^{-8} \text{M}\), and 160.1 ± 27.4 nM at \(10^{-7} \text{M}\) (Figure 2c). The maximum increase in peak \([Ca^{2+}]_i\) occurred at MaR1 10^{-8}M, thus this was the concentration of MaR1 used in subsequent experiments. In the cells from the same animals, histamine, the positive control, increased \([Ca^{2+}]_i\), by 305.7 ± 66.8. MaR1 increases \([Ca^{2+}]_i\), in conjunctival goblet cells.

### 3.3 MaR1-stimulated increase high molecular weight glycoprotein secretion is dependent upon \([Ca^{2+}]_i\) in rat conjunctival goblet cells

To determine if MaR1 utilizes the increase in \([Ca^{2+}]_i\) to stimulate HMWG secretion, rat conjunctival goblet cells were loaded with the intracellular calcium chelator BAPTA. First, to determine the appropriate concentration of BAPTA ester (BAPTA/AM) to use to chelate \([Ca^{2+}]_i\), cells were preincubated for 30 min with BAPTA/AM (10^{-4}M) and MaR1/uni2010 stimulated increase in \([Ca^{2+}]_i\) measured. MaR1 (10^{-9}M) increased \([Ca^{2+}]_i\), by 243.7 ± 57.5 nM (Figure S1; n = 5). When these cells were preincubated with BAPTA/AM, the response was significantly reduced to 82.8 ± 11.1 nM, a decrease of ∼66%. Similar results were obtained when cells were stimulated with LXA₄.

To determine if HMWG secretion is altered by chelation of Ca^{2+}, cells were incubated with BAPTA/AM (10^{-4}M) and MaR1/uni2010 stimulated HMWG secretion was measured. MaR1 (10^{-9}M) stimulated secretion by 1.6 ± 0.04 fold above basal (Figure 3; n = 3). Incubation with BAPTA/AM significantly decreased this response and was 0.91 ± 0.14 fold of basal (Figure 3). Cch/uni2010-stimulated increase in secretion was also significantly decreased and was below basal levels when incubated with BAPTA/AM. These data indicate that the MaR1 increase in \([Ca^{2+}]_i\) stimulates goblet cell secretion.

**FIGURE 2** Maresin 1 (MaR1) stimulates an increase in \([Ca^{2+}]_i\). Pseudocolor images of rat conjunctival goblet cells stimulated with MaR1 (10^{-8}M) (a) at t = 0, 30, 50, and 80 s. Changes in \([Ca^{2+}]_i\) over time with different concentrations of MaR1 are shown in (b). Change in peak \([Ca^{2+}]_i\) is shown in (c). Data are mean ± SEM from six experiments. *Significance above basal. Arrow indicates addition of MaR1. \([Ca^{2+}]_i\), intracellular Ca^{2+}; SEM, standard error of the mean

**FIGURE 3** Inhibition of \([Ca^{2+}]_i\), blocks maresin 1 (MaR1)-stimulated high molecular weight glycoprotein secretion. Rat conjunctival goblet cells were incubated with the Ca^{2+} chelator BAPTA/AM and secretion measured for 2 hr in response to MaR1 (10^{-9}M) or the cholinergic agonist carbachol (Cch, 10^{-4}M). Data are mean ± SEM from three experiments. *significance above basal. BAPTA, 1,2/bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; SEM, standard error of the mean
3.4 | MaR1 activates the phospholipase C pathway to increase [Ca\(^{2+}\)] and stimulate high molecular weight glycoprotein secretion in cultured rat goblet cells

The phospholipase C (PLC) pathway regulates cellular events such as exocytosis, fluid secretion, contraction, metabolism, proliferation, ion channel opening, and many other cellular processes (Berridge, 2009). The association between the PLC pathway and intracellular Ca\(^{2+}\) is well established (Hokin, 1966; Michell, 1975). To determine if MaR1 uses the PLC pathway to increase Ca\(^{2+}\), goblet cells were incubated with the PLC inhibitor U73122 (10\(^{-7}\) M) or the inactive control U73343 (10\(^{-7}\) M) for 30 min before MaR1 (10\(^{-8}\) M) stimulation. MaR1 significantly stimulated [Ca\(^{2+}\)] to a peak value of 213.5 ± 19.4 nM (Figure 4a; n = 3). The active inhibitor U73122 caused a significant decrease in stimulated [Ca\(^{2+}\)], to a peak of 55.4 ± 5.7 nM. Incubation with the inactive control U73343 caused an increase in [Ca\(^{2+}\)], by a maximum of 134.4 ± 31.1 nM which is unchanged from MaR1 alone. Cch, which is known to activate PLC in goblet cells (Dartt et al., 2000), also significantly increased [Ca\(^{2+}\)], which was blocked by U73122, but not U73343 (Figure 4a).

Similar to the increase in [Ca\(^{2+}\)], both the MaR1- and Cch-stimulated increase in secretion was significantly blocked by U73122 but not U73343 (Figure 4b; n = 3). This suggests that MaR1 activates PLC to increase [Ca\(^{2+}\)], and stimulate secretion.

3.5 | MaR1 releases Ca\(^{2+}\) from intracellular stores to increase [Ca\(^{2+}\)] and stimulate high molecular weight glycoprotein secretion in cultured rat goblet cells

It is well known that the PLC pathway generates inositol trisphosphate (IP\(_3\)) that binds to IP\(_3\) receptors on the membrane of an intracellular organelle, probably endoplasmic reticulum that stores intracellular Ca\(^{2+}\) (Williamson, 1986). This binding causes a release of Ca\(^{2+}\) from the intracellular stores into the cytoplasm to increase [Ca\(^{2+}\)]. To determine if MaR1 increases IP\(_3\) to raise the [Ca\(^{2+}\)], rat conjunctival goblet cells were incubated with the IP\(_3\) receptor inhibitor 2-APB (10\(^{-5}\) M) for 30 min before adding MaR1 (10\(^{-8}\) M). MaR1 significantly increased [Ca\(^{2+}\)], by 321.0 ± 100.3 nM (Figure 4c; n = 3). In the presence of 2-APB, the response was 44.2 ± 18.9 nM, a significant decrease from MaR1 alone. The positive control, Cch significantly increased [Ca\(^{2+}\)], that was blocked by preincubation with 2-APB.

The effect of 2-APB on glycoconjugate secretion was then explored. MaR1-increased secretion 2.2 ± 0.2 fold above basal. Treatment with 2-APB significantly decreased MaR1-stimulated response to 0.8 ± 0.1 fold above basal (Figure 4d; n = 3). Cch increased secretion 2.9 ± 0.4 fold above basal, which was also significantly inhibited by 2-APB. These data indicate that MaR1 generates IP\(_3\) to increase [Ca\(^{2+}\)], and stimulate secretion.

To investigate further MaR1’s effect on the release of calcium from intracellular stores, we used the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor thapsigargin. Thapsigargin depletes intracellular Ca\(^{2+}\) stores by preventing the reuptake of Ca\(^{2+}\) that passively leaks out. If MaR1 uses this store, addition of MaR1 after thapsigargin will not increase [Ca\(^{2+}\)]. Rat conjunctival goblet cells incubated with MaR1 alone stimulated an increase in [Ca\(^{2+}\)], to a maximum of 189.6 ± 36.7 nM (Figure 4e; n = 3). When cells were treated with thapsigargin (10\(^{-5}\) M) for 15 min, then stimulated with MaR1 (10\(^{-8}\) M), the peak response to MaR1 was decreased to 61.9 ± 6.5 nM. Cch-stimulated increase in [Ca\(^{2+}\)], the positive control, was also blocked by incubation with thapsigargin (Figure 4e). This is an additional support that MaR1 releases Ca\(^{2+}\) from intracellular stores.

3.6 | MaR1 does not activate influx of extracellular Ca\(^{2+}\)

To determine if the MaR1-stimulated increase in [Ca\(^{2+}\)], is dependent on the influx of extracellular Ca\(^{2+}\), goblet cells were incubated in KRB buffer with and without 1.0 mM CaCl\(_2\). In the presence of extracellular Ca\(^{2+}\), MaR1 (10\(^{-8}\) M) significantly increased [Ca\(^{2+}\)], by 191.5 ± 60.0 nM (Figure 4f; n = 5). When extracellular CaCl\(_2\) was removed, the increase in [Ca\(^{2+}\)], stimulated by MaR1 was 133.3 ± 41.7 nM, which was not a significant decrease. In contrast, Cch-stimulated increase in [Ca\(^{2+}\)], was significantly decreased by the removal of extracellular Ca\(^{2+}\) (Figure 4f). This suggests that MaR1 is mainly dependent on intracellular calcium stores, and not influx of extracellular calcium to increase [Ca\(^{2+}\)].

3.7 | MaR1 activates protein kinase C to increase [Ca\(^{2+}\)] and stimulate HMWG secretion in cultured rat goblet cells

Protein kinase C (PKC) was discovered by Nishizuka (1984). This enzyme can be activated by the PLC pathway, which generates diacylglycerol that in turn activates PKC. To determine if MaR1 stimulates PKC activity to increase [Ca\(^{2+}\)], rat conjunctival goblet cells were treated with the PKC inhibitor RO-317549 for 30 min, then stimulated with MaR1 (10\(^{-8}\) M). MaR1 significantly increased [Ca\(^{2+}\)], to a peak of 191.6 ± 6.1 nM (Figure 5a; n = 4). Incubation of MaR1 with RO-317549 (10\(^{-7}\) M) caused a significantly decreased response of 118.3 ± 25.9 nM. Cch was again used as a positive control as it is known to activate PKC in conjunctival goblet cells (Dartt et al., 2000). Cch-stimulated increase in [Ca\(^{2+}\)], was significantly reduced after incubation with RO-317549 (Figure 5a). Thus, MaR1 activates PKC to increase [Ca\(^{2+}\)].

The effect of RO-317549 on MaR1-stimulated increase in glycoconjugate secretion was investigated. MaR1 increased secretion 1.5 ± 0.1 fold above basal (Figure 5b; n = 3), and was significantly blocked by RO-317549 to 1.1 ± 0.1 fold above basal. Cch-stimulated increase in secretion was also significantly blocked by RO-317549 (Figure 5b). These results demonstrate that MaR1 activates PKC as a
FIGURE 4  Inhibition of phospholipase C (PLC) blocks maresin 1 (MaR1)-stimulated increase in \([\text{Ca}^{2+}]_i\). Goblet cells were incubated with the PLC inhibitor U73122 (10^-7 M), its negative control U73143 (10^-7 M, a and b) or (c and d) IP3 receptor inhibitor 2-APB (10^-5 M) for 30 min and stimulated with MaR1 at 10^-8 M for \([\text{Ca}^{2+}]_i\), or 10^-9 M for secretion or carbachol (Cch, 10^-4 M). (a) and (c) show the change in peak \([\text{Ca}^{2+}]_i\), while (b) and (d) show high molecular weight glycoprotein secretion. (e) Rat conjunctival goblet cells were preincubated with the SERCA-inhibitor thapsigargin (10^-5 M) for 15 min and stimulated with either MaR1 (10^-8 M) or carbachol (Cch, 10^-4 M). (f) Goblet cells were incubated with KRB with or without CaCl2 and stimulated with MaR1 (10^-8 M) or Cch (10^-4 M). (e and f) The change in peak \([\text{Ca}^{2+}]_i\). Data are mean ± SEM of three (a–e) or five (f) experiments. *Significance above basal. #Significant difference from MaR1 or Cch alone. 2-APB, 2-aminoethoxydiphenyl borate; \([\text{Ca}^{2+}]_i\), intracellular \([\text{Ca}^{2+}]\); KRB, Krebs-Ringer bicarbonate buffer; SEM, standard error of the mean; SERCA, sarco/endoplasmic reticulum.
component of the PLC signaling pathway to increase $[\text{Ca}^{2+}]_{i}$ and stimulate secretion.

### 3.8 MaR1 utilizes calcium/calmodulin-dependent protein kinase II to increase $[\text{Ca}^{2+}]_{i}$ and stimulate secretion in cultured rat goblet cells

In addition to PLC, other SPMs such as LXA$_4$ and RVD1 use calcium/calmodulin-dependent protein kinase II (CaMKII) to increase $[\text{Ca}^{2+}]_{i}$ (Hodges et al., 2017; Lippestad et al., 2017). To investigate if MaR1 also uses CaMKII to increase $[\text{Ca}^{2+}]_{i}$, we used the CaMKII inhibitor KN93 and its inactive control KN92. MaR1 (10$^{-8}$ M) significantly increased $[\text{Ca}^{2+}]_{i}$ by 219.2 ± 58.3 nM (Figure 5c; n = 5). Incubation with KN93 (10$^{-7}$ M) before addition of MaR1 (10$^{-8}$ M) caused a significantly inhibited response of 85.3 ± 17.9 nM. Preincubation with the inactive compound KN92 (10$^{-7}$ M) caused a MaR1-stimulated response of 137.2 ± 28.2 nM (Figure 5c), which is not a significant decrease. These data indicate that MaR1 uses CaMKII to increase $[\text{Ca}^{2+}]_{i}$.

Cch was again used as a positive control, and Cch significantly stimulated $[\text{Ca}^{2+}]_{i}$ (Figure 5c). The Cch-stimulated response was significantly blocked by KN93, but not KN92. Similar to the increase in $[\text{Ca}^{2+}]_{i}$, MaR1-stimulated secretion was inhibited by KN93, but not KN92 (Figure 5d; n = 3). MaR1 alone significantly stimulated secretion 1.8 ± 0.1 fold above basal, and was completely blocked to 0.8 ± 0.3 fold by KN93, but not KN92. Cch-stimulated increase in secretion was also blocked by KN93, but not KN92. These data indicate that MaR1 activates CaMKII to increase $[\text{Ca}^{2+}]_{i}$ and stimulate secretion.
MaR1 does not activate phospholipase D (PLD) nor phospholipase A2 (PLA₂) to increase \([\text{Ca}^{2+}]_i\), and high molecular weight glycoprotein secretion. Rat conjunctival goblet cells were preincubated with the PLD inhibitor 0.3% 1-butanol or the inactive control 0.3% t-butanol for 15 min and stimulated with MaR1 at \(10^{-8}\) M for \([\text{Ca}^{2+}]_i\), or \(10^{-9}\) M for secretion or carbachol (Cch, \(10^{-4}\) M). Changes in peak \([\text{Ca}^{2+}]_i\), are shown in (a) and secretion shown in (b). Goblet cells were incubated with the PLA₂ inhibitor aristolochic acid (AA; \(10^{-6}-10^{-5}\) M) for 30 min and stimulated with MaR1 at \(10^{-8}\) M for \([\text{Ca}^{2+}]_i\), or \(10^{-9}\) M for secretion or Cch (\(10^{-4}\) M). Data show changes in peak \([\text{Ca}^{2+}]_i\), (c) while secretion is shown in (d). Data are mean ± SEM of four (a), three (b), eight (c), and three (d) experiments. *Significance from basal. #Significance from MaR1 alone. [Ca²⁺], intracellular Ca²⁺; SEM, standard error of the mean.
Inhibition of extracellular-regulated kinase 1/2 (ERK 1/2) blocks maresin 1 (MaR1)-stimulated increase in \([\text{Ca}^{2+}]_i\) and high molecular weight glycoprotein secretion. Goblet cells were preincubated with the ERK 1/2 inhibitor UO126 (10^-8 M) for 30 min and stimulated with MaR1 at 10^-8 M for [Ca^{2+}]_i or 10^-7 M for secretion or carbachol (Ch, 10^-5 M). Changes in [Ca^{2+}]_i over time are shown in (a). Changes in peak [Ca^{2+}]_i are shown in (b). Secretion is shown in (c). Data are mean \pm SEM of three experiments. *Significance from MaR1 or Cch. Arrow indicates addition of MaR1. [Ca^{2+}]_i, intracellular Ca^{2+}; SEM, standard error of the mean.
These findings suggest that MaR1 does not activate cAMP/uni2010-dependent PKA to increase [Ca\textsuperscript{2+}]. To examine the dependency of MaR1 on PKA to stimulate secretion, rat conjunctival goblet cells were treated with H89 (10\textsuperscript{-7}−10\textsuperscript{-5} M) for 30 min before addition of MaR1. MaR1 increased secretion by 1.8 ± 0.14 fold above basal (Figure 8d; n = 7). Incubation with H89 did not significantly block MaR1-stimulated secretion and was 1.4 ± 0.5 fold above basal. VIP was used as a

FIGURE 8  Inhibition of the cAMP-dependent protein kinase A (PKA) does not block maresin 1 (MaR1)-stimulated increase in [Ca\textsuperscript{2+}], high molecular weight glycoprotein secretion, and MaR1 does not increase (cAMP). Goblet cells were preincubated with the cAMP-dependent PKA inhibitor H89 at 10\textsuperscript{-7}−10\textsuperscript{-5} M concentrations for 30 min and stimulated with MaR1 at 10\textsuperscript{-8} M for [Ca\textsuperscript{2+}], and cAMP levels or 10\textsuperscript{-9} M for secretion or VIP (10\textsuperscript{-8} M). Changes in [Ca\textsuperscript{2+}] over time is shown in (a) and (b), while changes in peak [Ca\textsuperscript{2+}] are shown in (c). Secretion is shown in (d). The amount of cAMP in goblet cells is shown in (e). Data are mean ± SEM of four (a–c), seven (d), and five (e) experiments. *Significance above basal. #Significance from MaR1 or VIP alone. Arrow indicates addition of MaR1 or VIP. cAMP, cyclic adenosine monophosphate; [Ca\textsuperscript{2+}], intracellular Ca\textsuperscript{2+}; SEM, standard error of the mean.
positive control and VIP-stimulated secretion was blocked by H89.

To confirm these results, the effect of MaR1 on cAMP levels on rat conjunctival goblet cells was determined. The basal level of cAMP was 0.75 ± 0.28 pg cAMP/μg protein (Figure 8c; n = 5). MaR1 caused an increase to 1.11 ± 0.20 pg cAMP/μg protein, which was not significantly different from basal. VIP was used as a positive control. VIP caused an increase to 2.20 ± 0.45 pg cAMP/μg protein, significantly different from basal (data not shown). This suggests that MaR1, in contrast to VIP, does not alter the level of cAMP and does not activate PKA to increase \([\text{Ca}^{2+}]_{i}\), or stimulate secretion in conjunctival goblet cells.

3.12 | Increase in \([\text{Ca}^{2+}]_{i}\) and the stimulation of HMWG secretion by histamine is counterregulated by MaR1 in rat conjunctival goblet cells

Histamine is a major mediator contributing to the symptoms of allergic conjunctivitis (del Cuvillo et al., 2009). To examine the effect of MaR1 on histamine stimulation in rat conjunctival goblet cells, the cells were treated with MaR1 (10^−8 M) for 30 min before stimulation with histamine (10^−5 M). Histamine caused an increase in \([\text{Ca}^{2+}]_{i}\), with a peak of 265.0 ± 45.6 nM (Figure 9ab; n = 8). When preincubated with MaR1 (10^−8 M), the peak in \([\text{Ca}^{2+}]_{i}\) was significantly reduced to 84.0 ± 18.8 nM.

To determine the effect of MaR1 on histamine-stimulated secretion, rat conjunctival goblet cells were incubated with MaR1 (10^−9 M) for 30 min before stimulation with histamine (10^−5 M). Histamine caused a secretory response above basal of 2.4 ± 0.32 (Figure 9c; n = 4). When incubated with MaR1 (10^−9 M), the histamine-induced secretory response was decreased to 0.87 ± 0.21. Thus, MaR1 counterregulates the stimulatory effect of histamine on \([\text{Ca}^{2+}]_{i}\) and secretion.

4 | DISCUSSION

In this study, we found that in rat conjunctival goblet cells MaR1 increases \([\text{Ca}^{2+}]_{i}\), and stimulates secretion. The increase in \([\text{Ca}^{2+}]_{i}\), and secretion occurs through the induction of PLC and its downstream effectors, namely IP3, PKC, ERK 1/2, and Ca2+/CaMK (Figure 10). MaR1 does not utilize PLA2 to stimulate an increase \([\text{Ca}^{2+}]_{i}\), or secretion. Interestingly stimulation of PLD activity by MaR1, leads to an increase in secretion only. MaR1 neither increases cAMP nor activates PKA to stimulate \([\text{Ca}^{2+}]_{i}\) or secretion. Similar to other SPMs such as LXα4, RvD1, and RvE1, MaR1 regulates \([\text{Ca}^{2+}]_{i}\), and stimulates secretion in conjunctival goblet cells which likely maintain optimal mucin layer on the ocular surface under physiological conditions (Hodges et al., 2016; Lippestad et al., 2017, 2018).

MaR1 stimulates conjunctival goblet cells similarly to LXα4 and RvD1; however, the pathways used by the three SPMs differ. Similar to MaR1, both LXα4 and RvD1 use the PLC pathway including using

FIGURE 9  Maresin 1 (Mar1) counterregulates histamine-stimulated increase in \([\text{Ca}^{2+}]_{i}\), and high molecular weight glycoprotein secretion. Rat conjunctival goblet cells were preincubated with MaR1 at 10^−9 M for \([\text{Ca}^{2+}]_{i}\), or 10^−9 M for secretion for 30 min, then stimulated with histamine (10^−5 M). Changes in \([\text{Ca}^{2+}]_{i}\), over time are shown in (a) and changes in peak \([\text{Ca}^{2+}]_{i}\) are shown in (b). Secretion is shown in (c). Data are mean ± SEM of eight (a and b) and four (c) experiments. *Significance from basal. #Significance between histamine alone. \([\text{Ca}^{2+}]_{i}\), intracellular Ca2+: SEM, standard error of the mean.
Ca^{2+}/CaMKII to increase [Ca^{2+}], and stimulate secretion. In contrast to MaR1, LXA4 and RvD1 also use PLD and PLA2 to increase [Ca^{2+}], and stimulate secretion (Hodges et al., 2017; Lippestad et al., 2017). Thus, the signaling pathways activated appear to be SPM-specific.

MaR1 uses the PLD pathway to stimulate glycoconjugate secretion, but not to increase [Ca^{2+}]. The signaling pathways activated by PLD are complex and can vary between agonists and cell type. There are two types of mammalian PLD, PLD1 and PLD2 (Gomez-Cambronero, 2014) and can interact with many other proteins. For example, PKC enhances the lipase activity of PLD1 and while PLD2 and PLCγ directly interact in an epidermal growth factor (EGF)-dependent manner. This interaction also links PLD to the mitogen-activated protein kinase and Ras/ERK pathways. In conjunctival goblet cells, MaR1 could activate PLD by this EGF-dependent pathway as MaR1 activates PKC and ERK1/2 to stimulate secretion.

MaR1 is mainly dependent on the activation of the PLC pathway to increase [Ca^{2+}], and stimulates secretion. All steps of the PLC pathway for which inhibitors were used in the present study were blocked indicated by a decrease in [Ca^{2+}], and in secretion. These steps included the activation of PLC, interaction of IP3 with its receptor on intracellular Ca^{2+} stores, release of intracellular Ca^{2+} stores, and activation of PKC. Furthermore, activation of PLC is a widely used mechanism for stimulating secretion by exocytosis.

MaR1 does not activate PKA to stimulate secretion. Herein, we used the PKA inhibitor H89 that did not alter MaR1-stimulated increase in [Ca^{2+}], and secretion. In addition, we measured cAMP levels that were not increased by MaR1. In contrast, Chiang et al. (2019) demonstrated that MaR1 activates the human LGR6 and the activation of this receptor-stimulated ERK1/2 and cAMP activity. In a work by Chiang et al. (2019), the actions of MaR1 were tested on macrophages, neutrophils (PMNs), fibroblasts, and in two epithelial cell lines CHO and HEK cells. In accordance with Chiang et al. (2019), MaR1 activated ERK 1/2 in the present study with rat conjunctival goblet cells. In contrast, MaR1 increased [Ca^{2+}], but did not alter cAMP levels nor activate PKA. Possible reasons for these differences between the two studies are (a) primary epithelial cells in culture have different responses and intracellular signaling than immune cells or cell lines, for example, cell-type specificity. (b) Rat LGR6 is coupled to a different G protein than human LGR6, or. (c) In rat conjunctival goblet cells, LGR6 is coupled to a different G protein than in other cell types. In future studies, we plan to determine if in rat conjunctival goblet cells, the LGR6 receptor is present, and if MaR1 also acts via this receptor to increase [Ca^{2+}], and stimulate secretion.

SPMs from both ω-3 fatty acids and the ω-6 fatty acid arachidonic acid each increase [Ca^{2+}], and stimulate secretion from rat conjunctival goblet cells in the absence of disease. Thus RvD1, RvD2,
and MaR1 derived from the ω-3 fatty acid DHA; RvE1 derived from precursor eicosapentaenoic acid (EPA); and LXA₄ biosynthesized from arachidonic acid each interact with their receptors, activate specific signaling pathways, increase [Ca²⁺], and stimulate secretion (Botten et al., 2019; Hodges et al., 2017; Lippestad et al., 2017, 2018). The diversity of the origin of different SPMs supports the hypothesis that if precursors of one of the biosynthetic pathways is depleted, others can potentially take over to maintain homeostasis in conjunctival tissue. Thus, MaR1 is similar to other SPMs in that it has a direct action on goblet cells alone in the absence of disease and can play a functional role in ocular surface homeostasis by regulating the mucous layer of the tear film.

The ω-6 fatty acid arachidonic acid and the ω-3 fatty acids DHA and EPA, which are the precursors of the SPMs, are detectable in human tears (Walter et al., 2016). The finding of precursors of SPMs, including precursors of MaR1, in human tears, supports that MaR1 plays a role in maintaining tissue homeostasis and stabilizing the tear film.

In addition to the actions of MaR1 alone, MaR1 also has an effect in disease conditions. When an allergic type 1 hypersensitivity reaction is initiated, a complex cascade is activated, with both cells and mediators involved. Histamine is a major mediator contributing to the symptoms of allergic conjunctivitis (del Cuvillo et al., 2009). Histamine is released from mast cells and basophils to cause an inflammatory response with increased vascular permeability and vasodilation (Thurmord, Gelfand, & Dunford, 2008). In conjunctival goblet cells histamine binds to its receptors (H1–H4) to increase [Ca²⁺], mainly through the PLC pathway (Li et al., 2012). Histamine also stimulates secretion (Hayashi et al., 2012). We previously showed that RvD1 and LXA₄ counterregulate the histamine receptors, thereby blocking histamine-stimulated increase in [Ca²⁺], and secretion (Hodges et al., 2016; Li et al., 2013). When rat conjunctival goblet cells are incubated with MaR1 before histamine stimulation, the histamine-stimulated increase in [Ca²⁺], and secretion are decreased. This suggests that MaR1 could be a candidate as novel treatment of allergic conjunctivitis.

Based on our findings, we conclude that MaR1 likely has two main functions; one in health and one in disease. First, during physiological conditions, MaR1 likely contributes to maintaining tissue homeostasis by activating the PLC pathway to increase [Ca²⁺], thereby regulating the amount of secretion from goblet cells. Second, during an ocular surface inflammatory disease, MaR1 likely stimulates the resolution of inflammation by inhibiting the overproduction of mucin by attenuating histamine-stimulated mucin secretion. Thus, based on these findings, MaR1 or its mimetic analogs could be a novel treatment for different inflammatory diseases spanning from allergic conjunctivitis to dry eye disease.

**ACKNOWLEDGMENTS**

The authors would like to thank Marie Shatos and Dayu Li for their helpful assistance. This study was supported by the Norwegian Research Council to Markus V. Olsen and by the National Institute of Health R01 EYO19470 to Darlene A. Dartt and R01GM038765 to Charles N. Serhan.

**CONFLICT OF INTERESTS**

The authors declare that there are no conflict of interests.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Olsen MV, Lyngstadaas AV, Bair JA, et al. Maresin 1, a specialized proresolving mediator, stimulates intracellular [Ca2+] and secretion in conjunctival goblet cells. J Cell Physiol. 2020;1–14. https://doi.org/10.1002/jcp.29846
Paper 2
Comparison of Signaling Pathways Used by the Specialized Pro-Resolving Mediators Maresin 2 and Maresin 1 to Regulate Goblet Cell Function

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Abstract

Purpose: Specialized pro-resolving mediators (SPMs) contribute to tear film homeostasis and resolution of inflammation in the conjunctiva. We reported that the SPM Maresin 1 (MaR1) increases the intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) and stimulates conjunctival goblet cell secretion through the PLC-pathway. The aim of this study is to determine if Maresin 2 (MaR2) uses the same signaling pathways as MaR1.

Methods: Goblet cells were cultured from pieces of rat conjunctiva. [Ca\textsuperscript{2+}] was measured using fura2/AM in cultured goblet cells stimulated by agonists and inhibitors. After incubation of goblet cells with agonists and inhibitors, high molecular weight glycoconjugate secretion was determined using an enzyme-linked lectin assay (ELLA).

Results: Treatment with MaR2 increased [Ca\textsuperscript{2+}], and secretion in a concentration dependent manner. Increase in [Ca\textsuperscript{2+}] stimulated by both MaR2 and the control MaR1 was blocked by a BLT1 receptor inhibitor LY29311, suggesting that MaR2 and MaR1 both activated the BLT1 receptor. MaR1, but not MaR2-stimulated glycoconjugate secretion was decreased by inhibition of the BLT1 receptor. The MaR1-stimulated, but not the MaR2-stimulated, increase in [Ca\textsuperscript{2+}] was reduced by the ALX/FPR2-receptor, BOC2, indicating that MaR1, but not MaR2 uses the ALX/FPR2 receptor. Stimulation with MaR1 blocked the [Ca\textsuperscript{2+}] response to MaR2, but stimulation with MaR2 did not attenuate the MaR1 response. This suggested that MaR1 activated a second receptor to block MaR2 response, or MaR1 and MaR2 interacted with overlapping regions on the BLT1 receptor. The actions of MaR2 were dependent on activation of phospholipase D (PLD) as 1-butanol blocked the stimulated increase in [Ca\textsuperscript{2+}] and glycoconjugate secretion, but only blocked MaR1 stimulation of secretion. Moreover, MaR2- and MaR1-stimulated increase in [Ca\textsuperscript{2+}] was dependent on intracellular Ca\textsuperscript{2+} stores as their stimulation was inhibited by the Ca\textsuperscript{2+} chelator BAPTA/AM and the SERCA inhibitor thapsigargin. MaR2 and MaR1 were unaffected by removing extracellular Ca\textsuperscript{2+}, indicating intracellular Ca\textsuperscript{2+} stores as the primary source of Ca\textsuperscript{2+}. In addition, MaR2 and MaR1 both activated protein kinase C (PKC) as their increase in [Ca\textsuperscript{2+}] and stimulated glycoconjugate secretion were blocked by the PKC inhibitor RO317549. In contrast, only MaR1 activated PLC, as the MaR1-, but not the MaR2-stimulated increase in [Ca\textsuperscript{2+}] was blocked by the PLC inhibitor U73122. Both the maresins, however, were dependent on PLC for stimulated glycoconjugate secretion. The PKA inhibitor H89 blocked MaR2-, but not MaR1- stimulated [Ca\textsuperscript{2+}] response, as well as glycoconjugate secretion, indicating that MaR2, but not MaR1, activates cAMP dependent PKA.

Conclusion: We conclude that in conjunctival goblet cells MaR2 and MaR1 both increase the [Ca\textsuperscript{2+}], and stimulate secretion. MaR2 and MaR1 use one set of different, but overlapping, signaling pathways to increase [Ca\textsuperscript{2+}], and another set to stimulate secretion.
Introduction

The ocular surface consists of the cornea and conjunctiva covered by a protective tear film. The innermost layer of the tear film is the mucous layer, which consists of electrolytes, water and mucins produced by conjunctival goblet cells (1). The mucins provide a critical line of defense from the external environment and play a role in maintaining health (2). A disturbance in the homeostasis of mucin secretion has been described in a variety of inflammatory ocular surface diseases, including allergic conjunctivitis, Sjogren’s syndrome and dry eye disease (2-7). Resolution of inflammation is an active process with production of pro-resolution mediators (8). A group of lipid mediators called specialized pro-resolving mediators (SPMs), including the maresins, counter regulate pro-inflammatory mediators in disease (9, 10).

The maresins, maresin 1 (MaR1) and maresin 2 (MaR2), are biosynthesized in macrophages and other tissues and are derived from the endogenous ω-3 fatty acid docosahexaenoic acid (DHA) (11). MaR1 and MaR2 are synthesized through multiple enzymatic steps. The synthesis is initiated by 12-lipoxygenase (12-LOX), which converts DHA to 14-hydroperoxydocosahexaenoic acid. The two maresins share synthesis pathway until 13S, 14S-epoxy-maresin. 13S, 14S-epoxy-maresin is enzymatically converted to MaR1 by a hydrolase and to MaR2 by a soluble epoxide hydrolase (11, 12). The maresins both consist of a carbon chain which is 22 carbons long, a carboxyl group, two hydroxyl groups and six double bonds, however, the placement of the hydroxyl groups and double bonds are dissimilar.

MaR1 and MaR2 function by limiting polymorphonuclear (PMN) infiltration and stimulating macrophage phagocytosis. By reducing the number of PMNs and removing apoptotic and necrotic cells the maresins act to resolve inflammation (11, 12). In addition to pro-resolving effects, MaR1 stimulates regeneration and reduces pain. After surgical decapitation of planaria, MaR1 is biosynthesized which accelerates regeneration (13). MaR1 reduces inflammatory and neuropathic pain by inhibition of TRPV1 (13). Furthermore, MaR1 is present in human lymphoid tissue (spleen and lymph nodes) and human serum, indicating a possible role in the immune system (14). Recent investigation of the actions of MaR1 on rat conjunctival goblet cells demonstrated that MaR1 increases \([\text{Ca}^{2+}]\) and stimulates glycoprotein secretion. MaR1 increased \([\text{Ca}^{2+}]\), and stimulated glycoprotein secretion by activating PLC and its downstream effectors, IP3, PKC, and by activation of PLD, Ca2+-calmodulin kinase (CaMK) II and extracellular regulated kinase (ERK) 1/2 (15).
In the present study, we compared the effect of MaR2 and MaR1 on cultured rat conjunctival goblet cells. To activate the goblet cell and stimulate mucin secretion, one of the main stimulating signals is an increase in the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]). We identified the intracellular pathways MaR2 uses by measuring [Ca\(^{2+}\)] and high molecular weight glycoprotein secretion including mucin secretion. We used pharmacologic inhibitors of different signaling pathways followed by addition of MaR2. Furthermore, the effect of MaR2 on histamine was investigated, because the maresins are thought to play a central role in allergy (15). MaR1 was used as a control.

Materials and Methods

2.1 Materials
RPMI-1640 cell culture medium, penicillin/streptomycin and L-glutamine were purchased from Lonza (Walkerville, IL). Fetal bovine serum (FBS) was ordered from Atlanta Biologicals (Norcross, GA). MaR2 and MaR1 were purchased from Cayman Chemical (Ann Arbor, MI), stored in an ethanol solution at -80°C. The solution was diluted immediately before use in 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\(_2\)PO\(_4\), 25 mM NaHCO\(_3\), 10 mM HEPES, and 5.5 mM glucose (pH 7.40-7.45)) to the desired concentrations and added to the cells. UEA-1 was obtained from Sigma-Aldrich (St. Louis, MO). Vasoactive intestinal peptide (VIP), U73122 and U73343 were purchased from Tocris Bioscience (Ellisville, MO). Histamine, carbachol (CCh), 2APB, 1-butanol (1-but) and tert-butanol (t-but) were obtained from Sigma-Aldrich (St. Louis, MO). Fura-2/AM was purchased from Life Technologies (Grand Island, NY). N-BOC-Phe-Leu-Phe-Leu-Phe (BOC2) was ordered from GenScript (Piscatawy, NJ, USA). BLT1 inhibitor U-75302 and LTB\(_4\) were ordered from Cayman Chemical (Ann Arbor, MI). Lipoxin A\(_4\), H89, thapsigargin and RO-317549 were ordered from EMD Millipore (Billerica, MA).

2.2 Animals
Male albino Sprague-Dawley rats from 4-6 weeks old (Taconic Farms, Germantown, NY) were anesthetized with CO\(_2\) for 5 min, decapitated, and the bulbar and fornical 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 of Committee.
2.3 Cell Culture
Goblet cells were cultured from male albino Sprague-Dawley rat conjunctiva. The conjunctival tissue was cut into pieces that were placed in 6 well plates with 0.5 ml RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine and 100 mcg/ml penicillin-streptomycin. The cells prepared for secretion experiments were plated in 24 well plates. RPMI media was changed every second day, and 2 ml media were used in each well. Cultured goblet cells were identified periodically by staining with anti-cytokeratin 7, anti-MUC5AC and the lectin UEA-1 directly conjugated to fluorophore. The cells were trypsinized and transferred to Ca2+ or secretion dishes 24 hours before the experiments were performed.

2.4 Measurement of [Ca2+]i.
Cultured rat conjunctival goblet cells were transferred after trypsinization to 35-mm glass bottom dishes and incubated in 37.0°C overnight. The cells were then incubated in 37.0°C for 1 hour with KRB-HEPES containing 0.5% BSA, 0.5 mM fura2/AM, 250 mM Sulfynpyrazone and 8 mM Pluronic acid F127. [Ca2+] was measured with a ratio imaging system (InCytIm2; intracellular imaging, Cincinnati, OH) using wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. A minimum of 10 goblet cells was selected and the Ca2+ response was followed for approximately 2 minutes. MaR2 was either added alone or treated with inhibitors before addition of either MaR2 or the positive controls MaR1, carbachol, or histamine. The inhibitors H89, BOC2, BLT1, RO 317549, 2APB, U73122, U73343 were added 30 minutes prior to the agonist. 1-butanol, t-butanol and thapsigargin were added 15 minutes prior to the agonist. Change in peak [Ca2+]i was calculated by subtracting the average basal [Ca2+]i from the peak [Ca2+]i.

2.5 Measurement of High Molecular Weight Glycoconjugate Secretion
Cultured rat conjunctival goblet cells were trypsinized and transferred to 24 well plates. The cells were serum starved in free RPMI 1640 media containing 0.5% bovine serum albumin (BSA) for 120 minutes. MaR2 (10^-10-10^-8 M) was then added alone or the cells were incubated with an inhibitor for 30 minutes, and then stimulated with MaR2 (10^-10-10^-8 M) or carbachol (10^-4 M) for 2 hr. The amount of goblet cell high molecular weight glycoconjugate secretion was measured using the lectin UEA-1 in an enzyme linked lectin assay (ELLA). Glycoconjugate secretion is shown as fold increase above basal (which was set to 1).

2.6 Statistical Analysis
Data are expressed as mean ± SEM. Data were analyzed by either Student's t-test or one-way ANOVA followed by Tukey test. p< 0.05 was considered significant.
Results

*Maresin 2 increases secretion in rat conjunctival goblet cells*

The SPMS, lipoxin (LX)A₄, resolvin (Rv)D₁, RvE₁ and MaR₁ stimulate secretion from rat conjunctival goblet cells (15-19). We compared the action of MaR₂ (10⁻¹⁰ M – 10⁻⁸ M) with that of the positive controls the cholinergic agonist carbachol (Cch) at 10⁻⁴ M and MaR₁ at 10⁻⁸ M that are known to increase rat goblet cell secretion (15, 20). Cultured rat goblet cells were stimulated for 2 hr with MaR₂, MaR₁ or Cch. MaR₂ significantly increased glycoconjugate secretion at 10⁻¹⁰ M (1.62 ± 0.12; p=0.0005) and 10⁻⁸ M (2.18 ± 0.34; p=0.006), but not at 10⁻⁹ M (1.38 ± 0.24; p=0.14) (Fig. 1; n=6)). MaR₁ and Cch each stimulated secretion.

![Figure 1: Maresin 2 (MaR2) stimulates glycoconjugate secretion. Rat conjunctival goblet cells were stimulated with either MaR2 (10⁻¹⁰–10⁻⁸ M) Maresin 1 (MaR1, 10⁻⁸ M), or carbachol (Cch, 10⁻⁴ M) for 2 hr. High molecular weight glycoprotein secretion was measured. Data are mean ± SEM from six experiments. *shows significance above basal. SEM, standard error of the mean.](image)

*Maresin 2 increases [Ca²⁺]ᵢ in rat conjunctival goblet cells*

Multiple SPMs that stimulate goblet cell secretion, also increase [Ca²⁺]ᵢ, in cultured rat conjunctival goblet cells (15-19). Cultured goblet cells were incubated in Fura2/AM for one hour and stimulated with MaR₂ (10⁻¹⁰ M – 10⁻⁸ M), MaR₁ at 10⁻⁸ M or histamine at 10⁻⁵ M. MaR₂ increases [Ca²⁺]ᵢ rat conjunctival goblet cells in a concentration-dependent manner.
MaR2-stimulated increase in $[\text{Ca}^{2+}]_i$ was $62.36 \pm 12.22$ nM ($p=0.007$) for $10^{-10}$ M, $93.48 \pm 12.66$ nM ($p=0.002$) for $10^{-9}$ M and $189.20 \pm 14.54$ nM ($p=0.029$) for $10^{-8}$ M, (Fig. 2C; $n=3$). The highest increase in peak $[\text{Ca}^{2+}]$ was triggered by MaR2 $10^{-6}$ M, thus this concentration was used in further experiments. MaR1 at $10^{-6}$M and histamine at $10^{-5}$ M also increased $[\text{Ca}^{2+}]$.

To determine if glycoconjugate secretion is dependent upon an increase in intracellular $\text{Ca}^{2+}$, cells were incubated with intracellular $\text{Ca}^{2+}$ chelator BAPTA/AM ($10^{-4}$ M) and MaR2-stimulated secretion was measured. MaR2 stimulated secretion by $1.7 \pm 0.2$ fold above basal ($p=0.0007$, Fig. 2D; $n=6$). When incubated with BAPTA/AM, the response was significantly decreased to $0.7 \pm 0.2$ fold above basal ($p=0.002$). MaR1-stimulated increase in secretion was also, significantly decreased by BAPTA/AM.

Figure 2: Pseudocolor images of rat conjunctival goblet cells stimulated with MaR2 ($10^{-6}$ M). (A) MaR1 ($10^{-8}$ M) is shown at four different times of stimulation. Panel I shows rat conjunctival goblet cell baseline $[\text{Ca}^{2+}]$ level before stimulation (AI), panel II 30 seconds after stimulation with MaR2 ($10^{-8}$ M) (AII); panel III 50 seconds after stimulation (AIII); and panel IV 80 seconds after stimulation (AIV). Changes in $[\text{Ca}^{2+}]$ over time at different concentrations of MaR2 ($10^{-8}$ M) are shown in (B). Changes in peak $[\text{Ca}^{2+}]$ after stimulation with MaR2 ($10^{-8}$ M) are shown in (C). Goblet cells were preincubated
with BAPTA/AM (10⁻⁴ M) and stimulated with MaR2 (10⁻⁸ M) or Maresin 1 (10⁻⁹ M) for glycoconjugate secretion (D). Data are mean ± SEM from three (B and C) and six (D) experiments. * shows significance above zero (basal). # shows significant difference between agonist and agonist + inhibitor.

Maresin 2 activates the BLT1 receptor and is not dependent on the ALX/FPR 2 receptor to increase [Ca²⁺].

The ALX/FPR2-receptor is a complex G-protein coupled receptor (GPCR) which is known to bind a variety of ligands, including peptides, lipids and small molecules. Bae et al. determined that this receptor may activate different intracellular pathways resulting in a variety of cellular responses (19, 21-23). We previously showed that MaR1 was dependent on the ALX/FPR-2 receptor to increase [Ca²⁺] and to stimulate glycoconjugate secretion in conjunctival goblet cells (15). Thus, we wanted to explore the role of the ALX/FPR2-receptor in MaR2-stimulated increase in [Ca²⁺]. We incubated rat conjunctival goblet cells with the ALX/FPR2 inhibitor N-BOC-Phe-Leu-Phe-Leu-Phe (BOC2) (10⁻⁴ M) for 30 minutes prior to stimulation with MaR2 (10⁻⁸ M). MaR1 (10⁻⁸ M) and LXA₄ (10⁻⁹ M) were used as positive controls (15, 19). MaR2 significantly increased [Ca²⁺] to 184.66 ± 18.00 nM (p=7xe⁻⁶) while the MaR2 10⁻⁸ M treated with BOC2 increased [Ca²⁺] to 182.33 ± 37.50 nM (p= 0.96, Fig. 3A; n=5). These results indicate that MaR2 is able to increase [Ca²⁺], independently of the ALX/FPR2-receptor. MaR1 added alone significantly increased [Ca²⁺] to 223.30 ± 47.88 nM (Fig. 3A). BOC2 significantly decreased the MaR1 response to 66.98 ± 8.95 nM. LXA₄-stimulated [Ca²⁺] increase was 178.10 ± 24.68 nM (Fig. 3A). In the presence of BOC2 the LXA₄ response was significantly reduced to 83.13 ± 29.00.

To explore the role of the ALX/FPR2-receptor in glycoconjugate secretion, goblet cells were incubated with BOC2 for 30 min prior to stimulation with MaR2, MaR1 and LXA₄. MaR2 increased secretion 1.8 ± 0.3 fold above basal (Fig. 3B; n=4). MaR2 was not significantly inhibited by BOC2, while secretion stimulated by the positive controls MaR1 was decreased and LXA₄ was significantly inhibited by the ALX/FPR2 inhibitor. These data indicate that MaR2 does not utilize the ALX/FPR2-receptor to increase [Ca²⁺], or stimulate secretion.

LTB₄ activates the GPCR receptor, BLT1, to cause chemotactic, pro-inflammatory actions (24). SPMs including RvE1 and MaR1 also bind to the BLT1 receptor (15, 25). To examine if MaR2 is using the BLT1 receptor to increase [Ca²⁺], rat conjunctival goblet cells were treated with an inhibitor of the BLT1 receptor, U-75302 (10⁻⁸-10⁻⁶) for 30 minutes. The MaR2
response was compared to the increase in $[\text{Ca}^{2+}]_i$ caused by LTB$_4$ at $10^{-9}$ M and MaR1 at $10^{-8}$ M. MaR2 $10^{-8}$ M significantly increased $[\text{Ca}^{2+}]_i$ to $240.96 \pm 55.34$ nM ($p=0.001$, Fig. 3C; n=6). Preincubation with the BLT1 receptor inhibitor U-75302 caused a MaR2 stimulated $[\text{Ca}^{2+}]_i$ increase to only $83.06 \pm 17.80$ (p=0.021), $113.05 \pm 29.34$ (p=0.07) and $62.88 \pm 10.55$ (p=0.01) (Fig. 3C) with $10^{-8}$ M, $10^{-7}$ M and $10^{-6}$ M inhibitor, respectively. The positive controls MaR1 and LXA$_4$ increased the $[\text{Ca}^{2+}]_i$, that was blocked by U-75302 (Fig. 3C). These results suggest that MaR2, similarly to the positive controls MaR1 and LXA$_4$, is dependent on the BLT1 receptor to increase $[\text{Ca}^{2+}]_i$.

To examine the dependency of MaR2 on the BLT1 receptor to stimulate secretion, rat conjunctival goblet cells were treated with U-75302 ($10^{-6}$ M) for 30 min before addition of MaR2. MaR2 increased secretion $1.8 \pm 0.3$ fold above basal (Fig. 3D; n=4). MaR2 was not significantly inhibited by U-75302. The positive controls MaR1 and LTB$_4$ were significantly inhibited by the U-75302. These data indicate that MaR2 does use the BLT1-receptor to increase $[\text{Ca}^{2+}]_i$, but not glycoconjugate secretion.
Figure 3: Inhibition of the ALX/FPR2- and the BLT1 receptors acts on stimulation of \([Ca^{2+}]_{i}\) and secretion by MaR2 at \(10^{-8}\) M. Goblet cells were treated with the ALX/FPR2 receptor inhibitor BOC2 (\(10^{-4}\) M) for 30 minutes and stimulated with MaR2 \(10^{-8}\) M (A and B), MaR1 \(10^{-8}\) M (A) MaR1 \(10^{-9}\) M (B), LXA₄ \(10^{-9}\) M (A) or LXA₄ \(10^{-8}\) M (B). Goblet cells were treated with the BLT1 receptor inhibitor U-75302 (\(10^{-8}-10^{-6}\) M) for 30 minutes and stimulated with MaR2 \(10^{-8}\) M (C and D), MaR1 \(10^{-8}\) M (C), MaR1 \(10^{-9}\) M (D) or LTB₄ \(10^{-9}\) M (C) LTB₄ \(10^{-8}\) M (D). Data are mean ± SEM of five (A), four (B), six (C) and four (D) experiments. * shows significance above basal. # shows significance between agonist and inhibitor + agonist.

**Maresin 1 inhibits Maresin 2-stimulated increase in \([Ca^{2+}]_{i}\) but Maresin 2 does not inhibit Maresin 1 stimulation**

Both MaR1 and MaR2 are SPMs derived from the \(\omega-3\) fatty acid docosahexaenoic acid (DHA). To determine if the two maresins desensitize each other and thus would activate the same receptor, we incubated rat conjunctival goblet cells two minutes with MaR2 \(10^{-8}\) M or MaR1 \(10^{-8}\) M alone. Then as a control to ensure that each of the maresins can desensitize themselves, cells were stimulated with MaR2 \(10^{-8}\) M or MaR1 \(10^{-8}\) M followed by MaR2 and MaR1, respectively. Then MarR1 was added before MaR2 to determine if MaR1 desensitized MaR2 and MaR2 before MaR1 to determine if MaR2 desensitized MaR1. Maresin 2 \(10^{-8}\) M caused an increase in peak \([Ca^{2+}]_{i}\) to 190.59 ± 21.78 (\(p=3.38 \times 10^{-5}\), Fig. 4 A and B; n=5). MaR2 addition before MaR2 caused an increase in peak \([Ca^{2+}]_{i}\) to 93.84 ± 25.20 that was significantly decreased compared to MaR2 alone (\(p=0.031\), Fig. 4 A and B; n=5). Addition of MaR1 before MaR2 caused an increase in peak \([Ca^{2+}]_{i}\) to 112.51 ± 7.02 that was significantly decreased compared to MaR2 alone (\(p=0.018\)).

MaR1 \(10^{-8}\) M caused an increase in \([Ca^{2+}]_{i}\) to 208.58 ± 44.19 (\(p=0.002\), Fig. 4 A and B; n=5). Addition of MaR1 before MaR1 caused an increase to 55.41 ± 8.61 that was significantly decreased compared to MaR1 alone (\(p=0.009\)). Addition of MaR2 before MaR1 caused an increase to 188.28 ± 26.07 that was not different from MaR1 alone (\(p=0.58\)). These results show that when MaR1 activates its receptor first, MaR2 cannot activate it, suggesting that MaR1 and MaR2 activate the same receptor. In contrast when MaR2 activates its receptor first, MaR1 can also activate it, suggesting that MaR2 and MaR1 are activating different receptors or overlapping areas on the same receptor.
Maresin 1 inhibits Maresin 2-stimulated increase in $[\text{Ca}^{2+}]_i$, but Maresin 2 does not inhibit Maresin 1 stimulation. Goblet cells were preincubated 2 minutes with either MaR2 (10^{-8} M) (first bar) or MaR1 (10^{-8} M (fourth bar), then stimulated with MaR2 (10^{-8} M) (second bar) or MaR1 (10^{-8} M (fifth bar), respectively or stimulated with MaR1 before MaR2 (third bar) or MaR2 before MaR1 (sixth bar).

Fig. A shows changes in $[\text{Ca}^{2+}]_i$ with time, while Fig. B show changes in peak of $[\text{Ca}^{2+}]_i$. Data are mean ± SEM of five experiments. * shows significance above basal. # shows significance between MaR2 and MaR2 after MaR2 or MaR1, and between MaR1 and MaR1 after MaR2 or MaR1.

*Maresin 2 increase in $[\text{Ca}^{2+}]_i$, but not secretion, is independent of the PLC-pathway in rat conjunctival goblet cells*

The PLC pathway is activated in a variety of cellular processes, including exocytosis and fluid secretion (26). We previously showed that this intracellular signaling pathway is essential for the function of MaR1 in rat conjunctival goblet cells (15). To determine if MaR2 uses the same pathway components to increase $[\text{Ca}^{2+}]_i$, we treated rat conjunctival goblet cells for 30 minutes with the PLC inhibitor U-73122 (10^{-7} M) or its inactive control U-73343 (10^{-7} M) before stimulating with MaR2 (10^{-8} M) (Fig. 5A), or the positive controls MaR1 (10^{-8} M) (Fig. 5A) or Cch 10^{-4} M (Fig. 5A). MaR2 10^{-8} M caused an increase in peak $[\text{Ca}^{2+}]_i$, to
196.87 ± 25.62 (p=0.0003, Fig. 5A; n=4). Treatment with U-73122 or U-73343 followed by MaR2 caused an increase in peak [Ca²⁺] to 99.28 ± 36.77 (p=0.072), and 118.95 ± 47.13 (p=0.20), respectively, that were unchanged when compared to MaR2 stimulation. MaR1 and Cch stimulation, in contrast to that of MaR1, is dependent on PLC to increase [Ca²⁺]; as their action on [Ca²⁺] was decreased by U-73122, but not by U-73343 (Fig. 5A).

The effect of PLC on MaR2-stimulated increase in glycoconjugate secretion was next investigated. MaR2 increased secretion 1.9 ± 0.1 fold above basal (Fig. 5B; n=4). The response was significantly blocked by U73122 to 0.9 ± 0.2 (p=0.003), but not by the inactive control U73343 (3.6 ± 2.1) (p=0.44). MaR1- and Cch-stimulated increase in secretion were also significantly blocked by U73122, but not by U73343 (Fig. 5B; n=4). We conclude that MaR2 is dependent upon activation of the PLC pathway to stimulate glycoprotein secretion, but not to increase [Ca²⁺].

Activation of the PLC pathway produces IP₃ which binds to its intracellular receptor on the ER causing release of Ca²⁺ from intracellular calcium stores increasing [Ca²⁺]. To determine if MaR2 is independent or not of the downstream molecules that activation of the PLC pathway produces, cells were treated with the IP₃-receptor inhibitor 2APB (10⁻⁵ M) and then stimulated with MaR2 10⁻⁸ M. MaR1 (10⁻⁸ M) and Cch (10⁻⁴ M) were used as positive controls. MaR2 caused an increase in peak [Ca²⁺] to 212.28 ± 69.55 (p=0.016, Fig. 5C; n=5). Treatment with 2APB (10⁻⁵ M) did not alter the increase in peak [Ca²⁺] of 125.37 ± 72.15 (p=0.41) compared with the action of MaR2 alone. In contrast treatment with 2-APB blocked the action of MaR1 and Cch on [Ca²⁺]. (Fig. 5C). We conclude that the action of MaR2 is independent of the action of IP₃ on its receptor to increase [Ca²⁺].

The effect 2APB on MaR2-stimulated increase in glycoconjugate secretion was then explored. MaR2-increased secretion was 3.3 ± 0.5 fold above basal (Fig. 5D; n=6). 2APB significantly decreased MaR2-stimulated response to 1.3 ± 0.2 (p=0.0002, Fig. 5D). The action of the positive control, Cch, on secretion was also significantly inhibited by 2APB (Fig. 5D). We conclude that the action of MaR2 on [Ca²⁺], but not secretion, is independent of the action of IP₃ on its receptor to increase [Ca²⁺]; and is in agreement with the action of PLC and these two functions.

To determine if MaR2 is using intracellular calcium stores to increase [Ca²⁺] by other mechanisms than PLC-IP₃ pathway, we used the sarco/endoplasmic reticulum Ca²⁺-ATPase
(SERCA) inhibitor thapsigargin. Thapsigargin blocks the uptake of Ca\(^{2+}\) into intracellular stores so that the cytoplasmic \([\text{Ca}^{2+}]_i\) increases by a passive leak from the ER. If an agonist uses the same intracellular Ca\(^{2+}\) store as thapsigargin, the increase in \([\text{Ca}^{2+}]_i\) by an agonist added after thapsigargin will be decreased. Conjunctival goblet cells were treated by thapsigargin (10\(^{-5}\) M) for 15 minutes that releases Ca\(^{2+}\) from the intracellular stores and then stimulated with MaR2 (10\(^{-8}\) M), or the positive controls or MaR1 (10\(^{-6}\) M) or Cch (10\(^{-4}\) M). MaR2 increased \([\text{Ca}^{2+}]_i\) to a peak of 219.10 ± 21.63 nM (p=0.00053, Fig. 5E and 5G; n=3). Treatment with thapsigargin caused an increase in \([\text{Ca}^{2+}]_i\) to a peak of 85.68 ± 4.02 nM that was significantly decreased compared to MaR2 alone (p=0.0037, Fig. 5F and 5G). A similar effect of thapsigargin was detected using MaR1 and Cch. Thus, MaR2 is dependent on a release of Ca\(^{2+}\) from intracellular calcium stores to increase \([\text{Ca}^{2+}]_i\).

**Figure 5:** Maresin 2 increase in \([\text{Ca}^{2+}]_i\), but not secretion, is independent of the PLC-pathway in rat conjunctival goblet cells. Goblet cells were treated with vehicle, the active PLC inhibitor U-73122 or the inactive PLC inhibitor U-73343 both at 10\(^{-7}\) M for 30 minutes and stimulated with MaR2 (10\(^{-8}\) M), MaR1 (10\(^{-8}\) M) or Cch (10\(^{-4}\) M). Fig. A shows changes in peak of \([\text{Ca}^{2+}]_i\) and B shows glycoconjugate secretion. Goblet cells were treated with vehicle or 2APB (10\(^{-5}\) M) and stimulated with MaR2 (10\(^{-8}\) M),
MaR1 (10⁻⁸ M) or Cch (10⁻⁴ M). C shows changes in peak of [Ca²⁺], and D shows glycoconjugate secretion. Goblet cells were stimulated with MaR2 (10⁻⁸ M), MaR1 (10⁻⁸ M) or Cch (10⁻⁴ M) alone or incubated with vehicle or thapsigargin (10⁻⁵ M) for 15 minutes and then stimulated with MaR2 (10⁻⁸ M), MaR1 (10⁻⁸ M) or Cch (10⁻⁴ M). E and F show changes in [Ca²⁺] with time, while G shows changes in peak of [Ca²⁺]. Data are mean ± SEM of four (A), four (B), five (C), six (D), and three (E, F and G) experiments. * shows significance above basal. # shows significance between Maresin 2 and inhibitor then Maresin 2 or control and inhibitor then control.

Maresin 2 stimulated increase in [Ca²⁺] is independent of the extracellular Ca²⁺ in rat conjunctival goblet cells

We published that MaR1 was not dependent on extracellular Ca²⁺ to increase [Ca²⁺](15). To explore if MaR2 is dependent on influx of extracellular Ca²⁺, we incubated rat conjunctival goblet cells in vehicle with or without CaCl₂ (1.0 mM). MaR2 with CaCl₂ increased [Ca²⁺] to a peak of 95.66 ± 28.34 nM (p=0.015, Fig. 6; n=4), while MaR2 without CaCl₂ increased [Ca²⁺] to a peak of 64.74 ± 15.25 nM, a non-significant decrease (p=0.37). A similar finding was detected for MaR1 (10⁻⁸ M) (Fig. 6). In contrast [Ca²⁺] stimulated by Cch (10⁻⁴ M) was significantly decreased in the absence of extracellular Ca²⁺ (Fig. 6). We conclude that MaR2 and MaR1 are independent of influx of extracellular Ca²⁺ to increase [Ca²⁺], but Cch is not.

Figure 6: Maresin 2 stimulated increase in [Ca²⁺] is independent of extracellular Ca²⁺ in rat conjunctival goblet cells. Goblet cells were incubated with KRB with or without CaCl₂, then stimulated with MaR2 (10⁻⁸ M), MaR1 (10⁻⁸ M) or Cch (10⁻⁴ M). Figure shows changes in peak of [Ca²⁺]. Data are mean ± SEM of four experiments. * shows significance above basal. # shows significance between agonist and extracellular Ca²⁺ removal then agonist.
Maresin 2 increases \([\text{Ca}^{2+}]_i\) and stimulates secretion by activation of protein kinase C (PKC)

Protein kinase C (PKC) is activated by diacylglycerol (DAG) produced by activation of PLC when PLC also produces IP3. PKC can also be activated by other signaling pathways. To determine if MaR2 is dependent on PKC to increase \([\text{Ca}^{2+}]_i\), we incubated conjunctival goblet cells with the PKC inhibitor RO317549 (10\(^{-7}\) M) for 30 minutes, then stimulated with MaR2 (10\(^{-8}\) M), MaR1 (10\(^{-8}\) M), or Cch (10\(^{-4}\) M). MaR2 increased \([\text{Ca}^{2+}]_i\) to a peak of 475.49 ± 125.41 nM (p=0.019, Fig. 7A; n=3), while treatment with RO317549 significantly decreased \([\text{Ca}^{2+}]_i\) to a peak of 76.67 ± 12.84 nM (p=0.034, n=3). Similar results were obtained with MaR1 and Cch (Fig. 7A). Thus, MaR2 is dependent on PKC to increase \([\text{Ca}^{2+}]_i\), as are MaR1 and Cch.

The effect of RO317549 on MaR2-stimulated increase in glycoconjugate secretion was determined after a 30 min incubation with RO317549 (10\(^{-7}\) M). MaR2 increased secretion 1.9 ± 0.1 fold above basal (p=1.44xe\(^{-5}\), Fig. 7B; n=4) and MaR2 stimulation was significantly blocked by RO317549 to 0.9 ± 0.2 fold above basal (p=0.001). The positive control, MaR1, increased secretion to 2.2 ± 0.8 fold above basal (p=0.03) and preincubation with RO317549 significantly decreased the response to 0.8 ± 0.2 fold above basal (p=0.03). Both MaR2 and MaR1 are dependent upon PKC to increase \([\text{Ca}^{2+}]_i\) and stimulate secretion.

Figure 7: Maresin 2 increases \([\text{Ca}^{2+}]_i\) and stimulates secretion by activation of protein kinase C (PKC). Goblet cells were incubated with RO317549 (10\(^{-7}\) M) for 30 minutes, then stimulated with MaR2 (10\(^{-8}\) M), MaR1 (10\(^{-8}\) M) or Cch (10\(^{-4}\) M) to measure the change in peak \([\text{Ca}^{2+}]_i\). A show changes in \([\text{Ca}^{2+}]_i\) with time, while B shows glycoconjugate secretion. Data are mean ± SEM of three (A) and four (B)
Maresin 2 has different dependency on Phospholipase D (PLD) and Phospholipase A2 (PLA2) to increase \([Ca^{2+}]\) and stimulate secretion in rat conjunctival goblet cells

Activation of Phospholipase D (PLD) is controlled by multiple mechanisms, activates distinct pathways and is important in cellular functioning (27). To explore if MaR2 uses PLD to increase \([Ca^{2+}]\), we used the PLD-inhibitor 1-butanol (1-but) at 0.3% and the inactive control t-butanol (t-but) at 0.3%. MaR2 (10^{-8} M) caused an increase in \([Ca^{2+}]\) to a peak of 475.49 ± 125.41 nM (p=0.019, Fig. 8A; n=3). MaR2 (10^{-8} M) added after t-butanol (inactive analog) increased \([Ca^{2+}]\) to a peak of 72.40 ± 17.05 nM that was significantly decreased from MaR2 alone (p=0.033). MaR2 (10^{-8} M) after 1-butanol (active analog) increased \([Ca^{2+}]\) to a peak of 97.89 ± 6.41 nM that was significantly decreased compare to MaR2 alone (p=0.040). For the positive controls, MaR1 and carbachol stimulation of peak in \([Ca^{2+}]\) was decreased by 1-butanol, but not by t-butanol (Fig. 8A). Although the peak in \([Ca^{2+}]\) was reduced by 1-butanol, we cannot conclude that the action of MaR2 is dependent on PLD, due to inhibition by the inactive control t-butanol. In contrast, MaR1 and Cch stimulation were dependent on the activation of PLD.

To explore the role of PLD in MaR2-stimulated glycoconjugate secretion, rat conjunctival goblet cells were incubated with 1-butanol or t-butanol then stimulated with MaR2. MaR2 increased secretion 3.5 ± 0.7 fold above basal (p=0.007, Fig. 8B; n=3). 1-butanol (p=0.02), but not t-butanol (p=0.50), significantly inhibited MaR2-stimulated secretion. The positive controls, MaR1 and Cch stimulated glycoconjugate secretion above basal and 1-butanol, but not t-butanol, significantly decreased the response for each agonist. This indicates that MaR2 utilizes PLD to stimulate glycoconjugate secretion, as do MaR1 and carbachol.

To examine if MaR2 is dependent on PLA2 to increase \([Ca^{2+}]\), we used the PLA2-inhibitor Aristolochic Acid (AA). MaR2 (10^{-8} M) caused an increase in \([Ca^{2+}]\) to a peak of 301.53 ± 17.7 nM (p=0.05, Fig. 8C; n=4). When incubated with AA 10^{-5} M or AA 10^{-6} M, MaR2 caused an increase in \([Ca^{2+}]\) to a peak of 108.77 ± 14.83 nM or 129.09 ± 25.71 nM, respectively values that were significantly decreased from MaR2 alone (p<0.001 for AA 10^{-5} M and p=0.001 for AA 10^{-6} M). The action of Cch on peak increase in \([Ca^{2+}]\) was blocked by AA at 10^{-5} M (Fig. 8C). Thus MaR2 activates PLA2 to increases \([Ca^{2+}]\).
To determine if the action of MaR2 is dependent on PLA$_2$ to stimulate glycoconjugate secretion, conjunctival goblet cells were preincubated with AA. MaR2 increased secretion 1.7 ± 0.2 fold above basal (p=0.003, Fig. 8D; n=6). When incubated with AA at $10^{-6}$ M MaR2 increased secretion to 1.7 ± 0.8 fold above basal (p=0.97), not a significantly different value from stimulation with MaR2 alone. The increase in glycoconjugate secretion stimulated by the positive control, Cch was significantly decreased by AA (Fig. 8D) This indicates that MaR2 is using PLA$_2$ to increase $[\text{Ca}^{2+}]$, but not to stimulate glycoconjugate secretion.

Figure 8: Maresin 2 has different dependency on Phospholipase D (PLD) and Phospholipase A$_2$ (PLA$_2$) to increase $[\text{Ca}^{2+}]$ and stimulate secretion in rat conjunctival goblet cells. Goblet cells were preincubated with the PLD inhibitor 0.3% 1-butanol or the inactive analog 0.3% t-butanol for 15 minutes and then stimulated with MaR2 ($10^{-8}$ M), MaR1 ($10^{-9}$ M) or Cch ($10^{-4}$ M) to measure the change in peak $[\text{Ca}^{2+}]$ (A), or secretion (B). Goblet cells were preincubated with the PLA$_2$ inhibitor aristolochic acid $10^{-6}$ M or $10^{-6}$ M for 30 minutes and stimulated with MaR2 ($10^{-8}$ M) or Cch ($10^{-4}$ M) to measure the change in peak $[\text{Ca}^{2+}]$ (C), or secretion (D). Data are mean ± SEM of three (A), three (B), four (C) and six (D) experiments. * shows significance above basal. # shows significance between agonist and inhibitor followed by agonist.
**Maresin 2 uses protein kinase A to increase [Ca$^{2+}$], and stimulate secretion in rat conjunctival goblet cells**

When a ligand activates $G_{\alpha}s$, adenyl cyclase (AC) catalyzes ATP to cAMP that in turn stimulates the activity of cAMP dependent protein kinase A (PKA). This is one among a variety of functions of cAMP (28). To explore if MaR2 uses PKA, we incubated rat conjunctival goblet cells with the PKA-inhibitor H89 ($10^{-5}$ M) for 30 minutes prior to stimulation with MaR2 ($10^{-8}$ M), or the positive control VIP ($10^{-8}$ M) or MaR1 ($10^{-8}$ M). MaR2 increased [Ca$^{2+}$], to a peak of 91.27 ± 17.97 nM ($p=0.00010$, **Fig. 9A**; n=5). Incubation with H89 increased [Ca$^{2+}$], to a peak of 40.66 ± 1.94 nM that was different from MaR2 alone ($p=0.023$). The action of MaR1 was not inhibited by H89, but of VIP was blocked (**Fig. 9A**). This indicates that MaR2 activates PKA to increase [Ca$^{2+}$].

To determine the dependency of MaR2 on PKA to stimulate glycoconjugate secretion, conjunctival goblet cells were incubated with H89 ($10^{-5}$ M) 30 minutes prior to addition of MaR2. MaR2 stimulated secretion to 3.4 ± 0.5 fold above basal ($p=2.2x10^{-5}$, **Fig. 9B**; n=6). Incubation with H89 significantly decreased MaR2-stimulated secretion to 1.8 ± 0.3 fold above basal ($p=0.004$). Secretion stimulated by the positive control VIP, but not MaR1, was also significantly inhibited by H89. These data indicate that MaR2, but not MaR1, is dependent on activation of PKA to increase [Ca$^{2+}$], and glycoconjugate secretion.
Figure 9: Maresin 2 uses protein kinase A to increase \([\text{Ca}^{2+}]_i\) and stimulate secretion in rat conjunctival goblet cells. Goblet cells were incubated with the protein kinase A (PKA) inhibitor H89 (10^{-5} M) for 30 minutes and then stimulated with MaR2 (10^{-8} M) or MaR1 (10^{-8} M) or VIP (10^{-8} M) to measure the change in peak \([\text{Ca}^{2+}]_i\) (A), or secretion (B). Note that MaR1 was not used as an agonist in B. Data are mean ± SEM of five (A) and six (B) experiments. * shows significance above basal. # shows significance between agonist and inhibitor followed by agonist.

Maresin 2 inhibits histamine-, but not LTB4-stimulated increase in \([\text{Ca}^{2+}]_i\) and histamine-stimulated glycoconjugate secretion in rat conjunctival goblet cells.
Histamine has a key role in inflammatory allergic diseases, and is mainly secreted by mast cells and basophils. Histamine acts on four receptors (H1-H4), causing vasodilatation and vascular permeability contributing to inflammation (29). In rat conjunctival goblet cells, histamine increases [Ca\(^{2+}\)]\(_i\), and stimulates glycoconjugate secretion (30). Other SPMs, including MaR1, inhibit histamine-stimulated increase [Ca\(^{2+}\)]\(_i\) and glycoconjugate secretion(15, 16). Histamine caused an increase in [Ca\(^{2+}\)]\(_i\) to a peak of 269.47 ± 28.92 nM (p=0.00074, Fig. 10A; n=3). When incubated with MaR2 for 30 min before addition of histamine, the increase in peak [Ca\(^{2+}\)]\(_i\) was attenuated to 116.79 ± 16.80 nM by MaR2 at 10\(^{-10}\) M (p=0.010), to 150.66 ± 50.78 nM by MaR2 at 10\(^{-9}\) M (p=0.11) and to 90.73 ± 5.55 nM by MaR2 10\(^{-8}\) M (p=0.0040). In addition, MaR1 at 10\(^{-8}\) M also blocked the histamine stimulated increase in [Ca\(^{2+}\)]\(_i\). We conclude that MaR2 similarly to MaR1 inhibits the histamine stimulated increase in [Ca\(^{2+}\)]\(_i\).

Dartt et al. demonstrated that histamine stimulates an increase in [Ca\(^{2+}\)]\(_i\) and glycoconjugate secretion in rat conjunctival goblet cells (30). To investigate if MaR2 inhibits histamine stimulated glycoconjugate secretion, rat conjunctival goblet cells were preincubated with MaR2 (10\(^{-8}\) M) for 30 minutes prior to stimulation with histamine (10\(^{-5}\) M). Histamine stimulated secretion to 1.75 ± 0.13 above basal (p=0.001, Fig. 10B; n=4). Preincubation with MaR2 decreased the response to 1.09 ± 0.19 above basal (p=0.029). This indicates that MaR2 inhibits histamine stimulated glycoconjugate secretion.

LTB\(_4\) is a chemoattractant involved in inflammation and immune response and activates inflammatory cells (24). LTB\(_4\) binds to the BLT1 receptor and to the ALX/FPR2-receptor (31). To determine if MaR1 and MaR2 act on LTB\(_4\)-stimulated increase in [Ca\(^{2+}\)]\(_i\), we preincubated rat conjunctival goblet cells with MaR1 (10\(^{-8}\) M) or MaR2 (10\(^{-8}\) M) for 30 minutes, then stimulated with LTB\(_4\) (10\(^{-9}\) M). LTB\(_4\) caused an increase in [Ca\(^{2+}\)]\(_i\) to a peak of 128.71 ± 27.51 nM (p=0.0034, Fig. 10C; n=4). Incubation with MaR2 caused a LTB\(_4\)-stimulated increase in [Ca\(^{2+}\)]\(_i\) to a peak of 89.62 ± 31.53 nM (p=0.39) that was not different from stimulation by LTB\(_4\) alone. Incubation with MaR1 significantly decreased the LTB\(_4\)-stimulated increase in [Ca\(^{2+}\)]\(_i\) to a peak of 47.65 ± 7.65 nM (p=0.030). We conclude that MaR1 inhibits LTB\(_4\)-stimulated increase in [Ca\(^{2+}\)]\(_i\), while MaR2 does not.
Figure 10: Maresin 2 inhibits histamine-, but not LTB4-stimulated increase in \([\text{Ca}^{2+}]\) and histamine-stimulated glycoconjugate secretion in rat conjunctival goblet cells. Goblet cells were incubated with MaR2 (10^{-8} M) and then stimulated with histamine (10^{-5} M). Goblet cells were incubated with MaR2 (10^{-10} to 10^{-8} M) or MaR1 (10^{-8} M) for 30 minutes, then stimulated with histamine (10^{-5} M). Changes in peak \([\text{Ca}^{2+}]\) are shown in (A) and changes in glycoconjugate secretion are shown in (B). Goblet cells were incubated with MaR2 (10^{-8} M) or MaR1 (10^{-8} M) for 30 minutes, then stimulated with LTB4 10^{-9} M. Changes in peak \([\text{Ca}^{2+}]\) are shown in (C). Data are mean ± SEM of three (A) and four (B) and four (C) experiments. * shows significance above basal. # shows significance between MaR2 or MaR1 treatment and histamine (A and B) or LTB4 (C) and histamine (A and B) or LTB4 (C) alone.

Discussion

In the present study we showed that MaR2 activates rat conjunctival goblet cells through an increase in \([\text{Ca}^{2+}]\), that stimulates secretion (Fig. 11). MaR2 uses the BLT1 receptor to increase \([\text{Ca}^{2+}]\), by activation of the cAMP-dependent PKA, PLD, PKC and PLA2 signaling pathways. None of the inhibitors of the signaling components, however, blocked MaR2-stimulated increase in \([\text{Ca}^{2+}]\), completely, indicating that multiple pathways/receptors were involved in cellular activation. Preincubation with thapsigargin decreased MaR2-stimulated
[Ca\(^{2+}\)]_i increase, suggesting that activated signaling pathways cause a release of Ca\(^{2+}\) from intracellular calcium stores. Similarly to other SPMs, such as MaR1, LXA\(_4\), RvD1, RvD2, and RvE1, MaR2 regulates [Ca\(^{2+}\)]_i and secretion including MUC5AC in rat conjunctival goblet cells (15, 17-19, 32). These actions likely contribute to optimal tear film function under normal, physiological conditions.

Although being of similar chemical structure, MaR2 and MaR1 activate different receptors. MaR2 to date only uses the BLT1 receptor and uses it only to increase [Ca\(^{2+}\)]. We found that MaR1 uses the BLT1- and the ALX/FPR2 receptor to increase [Ca\(^{2+}\)], but only the BLT1 receptor to stimulate secretion. Treatment with MaR1 desensitizes MaR2, while preincubation with MaR2 does not affect the MaR1 response. There are several possible mechanisms that might explain how MaR1 attenuates MaR2 response. A possible mechanism of inhibition of MaR2 actions by MaR1 is through activation of the ALX/FPR2 receptor or through other receptors, including the newly identified LGR6 receptor for MaR1 that MaR2 does not stimulate (33). It should be noted, however, that LGR6 was found in human, but not yet in rat, tissue. Furthermore, MaR1 may attenuate MaR2 by interacting with an overlapping or different region of the BLT1 receptor than MaR2 binds to. To support this hypothesis, we found that MaR1 decreases LTB\(_4\) induced increase in [Ca\(^{2+}\)], while MaR2 does not. We suggest that MaR1, but not MaR2, may attenuate LTB\(_4\)- and MaR2-dependent BLT1 responses by activating a protein kinase that phosphorylates the BLT1 receptor and counter-regulates it. MaR1, but not MaR2, could contribute to resolution of leukotriene-stimulated inflammation in ocular surface disease.

The BLT1 receptor is activated by the pro-inflammatory chemoattractant LTB\(_4\) (34). We found that MaR1 was dependent on the BLT-1 receptor to increase [Ca\(^{2+}\)], and to stimulate glycoconjugate secretion, while MaR2 was only dependent on the BLT1 receptor to increase [Ca\(^{2+}\)]. The fact that the pro-inflammatory mediator LTB\(_4\) and the pro-resolving mediators MaR1 and MaR2 are using the same receptor is an example of biased agonism. Biased agonism is when different ligands bind to a receptor to activate different signal transduction pathways, a phenomenon also found in receptors such as ALX/FPR2 (35). The BLT1 receptor is a GPCR primarily known to couple to the inhibitory protein of the adenyl cyclase, G\(_i\), and the stimulatory protein G\(_q\), the latter of which activates PLC, ultimately inducing chemotaxis (36). We found that MaR2 can activate the BLT1 receptor, while also increase cAMP levels and stimulate PKA, that are activated by the protein G\(_{as}\). BLT1 does not couple to G\(_{as}\) and does not activate adenyl cyclase suggesting that MaR2 could activate another receptor in rat conjunctival goblet cells to perform its actions. In support of
this suggestion another SPM derived from DHA, RvE1, is known to bind to both the ChemR23 and BLT1 receptors. A central role for BLT1 in SPM functioning in rat conjunctival goblet cells is emerging (25).

Consistent with MaR2 and MaR1, interacting with different receptors or different sites on the same receptor, these SPMs differ in the use of the cAMP/PKA signaling pathway. MaR2, but not MaR1, increases cAMP levels and activates PKA to increase \([\text{Ca}^{2+}]_i\) and stimulate secretion. The only other SPM published to date in rats to use cAMP and PKA to increase in \([\text{Ca}^{2+}]_i\) and stimulate secretion is RvD2 (32). Interestingly, in human immune cells MaR1 uses LGR6 to increase cAMP levels and activate PKA. RvD2 in rat and human conjunctival goblet cells uses the GPR18 receptor that activates adenyl cyclase, to increase cAMP levels and activate PKA. Activation of PKA by itself stimulates secretion, but also increases \([\text{Ca}^{2+}]_i\) by interacting with the \(\text{IP}_3\) receptors on intracellular \(\text{Ca}^{2+}\) stores, likely on endoplasmic reticulum. Vasoactive intestinal peptide (VIP) is a parasympathetic neurotransmitter that like MaR2 and RvD2 stimulates PKA (37). VIP activates the VPAC1- and the VPAC2 receptors causing activation of adenyl cyclase that increases levels of cAMP, ultimately activating PKA. The activated PKA increases \([\text{Ca}^{2+}]_i\) through a mechanism that is similar to that used by RvD2 and slightly different from that used by MaR2. The difference is that VIP and RvD2 stimulate PLC activity to produce \(\text{IP}_3\). \(\text{IP}_3\) then binds with its receptors on the ER to release \(\text{Ca}^{2+}\) and cAMP that interacts with the \(\text{IP}_3\) receptors to increase \(\text{Ca}^{2+}\). In contrast, MaR2 does not activate PLC, thus, no \(\text{IP}_3\) receptors should be involved. MaR2 activation of PKA would then increase \([\text{Ca}^{2+}]_i\) by a different mechanism. Further studies are warranted to determine the specifics of the MaR2 cAMP-dependent actions and to compare them with those of RvD2 and VIP.

In spite of MaR2 and MaR1 interacting with different receptors and interacting with different signaling pathways, MaR2 and MaR1 both use several similar \(\text{Ca}^{2+}\)-dependent signaling pathways. First, both agonists increase \([\text{Ca}^{2+}]_i\) by release of intracellular \(\text{Ca}^{2+}\) stores, confirmed by inhibition of secretion when the \([\text{Ca}^{2+}]_i\) was decreased by the \(\text{Ca}^{2+}\) chelator BATPA/AM and when MaR2 and MaR1 stimulated increase in \([\text{Ca}^{2+}]_i\) was blocked by the SERCA inhibitor thapsigargin that depletes intracellular \(\text{Ca}^{2+}\) stores (15). There are three main signaling pathways that SPMs use to increase \([\text{Ca}^{2+}]_i\) and stimulate secretion in conjunctival goblet cells PLA2, PLD and PLC. Neither MaR2 nor MaR1 activate PLA2 to increase \([\text{Ca}^{2+}]_i\) and stimulate secretion. Both MaR2 and MaR1 activate PLD to increase \([\text{Ca}^{2+}]_i\) and stimulate secretion, although the negative control for MaR1 and PLD increase in \([\text{Ca}^{2+}]_i\) was also inhibitory. MaR2 and MaR1 both activate components of the PLC pathway. MaR2 and MaR1 activate PLC and use the downstream molecule \(\text{IP}_3\)R, although only MaR2-
stimulated secretion is dependent on these components. Both MaR2 and MaR1 activated PKC to increase [Ca^{2+}] and stimulate secretion. In contrast, both MaR2- and MaR1-stimulated increase in [Ca^{2+}] are independent of extracellular Ca^{2+}. Thus, the PLC pathway has some differences between MaR2 and MaR1 activation, especially in the targets of PLC activation. Whereas MaR1 stimulates the increase in [Ca^{2+}] and secretion by the well-known PLC pathway that produces IP_3 that releases Ca^{2+} from intracellular stores and produces DAG to activate PKC to stimulate secretion, MaR2 only uses these processes to stimulate secretion. As MaR2 and MaR1 activate the PLD pathway, they could use PLD to activate PKC via an increase in Ca^{2+}. In contrast, MaR2 does not use PLC to increase Ca^{2+} and activate PKC, while MaR1 does. As there are multiple PKC isoforms in conjunctival goblet cells some of which are Ca^{2+}-dependent and Ca^{2+}-independent PKC isoforms, MaR2 and MaR1 may be activating different PKC isoforms to stimulate secretion (38). Identification of a MaR2-specific receptor and a more detailed investigation of the components of the signaling pathways could clarify some of the differences between MaR2 and MaR1 and their use of signaling pathways in conjunctival goblet cells.

A common type of chronic inflammation on the ocular surface is ocular allergy, a disease initiated by an allergic stimulus. Inflammatory ocular diseases usually cause hypersecretion of mucins. One of the central stimulatory mediators causing hypersecretion in allergic diseases is histamine (7). When rat conjunctival goblet cells are preincubated with MaR2 before stimulation with histamine, the increase in both [Ca^{2+}] and secretion decrease. This suggests that MaR2 can block the inflammatory effect of histamine on goblet cells decreasing mucin secretion. Many other SPMs, including LXA_4, RvD1, RvE1, and MaR1, similarly counter-regulated the effect of histamine in cultured rat conjunctival goblet cells (15, 16, 39, 40). Ours findings herein support a role of MaR2, in both health and disease.

Information about the function of MaR2 in disease in other organs is limited. MaR2 limits polymorphonuclear neutrophil (PMN) entry during inflammation and stimulates phagocytosis, similar to the actions of MaR1 (11, 12). Furthermore, the anti-inflammatory, pro-resolving and anti-atherosclerotic effects of MaR2 might be beneficial in diseases such as myocardial infarction and acute and chronic heart failure (11, 41). MaR2 is likely to be active in many additional diseases and tissues.

We conclude that MaR2 and MaR1 stimulate conjunctival goblet cell function especially secretion, by activating different, but overlapping GPCR and signaling pathways, and furthermore counter-regulate histamine stimulated increase in [Ca^{2+}]. Thus, MaR2 and MaR1 play a role in maintaining the ocular surface and tear film homeostasis in health and
As MaR2 and MaR1 modulate conjunctival goblet cell function, they have a potential as novel options for treatment of ocular surface inflammatory diseases including allergic conjunctivitis and dry eye disease.

Figure 11: Schematic diagram of signaling pathways activated by maresin 2 (MaR2) [orange arrows] compared to the pathways activated by maresin 1 (MaR1)[blue arrows]. MaR2 activates the BLT1 receptor activating PLD, AC, PLA₂ and PLC. PLD and AC activate downstream molecules that increase [Ca²⁺] causing glycoprotein secretion. PLA₂ and the PLC pathway stimulates glycoprotein secretion by another unknown mechanism than increasing [Ca²⁺].

**Funding**

Supported by the Norwegian Research Council (Oslo, Norway), Faculty of Medicine, University of Oslo, Department of Medical Biochemistry, Oslo University Hospital and by NIH R01 EY019470.

**Acknowledgements**
The authors would like to thank Drs. Marie Shatos and Dayu Li for their helpful assistance.

References

Figure S1: Inhibition of the ALX/FPR2- and the BLT1 receptors acts on stimulation of $[Ca^{2+}]_i$ by MaR2 at $10^{-8}$ M. **Fig. A**, **B**, and **C** show changes in $[Ca^{2+}]_i$ with time treated with the ALX/FPR2 receptor inhibitor BOC2 ($10^{-4}$ M) and then stimulated with MaR2 $10^{-8}$ M, MaR1 $10^{-8}$ M, and LXA$_4$ $10^{-9}$ M. **Fig. D, E and F** show changes in $[Ca^{2+}]_i$ with time treated with the BLT1 receptor inhibitor U-75302 ($10^{-8} – 10^{-6}$ M) and then stimulated with MaR2 $10^{-8}$ M, MaR1 $10^{-8}$ M, and LTB$_4$ $10^{-8}$ M. Data are mean ± SEM of five (**A, B, and C**) and six (**D, E, and F**).
Figure S2: Maresin 2 increase in $[\text{Ca}^{2+}]_i$ is independent of the PLC-pathway in rat conjunctival goblet cells. Changes in $[\text{Ca}^{2+}]_i$ with time are shown. In A, B and C, goblet cells were treated with vehicle, the active PLC inhibitor U-73122 or the inactive PLC inhibitor U-73343 both at $10^{-7}$ M for 30 minutes and stimulated with MaR2 ($10^{-8}$ M), MaR1 ($10^{-8}$ M) or Cch ($10^{-4}$ M). In D, E and F, goblet cells were treated with vehicle or 2APB ($10^{-5}$ M) and stimulated with MaR2 ($10^{-8}$ M), MaR1 ($10^{-8}$ M) or Cch ($10^{-4}$ M). Data are mean ± SEM of four (A, B, and C) and five (D, E, and F) experiments.

Figure S3: Maresin 2 stimulated increase in $[\text{Ca}^{2+}]_i$ is independent of extracellular Ca$^{2+}$ in rat conjunctival goblet cells. Changes in $[\text{Ca}^{2+}]_i$ with time are shown in goblet cells incubated with KRB with or without CaCl$_2$ and then stimulated with (A) MaR2 ($10^{-8}$ M), (B) MaR1 ($10^{-8}$ M) or (C) Cch ($10^{-4}$ M). Data are mean ± SEM of four experiments in each panel.
**Figure S4:** Maresin 2 increases $[\text{Ca}^{2+}]_i$ by activation of protein kinase C (PKC). Changes in $[\text{Ca}^{2+}]_i$ with time are shown in goblet cells incubated with RO317549 ($10^{-7}$ M) for 30 minutes and then stimulated with (A) MaR2 ($10^{-8}$ M), (B) MaR1 ($10^{-8}$ M) or (C) Chh ($10^{-4}$ M). Data are mean ± SEM of three experiments in each panel.

**Figure S5:** Maresin 2 has different dependency on Phospholipase D (PLD) and Phospholipase A$_2$ (PLA$_2$) to increase $[\text{Ca}^{2+}]_i$ and stimulate secretion in rat conjunctival goblet cells. Changes in $[\text{Ca}^{2+}]_i$ with time are shown. Goblet cells were preincubated with the PLD inhibitor 0.3% 1-butanol or the inactive analog 0.3% t-butanol for 15 minutes and then stimulated with (A) MaR2 ($10^{-8}$ M), (B) MaR1 ($10^{-8}$ M) or (C) Chh ($10^{-4}$ M). Goblet cells were preincubated with the PLA$_2$ inhibitor aristolochic acid $10^{-5}$ M or $10^{-6}$ M for 30 minutes and stimulated with (D) MaR2 ($10^{-8}$ M) or (E) Chh ($10^{-4}$ M). Data are mean ± SEM of three (A, B, and C) and four (D and E) experiments.

**Figure S6:** Maresin 2 uses protein kinase A to increase $[\text{Ca}^{2+}]_i$ and stimulate secretion in rat conjunctival goblet cells. Goblet cells were incubated with the protein kinase A (PKA) inhibitor H89 ($10^{-5}$ M) for 30 minutes and then stimulated with (A) MaR2 ($10^{-8}$ M), (B) MaR1 ($10^{-8}$ M) or (C) VIP ($10^{-8}$ M) to measure $[\text{Ca}^{2+}]_i$ over time. Data are mean ± SEM of five experiments in each panel.
**Figure S7:** Maresin 2 inhibits histamine-, but not LTB₄-stimulated increase in [Ca²⁺]ᵢ and histamine-stimulated glycoconjugate secretion in rat conjunctival goblet cells. Changes in [Ca²⁺]ᵢ with time are shown. (A) Goblet cells were incubated with MaR2 (10⁻¹⁰ to 10⁻⁸ M) or MaR1 (10⁻⁸ M) for 30 minutes, then stimulated with histamine (10⁻⁵ M) and (B) Goblet cells were incubated with MaR2 (10⁻⁸ M) or MaR1 (10⁻⁸ M) for 30 minutes, then stimulated with LTB₄ 10⁻⁹ M. Data are mean ± SEM of three (A) and four (B) experiments.
Paper 3
Pro-Resolving Mediator Annexin A1 Regulates Intracellular Ca\(^{2+}\) and Mucin Secretion in Cultured Goblet Cells Suggesting a New Use in Inflammatory Conjunctival Diseases

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The amount of mucin secreted by conjunctival goblet cells is regulated to ensure the optimal level for protection of the ocular surface. Under physiological conditions lipid specialized pro-resolving mediators (SPM) are essential for maintaining tissue homeostasis including the conjunctiva. The protein Annexin A1 (AnxA1) can act as an SPM. We used cultured rat conjunctival goblet cells to determine if AnxA1 stimulates an increase in intracellular \([\text{Ca}^{2+}]_i\) and mucin secretion and to identify the signaling pathways. The increase in \([\text{Ca}^{2+}]_i\) was determined using fura2/AM and mucin secretion was measured using an enzyme-linked lectin assay. AnxA1 stimulated an increase in \([\text{Ca}^{2+}]_i\) and mucin secretion that was blocked by the cell-permeant \(\text{Ca}^{2+}\) chelator BAPTA/AM and the ALX/FPR2 receptor inhibitor BOC2. AnxA1 increased \([\text{Ca}^{2+}]_i\) to a similar extent as the SPMs lipoxin A\(_4\) and Resolvin (Rv) D1 and histamine. The AnxA1 increase in \([\text{Ca}^{2+}]_i\) and mucin secretion were inhibited by blocking the phospholipase C (PLC) pathway including PLC, the IP\(_3\) receptor, the \(\text{Ca}^{2+}/\text{ATPase}\) that causes the intracellular \(\text{Ca}^{2+}\) stores to empty, and blockade of \(\text{Ca}^{2+}\) influx. Inhibition of protein kinase C (PKC) and \(\text{Ca}^{2+}/\text{calmodulin-dependent protein kinase}\) also decreased the AnxA1-stimulated increase in \([\text{Ca}^{2+}]_i\) and mucin secretion. In contrast inhibitors of ERK 1/2, phospholipase A\(_2\) (PLA\(_2\)), and phospholipase D (PLD) did not alter AnxA1-stimulated increase in \([\text{Ca}^{2+}]_i\), but did inhibit mucin secretion. Activation of protein kinase A did not decrease either the AnxA1-stimulated rise in \([\text{Ca}^{2+}]_i\), or secretion. We conclude that in health, AnxA1 contributes to the mucin layer of the tear film and ocular surface homeostasis by activating the PLC signaling pathway to increase \([\text{Ca}^{2+}]_i\), and stimulate mucin secretion and ERK1/2, PLA\(_2\), and PLD to stimulate mucin secretion from conjunctival goblet cells.

Keywords: ocular surface, annexin A1, secretion, specialized pro resolving mediators, mucin
INTRODUCTION

Inflammation is a component in common to both allergic conjunctivitis and dry eye disease—two frequently occurring diseases of the ocular surface (cornea and conjunctiva) (1). In allergic conjunctivitis allergens penetrate the conjunctival epithelium and initiate the production of pro-inflammatory mediators including histamine, leukotrienes, and prostaglandins. These compounds cause vasodilation, pain, edema and recruitment of neutrophils, macrophages, and mast cells into the conjunctiva (2). In acute inflammation, the infiltrating leukocytes switch from producing pro-inflammatory mediators to generate the specialized pro-resolving lipid mediators (SPMs) lipoxins, resolvins, protectins, and maresins (2–6). These SPMs actively terminate inflammatory processes by blocking the effects of the pro-inflammatory mediators on their target tissues, including the conjunctival goblet cells. The protein annexin A1 (AnxA1) is also pro-resolving (6, 7). In general, failed endogenous resolution mechanisms lead to uncontrolled and chronic inflammation (1). In addition to resolving inflammation, the SPMs also maintain homeostasis of the conjunctival epithelium to keep the ocular surface healthy.

Conjunctival goblet cells synthesize and secrete large, high molecular weight, gel forming mucins (8). These mucins make up the innermost layer of the tear film (9). MUC5AC, the major mucin secreted by conjunctival goblet cells, can trap and remove ocular allergens and airborne pathogens (8). An optimum amount of MUC5AC secreted into the tear film is critical for ocular surface health, as a depleted number of goblet cells and mucin secretion lead to corneal and conjunctival damage (8). Conversely, MUC5AC overproduction is a symptom of allergic conjunctivitis and unhealthy for the ocular surface (8). Lipid SPMs including D-series and E-series resolvins, lipoxins, and maresins act by blocking excess mucin secretion from conjunctival goblet cells during inflammation, as well as stimulating mucin secretion under physiological conditions, thus maintaining ocular surface homeostasis (10–12).

AnxA1 is an anti-inflammatory, pro-resolving protein originally described as a mediator of glucocorticoid action (13). The synthesis of AnxA1 is a primary anti-inflammatory mechanism of glucocorticoids. AnxA1 works at least partly by suppressing phospholipase A2 activity, thus blocking the production of pro-inflammatory eicosanoids (14). During inflammation, endogenous glucocorticoids are secreted from the adrenal gland to avoid an excessively vigorous inflammatory response that could potentially damage the host (15). Glucocorticoids can stimulate both the synthesis and secretion of AnxA1 (16). After being synthesized AnxA1 is mobilized to the plasma membrane and secreted by three different mechanisms: 1) the ATP-binding (ABC) transporter, 2) direct phosphorylation of serine-27 that localizes it to the plasma membrane, and 3) exocytosis by granule fusion with the cell membrane (17). The secreted AnxA1 can then interact with the ALX/FPR2 receptor in a paracrine, autocrine, or juxtacrine fashion.

As an effector molecule of glucocorticoids, AnxA1 has potential as an endogenous anti-inflammatory drug, and the use of AnxA1 therapeutically likely has fewer side effects than the use of glucocorticoids. AnxA1 contributes to the regulation of a wide variety of cellular events, including both acute and chronic inflammation, ischemic injury, fever, pain, and the release of arachidonic acid (17). AnxA1, as well as its N-terminal peptides, have also been found to potentially inhibit neutrophil trafficking in mice (7). AnxA1 could prevent development of pro-inflammatory disease in the conjunctiva.

To determine if AnxA1 plays a role in maintaining the mucous layer in ocular surface health we investigated if exogenous addition of AnxA1 can increase intracellular [Ca^{2+}] and stimulate MUC5AC secretion in cultured primary conjunctival goblet cells from rats whose signaling pathways AnxA1 activates in these cells.

MATERIALS AND METHODS

Materials

AnxA1 was obtained from MyBioSource (San Diego, CA). The compound was stored at -20°C. Prior to use, AnxA1 was immediately diluted in 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_{2}PO_{4}, 25 mM NaHCO_{3}, 10 mM HEPES, and 5.5 mM glucose [pH 7.45]) to the required concentration and added to the conjunctival goblet cells. RPMI-1640 cell culture medium, penicillin/streptomycin, trypsin, and L-glutamine were purchased from Lonza (Walkerville, IL). Fetal bovine serum (FBS) was from Atlanta Biologicals (Norcross, GA). Fura-2 was from Life Technologies (Grand Island, NY). U73122 and U73343, KN92 and KN93 were purchased from Tocris Bioscience (Ellisville, MO). The lectin Ulex Europaeus Agglutinin (UEA-1), histamine, BAPTA/AM, 2-aminoethyl diphenylborinate (2-APB), 1-butanol (1-but), t-butanol (t-but), carbachol, arachidonic acid (AA), thapsigargin and RO317549 were all purchased from Sigma-Aldrich (St Louis, MO). UO126 was obtained from R&D Systems (Minneapolis, MN). H89 and synthetic lipoxin A,

LXA_{4}, 5(S),6(R),15(S)-TriHETE were from EMD Millipore (Billerica, MA). Resolvin (Rv) D1 (7S,14R,17S-trihydroxy-4Z, 9E,11E,13Z,19Z-docosahexaenoic acid) was obtained from Cayman Chemical, Ann Arbor, MI. N-Boc-Phe-Leu-Phe-Leu-Phe (BOC2) was obtained from Genscript Corp in Piscataway, NJ.

Animals

Four- to eight-week-old male Sprague-Dawley rats (Taconic Farms, Germantown, NY, USA) were anesthetized with CO_{2} and decapitated. The bulbar and fornical conjunctival epithelium was surgically removed from both eyes. All experiments were in accordance with the US National Research Council’s Guide for the Care and Use of Laboratory Animals, the US Public Health Service’s Policy on Humane Care and Use of Laboratory Animals, and Guide for the Care and Use
of Laboratory Animals and were approved by the Schepens Eye Research Institute Animal Care and Use of Committee.

**Culture of Conjunctival Goblet Cells**

Goblet cells from rat conjunctiva were grown in organ culture as described previously (3). The conjunctiva was dissected free of underlying connective tissue. The conjunctival explants were grown on six-well plates in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 µg/mL penicillin-streptomycin for approximately one week. The goblet cells were then trypsinized and plated onto glass bottom petri dishes or 24 well plates, for [Ca^{2+}] measurements or mucin secretion measurements, respectively. First passage goblet cells were used in all experiments. The identity of the cultured cells was checked by evaluating staining with antibody to cytokeratin 7 (detects goblet cell bodies) and the lectin UEA-1 (detects goblet cell secretory products) to ensure that goblet cells predominated in the cultures.

**Measurement of cDNA Expression by Semi-Quantitative 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 AnxA1 using the Jumpstart REDTaq ReadyMix Reaction Mix (Sigma-Aldrich, St. Louis, MO, USA) in a thermal cycler (Master Cycler, Eppendorf, Hauppauge, NY). The forward primer was GTG ATC GCT GTG AGG ATA TGA G, and the reverse primer was TAC AGA GCA GTT GGG ATG TTT AG. These primers generated 504 bp fragments. β-Actin primers, the positive control, were forward CGT CAT ACT CCT GCT TGC TGA TCC A and the reverse primer was ATC TGG CAC CAC ACC TTC TAC AAT GG CT and generated a 790 bp fragment. The conditions were as follows: 5 min at 95°C followed by 35 cycles of 1 min at 94°C, 30 seconds 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.

**Measurement of Mucin Secretion**

Cultured rat goblet cells were plated in 24-well plates and grown to confluence (1, 10–12, 18, 19). After serum starving cultured goblet cells for 2 h, the goblet cells were stimulated with AnxA1 in increasing concentrations in serum-free RPMI 1640 supplemented with 0.5% BSA for 2 h. The inhibitors were added 30 minutes prior to stimulation with AnxA1. An enzyme-linked lectin assay (ELLA) with the lectin UEA-1 was used to measure high molecular weight glycoprotein secretion that included MUC5AC produced by goblet cells. The supernatants were collected and analyzed for the total amount of lectin-detectable glycoconjugates, which quantifies the amount of goblet cell secretion as described previously (1, 3, 10–12, 20–22). The cells were scraped, and the cell homogenate was analyzed for the total amount of protein by using the Bradford protein assay. High molecular weight glycoconjugate secretion was normalized to total protein in the homogenate. Secretion was expressed as fold increase above basal that was set to 1.

**Measurement of Intracellular [Ca^{2+}]**

Cultured rat conjunctival goblet cells were transferred to 35-mm glass bottom culture dishes and incubated overnight. The goblet cells were then incubated for 1 h at 37°C 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_{2}PO_{4}, 25 mM NaHCO_{3}, 10 mM HEPES, and 5.5 mM glucose) (pH 7.45) plus 0.5% BSA containing 0.5 fura-2/AM (Invitrogen, Grand Island, NY), 8 µM pluronic acid F127 (Sigma-Aldrich, St. Louis, MO, USA) and 250 µM sulfipyrazine (Sigma-Aldrich), followed by washing in KRB-HEPES containing sulfipyrazine. Ca^{2+} measurements were made with a ratio imaging system (InCyt Im2; Intracellular Imaging, Cincinnati, OH) using excitation wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. A minimum of 10 cells were selected in each experimental condition. AnxA1 was added either alone or after incubation with inhibitors for 15 or 30 min and intracellular [Ca^{2+}] ([Ca^{2+}]i) measured. After addition of agonist, the data were collected in real time. The data are presented as [Ca^{2+}]i, over time and peak [Ca^{2+}]i calculated by subtracting the average value before the agonist AnxA1 was added (basal) from the peak [Ca^{2+}]i.

**Statistical Analysis**

Results were expressed as the fold-increase above basal. Data are expressed as mean ± SEM. Data were analyzed by Student’s t-test. p < 0.05 was considered statistically significant.

**RESULTS**

**AnxA1 Is Expressed in Rat Conjunctival Goblet Cells**

The protein AnxA1 was detected in a wide range of tissues, including the lung, intestine and bone marrow (14). We used PCR to investigate the expression of AnxA1 in cultured rat conjunctival goblet cells (Figure 1). A single band at the correct size of 504 bp was found in cDNA isolated from the conjunctivas of three individual animals. Expression of β-actin was used as a control. As AnxA1 is a protein, this is the first pro-resolving mediator whose presence in rat conjunctival goblet cells was verified by PCR.

**AnxA1 Stimulates Mucin Secretion in Rat Conjunctival Goblet Cells**

Lipid specialized pro-resolving mediators (SPM) such as LXA₄, RvD1, and RvE1 stimulate mucin secretion from cultured rat conjunctival goblet cells (10–12). To explore if the protein AnxA1 stimulates secretion, goblet cells were stimulated for two h with AnxA1 (10^{-11}-10^{-8} M) or a positive control.
histamine (10^{-5} M) (18, 23) and the amount of secretion was then measured (Figure 2). Histamine is known to stimulate glycoconjugate secretion from conjunctival goblet cells (22, 24). AnxA1 increased mucin secretion in a concentration-dependent manner with a maximum increase at 10^{-10} M. Significant increases occurred from 10^{-11} M to 10^{-9} M AnxA1 and were 1.7 ± 0.2, 3.2 ± 0.3 and 2.0 ± 0 fold above basal, respectively from three independent experiments. In comparison histamine significantly increased mucin secretion from basal by 2.2 ± 0.4 fold.

AnxA1 Increases [Ca^{2+}]i in Rat Conjunctival Goblet Cells

As AnxA1 stimulates conjunctival goblet cells to secrete mucins, we determined if AnxA1 alters the [Ca^{2+}]i. Cultured goblet cells were incubated with fura-2/AM before stimulation with AnxA1 (10^{-11} - 10^{-8} M). AnxA1 increased [Ca^{2+}]i in a time and concentration-dependent manner (Figures 3A–C). All concentrations of AnxA1 significantly increased [Ca^{2+}]i, with the highest increase of 467.3 ± 95.1 nM occurring at 10^{-9} M (Figure 3C). These data are from five independent experiments. Therefore, AnxA1 increased [Ca^{2+}]i, in conjunctival goblet cells. To compare the [Ca^{2+}]i responses induced by other known mediators with that of AnxA1, goblet cells were stimulated with AnxA1, the lipid SPMs LXA4 and RvD1, and the allergic mediator histamine. All compounds were used at the concentrations previously found to result in the highest increase in [Ca^{2+}]i (10, 12). Each mediator significantly increased [Ca^{2+}]i over basal and to the same extent, as there was no significant difference between them (n=4, Figures 3D, E).

AnxA1 Uses Intracellular Ca^{2+} to Stimulate Secretion in Rat Conjunctival Goblet Cells

To determine if the AnxA1-stimulated increase in [Ca^{2+}]i leads to mucin secretion, rat goblet cells were incubated with the intracellular calcium chelator BAPTA/AM (10^{-4} M) for 30 minutes prior to AnxA1 (10^{-6} M) stimulation. The cholinergic agonist carbachol (Cch) at 10^{-4} M was used as a control. To ensure that BAPTA chelated [Ca^{2+}]i, and prevented the increase in [Ca^{2+}]i, induced by AnxA1 and Cch, [Ca^{2+}]i was measured (Supplemental Figure 1). AnxA1 significantly increased [Ca^{2+}]i, to a peak of 128.8 ± 11.5 nM. When goblet cells were preincubated with BAPTA/AM, the AnxA1-stimulated increase in [Ca^{2+}]i, was significantly inhibited to 30.1 ± 7.5 nM. The Ca^{2+} response to Cch, a positive control, was also significantly inhibited from 227.0 ± 54.8 nM to 29.3 ± 50.2 nM in the presence of BAPTA. These results were from 4 independent experiments.

We then investigated if AnxA1 uses Ca^{2+} to increase mucin secretion. Rat cultured goblet cells were treated with BAPTA/AM (10^{-4} M), stimulated by AnxA1 (10^{-10} M) and secretion measured. In three independent experiments, AnxA1 alone significantly increased glycoconjugate secretion by 1.5 ± 0.05 fold above basal (Figure 3F). BAPTA/AM significantly inhibited AnxA1-stimulated mucin secretion to 0.9 ± 0.06 fold above basal. Cch (10^{-7} M)-stimulated glycoconjugate secretion was also significantly inhibited from 1.7 ± 0.2 fold to 1.1 ± 0.1 fold above basal. These findings demonstrate that AnxA1 increases [Ca^{2+}]i, to stimulate mucin secretion in conjunctival goblet cells.

AnxA1 Uses Both Intracellular Ca^{2+} Stores and Extracellular Ca^{2+} to Increase [Ca^{2+}]i in Rat Conjunctival Goblet Cells

To examine the role that intracellular Ca^{2+} stores play in AnxA1 stimulation, goblet cells were incubated with the Ca^{2+} reuptake inhibitor thapsigargin. Thapsigargin depletes the ER of Ca^{2+} by blocking the Ca^{2+}/ATPase in the endoplasmic reticulum (ER) that inhibits Ca^{2+} from being taken up by the ER (25). The ER store of Ca^{2+} is depleted as Ca^{2+} passively leaks out of the ER and is not replaced as shown by the increase in [Ca^{2+}]i, over time (Figure 4A). AnxA1 added alone induced a peak increase in [Ca^{2+}]i of 166.8 ± 26.7 nM (Figures 4A, B, n=6). The AnxA1-induced Ca^{2+} response was significantly blocked by thapsigargin pretreatment and was 24.6 ± 9.7 nM. In the same cells, Cch was...
This increase in \([\text{Ca}^{2+}]\) and stimulated with increasing concentrations of Annexin A1 (AnxA1, 10^{-11} M to 10^{-8} M). Pseudo color images of \([\text{Ca}^{2+}]\) of goblet cells stimulated by AnxA1 10^{-9} M at four times are shown in (A). The increase in \([\text{Ca}^{2+}]\) over time with AnxA1 (10^{-11} M to 10^{-8} M) is shown in (B). The change in peak \([\text{Ca}^{2+}]\) after stimulation with AnxA1 (10^{-11} M to 10^{-8} M) is shown in (C). Increase in \([\text{Ca}^{2+}]\) over time in response to AnxA1 (10^{-5} M), LXA_{4} (10^{-8} M), RvD1 (10^{-5} M) and histamine (hist, 10^{-5} M) or histamine (hist, 10^{-5} M) is shown in (D). The change in peak \([\text{Ca}^{2+}]\), with AnxA1, LXA_{4}, LxA_{4} to Cch-stimulated increase in \([\text{Ca}^{2+}]\) was also significantly increased \([\text{Ca}^{2+}]\) for 30 minutes before stimulating the goblet cells with AnxA1 (10^{-5} M) or LXA_{4} (10^{-9} M), which is known to bind to ALX/FPR2 and is a positive control (12). AnxA1 added alone significantly increased \([\text{Ca}^{2+}]\), with a peak of 265.4 ± 61.0 nM (Figures 5A, C, n=5). BOC-2 significantly inhibited AnxA1’s increase in \([\text{Ca}^{2+}]\), to 54.1 ± 23.9 nM. The LXA_{4} response was 180.8 ± 52.4 nM (Figures 5B, C). In the presence of BOC-2, the LXA_{4} response was significantly reduced to 47.7 ± 20.0 nM.

To determine if AnxA1 uses the ALX/FPR2 receptor to stimulate high molecular weight glycoprotein secretion, cells were preincubated with BOC-2 (10^{-5} M) followed by AnxA1 (10^{-5} M) or BAPTA-AM (10^{-4} M) followed by carbachol (Cch, 10^{-4} M) or BAPTA-AM (10^{-4} M) followed by carbachol is shown in (F). Data are mean ± SEM from 4 independent experiments (A–E) or 3 (F) independent experiments. *indicates significant difference from basal; †indicates significant difference from AnxA1 or Cch alone.

**FIGURE 3 | AnxA1 elevates \([\text{Ca}^{2+}]\), to stimulate glycoconjugate secretion in cultured rat conjunctival goblet cells.** Cultured goblet cells were incubated with fura-2/AM and stimulated with increasing concentrations of Annexin A1 (AnxA1, 10^{-11} M to 10^{-8} M). Pseudo color images of \([\text{Ca}^{2+}]\) of goblet cells stimulated by AnxA1 10^{-9} M at four times are shown in (A). The increase in \([\text{Ca}^{2+}]\) over time with AnxA1 (10^{-11} M to 10^{-8} M) is shown in (B). The change in peak \([\text{Ca}^{2+}]\) after stimulation with AnxA1 (10^{-11} M to 10^{-8} M) is shown in (C). Increase in \([\text{Ca}^{2+}]\) over time in response to AnxA1 (10^{-5} M), LXA_{4} (10^{-8} M), RvD1 (10^{-5} M) and histamine (hist, 10^{-5} M) or histamine (hist, 10^{-5} M) is shown in (D). The change in peak \([\text{Ca}^{2+}]\), with AnxA1, LXA_{4}, LxA_{4} to Cch-stimulated increase in \([\text{Ca}^{2+}]\) was also significantly increased \([\text{Ca}^{2+}]\) for 30 minutes before stimulating the goblet cells with AnxA1 (10^{-5} M) or LXA_{4} (10^{-9} M), which is known to bind to ALX/FPR2 and is a positive control (12). AnxA1 added alone significantly increased \([\text{Ca}^{2+}]\), with a peak of 265.4 ± 61.0 nM (Figures 5A, C, n=5). BOC-2 significantly inhibited AnxA1’s increase in \([\text{Ca}^{2+}]\), to 54.1 ± 23.9 nM. The LXA_{4} response was 180.8 ± 52.4 nM (Figures 5B, C). In the presence of BOC-2, the LXA_{4} response was significantly reduced to 47.7 ± 20.0 nM.

To determine if AnxA1 uses the ALX/FPR2 receptor to stimulate high molecular weight glycoprotein secretion, cells were preincubated with BOC-2 (10^{-5} M) followed by AnxA1 (10^{-5} M) or BAPTA-AM (10^{-4} M) followed by carbachol (Cch, 10^{-4} M) or BAPTA-AM (10^{-4} M) followed by carbachol is shown in (F). Data are mean ± SEM from 4 independent experiments (A–E) or 3 (F) independent experiments. *indicates significant difference from basal; †indicates significant difference from AnxA1 or Cch alone.
FIGURE 4 | Annexin A1 (Anx1, 10−9 M) or carbachol (Cch, 10−4 M) (A top panel) or AnxA1 uses intracellular and extracellular Ca2+ to increase [Ca^{2+}]i in cultured rat conjunctival goblet cells. Cultured rat goblet cells were treated with thapsigargin (10−5 M) (first arrow) for 15 minutes before stimulation indicated by second arrow with annexin A1 (AnxA1, 10−9 M) or Cch (10−4 M) (A bottom panel). [Ca^{2+}]i over time is shown in (A) Change in peak [Ca^{2+}]i is shown in (B) Goblet cells were incubated in KRB solution with and without CaCl2 and stimulated with AnxA1 (10−9 M) or Carbachol (Cch, 10−4 M), [Ca^{2+}]i over time is shown in (C) Change in peak [Ca^{2+}]i is shown in (D) Data are mean ± SEM from 4 (A, B) or 6 (C, D) independent experiments. *indicates significant difference from basal; #indicates significance from AnxA1 or Cch alone.

FIGURE 5 | AnxA1 uses the ALX/FPR2 receptor to increase [Ca^{2+}]i and stimulate secretion in cultured rat conjunctival goblet cells. Goblet cells were preincubated with BOC2 (10−4 M) for 30 minutes and stimulated with vehicle or annexin A1 (AnxA1, 10−9 M) shown in (A, C) and vehicle or the control LXA4 (10−9 M) shown in (B, C) [Ca^{2+}]i over time is shown in (A, B) Change in peak [Ca^{2+}]i is shown in (C) Amount of glycoconjugate secretion is shown in (D) with AnxA1 at 10−10 M. B indicates basal (vehicle) in (D) Data in are mean ± SEM from 4 (A–C) or 3 (D) independent experiments. *indicates significant difference from basal; #indicates significance from AnxA1 or LXA4 alone.
trisphosphate (IP$_3$) and diacylglycerol (DAG) (27). IP$_3$ interacts with its receptors on the ER to release Ca$^{2+}$ from intracellular stores, while DAG activates protein kinase C (PKC). To explore if AnxA1 activates the PLC pathway in rat conjunctival goblet cells, these cells were preincubated with either the PLC inhibitor U73122 or its negative control U73343 at 10$^{-7}$ M, before addition of AnxA1 (10$^{-9}$ M) or a positive control Cch [10$^{-4}$ M] (28). AnxA1 added alone significantly increased [Ca$^{2+}$]$_i$ with a peak of 217.1 ± 34.6 nM (Figure 6A, n=3). The active inhibitor U73122 significantly decreased the AnxA1 stimulated increase in [Ca$^{2+}$]$_i$ to 63 ± 10.0 nM, while the inactive inhibitor U73343 did not significantly block AnxA1. The Cch response was also inhibited by U73122, but not U73343 (28).

The effects of U73122 and U73143 on secretion were then determined. AnxA1 (10$^{-10}$ M) increased secretion 2.0 ± 0.3 fold above basal (Figure 6B, n=3). AnxA1-stimulated response was completely inhibited by preincubation with U73122 (10$^{-7}$ M), but not U73143 (10$^{-7}$ M). U73122, but not U73143, also significantly inhibited secretion stimulated by Cch (10$^{-4}$ M), which was a positive control. These data show that AnxA1 stimulates PLC to increase [Ca$^{2+}$], and stimulate secretion.

To further explore the PLC pathway, goblet cells were incubated for 30 min with 2APB (10$^{-5}$ M), an inhibitor of IP$_3$ receptors on the ER (29). AnxA1 (10$^{-9}$ M) added alone significantly increased [Ca$^{2+}$]$_i$ by 160.6 ± 43.4 nM (Figure 6C, n=4). AnxA1-induced [Ca$^{2+}$]$_i$ increase was significantly
decreased to 35.7 ± 9.6 nM after 30 minutes of incubation with 2APB. Cch (10^{-4} M) was used as a positive control and its stimulation of [Ca^{2+}] was significantly blocked by 2APB as well.

A similar effect of 2APB was found when secretion was examined. AnxA1 (10^{-10} M) increased secretion 2.0 ± 0.3 fold above basal (Figure 6D, n=3). AnxA1-stimulated response was significantly inhibited by incubation with 2APB, which also significantly inhibited stimulation stimulated by Cch (10^{-4} M), the positive control.

Next, we investigated the effect of the PKC inhibitor RO317549. Goblet cells were incubated for 30 minutes with RO317549 (10^{-7} M) or a positive control Cch (10^{-4} M). [Ca^{2+}] was significantly increased when AnxA1 was added alone (263.7 ± 51.8 nM, Figure 6E, n=4). RO317549 significantly blocked the effect of AnxA1 on [Ca^{2+}] and was 27.0 ± 6.3 nM). Cch-stimulated increase in [Ca^{2+}] was also significantly inhibited.

Inhibition of PKC with RO317549 also blocked AnxA1-stimulated secretion. AnxA1 increased secretion by 1.9 ± 0.3 fold above basal (Figure 6F, n=3). RO317549 significantly reduced AnxA1-induced secretion to 0.7 ± 0.3 fold above basal. As a positive control, Cch stimulated secretion was also significantly inhibited.

In conjunctival goblet cells inhibitors of PLC activity, IP_{3} interaction with its receptors, and PKC each blocked the AnxA1-stimulated function measured. Based on these findings, AnxA1 activates the PLC pathway both to increase intracellular Ca^{2+} and stimulate secretion through the production of IP_{3} and activation of PKC.

**AnxA1 Increases [Ca^{2+}] and Secretion Through Activation of Ca^{2+}/CaMK II in Conjunctival Goblet Cells**

Free intracellular Ca^{2+} can bind to calmodulin, to create a calmodulin/calmodulin complex. This complex can bind to different proteins such as Ca^{2+}/calmodulin dependent protein kinase II (Ca^{2+}/CaMK) (30). We have previously shown that LXA_{4}, through the ALX/FP2 receptor, increases [Ca^{2+}], through Ca^{2+}/CaMK (12). To investigate if AnxA1 also activates Ca^{2+}/CaMK, goblet cells were preincubated with the Ca^{2+}/CaMK inhibitor KN93 or the inactive analog KN92 at 10^{-5} M for 30 minutes. AnxA1 (10^{-7} M) significantly increased [Ca^{2+}] to a peak of 235.6 ± 50.0 nM (Figure 7A). KN93 but not KN92 significantly blocked AnxA1-stimulated increase in [Ca^{2+}], and was 97.6 ± 19.6 (Figure 7A, n=4). A positive control Cch (10^{-4} M) significantly increased [Ca^{2+}], by 203.5 ± 40.2 nM. KN93, but not KN92, significantly decreased Cch-stimulated increase in [Ca^{2+}].

KN93 also significantly decreased 10^{-10} M AnxA1-stimulated secretion from 1.6 ± 0.1 to 0.9 ± 0.1 fold above basal (Figure 7B, n=3). KN92 had no effect on AnxA1-stimulated secretion. KN93, but not KN92, also significantly inhibited 10^{-4} M Cch-stimulated secretion. These data indicate that AnxA1 uses Ca^{2+}/CaMK to increase [Ca^{2+}] and stimulate glycoconjugate secretion.

**AnxA1 Does Not Increase [Ca^{2+}], But Does Increase Secretion Through Activation of ERK 1/2 in Rat Conjunctival Goblet Cells**

The ERK 1/2 pathway is a component of a signaling cascade that is activated in cells by a variety of stimuli (18, 31, 32). To determine if the ERK 1/2 pathway plays a role in AnxA1-stimulated increase in [Ca^{2+}], the ERK 1/2 inhibitor UO126 was used. Rat conjunctival goblet cells were incubated with UO126 (10^{-8}–10^{-6} M) for 30 minutes (Figure 8A, n=3). AnxA1 (10^{-9} M) added alone significantly increased [Ca^{2+}], by 152.5 ± 37.2 nM. AnxA1-induced [Ca^{2+}], increase was not significantly blocked by UO126 at any concentration. Histamine (10^{-5} M), which is known to activate ERK 1/2 was used as a control (22) and was significantly blocked by all three concentrations of UO126.

To examine the effects of UO126 on glycoconjugate secretion, goblet cells were incubated with AnxA1 with and without UO126 (10^{-7} M). AnxA1 (10^{-10} M) alone increased secretion by 2.0 ± 0.3 fold.
fold above basal (Figure 8B, n=3). UO126 significantly decreased AnxA1-stimulated response and was 0.6 ± 0.3 fold above basal. In contrast to previous studies (31) UO126 did not inhibit the carbachol-induced secretory response. These data indicate that AnxA1 does not activate ERK1/2 to increase \([\text{Ca}^{2+}]_i\), but does activate it to stimulate glycoconjugate secretion.

### AnxA1 Does Not Use PKA to Increase \([\text{Ca}^{2+}]_i\) in Rat Conjunctival Goblet Cells

To determine if AnxA1 increases cAMP levels to activate protein kinase A (PKA) to increase \([\text{Ca}^{2+}]_i\) and stimulate secretion, goblet cells were preincubated with the PKA inhibitor H89 (10^-5 M) for 30 minutes before stimulating the cells with AnxA1 (10^-9 M) (Supplemental Figure 2A, n=9). AnxA1 added alone significantly increased \([\text{Ca}^{2+}]_i\) by 420 ± 111.9 nM. H89 did not significantly inhibit AnxA1 stimulated increase in \([\text{Ca}^{2+}]_i\). Vasoactive intestinal peptide (VIP, 10^-8 M), which is known to increase cAMP (19), was used as a control. VIP-stimulated increase in \([\text{Ca}^{2+}]_i\) was 548.1 ± 110.6 nM. H89 significantly blocked VIP-stimulated increase in \([\text{Ca}^{2+}]_i\) and was 252.5 ± 39.6 nM.

Similar to the effects on \([\text{Ca}^{2+}]_i\), H89 did not significantly inhibit AnxA1-stimulated glycoconjugate secretion (Supplemental Figure 2B, n=3). However, the positive control, VIP-stimulated secretion was inhibited by H89. These data indicate that activation of PKA does not play a role in AnxA1-stimulated increase in \([\text{Ca}^{2+}]_i\), or secretion.

### AnxA1 Does Not Increase \([\text{Ca}^{2+}]_i\), But Does Stimulate Secretion Through the PLD Pathway in Rat Conjunctival Goblet Cells

Phospholipase D (PLD) is an important regulator of several critical aspects of cell physiology, and is involved in cell functions like endocytosis, exocytosis, cell migration and mitosis (33). We previously showed that the other SPMs such as RvD1, RvE1 and LXA4 work through PLD (10–12). To explore if AnxA1 uses PLD, cultured rat conjunctival goblet cells were incubated for 15 minutes with either the PLD inhibitor 1-but or the inactive inhibitor \(t\)-but, both at 0.3% (Figure 9A). AnxA1 (10^-9 M) alone significantly increased \([\text{Ca}^{2+}]_i\), to a peak of 141.2 ± 50.0 nM (n=4). Neither preincubation with 1-but or \(t\)-but altered the effect of AnxA1 on the \([\text{Ca}^{2+}]_i\) response. The increase in \([\text{Ca}^{2+}]_i\) stimulated by the positive control carbachol (10^-4 M) was significantly blocked by the active inhibitor 1-but, but not the inactive inhibitor \(t\)-but.
When secretion was measured, AnxA1 (10^{-10} M) alone increased secretion 1.9 ± 0.3 fold above basal (Figure 9B, n=3). Incubation with 1-but, but not t-but, significantly decreased this response that was 0.9 ± 0.1 fold above basal. Similarly to AnxA1, stimulation of secretion by Cch (10^{-4} M) was significantly decreased by 1-but, but not t-but. Based on these findings we conclude that AnxA1 does not activate the PLD pathway to stimulate an increase in [Ca^{2+}], but does activate PLD to stimulate secretion in cultured rat conjunctival goblet cells.

**AnxA1 Does Not Increase [Ca^{2+}] But Does Stimulate Secretion Through PLA2 Pathway in Cultured Rat Goblet Cells**

Another phospholipase that is present and can be activated in goblet cells is the PLA2 pathway (10–12). To determine if AnxA1 utilizes PLA2 to increase [Ca^{2+}], and secretion, goblet cells were incubated with the PLA2 inhibitor aristolochic acid (AA) for 30 min prior to stimulation with AnxA1 (Figure 10A). AnxA1 (10^{-9} M) significantly increased peak [Ca^{2+}] by 530.0 ± 109.2 nM (n=4). Preincubation with AA at 10^{-6} M significantly altered the AnxA1-stimulated response that was 228.1 ± 60.7 nM. AA at 10^{-5} M did not significantly alter the AnxA1 response. The positive control Cch (10^{-4} M)-stimulated response was significantly decreased with AA 10^{-6} and 10^{-5} M from 542.6 ± 78.6 nM to 217.7 ± 65.4 nM and 257.1 ± 52.6 nM, respectively.

AnxA1 (10^{-10} M) increased glycoconjugate secretion by 1.9 ± 0.3 fold above basal (Figure 10B, n=3). This response was significantly inhibited by AA (10^{-5} M) to 0.5 ± 0.4. Thus AnxA1 does not activate the PLA2 pathway to stimulate an increase in [Ca^{2+}], but does activate PLA2 to stimulate secretion in cultured rat conjunctival goblet cells.

**DISCUSSION**

In the present study, we showed that AnxA1 contributes to tear film and ocular surface homeostasis by activating the PLC pathway, including its downstream effectors IP3 and PKC, through interaction with the ALX/FPR2 receptor (Figure 11). This results agrees with findings in Hodges et al. (12) using a desensitization protocol that indicates that AnxA1 interacts with the same receptor as LXA4 and RvD1, the ALX/FPR2 receptor. This interaction increases [Ca^{2+}], and stimulates glycoconjugate secretion.
secretion including the protective mucin MUC5AC from conjunctival goblet cells. AnxA1-stimulated increase in [Ca\(^{2+}\)]\(_{i}\) is dependent on both intracellular Ca\(^{2+}\) stores and influx of extracellular Ca\(^{2+}\) consistent with an interaction with Orai and STIM-1 known to regulate these processes in most tissues in which stimulation of G-protein-coupled receptors activates PLC (34).

AnxA1 is present in a variety of other tissues like bone marrow, the intestine and lungs (14, 35). In this study, we showed by RT-PCR that AnxA1 is expressed in conjunctival goblet cells and thus is available to activate these cells. This indicates that AnxA1 not only works during ocular inflammation, but also under physiological conditions, by secreting a basal level of mucins to the tear film and thus maintaining ocular homeostasis. We have demonstrated this for other SPMs including LXA\(_4\), RvD1, RvD2, maresin 1, and RvE1 (10–12). Unlike the lipid SPMs AnxA1 is a protein, thus we were able to verify its expression by RT-PCR. In contrast, complex lipidomic measurements are needed to detect the presence of the lipid SPMs. As the lipid LXA\(_4\) is produced by the cornea, it is possible that LXA\(_4\) could diffuse via tears to goblet cells in order to stimulate glycoconjugate secretion. In contrast, AnxA1 is found in conjunctival goblet cells and could be secreted to act upon goblet cells or stratified squamous cells. As AnxA1 is both present and has a role in goblet cell function, AnxA1 likely plays a role in ocular health under physiological conditions as well as preventing or treating the multitude of ocular surface inflammatory diseases. Future study of the regulation of AnxA1 synthesis and secretion in conjunctival goblet cells is warranted.

The ALX/FPR2 receptor is a G-protein-coupled receptor and is the receptor to which AnxA1 binds (36). In addition to its presence on goblet cells (12) the ALX/FPR2 receptor has been identified in human neutrophils, epithelial cells, endothelial cells and monocytes (37, 38). Using ALX/FPR2, AnxA1 functions to resolve diverse inflammatory events, including reduction of joint injury in experimental arthritis (39), lessening of salivary gland inflammation (40), and contributes to corneal wound healing (41). AnxA1 in uterine epithelial cells also activates the ALX/FPR2 receptor to upregulate the membrane-spanning mucin MUC1, tight junction molecules claudin-1 and occludens 1 to increase adherence of trophoblast steroids, a model for embryo implantation (42). Herein we showed that AnxA1 activates the ALX/FPR2 receptor in rat conjunctival goblet cells and that this activation leads to the increase in [Ca\(^{2+}\)]\(_{i}\), and secretion of mucins into the tear film. We previously studied the SPMs LXA\(_4\) and RvD1, which also activate the ALX/FPR2 receptor in rats. Cooray et al. have shown that the ALX/FPR2 receptors can homodimerize or heterodimerize with FPR1 or FPR3 depending on which agonist is bound (43). In addition, ALX/FPR2 has multiple binding sites notably different lipid and protein sites and binds to multiple compounds (44). AnxA1, a protein, and RvD1 and LXA\(_4\), which are lipids, bind to different receptor regions in addition to inducing dissimilar receptor conformations (45). This differential binding and a potential difference in FPR receptor family dimerization could explain the complex desensitization we obtained with addition of AnxA1, LXA\(_4\), and RvD1 (46). These dissimilarities could make the three compounds differ in their effectiveness to treat ocular allergy and other forms of ocular inflammation.

This differential binding of AnxA1, LXA\(_4\), and RvD1 to bind to the same receptor in conjunctival goblet cells could also lead to ligand-specific activation of signaling pathways. In fact, the AnxA1, LXA\(_4\), and RvD1 activation of downstream signaling cascades are not fully similar. While AnxA1, LXA\(_4\), and RvD1 each activate the PLC pathway, only LXA\(_4\) and RvD1 activate PLD, PLA\(_2\), and ERK 1/2 (10, 12) to stimulate an increase both in [Ca\(^{2+}\)]\(_{i}\) and mucin secretion. This pattern of pathway activation is consistent with AnxA1 activating the peptide site on ALX/FPR2, whereas LXA\(_4\) and RvD1 activate the lipid site or overlapping sites.

In the present study, we found AnxA1 to have a peak increase in [Ca\(^{2+}\)]\(_{i}\) at a 10\(^{-9}\) M concentration, while the maximum
response for secretion was at $10^{-10}$ M. Furthermore, AnxA1 at $10^{-8}$ M did not even increase secretion. These findings could be due to several factors. The increase in $[\text{Ca}^{2+}]$ occurs very rapidly, within seconds, and is measured in the time frame of seconds. In contrast, secretion, although it can occur rapidly, is measured after 2 hours. The rapid increase in $[\text{Ca}^{2+}]$ may not directly link to secretion. The peak $[\text{Ca}^{2+}]$, measured is primarily a function of $\text{Ca}^{2+}$ release from intracellular stores whereas stimulation of secretion also is regulated by the subsequent influx of $\text{Ca}^{2+}$ as well as the size of the intracellular $\text{Ca}^{2+}$ stores. The maximum concentration of AnxA1 used to increase the $[\text{Ca}^{2+}]$ of in secretion also is regulated by the subsequent increase in $[\text{Ca}^{2+}]$ compared to that used for secretion may result from the differential dependence on the use of distinct mechanisms of $\text{Ca}^{2+}$ handling in the goblet cells.

In further support of the independence of AnxA1 concentration on peak $[\text{Ca}^{2+}]$ and secretion is that secretion, but not peak $[\text{Ca}^{2+}]$ is stimulated by AnxA1 activation of PLA$_2$, PLD, and ERK1/2. Thus the increase in peak $[\text{Ca}^{2+}]$ and secretion are differentially regulated by AnxA1. This is in contrast to the increase in peak $[\text{Ca}^{2+}]$ and secretion that when stimulated by LXA$_4$ or RvD1 are both dependent on activation of the PLC, PLD, and PLA$_2$ pathways. This finding is also consistent with binding of the protein and the lipids to different sites on ALX/FPR2.

In the present study we found that AnxA1 was able to significantly increase $[\text{Ca}^{2+}]$, at a notably low concentration ($10^{-12}$ M) in conjunctival goblet cells. In contrast, LXA$_4$ increased $[\text{Ca}^{2+}]$, at $10^{-6}$ M (10). Unfortunately the effect of RvD1 concentration on $[\text{Ca}^{2+}]$ was not performed at concentrations below $10^{-10}$ M (unpublished data, DA Dartt 2020). The differential effect of AnxA1 and LXA$_4$ concentration on $[\text{Ca}^{2+}]$ is consistent with their binding to different areas on the ALX/FPR2 receptor.

Furthermore, we found that AnxA1 was not dependent on either the ERK 1/2, PLA2, or the PLD pathway to increase $[\text{Ca}^{2+}]$, although AnxA1 does utilize these pathways to stimulate secretion. In conjunctival goblet cells AnxA1 did not activate ERK 1/2 pathway to increase $[\text{Ca}^{2+}]$, but rather more likely AnxA1 increased $[\text{Ca}^{2+}]$, to activate ERK1/2. ERK1/2 activity can be $\text{Ca}^{2+}$-dependent via activation of PLC by a G-protein-coupled receptor or can be independent of $\text{Ca}^{2+}$ when activated by $\beta$-arrestin-dependent down regulation of G-protein-coupled receptors (47). Published literature showed that in multiple tissues the binding of AnxA1 to the ALX/FPR2 receptor leads to the transient phosphorylation of ERK 1/2 (7, 13, 48, 49), which is associated with a prompt increase in $[\text{Ca}^{2+}]$ (7, 38, 50). This suggests that in goblet cells, the activation of ERK 1/2 occurs downstream of the rise of $[\text{Ca}^{2+}]$, after activation of PLC. Similarly for AnxA1 activation of PLD, the rise in $[\text{Ca}^{2+}]$ occurs before activation of PLD. An increase in $\text{Ca}^{2+}$ and activation of PKC as could occur by PLC stimulation could activate PLD in the goblet cells as documented in a mast cell line (51). The signaling pathways activated by AnxA1 are complex, cell specific, and species dependent.

The function of AnxA1 has been studied in a variety of disease models in order to explore this agonist’s potential as a novel anti-inflammatory therapeutic agent, including models of inflammatory bowel disease (52), rheumatoid arthritis (53), chronic atherogenesis (54), myocardial reperfusion injury (55), myocardial infarction (56), and now ocular inflammation. AnxA1 is thought to have potential as a new treatment option for inflammation by being an effector molecule of glucocorticoids (57). Synthetic glucocorticoids play an extensive and crucial role in the treatment of unwanted and uncontrolled inflammation but come with potentially harmful metabolic side effects with long-term use (57). It is believed that therapeutic application of endogenous anti-inflammatory mediators like AnxA1 will be effective and cause fewer side effects by inducing natural pathways of resolution of inflammation (58–60). AnxA1 could be used therapeutically to treat the multiple inflammatory diseases of the ocular surface especially dry eye and ocular allergy as well as to prevent these diseases or to maintain ocular surface homeostasis in health.

In conclusion, our findings demonstrate that the glucocorticoid effector protein AnxA1 has potential in prevention or as a new treatment for dry eye, allergic conjunctivitis and other forms of ocular surface inflammation through its effect on conjunctival goblet cells. AnxA1 works through activation of the ALX/FPR2 receptor to stimulate multiple signaling pathways, ultimately leading to the secretion of high molecular weight glycoconjugates including mucins into the ocular tear film. These mucins are a crucial component of a healthy tear film, and thus the regulation of mucin secretion from conjunctival goblet cells by AnxA1 is an important contributor to ocular surface health, and prevention of ocular surface disease and treatment of these diseases.

**AUTHOR’S NOTE**

This manuscript is dedicated to Robin R. Hodges who passed away on March 13, 2021. For over 30 years she managed the Dartt laboratory ensuring its excellence. She was respected and beloved by all.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

**ETHICS STATEMENT**

The animal study was reviewed and approved by Schepens Eye Research Institute Animal Care and Use of Committee.

**AUTHOR CONTRIBUTIONS**

AL, MO, JB, and RH performed experiments. AL, RH, TU, CS, and DD wrote the manuscript. All authors contributed to the article and approved the submitted version.
Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.618653/full#supplementary-material

Supplementary Figure 1 - Chelation of Ca^{2+} blocks ArxA1-stimulated increase in [Ca^{2+}] in cultured rat conjunctival goblet cells. Cultured rat goblet cells were preincubated with the Ca^{2+} chelator BAPTA/AM (10^{-4} M) prior to stimulation with either ArxA1 (10^{-9} M) or carbachol (Chc, 10^{-5} M) and change in peak [Ca^{2+}], shown. Data are mean ± SEM from 4 independent experiments. * indicates significant difference from basal; # indicates significant difference from either ArxA1 or Chc alone.

Supplementary Figure 2 - ArxA1 is not dependent on protein kinase A (PKA) to increase [Ca^{2+}] in cultured rat conjunctival goblet cells. Goblet cells were preincubated with the PKA inhibitor H89 for 30 minutes before stimulation with vehicle, annexin A1 (ArxA1, 10^{-9} M) in (A) or with ArxA1 (10^{-10} M) in (B), or vasoactive intestinal peptide (VIP, 10^{-7} M). Change in peak [Ca^{2+}] is shown in (A). Glycosylconjugate secretion is shown in (B). B indicates basal (vehicle in (B)). Data are mean ± SEM from 9 (A) or 3 (B) independent experiments. * indicates significant difference from zero; # indicates significance from ArxA1 or VIP alone.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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