Activation of professional phagocytes with emphasis on formyl peptide receptors

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Abstract

Phagocytic cells such as neutrophil granulocytes and monocytes are an essential part of our innate immune system and play an important role in the battle against pathogens. G-protein coupled receptors (GPCRs) and more specifically chemoattractant receptors are a vital part in guiding phagocytes towards the site of infection. Chemoattractant receptors are also involved in an effective activation of these cells.

This thesis investigates activating ligands and signalling properties of three different G-protein coupled receptors (GPCR) involved in innate immunity. Where the first two belongs to the formyl peptide receptor (FPR) family of chemoattractant receptors and the third is a non-chemotactic receptor expressed on monocytes.

The first paper describes the selective activation of the two receptors formyl peptide receptor 1 (FPR1) and formyl peptide receptor 2 (FPR2) by a synthetically derived hexapeptide with the sequence WKYMVm. We show that WKYMVm binds to both receptors but signal through FPR1 only when FPR2 is blocked. In paper number two we add the peptide MMK-1 to the list of FPR2 binding activators of the NADPH-oxidase. We also showed that calcium signalling induced by both FPR1 and FPR2 is dependent of release from intracellular stores and a subsequent opening of store operated calcium channels (SOCs) in the plasma membrane. Desensitization of chemotactic receptors is of importance for the termination of proinflammatory activities acted out by phagocytes. The third paper is a methodological study with the aim of solving problems associated with oxidation of stimulus in *in vitro* desensitization studies where intracellular calcium is measured. The solution put forward was to add serum proteins in the reaction mixture or to use a flow cytometry based method where the amount of reactive oxygen species (ROS) produced in the bulk could be reduced. In the fourth paper we identify a monocyte activating peptide, gG-2p19, derived from the secreted portion of the Herpes simplex virus type 2 (HSV-2) glycoprotein G. Monocytes produced ROS in response to stimulation with gG2p19 while neutrophils did not. The receptor for gG2p19 was shown to be a GPCR by its sensitivity to pertussis toxin, but the peptide could not induce chemotaxis through this receptor. It was determined that the receptor responsible for activation did not belong to the FPR family, but still share at least one common signalling pathway with FPR2.

List of publications

This thesis is based on the following papers referred to in the text by their roman numerals:

I. Karlsson J., Fu H., Boulay F., Bylund J., Dahlgren C.
The peptide Trp-Lys-Tyr-Met-Val-D-Met activates neutrophils through the formyl peptide receptor only when signalling through the formylpeptide receptor like 1 is blocked. A receptor switch with implications for signal transduction studies with inhibitors and receptor antagonists.
Biochem Pharmacol. 2006 May 14;71(10):1488-96

II. Karlsson J., Stenfeldt A., Rabiet M.J., Bylund J., Forsman H., Dahlgren C.
The FPR2 specific ligand MMK-1 activates the neutrophil NADPH-oxidase, but triggers no unique pathway for opening of plasma membrane calcium channels.
Cell Calcium 2009. In Press

III. Karlsson J., Bylund J., Movitz C., Björkman L., Forsman H., Dahlgren C.
A methodological approach to studies of desensitization of the formyl peptide receptor: Role of the read out system, reactive oxygen species and the specific agonist used to trigger neutrophils
Submitted

IV. Bellner L.*, Karlsson J.*, Fu H., Boulay F., Dahlgren C., Eriksson K., Karlsson A.
A monocyte-specific peptide from herpes simplex virus type 2 glycoprotein G activates the NADPH-oxidase but not chemotaxis through a G-protein coupled receptor distinct from the members of the formyl peptide receptor family
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Abbreviations

C3R	Complement receptor 3
C4R	Complement receptor 4
C5a	Complement factor 5a (split product from C5)
CGD	Chronic granulomatous disease
CHIPS	Chemotactic inhibitory peptide from *Staphylococcus aureus*
Cys H	Cyclosporin H
Cyt B	Cytochalasin B
DAG	Diacylglycerol
DAMP	Damage associated molecular patterns
DC	Dendritic cell
fMLF	N-formylmethionyl-leucyl-phenylalanine
FPR1 (FPR)	Formyl peptide receptor 1
FPR2 (FPRL1)	Formyl peptide receptor 2
FPR3 (FPRL2)	Formyl peptide receptor 3
GPCR	G-protein coupled receptor
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
HSV2	Herpes simplex virus type 2
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP_3	Inositol 1,4,5-triphosphate
LPS	Lipopolysaccharide
LXA_4	Lipoxin A_4
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
OH·	Hydroxyl radical
PAMP	Pathogen associated molecular pattern
phox	Phagocyte oxidase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol myristat acetate
PRR	Pathogen recognition receptor
ROS	Reactive oxygen species
SAA	Serum amyloid A
SNP	Single nucleotide polymorphism
SOC	Store operated calcium channel
TLR	Toll-like receptor
TNF	Tumour necrosis factor

Innate immunity

The human body is constantly exposed to potentially harmful microorganisms originating either from the surrounding world or from the normal flora, and still we very seldom suffer from microbial infections. The reason for this is contained within the functions of the different parts of the immune system. In mammals the immune system comprises innate and adaptive immunity, and both parts are built of a large number of collaborating cellular as well as humoral components (1). The immediate and very rapid innate immune response is a potent first line defence system against invading microorganisms and exists in all multicellular organisms. Many organisms rely on the innate immune reaction as their only protection. In higher organisms (i.e. vertebrates) additional mechanisms collectively composing the adaptive immune system have evolved and work in close collaboration with the innate immune mechanisms. As both parts of the immune system work hard to keep pathogens out, microbes work even harder to get in. The "success" of pathogens relies on their ability to avoid and manipulate host responses and defence mechanisms (2).

The main physical barrier against bacterial or viral attacks at mucosal membranes is the epithelium that also gets reinforcement from secretions lining the epithelium such as the mucus and saliva produced in the upper respiratory tract or acid secreted in the gut. One ancient and potent part of the secretions is antimicrobial peptides. Antimicrobial peptides are phylogenetically old and are for some invertebrates the only weapon against microbial attack.

The cells of the innate immune system cannot be selectively educated to recognize specific antigens, an important function of the adaptive part of the immune system. Instead the innate immune system relies on the recognition of certain conserved pathogen-associated molecular patterns (PAMPs). These PAMPs are conserved and essential structures or molecules expressed by the pathogen but not by the host, and can therefore be identified as a signal of danger by pattern recognition receptors (PRRs) on the innate immune cells. PRRs can also bind and be activated by for example endogenous products from damaged cells, damage-associated molecular patterns (DAMPs), which is an indication of peril. According to the danger hypothesis, PAMPs and DAMPs together signals danger and elicit similar responses in immune cells (3). This type of pattern recognition is found in both plants and animals, although PRRs in plants are not always structurally related to the animal equivalents (4, 5).

Acute inflammation

The physical barriers of the body are not always a sufficient defence, and thus as an invasive microbe passes the wall and enters the tissue it will meet a first wave of innate immune cells. The activities of cells that encounter the invading microbes will induce a reaction called acute inflammation. This reaction can be initiated not only by invading microorganisms but also by mechanical injury to a tissue such as a cut or burn, with or without a subsequent infection by bacteria or viruses. In addition, inflammation could also be induced by a non-invasive damage to the tissue like a sprained ankle. Looking at the "inflammation scene" from the outside there are five cardinal signs of inflammation that were described more than 2000 years ago and these are, rubor (redness), calor (rise in temperature), tumor (swelling), dolor (pain) and *functio laesa* (loss of function). These signs are the direct result of the higher capillary blood flow and increased vascular permeability caused by a release of pro-inflammatory mediators like histamine and bradykinin from cells in the affected tissue. Pro-inflammatory mediators from the damaged host cells also act as "call for help" signals for immune reactive cells that patrol the body. Substances released both from microbes and host cells that guide the cells towards the site of inflammation are called chemoattractants. Even though inflammation is the means by which the body is supposed to heal in an optimal situation, this is not always the case. The mechanisms used by immune cells to fight the intruding microbe can, like a double-edged sword, cause damage to the host (see NADPH-oxidase activation). Since acute inflammation is a powerful and potentially harmful process, tight regulation is of greatest importance. In the best-case-scenario, acute inflammation is a rapidly induced and terminated process that ends with eradication of microbes, resolution and healing (see apoptosis and clearance). If problems occur during resolution this could lead to chronic inflammation or autoimmunity (6).

Professional phagocytes in innate immunity

Neutrophil granulocytes

Being highly motile, neutrophil granulocytes are the first cells to migrate towards the site of tissue damage or infection. Neutrophils are important players in acute inflammation and are the most abundant white blood cell in circulation constituting approximately 50-70% of the total leukocyte count in human peripheral blood. The neutrophil is developed along the myeloid lineage in the bone marrow and the mature cell stays there until released to the blood stream (7). It is a short-lived cell designed to "seek and destroy", thus a "professional phagocyte". Once the neutrophil has left the bone marrow and entered the blood

stream , the lifespan is, on average, 25 hours (8), and for activated cells that have migrated into the tissue, several days. Circulating neutrophils have two possible fates: i) the cells age and are cleared from circulation by macrophages in the spleen or the liver or ii) they migrate into the tissue in response to chemokines or other inflammatory agents.
The presence of vast amounts of granules in the cytoplasm of certain blood leukocytes is the basis for naming them granulocytes. These cells can be divided into neutrophils, eosinophils and basophils through the use of laboratory dyes that differentially stain the cells and their granules. The neutrophil contains at least three different types of granules, azurophil, specific, and gelatinase and in addition also a fourth type of storage organelle of endocytic origin, the secretory vesicle. The different organelles are formed in the order azurophil first, then the specific and gelatinase, up to secretory vesicle that is formed last in the maturation process of the cell. Upon cellular activation these organelles are mobilized in an order opposite to their formation. Proteins are sorted into a defined granule type in relation to when they are formed i.e. the transcription of a granule protein destined to the azurophil granules occurs only during the time when this granule type is formed, the process being referred to as sorting by timing (9, 10). The granule content constitute an arsenal of potent microbicidal compounds that together with the reactive oxygen species generated by the so called NADPH-oxidase (see below) make up the weaponry against intruders. The dogma in the field has for a long time been, that once a neutrophil has left the bone marrow it is terminally differentiated with no or very low *de novo* synthesis of new proteins. All proteins needed for the neutrophil to fulfil its task are produced in the bone marrow and stored in the granules. In recent years it has, however, been made obvious that this theory was over-simplified -- mature neutrophils can produce and secrete large quantities of many proteins including the potent chemokine Interleukin (IL) 8 (11, 12).

Peripheral blood monocytes

Another leukocyte of the myeloid linage, the monocyte, is also a professional phagocyte with important functions in inflammation. Monocytes normally constitute 5-10% of the circulating white blood cells. After leaving the bone marrow these cells circulate for approximately 24-72 hours before they are cleared or transmigrate to a tissue (13).
Human peripheral blood monocytes show morphological heterogeneity, such as variability of size, granularity and nuclear morphology (14). Initially monocytes were identified by their high expression of CD14, which neutrophils lack. Later, the identification of a difference in surface antigen expression between groups of cells showed that monocytes in human peripheral blood are heterogeneous. Differential expression of CD14 and CD16 has rendered a division of monocytes

into two subsets: 90 % are $CD14^{+}CD16^{-}$ cells which resembles the original description of monocytes and are often referred to as “classical”; and 10% are $CD14^{+}CD16^{+}$ or “non-classical” monocytes (14-16). The latter subtype has also been called “pro-inflammatory”, due to the profile of cytokine production in response to bacterial products; lower levels of the anti-inflammatory cytokine IL-10 and higher amounts of the pro-inflammatory cytokine tumor necrosis factor α (TNF-α) (17, 18). The “classical” cells have a typical monocytic phenotype with high chemotactic and phagocytic activity, high cytotoxicity for tumor cells and suppression of lymphocyte proliferation.

Peripheral blood monocytes are, like neutrophils, a potent weapon against intruders, although not quite as short-lived. Following activation, monocytes migrate to the tissue and can when needed differentiate and reinforce the pool of tissue bound phagocytic cells.

Monocytes arrive at the scene of damage slightly later than neutrophils but remain for a longer period of time. Although both celltypes can produce new proteins, a significant difference is that neutrophils have a modest synthesis of new proteins compared to monocytes. This suggests that monocytes are potent in regulating the inflammatory response by production of cytokines while neutrophils rely on stored substances (7).

Macrophages and dendritic cells; Phagocytes derived from monocytes

This thesis is focused on professional phagocytes from peripheral blood, however there are tissue resident phagocytic cells derived from monocytes with crucial roles in infection and inflammation (reviewed in (14, 19)).

Tissue macrophages have a broad role in the maintenance of homeostasis, through the clearance of senescent cells and the remodelling and repair of damaged tissue. Since they reside in the tissue, they are among the first cells to become aware of tissue damage or infection. Upon activation macrophages produce and secrete pro-inflammatory cytokines and chemokines and have an important role in regulation of inflammation.

The macrophage population has a high degree of heterogeneity, which reflects the specialization adopted by macrophages in different tissues. Examples of diverse macrophage functions include: remodelling of bone by osteoclasts, removal of apoptotic T lymphocytes by tangible-body macrophages in the spleen and phagocytic and bactericidal activity in the gut. During homeostasis monocytes are slowly recruited to differentiate and refill the pool of macrophages, but under inflammatory conditions increased numbers of monocytes migrate and differentiate.

Monocyte derived dendritic cells (DCs) are professional antigen presenting cells in the tissue. Both DCs and macrophages act as a bridge between innate and

adaptive immunity by presenting antigens. While the MHC-II antigen-presenting capacity is gained by macrophages and DCs during differentiation from monocytes, some characteristics are also lost or dampened. One example of this is the lower ability to produce reactive oxygen species (ROS).
The "non-classical" monocyte described above is regarded as a possible precursor to macrophages and dendritic cells. These monocytes express higher levels of MHC-II and, *in vitro*, are more prone to become DCs than the "classical" subset of monocytes (14). What type of macrophage or DC a given monocyte will become depends ultimately on the substances and milieu that the cell meets in the tissue. *In vitro* studies of differentiation in human monocytes have shown that TNF-α skews the differentiation from macrophage to DC; IL-6 switches it in the opposite direction (20, 21). The effect of environment is also demonstrated by the *in vitro* protocol for polarization of macrophages towards different phenotypes. When cultured with Interferon γ (IFN γ) and lipopolysaccaride (LPS), macrophages show high microbicidal activity and produce ROS, but on the other hand, cells cultured with IL-4, IL-10, IL-13 or TGFβ show a phenotype that promotes tissue repair and suppresses inflammation (22).

Phagocyte functions in an inflammatory response

Extravasation

Extravasation is the process whereby circulating phagocytes leave the blood vessel and pass through the endothelium into the tissue. In circulation the major part of neutrophils and monocytes normally roll along the vessel wall at a rate slightly slower than that of the blood flow, this population constitutes the marginal pool (1, 7). Under inflammatory conditions cells residing in the tissue release cytokines and chemokines, which trigger endothelial cells to upregulate the cell adhesion molecules (CAMs) E-selectin and P-selectin. These adhesion molecules binds ESL-1 and PSGL-1 exposed on the surface of the circulating cells and, as a result, the adhesion between blood cells and the vascular endothelium is increased and the rolling speed of the cells is decreased. The expression of endothelial VCAM, which is involved in the later steps of firm adhesion and diapedesis, is also increased at this point (23). The process is further regulated through the release of vasodilators for e.g., histamine, which decreases the blood flow. After the cells arrest as a result of firm binding between VCAM and ICAM on the endothelium and integrins e.g. LFA-1 and CR3 on the leukocytes, extravasation into the tissue is initiated (24). Once out in the tissue the phagocytes will migrate towards the focus of inflammation via chemotaxis.

Chemotaxis

The directed movement of the cell towards a rising gradient of chemoattractants is called chemotaxis. This process is dependent on cytoskeletal reorganization and also on the interaction between integrins and the extracellular matrix (25). The cytoskeleton of eukaryotic cells is roughly divided into three components: actin filaments, intermediate filaments and microtubules. The actin filaments seem to be functionally most important in leukocyte locomotion (25, 26). Actin is present in the cell in two forms, a 42 kD monomer (G-actin) and a polymeric filament (F-actin). The rapid polymerization and depolymerization of actin in response to various stimuli permits the cell to reorganize the cytoskeleton and migrate. During chemotaxis the leukocyte becomes polarized with a broad lamellipodium in the front and a uropod at the rear end. As the lamellipodium extends forward, integrins adhere to the matrix ligands and the uropod is withdrawn into the cell body.

In the inflamed tissue, the immune cells are flooded with different types and concentrations of chemoattractants. In order to cope with this, the migration stimulated by chemoattractants has been shown to be hierarchal (27). The different chemoattractants are defined as intermediary or end-point attractants. It is intermediary chemoattractants like IL-8 and Leukotriene B_4 (LTB_4) that attract the phagocytes from the bloodstream. Later in the process, the end-point attractants, such as bacterial peptides and activated complement factors, take over in the tissue and guide the cells to their final destination. The signalling events from the receptors for these end-point attractants dominant to signals induced by intermediary chemoattractants. Another mechanism of importance for the cells to reach their targets is coupled to the desensitization of receptors, a process by which the receptor becomes refractory to stimuli as described below (see termination of signalling and desensitization).

Mobilization of granules

Neutrophils will mobilize their granules in a specific order starting with the secretory vesicle during extravasation. The secretory vesicle is of endocytic origin which means that it contains mainly plasma proteins, but the vesicle membrane also stores a pool of surface receptors and adhesion molecules ready to be exposed on the cell surface. Next in turn in the mobilization process are the gelatinase granules and a fraction of the specific granules that secrete matrix proteins and deliver membrane components to the plasma membrane during chemotaxis. When the cell has reached the site of infection and engulfed invading microbes, the remaining pool of specific granules are needed together with the azurophil granules. These granules fuse with the phagosome and the so formed phagolysosome will be filled with pre-formed microbicidal substances which include for e.g., BPI (bacterial permeability increasing factor), lactoferrin,

lysozyme and antimicrobial peptides. The specific granules also provide the membrane bound subunits of the NADPH-oxidase while the azurophil granules contain myeloperoxidase (MPO) that takes part in the oxygen-dependent microbial killing (28). The mechanism underlying the well ordered degranulation in neutrophils is yet to be described, but the leading hypothesis states that it could depend on granule differences in size and density, or different sensitivity to the level of intracellular Ca^{2+} (29, 30).

The granule composition and the degranulation process are not as well characterized in monocytes as it is in neutrophils. The major granule populations are also present in monocytes and even though they differ somewhat morphologically from their counterparts in neutrophils (31), it is tempting to assume that the basic functions are similar in the two cell types.

Phagocytosis

Engulfment of particles, phagocytosis, is for many unicellular organisms simply a process for intake of nutrients, but for phagocytes of the innate immune system it is an effective strategy for the removal, and subsequent killing, of microbes. When a phagocyte has reached the source of infection, a direct interaction between pathogen and host cell is required for initiation of phagocytosis. The microbe can be recognized by the phagocyte directly via lectin/glycoconjugate receptor interactions. Yet, a more effective and common way to engage pathogens is via recognition of endogenous opsonins, such as complements and antibodies, that cover the surface of the prey. Opsonins function as bridging molecules, between the pathogen and phagocyte, recognized by Fc-receptors or CR (complement receptors) on the phagocyte (32, 33) (see general overview below). All these different interactions generate a host cell signalling cascade that promotes the phagocytic machinery. Actin rearrangement is initiated and followed by formation of pseudopods, which will reach out, fuse and enclose the microbe in the phagocytic vacuole.

Activation of the NADPH-oxidase

The production of reactive oxygen species (ROS) is mainly regarded as unwanted and harmful consequence of mitochondrial respiration, but professional phagocytes are equipped with an enzyme system, the NADPH-oxidase complex, that functions to produce ROS as a weapon against pathogens.

The phagocyte NADPH-oxidase is an enzymatic complex consisting of several subunits. Two of these subunits, $gp91^{phox}$ and $p22^{phox}$, are membrane proteins present both in the plasma membrane (5-10%) and in the membranes of the easily mobilized granules (34, 35). These two membrane bound subunits is together called cytochrome b. The other three subunits, $p67^{phox}$, $p47^{phox}$ and $p40^{phox}$, are cytosolic thus separated from the membrane components in a resting

cell (**Fig. 1**). Upon cell activation the cytosolic components are recruited to the membranes and assembled to form a functional electron transporting enzyme system. The NADPH-oxidase can be assembled in the phagosomal membrane and the respiratory burst products are used to eradicate the microbial prey enclosed in the phagolysosome. The NADPH-oxidase can be assembled also in the plasma membrane and the generated ROS are released from the cell. This could be beneficial for killing non engulfed microbes, but at the same time these radicals might cause tissue destruction to the host.

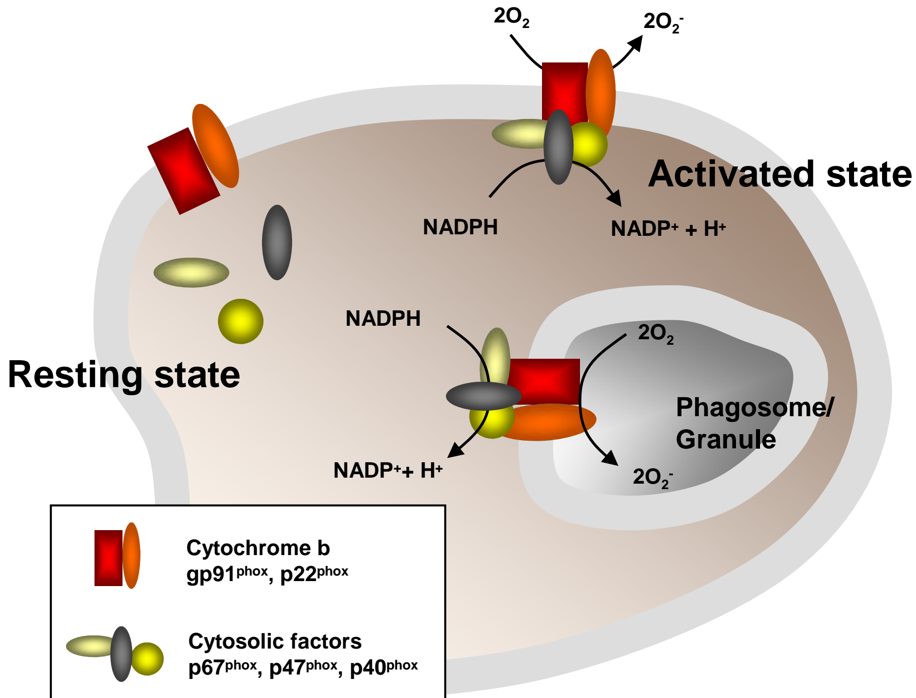

Figure 1. The phagocyte NADPH-oxidase in its resting and activated state. In the resting state the NADPH-oxidase is divided into the membrane bound factors ($gp91^{phox}$ and $p22^{phox}$) and the cytosolic factors ($p67^{phox}$, $p47^{phox}$ and $p40^{phox}$). The major part of the membrane bound factors is localized to granule membranes and only a smaller part to the plasma membrane. Upon cell activation the cytosolic factors are recruited to the membranes, together with the membrane bound factors they form a functional electron transporting enzyme system. The NADPH-oxidase can be assembled both in the phagosomal/granule membrane but also in the plasma membrane.

The assembled and activated NADPH-oxidase ferries two electrons from NADPH in the cytoplasm over the membrane and reduce two oxygen molecules (O_2) to superoxide anions (O_2^-) on the other side of that membrane. The reduced

oxygen molecules are short-lived and the first in a cascade of different toxic compounds. Superoxide anions can dismutate to H_2O_2, and this reaction occurs spontaneously but can also be catalysed by the enzyme superoxide dismutase (SOD). H_2O_2 can be used by the cell to generate a number of other ROS via a variety of pathways. The azurophil granule enzyme MPO catalyses the halogenation of hydrogen peroxide, to form hypochlorous acid ($HClO^-$). H_2O_2 can also be reduced further to hydroxyl radicals (OH·), one of the most potent antimicrobial ROS.

The importance of ROS for microbial killing could best be illustrated by the chronic granulomatous disease (CGD). People with CGD lack a functional NADPH-oxidase and in two thirds of the patients this is a consequence of mutations in the X-chromosome linked gene for $gp91^{phox}$. This results in failure of affected phagocytes to produce ROS, often with recurrent severe infections as the outcome (36). Oxygen radicals produced by phagocytes have also been shown to have a broader immuno regulatory role both in healthy individuals and CGD patients. After performed duty the natural path for neutrophils is to go into apoptosis. ROS has been shown to act as signalling molecules to initiate and accelerate apoptotic pathways, and in accordance with this neutrophils from CGD patients have a prolonged survival (37, 38). Besides severe infections CGD patients are also predisposed for inflammatory responses that are out of proportion and results in complications. One reason for this may be that leukocytes from CGD patients have a hyperinflammatory phenotype with exaggerated production of pro-inflammatory cytokines in response to stimuli (39, 40). The formation of neutrophil extracellular traps (NETs) is a suggested bactericidal mechanism important for extracellular killing that also has been shown to be dependent on oxygen radicals (41, 42). NETs are extracellular fibers consisting of chromatin decorated with granular proteins, which are released upon activation to bind Gram-positive and –negative bacteria both *in vivo* and *in vitro*. The release of NETs after activation has been shown to be a process following a type of ROS dependent cell death distinct from apoptosis or necrosis. The formation of NETs is abundant in neutrophils from healthy individuals while CGD neutrophils do not shown signs of NETs after activation *in vitro*.

Induction of ROS production has also been proposed as an immune escape mechanism for viruses, for e.g. Herpes virus, releasing phagocyte activating factors (43), and thereby inhibiting natural killer (NK) cell activity. The NK cell is the innate immune cell especially important in the defence against viral infections, therefore it is not surprising that viruses are constantly evolving to develop mechanisms for evasion of NK cell killing (44). Not long after the discovery and characterisation of NK cells, it was described that monocytes had an inhibitory effect on NK cell function including induction of apoptosis. The

responsibility for this has later on been ascribed to the radicals produced by phagocytes (45).

Apoptosis and clearance

Neutrophils arrive at the site of infection in order to phagocytose and eradicate pathogens as described earlier. When they have fulfilled their duties they go into apoptosis (programmed cell death) and are disposed of by other celltypes, most commonly macrophages (6). During apoptosis neutrophils loose their ability to degranulate (46), they keep intact and go quietly without releasing harmful substances to the surrounding tissue. However, if macrophages are not available or the environment gets too destructive neutrophils can undergo primary or secondary necrosis, a more inflammatory type of cell death. The membrane loses its integrity and toxic cellular contents reach the surroundings. Necrotic cell death is thought to be coupled to tissue damage and possibly chronic inflammation (6). Apoptosis and subsequent clearance by macrophages is important for a proper resolution of the acute inflammation. It has been shown that macrophages ingesting for example yeast particles produce pro-inflammatory cytokines (47) while cells phagocytosing apoptotic neutrophils do not (48). The process of engulfing apoptotic bodies is not entirely silent; the macrophages seem to release agents that have suppressing influence on inflammation (48). The macrophage response is also dependent on how the granulocyte apoptosis has been induced. Engulfment of *Mycobacterium tuberculosis*-induced apoptotic neutrophils triggers TNF-α production in macrophages (49). Thus apoptosis itself as well as the phagocytosis of apoptotic neutrophils, but not microbes, promote resolution of inflammation.

Receptors involved in the activation of professional phagocytes

In order to sense danger, phagocytes are equipped with PRRs able to identify different molecular patterns as described earlier. They also express a multitude of other receptors involved in for example adhesion and phagocytosis. This chapter will give a general overview of receptors important in activation of phagocytes, but it is by no means a complete listing.

General overview

In the late 1990s a mammalian homologue of the fruit fly (*Drosophila melanogaster*) receptor named Toll was identified (50). The Toll receptors were initially described to have a role in host defence against fungal infections, and the family of human Toll-like receptors (TLRs) has in recent years been shown to be of outmost importance in innate immune recognition. The TLR family has

to date 11 members (TLR1-11) of which TLR1, 2 and 4 are expressed by neutrophils and all except TLR 3 by monocytes (51, 52). Although there are a great deal of contradictory data about the exact expression patterns of TLRs on leukocytes, scientists seem to agree that monocytes express higher numbers of TLRs compared to neutrophils (7). The TLRs are able to sense/recognize a large variety of structures derived from microbes including certain proteins, lipoproteins, LPS, bacterial DNA and cell wall peptidoglycans, but they can also recognize host proteins such as the heat shock proteins, well known endogenous danger signals. Activation of TLRs does not directly induce effector functions such as ROS production or chemotaxis but ligation of a TLR can pre-prime the cells. This means that after TLR activation by for example LPS the cells will respond more vigorously to other stimuli that directly activate the NADPH-oxidase (53, 54). The degranulation induced by LPS is one part of the priming process but also other events, such as increase of plasmamembrane G_i levels, participates to accomplish a fully primed cell.

Cells of the innate immune system lack the type of receptor variability that is an important character for the adaptive part of the immune system. Despite this, the functions of these cells are of importance for a proper elimination of specific antigens recognized by the adaptive system. This elimination is achieved through the immunoglobulin- or Fc-receptors (FcR) exposed on the cell surface of phagocytes. This is an indirect way of sensing certain antigens, as antibodies bind to the foreign antigen and the FcRs bind to the Fc domain of these antibodies. The FcRs are named after the subclass of immunoglobulins they identify, FcγR bind IgG, FcαR bind IgA, FcεR bind IgE, FcμR bind IgM and FcδR bind IgD. The majority of receptors that belong to this family trigger signals that lead to internalization of their ligands, a process that results in endocytosis, phagocytosis or transcytosis. In phagocytes the most prominent task for FcRs is binding to particles opsonized with antibodies to enhance phagocytosis. Interaction between immunoglobulins bound to their specific antigen and FcRs starts also a more general activation pathway in the cell with ROS production as a result (55).

Several different surface molecules are responsible for the direct interaction between neutrophils and extracellular matrix, endothelium or microbes, integrins being the most important class. The integrins are a large group of adhesion molecules ancient in origin. There are three main families, each defined by a common β subunit that can be combined with a variety of α subunits. The β_2 family of integrins is expressed exclusively on hematopoetic cells. Analysis of leukocytes from patients with different inherited disorders of β_2 integrin expression or signalling has shown that these adhesion molecules are important in cell adhesion, diapedesis and phagocytosis (56, 57). This is in line with the fact that the complement receptors CR3 (Mac-1, CD11b/CD18) and CR4

(p150,95, CD11c/CD18), which are involved in phagocytosis, belong to this family. CR3 and CR4 can both interact with complement factors C3b and C3bi that coat and opsonize the surface of an object destined for phagocytosis. These objects include not only microbes of different origin, but also aged erythrocytes containing valuable iron that is recycled by macrophages through phagocytosis. The contribution of CR4 to C3bi binding by neutrophils and monocytes is however much smaller than that of CR3 partly because of a 10-fold lower expression on the surface (58). Binding of a complement ligand to CR3 gives rise to multiple signal transduction events in the leukocyte, many of them acting to stimulate phagocytosis.
Whereas the CR3 and CR4 mainly interact with opsonins coating a prey, another class of surface receptors is chiefly responsible for adhesion to non-opsonized objects, the lectins. Lectins are classified according to which monosaccharide the binding lectin has the highest affinity for. Lectins are found in a variety of species of plants, bacteria and mammals (59). In bacteria, lectins are of great importance for the colonization of mucosal surfaces in mammals. To establish a successful infection, the microbe first has to adhere to the physical barriers of the host. That is an interaction which can be partially mediated by lectins. The fact that bacteria are rich in surface lectins is also used by phagocytic cells of the immune system. In areas of the body where opsonins are scarce, for e.g., the urinary tract, adhesion before phagocytosis is the result of interactions between glycoconjugates on the phagocyte cell surface and lectins on the bacterium (33). There are also examples where lectins on the phagocyte mediate attachment to microbial carbohydrates.

G-protein coupled receptors

Phagocytes are also equipped with a large number of receptors that belong to the G-protein coupled receptor (GPCR) family. These receptors are all seven-transmembrane-spanning proteins that transduce information to the cell interior, upon binding a variety of extracellular ligands.
Generally, receptors belonging to this family recognize ligands of great diversity such as odour, light, hormones, pheromones, neuro-transmittors and substances produced by immune competent cells. The cytoplasmic parts of a GPCR protein (three intracellular loops and the C-terminal tail) can interact with a heterotrimeric, guanine nucleotide-binding protein (G-protein). The task of the G-protein is to transfer the ligand-induced signal into a wave of second messengers and expansion of downstream signalling. The G-protein is composed of three subunits, α, β and γ, where the α unit has a binding site for GDP/GTP and possesses the GTPase activity that enables the transformation of GTP to GDP. Activation of the GPCR induces a conformational change in the receptor that is followed by an exchange of GDP for GTP bound to G_{α}. Subsequently, the

G-protein dissociates into G_{α}- and $G_{\beta\gamma}$-subunits that proceeds to activate or inhibit different signalling pathways. After passing the signal on to effectors elsewhere in the cell, bound GTP is hydrolyzed to GDP and the G_{α} subunit is recirculated to the membrane to participate in the formation a new G-protein/receptor complex (**Fig. 2**). Four main families of G-proteins have been characterized based on the classification of α subunits, $G_{i/o}$, G_s, $G_{q/11}$ and $G_{12/13}$(60).

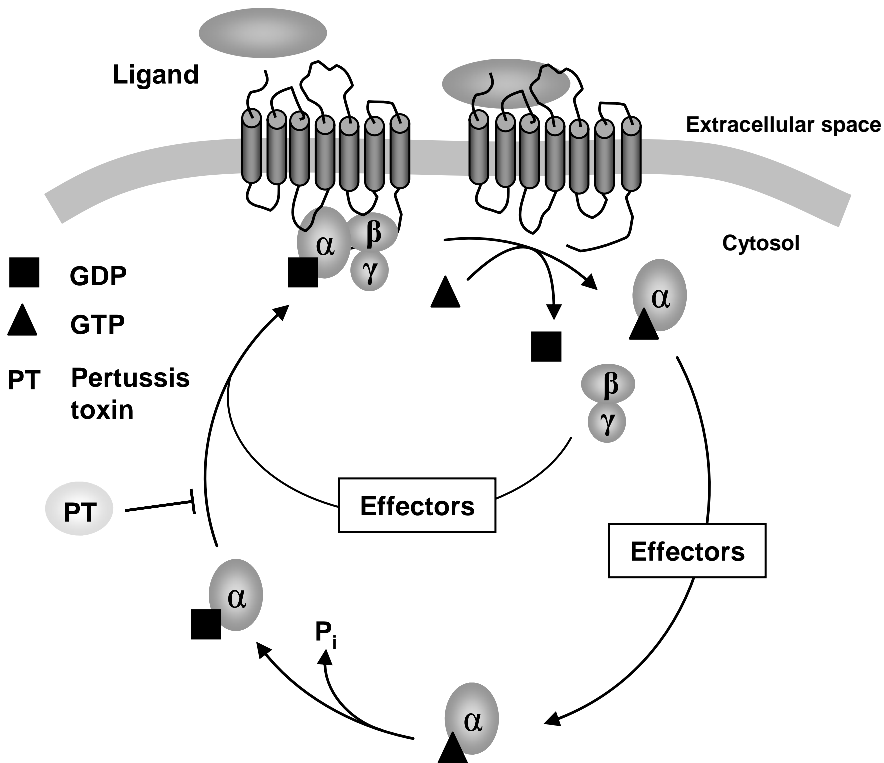

Figure 2. Activation of a GPCR with subsequent dissociation and recirculation of the G-protein. Pertussis toxin has an inhibiting effect through a covalent modification of G_{α} and thereby prevent association to the receptor.

Chemoattractant receptors

In many chemotaxing cells, the signal that regulates movement is initiated by GPCRs on the surface that bind to specific chemoattractants. The classic chemoattractant receptors on phagocytes are all members of the GPCR family. Chemical attractants that activate GPCRs exhibit a wide range of sizes and molecular properties, from small formylated peptides like the prototypic N-formyl-Met-Leu-Phe (fMLF) and larger proteins like complement factor 5a

(C5a) or chemokines. There are two classes of chemokines, CXC and CC, which are distinguished by the number of residues between their two N-terminal cysteins. The two types of chemokines bind to their respective receptor families, CXCR or CCR. Non-chemokine GPCRs involved in chemotaxis, like C5aR or formyl peptide receptor 1 (FPR1, discussed below), share some sequence similarities with each other and also with the chemokine receptors. Still, the homology is higher within the chemokine receptor family than compared with any other chemotactic receptors (61).
Phagocyte chemoattractants transduce their signals mainly through G_i-coupled GPCRs. Receptors coupled to G_i-proteins have often been characterized with respect to their sensitivity to pertussis toxin, produced by the bacterium *Bordetella pertussis*. Pertussis toxin catalyses an ADP-ribosylation of G_α, and this covalent modification prevents association between the G-protein and the receptor. The modification arrests G_α in the GDP-bound state and by doing so, the protein is unable to transduce signals (61, 62) (**Fig. 2**). All the chemoattractant receptors mentioned above are unable to induce chemotaxis or ROS production after treatment with pertussis toxin.

A pertussis toxin sensitive receptor without chemotactic activity?

There are exceptions to the rule that chemoattractant receptors always utilize G-proteins of the G_i type and chemotactic receptors that are able to bind G-proteins from the G_q subfamily (63, 64), have been described. In addition to chemoattractant receptors that use other G-proteins than G_i, there may also be receptors coupled to G_i-proteins that are not chemotactic.
We have studied, but not yet identified, a receptor that binds the peptide gG2p19. This peptide, gG2p19, is derived from the secreted portion of glycoprotein G2 (sgG2) of Herpes virus type 2 (HSV2). Projects initiated by Kristina Eriksson and co-workers, aimed at investigating possible functions and inflammatory properties of sgG2. For this purpose, over 70 different peptides were synthesized and screened for activity. The peptides were 15 amino acids (a.a.) in length with a 5 a.a. overlap and spanned the entire 321 a.a. sequence of the protein. Two of these peptides, gG2p19 and gG2p20, displayed an ability to activate the NADPH-oxidase of monocytes. Whereas gG2p20 was shown to induce phagocyte ROS-production through FPR1 (and also activated neutrophils) to inhibit NK-cell activity (43), a different activation pattern is seen for gG2p19 (**Paper IV**) that fails to activate neutrophils. This finding alone indicate differential receptor specificities and in addition, gG2p19 is completely unable to mediate monocyte chemotaxis. The cellular responses induced by gG2p19 are totally inhibited by pertussis toxin which defines G_i coupling of the responsible receptor. Despite being Gi coupled, the receptor is apparently

completely unable to transduce the signals needed for chemotaxis (**Paper IV**). In an attempt to identify this unique receptor, we have excluded all members of the FPR family (see below) and also tested a peptide corresponding only to the 5 overlapping a.a. (**Fig. 3**). Interestingly, in contrast to gG2p19 the pentapeptide selectively binds FPR1 (Karlsson, unpublished data). Thus, the 10 additional a.a. in gG2p19 switches the receptor specificity from FPR1 to another receptor. We also show that, apart from chemotaxis, the functional responses elicited in monocytes by gG2p19 are very similar to the ones induced by chemoattractants such as gG2p20 or fMLF. The kinetics of calcium responses as well as ROS production induced by gG2p19 and gG2p20 are almost indistinguishable (**Paper IV** and unpublished data).

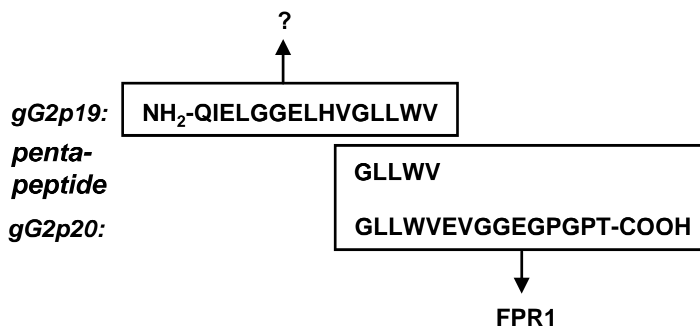

Figure 3. Peptides derived from the secreted portion of HSV2 glycoprotein G have different receptor preferences

It is very uncommon for a G_i-protein coupled receptor with activating properties to be completely devoid of chemotactic activity. The explanation for differential effects on chemotaxis between the five a.a. peptide and gG2p19 is still obscure, as is the identity of the gG2p19 receptor. It should be noticed, however, that it *may* be possible for the gG2p19 receptor to induce chemotaxis when activated by another ligand. Synthetically modified formylated peptides (binding to FPR1) have been shown to induce either a full response (chemotaxis, superoxide production and granule secretion), a pure chemotactic response, or activation of the oxidase and granule secretion only. The pattern of responses has been suggested to rely on ligand specific intracellular signals generated by the receptor, but the mechanistic details remain unclear (65).

The formyl peptide receptor family

N-formylated peptides from bacteria were the first chemotactic factors to be structurally defined. The corresponding receptor, the formyl peptide receptor 1 (FPR1), has become the most widely studied of the GPCRs. During the years

members of the formyl peptide receptor family have been shown to be of importance in many aspects of phagocyte activation, both in health and disease.
It was discovered in the middle of the 1970s that granulocytes could be activated by and chemotactically migrate towards formylated peptides released by bacteria (66). The receptor responsible was not identified until 1990, when it was cloned and sequenced (67). The formyl peptide receptor family has, to date, three human members, FPR1, FPR2 and FPR3. From the point of discovery, there has been some confusion regarding naming of the receptors. They were originally named formyl peptide receptor (FPR), formyl peptide receptor like-1 (FPRL1) and formyl peptide receptor like-2 (FPRL2), but the naming have recently been changed to FPR1, FPR2 and FPR3 respectively. Except for papers I and IV, which were published before 2008, the new nomenclature will be used throughout this thesis.
Shortly after the revealing of the FPR1 sequence the two related receptors, FPR2 and FPR3, were cloned using low stringency hybridisation with the FPR1 cDNA as a probe (68-70). Although the FPRs were initially found on leukocytes the list of cells expressing these receptors has grown over the years (71, 72) (**Table 1**).
Also FPR2 can interact with formylated peptides but the binding affinity to this receptor is much lower compared to that of FPR1 (69). The sequence similarity between FPR1 and FPR2 is high, 69% at the a.a. level. Although there is a large sequence similarity also between FPR2 and FPR3 (83% identity at the a.a. level) (73) the latter can not bind formylated peptides. The similarities between the receptors are even larger when only the intracellular signalling transducing domains are compared (73). Studies with chimeric receptors and receptor derived peptides have been done to characterize the ligand binding and G-protein binding domains of FPR1 and FPR2 (74-77). One general conclusion has been that multiple domains are required for ligand binding of both receptors. More specifically for FPR1, all three extracellular loops seemed to be of importance for the binding to fMLF. The crucial domains for FPR2 binding to the ligands MMK-1 and Aβ42 (described below), lay in the sixth transmembrane domain and the third extracellular loop. The interaction between FPR1 and the G-protein has been ascribed to the second intracellular loop and the membrane proximal part of the carboxy-terminal tail and not the third intracellular loop that is important in many other GPCRs.
The interaction between fMLF and FPR1 triggers a cascade of multiple second messengers through the activation of PLC, PLD and PLA_2. This signalling cascade culminates in cell chemotaxis (66), phagocytosis (78), production of proinflammatory mediators (79), production of ROS (80) and activation of transcription factors (81).

Table 1. Celltypes expressing FPRs and microbial ligands for different family members. References, unless given, are found in (71,72) from which the table is adapted.

Receptor	Cell expression	Microbial ligands (Origin)
FPR1 former FPR	Neutrophils Monocytes/Macrophages Immature dendritic cells Eosinophils (93) Platelets Endothelial cells Astrocytes Hepatocytes Microglial cells Fibroblasts	fMLF and analogues (bacteria) T20 (HIV) T21 (HIV) gG2p20 (HSV2)
FPR2 former FPRL1	Neutrophils Monocytes/Macrophages Immature dendritic cells Eosinophils (93) T and B lymphocytes Endothelial cells Epithelial cells Astrocytes Hepatocytes Microglial cells Fibroblasts	fMLF and analogues (bacteria) Hp2-20 (H. pylori) N36 (HIV) F peptide (HIV) T21 (HIV) V3 peptide (HIV)
FPR3 former FPRL2	Monocytes/Macrophages Immature/mature dendritic cells	Hp2-20 (H. pylori)

FPR1 is of utmost importance for host defence and this is evident by the observation that mice that are devoid of FPR expression are unable to respond to an infection by *Listeria monocytogenes* (82). The fact that the mouse ortholog of FPR2 cannot compensate for the loss is consistent with the lower affinity of FPR2 for formylated peptides in general and particularly *Listeria* peptides (83). Another specific example of the significance of FPR1 is a virulent peptide produced and secreted by *Staphylococcus aureus*. The chemotactic inhibitory peptide of *S. aureus* (CHIPS) is an inhibitor of FPR1 and C5aR and can thereby be speculated to act as a virulence factor for the bacterium (84). The FPRs have also been studied in human patophysiological contexts. Neutrophils from

patients with localized juvenile periodontitis have a decreased binding and responsiveness to fMLF, which is caused by one or two single nucleotide polymorphisms (SNPs) in the gene for FPR1 (85). The SNPs result in a.a. changes in the second intracellular loop of the receptor that has been shown to be important for G-protein binding. An evolving field is the characterisation of receptor function for FPRs expressed on cells involved in brain function and disease. FPR1 is expressed in highly malignant, human glioma cells, and is thought to be responsible for mediating motility, growth and angiogenesis of the glioblastoma (86, 87). Peptides derived from the protein amyloid β have been shown to stimulate the release of neurotoxic substances from monocytes via FPR2 (88). These infiltrating activated monocytes are an important part of the pathology of Alzheimer's disease.

FPR1, FPR2 and FPR3 agonists

FPR1

Although the formylated peptide fMLF, isolated from growing *E. coli* bacteria, was the first described ligand for FPR1, formylated peptides from other bacterial strains are effective at activating phagocytes via FPR1 (89, 90). It was shown that the formyl group is the key to a biologically active peptide, but certain peptides longer than three a.a. retain activity without the formylated N-terminus (66). Like bacteria, mitochondria start protein synthesis with a formylated methionyl group, thus there exists an endogenous source of N-formylated peptides that can bind to FPR1 and FPR2 (91). If these mitochondrial-derived peptides are found outside the cell, they could act as signals of tissue damage via FPR1.

Today, the list of ligands for FPR1 is immense. It contains many microbial agonists derived from both bacteria and viruses (71, 72) (**Table 1**), as well as endogenous substances (92) and allergens (93).

During recent years, many synthetic agonists, as well as some antagonists, have been described with specificities that are either fairly narrow or very broad. The hexapeptide WKYMVm, in which the last a.a. (at the C-terminus) is D-methionine, is a potent agonist for all three members of the FPR family. Although WKYMVm binds all three of the FPRs (94, 95), we have shown that the peptide only signals through FPR1 when FPR2 is blocked (**Paper I**). WKYMVm induces a strong ROS production in neutrophils. We could see that when cyclosporine H (Cys H, see below) was used to block FPR1 the magnitude of the response was not diminished. Blocking of FPR2 could inhibit the ROS production partially, and despite the earlier inefficiency of FPR1 inhibition the remaining response could now be silenced by CysH. The FPRs have been discussed as potential therapeutic targets to control unwanted inflammatory responses. The results discussed in paper I may have implications for signal

transduction studies, performed in the search for specific receptor antagonists and inhibitors. The potential of developed inhibitors could be neglected if the activating agonist is promiscuous with respect to receptor binding and activation, as seen in paper I. If the agonist can switch to another receptor during the use of a possible pharmaceutical substance, the effect of the inhibitor would be lost.

FPR2

FPR2 was initially described as an orphan receptor, with low affinity for fMLF (69). At present FPR2 has been proven to be a very promiscuous receptor that can bind ligands of great diversity in both origin and structure. LXA_4 was the first high affinity ligand reported for FPR2 (96). The most pronounced effect of LXA_4 was that it *inhibited* neutrophil function through FPR2, a finding that has been debated in later years (97).

A number of synthetic peptides are agonists for FPR2, and these include WKYMVM, WKYMVm and MMK-1. Whereas WKYMVM has affinity mainly for FPR2 and to a lesser degree for FPR3, the replacement of the C-terminal L-Met for the D-variant of the a.a., broadens the specificity to include all three receptors in the family. MMK-1 is a 13 a.a. long peptide, able to induce monocyte/neutrophil chemotaxis and a rise in intracellular calcium (98) (see below and **Paper II**).

From the pathogenic point of view, amyloidogenic molecules have been studied as interesting ligands for FPR2. This group comprises the acute phase protein serum amyloid A (SAA) (99), β-amyloid peptide 42 (Aβ42) (100) and the prion protein-derived peptide PrP106-126 (101). The definition of an amyloidogenic molecule is any polypeptide which polymerizes to form deposits with cross-beta structure, *in vivo* or *in vitro*. SAA can form amyloid plaques in tissues of patients with rheumatoid arthritis. There have been opposing views regarding FPR2 as the sole receptor for SAA. This is illustrated in a recent publication where we show that SAA can bind and signal through FPR2 when the receptor is over expressed in a transfected cell line, but clearly utilize another (yet unknown) receptor for activation of primary human neutrophils (102).

Another pathogenically important amyloidogenic protein is amyloid beta, which is present in high concentrations in brain tissue of Alzheimer's patients. Large amounts of Aβ42, a peptide cleaved from amyloid beta, are found in senile plaques from these patients. The fact that Aβ42 is chemotactic and activating for monocytes and microglia cells through FPR2, suggests that this receptor is at least partly responsible for proinflammatory destructive activity in brain tissue during Alzheimer's disease.

FPR3

WKYMVM and WKYMVm were the first compounds suggested as agonists for FPR3 (95). These peptides have been shown to activate FPR3 expressed both in HL-60 and RINm5F cells (95) (**Paper IV**). To date, the only FPR3 specific ligand presented is F2L, a peptide derived from the aminoterminal end of an intracellular heme-binding protein (103). F2L has been claimed to induce chemotaxis as well as a calcium response in some monocytes and dendritic cells (103). In higher concentrations F2L will activate also FPR2.

FPR1, FPR2 and FPR3 antagonists

To be able to perfom functional characterisation coupled to an identification of the receptor(s) involved, there is a requirement for well defined receptor antagonists and inhibitors. Already 25 years ago it was shown that replacing the formyl group in fMLF by a t-butyloxycarbonyl (tBOC) group transfers the agonist to an antagonist. BOC-MLF has been commonly used and several other BOC-modified peptides such as BOC-FLFLF have also turned out to be FPR1 selective antagonists. We have preferred to use the cyclic undecapeptide Cys H (**Paper I, II, IV**), since it has a slightly higher specificity for FPR1 compared to the BOC compounds (104, 105). For FPR2 the first specific antagonist, WRWWWW (WRW_4) (106), was described in 2004 and later this peptide has been defined as a blocker also for FPR3 (107). One should remember that the specificity/selectivity analyses of these antagonists have been limited to the members of the FPR family, but it may well be that the antagonists influence also other not yet characterized receptors (**Paper IV**). As an additional inhibitor in receptor studies we have utilized the phosphoinositide binding peptide PBP10. This is a 10 a.a. long peptide derived from the PIP_2 binding domain of gelsolin and the peptide has been coupled to Rhodamine B. The presence of the fluorophore makes the peptide cell permeable and this is needed for the inhibitory activity of PBP10. We have shown that PBP10 has receptor selectivity in that it inhibits signalling through FPR2 but not through FPR1 (108). Although the underlying mechanism for PBP10 function and selectivity is not clear, our preliminary data suggests that it interferes with the early events of signalling. An involvement in the interaction between receptor and G-protein could be possible. The C-termini of the receptors are important for signalling, and in this region FPR1 and FPR2 differ in some a.a.. In order to determine the importance of the receptor C-terminus for PBP10 sensitivity, we have investigated the effect of PBP10 on cells expressing a chimeric receptor. A 56 a.a. long sequence in the C-terminal of FPR2 was exchanged for the corresponding a.a. from FPR1. Signalling in cells expressing the chimeric receptor was also inhibited by PBP10 with an IC_{50} value similar to that obtained with the wild type FPR2 (Karlsson, unpublished data in collaboration with Francois Boulay and Marie-Josèphe

Rabiet, **Fig. 4**). These results indicate that PBP10 selectivity is not conclusively determined by the C-terminal, but does not completely rule out interference in coupling to the G-protein. Other possibilities have to be investigated to fully map the mechanisms involved.

In addition to its effect on FPR2, PBP10 has also been shown to partially inhibit signalling triggered by some other ligands with known or unknown receptor preferences (102). We show for instance that activation of the monocyte NADPH-oxidase by gG2p19, mediated via an unknown receptor distinct from FPR2, is blocked by PBP10 (**Paper IV**). We have also seen that PBP10 partly inhibits ROS production in eosinophil granulocytes triggered by RANTES (CCL5) (Stenfeldt, unpublished data in collaboration with Christine Wennerås), a chemokine mediating its activity on eosinophils mainly through the well characterized receptor CCR3 and possibly to a lesser extent through CCR1. PBP10 thus shows a high selectivity *within* the FPR family of receptors, but its promiscuity among other, unrelated receptors could imply that inhibition occurs in a pathway that is more general than previously thought.

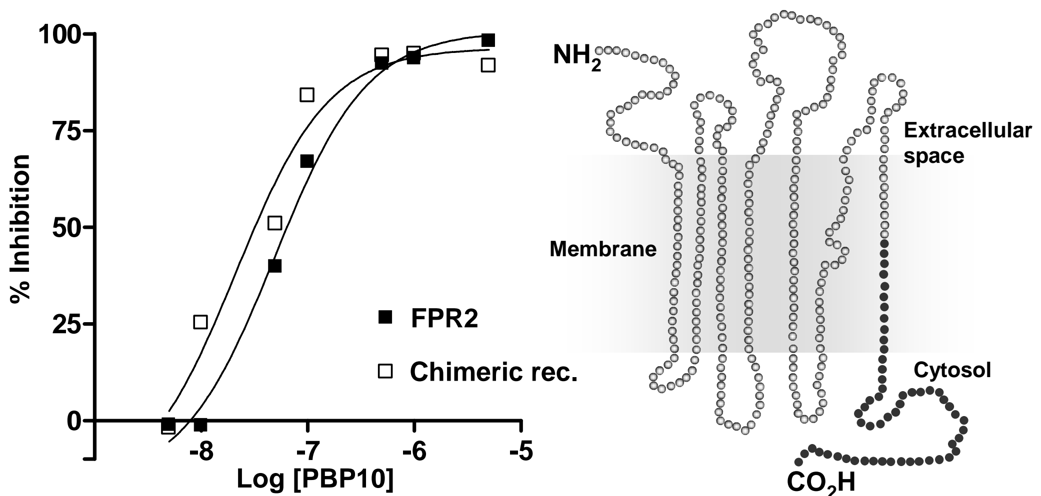

Figure 4. (A) Dose-response plot for the effect of PBP 10 on receptor response in HL-60 cells expressing FPR2 (■) or a chimeric receptor (□). The agonist used was WKYMVM [10^{-7}].
(B) Schematic picture of the chimeric receptor. The 56 most C-terminal a.a. in FPR2 has been replaced by the corresponding in FPR1.

Ca^{2+} signalling

The calcium ion is nature's favourite among signalling ions. It controls cellular processes from the beginning of life until the end, such as fertilization, mitosis, differentiation, transcription, exocytosis, contraction, nerv impulses and cell death. Calcium signalling is also a part in the events after GPCR activation in

phagocytes. Ligand binding to a GPCR activates the heterotrimeric G-protein that dissociates into G_{α}- and $G_{\beta\gamma}$-subunits. These subunits can then activate multiple downstream signal transducers e.g. phospholipases and protein kinases. The $G_{\beta\gamma}$ subunit activates phospholipase Cβ2 (PLCβ2) which cleaves membrane phosphatidylinositol 4, 5 bisphosphate (PIP_2) and generates inositol 1,4,5-trisphosphate (IP_3). The soluble IP_3 binds the IP_3R localized in calcium storage organelle and this binding triggers a release of Ca^{2+} from the stores (possibly linked to the endoplasmic reticulum). In addition to IP_3 there is also another second messenger that can induce a release of calcium from intracellular stores upon GPCR activation, cyclic ADP ribose (cADPR). The nicotinamide adenine dinucleotide (NAD^+) metabolite cADPR is like IP_3 believed to act on specific intracellular receptors to open channels in the ER membrane (109). It is generally thought that cADPR binds and opens ryanodin receptor regulated channels in mammalian neutrophils and monocytes/macrophages (110, 111).
The initial rise in intracellular Ca^{2+} after IP_3 formation is followed by a calcium entry through store operated calcium channels (SOCs) in the plasma membrane (**Fig. 5**). The complete mechanism for ion entry through SOCs has not yet been unfolded but different models have been put forward. The task of signalling from stores to SOCs has recently been ascribed to the protein STIM1 that was identified as a Ca^{2+} level sensor in the ER membrane (112-114). The protein Orai1 has been identified as a regulator of entry through SOCs, but it remains to be further clarified if Orai1 is a subunit of the channel or a membrane docking protein for the sensor STIM1 (115).
The release of Ca^{2+} from intracellular stores gives rise to rapid peak of free calcium in the cytoplasm, and the initial phase is followed by a longer slowly declining response that is the result of an opening of SOCs. The initial rise in the free Ca^{2+} levels in the cell (μM levels) is transient and returns very rapidly to nM levels through a removal by the sarco endoplasmic reticulum Ca^{2+}-ATPase (SERCA). The pump forces Ca^{2+} back into the storage organelle (116). This process is vital to the cell, since persisting high levels of calcium is a potent signal that gives rise to permanent activation of proteases and Ca^{2+} dependent cytoskeletal modulating proteins. These events, together with deposition of hydroxyapatite due to high Ca^{2+} concentration in the mitochondria, cause impaired mitochondrial function, perturbation of cytoskeletal organization and induction of apoptosis or necrosis (117-119).
The transient rise in intracellular calcium has been regarded as a crucial factor for the responses induced by neutrophil chemoattractants. It should be noticed, however, that phagocytosis and chemotaxis can proceed also in calcium depleted cells despite the fact that calcium is supposedly needed for proper function of cytoskeleton remodelling proteins (120). In the case of NADPH-oxidase

activation, calcium elevation is not sufficient for activity; ROS production can also be induced without a simultaneous Ca^{2+} signal (121, 122).

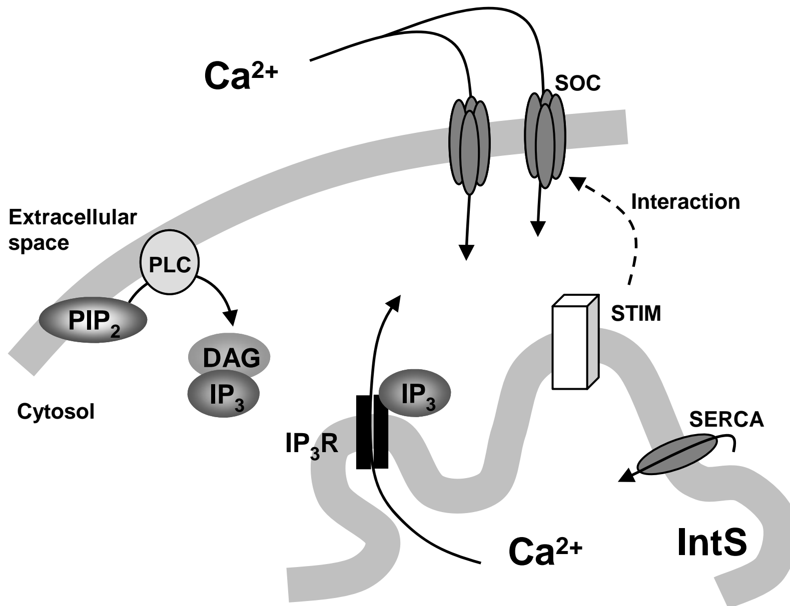

Figure 5. Schematic picture of store operated calcium entry. Phospholipase C cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to the IP_3 receptor and mediates release of Ca^{2+} from intracellular stores (IntS). STIM senses empty stores and mediates a signal that is followed by opening of store operated Ca^{2+} channels (SOCs) in the plasma membrane.

The process of Ca^{2+} signalling following FPR1 activation has been extensively characterized (62, 110, 123), in contrast little is known about FPR2. It is reasonable to believe that the two receptors use very similar signalling pathways. This reasoning is based on the facts that they induce comparable responses in the cell and are structurally very similar. We have shown, using the agonist MMK-1 and two different methods for detection of intracellular calcium, that signalling through both receptors involves a release from intracellular stores as well as subsequent opening of SOCs (**Paper II**). The removal of extracellular calcium by EGTA resulted in elimination of the later phase in the transient and was equally pronounced for both receptors. Interestingly, it has been shown that intracellular calcium rise after FPR2 stimulation is dependent on cADPR signalling while FPR1 induced is not (124). Somewhat surprisingly it has also been suggested that signalling through FPR2 totally rely on an entry of extracellular calcium without any emptying of intracellular stores (124). Our

study did thus not confirm this, but showed a pattern of calcium signalling similar for both FPR1 and FPR2.

Termination of signalling and desensitization

The ability of phagocytic cells to become non-responsive (desensitized) to chemoattractants is a crucial process. In order to be able to migrate in a gradient of a chemoattractant, myeloid cells gradually become desensitized to lower or unchanged concentrations of the guiding substance. Desensitization and termination of the receptor mediated signal is also of importance for the limitation and resolution of inflammation.

Receptor activation is a transient process. Binding of an agonist results in G-protein coupling/activation and generation of signals, but the occupied receptor is then fairly rapidly transferred to a refractory state that lacks signalling capacity. Since the cells may be fully responsive to an unrelated agonist, the phenomenon is referred to as homologous desensitization. The molecular mechanism behind desensitization involves several different parts and the time frame during which it happens range from seconds to minutes.

The phenomenon of desensitization is not only essential for the regulation of the inflammatory response; it has also been an important tool in many *in vitro* experiments to determine for example receptor specificity. Neutrophil ROS production has been used regularly by us, as a read out system for *in vitro* studies of desensitization (102) (**Paper IV**). When monitoring ROS production, homologous desensitization by repeated fMLF stimulations is very obvious (**Paper III**). We were thus surprised by the complete lack of homologous desensitization when we instead used intracellular calcium levels as a read-out system (**Paper III**). The explanation to the apparent paradox was that the ROS, produced during activation of neutrophils, inactivated the agonist quickly and the receptors were left free to be re-stimulated. This lack of desensitization was only apparent when methionine containing stimuli were used, which is in accordance with the fact that MPO derived ROS can inactivate peptides containing methionine (125). It is possible to design systems that monitor calcium levels, provided that such experimental set ups take into account that the triggering agonist may be inactivated by ROS produced by the activated cells. (**Paper III**). Desensitization most probably occurs *in vivo*, it is therefore important to be aware that potential problems with detection of desensitization could be strictly an *in vitro* phenomenon that is overcome by using the methods described in paper III.

The mechanism behind the desensitization phenomenon differs within the family of GPCRs, but with respect to the FPRs there is no reason to believe that there should be any difference with respect to the mechanism (126, 127). When activated, FPR1 becomes phosphorylated primarily by G-protein coupled

receptor kinase 2 (GRK2) (127, 128). There are 8 potential phosphorylation sites in the C-terminus of FPR1 where the phosphorylation of importance for desensitization occurs. FPR2 is also phosphorylated but the kinase(s) responsible for this phosphorylation is not known. In contrast to the other receptors in the FPR family, FPR3 is highly phosphorylated also without any ligand binding (127). Phosphorylation of FPR1 increases the affinity of the receptor for β-arrestins. Arrestins preferentially bind GRK phosphorylated FPR1 but can also bind non-phosphorylated receptors. The receptor-arrestin complex is then finally internalized. It has been shown that phosphorylation of the receptor is necessary for the internalisation but not for chemotaxis (129).
Another process important for termination of the signal generated by FPRs is the binding of the receptor to the cytoskeleton. Cytochalasin B (Cyt B) is a fungal metabolite that binds to the plus-end of the actin filaments, and this binding prevents actin polymerization and severing of existing filaments. When cells treated with Cyt B are activated through FPR1 or FPR2 the ROS production is increased and prolonged (**Paper II**), whereas ROS production induced by a stimulus that bypasses membrane receptors (i.e. the PKC activator phorbol myristat acetate) is unaffected by Cyt B treatment (130). Cytochalasins can also reactivate FPRs that has been desensitized but not internalized, suggesting that the actin cytoskeleton has a role in the termination of cellular responses triggered by the FPRs.
The desensitized state can be induced also in neighbouring non-occupied receptors with specificity for other agonists. This is a phenomenon that has an inbuilt receptor hierarchy and is referred to as heterologous desensitization. Binding of the formylated peptide fMLF to its receptor, FPR1, leads to desensitization not only of this receptor, but also of CXCR1. Binding of IL-8 to CXCR1 desensitizes this particular receptor, but not FPR1 which illustrates the hierarchy (130). This type of desensitization is thought to be the consequence of phosphorylation of the non-occupied receptor by PKC and PKA (131).

Model cell systems to study FPRs

As described above a myriad of effector functions are elicited upon activation of phagocytes. Activation of primary cells via FPRs is a useful tool in studies of functionality of these cells. In the case of specific receptor studies however, the use of cell lines could facilitate both performance of experiment and interpretation of results. By using cultured cells transfected with the receptor(s) in focus, binding assays and antagonist effects are easier to attribute to one specific receptor. Other obvious advantages with transformed cell lines compared to primary cells are simplicity, less variability and supply.
One approach commonly used by us and others, is to transfect a cell line with one or more members of the FPR family and then use the rise in intracellular

calcium as a read-out system for activation. Several different cell lines have been used for this purpose, non-hematopoetic cell strains like RINm5F (95), HEK 293 (74, 98) and CHO cells (96) but also the undifferentiated promyelocytic HL-60 cell line (95, 102, 132, 133) (**Paper I, II, IV**). This approach is often very suitable for determination of receptor specificity.
Another way to study receptors in a setting that more resembles primary cells, regarding for example signalling machinery, is to use differentiated cells from the myelocytic cell lines HL-60 or PLB 985 (134, 135)(**Paper III**). These cells are arrested in the promyelocytic stage, but can be differentiated by addition of DMSO, ATRA or Bt_2cAMP towards the granulocytic lineage. Chemically differentiated cells differ from primary cells with respect to granule content (136). Azurophil granules and granule proteins is present in the undifferentiated promyelocytic cell but during differentiation the cell goes through maturation without the development of other granules and vesicles. The differentiated HL-60 cell also differs from granulocytes in receptor expression e.g., CXCR1 (137) but have preserved the ability to phagocytose (138, 139) and to assemble a functional NADPH-oxidase (140). Although the advantages by using cell lines are obvious as described above, one should bear in mind that differences exist in features connected with chemoattractant receptors and signalling (62, 141). Differences in ROS production, possibly due to divergence between MPO content in primary and cultured cells could also give results *in vitro* that are misleading (**Paper III**).

Concluding remarks

Phagocytic cells are an essential part of our innate immune system and play an important role in the battle against pathogens. GPCRs and more specifically chemoattractant receptors are a vital part in an effective activation of these cells. Since formylated peptides from bacteria were the first chemoattractants to be defined, FPR1 has been the most intensively dissected of the chemoattractant receptors. Together with the two other members of the FPR family it clearly has a task in the regulation of immune functions. Complex functional properties are displayed by these receptors and this is in part due to their promiscuity among ligands. Many of the ligands defined for the FPRs do not share significant sequence homology, thus these receptors behave as PRRs that can be activated by a wide variety of unrelated ligands. Formyl peptide receptors are involved in host response to microbes and cell necrosis, but also in pathological contexts such as amyloidogenic disease, viral infections as well as cancer. It is therefore hard to tell if the nature of these receptors is "good or bad", but it does put the family in the spotlight as pharmacological targets.

The broad spectra of synthetic peptides that are recognized by the FPRs are useful tools in pharmacological studies and characterization of signalling elicited by the receptors. This will hopefully help us to better understand the role of these receptors in health and disease.

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Populärvetenskaplig sammanfattning

Vi befinner oss i en omgivning där vi konstant är utsatta för mikroorganismer (bakterier och virus) vilka skulle kunna orsaka skada. Trots detta faktum är det inte särskilt ofta som vi faktiskt blir allvarligt sjuka. Anledningen till det är att människan har utvecklat ett effektivt immunförsvar. Vår första försvarsbarriär mot mikroorganismers intrång utgörs av ett antal rent fysiska hinder. Denna barriär består av hud och slemhinnor tillsammans med t.ex. saliv och sekret i mun och övre luftvägar samt magsyra som utgör den ogästvänliga miljön i magsäcken. Om de fysiska barriärerna förblir intakta skyddas kroppen bra mot infektion, men vid ett brott i barriären finns det risk för invasion av bakterier. När barriärbrottet sker aktiveras de delar av immunförsvaret som finns inne i kroppen. Det utgörs av celler (t.ex. vita blodkroppar) men också olika lösliga faktorer, vilka startar den process som kallas akut inflammation. En aktivering av immunförsvaret sker även vid en skada utan efterföljande infektion av mikroorganismer som t.ex. vid en stukning.
Immunförsvaret kan delas in i två delar, det medfödda och det förvärvade. Cellerna i det förvärvade immunförsvaret kan ”utbildas” till att känna igen vissa potentiellt skadliga mikroorganismer, för att väldigt specifikt kunna avvärja ett hot. Detta kan dock ta upp till flera dagar, och processen kan inte på börjas förrän efter det att invasionen av mikrober har startat. Det medfödda immunförsvaret är sett till evolutionen en gammal mekanism som inte bara finns i däggdjur utan också i andra organismer. Cellerna som tillhör det medfödda immunförsvaret har till uppgift att agera snabbt vid en skada eller infektion, och de behöver ingen ”utbildning” för att fungera. De kan inte registrera exakt vad de stöter på utan känner istället igen återkommande molekylära mönster som indikerar fara, på/från bakterier men också från våra egna skadade celler generellt.
De så kallade "professionella fagocyterna" (granulocyter / monocyter / makrofager) är viktiga celler i vårt medfödda immunförsvar och har till uppgift att eliminera invaderande bakterier. Granulocyterna utvecklas från celler i benmärgen och tidigt under sin mognadsprocess producerar dessa celler en mängd olika proteiner som förpackas i speciella granule. När cellerna är mogna skickas de ut för att cirkulera med blodet. Vid en skada producerar närliggande celler substanser för att locka till sig granulocyter och monocyter som vandrar från blodet till centrum för inflammationen, en process som kallas för kemotaxi. Invaderande mikroorganismer utlöser vid interaktion med receptorer på cellens plasmamembran en mängd olika signaler i cellen. Dessa processer syftar bl.a. till att inducera fagocytos (cellätande) och innesluta den främmande partikeln. Därefter ska cellen snabbt kunna döda och/eller bryta ned angriparen med hjälp av giftiga ämnen som har lagrats i granule men också nyproducerats. Om det

medfödda immunförsvarets ansträngningar inte är tillräckliga för att lösa situationen kommer en aktivering av det förvärvade immunförsvaret att ske. Som tidigare nämts tar det dock en tid innan det förvärvade immunförsvaret är redo för strid.
De bakteriedödande ämnen som frisätts från fagocyter har tyvärr också negativa effekter på de egna cellerna, vilket kan resultera i vävnadsskada. I ett optimalt scenario elimineras bakterierna av fagocyter som därefter går i programmerad celldöd, de städas sedan bort av andra fagocyter (makrofager) som också gynnar läkningsprocessen. Om det förekommer störningar i utläkningen av den akuta inflammationen med en kvarstående aktivering av celler, kan detta leda till en kronisk inflammation eller autoimmunitet.

Fagocyter använder speciella receptorer på sin yta för att hitta fram till sitt byte. Dessa receptorer känner igen substanser (kemoattraktanter) som utsöndras av bakterier eller skadad vävnad och man kan säga att fagocyterna ”luktar” sig fram till infektions-/inflammationsstället. Inbindningen av kemoattraktanter till receptorer på cellytan kan också starta andra aktiviteter utöver kemotaxi t.ex. produktion av toxiska s.k. fria syreradikaler.
I mitt avhandlingsarbete har jag studerat receptorer vars signaler kan aktivera professionella fagocyter och mer specifikt en familj av kemoattraktantreceptorer som heter formyl peptid receptorer. Den här receptorfamiljen har tre medlemmar FPR1, FPR2 och FPR3 som har besläktade strukturer men inte alltid aktiveras av liknande substanser. Jag har undersökt vad de här receptorerna aktiveras av, vilken reaktion det utlöser i cellen och hur avstängningsmekanismen fungerar.

I arbete **I** undersöks en sex aminosyror lång peptid som har visat sig kunna binda till både FPR1 och FPR2. Syftet var att studera om peptiden aktiverade fagocyter genom en eller eventuellt båda receptorerna. Vi visar att peptiden kan aktivera båda receptorerna men signalerar enbart genom FPR2 så länge den finns tillgänglig. Om FPR2 blockeras växlar receptorpreferensen och signalen går via FPR1 istället. Formyl peptid receptorerna har visat sig vara viktiga i vissa sjukdomstillstånd, och det har därför funnits ett visst experimentellt intresse att utveckla hämmare för dessa receptorer. Det är i sådana studier då viktigt att ta hänsyn till om det svar man vill blockera induceras av ett stimuli som kan växla receptorpreferens som i vårt arbete.

I arbete **II** karaktäriseras syreradikalproduktion inducerad av en 13 aminosyror lång peptid, MMK-1, via FPR2. Den intracellulära kalciumfrisättningen som induceras av MMK-1 jämförs också med den som ges av en bakteriell peptid via FPR1. De två receptorerna FPR1 och FPR2 är strukturellt lika och ger upphov till liknande effekter i cellen, dock har det visat sig att de skiljer sig åt med

avseende på vissa signaler. I det här arbetet visar vi att signalering via de två receptorerna är beroende av frisättning av lagrat kalcium i cellen men också av insläpp av kalcium från omgivningen genom kanaler i plasmamembranet.

I arbete **III** beskrivs ett metodologiskt problem angående mätningar av avstängningsmekanismen hos FPR1. Vi ger förslag till förbättringar av metoden för att undvika problem. En lösning är att utföra experimentet i en miljö som mer liknar kroppens egen genom att tillsätta serum eller serumproteiner.

I det avslutande arbetet **IV** beskrivs en ny peptid som kommer från Herpes simplex virus typ 2 (HSV2). Peptiden är en del av glykoprotein G2 och den kallas gG2p19. Vi visar att monocyter aktiveras av gG2p19 via en receptor som inte tillhör FPR familjen men delar vissa egenskaper med denna receptorgrupp. Den ännu okända receptorn inducerar produktion av syreradikaler men kan inte inducera kemotaxis.

Paper I

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The peptide Trp-Lys-Tyr-Met-Val-D-Met activates neutrophils through the formyl peptide receptor only when signaling through the formylpeptde receptor like 1 is blocked A receptor switch with implications for signal transduction studies with inhibitors and receptor antagonists

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ABSTRACT

Neutrophils express the G protein-coupled N-formyl peptide receptor (FPR) and its homologue FPRL1. The hexapeptide Trp-Lys-Tyr-Met-Val-D-Met-NH_2 (WKYMVm) activates HL-60 cells transfected either with FPRL1 or with FPR. The signaling through the stably expressed receptors was inhibited by specific receptor antagonists, cyclosporine H and WRWWWW (WRW_4) for FPR and FPRL1, respectively. The neutrophil release of superoxide was used to determine receptor preference, when these cells were triggered with WKYMVm. The response was not affected by the FPR specific antagonist suggesting that no signals are transduced through this receptor. The response was only partly inhibited by WRW_4, but this antagonist induced a receptor switch, perceptible as a change in sensitivity to the FPR antagonist. The activity remaining in the presence of WRW_4 was inhibited by cyclosporine H. A cell permeable peptide (PBP10) corresponding to the phosphatidyl-inositol-bisphosphate binding region of gelsolin, inhibited the FPRL1-, but not the FPR-induced cellular response and induced the same type of receptor switch. We show that an agonist that has the potential to bind and activate neutrophils through FPRL1 as well as through FPR, uses the latter receptor and its signaling route, only when the activating signal generated through FPRL1 is blocked. The receptor switch is achieved when signaling through FPRL1 is inhibited both by a receptor antagonist, and by an inhibitor operating from the inside of the plasma membrane. The phenomenon described is of general importance for proper interpretation of results generated through the use of different ''silencing technologies'' in receptor operated signaling transduction research.

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1. Introduction

The extravasation of leukocytes from the peripheral blood stream to inflammatory sites is a key feature in the innate immune response to infection [1,2]. Different chemoattractants (e.g. *N*-formylated peptides, C5a, IL-8, LTB_4 and PAF) and chemokines induce leukocyte infiltration and activation through binding to G protein-coupled seven-transmembrane cell surface receptors (GPCRs) [3,4]. The general scheme for chemoattractant-signaling involves a dissociation of $G_{\alpha i2}$ from the $G_{\beta\gamma}$ subunit complex results in the activation of several downstream signaling effector enzymes that promote intracellular calcium mobilization, modifications in the metabolism of phosphoinositides, and activation of mitogen-activated protein kinases [5]. The integration of the different chemoattractant-activated signaling pathways results in directed cell migration, recruitment of new receptors from the granules to the cell surface, release of proteolytic enzymes, production of large amounts of superoxide by the neutrophil NADPH-oxidase, and increased gene transcription [6–9]. The extent of the cellular response is dependent on the identity of the agonist and on the level of expression and desensitization of the receptors involved in the activation process [6–9].

Neutrophils express two very similar members of the seven-transmembrane GPCR super family, the formyl peptide receptor (FPR) and the formyl peptide receptor like 1 (FPRL1) (for a review see [10]). FPRL1 was originally cloned from human phagocytes by low-stringency hybridization of a cDNA library with the formyl peptide receptor (FPR) sequence and initially defined as an orphan receptor [1,11]. The fact that the cytoplasmic regions of FPRL1 share around 80% identity with FPR suggests that signaling from the two receptors should be very similar. During the past few years several different peptides/proteins have been reported to function as agonists for FPRL1 and in accordance with the great similarities of this receptor with FPR, most of these agonists trigger the same neutrophil functions as the prototype FPR agonist fMLF [12]. The neutrophil activating FPRL1 agonists include HIV derived peptides, antimicrobial peptides, the acute phase protein serum amyloid A (SAA), the neurotoxic prion peptide fragment 106–126, mitochondria derived peptides, and the synthetic peptide Trp-Lys-Tyr-Met-Val-Met-NH_2 (WKYMVM) [13–16]. Even though no defined structure has been identified to be the determinant for FPRL1 binding and activation, the close relationship between structural variation and function is illustrated by the fact that an exchange of the carboxyterminal L-methionine in WKYMVM for the D-isomer (indicated by lower case m as opposed to the capital M), broadens the binding characteristics of the peptide to include also FPR as a high affinity receptor [13,17].

The WKYMVm peptide is a very potent activator of several leukocyte effector functions [17,18] and since the peptide has the ability to bind and activate both FPR and FPRL1, the question arises whether the peptide uses one or the other or both of these two receptors during activation of neutrophils. Recently described peptides that selectively interfere with FPRL1-triggered responses [19,20] were used to determine the receptor involved in WKYMVm induced activation of the neutrophil superoxide anion generating oxidase. We could show that the FPR specific antagonist cyclosporine H inhibited the triggering of the oxidase only when signaling through FPRL1 was blocked.

2. Experimental procedures

2.1. Isolation of human neutrophils

Blood neutrophils were isolated from buffy coats from healthy blood donors, using dextran sedimentation and Ficoll–Paque gradient centrifugation [21]. All cells were washed and resuspended (1×10^7/ml) in Krebs–Ringer phosphate buffer containing 10 mM glucose, 1 mM Ca^{2+}, and 1.5 mM Mg^{2+} (KRG, pH 7.3).

2.2. Peptides and peptide receptor antagonists

The hexapeptides Trp-Lys-Tyr-Met-Val-Met-NH_2 (WKYMVM/m) were synthesized and HPLC purified by Alta Bioscience (University of Birmingham, UK). The formylated peptide *N*-formylmethionyl-leucyl-phenylalanine (fMLF) was from Sigma Chemical Co. (St. Louis, MO). The receptor antagonist Arg-Trp-Trp-Trp-Trp-$CONH_2$ (WRW_4) was from GenScript Corp. (Piscataway, NJ) and cyclosporin H was kindly provided by Novartis Pharma (Basel, Switzerland). The peptides/receptor antagonists were dissolved in dimethyl sulfoxide to 10^{-2} M and stored at -70 °C until use. Further dilutions were made in KRG.

The peptide QRLFQVKGRR (gelsolin residues 160–169), prepared by solid phase peptide synthesis and coupled to rhodamine as described [22], was a generous gift from Dr. Paul Janmey.

2.3. Stable expression of FPR and FPRL1 in undifferentiated HL-60 cells

The stable expression of FPR and FPRL1 in undifferentiated cells has been previously described [17]. Transfection of HL-60 cells was performed by electroporation with a Bio-Rad Gene Pulser apparatus, according to a slightly modified version [23] of the technique described by Tonetti et al. [24]. Following electroporation, cells were allowed to recover in 20 ml of culture medium for 48 h prior to selection in a medium containing G418 (1 mg/ml) (Gibco, Invitrogen). Cells were cultured in RPMI 1640 (PAA Laboratories GmbH, Austria) containing FCS (10%) (PAA Laboratories GmbH, Austria), PEST (1%) (PAA Laboratories GmbH, Austria) and G418 (1 mg/ml). The maximal density was maintained below 2×10^6 cells/ml. The cells were passaged to a concentration of 5×10^5 cells/ml approximately 24 h prior to use in assays.

2.4. Neutrophil NADPH-oxidase activity

The NADPH-oxidase activity was determined using an isoluminol-enhanced chemiluminescence (CL) system [25]. The CL activity was measured in a six-channel Biolumat LB 9505 (Berthold Co. Wildbad, Germany), using disposable 4-ml polypropylene tubes with a 1000 μl reaction mixture containing 5×10^5 neutrophils/ml, horseradish peroxidase (HRP; 4U) and isoluminol (2×10^{-5} M). The tubes were equilibrated in the Biolumat for 5 min at 37 °C, after which the stimulus (0.1 ml)

was added. By a direct comparison of the superoxide dismutase (SOD) inhibitable reduction of cytochrome C and SOD inhibitable CL, 7.2×10^7 cpm were found to correspond to a production of 1 nmol of superoxide (a millimolar extinction coefficient for cytochrome C of 21.1 was used). Details about the CL technique is given in [25].

2.5. *Determination of changes in cytosolic calcium in HL-60 cells expressing FPR or FPRL1*

HL-60 cells at the density of $1–3 \times 10^6$ cells/ml were washed with KRG without Ca^{2+}. The cell pellets were resuspended at a density of 2×10^7 cells/ml in KRG without Ca^{2+} containing 0.1% BSA and loaded with 2 μM Fura 2-AM (Molecular Probes, Eugene, OR) for 30 min, at RT. Cells were then diluted to twice the original volume with RPMI 1640 culture medium without phenol red (PAA Laboratories GmbH, Austria) and centrifuged. Finally the cells were washed once in KRG (with 1.0 mM Ca^{2+} from here on), and resuspended in KRG at a density of 2×10^7 cells/ml. Calcium measurements were carried out with a Perkin-Elmer fluorescence spectrophotometer (LC50) with an excitation wavelength of 340 nm, an emission wavelength of 505 nm and slit widths of 5 and 10 nm, respectively. Intracellular free calcium concentrations were calculated using the following formula: $(Ca^{2+})_i = K_D(F - F_{min})/(F_{max} - F)$ with a K_D for Fura-2 of 224 nM; F_{max} is the fluorescence in the presence of 0.04% Triton X-100 and F_{min} the fluorescence obtained after addition of 5 mM EGTA plus 30 mM Tris–HCl, pH 7.4.

3. Results

3.1. *Peptide-induced mobilization of intracellular Ca^{2+} in transfected HL-60 cells expressing the N-formyl peptide receptor (FPR) or its homologue FPRL1*

In addition to the high-affinity *N*-formyl peptide receptor (FPR), human neutrophils express a structurally related receptor originally known as FPRL1. These receptors are specifically activated by the chemoattractants fMLF (activates primarily FPR) and WKYMVM (activates FPRL1 [13]). When triggering a response in undifferentiated HL-60 cells stably expressing one or the other of the receptors, the FPR specific agonist cyclosporine H inhibits the fMLF induced mobilization of intracellular Ca^{2+} in FPR expressing cells (Fig. 1A) but not the WKYMVM induced response in FPRL1 expressing cells (Fig. 1B). Likewise, the FPRL1 specific antagonist WKW_4 inhibits the WKYMVM induced mobilization of intracellular Ca^{2+} in FPRL1 expressing cells (Fig. 1B) but not the fMLF induced response in FPR expressing cells (Fig. 1A). The D-methionyl containing hexapeptide WKYMVm is somewhat more potent than its L-conformer for the activation of FPRL1. However, it can also activate cells through FPR [17]. The specificities of the receptor antagonists were retained with WKYMVm as the triggering agonist, i.e. the WKYMVm-mediated calcium mobilization was inhibited by cyclosporine H in FPR-expressing cells (Fig. 2A) and by WKW_4 in FPRL1-expressing cells (Fig. 2B). In accordance with the receptor specificities of the antagonists no effect was obtained with WKW_4 when FPR expressing cells were triggered by WKYMVm or with cyclosporine H when FPRL1 expressing cells were triggered with the same agonist (data not shown).

3.2. *Peptide-induced NADPH-oxidase activity in neutrophils—effects of the receptor antagonists*

The chemotactic peptides fMLF and WKYMVM are potent activators of the neutrophil NADPH-oxidase and the generated reactive oxygen species are secreted from the cells. According to its receptor specificity, cyclosporine H blocked the NADPH-oxidase activity induced by fMLF while the inhibitor had no effect on the response induced by WKYMVM (Fig. 3). To further verify the specificity in inhibition, the effects of WKW_4 were investigated. The FPRL1 antagonist inhibited the WKYMVM- but not the fMLF-induced response (Fig. 3).

When exposing neutrophils to WKYMVm, a similar superoxide production was achieved and the response was

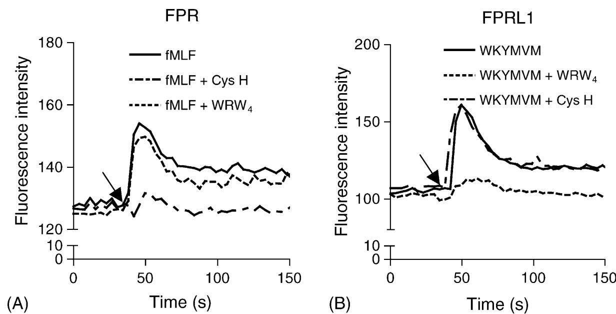


Fig. 1 – Effects of the receptor antagonists cyclosporine H (cys H; FPR specific) and WRWWWW (WRW_4; FPRL1 specific) on the intracellular Ca^{2+} transient in HL-60 cells expressing FPR or FPRL1. Stably transfected HL-60 cells expressing either FPR or FPRL1 were loaded with Fura-2 and incubated (at 37 °C for 5 min) in the absence or presence of the antagonists (1 μM cys H or 5 μM WRW_4). The cells were then triggered with the FPR agonist fMLF (A; 10^{-7} M final concentration) or the FPRL1 agonist WKYMVM (B; 10^{-7} M final concentration). The time point for addition of agonist is indicated by an arrow. The changes in cytosolic Ca^{2+} were determined through measurement of the fluorescence emitted at 510 nm, during excitation at 340 nm. The levels of intracellular Ca^{2+} are expressed as the fluorescence change and representative experiments out of at least three are shown.

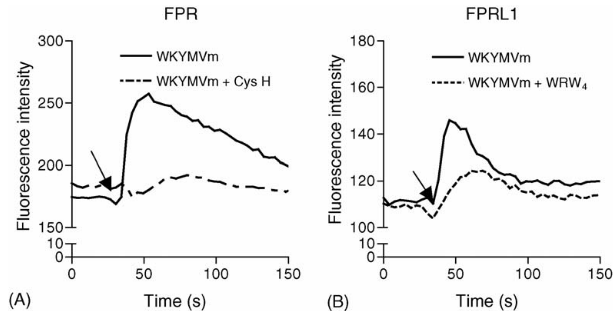


Fig. 2 – Effects of the receptor antagonists cyclosporine H (cys H; A) and WRWWWW (WRW$_4$; B) on the response in receptor expressing HL-60 using WKYMVm as triggering agonist. Stably transfected HL-60 cells expressing either FPR or FPRL1 were loaded with Fura-2 incubated (at 37 °C for 5 min) in the absence or presence of the antagonists (1 μM cys H or 5 μM WRW$_4$). The cells were then triggered with WKYMVm (10^{-8} M final concentration). The time point for addition of agonist is indicated by an arrow. The changes in cytosolic Ca^{2+} were determined through measurement of the fluorescence emitted at 510 nm, during excitation at 340. The levels of intracellular Ca^{2+} are expressed as the fluorescence change and representative experiments out of at least three are shown.

insensitive to cyclosporine H, showing that FPR is not involved in signaling and suggesting that the signals go through FPRL1. The FPRL1 antagonist had, however, no effect when a high concentration of WKYMVm was used (10^{-7} M; data not shown) and only partly inhibited the response when a lower, suboptimal, concentration (2×10^{-8} M or lower) was used to trigger the cells (Fig. 4).

3.3. *Combined effects of the receptor antagonists*

On the one hand, the observation that cyclosporine H was without effect on the WKYMVm induced respiratory burst activity suggests that FPR is not involved in transmitting the activating signals. On the other hand, the observation that the FPRL1 antagonist reduces the response with only around 50% suggests that FPR indeed is involved in the activation process, or that a third (unknown) receptor is involved. The involvement of FPR was evident, as the NADPH-oxidase activity remaining in the presence of WKW$_4$ was inhibited by cyclosporine H (Fig. 4). The fact that all activity was inhibited when the two antagonists were combined also excludes the possible involvement of a third receptor in the activation process.

The same pattern of inhibition with the antagonists alone and in combination, was evident when the change in intracellular Ca^{2+} was determined instead of oxidase activity (Fig. 5). Moreover, the pattern of inhibition with the antagonists alone and in combination, was the same also when differentiated HL-60 cells were triggered with WKYMVm (data not shown).

3.4. *Effects of PBP10 on NADPH-oxidase activity*

A cell permeable 10 amino acid peptide (PBP10) derived from the phosphatidylinositol 4,5-bisphosphate (PIP$_2$) binding region of gelsolin blocks activation of the oxidase and the subsequent secretion of oxygen radicals. The inhibitory effect of PBP10 is receptor specific and affects the WKYMVM but not

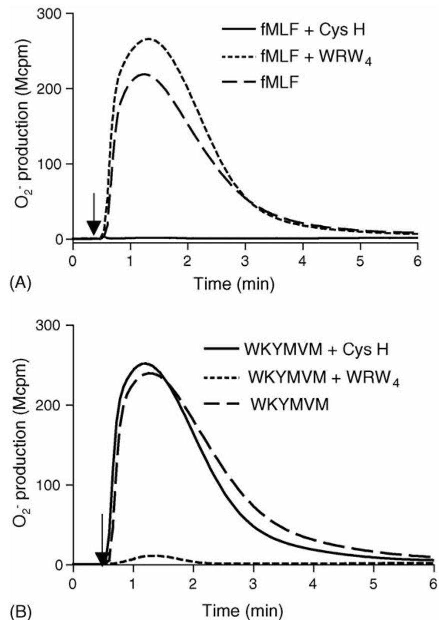


Fig. 3 – Superoxide production in neutrophils triggered with fMLF (A) or WKYMVM (B) and the effects of receptor specific antagonists. Neutrophils were pre-incubated at 37 °C for 5 min in the absence or presence of the antagonists, Cys H (1 μM) or WRW$_4$ (5 μM). The cells were then challenged with fMLF (10^{-7} M) or WKYMVM (10^{-7} M) and the release of superoxide anion was monitored. The time point for addition of the agonist is indicated by an arrow and the amount of superoxide is expressed in arbitrary units. Abscissa; time of study (min); ordinate; light emission expressed in Mcpm.

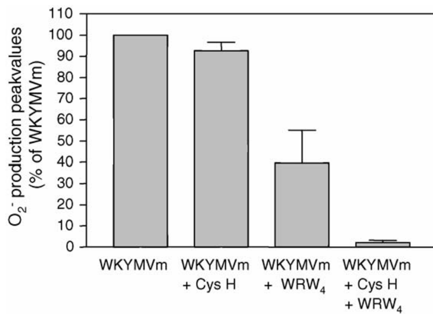


Fig. 4 – Superoxide production in neutrophils triggered with WKYMVm and the effects of receptor specific antagonists. Neutrophils were pre-incubated at 37 °C for 5 min in the absence or presence of the inhibitors, Cys H (1 μM), WRW_4 (5 μM) or a mixture of both. The cells were then challenged with WKYMVm (10^{-8} M) and the release of superoxide anion was monitored. The amounts of superoxide (peak values) produced are expressed as percent of control without any antagonist (mean ± S.E.M.; *n* = 3).

the fMLF induced cellular response (Fig. 6) [20]. The NADPH-oxidase induced by WKYMVm was only partly inhibited by PBP10 unless the signaling through FPR was also inhibited through a simultaneous addition of cyclosporine H (Fig. 7). The same inhibition patterns with the inhibitor, were obtained when differentiated, HL-60 cells were triggered with WKYMVm (Fig. 7 inset). The differentiated HL-60 cells lack specific granules [26] and are thus unable to recruit new

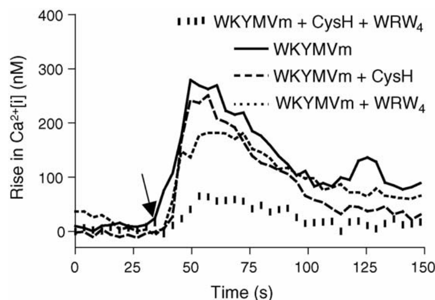
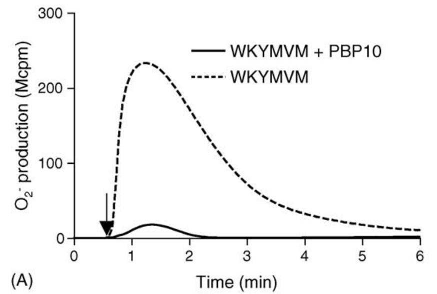


Fig. 5 – Effects of the receptor antagonists cyclosporine H (cys H; FPR specific) and WRWWWW (WRW_4; FPRL1 specific) on the intracellular Ca^{2+} transient in neutrophils. Cells were loaded with Fura-2 and incubated (at 37 °C for 5 min) in the absence or presence of the antagonists (1 μM cys H or 5 μM WRW_4). The cells were then triggered with WKYMVm (5×10^{-9} M final concentration). The time point for addition of agonist is indicated by an arrow. The changes in cytosolic Ca^{2+} were determined through measurement of the fluorescence emitted at 510 nm, during excitation at 340 nm. The levels of intracellular Ca^{2+} are expressed as the rise in the concentration from the resting level of around 100 nM.



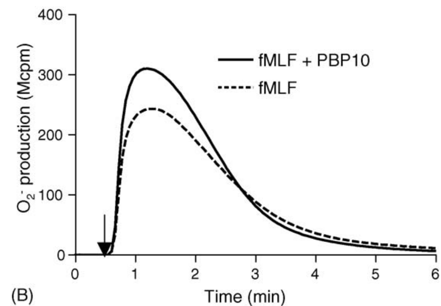


Fig. 6 – Effects of the PIP_2 binding peptide PBP10 on superoxide anion production in neutrophils triggered with WKYMVM (A) or fMLF (B). Neutrophils were pre-incubated at 37 °C for 5 min in the absence or presence of PBP10 (1 μM). The cells were then challenged with WKYMVM (A; 10^{-7} M) of fMLF (B; 10^{-7} M) and the extracellular release of superoxide anion was monitored with the use of an isoluminol amplified chemiluminescence system. The time point for addition of the agonist is indicated by an arrow and the amount of superoxide produced is expressed in arbitrary units. Abscissa; time of study (min); ordinate; light emission expressed in Mcpm.

plasma membrane receptors from granule stores [27,28], showing that the inhibition pattern is independent of ligand triggered receptor mobilization.

3.5. *Activation through FPRL1 does not block signaling by the FPR agonist fMLF*

Receptor desensitization experiments of neutrophil chemotactc receptors suggest the existence of a hierarchial receptor cross-talk between different families of receptors [29]. This hierarchy has been shown to be operating for activation with FPR/FPRL1 agonists as the primary event and the CXC receptor agonist IL-8 as the second receptor/ligand pair [12,30].

A receptor hierarchy within the formyl peptide receptor family could be a mechanism by which WKYMVm "chooses" to work through only one receptor despite the inherent potential to activate neutrophils through both FPR and FPRL1. The FPR was, however, able to add to the activating signal generated through FPRL1. The neutrophil NADPH-oxidase activity was more potent when the cells were triggered by a

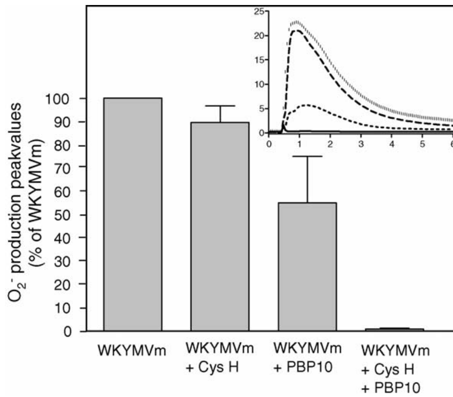


Fig. 7 – Effects of the PIP_2 binding peptide PBP10 and Cys H on superoxide anion production in neutrophils and differentiated HL-60 cells (inset) triggered with WKYMVm. The cells were pre-incubated at 37 °C for 5 min in the absence or presence of the inhibitors, PBP10 (1 μM), Cys H (1 μM) or a mixture of both. The cells were then challenged with WKYMVm (10^{-8} M) and the release of superoxide anion was monitored. The amounts of superoxide (peak values) produced are expressed as percent of control without any antagonist (mean ± S.E.M.; *n* = 3). Inset: Time course of the response in HL-60 cells triggered with WKYMVm alone (10^{-8} M; - - - -) and in the presence of Cys H (1 μM; ▬), PBP 10 (1 μM; ·······), and both Cys H and PBP10 (——).

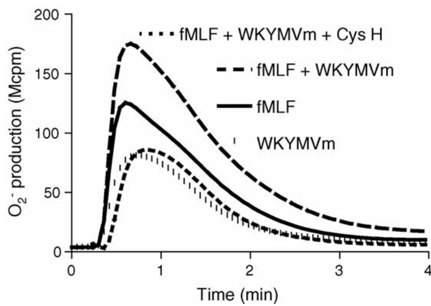


Fig. 8 – Effect of Cys H on neutrophil superoxide production in cells triggered simultaneously with WKYMVm and fMLF, two agonists with overlapping receptor specificities. Neutrophils were pre-incubated at 37 °C for 5 min in the absence or presence of Cys H (1 μM). The cells were then challenged with WKYMVm (2×10^{-9} M) and fMLF (10^{-7} M) alone or by the two agonists together and the release of superoxide anion was monitored. The time point for addition of the agonist is indicated by an arrow and the amount of superoxide is expressed in arbitrary units. Abscissa; time of study (min); ordinate; light emission expressed in Mcpm.

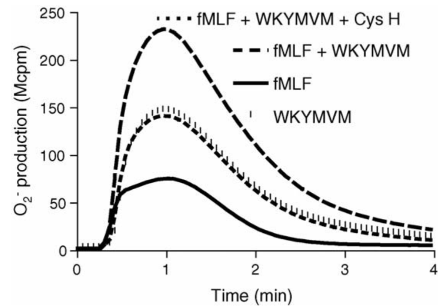


Fig. 9 – Effect of Cys H on neutrophil superoxide production in cells triggered simultaneously with WKYMVM and fMLF, two agonists with different receptor specificities. Neutrophils were pre-incubated at 37 °C for 5 min in the absence or presence of Cys H (1 μM). The cells were then challenged with WKYMVM (4×10^{-8} M) and fMLF (2×10^{-8} M) alone or by the two agonists together and the release of superoxide anion was monitored. The time point for addition of the agonists is indicated by an arrow and the amount of superoxide is expressed in arbitrary units. Abscissa; time of study (min); ordinate; light emission expressed in Mcpm.

mixture of WKYMVM/m and fMLF, compared to the activity induced by WKYMVM/m alone, and the increase was indeed inhibited by the FPR agonist cyclosporine H (Figs. 8 and 9).

4. Discussion

The G protein-coupled seven-transmembrane neutrophil receptors, FPR and FPRL1, belong to the *N*-formyl peptide chemoattractant receptor family [14,16]. The two receptors share a large sequence similarity, they have the same subcellular distribution, the major portions localized in mobilizable organelles, and despite the fact that the two highly related receptors recognize ligands with large structural diversities, the receptors induce almost indistinguishable cellular responses [10]. The first prototypical chemoattractant fMLF binds to FPR with high affinity, whereas the homologue FPRL1 binds the same agonist with very low affinity. We have earlier shown that the hexapeptide WKYMVM selectively activates neutrophils via FPRL1 [13]. This peptide therefore emerges as a very useful agonist to study the downstream signaling of FPRL1 without interference emanating from the activation of FPR, and this has made it possible to identify receptor antagonists [19] and also a specific inhibitor of signal transduction [20]. Even though no defined structure or sequence has been identified to be the determinant for FPR and FPRL1 binding and activation, there is obviously a close relationship between structural variation and function. This is clearly illustrated by the fact that an exchange of the carboxyterminal L-methionine in WKYMVM for the D-isomer, generates a peptide that increases its binding to FPRL1 but at the same time the WKYMVm peptide is an

agonist also for FPR with roughly the same receptor affinity as fMLF [17]. In this study we used receptor specific antagonists (WKW_4 for FPRL1 and cyclosporine H for FPR) and an earlier described receptor specific inhibitor (the gelsolin derived peptide PBP10) to determine whether WKYMVm uses one or the other, or both of the formyl peptide receptor family members to trigger the neutrophil NADPH-oxidase. We show that WKYMVm has the ability to activate neutrophils through both receptors, but the FPR signaling route is used only when the FPRL1 signaling pathway is blocked.

We used cyclosporin H as an FPR antagonist. This peptide has been shown to be around 10 times more potent and more specific than earlier described antagonists in inhibiting FPR mediated functions [31,32], and we could confirm its selectivity using fMLF and WKYMVM as agonists and HL-60 cells expressing FPR or FPRL1 or normal neutrophils as target cells. It is reasonable to assume, that although WKYMVm can activate both FPR and FPRL1 expressing cells, this activation involves different parts (binding sites) on these receptors. This assumption is based the fact that cyclosporine H inhibits the activity transmitted in HL-60 cells stably expressing FPR but has no effect on the signal induced in those expressing FPRL1. Recently several novel peptides were identified that inhibit agonist binding to FPRL1 [19] and the most potent of these, WRW_4 was used in our experiments. The same experimental set up as that used for characterization of cyclosporine H was used to define the inhibition profile of the FPRL1 antagonist and, as expected, we could show that it blocks WKYMVM induced activity in FPRL1 expressing cells but not the fMLF induced activity induced in FPR expressing cells. Moreover the ability to antagonize the activity was related to the receptor and not to the stimulus as WKW_4 reduced also the WKYMVm response in FPRL1 but not in FPR expressing cells. No Ca^{2+} response is obtained with WKYMVm in undifferentiated non-transfected HL-60 cells or in cells stably expressing IL-8 receptors [17], showing that the WKYMVm response is mediated through FPRL1 and not through another unidentified receptor. The reason why WRW_4 only partly inhibited the WKYMVm induced Ca^{2+} response, may be related to a difference in affinity, between the agonist and the antagonist. More important is, however, that WRW_4 has no effect on the response induced by WKYMVm in FPR expressing cells.

The neutrophil NADPH-oxidase activity induced by WKYMVm is very similar, with respect to kinetics and magnitude, to those induced by the specific FPR and FPRL1 agonists. Neutrophils exposed to WKYMVm for 10 min are desensitized to both WKYMVM (working through FPRL1) and fMLF (working through FPR) [17], suggesting that both members of the formyl peptide receptor family are involved in the activation process. According to this, cyclosporin H should block the activity; this antagonist was, however, completely without effect on the response induced by WKYMVm regardless of agonist concentration. Although these results suggest that WKYMVm act solely through FPRL1, the response was only partly inhibited by the FPRL1 antagonist WKW_4. This could imply a third receptor, but when combined, the two antagonists inhibited the WKYMVm induced neutrophil activity totally. It has been shown that FPR as well as FPRL1, dominate over the IL-8 signaling through CXC receptor [12,29], and the mechanism suggested to be responsible for this hierarchical deactivation, has been receptor phosphorylation. One explanation to the finding described here could be the existence of a hierarchical cross-talk also between the two formyl peptide receptor family members. We found, however, that when fMLF was added to the cells together with WKYMVm or WKYMVM, the neutrophil response was increased and this part of the response was cyclosporine H sensitive, suggesting that FPR has the capacity to signal despite a simultaneous activation of FPRL1. A type of hierarchy might still exist, but at some type of signaling level, rather than on a direct deactivation of the receptors. Such a mechanism could possibly involve a threshold for one of the signals generated. According to such a ‘‘threshold dependent mechanism’’, signals generated simultaneously by two different receptors influence the cellular activity differently in that only one signal (the strongest generated by the receptor with the higher affinity?) reaches the effector function whereas the other (the weaker generated by the receptor with the lower affinity?) is not allowed to pass the ‘‘threshold’’. There is normally a direct dose-response relationship between superoxide production and the amount of peptide used to trigger the neutrophils, and this is true both for fMLF and for WKYMVM(m) [13,17]. This suggests that in order for a signal from one receptor (FPR) to be bypassed when the cells are triggered with WKYMVm, which has the ability to activate two receptors (FPR and FPRL1) simultaneously, the two receptors must operate through different signaling pathways. The two neutrophil formyl peptide receptors fulfill this requirement (see below) but it should be noticed that the signals generated by FPR, when this receptor is occupied by a high affinity ligand such as fMLF, are strong enough to overcome the threshold level inflicted by the signals from FPRL1. We can at present not rule out that the activation of FPR by WKYMVm induces a conformational change that is slightly different from that triggered by fMLF. This may result in a lower affinity for the G-protein that, in the case where both receptors are occupied by WKYMVm, preferentially will bind to FPRL1. Accordingly, when the two agonists are added together, FPR will be occupied primarily by fMLF whereas FPRL1 will be occupied by WKYMVm, and both receptors will bind to the G-protein and add to the response. The fact that PBP10, which is an intracellular inhibitor rather than a receptor antagonist, promotes WKYMVm usage of FPR suggests that our findings are not solely dependent on receptor/ligand affinities.

Even though the two formyl peptide receptors in neutrophils share a high degree of amino acid identity, also in the signaling cytoplasmic domains, the cell permeable PIP_2-binding peptide PBP10 blocks FPRL1-mediated signaling. This blockage is specific for FPRL1 as illustrated by the fact that it has no effect on the neutrophil response to FPR, C5aR or CXCR agonists [20]. The PIP_2-binding peptide inhibited, however, only partly the WKYMVm induced superoxide production, but PBP10 shifted the activity to become sensitive to cyclosporine H. Taken together, these data clearly demonstrate that a fundamental difference exists in intracellular signaling between the two very closely related neutrophil formyl peptide receptor members. The precise mechanism by which PBP10 selectively interferes with FPRL1-signaling pathways and how the receptor shift is achieved at a molecular level remains to be determined in detail.

It should also be noticed that the results presented here may have implications for all signal transduction studies performed, in which specific receptor antagonists or inhibitors are used. The constitutive and ubiquitous expression of cell surface receptors in neutrophils as well as other cells, implies the potential to target these molecules for control of unwanted inflammatory reactions or some other disease state, and one approach is to develop specific antibodies or receptor antagonists. The potential of such inhibitors might be neglected if the receptor agonist is promiscuous with respect to receptor binding and activation. Intracellular signal transduction pathways provide another rich source of potential points for intervention in a huge number of cellular responses and disease states [33], but conclusions drawn from studies using signal transduction inhibitors always suffer from the uncertainty of their specificity. It is obvious that conclusions drawn regarding the potential of this type of inhibitors might also be neglected not only if the receptor agonist is promiscuous but also if multiple signaling pathways are triggered through the same receptor.

Acknowledgements

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Paper II

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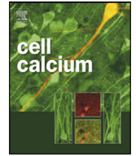

The FPR2-specific ligand MMK-1 activates the neutrophil NADPH-oxidase, but triggers no unique pathway for opening of plasma membrane calcium channels

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Human neutrophils express formyl peptide receptor 1 and 2 (FPR1 and FPR2), two highly homologous G-protein-coupled cell surface receptors important for the cellular recognition of chemotactic peptides. They share many functional as well as signal transduction features, but some fundamental differences have been described. One such difference was recently presented when the FPR2-specific ligand MMK-1 was shown to trigger a unique signal in neutrophils [S. Partida-Sanchez, P. Iribarren, M.E. Moreno-Garcia, et al., Chemotaxis and calcium responses of phagocytes to formyl peptide receptor ligands is differentially regulated by cyclic ADP ribose, J. Immunol. 172 (2004) 1896–1906]. This signal bypassed the emptying of the intracellular calcium stores, a route normally used to open the store-operated calcium channels present in the plasma membrane of neutrophils. Instead, the binding of MMK-1 to FPR2 was shown to trigger a direct opening of the plasma membrane channels. In this report, we add MMK-1 to a large number of FPR2 ligands that activate the neutrophil superoxide-generating NADPH-oxidase. In contrast to earlier findings we show that the transient rise in intracellular free calcium induced by MMK-1 involves both a release of calcium from intracellular stores and an opening of channels in the plasma membrane. The same pattern was obtained with another characterized FPR2 ligand, WKYMVM, and it is also obvious that the two formyl peptide receptor family members trigger the same type of calcium response in human neutrophils.

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1. Introduction

Neutrophil granulocytes are of great importance for the outcome of the “battle” between the innate immune system and foreign organisms or damaged tissue at sites of infection and inflammation. These inflammatory cells recognize a large variety of endogenous and exogenous chemoattractants, and in addition to the locomotory response induced by the attractants, a cell–ligand interaction lead to up-regulation of adhesion molecules, secretion of granule constituents and production of reactive oxygen species (ROS) generated by the electron transporting NADPH-oxidase system [2–6]. The molecular basis for recognition of chemoattractants is their binding to specific cell surface receptors [7–10]. Despite large structural differences between the huge number of extracellular ligands, many bind to (and activate) specific receptors belonging to a large family of pertussis toxin-sensitive G-protein linked receptors (GPCRs). These receptors possess a high degree of similarity and although activated by different agonists they transduce downstream signals that have many common features. Nevertheless, it is clear that there are also important differences between receptor–ligand pairs regarding the functional repertoire triggered [11,12]. The pattern recognition formyl peptide receptor (FPR) family belongs to the larger GPCR group of chemoattractant receptors [13–15]. Human neutrophil granulocytes express two FPR members, formyl peptide receptor 1 and 2 (FPR1 and FPR2, respectively, former FPR and FPRL1). The latter was originally defined as an

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orphan receptor, cloned from an HL-60 cell cDNA library by low-stringency hybridization with the FPR1 sequence [13]. In the past few years several ligands for FPR2 have been identified [15], including mitochondrial and bacterial derived peptides [16,17], different antimicrobial peptides [18], the acute phase protein serum amyloid A (SAA) [19], the neurotoxic prion peptide fragment 106–126 [20], and synthetic peptides such as WKYMVM [21] and MMK-1 [22]. No defined structure has so far been identified to be the determinant for FPR2 binding and activation, but the close relationship between structural variation and function is illustrated by the fact that an exchange of the carboxy terminal L-methionine in WKYMVM for the D-isomer, broadens the specificity of binding from being selective for FPR2 to include both FPR2 and FPR1 [23].

A large number of studies on FPR1 induced cell function and signaling have been performed, and these studies reveal that FPR1 signaling fulfills all the basic characteristic of a pertussis toxin sensitive GPCR. The activated receptor initiates a chain of signaling events starting with dissociation of the receptor associated G-protein and an activation of a number of downstream signaling routes [24,25]. In one of these, the activation of a phosphoinositide-specific phospholipase C (PLC) generates a second messenger upon cleavage of PIP_2, and this is the starting signal for a transient increase in cytosolic free calcium. Binding of the cleavage product, IP_3, to its receptor on storage organelles results in release of calcium from these intracellular organelles and an elevation in the concentration of free calcium ions in the cytoplasm is achieved [26]. The emptying of the storage organelles then leads to an entry of extracellular calcium through store-operated calcium channels in the plasma membrane that causes a prolonged rise in intracellular free calcium [27–29]. Knowledge of the signal transduction pathways utilized by FPR2 are still somewhat limited but since there is a large degree of homology between FPR2 and FPR1 (69% at the amino acid level; [15]) it could be assumed that the two receptors share many functional as well as signal transduction features. Accordingly, we have earlier shown that the functional responses induced by the FPR2-specific agonist WKYMVM is in most respects similar to (or even indistinguishable from) those induced by the prototype FPR1 agonist fMLF [21,30–32]. Fundamental differences in signaling have, however, been described between the two receptors. One prominent example being that lipoxin A4, a suggested FPR2 agonist, has been shown to inhibit neutrophil functions such as chemotaxis, mobilization of adhesion molecules and calcium flux [33,34]. Even though we have suggested that the LXA_4 mediated effects are triggered through some other receptor [35], there are in addition to the similarities also important differences between FPR1 and FPR2. Accordingly we recently showed that a PIP_2-binding peptide (PBP10) uncover a difference between the two receptors with respect to the generation of neutrophil activating signals, one route being sensitive to PBP10 (the FPR2) the other insensitive (the FPR1) [36,37]. A cADPR antagonist was recently shown to have the same receptor selectivity [1], and the selectivity was in a direct way linked to the calcium response. The FPR2-specific ligand MMK-1 was shown to induce an influx of calcium over the plasma membrane, using a channel that was not operated by the intracellular stores and by that not preceded by a rise in intracellular calcium ($[Ca^{2+}]_i$) originating from the release of calcium from the storage organelles [1]. These results suggest that FPR2, in contrast to FPR1, triggers a unique signal, independent of intracellular stores, that allows a direct influx of extracellular calcium over the plasma membrane.

In this study we have characterized the neutrophil response induced by the FPR2-specific agonist MMK-1, the peptide suggested to trigger a unique signalling pathway (see above [1,22]). We show that MMK-1 share the ability of another FPR2 agonist as well as the prototype agonist for FPR1, to activate neutrophils to produce and release superoxide anions. The rise in $[Ca^{2+}]_i$ induced by MMK-1 (and another FPR2-specific agonist), is characterized by a release from intracellular stores that occurs also without any extracellular calcium, and the initial rise is followed by an opening of ion channels in the plasma membrane. We thus conclude that the mechanism by which FPR2 triggers a rise in intracellular calcium is by no means unique for this receptor.

2. Materials and methods

2.1. Peptides and reagents

MMK-1 (LESIFRSLLFRVM) was synthesized and HPLC purified by KJ Ross-Pedersen AS (Klampenborg, Denmark). The hexapeptide WKYMVM were synthesized and purified by Alta Bioscience (University of Birmingham, UK). The formylated peptide fMLF was from Sigma Chemical Co. (St. Louis, MO). The receptor antagonist WRWWWW (WRW_4) was from Genscript Corporation (Scotch Plains, NJ, USA) and cyclosporin H was kindly provided by Novartis Pharma (Basel, Switzerland). The peptides/receptor antagonists were dissolved in dimethyl sulfoxide to 10^{-2} M and stored at $-70\,^{\circ}$C until use. Further dilutions were made in KRG (Krebs Ringer buffer with glucose). EGTA, Cytochalasin B and human recombinant TNF-α were obtained from Sigma Chemical Co. (St. Louis, MO).

2.2. Stable expression of FPR1 or FPR2 in undifferentiated HL-60 cells

The stable expression of FPR1 and FPR2 in undifferentiated cells has been previously described [23]. Transfection of HL-60 cells was performed by electroporation with a Bio-Rad Gene Pulser apparatus, according to a slightly modified version [38] of the technique described by Tonetti et al. [39]. Following electroporation, cells were allowed to recover in 20 ml of culture medium for 48 h prior to selection in a medium containing G418 (1 mg/ml) (Gibco, Invitrogen). G418-resistant clones were obtained by limited dilution into 24-well microtiter plates and FPR2-expressing clones were identified by their ability to mobilize intracellular calcium upon addition of $WKYMVmNH_2$ (100 nM final concentration). Cells were cultured in RPMI 1640 (PAA Laboratories GmbH, Austria) containing FCS (10%) (PAA Laboratories GmbH, Austria), PEST (1%) (PAA Laboratories GmbH, Austria) and G418 (1 mg/ml). The maximal density was maintained below 2×10^6 cells/ml. The cells were passaged to a concentration of 5×10^5 cells/ml approximately 24 h prior to use in assays.

2.3. Determination of changes in cytosolic calcium by fluorometry

Cells at the density of $1–3 \times 10^6$ cells/ml were washed with KRG without Ca^{2+}. The cell pellets were resuspended at a density of 2×10^7 cells/ml in KRG containing 0.1% BSA and loaded with 2 μM Fura 2-AM (Molecular Probes, Eugene, OR) for 30 min, at RT. Cells were then diluted to twice the original volume with RPMI 1640 culture medium without phenol red (PAA Laboratories GmbH, Austria) and centrifuged. Finally the cells were washed once in KRG, and resuspended in KRG at a density of 2×10^7 cells/ml. Calcium measurements were carried out with a PerkinElmer fluorescence spectrophotometer (LC50) with excitation wavelengths of 340 and 380 nm, an emission wavelength of 509 nm and slit widths of 5 and 10 nm, respectively. The transient rise was presented as a ratio between the fluorescence intensities received, the values for 340 nm as numerator and 380 nm as denominator. The maximal peak values shown correspond to approximately 600 nM free calcium.

The concentration of EGTA required to achieve a calcium-free extracellular environment was determined by titration in the Fura

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2-AM system. The basal level of fluorescence of Fura-2-labelled neutrophils in KRG containing 1 mM Ca^{2+} was determined continuously. A small volume (10 μl) of an EGTA containing buffer was added to the measuring vial, and 20 s later the cells were lysed by the addition of Triton X-100. The lowest amount of EGTA was determined, that was without effect on the basal level of fluorescence when added to the cells and at the same time immediately and completely abolished the fluorescence when the cells were lysed with the detergent. No free Ca^{2+} was, thus, available that could bind to the Fura-2 released from the lysed cells.

2.4. Determination of changes in cytosolic calcium by flow cytometry

Intracellular calcium mobilization was measured essentially according to the method developed by Partida-Sanchez et al. [1]. Cells (3×10^6) were resuspended in cell-loading medium (KRG with Ca^{2+} + 1% FCS) and loaded with the fluorescent dyes Fluo-3 AM (4 μg/ml) and Fura Red AM (10 μg/ml) (Molecular Probes, Eugene, OR) for 30 min at 37 °C. Intracellular Ca^{2+} levels were monitored by dual flow cytometry (FACScan). The fluorescence emission of Fluo-3 was recorded in the FL-1 channel and that of Fura Red in the FL-3 channel. Prior to use in the assay, cells were warmed to 37 °C in a water bath. First, baseline fluorescence was established (20 s) in a tube containing 5×10^5 cells/ml. Next, the tube was rapidly removed, the stimulant added, and the tube was returned to the flow cytometer for further measurement. The relative Ca^{2+} concentration was expressed as the ratio between Fluo-3 and Fura Red (FL-1/FL-3) MFI (mean fluorescence intensity) over time.

2.5. NADPH-oxidase activity and neutrophil priming

Neutrophils (5×10^6 cells/ml) were incubated at 37 °C for 20 min in the presence of a priming agent or left untreated (control). The NADPH-oxidase activity of these cells was then recorded using an isoluminol-enhanced chemiluminescence (CL) system [40,41]. The extracellular CL activity was measured in a six-channel Biolumat LB 9505 (Berthold Co., Wildbad, Germany), using disposable polypropylene tubes with a 0.90-ml reaction mixture. The mixture contained neutrophils 5×10^5 cells/ml, horseradish peroxidase (HRP; a cell impermeable peroxidase; 4 U) and isoluminol (a cell impermeable CL substrate; 2×10^{-5} M). The tubes were equilibrated at 37 °C for 5 min in the absence or presence of inhibitor, after which the stimulus (0.1 ml) was added. The light emission was recorded continuously.

3. Results

3.1. Confirmation of the receptor preference for the peptide MMK-1 using FPR1- or FPR2-expressing transfected HL-60 cells

In order to confirm the reported receptor preference for MMK-1, the peptide was used to trigger a rise $[Ca^{2+}]_i$ in cells stably expressing one or the other of the formyl peptide receptors. The two neutrophil members of the FPR-family, FPR1 or FPR2, were expressed in undifferentiated HL-60 cells and a rise in intracellular Ca^{2+} was used as readout system for binding and activation. Each of the two transfected cell clones was challenged with MMK-1 and we could confirm earlier findings, that the peptide is a ligand for FPR2 with only a minimal effect on FPR1 (Fig. 1A and inset).

3.2. The MMK-1 peptide induced neutrophil superoxide production through FPR2

Neutrophils produced and released superoxide anions when triggered with the MMK-1 peptide (Fig. 1B). The kinetics of the

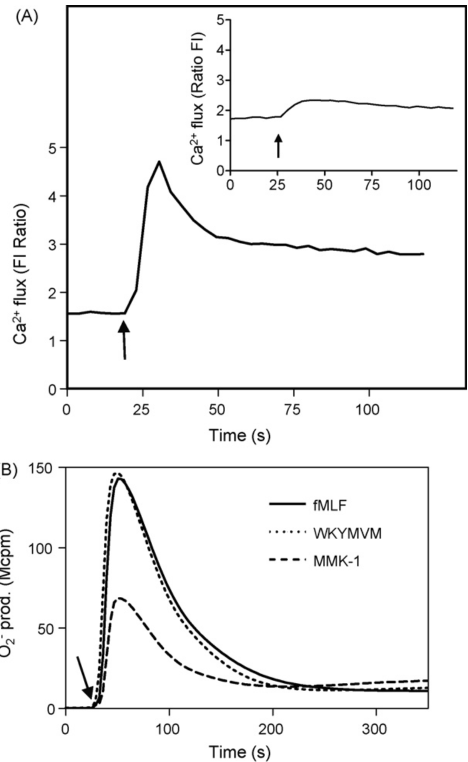

Fig. 1. Transient calcium rise in HL-60 cells expressing FPR1 or FPR2 and the activation of the neutrophil NADPH-oxidase in response to MMK-1. (A) Stably transfected HL-60 cells expressing either FPR1 or FPR2 loaded with Fura-2 were incubated at 37 °C for 5 min. The cells were then stimulated with MMK-1 (10^{-7} M final concentration). The time points for the addition of agonist are indicated by arrows. MMK-1 induced a Ca^{2+} rise in FPR2 (main figure) expressing cells but not in cells expressing FPR1 (inset). The changes in cytosolic Ca^{2+} levels were determined through the measurement of the fluorescence, emitted at 509 nm, during excitation at 340 and 380 nm. The results are presented as a ratio between the fluorescence intensities at 340 and 380 nm. (B) Neutrophils were pre-incubated at 37 °C for 5 min and then challenged with MMK-1 (broken line), WKYMVM (dotted line) or fMLF (solid line). The extracellular release of superoxide anion was monitored with the use of an isoluminol amplified chemiluminescence system. The time point for addition of the agonist is indicated by an arrow and the amount of superoxide is expressed in arbitrary units. Abscissa; Time of study (min); ordinate; superoxide production given as light emission and expressed in cpm $\times 10^{-6}$.

NADPH-oxidase response triggered by MMK-1 was very similar to that induced by the earlier characterized FPR2-specific agonist WKYMVM and the FPR1 specific agonist fMLF with a fast response that peaks after around 1 min and then rapidly declines. At equimolar concentrations (a final concentration of 10^{-7} M chosen for comparison) of the peptides the neutrophil response to MMK-1 was somewhat lower than that induced by WKYMVM (Fig. 1B), and the EC_{50} values for the two peptides were 2×10^{-7} M and 4×10^{-8} M for MMK-1 and WKYMVM, respectively. The neutrophil NADPH-oxidase response induced by MMK-1 and WKYMVM was further compared with respect to priming. Neutrophils pre-treated with TNFα were primed in their response to both MMK-1 and WKYMVM, and the increase was of the same magnitude (Fig. 2A). Termination of signalling from many GPCRs, including both FPR2 and FPR1 involves coupling of the occupied receptor to the cell cytoskeleton, a process that blocks the binding of the active receptor to the G-

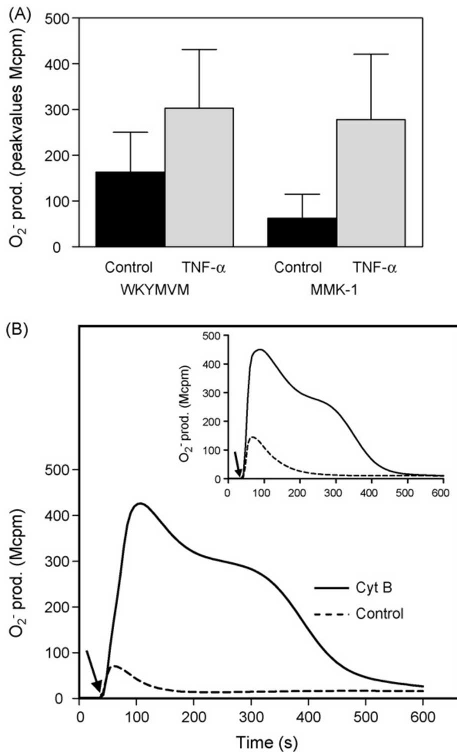

Fig. 2. The effect of TNF-α priming (A) and cytochalasin B (B) on superoxide anion production in neutrophils induced by MMK-1 and WKYMVM is similar. (A) Neutrophils were incubated at 37 °C for 20 min in the presence of a priming agent or kept on ice (control). The cells were then pre-incubated at 37 °C for 5 min before stimulation with WKYMVM (10^{-7} M) or MMK-1 (10^{-7} M). The extracellular release of superoxide anion was monitored with the use of an isoluminol amplified chemiluminescence system. The amount of superoxide produced is expressed as peak values (mean ± S.D., $n = 3$). (B) Neutrophils were pre-incubated at 37 °C for 5 min in the presence or absence of cytochalasin B (2.5 μg/ml final concentration) and then challenged with the agonist MMK-1 or WKYMVM (inset). The extracellular release of superoxide anion was monitored with the use of an isoluminol amplified chemiluminescence system. The cellular responses induced in the presence of cytochalasin B are shown as solid lines and the responses in control cells are shown as broken lines. A control experiment with WKYMVM is shown as an inset. Abscissa; time of study (min); ordinate; superoxide production given as light emission and expressed in cpm × 10^{-6}.

protein and by that the down stream signalling is blocked. The actin binding drug cytochalasin B disrupts the cytoskeleton and by that the binding to, and inactivation of, the receptor–ligand complex [42]. In the presence of cytochalasin B the occupied receptors are active for a longer time, and the response induced is thus enhanced and prolonged [43]. Accordingly, the neutrophil release of superoxide anions induced by both fMLF and WKYMVM (shown for the latter in Fig. 2B) had a higher magnitude and a longer duration in the presence of cytochalasin B, and the MMK-1 induced oxidase activity resembled that of WKYMVM (Fig. 2B).

As shown above (Fig. 1A) using transfected cells expressing a defined receptor, the MMK-1 peptide has the ability to bind and activate FPR2 [22]. However, this does not necessarily mean that this is the only one used in neutrophils that express also other receptors [44,45]. In order to determine the receptor involved in MMK-1 induced activity, we used earlier described receptor antagonists and a receptor selective inhibitor. In accordance with the assumed receptor specificity, MMK-1 triggered neutrophil oxidase activity was insensitive to cyclosporine H, an FPR1 specific antagonist, whereas WRW_4, an FPR2 antagonist inhibited the response (Fig. 3). We have previously shown that the membrane permeable polyphosphoinositide-binding peptide rhodamine-B-QRLFQVKGRR (PBP10) is an FPR2-selective inhibitor. This is illustrated by the fact that the neutrophil NADPH-oxidase activity was completely inhibited by PBP10 when activation was triggered with WKYMVM (the FPR2-specific agonist; Fig. 4), whereas there was no effect of the peptide on the fMLF-induced, FPR1-mediated neutrophil response (Fig. 4). The receptor preference of MMK-1, for FPR2, was confirmed in the inhibition experiments with PBP10 (Fig. 4).

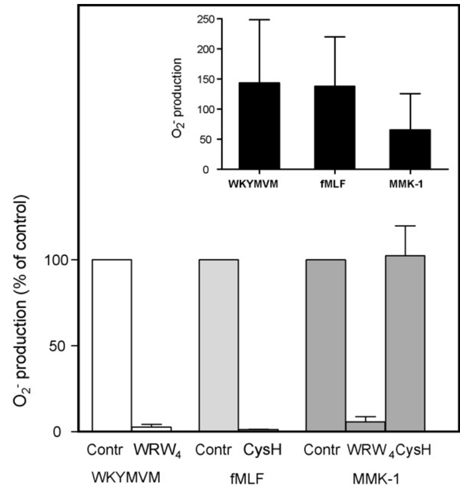

Fig. 3. Effects of receptor specific antagonists on superoxide production in neutrophils activated by MMK-1, fMLF or WKYMVM. Neutrophils were pre-incubated at 37 °C for 5 min in the absence or presence of the antagonists, Cys H (1 μM) or WRW_4 (5 μM). The cells were then challenged with MMK-1 (10^{-7} M), fMLF (10^{-7} M) or WKYMVM (10^{-7} M) and the extracellular release of superoxide anion was monitored with the use of an isoluminol amplified chemiluminescence system. Peak values for the amount of superoxide produced are expressed as % of control and the absolute values (mean ± S.D., $n = 3$) are presented as an inset.

3.3. The transient rise in intracellular Ca^{2+} triggered through FPR2 and FPR1 is linked to a mobilization of ions from intracellular storage organelles

It is well known that binding of a specific ligand to FPR1 results in a PLC-dependent cleavage of PIP_2, and the product generated, IP_3, mobilizes calcium from intracellular storage organelles [46]. This transient rise is achieved also in the absence of extracellular calcium but it is sustained and prolonged in the presence of extracellular calcium, through an activation of the so-called store-operated channels in the plasma membrane [28]. The activation of cells with the FPR2-specific ligand MMK-1 has been shown to induce a totally different response that according to the model presented relies directly on an opening of ion channels in the plasma membrane [1]. These ion channels are thus regulated in a totally different manner than the store-operated channels known to be activated (in one way or the other) by an emptying of the intracellular stores. As mentioned, when the rise in intracellular calcium is dependent on an initial release from storage organelles, the rise should be obtained also in the absence of extracellular calcium. In order to visualize the direct role of the non-store-operated channels, the calcium ions

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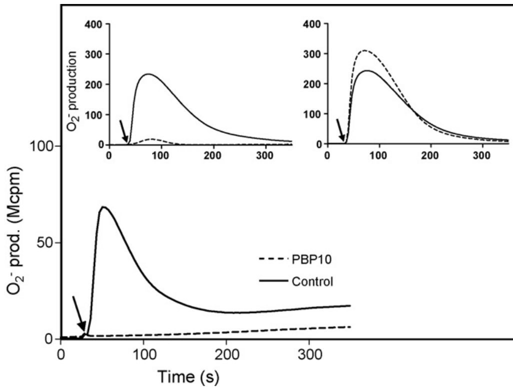

Fig. 4. PBP10 inhibits the activation of the neutrophil NADPH-oxidase triggered by MMK-1. Neutrophils were pre-incubated at 37 °C for 5 min in the absence (solid lines) or presence (broken lines) of the inhibiting peptide PBP10 (1 μM final concentration). The cells were then challenged with MMK-1 (10^{-7} M) and the extracellular release of superoxide anion was monitored with the use of an isoluminol-amplified chemiluminescence system. One representative experiment out of three is shown. The time point for the addition of the agonist is indicated by an arrow. The effects of PBP10 on WKYMVM (left inset) and fMLF (right inset) on neutrophil oxidase activity is shown as control. Abscissa; time of study (min); ordinate; superoxide production given as light emission and expressed in cpm × 10^{-6}.

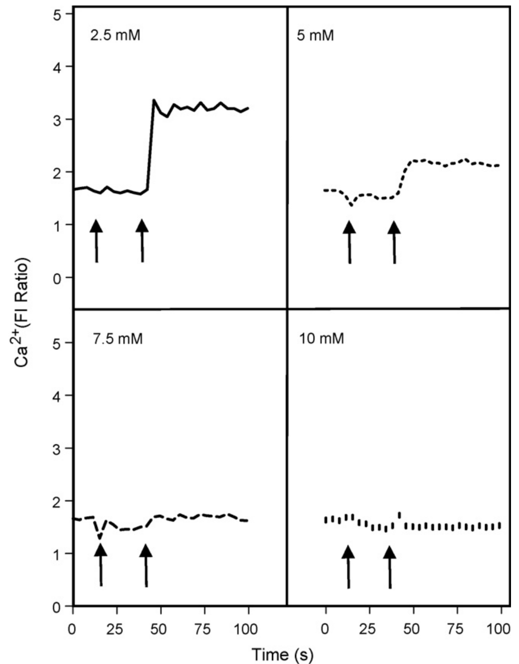

Fig. 5. Titration of the EGTA concentration needed to achieve a calcium-free environment. The concentration of EGTA required to achieve a calcium-free extracellular environment was determined by titration in the Fura 2-AM system. The basal level of fluorescence of Fura-2-labelled neutrophils in KRG containing 1 mM Ca^{2+} was determined continuously. A small volume (10 μl) of an EGTA containing buffer was added to the measuring vial (indicated by the first arrow), and 20 s later the cells were lysed by the addition of Triton X-100 (indicated by the second arrow). The EGTA concentrations used was 2.5 mM (solid line), 5 mM (broken line), 7.5 mM (dotted line) and 10 mM (vertical lines). The lowest amount of EGTA was determined (10 mM), which was without effect on the basal level of fluorescence when added to the cells and at the same time immediately and completely abolished the fluorescence when the cells were lysed with the detergent. No free Ca^{2+} was, thus, available that could bind to the Fura-2 released from the lysed cells.

present in the extracellular medium were chelated with EGTA prior to activation of the cells. The chelator, was added to the cells 10–15 s before the addition of the receptor specific peptide agonist, and the minimum amount of EGTA required to chelate extracellular calcium was determined (Fig. 5; described also in Section 2), In agreement with earlier findings the fMLF induced transient rise in intracellular free calcium (representing in large the mobilization from intracellular stores) was present also when extracellular calcium was removed. The removal of extracellular calcium resulted in a small change in the kinetics of the responses (Fig. 6A), a change due to the influx through storage-operated plasma membrane channels. Unexpectedly, the same pattern of response was obtained when neutrophils were triggered through FPR2. This was irrespective of if the MMK-1 or the WKYMVM peptide was used as stimulus. This is not in agreement with the suggestion that FPR2 rely solely on an opening of ion channels in the plasma membrane [1]. In our hands both peptides induced intracellular calcium rises that rely on emptying of the intracellular stores (Fig. 6B and C).

In order to exclude the possibility that the technique used to measure intracellular calcium influences the results obtained, we monitored calcium levels using flow cytometry [1] instead of the bulk system described so far. The picture appeared the same also when this technique was applied. There was a transient rise in intracellular free calcium also in the absence of extracellular Ca^{2+} not only with fMLF but also with MMK-1 or WKYMVM (Fig. 7). The pattern of response triggered through FPR2 was, thus, identical to that through FPR1, and this was evident for both FPR2 agonists, MMK-1 and WKYMVM.

4. Discussion

Human neutrophils express two members of the formyl peptide receptor family (FPR1 and FPR2) that possess a high degree of amino acid identity. Despite the large degree of similarity between the two receptors, signaling events following receptor occupancy and activation have been shown to differ, and these differences may be related either to the specific agonist or a distinct difference between the receptors [36]. We and others have shown that both FPR1 and FPR2 can mediate an inhibitory signal (as illustrated by the lack of responsiveness to IL-8 induced by a FPR1/FPR2 agonist) as well as a direct activating signal in neutrophils [33,44,47]. In this study we add the synthetic peptide MMK-1 to the group of direct activating FPR2-specific agonists that triggers the assembly of the neutrophil NADPH-oxidase. With respect to the different signaling properties of the two neutrophil FPRs, it was recently shown, using MMK-1 and fMLF as the receptor specific agonists for FPR2 and FPR1, respectively, that two different signals are generated by the receptors. Both these signals mediate a rise in intracellular calcium through an influx of ions over the plasma membrane, but whereas one of the receptors operates through an emptying of intracellular stores (FPR1), the other operates directly on the plasma membrane and independently of the stores [1]. Our original hypothesis was that the two receptors possess two distinct signaling properties, but we could not verify such a difference. Instead we show that the signals from both FPR1 and FPR2 initiate a rise in cytosolic free calcium derived from an emptying of intracellular stores.

We used receptor specific agonists and antagonists to confirm the receptors involved in MMK-1 signaling. The peptide WKYMVM, a high affinity ligand for the chemoattractant receptor FPR2, was used together with the receptor specific antagonist WRW_4 and the receptor specific inhibitor PBP10, as tools to determine the

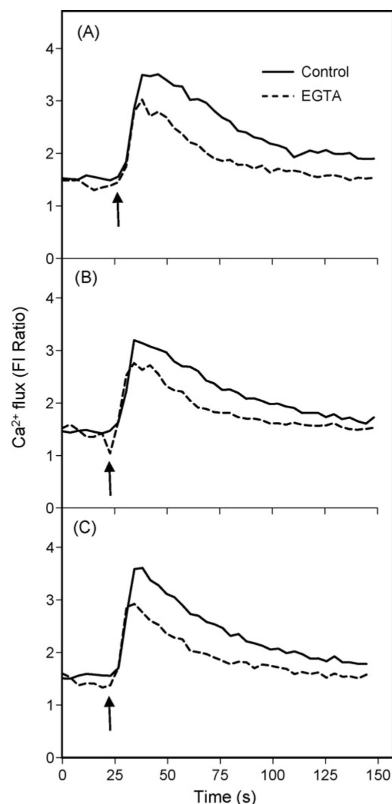

Fig. 6. A rise in intracellular calcium is induced by fMLF (A), MMK-1 (B) or WKYMVM (C) also when extracellular calcium is removed. Neutrophils loaded with Fura-2 AM were incubated at 37 °C for 5 min. Removal of extracellular calcium was achieved by the addition of EGTA (10 mM; broken lines) 20 s prior to the addition of the agonist. The cells were then stimulated with fMLF (A), MMK-1 (B) or WKYMVM (C) (10^{-7} M final concentration). The response in control cells (in the presence of 1 mM calcium) are presented as a solid lines and the time points for the addition of agonist are indicated by arrows. The changes in cytosolic Ca^{2+} levels were determined through measurement of the fluorescence, emitted at 509 nm, during excitation at 340 and 380 nm. The results are presented as a ratio between the fluorescence intensities at 340 and 380 nm.

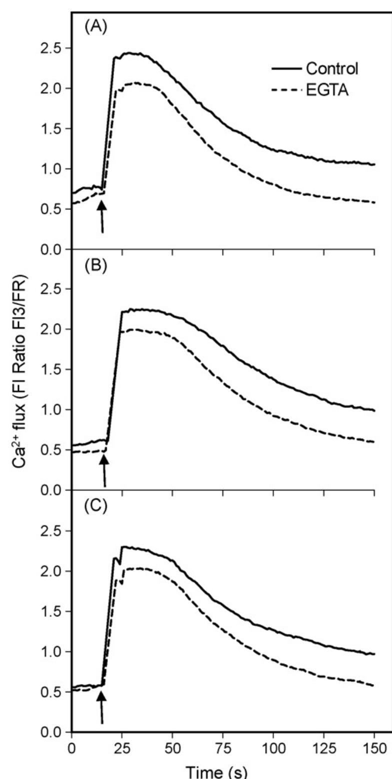

Fig. 7. A rise in intracellular calcium is induced by fMLF (A), MMK-1 (B) or WKYMVM (C) in the presence of EGTA when studied by FACS analysis. Neutrophils loaded with Fluo-3 AM and Fura red AM were incubated at 37 °C for 5 min. Removal of extracellular calcium was achieved by the addition of EGTA (10 mM; broken lines) 20 s prior to the addition of the agonist. The cells were then stimulated with fMLF (A), MMK-1 (B) or WKYMVM (C) (10^{-7} M final concentration). The response in control cells (in the presence of 1 mM calcium) are presented as a solid lines and the time points for the addition of agonist are indicated by arrows. The changes in cytosolic Ca^{2+} levels were determined through measurement of the emitted fluorescence by FACS analysis. A ratio was then calculated using the mean fluorescence for Fluo-3 as the numerator and Fura red as the denominator.

receptor specificity of the new NADPH-oxidase activating ligand [36,48]. For comparison the formylated peptide fMLF, a classical high-affinity ligand for the chemoattractant receptor FPR1, was used together with the antagonist cyclosporine H [49]. The FPR2 antagonist as well as the inhibitor blocks the MMK-1induced respiratory burst, whereas cyclosporine H was without any effect. These results were in agreement with the results obtained with FPR1- and FPR2-expressing undifferentiated HL-60 cells.

A large number of ligands have been identified for FPR2 (see [15] for a review). All those that have been investigated, with one exception in lipoxin A4, have in common that they trigger an assembly of the neutrophil NADPH-oxidase which gives rise to secretion of superoxide ions. We now add MMK-1 to the list of FPR2 agonists that effectively activates the NADPH-oxidase, and the basic characteristics of the response resemble that of the established FPR2 ligand WKYMVM.

One of the early signals generated by activated neutrophil GPCRs is the rapid alteration of free $[Ca^{2+}]_i$ that secondarily results in the activation of different cellular functions. In resting neutrophils, the free cytosolic calcium concentration is kept at very low levels (around 100 nM) as compared to the level outside the cells, which reaches four orders of magnitude higher [50,51]. To counter this strong concentration gradient the cells are equipped with ion pumps that transport Ca^{2+} out of the cells as well as to the intracellular calcium storing organelles containing an abundance of the calcium binding protein calreticulin. The main and generally accepted pathway for the control of rapid changes of $[Ca^{2+}]_i$ in neutrophils is the capacitive model starting with the production of inositol-1,4,5-trisphosphate (IP_3). The main pathway for this production involves the phospholipase C mediated hydrolysis of membrane PIP_2, and the generated IP_3 releases calcium from intracellular stores by binding to specific receptors on the intracellular storage organelles. The filling state of the stores then exerts control over ion channels (store-operated channels; SOCs) in the plasma membrane. An emptying of the intracellular stores thus regulates the opening of SOC that provide a rich source of capacitive entry of calcium ions originating from the extracellular space [28,29]. A release from intracellular stores can be measured without any influence of SOC if extracellular calcium is chelated, and

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using this experimental approach, FPR2 triggered calcium mobilization has been suggested to be due solely to extracellular calcium influx [1] whereas FPR1 triggered calcium mobilization was in large dependent on the stores. Using two different FPR2-specific ligands, WKYMVM and MMK-1, the latter earlier used in identifying the distinct and unique fashion for calcium entry in neutrophils, we could show that the responses induced by the two highly related human chemoattractant receptors, FPR2 and FPR1, are identical and follow the classical calcium response pattern described above. The results suggesting a unique entry of Ca^{2+} were generated in an FACS based single cell assay system [1]. In order to determine if the data was depending on different sensitivities in the two methods we also used the FACS-based system. However, in our hands the same results were obtained when using this approach.

As previously stated, FPR2 is a receptor with known ligands of many different origins and structures. It is interesting that a receptor that responds so effectively to small peptides like WKYMVM and MMK-1 also binds larger bulkier molecules like Aβ42 [52]. It is not yet known exactly which ligand epitopes that bind to what sites on the receptor. The work with receptor chimeras however, has mapped the regions on FPR2 important for ligand binding [53]. It has also been concluded that small conformational changes in a ligand, such as exchange of the N-terminal L-Met for a D-Met in WKYMVM, can alter FPR2-specific properties towards a more general FPR family specificity [23]. Despite differences in binding to the receptor, the activating peptide ligands of FPR2 all seem to elicit the same intracellular signalling pathways and cellular responses [53], and this is consistent with the great similarities between MMK-1 and WKYMVM in this study.

Conflict of interest

All authors state that there are no conflict of interest for this work.

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Paper III

A Methodological Approach to Studies of Desensitization of the Formyl Peptide Receptor: Role of the read out system, reactive oxygen species and the specific agonist used to trigger neutrophils

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Neutrophil accumulation at an inflammatory site or an infected tissue is dependent on the recognition of chemotactic peptides that bind to G-protein coupled receptors (GPCR's) exposed on the surface of the inflammatory cells. A GPCR activated by a chemoattractant quickly becomes refractory to further stimulation by ligands using the same receptor. This desensitization phenomenon has been used frequently to characterize new receptor agonists and to determine receptor hierarchies.

In this study we describe a difference in the pattern of desensitization using different techniques to follow neutrophil activities. The desensitized state seen with the prototype agonist formylmethionyl-leucyl-phenylalanine (fMLF) when the release of reactive oxygen species generated by the NADPH-oxidase was determined, was not seen when the rise of intracellular calcium ($[Ca^{2+}]_i$) was followed. We show that the difference between the two systems is dependent on an inactivation of the agonist in one system but not in the other, and we suggest several different solutions to the problem. Agonist inactivation occurs through a myeloperoxidase (MPO)/hydrogen peroxide catalyzed reaction, and the problem could be avoided by using a FACS based technique to measure the change in $[Ca^{2+}]_i$, by the use of an agonist insensitive to the MPO/hydrogen peroxide-system or, by adding an MPO inhibitor or a scavenger that removes either superoxide/hydrogen peroxide or the MPO-derived metabolites.

Neutrophils are the first cells to be recruited to the site of a microbial infection as well as in the early phase of an ongoing inflammation, and the cells play important roles in our innate immune defence system. The recruitment of neutrophils by different types of chemoattractants is a key event in the inflammatory process, and this response relies on the recognition of the attractants by specific neutrophil cell surface receptors [1]. These receptors trigger not only migration, but also a wide range of other responses such as a transient rise in intracellular calcium [2], cleavage of L-selectin from the cell surface [3, 4], mobilization of granules and secretion of reactive oxygen species (ROS) generated by a membrane localized electron transporting system, the NADPH-oxidase [5]. Most chemoattractant receptors are members of the large family of G-protein coupled receptors (GPCR), and these all have in common that they are membrane spanning (seven times) and signalling is dependent on binding of an agonist to the extracellular domains. This binding triggers a coupling of the intracellular parts of the receptors to a signalling heterotrimeric G-protein. A large number of endogenous neutrophil chemoattractants have been described, including e.g., the cytokine IL-8, the complement cleavage product C5a and the acute phase reactant serum amyloid A (SAA). The cellular responses could be triggered also by microbial specific molecules such as peptides with an N-terminal methionyl group that is formylated [1].

Receptor activation is a transient process; binding of an agonist results in G-protein coupling and signal is generated, but the occupied receptor is then fairly rapidly transferred to a refractory state that lacks signalling capacity [6]. The time required for the occupied receptor to be switched from a signalling to a non-signalling state is relatively short (seconds to minutes) and the phenomenon is referred to as homologous desensitisation. The molecular mechanism behind desensitisation differs within the family of GPCRs, but has been shown to include phosphorylation [7], arrestin binding [6, 8] and direct binding of the receptor to the cell cytoskeleton and by that a physical separation from the G-protein [5, 9]. The desensitized state can be induced also in neighbouring non-occupied receptors with different agonist specificity, a phenomenon that has an inbuilt receptor hierarchy and is referred to as heterologous desensitisation [5, 10]. The hierarchy is illustrated by the fact that binding of the formylated peptide f-Met-Leu-Phe (fMLF) to its receptor, FPR1, leads to a desensitization not only of this receptor, but also of the receptors for IL-8 [5, 10]. Desensitisation is believed to be of prime importance for recruitment of cells to inflammatory sites as well as for termination of the inflammatory response [10].

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The desensitization phenomenon has been widely studied and used/exploited to characterize new receptor agonists and to determine receptor hierarchy. We have, in a series of studies that characterize new neutrophil receptor agonists [11, 12], determined desensitization using release of superoxide anions as the read out system. This method has also been used to determine receptor preferences for known agonists [13] or to disclose receptor hierarchies among the different receptors [5]. The transient rise in intracellular Ca^{2+}($[Ca^{2+}]_i$) induced by occupied chemoattractant receptors is another, more commonly used, read out system in these type of studies [14, 15]. In our hands neutrophil desensitization in the NADPH-oxidase read out assay systems can, however, not always be reproduced in the $[Ca^{2+}]_i$ read out system. Our aims with this study were to exhibit the differences between desensitization experiments using superoxide production and changes in $[Ca^{2+}]_i$ as read-out systems, and to disclose the mechanism responsible for the discrepancy between the systems. We show that the difference between the two systems is dependent on the choice of agonist, and that inactivation of the agonist in one system but not the other leads to contrasting results. Agonist inactivation is achieved through a myeloperoxidase (MPO)/hydrogen peroxide catalyzed reaction, and radical scavengers or an inhibitor of MPO could be added to the $[Ca^{2+}]_i$ measuring system, in order to eliminate this problem.

Material and methods

Peptides and reagents

The formylated peptide N-formylmethionyl-leucyl-phenylalanine (fMLF) was from Sigma Chemical Co. (St. Louis, MO). A FPR1-activating hexapeptide derived from annexin-1 (unpublished data) was synthesised by and HPLC purified by KJ Ross-Pedersen AS (Klampenborg, Denmark). The peptides were dissolved in dimethyl sulfoxide (DMSO) and stored at -70°C until use. Further dilutions were made in H_2O and buffer for working solutions. Isoluminol and sodium azide was from Sigma Chemical Co. (St. Louis, MO). HRP, superoxide dismutase (SOD), catalase and bovine serum albumin (BSA) were from Roche diagnostics GmbH (Mannheim, Germany). MPO was a generous gift from Inge Olsson, Department of Hematology, Lund University.

Isolation of neutrophils

Neutrophils were isolated from freshly prepared buffy coats (The Blood Center, Sahlgrenska University Hospital, Gothenburg) obtained from healthy blood donors. After removal of erythrocytes through dextran sedimentation, the leukocyte-rich supernatant was carefully layered onto Ficoll-Paque (Lymphoprep, Nyegaard, Norway). After centrifugation at 380 × g for 30 minutes, the pellet was washed two times in buffer without calcium and re-suspended in Krebs-Ringer phosphate buffer (KRG, pH 7.3; 120 mM NaCl, 5 mM KC1, 1.7 mM KH_2PO_4, 8.3 mM $NaHPO_4$ and 10 mM glucose) supplemented with Ca^{2+} (1 mM) and Mg^{2+} (1.5 mM). The cells were kept on ice until used in experiments.

Culturing of PLB-985 and PLB-985 X-CGD cells

PLB-985 is a human diploid myeloid leukemia cell line with granulocytic and monocytic differentiating capacity [16]. The PLB-985 cells used in this work were kindly provided by Mary Dinauer (Indianapolis, IN, USA). The PLB-985 X-CGD cells were prepared by homologous recombination as described in [17]. The cells were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum, penicillin (100 units/ml), streptomycin (100 ug/ml) and L-glutamine (2 mM), in a humidified incubator at 37°C under an atmosphere of 5% CO_2/ 95% air. Differentiation towards the granulocytic phenotype was carried out by exposing cells at a density of 2×10^5 /ml to 1% DMSO for 5 days. Differentiated cells were washed and counted before use in various assay systems.

NADPH-oxidase activity measurements

The NADPH-oxidase activity of cells was recorded using an isoluminol-enhanced chemiluminescence (CL) system [18, 19]. The extracellular CL activity was measured in a multilabel reader LB940 Mithras (Berthold Technologies, Bad Wildbad, Germany) using 96 well plates with 200 uL reaction mixture per well. The mixture contained neutrophils or PLB-985 cells (10^6 cells/well), horseradish peroxidase (HRP; a cell impermeable peroxidase; 4 U) and isoluminol (a cell impermeable CL substrate; 2×10^{-5} M). The plates were equilibrated at 37°C for 5 min, after which the stimulus (10 µL) was added by programmed injection. The light emission was recorded continuously.

Determination of changes in cytosolic calcium by fluorometry

Cells at the density of 1-3 × 10^6 cells/ml were washed with KRG without Ca^{2+}. The cell pellets were resuspended at a density of 2×10^7

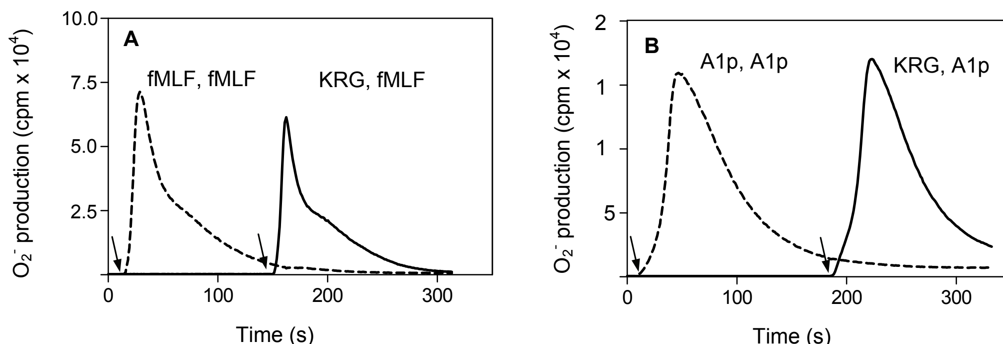

Figure 1. Desensitisation of the neutrophil N-formyl peptide receptor measured by oxygen radical production. Neutrophils were preincubated for 5 min in 37°C. The cells were then stimulated with (A) fMLF (10^{-7} M; broken line) (B) A1p (5 µM; broken line) or a buffer control (solid line). When the radical production of the stimulated cells returned to a background level these cells and the control cells were re-stimulated with (A) fMLF (10^{-7} M) or (B) A1p (5 µM). This second addition of stimulus induced an oxidative burst in the control cells, but the cells that responded to the first challenge were desensitized to further activation. The extracellular release of superoxide anion was monitored with the use of an isoluminol amplified chemiluminescence system.

cells/ml in KRG containing 0.1 % BSA and loaded with 2 μM Fura 2-AM (Molecular Probes, Eugene, OR) for 30 min, at RT. Cells were then diluted to twice the original volume with RPMI 1640 culture medium without phenol red (PAA Laboratories GmbH, Austria) and centrifuged. Finally the cells were washed once in KRG, and re-suspended in KRG at a density of 5×10^7 cells/ml. The amount of cells used in the assay was 10^6 cells/well in a 96 well plate. Calcium measurements were carried out with a multilabel reader LB940 Mithras (Berthold Technologies, Bad Wildbad, Germany) with excitation wavelengths of 340 and 380 nm, an emission wavelength of 509 nm. The transient rise was presented as a ratio between the fluorescence intensities received, the values for 340 nm as numerator and 380 nm as denominator.

Determination of changes in cytosolic calcium by flow cytometry

Intracellular calcium mobilization was measured essentially according to the method described by Partida-Sanchez *et al.* [20]. Cells (3×10^6) were re-suspended in cell-loading medium (KRG with Ca^{2+} + 1% FCS) and loaded with the fluorescent dyes Fluo-3 AM (4 μg/mL) and Fura Red AM (10 μg/mL; Molecular Probes) for 30 min at 37°C. The cells were then washed and resuspended in KRG with Ca^{2+}. For 5 min prior to analysis, the cells were warmed to 37°C in a water bath. Intracellular Ca^{2+} levels were assessed by dual flow cytometry (FACScan). The fluorescence emission of Fluo-3 was monitored in the FL-1 channel and that of Fura Red in the FL-3 channel. First, baseline fluorescence was established. Next, the tube was rapidly removed, the stimulant added, and fluorescence emission was recorded in the tube restored to the flow cytometer. The relative Ca^{2+} concentration was expressed as the ratio between Fluo-3 and Fura Red (FL-1/FL-3) MFI over time.

Results

Desensitization of the N-formyl peptide receptor (FPR1) determined as a loss in the ability of fMLF to trigger production of superoxide anions

Activation of the neutrophil NADPH oxidase, induced by agonists to FPR1, leads to a production and secretion of superoxide anions (Fig 1). The burst in activity induced by the formylated peptide fMLF persists at the most for 3 to 4 minutes, and the rapid termination of radical release is partly due to desensitisation of the receptor involved [6]. The desensitization phenomenon can be illustrated by a sequential stimulation i.e., adding a second dose of the agonist to cells that have just responded with radical release to fMLF. A second dose of fMLF to cells that have returned to base line, does not induce a new burst in activity; the cells are non-responding -- desensitized (Fig 1a). The desensitized state was also induced when fMLF was exchanged for another FPR1 selective agonist (Fig 1b), a hexapeptide derived from the calcium regulated protein annexin I (A1p) [21] (Movitz *et al.* unpublished data).

Desensitization determined with the rise in intracellular calcium as the read-out system

In order to determine if desensitization, so obvious when using activation of the NADPH-oxidase as the read-out system (Fig 1), can be observed also when a rise in intracellular calcium ($[Ca^{2+}]_i$)is used as the read-out system, Fura 2 loaded neutrophils were stimulated in sequence with two doses of fMLF. The changes in fluorescence were recorded, and the initial rise in $[Ca^{2+}]_i$ induced by fMLF rapidly declined but the non-responding state characterizing desensitized cells was not induced. Contrary to what is described above, fMLF induced a legible calcium response also when the cells had experienced the same peptide a couple of minutes earlier (Fig 2).

When neutrophils were triggered with the annexin I peptide, the cells were desensitized, but not only to this agonist but also to fMLF (shown for repeated additions of A1p in the inset of Fig 2). This suggests that induction of the desensitized state is dependent not only on the read out system but also on the agonist used to obtain desensitization.

Desensitization and the role of oxidants

The two measuring systems used are very similar, but there is one fundamental difference between the two systems; the system designed to follow production of superoxide anions contains an enzyme (horseradish peroxidase; HRP) and an amplifier of the luminescence reaction (isoluminol) in addition to cells, buffer and stimulus. These two additives (HRP and isoluminol) are required to determine NADPH-oxidase activity, but possibly at the same time consume and neutralize the ROS generated by the neutrophils. An experimental system in which the change in $[Ca^{2+}]_i$ was determined in the presence of HRP and isoluminol, differed from the

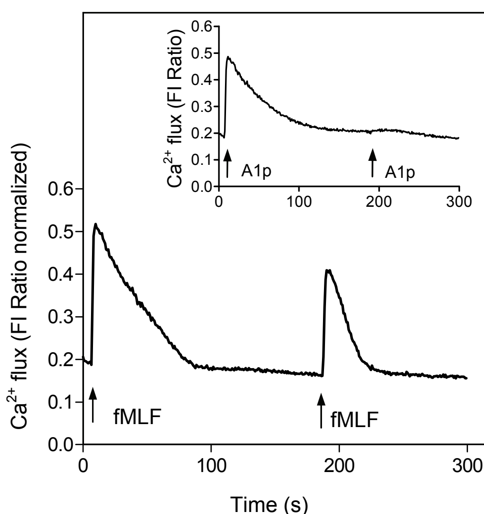

Figure 2. Desensitisation of the neutrophil N-formyl peptide receptor measured by rise of intracellular calcium. Neutrophils were loaded with Fura 2-AM for 30 min in room temperature, washed and resuspended in KRG. After incubation in 37ºC for 5 min the cells were stimulated with fMLF (10^{-7} M) or a hexapeptide derived from Annexin-1 (A1p; 5 μM; inset) which are both agonists for FPR1. The fluorescence ratio was monitored, and both stimuli gave a transient rise in intracellular calcium in response to the first stimulation. After 180 s the cells were again exposed to the same peptides. In the case of the Annexin hexapeptide the second stimulation of the cells gave a clearly diminished response, which indicates a desensitisation of the receptor. This was not seen for fMLF. One reperesentative experiment out of six is shown.

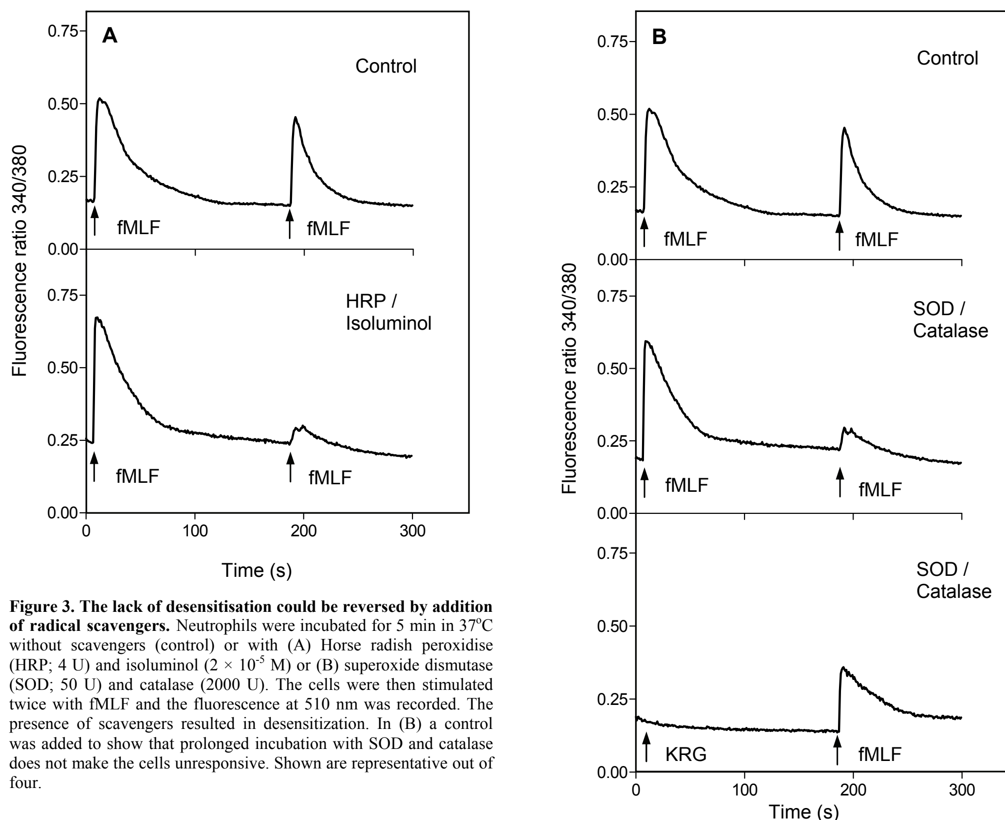

Figure 3. The lack of desensitisation could be reversed by addition of radical scavengers. Neutrophils were incubated for 5 min in 37°C without scavengers (control) or with (A) Horse radish peroxidise (HRP; 4 U) and isoluminol (2×10^{-5} M) or (B) superoxide dismutase (SOD; 50 U) and catalase (2000 U). The cells were then stimulated twice with fMLF and the fluorescence at 510 nm was recorded. The presence of scavengers resulted in desensitization. In (B) a control was added to show that prolonged incubation with SOD and catalase does not make the cells unresponsive. Shown are representative out of four.

standard system in that the desensitized state was induced by fMLF (Fig 3a). Taken together the results imply that the production of oxygen radicals is a key factor that could explain the discrepancy between the two readout systems.

Reactive oxygen species are of importance, but not solely responsible for the lack of desensitisation

It has been shown that methionyl-containing peptides can be oxidized by ROS and such a modification inactivates the peptides [22, 23]. To determine the role of ROS as the basis for the lack of desensitisation, two different experimental approaches were used. In the first experimental set up, neutrophils were sequentially activated with fMLF in the presence of the ROS scavengers superoxide dismutase (SOD; an enzyme that dismutates superoxide to hydrogen peroxide) and catalase (an enzyme that removes hydrogen peroxide). Neutrophils that were re-activated by fMLF in the presence of SOD and catalase regained the pattern of desensitisation in the $[Ca^{2+}]_i$ assay system, and these cells were thus non-responding to the second dose of fMLF (Fig 3b).
The second approach to disclose the role of ROS was to use a variant of the cell line PLB-985 [16], that lacks a functional NADPH-oxidase due to a mutation in one of the oxidase components, $gp91_{phox}$ (PLB-985 X-CGD) [17]. The differentiated "wild type" cells express both FPR1 and a functional oxidase (Fig 4a). On the other hand, PLB-985 X-CGD cells express the receptor but lack the ability to produce oxygen radicals through the NADPH-oxidase (Fig. 4a). When the PLB-985 X-CGD cells were triggered repeatedly with fMLF, the cells responded with a transient $[Ca^{2+}]_i$ to the first dose, but were desensitized and therefore non-responding to the second dose of the agonist (Fig 4b). The desensitized state was, however, induced also with the wild-type, superoxide producing, PLB-985 cells (Fig 4b).
Earlier findings show that the ROS work together with MPO (an azurophil granule constituent) to inactivate fMLF [22, 23]. This suggests that MPO might be a limiting factor in our experimental system and in order to determine this, the purified enzyme was added to PLB-985 cells prior to activation with fMLF. The pattern of the $[Ca^{2+}]_i$ response was now almost identical to that of normal neutrophils (Fig 5a), when the PLB-985 cells were triggered with fMLF in the presence of MPO. The ROS-deficient cells were still desensitized to fMLF, despite the presence of MPO (Fig 5b).

Inhibiting MPO in neutrophils reveals the desensitisation pattern

In order to determine the role of MPO in the calcium measuring system, sodium azide (an inhibitor of the

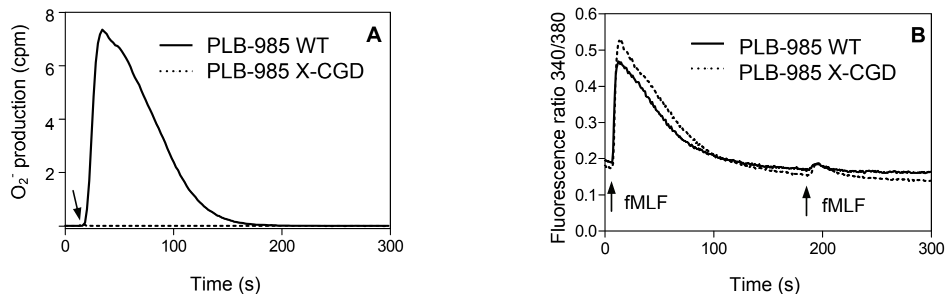

Figure 4. Desensitisation of FPR1 in PLB-985 and PLB-985 X-CGD cells differentiated towards the granulocytic lineage. (A) After washing the differentiation status of the cells were verified by measuring of radical production. The PLB-985 (solid line) and PLB-985 X-CGD (broken line) cells were pre-warmed and stimulated with fMLF (10^{-7} M) and the extracellular release of radicals was recorded. The PLB 985 X-CGD cells were

enzyme [24]) was included in the measuring system with normal neutrophils. In the presence of azide the cells were desensitized to fMLF after the initial challenge with this agonist (Fig 6), and the response pattern thus resembles that seen in the presence of radical scavengers (Fig. 3b).

A change in the state of desensitization is achieved by lowering the cell numbers

In order to be able to follow the change in $[Ca^{2+}]_i$ in our standard assay system with Fura 2 loaded cells, we have to use a rather dense neutrophil population (more than 10^6 cells/ml), and in such a population the local concentrations of ROS and MPO can be expected to be rather high. By using another labeling technique combined with an analysis of the response in individual cells [20] we could determine the response also in dispersed cell populations. The same pattern (lack of desensitization) was obtained with this technique when a dense neutrophil sample was activated with fMLF (Fig 7a), but there was a shift towards desensitization when the concentration of cells in the measuring vial was lowered (Fig 7a-c).

MPO-derived ROS can be neutralized/scavenged by serum proteins

We found that an alternative approach to eliminate the described experimental problem is to add a competitive substrate for the MPO-derived ROS. We show that addition of BSA (0.5% w/v) or normal human serum (NHS; 10% v/v) to the calcium measuring system, allows for an induction of a desensitized state also in the

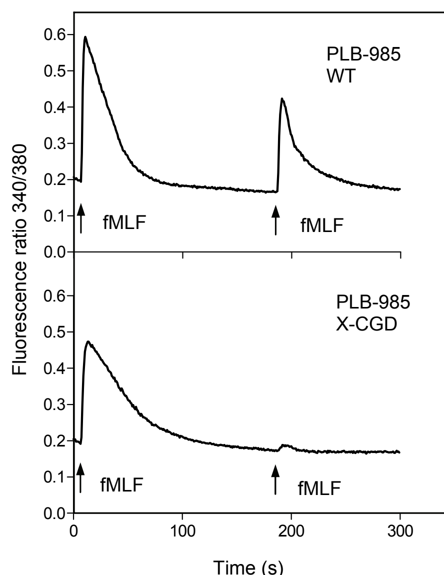

Figure 5. Addition of MPO prevents desensitisation of FPR1 in PLB-985 but not PLB-985 X-CGD cells. After loading with Fura 2-AM the cultured cells were washed and incubated with MPO (100 µg/ml) for 5 min in 37°C. The PLB-985 and PLB-985 X-CGD cells were then stimulated with fMLF (10^{-7} M). When the fluorescence ratio returned to baseline the cells were challenged once more with the same concentration of fMLF. Only the PLB 985 X-CGD cells were desensitized to the second stimulation; shown are one representative experiment out of three.

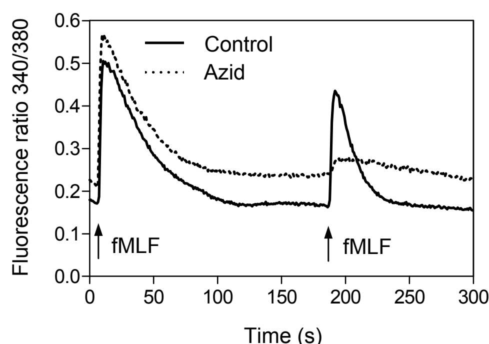

Figure 6. Azide treatment restores desensitisation of FPR1 in neutrophils by inhibiting production of MPO-derived ROS. Neutrophils were isolated according to protocol and then loaded with Fura2-AM. After washing with KRG the cells were incubated in the presence or absence of azide (0.5 mM) and then stimulated twice with fMLF (10^{-7} M). The untreated neutrophils responded to the second activation while the azide treated cells were desensitized and did not give a radical burst. Shown are representative out of three.

fluorometry $[Ca^{2+}]_i$ measuring system with a dense population (Fig 8).

Discussion

The ability of cells to become non-responsive (desensitized) to chemoattractants is a crucial process *in vivo*. In order to be able to migrate chemotactically in a gradient of an attractant, phagocytic cells gradually become non-responsive to lower or unchanged concentrations of the guiding chemoattractant. Desensitisation is also of importance for the limiting and resolution of inflammation. In the field of receptor biology desensitisation is frequently used as an effective *in vitro* tool [13, 14, 25]. Here we describe some difficulties in such *in vitro* studies, related to the technique and agonist used to study neutrophil desensitization. We disclose the background to the problem, and we also point out different solutions by which the problem can be avoided.

The formylpeptide receptor (FPR1) expressed in neutrophils recognises the prototype agonist fMLF as well as a hexapeptide derived from the calcium regulated protein annexin I [26]. Neutrophils activated with the annexin I peptide become desensitized to a second stimulation with the same peptide or with fMLF. When cells are first triggered with fMLF, they become desensitized to a second dose of agonist when using one read out system (release of ROS) but not when using another (the transient rise in intracellular calcium). The lack of desensiti-

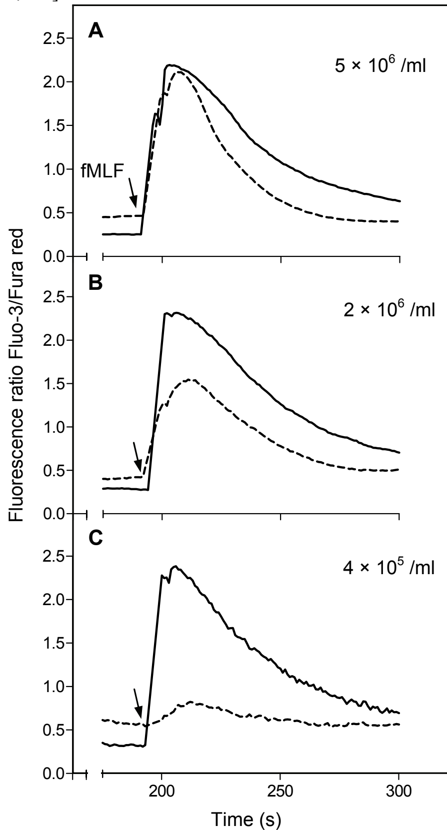

Figure 7. Desensitisation in neutrophils in a flow cytometry based method with decreased cell concentrations. Fluo-3 AM and Fura red AM loaded neutrophils 5 × 10^6 /ml (A), 2 × 10^6 /ml (B), or 4 × 10^5 /ml (C) were incubated at 37°C for 5 min. The baseline fluorescence was established for 20s, and then the first treatment by fMLF (10^{-7} M) (broken line) or buffer control (solid line) was performed (not shown in figure). After 3 min both control cells and fMLF stimulated cells were challenged with fMLF (10^{-7} M) and changes in cytosolic Ca^{2+} levels were determined through measurement of the emitted fluorescence by FACS analysis. A ratio was then calculated using the mean fluorescence for Fluo-3 as the numerator and Fura red as the denominator. The figure contains data obtained from the second stimulation for every cell concentration. Shown are representative out of three.

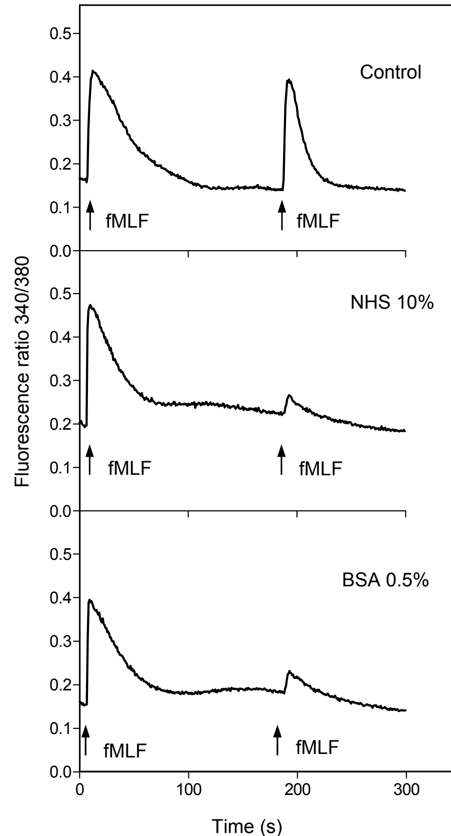

Figure 8. The lack of desensitisation could be reversed by addition of proteins. Neutrophils were incubated for 5 min in 37°C without additions (control), with bovine serum albumin (BSA; 0.5%w/v) or normal human serum (NHS; 10% v/v). The cells were then stimulated twice with fMLF (10^{-7}M) and the fluorescence at 510 nm was recorded. In the presence of proteins cells were desensitized to the second stimulation. Shown are representative out of three.

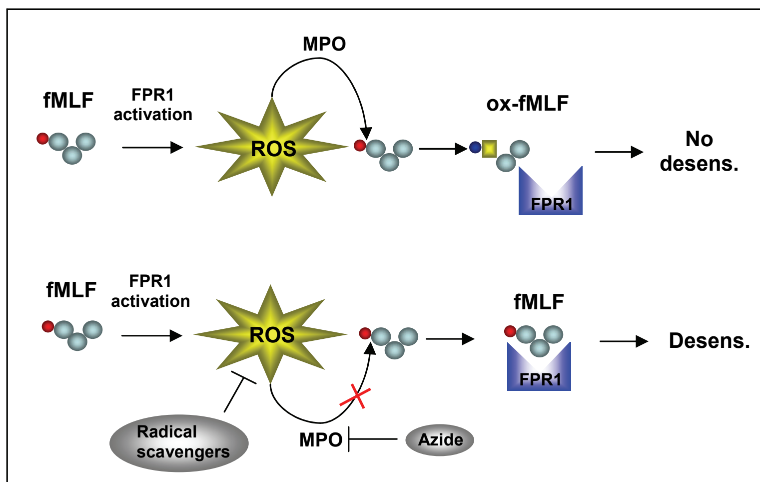

Figure 9. Suggested mechanism behind the lack of desensitisation in *in vitro* calcium studies.

zation was found to be due to inactivation of the formylated peptide by the MPO/ hydrogen peroxide system (Fig 9). It has been shown earlier that fMLF can trigger its own inactivation by a mechanism that involves both the granule protein MPO and hydrogen peroxide generated by the superoxide releasing NADPH-oxidase [22]. The annexin I peptide used in this study triggers neutrophils to generate superoxide and hydrogen peroxide, but the suggested target for the MPO/hydrogen peroxide -system (a methionyl group) is missing in this peptide. The molecular background to why cells become desensitized when triggered with the annexin I peptide but not fMLF, is that the agonist is present and active also when the direct cellular response has ceased (Fig 9). In order to detect the desensitized state in an experimental system in which the agonist trigger its own inactivation is thus to, in one way or another, neutralize the MPO/ hydrogen peroxide -system. This is by necessity achieved in an experimental system in which measurement of the released reactive oxygen species is the basis for detection of the cellular response.

Taken together all our data suggest that the difference between the two read-out systems used is due to the ability of the MPO/hydrogen peroxide to inactivate methionyl-containing peptides. In support for this is that the same pattern of desensitization, was obtained in the two read-out systems if an inhibitor/scavenger such as SOD/catalase or azide was included in the $[Ca^{2+}]_i$ read out system (Fig 9). The effects of the MPO/hydrogen peroxide system can also be minimized by performing the experiments with a very sparse cell suspension, in which no inhibitory concentrations of MPO/oxidants are reached. It is thus possible to obtain the same desensitization pattern without any addition of scavengers/ inhibitors as illustrated with the $[Ca^{2+}]_i$ – measurements using Fluo-3/Fura red labeled cells and flow cytometry. The addition of (serum) proteins as substituting targets for oxidation also reduces the influence of the MPO/ hydrogen peroxide system.

Going through published data on chemoattractant receptor desensitization, it is obvious that when the read out system used is the transient rise in $[Ca^{2+}]_i$, it is quite uncommon that neutrophils have been the preferred cell. The experiments have instead been performed with monocytes or cells that express a cloned receptor [14, 15, 27]. These cells with cloned receptors in most cases lack the basic functional repertoire of the neutrophil and in most cases lack MPO. It has been shown that also monocytes contain lower amounts of MPO [28], which might indicate that the problem described would be less pronounced with these cells. Experiments performed a mixed peripheral blood mononuclear (PBMC) population of cells confirmed this; the results obtained with these cells were similar to those obtained with the wild type PLB cells. The fMLF triggered cells were desensitisation to a second stimulation with the second dose of the same agonist, but when MPO was added to the system, the desensitized state was abolished (data not shown).

In summary, we describe a major discrepancy in the pattern of desensitization using two common techniques to follow neutrophil responses to a set of receptor specific agonists *in vitro*. We also give some alternative solutions to the problem by showing that comparable results are obtained with the NADPH-oxidase read out system and the $[Ca^{2+}]_i$ read out system if the effects of the MPO-derived oxygen radicals are reduced/ eliminated in the latter system. This could be achieved either by use of a method where the sensitivity allows a lowering of cell concentration in the bulk or by addition of (serum) proteins to the experimental setup.

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Paper IV

A Monocyte-Specific Peptide from Herpes Simplex Virus Type 2 Glycoprotein G Activates the NADPH-Oxidase but Not Chemotaxis through a G-Protein-Coupled Receptor Distinct from the Members of the Formyl Peptide Receptor Family[1]

Lars Bellner,[2,3]* Jennie Karlsson,[2]* Huamei Fu,* François Boulay,† Claes Dahlgren,* Kristina Eriksson,[4]* and Anna Karlsson*

We have recently identified a peptide derived from the secreted portion of the HSV-2 glycoprotein G, gG-2p20, to be proinflammatory. Based on its ability to activate neutrophils and monocytes via the formyl peptide receptor (FPR) to produce reactive oxygen species (ROS) that down-regulate NK cell function, we suggested it to be of importance in HSV-2 pathogenesis. We now describe the effects of an overlapping peptide, gG-2p19, derived from the same HSV-2 protein. Also, this peptide activated the ROS-generating NADPH-oxidase, however, only in monocytes and not in neutrophils. Surprisingly, gG-2p19 did not induce a chemotactic response in the affected monocytes despite using a pertussis toxin-sensitive, supposedly G-protein-coupled receptor. The specificity for monocytes suggested that FPR and its homologue FPR like-1 (FPRL1) did not function as receptors for gG-2p19, and this was also experimentally confirmed. Surprisingly, the monocyte-specific FPR homologue FPRL2 was not involved either, and the responsible receptor thus remains unknown so far. However, the receptor shares some basic signaling properties with FPRL1 in that the gG-2p19-induced response was inhibited by PBP10, a peptide that has earlier been shown to selectively inhibit FPRL1-triggered responses. We conclude that secretion and subsequent degradation of the HSV-2 glycoprotein G can generate several peptides that activate phagocytes through different receptors, and with different cellular specificities, to generate ROS with immunomodulatory properties. ***The Journal of Immunology,* 2007, 179: 6080–6087.**

Herpes simplex virus 2 is a sexually transmitted pathogen that infects the genital tract mucosa and is the most common causative agent of genital ulcer disease in humans. Once HSV-2 infects the epithelium and replicates, it can be transmitted to the sacral ganglia via nerve axons and establish a latent infection, which in association with factors such as stress, fever, UV-irradiation, and immunosuppression can reactivate and cause recurrent disease (1, 2). Professional phagocytes (neutrophil granulocytes and monocytes/macrophages) constitute an important first line of defense against microbial intruders, including many viruses. Their potential role in HSV infection is suggested by studies showing that reduced numbers of granulocytes, or functional defects in these cells, may lead to severe and recurrent infections due to a deficiency in the control of HSV replication (3, 4). Functional restoration of the impaired cells is also associated with a recovery of the infected host (4).

We recently identified an HSV-2 peptide, named gG-2p20, which possesses proinflammatory properties in being an activator of professional phagocytes (5). Based on our results, we introduced a new concept for the role of inflammatory cells in HSV-2 pathogenesis; the fact that gG-2p20-activated phagocytes could impair NK-cell function suggested that this HSV-2-derived proinflammatory peptide may contribute to the survival of virus-infected cells and thereby be of immunomodulatory importance in HSV-2 pathogenesis (5). We were also able to identify both the receptor through which the HSV-2 peptide triggered its proinflammatory activity and the mechanism by which the NK cell function was impaired. The triggering receptor was identified as one of the classical pertussis toxin sensitive G-protein coupled chemoattractant receptors (GPCR)[5] known as the formyl peptide receptor (FPR) (6). This is a high-affinity pattern recognition receptor with the ability to track not only formylated peptides released from bacteria, but also virus-derived proteins, e.g., peptides from the envelope proteins of HIV-1 (7) or the earlier mentioned peptide from HSV-2 (5), as well as a number of different endogenous agonists (8). In addition to FPR, there are two other members of the formyl peptide receptor

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[5] Abbreviations used in this paper: GPCR, G-protein coupled chemoattractant receptors; CL, chemiluminiscence; FPR, formyl peptide receptor; FPRL1, FPR like-1; FPRL2, FPR like-2; KRG, Krebs-Ringer phosphate buffer; N-t-Boc-MLF, N-t-butoxycarbonyl-methionyl-leucyl-phenylalanine; ROS, reactive oxygen species; sgG-2, secreted portion of HSV-2 glycoprotein G.

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family, formyl peptide receptor like-1 and -2 (FPRL1, FPRL2). The FPR family members are differently expressed in phagocytes, with neutrophils expressing FPR and FPRL1, and monocytes/macrophages expressing all three. A number of agonists that trigger cells through FPRL1 have been described whereas the monocyte-specific family member FPRL2 was until very recently considered an orphan receptor (9).

Ligation of the FPR family receptors with specific agonists (including gG-2p20 for FPR) results in oxygen radical generation by activation of the NADPH-oxidase, an electron transport system assembled in the plasma membrane during cell activation in both neutrophils and monocytes/macrophages (10). The primary products of the assembled oxidase are superoxide anions and hydrogen peroxide, and these reactive oxygen species (ROS) can function as regulators of other immune reactive cells, providing a molecular explanation behind the functional changes seen as apoptotic/necrotic processes in NK cells exposed to activated phagocytes (11–14).

The HSV-2 glycoprotein G, from which the proinflammatory peptide gG-2p20 was derived, is the only HSV-2 envelope protein to be cleaved post-translationally, generating two proteins of which one has the unique property of being secreted from the infected cells (sgG-2) (15–18). Previous to our study of the sgG-2-derived peptide, indicating proinflammatory activity, the secreted protein had not been ascribed a specific function. In this study, we have continued our investigation on the immunomodulatory properties of peptides derived from the sgG-2 amino acid sequence. In addition to the earlier identified neutrophil/monocyte activating peptide, we found one peptide, gG-2p19, which induced NADPH-oxidase activation in monocytes, but not neutrophils. Although the activity induced by gG-2p19 was monocyte specific and mediated through a pertussis toxin-sensitive GPCR, it was not triggered by FPRL2 (nor by FPR or FPRL1), which is the only member of the FPR family that is expressed solely in monocytes. Intriguingly, gG-2p19 was not a monocyte chemoattractant, suggesting that the receptor involved has a more restricted functional spectrum compared with other GPCRs.

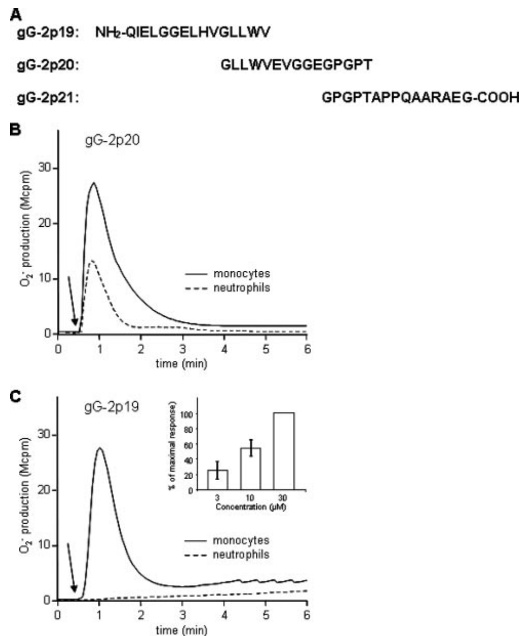

FIGURE 1. The gG-2p19 induces activation of the monocyte (but not neutrophil) NADPH-oxidase. *A*, Schematic representation of the amino acid sequences of gG-2p19, gG-2p20, and gG-2p21. *B* and *C*, Monocytes (solid lines) and neutrophils (dashed lines) were activated with gG-2p20 (30 μM) (*B*) and gG-2p19 (30 μM) (*C*) and the following superoxide anion release was measured continuously for 4 min by isoluminol-ECL (CL). The amount of superoxide anion is presented as 10^6 cpm (Mcpm). Arrows indicate the time of addition of peptide. The figure shows one representative experiment of three. The amount of superoxide released from monocytes in response to different concentrations of gG-2p19 was measured (n = 3–4; mean ± SD) (*C*, *inset*). No NADPH-oxidase activity was detected for gG-2p21 (data not shown).

Materials and Methods

Peptides and reagents

Three 15-mer synthetic peptides (Fig. 1*A*) from the secreted portion of the HSV-2 glycoprotein G (sgG-2) of strain HG52 (19) were synthesized as described earlier using F-moc synthesis chemistry (20) and were from two different sources, KJ Ross-Petersen (used in Fig. 1) and GenScript (used in Figs. 2–8). The purity of the peptides was found to be ≥95% by reverse phase HPLC. The HSV-2 gG-2 peptides were dissolved in either water, sodium bicarbonate ($NaHCO_3$ 0.02 M in H_2O), or DMSO to 6 mM and stored at −70°C until use.

The hexapeptides WKYMVM and WKYMVm, which were synthesized and HPLC purified by KJ Ross-Petersen, and the formylated tripeptide formyl-methionyl-leucyl-phenylalanine (fMLF), which was purchased from Sigma-Aldrich, were dissolved in DMSO (0.01 M in H_2O) to 0.1 mM and stored at −70°C until use. Subsequent dilutions of all peptides were made in Krebs-Ringer phosphate buffer (KRG (pH 7.3); 120 mM NaCl, 5 mM KCl, 1.7 mM KH_2PO_4, 8.3 mM NaH_2PO_4, and 10 mM glucose) supplemented with Ca^{2+} (1 mM) and Mg^{2+} (1.5 mM).

Cyclosporin H was provided by Novartis Pharma. Ficoll-Paque was obtained from Amersham Biosciences *N-t*-butoxycarbonyl-methionyl-leucyl-phenylalanine (*N-t*-Boc-MLF), isoluminol, and PMA were obtained from Sigma-Aldrich, and HRP was obtained from Boehringer Mannheim. The WRW_4 was from GenScript. The gelsolin-derived peptide PBP10 (residues 160–169, aa sequence QRLFQVKGRR) was prepared by solid phase peptide synthesis and coupled to rhodamine as described earlier (21).

Separation of human monocytes

Human PBMC were separated from buffy coats from healthy blood donors. After Ficoll-Paque centrifugation, mononuclear cells were separated into lymphocytes and monocytes using counter current centrifugal elutriation as described in detail elsewhere (22). This procedure yielded a fraction with >90% monocytes at a flow rate of 20–22 ml/min. The cells were washed and resuspended in KRG and stored on melting ice until use.

Separation of human neutrophils

Human peripheral blood neutrophils were isolated from buffy coats from healthy blood donors using dextran sedimentation and Ficoll-Paque gradient centrifugation as described (23). The cells were washed and resuspended in KRG and stored on melting ice until use.

Stable expression of FPR, FPRL1, and FPRL2 in undifferentiated HL-60 cells

The stable expression of FPR, FPRL1, and FPRL2 in undifferentiated HL-60 cells has been described previously (24, 25). Transfected cells were cultured in RPMI 1640 (PAA Laboratories GmbH) containing FCS (10%) (PAA Laboratories GmbH), penicillin/streptomycin (1%) (PAA Laboratories GmbH), and G418 (1 mg/ml). The maximal density was maintained below 2×10^6 cells/ml. The cells were passaged to a concentration of 5×10^5 cells/ml ~24 h before use in assays.

Measurement of superoxide release

The NADPH-oxidase activity was determined using isoluminol-ECL chemiluminescence (CL) (26). The CL activity was measured in a six-channel Biolumat LB 9505 (Berthold), using disposable 4-ml polypropylene tubes with a 400-μl reaction mixture containing 4×10^4 cells. The tubes were equilibrated in the Biolumat for 5 min at 37°C, after which the

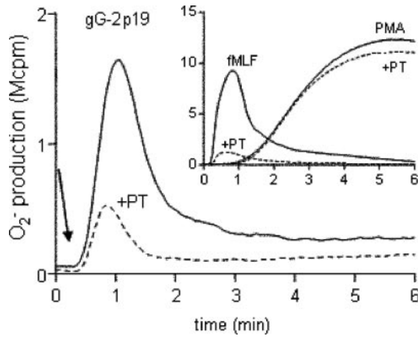

FIGURE 2. The gG-2p19-induced NADPH-oxidase activation in monocytes is pertussis toxin sensitive. Monocytes were preincubated at 37°C for 90 min in the absence (solid line), or presence (dashed line) of pertussis toxin (PT, 500 ng/ml) and were then stimulated with gG-2p19 (30 μM). The superoxide anion release was measured continuously for 6 min, by isoluminol-enhanced CL. To ensure that the PT treatment was successful in inhibiting GPCR-triggered NADPH-oxidase activation without affecting the overall condition of the cells, control experiments were performed (*inset*). The PT treatment successfully inhibited the fMLF-induced response (0.1 μM), while the PT-insensitive PMA-induced response (50 nM) was unaffected. The amount of superoxide anion is presented as 10^6 cpm (Mcpm). The figure shows one representative experiment of three.

stimulus (20 μl) was added. The light emission was recorded continuously, and the light intensity was measured 15 times per minute. The relation between the amount of superoxide produced and the amount of detected light is dependent on the luminometer, but it can easily be determined by a direct comparison of the superoxide dismutase-inhibitable reduction of cytochrome *c* with the integrated value of the superoxide dismutase-inhibitable chemiluminescence (27, 28). With the equipment used, 7.2×10^7 counts were found to correspond to the production of 1 nmol superoxide (using a millimolar extinction coefficient for cytochrome *c* of 21.1).

When experiments were performed with antagonists and inhibitors such as N-t-Boc-MLF, cyclosporin H, WRW_4, or PBP10, these substances were included in the reaction mixture during the 5 min equilibration period. In desensitization experiments, the primary stimulus was added to the reagent tubes on ice or at 37°C, followed by incubation in the Biolumat for 10 min before addition of the second stimulus.

Monocyte chemotaxis

Monocyte chemotaxis was determined using ChemoTX multiwell chambers (Neuro Probe) with a membrane pore diameter of 5 μm, according to

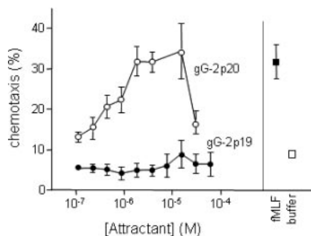

FIGURE 3. The gG-2p19 does not induce chemotaxis in monocytes. Monocyte transmigration in response to gG-2p19 (●), gG-2p20 (○), buffer (□), and fMLF (10 nM, ■) was assayed using a ChemoTX multiwell chamber system. Migration was determined after 90 min incubation by measuring the amount of myeloperoxidase in lysates of migrated cells. Data are expressed as mean per cent migrated cells ± SEM for different peptide concentrations, where maximum is the OD_{450} value obtained from lysates of 10^5 monocytes added directly to the lower chamber.

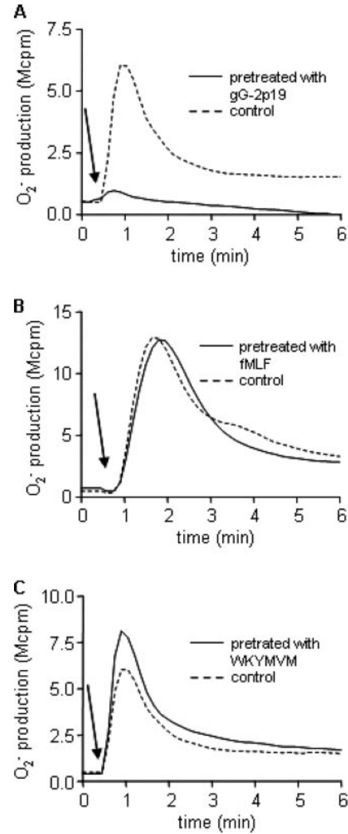

FIGURE 4. Desensitization of the gG-2p19-induced oxidative response. Monocytes were preactivated with gG-2p19 (30 μM; *A*), fMLF (0.1 μM; *B*), or WKYMVM (0.1 μM; *C*), and subsequently challenged with gG-2p19 (30 μM). The oxidative response was followed by isoluminol-amplified CL. The curves show that gG-2p19 is homologously desensitized (*A*) but not heterologously desensitized by neither fMLF (agonist for FPR; *B*) or WKYMVM (agonist for FPRL1/FPRL2; *C*). The amount of superoxide anion is presented as 10^6 cpm (Mcpm). The figure shows one representative experiment of three.

instructions provided by the manufacturer. Cells were allowed to migrate through the membranes toward stimulus added to the lower chambers during a 90-min incubation at 37°C, after which the cells in the lower chamber were lysed with 0.2% cetyltrimethylammonium bromide in PBS with 0.2% BSA. Twenty microliters of each lysate were transferred into a 96-well ELISA plate (MaxiSorp, Nunc) and quantified based on myeloperoxidase activity by the addition of 45 μl peroxidase substrate solution containing 0.1 mg/ml tetramethylbenzidine and 0.04% H_2O_2 in 0.2 M phosphate-citrate buffer (pH 5). The reaction was stopped after 2 h by the addition of 25 μl 1M H_2SO_4 and the absorbance (OD) was measured at 450 nm. Relative migration (%) was obtained by dividing the sample OD_{450} with the OD_{450} of cells added directly to the lower compartment of the multiwell chamber (representing 100% migration). Spontaneous movement, i.e., OD_{450} values obtained from samples with no added stimulus, was used as negative control. As positive control, migration toward fMLF (10^{-8} M) and gG-2p20 (varying concentrations) were used. The OD_{450} values used are means of duplicates.

Cytosolic calcium mobilization

Monocytes or undifferentiated HL-60 cells (2×10^7/ml) stably expressing FPR, FPRL1 (26), or FPRL2 (29) were incubated with fura-2AM (2 μM)

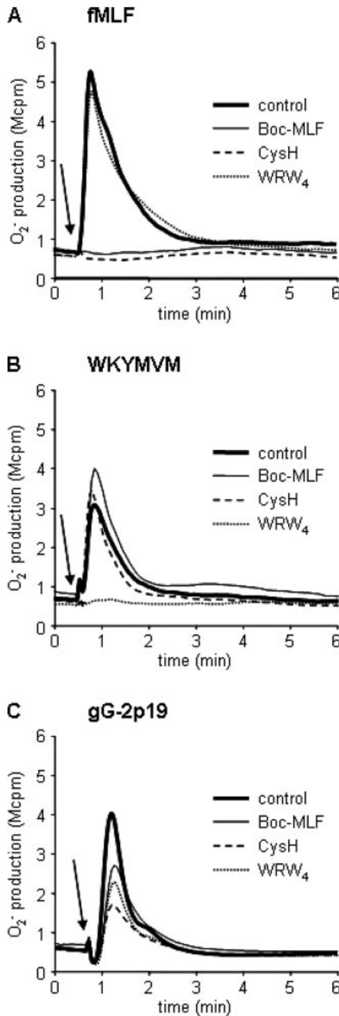

FIGURE 5. Effect of receptor antagonists and inhibitors on the monocyte NADPH-oxidase response to gG-2p19. Cells were preincubated at 37°C for 5 min in the absence (solid line) or presence (dashed line) of the FPR antagonist N-t-Boc-MLF (10 μM; dashed line), the FPR inhibitor Cyclosporin H (0.25 μM; dotted line), or the FPRL1 antagonist WRW_4 (0,5 μM; thin line) and were subsequently stimulated with fMLF (4 nM; *A*), WKYMVM (4 nM; *B*), or gG-2p19 (30 μM; *C*). The amount of superoxide anion is presented as 10^6 cpm (Mcpm). The figure shows one representative experiment of three.

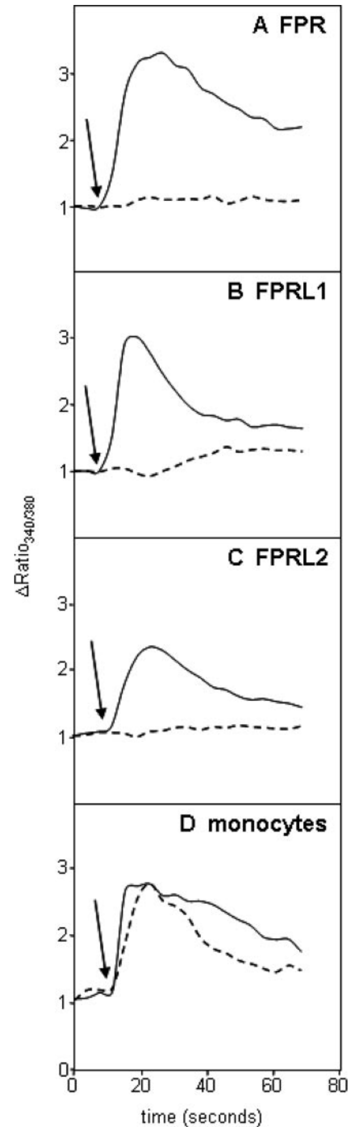

FIGURE 6. The gG-2p19 is not a ligand for any of the formyl peptide receptor family receptors. Fura-2 loaded nondifferentiated HL-60 cells (10^6/ml) overexpressing either FPR (*A*), FPRL1 (*B*), FPRL2 (*C*), or monocytes (*D*; 10^6/ml) were analyzed for mobilization of intracellular Ca^{2+} in response to gG-2p19 (30 μM; bold lines). The FPR/FPRL1/FPRL2 agonists fMLF and WKYMVM (0.1 μM) were used as positive controls (thin lines). Representative Ca^{2+} measurements ($n = 3$) are shown, given as the relative change in Ex_{340}/Ex_{380} ratio.

in calcium free KRG supplemented with BSA (0.1%) at room temperature for 30 min in the dark. Cells were then diluted to twice the original volume with RPMI 1640 culture medium without phenol red (PAA Laboratories GmbH) and centrifuged. The cells were washed once and resuspended to a density of 2×10^7/ml in KRG containing 1.0 mM Ca^{2+}. Cells were equilibrated for 5 min at 37°C, after which gG-2p19 or positive control peptides (fMLF for FPR and WKYMVm for FPRL1 and FPRL2) were added. The fura-2 fluorescence was measured by a luminescence spectrometer (LS50B; PerkinElmer) using excitation wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. Maximal and minimal fluorescence were determined in the presence of Triton X-100 and EGTA/Tris-HCl, respectively. The intracellular calcium levels are shown as $Ex_{340}:Ex_{380}$ ratios (25).

Results

An HSV-2 glycoprotein G-derived peptide triggers the monocyte NADPH-oxidase to produce superoxide anions

Through screening of peptides spanning the whole amino acid sequence of the secreted portion of HSV-2 glycoprotein G, we have

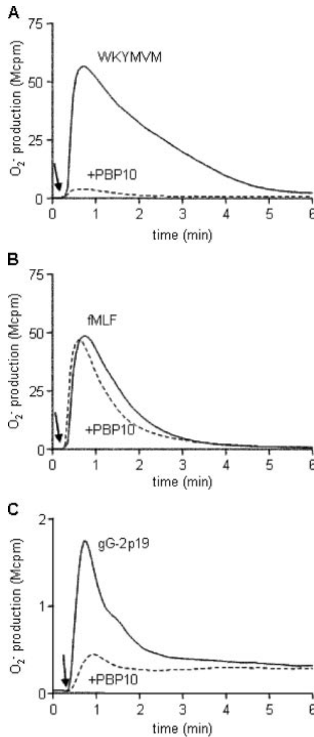

FIGURE 7. The monocyte NADPH-oxidase response to gG-2p19 is inhibited by the gelsolin-derived peptide PBP10. Monocytes were preincubated at 37°C for 5 min in the absence (solid line), or presence (dashed line) of PBP10 (1 μM; inhibitor of the FPRL1-, but not the FPR-induced signaling pathway) and were subsequently stimulated with WKYMVM (0.1 μM; *A*), fMLF (0.1 μM; *B*), or gG-2p19 (30 μM; *C*).The amount of superoxide anion is presented as 10^6 cpm (Mcpm). The figure shows one representative experiment of three.

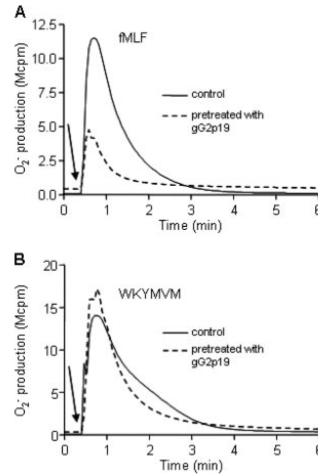

FIGURE 8. Desensitization of the fMLF and WKYMVM responses by gG-2p19. Monocytes were preactivated with gG-2p19 (30 μM; added on ice and incubated at 37°C for 10 min) and subsequently challenged with fMLF (0.1 μM; *A*) or WKYMVM (0.1 μM; *B*). The oxidative response was followed by isoluminol-amplified CL. The curves show that gG-2p19 partially desensitized the fMLF-induced response, whereas the WKYMVM-induced response was unaltered. The amount of superoxide anion is presented as 10^6 cpm (Mcpm). The figure shows one representative experiment of three.

previously identified a peptide, gG-2p20, that exerted downregulatory functions on human NK cells by activating phagocytes to produce ROS (5). In this study, we analyze two neighboring peptides derived from the same protein, overlapping the gG-2p20 sequence with 5 aa in either end of the peptide, and extended with 10 aa to the N-terminal side (gG-2p19) or the C-terminal side (gG-2p21) (Fig. 1*A*). When exposing neutrophils or monocytes to these peptides we found that whereas gG-2p20 activated a response in both monocytes and neutrophils (Fig. 1*B*), gG-2p19 activated a respiratory burst response in monocytes but was without effect on neutrophils (Fig. 1*C*). The gG-2p19-induced oxidase activation in monocytes was concentration dependent (Fig. 1*C*, *inset*). No respiratory burst activity was triggered by gG-2p21 in either of the cells (data not shown).

The NADPH-oxidase activity triggered in monocytes by gG-2p19 is pertussis toxin sensitive

The gG-2p19 induced production of ROS in monocytes (Fig. 1 and 2) with a time course resembling that induced by the earlier described FPR agonists gG-2p20 (Fig. 1 and (5)) and fMLF (Fig. 2; *inset*). The ROS production induced by gG-2p19 was inhibited by preincubation of the monocytes with pertussis toxin, known to specifically block signaling induced by G_i GPCRs (30), suggesting that also gG-2p19 induces activation through a GPCR.

The gG-2p19 is not chemotactic for monocytes

The signals generated by GPCRs in phagocytes usually mediate a chemotactic response. No such response was however triggered by the gG-2p19 peptide, irrespective of the concentration used to trigger the response (Fig. 3). The earlier-described FPR ligands fMLF and gG-2p20 were used as controls, both inducing a potent chemotactic response in monocytes (Fig. 3).

The gG-2p19 does not interact with FPR or FPRL1

The gG-2p19-induced NADPH-oxidase response closely resembled responses induced by other peptides (bacterial and synthetic) acting through different GPCRs, including those of the FPR family. This, together with the fact that the earlier-described HSV-2 peptide gG-2p20 is an FPR agonist, gave us cause to investigate the involvement of FPR family members in the gG-2p19-induced monocyte response. The basis for the first part of this investigation was desensitization protocols using gG-2p19, the FPR agonist fMLF, and the FPRL1/FPRL2 agonist WKYMVM. In these experiments we exploit the fact that agonist binding to a particular receptor induces receptor-specific desensitization and subsequent inability of the cells to respond to the same or a second agonist that ligates the same receptor (29).

Attempts to challenge gG-2p19-pretreated monocytes with the same agonist a second time failed, meaning that the HSV-2 peptide acts through a receptor that can be homologously desensitized (Fig. 4*A*). Cells pretreated with the FPR agonist fMLF were however still able to mount an oxidative response when stimulated with gG-2p19 (Fig. 4*B*), i.e., fMLF did not heterologously desensitize the cells to the HSV-2 peptide. Furthermore, preincubation of the cells with the FPR receptor antagonists N-t-Boc-MLF or

cyclosporin H, both of which completely abrogate fMLF-induced responses (Fig. 5*A*), did not prevent the cells from responding to gG-2p19 (Fig. 5*C*), even though both N-t-Boc-MLF and cyclosporin H pretreatment did affect the magnitude of the gG-2p19-induced responses (Fig. 5*C*). Taken together, we conclude that FPR is not involved in the response to gG-2p19.

No substantial desensitization of the gG-2p19-induced activation could be seen in monocytes first treated with the FPRL1/FPRL2 agonist WKYMVM (Fig. 4*C*), suggesting that the peptide uses neither FPRL1 nor FPRL2. For FPRL1, this is supported by inhibition experiments using the FPRL1-specific antagonist WRW_4 (Fig. 5*B*), which did not prevent a gG-2p19-induced response (Fig. 5*C*).

The gG-2p19 does not trigger any response in transfected cells expressing FPRL2

Because gG-2p19 was a potent activator of the NADPH-oxidase in monocytes (expressing FPR, FPRL1, and FPRL2) but not in neutrophils (which express FPR and FPRL1), FPRL2 was more plausible as a receptor for gG-2p19 than FPR and FPRL1. To determine whether gG-2p19 could activate cells specifically through FPRL2, we investigated the triggering potential of the peptide in HL-60 cells stably transfected to express one or the other of the three FPR family members. In accordance with our desensitization data, gG-2p19 could not trigger any transient Ca^{2+} response in cells expressing either FPR (Fig. 6*A*) or FPRL1 (Fig. 6*B*). The same was true also for FPRL2, i.e., gG-2p19 could not activate the FPRL2-expressing cells (Fig. 6*C*). Undifferentiated HL-60 cells transfected with an irrelevant receptor, CXCR2, do not show a Ca^{2+} response to FMLF or WKYMVm (25). In contrast, gG-2p19 did induce a Ca^{2+} response in purified monocytes (Fig. 6*D*). All taken together, our data indicate that gG-2p19 interacts with a GPCR that is not a member of the formyl peptide receptor family.

The gG-2p19 activation of monocytes is inhibited by PBP10

We have recently shown that the signaling cascades induced in neutrophils by agonists binding to FPR and FPRL1 differ in their sensitivity to the gelsolin-derived, PIP_2-binding peptide PBP10 (31). This membrane-permeable gelsolin-derived peptide specifically inhibits signaling through FPRL1. In an attempt to determine whether the signaling route induced by the gG-2p19 receptor was in any way similar to that induced by FPRL1, we investigated the inhibitory effect of PBP10 on the gG-2p19-induced response in monocytes. The inhibitory profile of PBP10 with respect to FPR and FPRL1 was the same in monocytes as in neutrophils, i.e., the response induced in monocytes by WKYMVM (FPRL1/FPRL2 agonist) was completely inhibited in presence of PBP10 (Fig. 7*A*), whereas the response induced by fMLF (FPR agonist) was unaffected (Fig. 7*B*). Interestingly, the monocyte NADPH-oxidase response induced by gG-2p19 was substantially inhibited in the presence of PBP10 (Fig. 7*C*), indicating that the gG-2p19-binding GPCR shares the PBP10 sensitive signaling route with FPRL1.

Receptor cross-talk and hierarchy

There is a hierarchical receptor cross-talk within the phagocyte GPCR family. Cells desensitized to FPR or FPRL1 agonists are down-regulated not only in the response to their respective receptor-specific agonists but also to a second stimulation with the GPCR agonists IL-8 or platelet-activating factor (32, 33). The fact that desensitization of FPR or FPRL1 does not affect the gG-2p19-induced response suggests not only that monocyte activation by gG-2p19 does not involve the FPR family receptors, but also that the gG-2p19 receptor is not subordinated FPR or FPRL1. However, desensitization experiments, in which the order of the agonists was changed, revealed receptor cross-talk between the gG-2p19 receptor and FPR. We found that the FPR-triggered response was reduced when monocytes were desensitized with gG-2p19 before stimulation with fMLF (Fig. 8*A*). No such cross-talk or inhibition was seen when fMLF was replaced by the FPRL1 agonist WKYMVM (Fig. 8*B*). Our interpretation of these results is that activation of the gG-2p19 receptor cross-desensitizes the FPR, i.e., that the gG-2p19 receptor is hierarchically above the FPR. The fact that FPRL1 differs from FPR by being unaffected by desensitization of the gG-2p19 receptor gives support to the notion that these two receptors, although closely related, differ in their intracellular signaling.

Discussion

A peptide derived from the secreted portion of HSV-2 glycoprotein G has been identified that selectively activates monocytes through a pertussis toxin-sensitive receptor. The peptide, denoted gG-2p19, triggered activation of the superoxide-generating NADPH-oxidase, but was not able to induce monocyte chemotaxis. The main receptor candidate, FPRL2, the monocyte-specific member of the formyl peptide receptor family, was found not to be involved in cell activation. The peptide used a receptor distinct from the formyl peptide receptor family, but the receptor appeared to share at least one crucial step in its signaling pathway with one of the family members, FPRL1, based on the inhibition of the response to gG-2p19 by the PIP_2-binding, gelsolin-derived peptide PBP10, known to block FPRL1 signaling (31).

The strategy used to identify gG-2p19 as a monocyte-activating peptide has been used earlier in the search for neutrophil-activating peptides from the HSV-2 glycoprotein G (5). We then identified gG-2p20 as a chemotactic peptide for both neutrophils and monocytes and we were also able to disclose the identity of the gG-2p20 binding receptor, which was the FPR. We originally used thirty-three 15-mer, partly overlapping peptides covering the entire secreted portion of the HSV-2 glycoprotein G. The fact that the only two proinflammatory peptides, gG-2p19 and gG-2p20, originate from the same part of the protein and contain an identical sequence of five amino acids, suggested to us that they should bind to two closely related receptors.

Because gG-2p19 did not activate neutrophils, the receptor could be differently expressed in neutrophils and monocytes, which made FPRL2 the most obvious candidate. The FPRL2 was originally cloned from a promyelocyte cDNA library using low-stringency hybridization with the FPR cDNA as a probe (34, 35), and it was for a long time regarded as an orphan receptor. In contrast to the other two members of the receptor family (FPR and FPRL1), the expression of FPRL2 is restricted to cells in the monocyte/macrophage linage.

From our data we conclude, however, that the activating signal in monocytes induced by gG-2p19 is not transduced through FPRL2. This conclusion is based on the facts that no signal is generated by gG-2p19 in HL-60 cells transfected to express FPRL2 and that the FPRL2 agonist WKYMWM did not desensitize monocytes to the gG-2p19 peptide. The fact that no activity is induced in FPRL2-expressing HL-60 cells by gG-2p19 strongly indicates that another monocyte-specific receptor is involved, but desensitization or binding inhibition experiments with an FPRL2-specific ligand would of course add strength to the conclusion. Such an agonist has been described (9). We have tried to use the FL2 peptide (with the sequence MLGMIKNSLFGSVETWP WQVL), however, in our experimental systems with FPRL2-expressing cells or monocytes this peptide was without effect (our unpublished data). We can at present only speculate on the reason for this but it has been shown that FL2 induces a strong response

in around 50% of the blood cell donors suggesting that we might have obtained all our samples from nonresponders. More importantly, however, is that these cells still responded to gG-p2–19. We cannot at present explain why FL2 fail to trigger a response in FPRL2 expressed in HL-60 cells, but the receptor might look a bit different when expressed in another cell line such as cho-K1 cells. Even though the identity of the gG-2p19 receptor remains to be disclosed, our results suggest that it is a receptor distinct from FPRL2.

However, we have clues to some characteristics of the gG-2p19 receptor. Firstly, the receptor is a pertussis toxin-sensitive receptor. Pertussis toxin inhibits the G_i group of GPCRs in phagocytes, and cell activation through members of this receptor family normally provokes various biochemical or functional responses which contribute to the defense as well as the immunoregulatory functions of these cells. It is well known that the signals underlying the chemotactic behavior differ from those that regulate granule mobilization and secretory events (36). This is clearly illustrated by the fact that the LTB_4-specific GPCR has the capacity to transduce ligand binding into a chemotactic response without any concomitant secretion of ROS (37). Furthermore, depending on the structure of the ligand, the occupied FPR can transfer the chemotactic signal alone, or combined with the signal responsible for superoxide secretion (36). We found that the gG-2p19 peptide was able to induce ROS production without triggering a chemotactic response, a combination of signals unique for this receptor-ligand pair. Whether the receptor entirely lacks the ability to mediate a chemotactic response, or if the ligand (gG-2p19) triggers a very specific signaling pathway that bypasses the route of chemotaxis, cannot be determined until other ligands for the receptor has been identified.

Secondly, the unknown receptor shares signaling properties with FPRL1 in that it is sensitive to the inhibitory activity of PBP10, a membrane-permeable polyphosphoinositide-binding peptide derived from the cytoskeletal protein gelsolin (21). We have previously shown that PBP10 blocks FPRL1-induced ROS secretion in neutrophils, while having no effect on the neutrophil response to agonists for FPR, C5aR, PAFR, or CXCR (31). The precise mechanism by which PBP10 selectively interferes with signaling by some receptors but not by others remains to be determined in detail, but it is reasonable to believe that the signaling intracellular parts of the two sensitive receptors, FPRL1 and the gG-2p19 receptor, contain structural similarities.

Thirdly, the gG-2p19 receptor cross-talks with other phagocyte GPCRs. The process known as desensitization (homologous as well as heterologous) is most probably important for the limitation or termination of the cellular response to high concentrations of a chemoattractant, avoiding prolonged activation and by that limiting the inflammation-associated tissue damage at an inflammatory site (8). With respect to cross-talk hierarchy, it is obvious that some activators are strong whereas others are weak. The fact that ligation of the gG-2p19 receptor desensitized the hierarchically strong FPR, indicates that the novel receptor is even stronger. Further desensitization studies have to be performed to more precisely place this receptor in the GPCR hierarchy. It should also be kept in mind that yet other gG-2p19 receptor(s) may be present that is responsible for inhibitory signal(s). We have shown earlier this to be the case for a peptide derived from the N terminus of annexin I, a peptide that triggers NADPH-oxidase activation through one receptor, concomitantly with eliciting inhibitory signals through another receptor (38).

We can only speculate on whether gG-2p19 could exert any effects in vivo. However, there should be high levels of sgG-2 at sites with massive local viral replication, i.e., in herpetic lesions, where $>10^{10}$ viral particles are found to be present (5). In such an environment, gG-2p19-induced ROS has the potential to eliminate viral particles, but could also lead to local tissue destruction, enhancing the damage induced by the virus itself. However, ROS secreted from neutrophils and monocytes also have a strong negative impact on cell-mediated immunity. Numerous studies have shown that both the cytotoxic capacity and the survival of NK cells and CTL are impaired in the presence of activated phagocytes, in a process that is reversed by ROS scavengers (11–14). Thus, sgG-2 released from HSV-2-infected cells could, through its interaction with a GPCR (as suggested in this study and in (5)) and subsequent activation of the NADPH-oxidase, contribute to the documented down-regulation of the cellular immune response seen during HSV infection (39–41).

In summary, we have identified a peptide derived from HSV-2 glycoprotein G that induces release of ROS from monocytes through binding to a GPCR. In vitro experiments showed that the gG-2p19 peptide did not induce a chemotactic response, indicating that the receptor involved has a more restricted functional spectrum compared with other GPCRs. Furthermore, we were able to show that the gG-2p19-interacting GPCR shares at least one common signaling pathway with FPRL1 because both these receptors, as opposed to, for instance, FPR, are sensitive to the PIP_2-specific gelsolin-derived peptide PBP10. This is of considerable interest for further elucidation of chemoattractant signal transduction, because FPRL1 is a chemotactic receptor whereas the gG-2p19-binding GPCR is not.

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Disclosures

The authors have no financial conflict of interest.

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