REVIEW

Turning chemoattractant receptors on and off with conventional ligands and allosteric modulators: recent advances in formyl peptide receptor signaling and regulation

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Received: January 27, 2014
Published online: July 15, 2014

Recruitment and activation of neutrophils at sites of infection/inflammation relies largely on the surface expression of G-protein coupled receptors (GPCRs) that recognize chemoattractants. One of these receptors, FPR1, for which formylated peptides generated by bacteria and mitochondria are high affinity agonists, was among the first human neutrophil GPCRs to be cloned. This receptor shares large sequence homologies with FPR2, another neutrophil member of the FPR-family, but with a distinct ligand binding profile. The two FPRs transduce very similar neutrophil responses but possess somewhat different regulatory profiles. The FPRs have served as excellent model receptors in studies attempting to understand not only GPCR related regulation in general, but also receptor signaling in relation to innate immune reactivity and inflammation. Recent research has identified not only a large number of conventional ligands (agonist/antagonists) that regulate FPR activities by binding to surface exposed parts of the receptors, but also a number of membrane penetrating molecules that allosterically modulate receptor function after passing the membrane and interacting with the receptor on the cytosolic side. After activation, FPR signaling is rapidly terminated and the receptors become desensitized, a dormant state that can be achieved by multiple mechanisms. A coupling of the activated receptors to the actin cytoskeleton in a process that physically separates the receptors from the signaling G-protein is one such mechanism. Traditionally, the desensitized state has been viewed as a point of no return, but recent findings challenge this view and demonstrate that desensitized FPRs may in fact be reactivated to resume active signaling. The FPRs have also the capacity to communicate with other receptors in a hierarchical manner and this receptor cross-talk can both dampen and amplify neutrophil responses. In this review, we summarize some recent advances in our understanding of how the FPRs can be turned on and off and discuss some future challenges, including mechanisms of allosteric modulation, receptor cross-talk, and FPR reactivation.

Keywords: FPRs; neutrophils; GPCRs; cross-talk; desensitization

Abbreviations: GPCRs, G-protein coupled receptors; FPRs, formyl peptide receptors; fMet, formylated methionine; GPCRs, G-protein coupled receptors; PAFR, platelet activating factor; ROS, reactive oxygen species; SAA; serum amyloid A; HIV-1, human immunodeficiency virus type 1; PSMs, phenol soluble modulins; SDS, sodium dodecyl sulfate; LXA4, lipoxygenase-derived eicosanoid lipoxin A4.

To cite this article: Johan Bylund, et al. Turning chemoattractant receptors on and off with conventional ligands and allosteric modulators: recent advances in formyl peptide receptor signaling and regulation. Inflamm Cell Signal 2014; 1: e73. doi: 10.14800/ics.73.

Neutrophil chemoattractant receptors - focusing on the formyl peptide receptors (FPRs)

The chemoattractant receptors and neutrophil function

Based on the fact that prokaryotes initiate protein synthesis with a formylated methionine (fMet) residue, it was anticipated almost 40 years ago that peptides starting with fMet should be recognized by the immune

system, and cells equipped with proper recognition structures (receptors) should be able to find the peptide source through chemotactic migration[1]. In accordance with this, chemotactic fMet-containing peptides were also identified as pro-inflammatory mediators released by growing *Escherichia coli*[2]. Subsequently, the application of molecular biology techniques led to the identification and characterization of the formyl peptide receptor (FPR1)[3]. This was among the first neutrophil receptors belonging to the large family of G-protein coupled receptors (GPCRs) that was cloned, but soon after two additional FPR1-like receptors, now known as FPR2 (previously FPRL1) and FPR3 (previously FPRL2), were cloned from a promyelocyte cDNA library using low-stringency hybridization with the FPR1 cDNA as a probe[4, 5]. The last decade's research has highlighted a role of the FPRs not only in host defence against bacterial infections, but also in immune regulation[5]. The three FPRs (FPR1, FPR2, and FPR3) belong to the subfamily of chemoattractant GPCRs that also includes, e.g., receptors for the complement component C5a (C5aR), the lipid metabolite platelet activating factor (PAFR) and the chemokine IL-8 (CXCR1/2)[6]. These chemoattractant receptors have in common that they comprise a single 350-370 amino acids long polypeptide chain that spans the cell membrane seven times. The amino terminus and three extracellular loops (facing the cell exterior) are believed to be essential for interaction with the chemoattractant, while the carboxyl terminus and the three intracellular loops (facing the cytosolic side of the membrane) are of importance for signaling[6-8]. The transmembrane regions as well as the cytosolic signaling domains of the chemoattractant GPCRs share certain sequence similarities whereas the degree of sequence similarity is less obvious in the extracellular domains supposed to contain agonist recognition sites[9]. Receptor recognition of a chemoattractant in neutrophils typically induces not only chemotactic migration, but also exocytosis leading to mobilization of receptors and adhesion molecules from intracellular storage granules, secretion of proteolytic enzymes and inflammatory mediators, and production of reactive oxygen species (ROS) by the activated NADPH-oxidase[10].

The human FPRs

It was for many years believed that the expression/distribution of FPRs was restricted to phagocytes, a view that has been reconsidered and we now know that these receptors are expressed also in a variety of non-immune cell types[11]. Regarding the expression in professional phagocytes, human neutrophils express FPR1 and FPR2 whereas monocytes/macro-phages express all three family members[5]. FPR1 and FPR2 exhibit an amino acid sequence similarity of 69% with a higher degree of identity in the cytosolic loops and a lower in the extracellular domains and carboxyl tail[5] (Fig 1). This suggests that the two receptors differ more with regards to ligand binding than in what type of intracellular signals they transmit. Further, FPR1 displays a high degree of single nucleotide polymorphism, whereas no polymorphisms have been described for FPR2[12]. This indicates that although these receptors are quite similar, they have been subjected to distinctly different evolutionary events. The two neutrophil FPRs are expressed fairly late during neutrophil differentiation (after the promyelocyte stage)[13], they mediate very similar cellular responses[14], and have the same subcellular distribution in mature neutrophils, i.e., the major part is stored in easily mobilizable granules/secretory vesicles, as reviewed by subcellular fractionation studies[15, 16]. The stored FPRs can be mobilized to the cell surface by priming agents such as bacterial endotoxin and TNF-α, but they also become surface-exposed during the process of extravasation from the blood stream to the tissue[15, 17, 18].

The murine Fprs

The FPR gene cluster has undergone differential expansion in mammals[19]. The murine genome comprises eight distinct Fprs (rather than three as in humans) with unique functional and distribution characteristics [19-21]. It is also worth mentioning that the prototypic FPR1 agonist fMLF is a much less potent stimulus of murine neutrophils[22-24]. The evolutionary relationship and functional correlation between human FPRs and their mouse counterparts remain incompletely understood, but the available data suggest that the murine Fpr1 is an ortholog of human FPR1 but with much lower affinity for formyl peptides[21, 25]. The observation that the WKYMVm peptide, a potent FPR1/2 dual agonist[26], activates transfected cells expressing murine Fpr-rs2, suggests that this receptor may be the mouse ortholog of FPR2, but cross desensitization experiments indicate that also other murine receptors may recognize this agonist[27]. Despite the insufficient knowledge about the murine Fprs in general, it is however evident that activation of these receptors induces similar cellular responses as their human counterparts [22, 23, 28-30]. An additional similarity is that exudate murine neutrophils are primed in their response to WKYMVm and this FPR1/2 dual agonist desensitizes the murine cells for subsequent stimulation with several FPR1
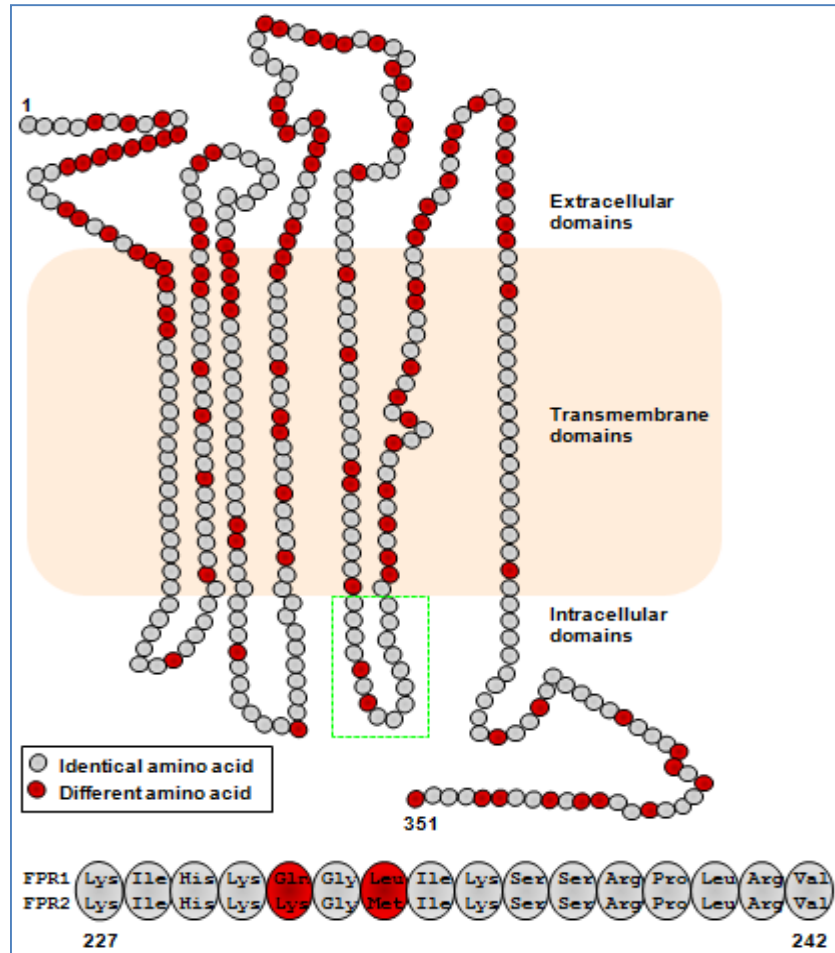
Figure 1. Schematic drawing of FPR. The amino acid sequences for FPR1 (350 amino acids) and FPR2 (351 amino acids) are very similar and the majority of differences are located in the extracellular domains. Grey circles denote amino acids that are identical between FPR1 and FPR2, red circles represent residues that are different between FPR1 and FPR2. The third intracellular loop is framed in green and the sequences are specified below. This sequence (FPR2) is the basis for activating FPR2 pepducins as described in the text.

agonists as well as the specific FPR2 agonist WKYMVM[31, 32]. The priming process of murine neutrophils involves mobilization of subcellular granules, suggesting that the subcellular localization profiles for the receptors in human and murine neutrophils are similar[27].

Ligand specificity and selectivity for FPR1 and FPR2

Conventional FPR agonists

FPR1 is a high affinity receptor involved in guiding neutrophils to bacteria or damaged tissue through recognition of formyl peptides[1, 33]. In addition to formyl peptides, a number of different FPR1 agonists have been identified including non-formylated host-derived peptides, as well as non-peptide agonists identified from library screens. The reader is referred to other recent reviews for a full description of FPR specific/selective ligands[10, 11]. When comparing FPR1 and FPR2, it is clear that the latter

displays a much more diverse ligand profile and this receptor recognizes a broad range of molecules including the GP-41 envelope protein of the human immunodeficiency virus type 1 (HIV-1)[34], a peptide from glycoprotein G of herpes simplex virus type 2[35], Hp2-20 from *Helicobacter pylori*[36], and the synthetic peptide WKYMVM/m[18]. Also host-derived molecules have been suggested to be FPR2 ligands, most notably the acute phase protein serum amyloid A (SAA) [37]. However, most (if not all) studies on the SAA-FPR2 link have been performed with a recombinant protein that is a hybrid of two human SAA isoforms (SAA1 and 2) that does not exist *in vivo*. It is thus highly debatable if the notion that acute phase SAA is a cytokine-like protein with pro-inflammatory properties, really reflect the true biological activity of endogenous SAA. We recently showed that the native protein, obtained from patients with inflammatory arthritis, is remarkably inert and do not share the neutrophil activating properties
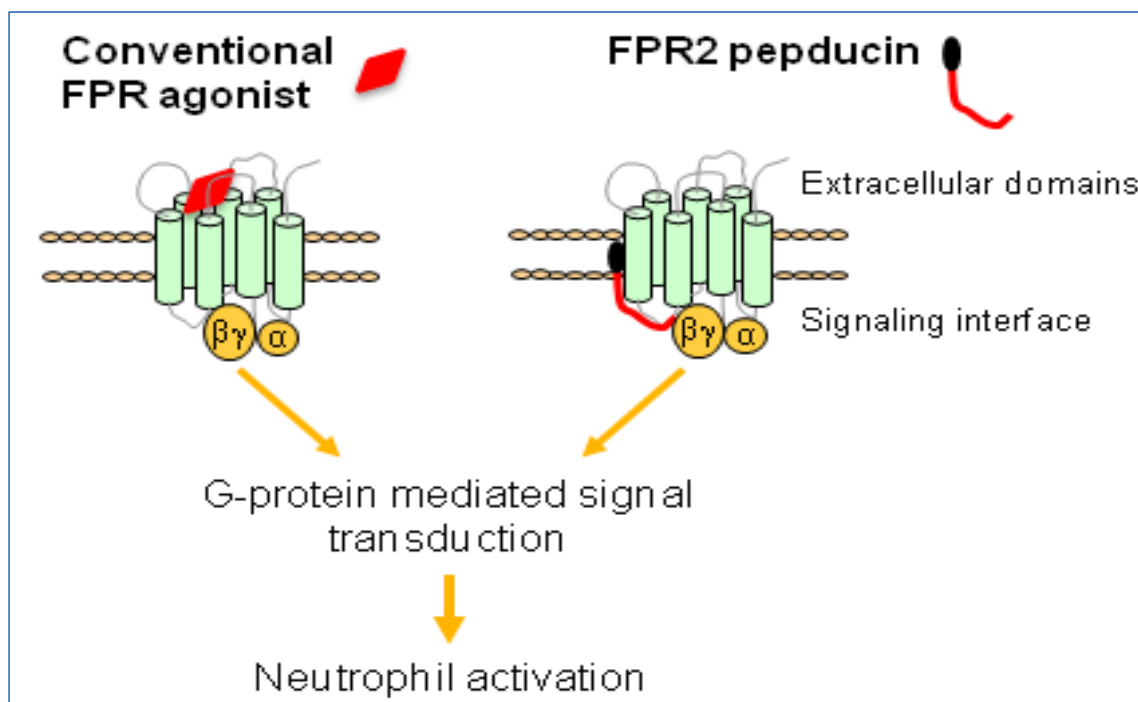
Figure 2. FPR-mediated neutrophil activation upon stimulation with a conventional agonist (left) that binds to the extracellular receptor domains, or by a pepducin (right) that anchors to the membrane through its palmitoyl moiety (black) and interacts with the receptor at the signaling interface on the cytoplasmic side. Both agonists trigger G-protein-dependent signaling and induce very similar cellular responses.

of recombinant SAA[38, 39]. Thus, the recombinant forms of SAA are not valid substitutes for the native protein, but since they are indeed capable of interacting with FPR2[39, 40], they may still be of value for studying receptor biology.

Another host-derived molecule that interacts with the FPRs is annexin I, a member of the annexin family of calcium-regulated phospholipid binding proteins involved in regulation of innate and adaptive immunity [41]. Peptides derived from the N-terminus of annexin I have both pro- and anti-inflammatory activities[29, 42-44]. All three FPRs recognize different and possibly overlapping parts of the protein with a core structure for FPR1 interaction identified as Gln(9)-Ala(10)-Trp(11)-Phe(12) corresponding to the annexin I$_{9-12}$ sequence[45]. A structural model of this peptide agonist-FPR1 complex suggests that the transmembrane part of the binding pocket of the receptor binds optimally to this tetrapeptide[45]. According to the model, the N-terminal glutamine of the peptide is located close to the bottom of the binding cleft, leaving for steric reasons insufficient space to extend the peptide at the N-terminus, information that may be helpful for the development of specific FPR1 ligands[45].

Although several peptide/protein agonists for the FPRs have been identified, more stable and selective small-molecule ligands should be valuable tools for a better understanding of the physiologic roles played by these receptors. When it comes to the identification non-

peptide/protein agonists for FPRs, high throughput screening of small compound libraries in cells over-expressing FPR1 or FPR2 has been the system of choice for rapid data acquisition and ligand identification. Several high-affinity agonists (and some antagonist/inhibitors) have been identified and characterized through such screening approaches[46-48], and some potent agonists originally identified with FPR2-expressing cells have later been demonstrated to interact preferentially with FPR1[48, 49]. The small-molecules identified as FPR agonists using transfected cells were shown to activate also the neutrophil receptors and comparative docking studies show that agonists with dual specificity (FPR1/FPR2) bind to similar binding pockets as high affinity FPR1 and FPR2 peptide agonists, respectively[50, 51]. Many of the identified small-molecule compounds could possibly be used as potential lead compounds for further development of highly potent and selective FPR agonists/antagonists.

Peptides starting with an N-formyl methionyl group constitute a unique pattern of bacterial as well as mitochondrial metabolism[33], but since FPR2 hardly recognizes the prototypic bacterial chemoattractant fMLF, FPR1 was for a long time regarded as the sole receptor capable of recognizing formyl peptides. However, this notion has been challenged by the recent finding that community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA) secrete a group of FPR2 binding
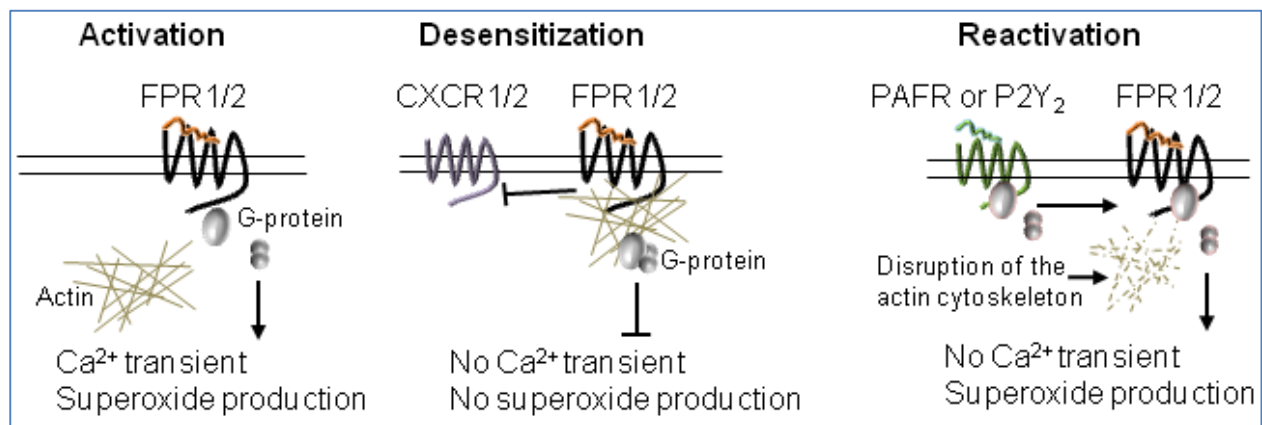
Figure 3. Schematic drawing of FPR activation (left), desensitization (middle), and reactivation (right). The FPRs (both FPR1 and FPR2), subsequent to agonist binding, rapidly undergo homologous desensitization by coupling to the actin cytoskeleton. FPR agonist induce also heterologous desensitization of e.g., CXCR1/2, no superoxide is induced by a second stimulation CXCR1/2 agonist. The desensitized FPRs can be reactivated by either disruption of the actin cytoskeleton, or by cross-talk signals triggered through PAFR or the ATP receptor $P2Y_2$. Reactivated FPRs transmit signals that activate neutrophils to produce superoxide, but no Ca^{2+} transients are induced by reactivation.

peptides, the so called phenol soluble modulins (PSMs)[52, 53]. These PSM peptides are secreted by the recently identified PSM transporter from the bacteria without removal of the formyl group at the N-terminal methionine (deformylation) and they all have in common that they are α-helical molecules with a high degree of amphipathicity[54]. All PSMα peptides (grouped by their size, physicochemical properties and genomic arrangement) investigated promote virulence through effects on discrete neutrophil functions (i.e., chemotaxis) and by being cytotoxic at higher concentrations[55-57]. The physicochemical properties of their C-terminus are crucial not only for the cytotoxic activity but also for the receptor activation potency of the peptide. Regardless of the C-terminal sequence these formyl peptides always seem to prefer FPR2 over FPR1[52]. This suggests that the name FPR2 is fitting not only based on the close structural similarity with FPR1, but also based on the ability to recognize fMet-containing peptides.

FPR antagonists and non-conventional FPR agonists

Several ligands have been described that interact with FPR and inhibit the cellular responses more or less specifically[58, 59]. Antagonism of GPCRs can be achieved through different mechanisms, e.g., by binding of an inhibitor to a receptor site different from that used by conventional agonists (allosteric inhibition), or by binding of an inhibitor to the same receptor site as conventional agonists (orthosteric inhibition)[60]. The latter type of inhibitors could exert their effect either by competitive binding (a neutral antagonist), or by being an inverse agonist that inhibits the basal activity of a constitutively active receptor. The FPR1 selective inhibitor cyclosporin H, a cyclic undecapeptide, has been shown to be an inverse agonist[61, 62]. No precise inhibitory mechanisms have been

defined for the identified FPR2 inhibitors (described below) and whether also FPR2 is constitutively active in the absence of agonist is not known.

Despite the fact that cyclosporin H is the most potent and selective FPR1 inhibitor described, several other more or less specific FPR1 inhibitors have been identified. Replacement of the formyl group in the prototype agonist fMLF with a bulky t-butyloxylcarbonyl (Boc)- or isopropylureido-groups yields FPR1 antagon-ists, and the potency of such antagonists can be changed through a replacement of the individual amino acids in the peptide[58]. Moreover, the anionic amphiphil sodium dodecyl sulfate (SDS) was recently shown to act as an FPR1 antagonist with fairly high receptor specificity; signaling through FPR2 was actually slightly primed by SDS[63]. The precise mechanism behind this is not known, but might be related to effects earlier described for SDS on the small G-protein Rac, that is of importance for a proper transduction of the down-stream signals from the occupied receptor[64]. With respect to the antagonistic effect of SDS, both the length of carbohydrate chain and the net charge of the molecule are of importance[63].

A recent search for new FPR antagonists, using a ligand-based virtual screening approach, identified 30 different FPR antagonistic compounds belonging to 9 distinct chemical families[65], suggesting that there will be an increasing number of antagonists identified also for FPR2. The FPR2-specific antagonists/inhibitors described so far are the recently identified hexapeptide WRWWWW[66] and the cell permeable allosteric modulator PBP10 ([67], described below). Both WRWWWW and PBP10 block FPR2-mediated responses without affecting FPR1 signaling [66-69]. However, these antagonists/inhibitors are

not entirely FPR2-specific since both inhibit also FPR3 signaling (our unpublished observations).

Among the more mysterious inhibitors for the FPRs are the endogenous lipid mediators, lipoxins and resolvins. The formation and activity of lipoxins and resolvins have been shown to be of prime importance for the resolution of inflammation in a great number of murine models and this inhibitory approach is currently being developed for anti-inflammatory therapies[70, 71]. The lipoxygenase-derived eicosanoid lipoxin A_4 (LXA_4) was the first specific agonist shown to bind to FPR2 with high affinity[72], and resolvin D1 (belonging to a group of non-classical eicosanoids) has recently been shown to trigger the same receptor[73]. In contrast to the large number of non-lipid FPR2 agonists identified in recent years, the lipids have been suggested to use some of the signaling pathways commonly used by classical agonists, but at the same time *inhibit* neutrophil functions[74]. Based on these observations the lipoxins/resolvins should be classified as biased agonists, but it should be noticed that several studies clearly show that LXA_4 neither inhibits neutrophil functions, nor triggers the signaling pathways commonly used by classical agonists[32, 75-77]. These results imply that the anti-inflammatory actions of LXA_4 may be FPR2-independent.

Intracellular allosteric modulation of the FPRs

Intracellular allosteric activation by FPR-derived pepducins

The development of a group of lipidated peptides (pepducins) has recently introduced a new concept for the regulation of GPCR activity[78]. Members of this group of GPCR regulators affect receptor function through unique allosteric mechanisms that basically involve cytosolic parts of sensitive receptors, and not the receptor domains typically used by conventional agonists that bind to the extracellular loops of the receptors and possibly also to the transmembrane regions localized close to the cell surface[78-80]. In contrast to conventional agonists, the activity of pepducins depends on their capability to pass through membranes and affect receptor activities from the cytosolic side of the membrane[81] (Fig 2). A pepducin typically comprises a fatty acid (normally palmitic acid) linked to a short peptide chain with an amino acid sequence identical (or very similar) to one of the intracellular loops or the cytoplasmic tail of the targeted GPCR. The N-terminally linked palmitoyl group acts as a hydrophobic anchor and is of prime importance for the ability of the pepducin molecule to pass the membrane and allow the peptide to target the receptor from the cell interior[79]. It should be noticed that pepducins may either activate or inhibit receptor function and the signals that are generated are very similar to those induced by conventional activating/inverse agonists[82]. Pepducins with amino acid sequences originating from the third intracellular loop of FPR2 have the capacity to activate neutrophils through an interaction with this receptor[82, 83] (Fig 2). Although FPR1 and FPR2 are very similar at the amino acid level, particularly in the third intracellular loop of the receptors (Fig 1), the corresponding FPR1-like pepducin is not able to activate this receptor[82], showing that there are indeed clear differences in signaling between these very closely related receptors.

Although it is hard to understand why/how pepducins with amino acid sequences identical to a cytosolic part of a GPCR specifically interact with the receptor from which it is derived, it is clear that an FPR2-specific pepducin solely activates cells that express FPR2[82]. The precise mechanism by which pepducins transduce signals in a receptor specific manner require further studies, but different, and possibly unique, modes of action may be utilized depending on the particular experimental set up and the individual pepducin/receptor pair examined. As of yet, no inhibitory pepducins, based on sequences from FPRs linked to a palmitoyl group has been described.

Intracellular allosteric inhibition by gelsolin-derived PIP_2-binding peptides

Another allosteric modulator, distinct from the pepducins, is the FPR2-selective inhibitor PBP10 that contains a peptide sequence identical to one of the PIP_2-binding domains of the cytoskeletal protein gelsolin[69, 84]. The PBP10 peptide has a rhodamine group conjugated to its N-terminus, and this is apparently required for the peptide to pass the plasma membrane[85], and for the inhibitory function of the peptide[67]. A core PBP peptide for FPR2 inhibition has been identified, and the fact that this shorter peptide partly inhibits also FPR1[69], suggests that a structure of importance for inhibition is present also in FPR1 but this is obviously not accessible for the longer peptide.

It is hard to conclusively show that the allosteric modulators described interact with its specific receptor from the inside of the plasma membrane, but it is clear that PBP10 as well as the FPR2-derived pepducins indeed have the capacity to pass the plasma membrane and that the physicochemical properties (charge and hydrophobicity) that permit them to enter the cytoplasm are also required for proper function. In accordance with this, the naked peptides are completely devoid of biological activity[82, 85].

FPR desensitization and reactivation

Homologous and heterologous desensitization

As reviewed elsewhere, agonist-induced activation of FPRs is regulated at multiple levels; receptor to G-protein activation, transduction and amplification of signals from activated G-proteins to effectors including kinases and

small GTPases, and integration of effector signals leading to phagocyte responses such as chemotaxis, degranulation, and superoxide generation[10, 11]. One particularly important regulatory process is receptor desensitization, a process whereby GPCRs lose the ability to signal and this is thus a mechanism whereby prolonged activation can be avoided. Homologous desensitization is a process by which neutrophils interacting with a receptor-specific agonist gradually become non-responsive to further stimulation by the same agonist as well as to other agonists employing the same receptor[86]. Activation of e.g., FPR1 will lead to desensitization and unresponsiveness to subsequent stimulation with FPR1 agonists[18]. However, agonist binding to FPRs triggers also the desensitization of other unrelated receptors including CXCR1/2 (Fig 3). This type of receptor cross-talk is known as heterologous desensitization[87]. Heterologous desensitization occurs in a hierarchical manner and may be of importance for directional migration to the focus of infection and/or inflammation when cells are facing multiple gradients of different chemoattractants[88]. Interestingly, no cross desensitization is observed with specific FPR1 and FPR2 agonists, which suggest that these two receptors are hierarchically equally strong[18]. The desensitization phenomenon can be used to characterize new receptor agonists and to determine receptor hierarchy but it is of importance to notice that desensitization patterns may differ depending on the read-out system. The background to this is that certain agonists (e.g., fMLF) may be inactivated by a myeloperoxidase/hydrogen peroxide catalyzed reaction and by that the desensitization is broken[89]. Several approaches can, however, be used to avoid the influence of agonist inactivation[90].

Many desensitization mechanisms have been proposed including the phosphorylation of occupied GPCRs by specific GPCR kinases, second messenger-dependent protein kinases, β-arrestin binding, and physical coupling of the receptor to the cytoskeleton[9, 91]. It is clear that the actin cytoskeleton beneath the neutrophil cell membrane (also known as the cortical cytoskeleton) plays an important role at least in homologous desensitization of FPRs. As an example, a disruption of the actin cytoskeleton with cytochalasin B or latrunculin A prolongs the FPR signaling without affecting signaling stemming from GPCR-independent activation, e.g., by PMA (a direct PKC activator)[92]. Similar to cytoskeletal disrupting agents, the inhibition of cellular phosphatases by treatment with okadaic acid or calyculin A also delays termination of FPR signaling, an effect that is accompanied with reduced association of the receptor-ligand complex to the actin cytoskeleton[93]. Jesaitis *et al.* have demonstrated that upon formyl peptide binding to FPR1, the formation of Triton X-100 resistant FPR1-cytoskeleton complexes occurs before receptor internalization [94]. The basis for cytoskeleton-dependent GPCR desensitization is thus proposed to involve a physical segregation of the receptor from the signaling G-protein and coupling to the actin cytoskeleton [91] (Fig 3). The precise mechanism for how activated FPRs display binding affinity for the actin cytoskeleton and how this association separates the receptor from the signaling G-protein is not yet understood. The fact that the cytoplasmic signaling/phosphorylation regions of FPR1 shares 40-50% identity with actin-binding proteins such as villin suggests that FPRs may directly bind to the actin cytoskeleton[95].

FPR reactivation involving the actin cytoskeleton

A key role of the actin cytoskeleton in FPR desensitization gains further support from the fact that desensitized neutrophils can be reactivated to produce superoxide by the secondary addition of a cytoskeleton-disrupting agents[67, 96]. When the agonist-bound FPRs have terminated signaling, these receptors are homologously desensitized and do not respond to subsequent doses of the same agonists. This desensitized state had for a long time been considered a stable point of no return, i.e., that it would not be possible to transfer the desensitized receptors back into signaling mode again. However, by addition of pharmacological agents that disrupt the cytoskeleton (cytochalasin B or latrunculin A) to FPR-desensitized cells, these receptors rapidly resume signaling again, a process termed receptor reactivation [96] (Fig 3). The actin-dependent desensitization mechanism is not universal for all GPCRs as evidenced by the fact that cells desensitized by IL-8 (CXCR1/2) or PAF (PAFR) cannot be reactivated to produce superoxide anion by cytochalasin B or latrunculin A[97].

Whereas pharmacological disruption of the cytoskeleton may be a rather artificial way to achieve reactivation of desensitized FPRs, we have recently found that a similar process can be triggered by stimulation of PAFR or the ATP receptor P2Y$_2$ that transmits cross-talking signals to the desensitized FPRs ([98, 99] and described below). This indicates that FPR reactivation could actually take place and be of importance during physiological settings. Taken together, this clearly indicates that the block, e.g., actin cytoskeleton, put on desensitized FPRs and that refrain them from transmitting their signals is not permanent, but can in fact be removed.

FPR reactivation induced by receptor cross-talk

Recent research has demonstrated that receptor cross-talk can not only induce desensitization, but as mentioned above it can also trigger reactivation of desensitized FPRs when FPR-desensitized neutrophils are stimulated with ATP (that binds to the ATP receptor P2Y$_2$[99]), or with PAF (that binds PAFR expressed in the plasma membrane)[98, 100] (Fig 3). This shows that stimulation of unrelated GPCRs (e.g., PAFR) generates signals that are capable of lifting the

block put on desensitized FPRs and turn the dormant receptor-ligand complex back into signaling mode again. Interestingly, this PAFR-mediated reactivation of FPRs takes place even if the cytoskeleton has been disrupted prior to PAF stimulation, suggesting the existence of non-actin mediated reactivation mechanisms. Also, the cross-talk between PAFR or $P2Y_2$ and FPRs is unidirectional, i.e., FPR ligation cannot reactivate desensitized PAFR or $P2Y_2$[98, 99]. The mechanistic details of the reactivation signal are still obscure, but whereas both neutrophil FPRs (FPR1 and FPR2) are susceptible to reactivation, other GPCRs, e.g., CXCR1/2 cannot be reactivated[97, 98], indicating that there is some kind of specificity involved in FPR cross-talk with other GPCRs.

Signaling through neutrophil FPRs

Common signaling pathways trigger a transient rise in intracellular Ca^{2+}

The signaling pathways downstream FPR1 have been extensively studied, and details about the signal transduction pathways for the onset of discrete neutrophil functions can be found in several recent reviews[9, 95, 101, 102]. Once activated, the dissociated G-protein subunits activate multiple downstream second messengers including various phospholipases and protein kinases[9, 103]. In contrast, less is known about FPR2 signaling, but it is generally assumed that they use very similar signaling pathways based on the fact that the two receptors are structurally very similar in their signaling domains and they also mediate very similar cellular responses[14]. Both FPRs, upon binding by a large array of ligands, adopt a conformational change that induces a dissociation of pertussis toxin-sensitive G-proteins into two parts, the α and βγ subunits[104], and based on results obtained using a simple and straightforward system to measure β-arrestin binding, it is clear that both FPR1 and FPR2 trigger a translocation of β-arrestin[32].

In resting neutrophils, the cytosolic Ca^{2+} concentration is kept at very low levels (about 100 nM) as compared to the level outside of the cells, and in a general signaling transduction scheme an immediate downstream consequence of GPCR activation is the transient elevation of intracellular Ca^{2+}. The Ca^{2+}-response triggered by FPR1 agonists is characterized by an initial release of Ca^{2+} from intracellular storage organelles exposing IP_3 receptors[105]. The emptying of the storage organelles then leads to the entry of extracellular Ca^{2+} through store-operated calcium channels in the plasma membrane, thereby prolonging the increase in intracellular Ca^{2+}. A fundamental difference between the signaling profiles of FPR1 and FPR2 was recently described when FPR2 was shown to trigger a unique type of Ca^{2+} influx across the plasma membrane and it was without involvement of the intracellular storage organelles[106]. Thus, the influx of Ca^{2+} across the plasma

membrane was not preceded by Ca^{2+} release from the intracellular stores. We have found that all specific FPR2 agonists tested induce an increase in intracellular Ca^{2+} that also rely on a release from intracellular stores, clearly showing that Ca^{2+} signaling mediated *via* FPR2 in fact follows the same route as that of FPR1[107].

The transient rise in intracellular Ca^{2+} is not required for neutrophil activation

The temporary rise in Ca^{2+} described above has long been considered essential for various neutrophil functions. There are, however, studies showing that an elevation of Ca^{2+} alone is neither sufficient nor required for certain FPR-mediated neutrophil responses. It is clear that although many cytoskeleton-remodeling proteins require Ca^{2+} for proper function[108], cytoskeleton dependent cellular processes such as neutrophil polarization, membrane ruffling, chemotaxis and phagocytosis can take place also in Ca^{2+} depleted cells[109]. Also activation of the superoxide generating system can occur without an elevation of cytosolic Ca^{2+} and superoxide produced as a result of FPR reactivation occurs without any transient Ca^{2+} rise[87, 96, 97] (Fig 3). The experimental evidence suggesting that Ca^{2+} elevations activate downstream effector functions (i.e. granule mobilization and activation of the NADPH-oxidase), directly or in synergy with other signals[110, 111], relies on methods that cannot distinguish a dependence of basal Ca^{2+} levels from a requirement for a Ca^{2+} transient. Our data showing that neutrophils can be activated without any cytosolic Ca^{2+} transients are thus in contradiction with the prevailing view.

Future Perspectives

The last decade's research, with advanced cellular and molecular biology technologies, has generated an impressive amount of knowledge to increase our understanding of the members of FPR family. The recent progress in using allosteric modulators (activating as well as inhibitory) that interact with the FPRs from inside the plasma membrane is wildly different from the conventional scenario where GPCRs can only be targeted from the outside. This progress and the fact that desensitized FPRs can be reactivated to resume signaling add an additional layer of complexity to the classical view of FPR activation. Especially the finding that reactivation of desensitized FPRs can be achieved through GPCR cross-talk could have bearing on how neutrophils react to multiple gradients of different chemoattractants and the detailed nature of the cross-talk signal, and whether this mechanism applies also to other GPCRs (and other cell types) awaits further investigation. Most of the FPR agonists/antagonists described are related to infections or inflammatory processes, indicating that this group of GPCRs plays critical roles in host defense as well as for immune

regulation. This notion gains further support from *in vivo* studies using mice deficient in individual receptor subtypes showing that these mice display dysregulated inflammatory responses. However, when translating data from murine models into a human setting, it is important to be aware that many potent human FPR agonists/antagonists display very low affinity (or are even inactive) for the murine Fprs. Finally, the GPCR research has entered a new era due to the recent explosion in available GPCR crystal structures. Once proper structures of the FPRs have been resolved, these structures should open up new opportunities to study their function and offer conformational insights into FPR activation by conventional ligands as well as allosteric modulators. Hopefully, such knowledge would facilitate the elucidation of mechanisms underlying FPR desensitization and reactivation. Understanding of how FPR activity is regulated is of direct importance in physiological as well as pathological settings. Increased knowledge in these matters should facilitate the development of novel prophylactic and/or treatment strategies for autoimmune and inflammatory diseases.

Conflicting interests

The authors have declared that no competing interests exist.

Acknowledgments

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