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$P2Y_2$ RECEPTOR PEPDUCINS HIJACK AND ACTIVATE THE FORMYL PEPTIDE RECEPTOR 2 IN HUMAN NEUTROPHILS

Master's thesis in biotechnology

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Abstract

Neutrophils are the most abundant phagocytic cell type and have a critical role in the innate immune system. They are attracted to the site of infection/inflammation through a number of surface expressing chemoattractant G-protein coupled receptors (GPCRs). Activation of neutrophil GPCRs mediates not only directional migration, but also triggers the release of reactive oxygen species and granule stored enzymes. The formyl peptide receptor 1 (FPR1), displaying high binding affinity for the “danger signal” formyl peptides derived from bacteria or damaged mitochondria, was the first chemoattractant GPCRs cloned and has therefore served as a model receptor for our understanding of neutrophil physiology. In addition to FPR1, neutrophils express also the closely related formyl peptide receptor 2 (FPR2) and the danger signaling ATP recognition receptor ($P2Y_2$-R). All GPCRs share a similar seven transmembrane helical structure in which the extracellular domains are involved in ligand binding whereas the intracellular parts are engaged in G-protein coupling and signaling transduction. Recent research has proposed a group of cell penetrating molecules, so called pepducins that can modulate GPCR signaling from the inside. Pepducins are lipopeptides derived from one of the intracellular lopes of a GPCR, they are supposed to allosterically activate or inhibit the receptor from it is derived. In this study, we investigated the ATP receptor signaling by applying pepducins from the $P2Y_2$ receptor. Our data show that these pepducins do not target their cognate receptor as the currently “pepducin model” proposed and instead activate FPR2. This “model” has also been challenged by the recent findings from pepducins from FPR1 and β2-adrenergic receptor.

Keywords: neutrophils, ROS, Ca^{2+} transient, GPCRs, FPRs, $P2Y_2$-R, pepducins

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1 Background

The immune system has evolved to protect eukaryotic life against fungal and prokaryotic invaders. Neutrophils are part of the innate immune system and form the first line of defense (1). The second line is the adaptive immune system comprising the activation of killer cells and specific antibodies (2). The inflammation response to a microbial invasion or tissue damage is literally a matter of life and death. During microbe invasion of healthy tissue are professional phagocytes including neutrophils and monocytes/macrophages attracted to the site of infection by gradients of the released chemoattractant. The most abundant cell type in the blood is the neutrophil that account for 40-60% (3). They are formed (1-2 x 10^{11}/day) and matured in the bone marrow (1). They have a short life span, in blood circulation a half-life of 5-90 hours and if transmigrated to tissue a maximum of 48 h (4)(5). To avoid tissue damage to the host, neutrophils are resting (naive) until an encounter with a priming agent. They can be primed by either chemoattractants, chemokines or cytokines like TNFα. There are two forms of priming, the fast one which will mobilize chemoattractant receptors (FPRs) and proteolytic enzymes stored in granules and vesicles (see **Figure** 1).

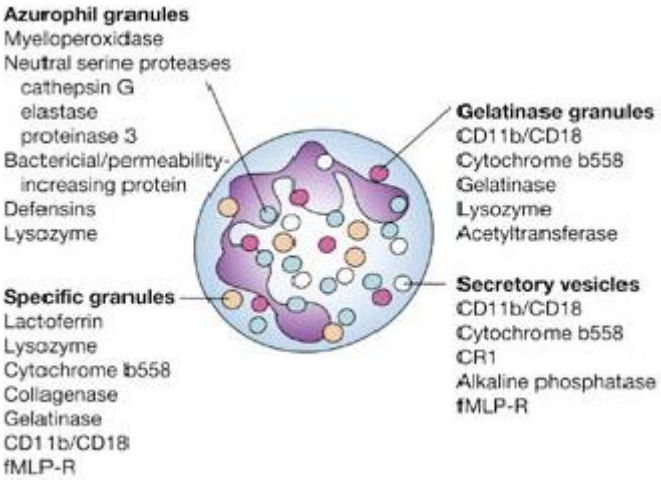

Figure 1 neutrophil with granules and vesicles (6)

The slow priming will induce transcription factors for the production (de novo) of cytokines and receptors that will enhance the neutrophils killing capacity (3). Once in the tissue, they try to eliminate the threat by releasing bactericides through the oxygen dependent reactive species (ROS) and oxygen-independent proteolytic enzymes (7). NADPH-oxidase is a membrane multi-component enzyme complex comprising both in the membrane and cytosolic parts (7)(8). During phagocyte activation with appropriate stimuli, the intracellular

parts will translocate to the membrane and assemble the active complex (see **Figure** 2) (9).

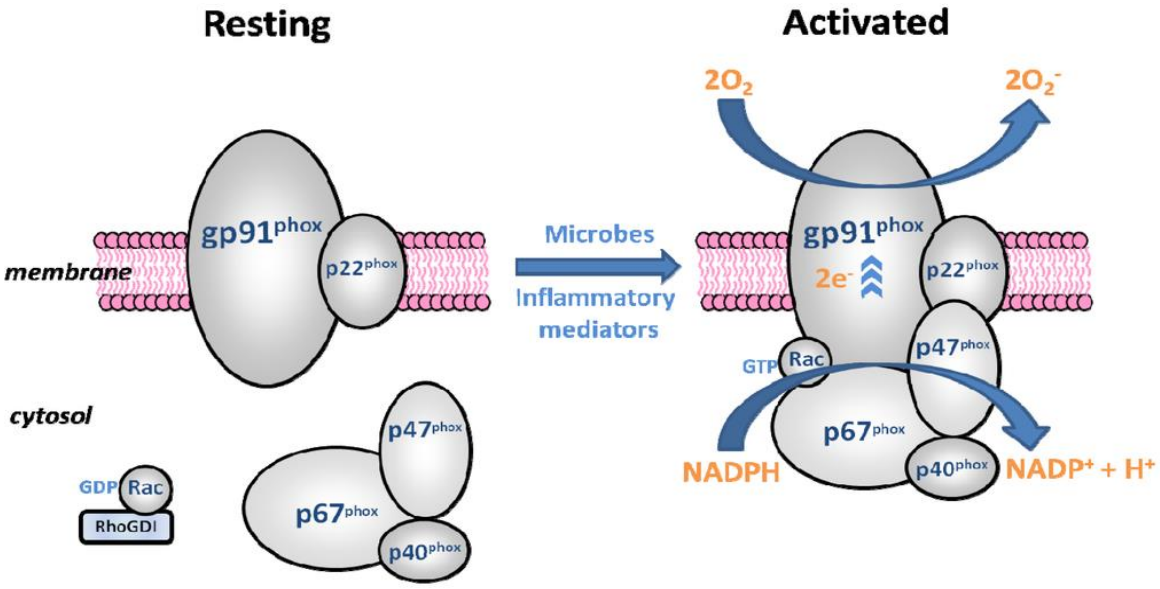

Figure 2 the activation of the NADPH-oxidase complex (10)

This complex catalyzes the reaction of two electrons from NADPH to O_2, forming the O_2^- (superoxide anion). The superoxide anion will dismutate and form hydrogen peroxide and also even more bactericidal radicals. Myeloperoxidase (MPO) is an enzyme localized in azurophil granules (primary granules) that are fused with the phagosome during phagocytosis (9). They catalyze in the presence of H_2O_2 (hydrogen peroxide) the oxidation of the halide anions (F^-,Cl^-,Br^-,I^-) to the bactericidal HClO (hypochlorus acid) (11). In order to limit the production of an excess of radicals that may damage the tissues, the processes are negatively regulated by a protein called NROS (negative regulator of ROS). The proposed mechanism is that it helps degrade one of the subunit of the NADPH-oxidase complex (12). The importance of ROS in host defense during infection becomes obvious, when the disease chronic granulomatous disease was discovered. A disease caused by an impaired ability to produce ROS due to mutation of NADPH-oxidase subunits caused non-functional NADPH-oxidase (7)(13). In addition mice that lack a functional NADPH-oxidase are more susceptible to bacterial infection compare to their littermate controls (8)(12). The azurophil granules contain hydrolytic proteins that are bactericidal and decrease the bacterial growth rate (11). The inflammatory responses need to be tightly regulated otherwise the host will suffer serious harm or even death. There are a number of serious diseases associated with ongoing inflammation, for example Type 1 diabetes and rheumatoid arthritis (RA) (3). This makes it important to understand the inflammation process and thereby gain more knowledge to develop new anti-inflammatory drugs.

2 G-protein coupled receptors (GPCRs)

GPCRs are the largest family of membrane receptors (~ 800 genes). They are involved in everything from olfactory, hormone responses, opioid response to act as neurotransmitter. The common structure similarities are seven transmembrane helicies that span the cell membrane (14) (see **Figure** *3*).

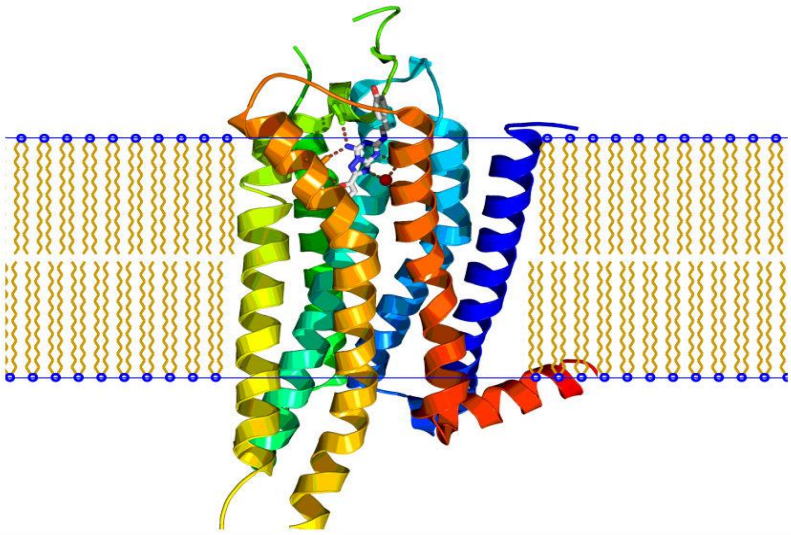

Figure 3 GPCRs (15)

They are coupled to different G-proteins; depending on the subtype of G-protein the cellular response will be altered. Simplified, the general mechanism is that an agonist binds to the active site located to the extracellular part and induce a conformational change of the

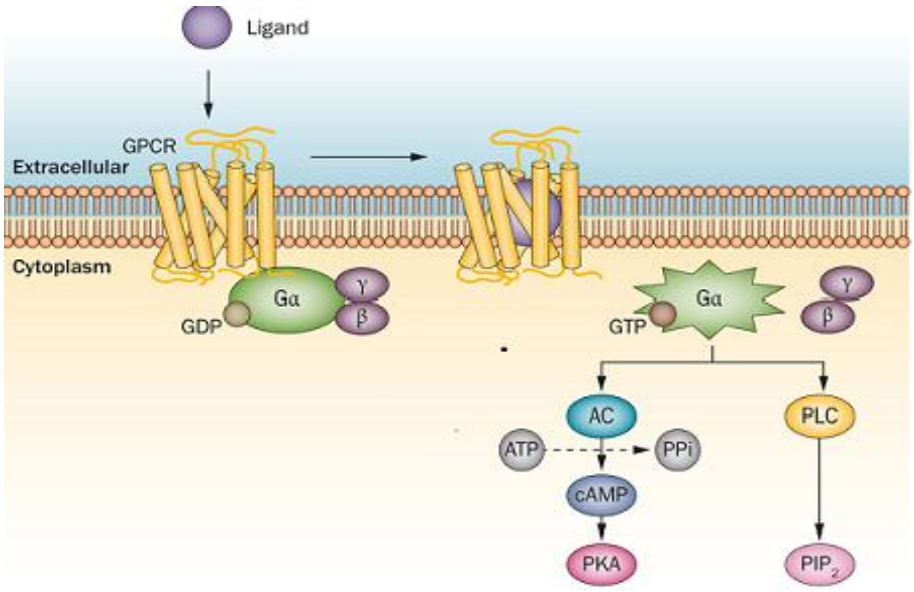

Figure 4 GPCR activation (66)

receptor. This will lead to the G-protein exchange of GDP to GTP and active a dissociation of the α-subunit and βγ-unit (16) (see **Figure** 4).

Those subunits will activate or inhibit other proteins that act as secondary messengers and by that activate different cellular responses (17). The current view is that GPCRs can exist in different active conformations depending on the agonist examined, a phenomena s.c "bias signaling" or "functionally selectivity". In other words some ligands activate certain signaling pathways over others (18). This is extremely important in "drug discovery" to find a ligand that maximize the beneficial effects and minimize the side effects (19). This is also important when trying to block the signal by using antagonists, blocking more receptors than the intended one leads to side effects. Antagonists can compete in a dose dependent manner with the agonist for the "active site" or they bind allosteric, induce an conformational change that make it impossible for the agonist to bind. Inverse agonist function as orthosteric agonist with the different that they also terminate the basal activity of the receptor, instead of just blocking the receptor for agonists (20).

2.1 Signaling pathways

There are a number of signaling pathways to mention some of the more common ones: cAMP dependent one, protein kinase C (PKC) dependent one and the MAPK (21). Cyclic adenosine mono phosphate (cAMP) is a secondary messenger that get produced by the enzyme adenylate cyclase from ATP. Activation of this protein by a Gs-protein α-subunit or inhibition by Gi α-subunit will induce a signaling cascade (22). PKC is family of kinase enzymes that start a signaling cascade by phosphorylation of OH-groups that are localized on serine and threonine residues on specific target proteins. The activation of PKC can be triggered in a diverse way dependent on family member (18). Two common activation mechanism of PKC is the rise of intracellular Ca^{2+} and diacyl-glycerol (DAG). DAG is one of the product when the membrane bound lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) is hydrolyzed by the enzyme phospholipase C (PLC), an enzyme that cleaves phospholipids. The activation of PLC is mediated through many cell surface receptors including GPCRs (23). Some stimuli like phorbol myristate acetate (PMA) can bypass the G-protein signaling and activate PKC directly (24). This is proven by pertussis toxin experiment, a substance that inactivates G_i-protein (25). The other product of the hydrolysis is Inositol triphosphate (IP_3), a classical second messenger that leaves the membrane and diffuses to the endoplasmic reticulum (ER), binds to its receptor (a Ca^{2+} channel) and induces an influx of Ca^{2+} as a result of release from ER (see **Figure 5**) Depletion of Ca^{2+} from the ER and a transient cytosolic Ca^{2+} rise will lead to extracellular Ca^{2+} entry from outside (26).

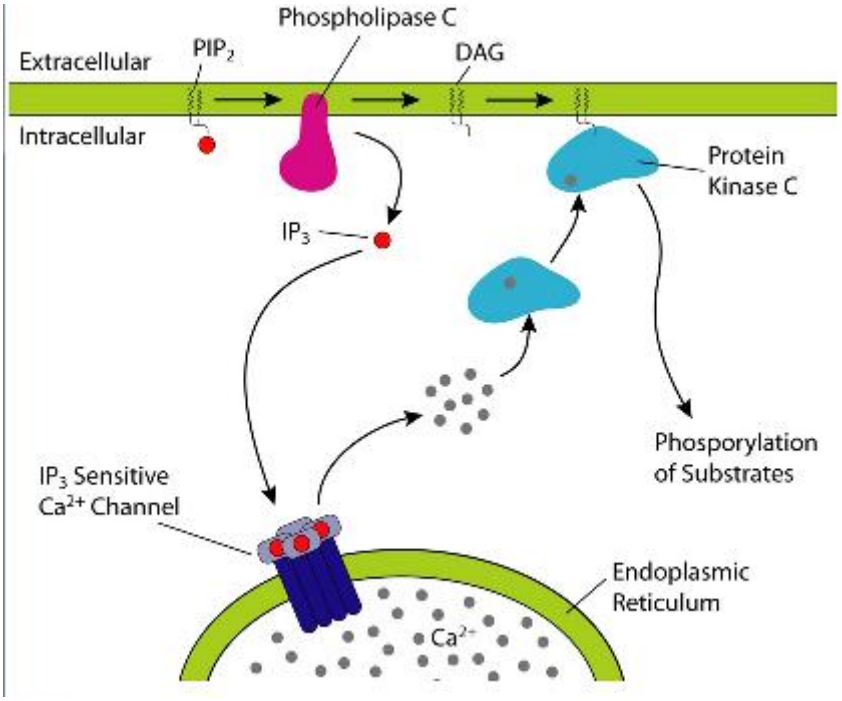

Figure 5 cytosolic rise of Ca^{2+} (67)

The MAPK cascade pathway is crucial in eukaryotes and it is involved in everything from: Inflammation, cell growth and migration, cancer, apoptosis and altered gene expression(21). Some of the stimuli include: chemoattractants, cytokines and mitogens. These stimuli activate specific MAPKs. The pathway is kinase dependent and the signal is transduced by a phosphorylation cascade (see **Figure** 6) (21) (26).

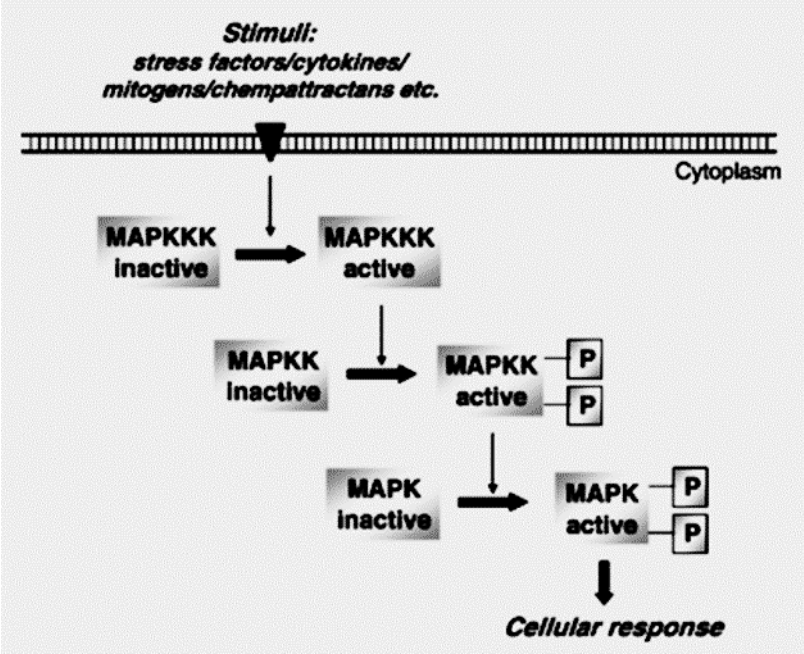

Figure 6 the MAPK cascade (21)

3 The FPR family

Chemoattractant (or synthetic agonist/antagonist) binds to chemoattractant GPCRs that are located on the membranes of phagocytes including the Formyl peptide receptors (FPRs) (27). They are coupled to Gi protein that are pertussis toxin sensitive (28). The human genome encodes three protein members called FPR1, FPR2 and FR3 of which neutrophils express two (FPR1 and FPR2) (16). Murine receptor biology with respect to FPRs is less well studied despite their use as animal models. They differ a lot comparing to human and express at least eight members. Human FPR1 and FPR2 are orthologs with murine fpr1 and fpr2, respectively (29). The murine fpr1 has 100 fold lower affinity for fMLF, the prototype agonist for human FPR1 (8). The difference between men and mice is likely due to different evolutionary pressure. It is important to understand the differences with respect to receptor expression profile and ligand binding across species, when using murine models in inflammation research. The FPR family is pattern recognition receptors evolved to recognize formylated peptides. These peptides are a hallmark for bacterial synthesis and mitochondrial damage. Upon activation, the receptors induce phagocyte activation and migration through the chemoattractant gradient. Upon arrival, a deadly load of bactericidal agents will be released (30). Because of their role in autoimmune diseases, they are an attractive target for development of new drugs, for example antagonist that blocks the response by the receptors (3). The major challenges for potential drug candidates are the selectivity and the stability of the compounds (19).

3.1 FPR1

FPR1 (first named fMLP-R because of its high affinity to the peptide) primary structure was disclosed in 1990 (31) by the breakthrough of molecular cloning and has been extensively studied since then. This has to large extent been done by the high affinity formylated tri-peptide agonist fMLF (old literature fMLP) (32). fMLF binding to the receptor will induce granule mobilization, chemotaxis and release of intracellular Ca^{2+} from organelles, this will disclose the IP_3 receptor and the opening of membrane channels leading to an influx of extra cellular Ca^{2+} (33). This influx is needed for neutrophil activation and the proper function of some proteins but is has recently been shown that maybe it is not crucial, because cytoskeleton processes that was believed to be Ca^{2+} dependent and radical production can occur in neutrophils depleted of Ca^{2+} (34). It will also lead to an activation of NADPH-oxidase that will produce radicals that are highly bactericidal (11). After activation, the receptors rapidly terminate the signaling, a process known as receptor homologous desensitization. In other words the receptor does not respond to continuous stimulation with the same agonist (35). This is a process that is still elusive in many ways. One major mechanism proposed is the regulation by the actin cytoskeleton. The receptor with the bound ligand forms a complex with the actin cytoskeleton and physically separates the receptor from the signaling G-protein (30). This has been proven by the use of actin disrupting agents like latrunculin A, that hinders actin polymerization and return the receptor to a signaling state (36). This state will also terminate after a while, so it has to be more regulatory mechanism/s involved. An important note is that some responses are much stronger and longer. For example the radical production by NADPH-oxidase gets both prolonged and higher (36). The actin cytoskeleton do not to seem to have a regulatory role in Ca^{2+} signaling because disturbing it with Latrunculin A, will not lead to an new response (34).

A valuable tool to study FPR1 signaling are the FPR1 selective antagonists. A problem has been to find them, one reason might be that the structure seems to differ a lot between agonist/antagonist, making screening hard. The most potent to date is cyclosporine H (a cyclic undecapeptide), that has no structure resemblance of any known agonist. Cyclosporine H (CysH) is potent with a IC50 ~ 100 nM (37) and has no effect on FPR2 < 2,5 µM (38).

Heterologous desensitization is the process when another receptor becomes desensitized. This is because of receptor crosstalk, an important feature to optimize the inflammation response (34)(27). There seems to be a hierarchy among the chemoattractant receptors, on the top are FPR1 and FPR2 (at least they cannot desensitize each other). An example is desensitization of FPR1 will lead to desensitization of the chemokine receptors (CXCR1/2) (34).

Beta-arrestin is a small protein that regulates GPCRs desensitization and internalization. Its regulatory role was first shown for rhodopsin (GPCR involved in vision) in 1986 by Herman Kuhn and his team (39). Following the stimuli are the receptors phosphorylated on the third intracellular lope (ICL3) by a group of serine and threonine G-protein coupled kinases called

GRKs. The phosphorylation leading to that arrestin can bind with high affinity to the receptor. The receptor ligand- arrestin complex is then directed to a protein called clathrin (a protein that can form vesicles). There the receptor get dephosphorylated and either internalized to the membrane or degraded in the lysosome and then recycled to the membrane (40).

3.2 FPR2

FPR2 was originally mentioned as the receptor for lipoxin A_4 (see **Figure** 7). This molecule belongs to a class of compounds called non classic- eiconosanoids. They are formed by oxygenasation of Arachidonic acid and act as anti-inflammtory modulators on the inflammatory response (41). An important note is that there is a controversy if lipoxin A_4 really is an FPR2 agonist. A research paper has provided compelling evidence that lipoxin A_4, does not act through FPR2 and not having an inhibitory effect on the pro-inflammatory cytokine TNF-α (42).

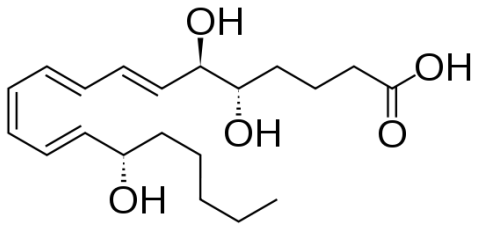

Figure 7 LipoxinA$_4$ (68)

Anyway it soon becomes obvious it was more much more diverse than that, by the finding of lipids, bacterial, mitochondrial and synthetic peptides that acts as agonist through the receptor(23). One of the most potent and selective agonist of FPR2 is WKYMVM, a synthetic hexapeptide. This hexapeptide will induce granule mobilization, chemotaxis and release of intracellular Ca^{2+} from organelles in neutrophils, in a similar fashion as fMLF the potent FPR1 agonist (43). There are a number of FPR2 antagonist that have been characterized some of the more potent are: WRW^4 (44), HF-965A (45) and PBP10 (46). WRW^4 is a hexapeptide that was found during a high through put screening of hexapetides, that could inhibit binding of the FPR2 agonist Trp-Lys-Tyr-Met-Val-D-Met-$CONH_2$ (WKYMVm) (47). Another study confirmed the specificity of WRW4 for FPR2 but it also points out that in higher concentrations (>1 µM) it will also bind to FPR1 (38). WRW4 consists of 5 bulky and hydrophobic (tryptophan) amino acids and an arginine a positive charged amino acid (44). This amino acids is ideally for forming hydrogen bond and bind to negative charged entities in polar environments. HF-965A is the first peptidomimetic that been published as an antagonist for FPR2, It is potent with an IC50 ~ 50 nM (45). PBP10 is an supposed intracellular allosteric potent (IC50 ~ 50 nM) and selective antagonist derived from the actin binding protein Gelsolin (46).

FPR1 and FPR2 share high degree of homology (69%) and they have highest homology in the intracellular parts and differ quite a bit in the extracellular parts. This is in line with experimental data that shows that they differ in agonist preferences but have similar signaling pathways (36).

4 The $P2Y_2$ receptor

$P2Y_2$-R belongs to the GPCR family and is widely expressed in main organs like: heart, kidneys, brain and in the soft tissue (48). They exert many functions ranging from maturation of stem cells, cytokine secretion to apoptosis (34) but the specific mechanisms are still elusive. It is also associated to cancer because it is overexpressed in some cancer forms (49). A recent study in hepatocellular carcinoma (most common form of liver cancer), has indicated that the receptor plays an important role in the proliferation and migration of the cancer, suggesting that the receptor might be a therapeutic target in carcinoma (49). The endogenous agonist for $P2Y_2$ is adenosine triphosphate (ATP) the dominant energy molecule of living systems and uridine triphosphate (UTP). An synthetic orthosteric antagonist is AR-C118925 (ARC) (48). $P2Y_2$ is expressed in high density in neutrophils but is not a chemoattractant receptor on its own (50). However it seems to play an important role in the inflammation response anyway. ATP does not activate naïve neutrophils to assemble the NADPH-oxidase system for the production of superoxide anion. However, it is a potent NADPH-oxidase activator when the actin cytoskeleton is disrupted prior to ATP stimulation (34). Novel cross-talk mechanism between $P2Y_2$ and FPRs has recently been demonstrated. This cross talk induces reactivation of NADPH radical production of agonist desensitized FPRs, through ATP activation of $P2Y_2$. This does not work the other way around, indicating that the cross-talk is unidirectional (34) .

5 Pepducins

Pepducins are a relative new class of lipopeptides (51). They consist of a fatty acid head and a tail of an amino acid sequence derived from one of the intracellular loops of a GPCR. One interesting part of this class of compounds is that they are supposed to allosterically activate or inhibit the GPCRs from which their peptide sequence is derived (52). It is proposed that they interact with the signaling domain on the cytosolic part of the receptor. Two of the proposed mechanism is: When it passed the membrane the pepducin stabilizes receptor/G-protein conformation by mimicking a receptor dimerization or it interacts with the receptor allosterically on the cytosolic part and induces a receptor conformation that activates the coupled G-protein (53). They have also been shown to have bactericidal properties and hopefully they can be potentially designed as a novel class of drugs with functional dualism during infection, in the ongoing life and death struggle against antibiotic resistant bacteria (54).

6 Aim of the study

The aim was to investigate the effect of three pepducins designed from ICL1-ICL3 of the $P2Y_2$ receptor on neutrophil activation. The main objective was to characterize Ca^{2+} signaling and ROS production by NADPH-Oxidase activation in neutrophils.

7 Methods/Experimental setup

7.1 Pepducins

Pepducins from intracellular lope 1-3 (ICL1-ICL3) of the $P2Y_2$ receptor (see **Figure** 8) were synthesized by Caslo (Ljungby ,Denmark) and have the following sequences:

ICL1-11 (60-70*):	Pal-CRLKTWNASTT	11 amino acids
ICL2-23 (130-152*):	Pal-HRCLGVLRPLRSLRWGRARYARR	23 amino acids
ICL3-26 (221-246*):	Pal-MARRLLKPAYGTSGGLPRAKRKSVRT	26 amino acids

* Numbers indicate the amino acid position in the $P2Y_2$ receptor

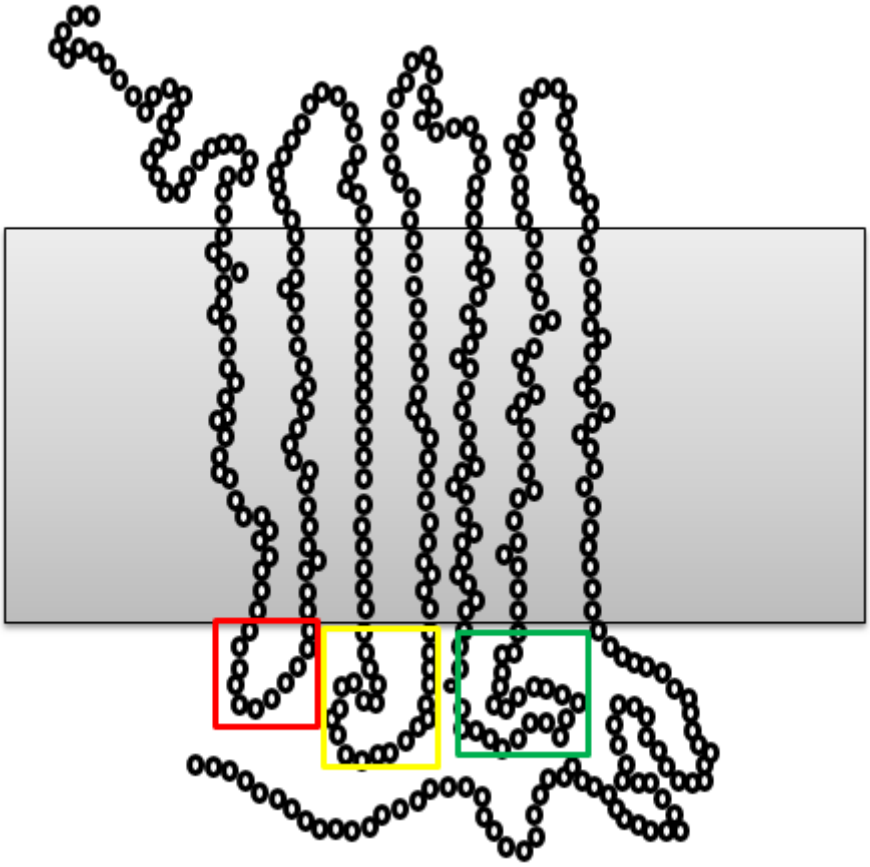

Figure 8 schematic drawing of $P2Y_2$-R. Red box = ICL1-11, yellow box = ICL2-23 and green box ICL3-26

7.2 Neutrophil isolation

Neutrophils were isolated from buffy coats from apparently healthy adults from Sahlgrenska blood center. The erythrocytes were separated by dextran (1%) sedimentation (~ 25-35 min). The monocytes and lymphocytes was removed by Ficoll-Paque (+375mg NaCl) centrifugation. The solution where then washed with Krebs-Ringer phosphate buffer (KRG) and the remaining erythrocytes was lysed with H_2O (6 ml for 30 sec) and the lysis were terminated by PBS- buffer (2 ml). The purified (~ 90%) neutrophils were suspended in KRG with Ca^{2+} to a concentration of $1x10^7$/ml and kept on ice until use.

7.3 FPR1/FPR2 overexpressed cell culturing

How the cell lines were created is described in (43). FPR1/FPR2cell lines were cultured in a RPMI media containing: fetal calf serum (10%), Penicillin/Streptomycin solution (1%), L-glutamine (10%) and the selective antibiotic marker G418 (1mg/ml). To avoid spontaneous differentiation were the cells passed every fifth day. During these passages were an aliquot of the cell culture centrifuged and the resulting pellet resuspended in fresh media (55).

7.4 Quantifying NAPDH -oxidase ROS production in phagocytes by Isoluminol enhanced Chemiluminscence, catalyzed by horse radish peroxidase (HRP).

The emission of light caused by a chemical reaction is called Chemiluminscence. Luminol and isoluminol (see **Figure** 9**)** are two common reagent to quantify this phenomena in radical biology. They share the same molecular formula and only differ in the position of the amino group in the phthalate-ring. The position changes of the electronegative amino group on luminol to isoluminol make the molecule more polar. As a consequence of this, isoluminol can not pass through the cell membrane (56) .

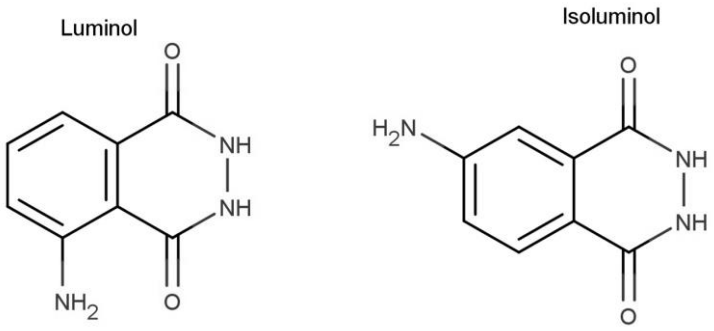

Figure 9 molecular structure of luminol and isoluminol (56)

This is important because it is now possible to quantify extracellular or intracellular ROS. The chemical reaction is catalyzed by horse radish peroxidase (due to the lack of extracellular MPO). The O_2^- (super oxide anion) reacts with isoluminol and forming an intermediate-state. This state will emitt light when returning to the quantum ground state. The amount of light emitted when returning to ground state will be quantified with Biolumat LB 9595 from Berthold (57).

Experimental procedure:

A total reaction mixture of 900 µL was incubated for 5 min and 37^0C in one of the six channels. The mixture contained: 650 µL KRG with Ca^{2+} (Reduced if antagonist or Latrunculin A was used to always keep the total reaction to 900 µL), 100 µL isoluminol, 50 µL HRP, 100 µL neutrophils ($1x10^5$/ml) and optionally 100 µL antagonist and/or latrunculin A 100 µL (25 ng/ml). After incubation the samples were stimulated with 100 µL agonist and the light emission was quantified continuously during the set time.

7.5 Measuring of intracellular Ca^{2+} by a Perkin Elmer fluorescence spectrophotometer

Neutrophils or overexpressed FPR1/FPR2 HL-60 cells ($5x10^7$/ml) was resuspended in 2,5 ml KRG with bovine serum albumin (0,1%) and labeled (30 min, dark, RT) with 5 µl FURA 2-AM (see **Figure 10**)

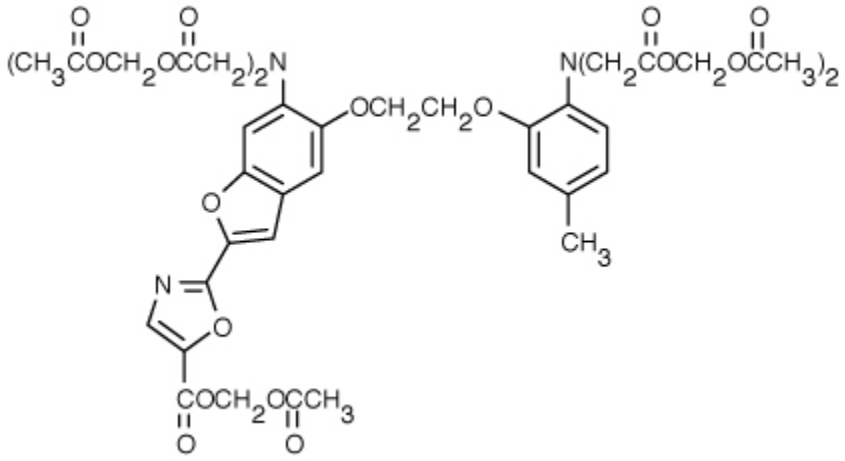

Figure 10 FURA2-AM (69)

FURA 2-AM is an intracellular Ca^{2+} fluorescence dye with two excitation wavelengths 340 nm and 380 nm that emit light at 510 nm. The acetoxymethyl group makes the molecule cell permeable and fluorescence inactive. Inside the cell will esterases cleave the acetoxymethyl groups and make it fluorescence active (58).

After the labeling the cells where diluted in 2.5 ml cell culture medium (RPMI) and centrifuged. The cells were then washed with KRG containing Ca^{2+} and counted in a Bürkerchamber. The cells were finally diluted to a concentration of $2x10^7$/ ml and kept on ice and protected from light until use.

Experimental procedure:

A sample (2475 µL) in a cuvette containing: 2225 µL KRG (RT) with Ca^{2+} (2200 µL if antagonist was used), 250 µL labeled cells and optionally 25 µL antagonist. The sample were then incubated (10 min, 37^oC) in the machine. After the incubation the sample was stimulated with an agonist and the ratio of the two excitation wavelengths (340 nm and 380 nm) when emitted at 510 nm was measured. This ratio corresponds to the rise of intracellular Ca^{2+} (33) The ratio is used to reduce the danger from artifacts.

7.6 Competitive receptor binding assay by flow cytometry

Flow cytometry is a powerful laser based technique used to measure the properties of

individual particles. In biological research these are often cells or bacteria. The sample is injected in the flow cytometer and are ordered in to a single stream of particles by hydrodynamic focusing. The single particle will now pass a laser, the laser will create two forms of light scatter. These are called forward scatter and side scatter. The intensity of the forward scatter corresponds crudely to the particle size. The side scatter provide data of the granular content of the cell. The sample is often labeled with a fluorochrome to provide additional information (59).

Neutrophils 198 µL / sample ($1x10^6$/ml) in ice cold KRG+Ca^{2+} were kept on ice and then incubated with 2 µl ICL2-23 (500 nM,50 nM,5 nM and 1 nM) ICL3-26 (500 nM,50 nM,5 nM and 1 nM), 2 µl WKYMVM (100 nM) or 2 µl Cy5-WKYMVM (1 nM) for 10 minutes. Followed by the addition of Cy5-WKYMVM to all samples except the sample has been pre-incubated with Cy5-WKYMVM and the incubation was continued for 1 hour on ice. After the second incubation, the samples were analyzed for fluorescence by an Accuri flow cytometer. The sample with Cy5-WKYMVM only is the positive control (total binding with maximum fluorescence) and Cy5-WKYMVM in the presence of the WKYMVM (100 nM) is the negative control (minimum fluorescence reflecting non-specific binding, due to the competitive binding).

Data analysis

The data and the statistically analysis was processed by the program GraphPad Prism 6.

8 Results

8.1 ICL2-23 and ICL3-26 induce an intracellular Ca^{2+} transient in neutrophils

To elucidate if the pepducins derived from the $P2Y_2$ receptor activate neutrophils, we examined their ability to induce Ca^{2+} intracellular transient, an early signal downstream GPCR upon neutrophil activation. The assay system used was the FURA-2 fluorescence system described in material and methods. Both ICL2-23 and ICL3-26 induced an intracellular Ca^{2+} transient in neutrophils whereasICL1-11 had no effect (see ***Figure 11***). The concentrations shown in ***Figure 11*** correspond to ~ 80-90% of the individual maximum response. They were also tested in cells pre-treated with the non/slow-membrane permeable Ca^{2+} chelator agent ethylene glycol tetraacetic acid (EGDTA) which removes all the extracellular Ca^{2+} to be sure that the Ca^{2+} response was from intracellular stores. The Ca^{2+} chelator agent was without any effect indicate that the response was indeed from intracellular stores (data not shown).

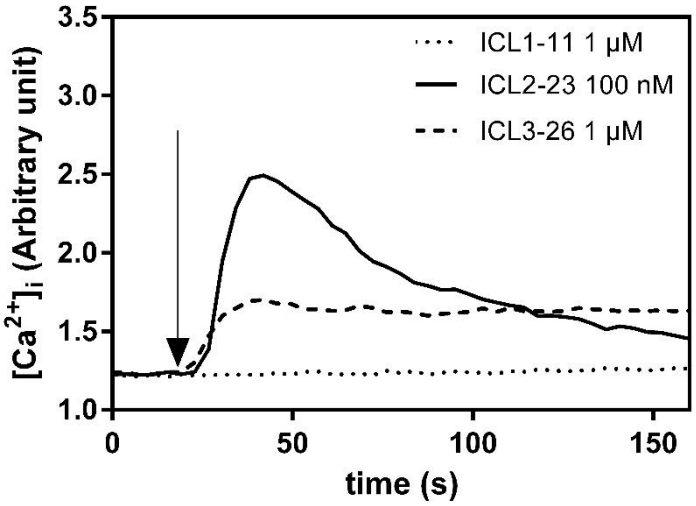

Figure 11 ***transient changes in the neutrophils cytosolic Ca^{2+} levels induced by the pepducins from the second and third intracellular loop of $P2Y_2$-R.***

Neutrophils labeled with the fluorophore FURA-2 were stimulated with the three individual pepducins corresponding to the three intracellular parts of $P2Y_2$-R (ICL1-11, ICL2-23 and ICL3-26). The arrow indicates the time point for addition of the pepducins. The change in cytosolic Ca^{2+} levels were measured as change in FURA-2 fluorescence. One representative experiment is shown out of at least three.

To investigate if ICL1-11 has any inhibitory effect on $P2Y_2$-mediated response, the ICL1-11 pepducin was incubated with the $P2Y_2$ agonist ATPγS (more stable analogue) and the effect was compared to ARC, a characterized $P2Y_2$ antagonist. ICL1-11 had no effect on the ability of

ATPγS to induce a Ca^{2+} transient through the $P2Y_2$-R (**Figure 12**). The well characterized $P2Y_2$-R antagonist ARC totally abolished the response and served as a control (see **Figure** 12).

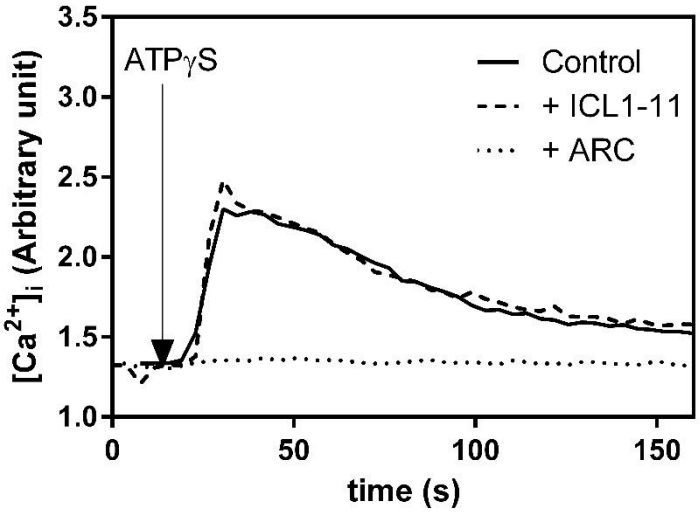

Figure 12 ICL1-11 has no inhibitory effect on the ATP analog ATPγS, while the $P2Y_2$ antagonist ARC totally abolished the response.

Neutrophils labeled with the fluorophore FURA-2 were pre-incubated with ICL1-11 (1 µM) or ARC (1 µM) 1 minute before addition of the stimulus ATPγS (10 µM). The arrow indicates the time point for agonist addition. The change in cytosolic Ca^{2+} levels were measured as change in FURA-2 fluorescence. One representative experiment is shown out of a least three.

We next examined whether ICL2-23 induced Ca^{2+} transient was sensitive to ARC or ICL1-11. Neither ICL1-11 nor ARC had effect on the ability of ICL2-23 to induce a Ca^{2+} transient (see **Figure** 13), suggesting that ICL2-23 does not signal through $P2Y_2$-R. ICL3-26 was also tested with the same results (data not shown)

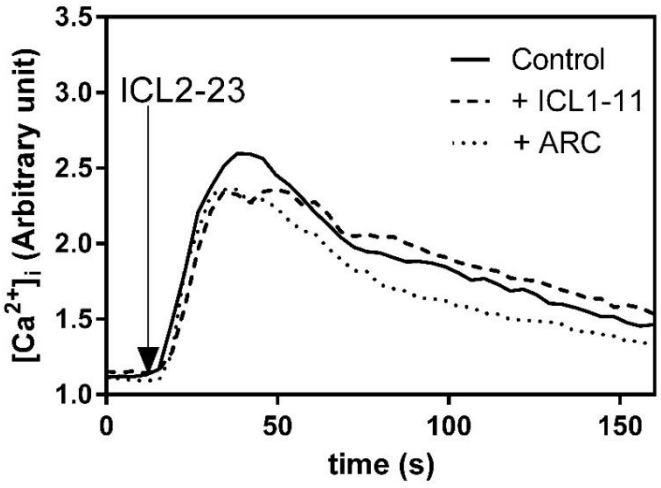

Figure 13 the extracellular antagonist ARC or the first intracellular loop pepducin of $P2Y_2$–R ICL1-11 has no effect on ICL2-23 ability to induce a Ca^{2+} transient.

The experiment was executed as in **Figure 11** *, with the difference that ICL1-11 (1 µM) or ARC (1 µM) were added 1 minute before ICL2-23 (100 nM) stimulation. One representative experiment is shown out of a least three.*

8.2 NADPH-oxidase activation measured by chemiluminscence assay

To investigate if the pepducins were able to mobilize and activate the NADPH-oxidase and produce the superoxide anion (O_2^-), the pepducins were investigated in the Chemiluminscence system previously described. Both ICL2-23 and ICL3-26 activated naive neutrophils while ICL1-11 was inert, following the pattern observed in the Ca^{2+} assay. **Figure *14*** shows the dose response curve (left) and representative kinetic (right).

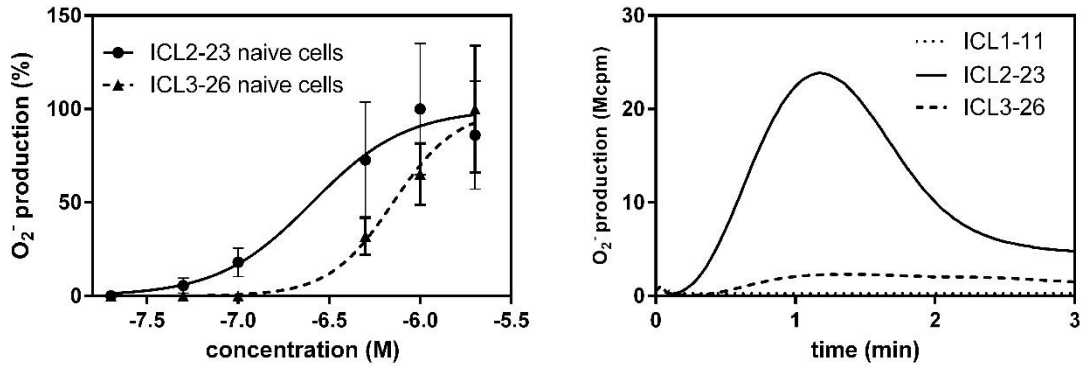

Figure 14 ICL2-23 is a full agonist and ICL3-26 is a weak agonist.

The dose response curve (left figure) is made out of 4 experiments with concentrations (2 µM, 1 µM, 500 nM, 100 nM, 50 nM and 20 nM) in the Chemiluminscence system previously described. The error bars are SEM. The representative kinetic figure (right figure) is from one experiment (ICL1-11 2 µM, ICL2-23 500 nM and ICL3-26 1 µM).

To see how the $P2Y_2$-R derived pepducins responded on primed neutrophils, the effect of pepducins was examined on TNFα primed cells and on Latrunculin primed cells (see ***Figure 15)***. $P2Y_2$-R endogenous ligand ATP (ATPγS was used) was included for comparison. ICL1-11 was also inert on primed neutrophils whereas ICL2-23 responded strongly to the priming and ICL3-26 responded but with less degree (see ***Figure 15***). This data corresponds to the naïve response where ICL2-23 was the most potent among the three pepducins examined. In contrast, ATPγS activates the NADPH-oxidase only in latrunculin A treated cells but not in naive cells (see ***Figure 15***) suggesting that ATP has different activation mechanism than the $P2Y_2$-R derived pepducins. This indicates that they might target different receptors.

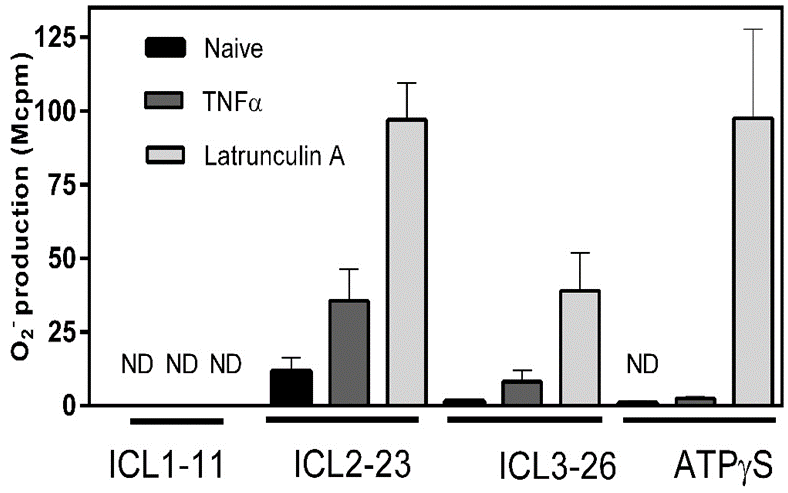

Figure 15 Both ICL2-23 and ICL3-26 respond to the priming. The endogenous ligand ATP only respond to Latrunculin priming.

Neutrophils naive or primed (Latrunculin 5 minutes or TNFα 20 minutes before stimulation) were stimulated with the pepducins (ICL1-11 2µM, ICL2-23 500 nM, ICL3-26 1 µM) or ATPγS 50 µM and the maximal response were recorded (ND = not detectable). The figure represent 3 individual experiment and the error bars are the standard deviation.

8.3 ICL2-23 response seems to mediated through FPR2 but not $P2Y_2$-R as expected

To determine which receptor was responsible for the response of the full agonist ICL2-23, different approaches were used. One approach is receptor desensitization and the other approach is to employ receptor specific antagonists including ARC (the $P2Y_2$-R), CysH (FPR1)

and PBP10, WRW4 (FPR2). To test for receptor desensitization, agonists for $P2Y_2$-R (ATPγS) and FPR2 agonists including an extracellular hexapeptide WKYMVM (L-peptide) and F2Pal10, a pepducin derived from the third loop (10 amino acids) of the FPR2 receptor (55). Both agonist (ATPγS) and to less extent the antagonist (ARC) from the $P2Y_2$-R primed the NADPH-oxidase response induced by ICL2-23. The FPR1 selective antagonist CysH was without any effect. Both the FPR2 allosteric and selective antagonist PBP10 and the extracellular antagonist WRW4 reduced the response but PBP10 was more efficient. In addition, both the FPR2 selective extracellular agonist WKYMVM and the FPR2 selective pepducin agonist F2Pal10 desensitized the response from ICL2-23. These results strongly indicate that the ICL2-23 response is mediated through FPR2 but not $P2Y_2$-R. See **Figure *16*** for a summary.

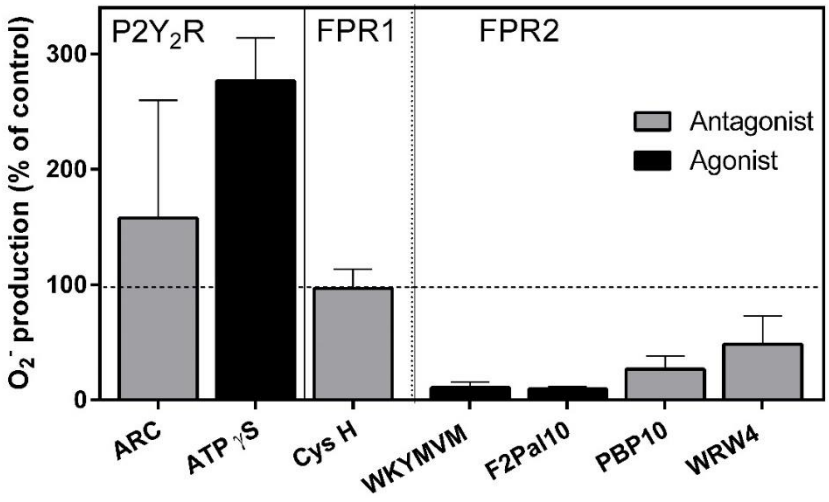

Figure 16 Effect of FPR2 and $P2Y_2$-R agonists and antagonists on the ICL2-23 response.

Antagonist and agonist were added 5 minutes prior to ICL2-23 (500 nM) stimulation and the response was compared to control received no previously treatment. All experiment were repeated at least 3 times, the error bars corresponds to the standard deviation.

To further examine the priming effect (ATPγS) and the desensitization effect (F2Pal10), double stimulation experiment was executed (see ***Figure 17***). The results point strongly to ATPγS primes the ICL2-23 response and vice versa. This priming effect was PBP10 sensitive (data not shown) indicating the engagement of FPR2. This is in line with previous studies showing cross-talk between FPRs and $P2Y_2$-R trigger FPR reactivation which results in a primed ATP response (34). F2Pal10 on the other hand desensitized the ICL2-23 response indicating that the two agonists probably target the same receptor.

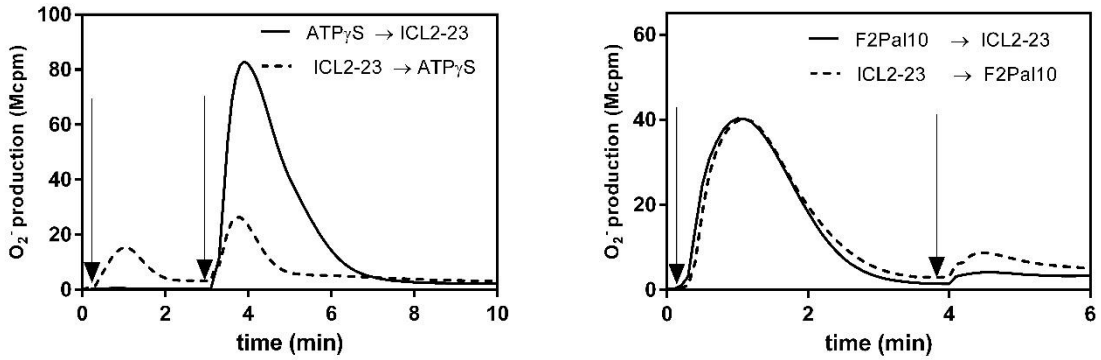

Figure 17 ATP primes the ICL2-23 response through heterologous receptor reactivation and ICL2-23 and the FPR2 pepducin agonist F2Pal10 desensitize each other.

The arrows indicate the time points for adding the stimuli/s ATPγS (50 µM) and ICL2-23 (500 nM) (left figure) and ICL2-23 (500 nM) and F2Pal10 (500 nM) (right figure).

8.4 ICL3-26 response is also mediated through FPR2

Because ICL3-26 naive response is so low, it was necessary to prime the cells with latruculin A before testing for receptor specificity by employing antagonists. The response was sensitive to PBP10 and the degree of inhibition was dependent on the ICL3-26 concentration used (see **Figure 18**). This indicates that the receptor involved is FPR2 also for the ICL3-26 response. ARC had no effect, indicating that $P2Y_2$-R is not targeted.

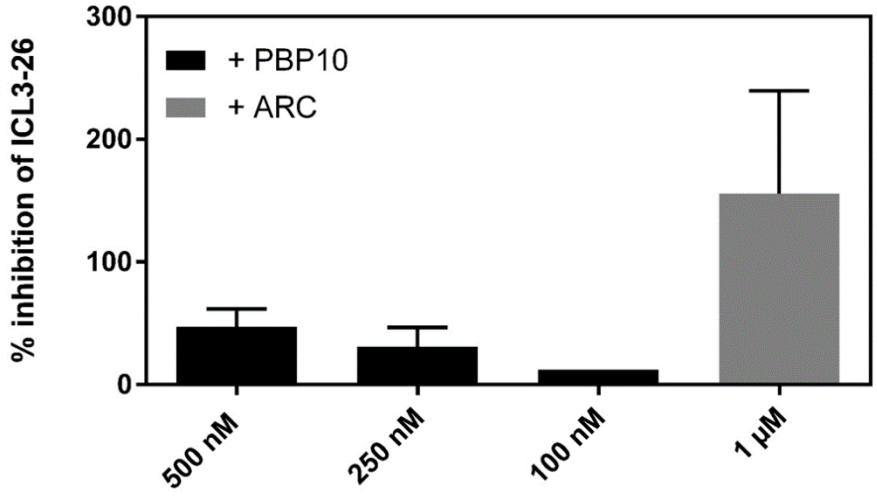

Figure 18 the ICL3-26 response is PBP10 sensitive and not reduced by ARC

The antagonists PBP10 (1 µM) or ARC (1 µM) together with the priming agent Latrunculin were incubated for 5 minutes before ICL3-26 stimulation and the response was compared to control received no antagonist but treated with latruculin A. The error bars corresponds to the standard deviation. The number of times the experiments where repeated: 500 nM = 3 times, 250 nM = 2 times, 100 nM = 1 time and ARC = 2 times.

8.5 ICL2-23 induced intracellular Ca^{2+} transient in neutrophils is dependent on FPR2

To investigate if the Ca^{2+} transient was also mediated through FPR2, ICL2-23 was tested with the FPR1 specific antagonist CysH and the FPR2 specific antagonist PBP10. The FPR1 antagonist CysH had no effect but the FPR2 antagonist PBP10 completely abolished the response (see ***Figure 19***). This strongly indicates that the Ca^{2+} transient induced by ICL2-23 is mediated selectively through FPR2.

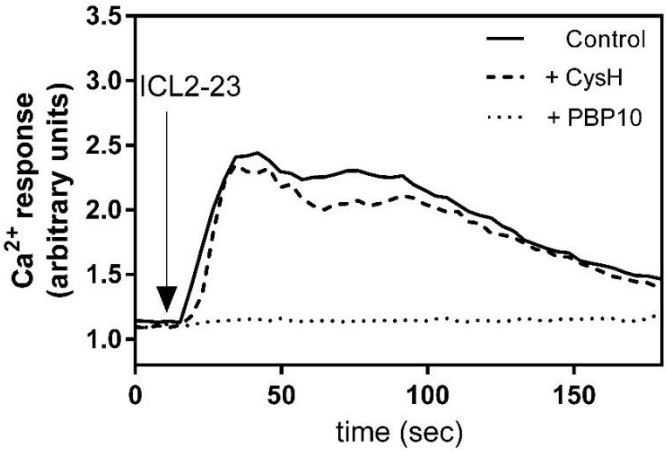

Figure 19 ICL2-23 Ca^{2+} transient is sensitive to the FPR2 specific inhibitor PBP10

The experiment was executed as in **Figure 11** *, with the difference that CysH (1 µM) or PBP10 (1 µM) was added 1 minute before ICL2-23 (100 nM) stimulation. One representative experiment is shown out of at least three.*

8.6 ICL3-26 induced intracellular Ca^{2+} transient is also mediated through FPR2

To determine if the partial agonist ICL3-26 was also PBP10 sensitive with respect to the intracellular Ca^{2+} transient, its ability to induce an intracellular Ca^{2+} transient was examined in the presence of PBP10 and compared to a naive control. The neutrophils that were incubated with PBP10 had almost lost all response to ICL3-26 (see ***Figure 20***). This indicates that ICL3-26 induced intracellular Ca^{2+} transient is mediated through FPR2.

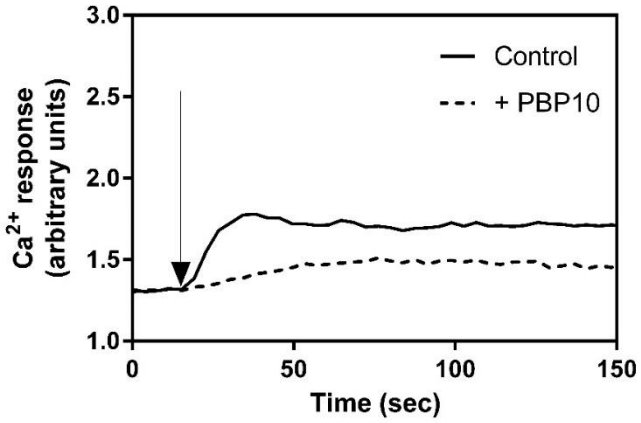

Figure 20 the ICL3-26 induced intracellular Ca^{2+} transient is PBP10 sensitive

The experiment was executed as **Figure 11***, with the difference that PBP10 (1 µM) was added 1 minute before ICL3-26 (1 µM) stimulation. One representative experiment is shown out of at least three.*

8.7 ICL2-23 and ICL3-26 induce an intracellular Ca^{2+} transient in FPR2 but not FPR1 overexpressingHL-60 cells

To confirm that FPR2 is responsible for ICL2-23 and ICL3-26 to induce an intracellular Ca^{2+} transient, we used genetically modified HL-60 cells that overexpress either FPR1 or FPR2. Since the cell lines overexpressing FPRs are undifferentiated, the NADPH-oxidase activation can thus not be studied as not all subunits in the NADPH-complex are formed. If the cell-lines are differentiated by for example DMSO, will FPR1 start expressing FPR2 and vice versa. The potency of ICL3-26 was much higher than in neutrophils so only 200 nM was needed to induce a good response in FPR2 overexpressed cell lines, compared to 1 µM in neutrophils. Both cell lines were tested and the receptor expression profile was confirmed with the FPR2 specific agonist WKYMVM and the FPR1 specific agonist fMLF before use.

Both ICL2-23 and ICL3-26 failed to induce an intracellular Ca^{2+} transient in FPR1 overexpressing HL-60 cells but succeeded in FPR2 overexpressing cell lines (see ***Figure 21*** and ***Figure 22***). This confirms previous results with PBP10 that the signaling is through FPR2 (46).

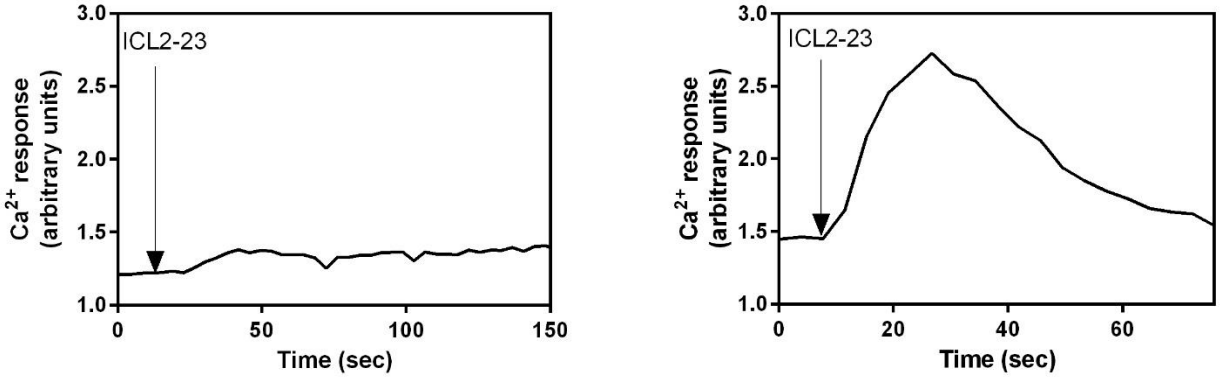

Figure 21 ICL2-23 does not induce a intracellular Ca^{2+} transient on FPR1 overexpressing HL-60 cells (left figure) but it does on FPR2 overexpressing cells (right figure).

HL-60 cells overexpressing FPR1 (left figure) or FPR2 (right figure) were labeled with the fluorophore FURA-2 and stimulated with ICL2-23 (100 nM). The arrow indicate the time point for adding the pepducin. The change in cytosolic Ca^{2+} levels were measured as change in FURA-2 fluorescence. One representative experiment is shown out of at least three

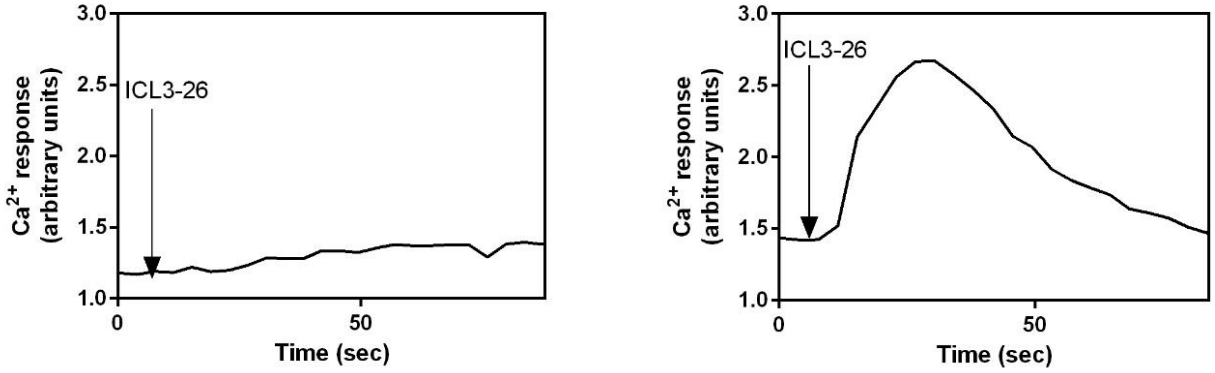

Figure 22 **ICL3-26 does not induce a intracellular Ca^{2+} transient on FPR1 overexpressing cells (left figure) but it does on FPR2 overexpressing cells (right figure).**

HL-60 cells overexpressing FPR1 (left figure) or FPR2 (right figure) were labeled with the fluorophore FURA-2 and stimulated with ICL3-26 (200 nM). The arrows indicate the time point for addition of the pepducin. The change in cytosolic Ca^{2+} levels were measured as change in FURA-2 fluorescence. One representative experiment is shown out of at least three

8.8 ICL2-23 and ICL3-26 compete with a fluorescence labeled orthosteric FPR2 agonist WKYMVM

To investigate if ICL2-23 and ICL3-26 compete with the binding of WKYMVM, an orthosteric FPR2 agonist, the competitive receptor binding assay was used by flow cytometry as described previously. Both ICL2-23 and ICL3-26 inhibited the Cy5-WKYMVM binding in dose dependent manner (see ***Figure 23***). This confirms the earlier experiment that both pepducins derived from the second and third intracellular loop of $P2Y_2$-R, mediates their response through FPR2. It also indicates that the binding site of ICL2-23 and ICL3-26 might be orthosteric.

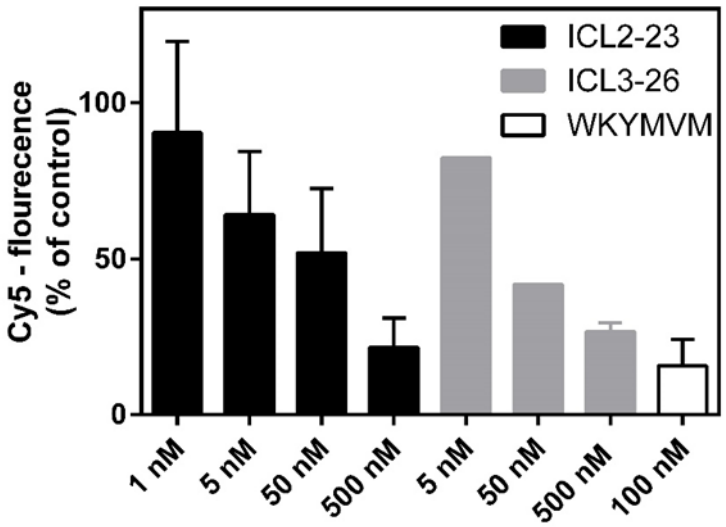

Figure 23 Both ICL2-23 and ICL3-26 compete with fluorescence labeled WKYMVM binding to neutrophils in a dose dependent manner

Neutrophils stored on ice were incubated (10 minutes) with ICL2-23, ICL3-26 or no addition (controls) and then incubated (1 hour) a second time by addition of fluorescence labeled Cy5-WKYMVM. The samples were then analyzed for fluorescence by an Accuri flow cytometer and compared to the positive control (Cy5-WKYMVM binding in the absence of pepducins). The error bars are the standard deviation and n=3 for ICL2-23 and n=1 for ICL3-26.

9 Discussion

Researchers working with pepducins have proposed that the pepducin derived from one of the intracellular signaling domains of the receptor either activate or inhibit the receptor. The

mode of action is suggested to be mediated through an allosteric interaction between the pepducin with the cognate receptor/G-protein on the cytosolic part after passing through the cell membrane (51). According to this proposed model, pepducins should be insensitive to conventional antagonists and not compete with conventional agonists for binding to the orthosteric site (the extra cellular active site) (60). Recent data indicates that this is a simplification and generalization and only valid to some pepducins (61)(62). It has recently been shown that pepducin derived from the β2-adrenergic receptor can act through s.c "bias" or receptor independent signaling (63). Two classes of β2-adrenergic pepducins were found, receptor independent and receptor dependent pepducin. The receptor independent one acts directly through the G_s protein. The receptor dependent one seemed to stabilize different receptor conformation. One conformation promotes β-arrestin recruitment and internalization. Another conformation does not recruit β-arrestin and the receptor undergoes no conventional desensitization. This is a breakthrough and in the case with β2- adrenergic receptor the finding should lead to the rise of a new generation of asthma drugs (64). The ICL1-11 one is inert in all systems examined. This is probably due to its reduced number of positive charged amino acids and shorter length which are required for receptor interaction. Data obtained from the pepducins from ICL2-23 and ICL3-26 of the $P2Y_2$ receptor challenges the pepducin mechanism of action proposed model. They do not interact with the $P2Y_2$ receptor instead they surprisingly target FPR2, ICL2-23 as a full agonist and ICL3-26 as partial agonist. The sensitivity to extracellular FPR2 antagonist like WRW4 makes it possible that another biochemical mechanism is at work.

Recent work on pepducins from FPR1 ($F1Pal_{16)}$) and FPR2 ($F2Pal_{16}$) also challenged the pepducin concept. $F1Pal_{16}$ is inert on FPR1 but is a potent selective FPR2 antagonist. F2Pal16 and the more potent $F2Pal_{10}$ are FPR2 agonists, display however receptor specificity for the receptor from which the pepducins are derived. Nevertheless, in discrepancy with the current pepducin concept, the FPR2 pepducins compete with conventional FPR2 agonist, that bind to the orthosteric site and it is sensitive to an extracellular antagonist (62)(55).

The fatty acid linked to the peptide is proposed to function as an anchor to the cell membrane. This "anchor" is proposed to allow the peptide part to penetrate the membrane(65). There is no question regarding the importance of the fatty acid, as illustrated by the complete lack of function if removed(61). This is however no proof that it interacts with the receptor on the cytosolic part, and our data suggest an alternative model that the fatty acid allows it to anchor to the cell membrane of peptides which otherwise may not get close contact with the membrane bound receptor and interact with the extracellular parts. The precise biochemical mechanism of pepducins seems to be much more diverse and complex than first thought. New findings in the field indicates that the "pepducin concept" is not entirely valid and pepducin seems to interact with the receptor in a number of different ways. This is depending of which ICL and the length (number of amino acids) the pepducin is derived from and from which

receptor. This is not so strange considering the difference in colulombian and hydrophobic forces at work, this is especially true when considering pepducins from different receptors.

10 Acknowledgment

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