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The leukocyte chemotactic receptor FPR2, but not the closely related FPR1, is sensitive to cell-penetrating pepducins with amino acid sequences descending from the third intracellular receptor loop

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Running title: Receptor specific immunomodulatory effects of pepducins

SUMMARY

Lipidated peptides (pepducins) can activate certain G-protein coupled receptors (GPCRs) through a unique allosteric modulation mechanism involving cytosolic receptor domains. Pepducins with the amino acid sequence of the third intracellular loop of the neutrophil formyl peptide receptors (FPRs) as a common denominator were N-terminally conjugated with palmitic acd. F2Pal$_{16}$, containing the 16 amino acids present in the third intracellular loop of FPR2, induced superoxide production in human neutrophils and the activity was sensitive to FPR2 antagonists. Cells over-expressing FPR2 were similarly responsive and responded with a transient increase in cytosolic calcium. No such effects were observed with the corresponding FPR1 pepducin. The peptide alone, lacking palmitic acid, did not activate neutrophils. A ten amino acids long pepducin F2Pal$_{10}$, that was a more potent neutrophil activator than F2Pal$_{16}$ and was used for amino acid substitution studies. The sequences of FPR1 and FPR2 in the third intracellular loop differ by only two amino acids, and a pepducin with the FPR2-specific K_{231} replaced by the FPR1-specific Q_{231} lost all activity. The active F2Pal$_{10}$ pepducin also triggered a response in cells expressing a mutated FPR2 with the third intracellular loop identical to that of FPR1. The data presented suggest that the same signaling pathways are activated when the signaling cascade is initiated by a classical receptor agonist (outside-in signaling) and when signaling starts on the cytosolic side of the membrane by a pepducin (inside-in signaling). A fundamental difference is also disclosed between the two neutrophil FPRs regarding their sensitivities to third intracellular loop pepducins.

1. INTRODUCTION

Lipidated peptides (pepducins) activate G-protein coupled receptors (GPCRs) through a unique allosteric activation mechanism that involves cytosolic parts of the receptor, but not the extracellular domains typically used as binding sites for classical receptor agonists [1]. The GPCRs represent a very large family of cell-surface receptors with many common structural and functional characteristics. They all have a conserved molecular structure with the N-terminus and C-terminus exposed on different sides of the cell membrane which is transversed seven times by the receptor peptide chain. From a functional point of view, conventional agonists in the form of hormones, peptides, carbohydrates, lipids, or large proteins interact with the extracellular loops of the recognizing receptor as well as with transmembrane domains, in regions localized closer to the cell surface. The agonist occupied receptor then transfers the primary signal to the G-protein binding structures present in the cytosolic part of the receptor [2]. The activated receptor binds a heterotrimeric G-protein that when dissociated transfers the signal further to different second messenger systems which regulate functional responses of the cells expressing the receptor.

In contrast to conventional agonists, the activity of pepducins depends on their capability to pass membranes and activate receptors from the inside of cells [3]. In general, a pepducin comprises a short peptide chain with an amino acid sequence identical to one of the intracellular loops (typically the third) or the cytoplasmic tail of the receptor to be targeted. The peptide is then N-terminally lipidated with a palmitoyl group that acts as a hydrophobic anchor. Such an anchor is critical for the ability of pepducins to gain access to the cell interior where they supposedly activate signaling through an interaction with the cytosolic parts of the receptor [1]. According to the predominant view regarding the mechanism of

entrance, the palmitoyl group binds to the membrane lipid bilayer allowing the peptide to "flip" across the membrane and face the cytosolic side of the membrane. It is believed that once on the inside of a membrane exposing the right receptor, the peptide part of the pepducin targets the interface between the receptor and the G-protein and signaling is achieved through allosteric modulation. This triggers a signal very similar to that induced by an agonist occupied receptor. Accordingly the signal is transferred to the G-protein in the case of an activating pepducin, whereas the "allosterism" induces a block of the physical contact between the receptor and the G-protein in the case of an antagonistic/inhibitory pepducin [4]. Several immune regulatory GPCRs including the CXCR1, CXCR2 and CXCR4 have successfully been activated/inhibited by specific pepducins [5]. Recently the Formyl Peptide Receptor 2 (FPR2) was added to the group of receptors that can be activated by a pepducin [6, 7].

The FPR2 belongs to the formyl peptide receptor (FPR) family of pattern recognition receptors used by the innate immune system to sense bacterial invasion as well as destructed tissues through the production/release of N-formylated peptides, a unique hallmark of bacterial and mitochondrial metabolism [8]. The FPRs are exposed on cells that form our first line of defense against infection such as neutrophil granulocytes and professional phagocytes of the monocyte/macrophage lineage [9]. Another family member, FPR1, for which an N-formylated methionyl group is a critical determinant of ligand binding, is the best characterized receptor among all the neutrophil chemoattractant receptors. Together with other receptors with similar functions such as the C5a-receptor, the LTB_4-receptor, and the PAF-receptor, the FPRs belong to the larger family of G-protein coupled receptors (GPCRs) [10].

The two FPRs expressed on human neutrophils (FPR1 and FPR2) have very similar primary sequences, but they bind and are activated by different agonists [9]. For example, the prototypical bacterial chemoattractant peptide fMLF starting with a formylated methionyl group that is suggested to bind deep inside the receptor [11-13] is recognized by FPR1 but not by FPR2. In contrast, certain peptides generated by community associated methillicin resistant *Staphylococcus aureus* strains, starting with the same formylated amino acid, are specifically recognized by FPR2 rather than FPR1 [14, 15]. Moreover, some *N*-formylated peptides derived from mitochondrial protein synthesis have been shown to be equally potent for FPR1 and FPR2. Some of these are also low-affinity agonists for FPR3 [8], the third family member expressed in monocytes, which still lacks known, specific, high affinity agonists [16, 17].

The precise biological roles of the FPRs are not completely understood, but the identification of both exogenous and endogenous ligands involved in inflammation strongly indicates a pivotal role of these receptors in regulating inflammatory defense reactions [16]. FPR1 and FPR2 share a high degree of amino acid sequence similarity particularly in the cytosolic (signal transducing) domains and the functional repertoire induced by the two receptors are almost identical. Despite these signaling similarities, there are some fundamental differences between the two receptors. This is illustrated by the fact that a gelsolin-derived phosphoinositol-binding peptide (PBP10) specifically inhibits FPR2-triggered neutrophil activation [6, 18, 19]. Although the precise signal transduction step that is disrupted by PBP10 remains to be elucidated, it is intriguing that the inhibitory effect of this peptide is, in contrast to the conventional antagonists, dependent on its ability to pass membranes and the inhibitory effect is thus likely to be mediated from the cytosolic side of

the membrane, a mechanism similar to that used by pepducins [3, 20].

In this study we show that FPR2, but not FPR1, can be activated by pepducins with amino acid sequences corresponding to the third intracellular loop of the targeted receptor. The peptide alone (without the lipid tail) did not activate the receptor and the signals induced by the pepducins were indistinguishable from those induced by conventional FPR2 agonists. The FPR2 pepducin-induced response was inhibited by FPR2 specific antagonists, and on the receptor level, sequences/regions apart from those in the third intracellular loop appears to determine sensitivity to pepducins.

2. MATERIAL AND METHODS

2.1. Reagents

The chemoattractant fMLF, and isoluminol, superoxide dismutase (SOD), as well as catalase were purchased from Sigma-Aldrich and the horse radish peroxidase (HRP) was from Roche Diagnostics (Bromma, Sweden). The chemoattractants WKYMVM was synthesized and purified by HPLC by Alta Bioscience (University of Birmingham, Birmingham, United Kingdom). The pepducins and the non-palmitoylated control peptide with free termini as well as the FPR2 specific gelsolin-derived inhibitory peptide PBP10 were synthesized by CASLO Laboratory (Lyngby, Denmark). Pepducins were synthesized by Fmoc solid phase peptide synthesis and N-terminal palmitoylation were made on the resin as the last step before deprotection of side chains. Peptides were purified by HPLC on a C18 column and the correct sequence of each peptide was verified by MALDI-TOF Mass Spectrometry. All peptide stocks were made in DMSO and further dilutions were made in Krebs-Ringer phosphate buffer containing glucose (10 mM), Ca^{2+} (1 mM) and Mg^{2+} (1.5 mM) (KRG, pH 7.3). WRWWWW (WRW_4) was from Genscript Corporation (Scotch Plains, NJ, USA) and cyclosporin H was kindly provided by Novartis Pharma (Basel, Switzerland). The FPR2 specific receptor antagonist Flipr, derived from *Staphylococcus aureus* was kindly provided by Dr JA van Strijp (Utrecht, The Netherlands). Ficoll-Paque was obtained from Amersham Biosciences. RPMI 1640, fetal calf serum (FCS), PEST and G418 were from PAA Laboratories GmbH, Austria.

2.2. Expression of formyl peptide receptors in HL-60 cells

The procedures used to obtain stable expression of FPR1 and FPR2 in undifferentiated HL-60 cells have been previously described [21]. Control experiments with the specific agonists (fMLF for FPR1 and WKYMVM for FPR2) were performed at each experimental event. The wild type form of 3HA-tagged FPR2 was purchased from UMR cDNA Resource

Center, University of Missouri-Rolla. A mutant 3HA-tagged FPR (3HA-FPR2loopR1) in which the sequence of the third intracellular loop of FPR2 (KIHKKGMIKSSRPLRV) has been mutated to the sequence of the third intracellular loop of FPR1 (KIHKQGLIKSSRPLRV) was created by using the PCR strategy described by Yon and Fried [22]. Briefly, using 3HA-tagged FPR2 in pCDNA3.1 as a template, two intermediary polymerase chain reaction fragments, PCR1 and PCR2, were generated with two couples of primers. PCR1 was produced with a sense primer that is located upstream the start codon. The 5'-end of the primer contained an extension with an appropriate restriction site for further ligation in an expression vector, whereas the reverse primer carried the desired mutations. Likewise, PCR2 was created with a sense primer, which is complementary to the reverse primer used for the synthesis of PCR1, and a reverse primer, located downstream from the stop codon, with an appropriate restriction site. After purification, the two PCR fragments were mixed, denatured, and hybridized. The mixture was used to generate PCR3, which contains the mutated sequence. Ten cycles of amplification were first performed in the absence of primers in order to increase the number of copies of full length cDNA carrying the mutations on both strands. Then, 35 cycles of amplification were carried out in the presence of the sense and reverse primers used to generate PCR1 and PCR2. PCR3 was cleaved with the appropriate restriction enzymes for ligation in pCDNA3.1 (Invitrogen, Cergy Pontoise, France). The entire sequence of the 3HA-tagged mutant was cloned in pEF-neo for stable expression in HL-60 cells. The open reading frame was entirely sequenced.

To prevent possible auto-differentiation due to the accumulation of differentiation factors in the culture medium, cells were passed twice a week before they reached a density of 2×10^6 cells/ml. At each passage, an aliquot of the cell culture was centrifuged, the supernatant was

discarded and the cell pellet was resuspended in fresh medium RPMI 1640 containing FCS (10%), PEST (1%), and G418 (1mg/ml).

2.3. Isolation of human neutrophils from peripheral blood

Blood neutrophils were isolated as described by Böyum [23] from buffy coats from healthy volunteers. After dextran sedimentation at 1 x g, hypotonic lysis of the remaining erythrocytes, the neutrophils obtained by centrifugation in a Ficoll-Paque gradient were washed twice in KRG. The cells were resuspended in KRG (1 x 10^7/ml) and stored on ice until use.

2.4. Measurement of superoxide anion production

The production of superoxide anion by the neutrophil NADPH-oxidase was measured by isoluminol-amplified chemiluminescense (CL) in a six-channel Biolumat LB 9505 (Berthold Co, Wildblad, Germany) as described earlier [24, 25]. In short $2x10^5$/ml neutrophils were mixed (in a total volume of 900 μl) with HRP (4 U), and isoluminol ($6x10^{-5}$M) in KRG, pre-incubated at 37°C after which the stimulus (100 μl) was added. The isoluminol/HRP technique detects the release of superoxide independent of H_2O_2 [24, 25]. The light emission was recorded continuously. When required, the specific receptor inhibitors were included in the CL mixture for 5 min at 37°C before stimulation. By a direct comparison of the SOD inhibitable reduction of cytochrome C and SOD inhibitable CL, 7.2 x 10^7 counts were found to correspond to a production of 1 nmol of superoxide (a millimolar extinction coefficient cytochrome C of 21.1 was used).

2.5. Determination of changes in cytosolic calcium

Cells were resuspended at a density of $2x10^7$ cells/ml in KRG containing 0.1 % BSA and loaded with 2 μM Fura 2-AM (Molecular Probes, Eugene, OR) for 30 min, at room temperature. The cells were then washed and resuspended in KRG at a density of 2×10^7 cells/ml. The amount of cells used in the assay was $2x10^6$ cells/measuring cuvette. Calcium measurements were carried out with a Perking Elmer fluorescence spectrophotometer (LS50) with excitation wavelengths of 340 and 380 nm, and an emission wavelength of 509 nm. The transient rise in intracellular calcium is presented as ratio of fluorescence changes (340:380nm) [26].

2.6. Determination of agonist binding by FACs analysis

To determine the effect of $F2Pal_{10}$ on ligand binding to FPR2, a Cy5-conjugated hexapeptide (Cy5-WKYMVM; 10^{-9} M final concentration) was added to neutrophils stored on ice. The fluorescent labeled agonist was added to the neutrophils shortly after the addition of $F2Pal_{10}$ or non-labeled WKYMVM (10^{-7}M) and the cell/peptide mixtures were then incubated at 4°C for 30 min. When the replacement experiments were preformed, Cy5-WKYMVM was added to the cells and binding was allowed to equilibrate at 4°C for 45 min before the addition of $F2Pal_{10}$ or non-labeled WKYMVM. The amounts of bound peptides were determined as the mean fluorescence intensity (MFI) using an Accuri C6 flow cytometer equipped with two laser lines (488 and 640 nm; Becton Dickinson Sparks, MD, USA).

2.7. Statistical analysis

One-way determinations of variance (ANOVA) were performed for statistical analysis. A *p* value less than 0.05 was considered statistically significant.

3. RESULTS

3.1. An FPR2 pepducin (F2Pal$_{16}$; Pal- KIHKKGMIKSSRPLRV) with an amino acid sequence identical to that of the third intracellular loop of FPR2 activates primary human neutrophils

Membrane penetrating pepducins are suggested to target the interface between the receptor and the signaling heterotrimeric G-protein through some type of allosteric mimicking, and the same signal as that induced by conventional agonists is transferred downstream of the G-protein (Fig 1A). We investigated the effect on neutrophils of such a pepducin earlier identified through its ability to activate monocytes, the F2Pal$_{16}$ [7]. The amino acids in this peptide, F2Pal$_{16}$ (Pal-KIHKKGMIKSSRPLRV), correspond to those present in the third intracellular loop of FPR2 (spanning from K_{227} to V_{242}). When exposed to F2Pal$_{16}$ primary human neutrophils responded with a robust respiratory burst (Fig 1B). The amount of released superoxide (O_2^-) increased with increasing concentrations of the pepducin reaching a maximum value at 5 μM. As compared to the classical FPR2 agonist WKYMVM, the maximal amount of O_2^- release induced by F2Pal$_{16}$ was lower but the time course of the pepducin response was fairly similar to that induced by WKYMVM (inset in Fig 1B), reaching a peak value after around a minute and then declined.

The amino acid sequence in the third intracellular loop of FPR1 is almost identical to that in FPR2 and the difference is limited to two amino acids. FPR1 contains a Q_{231} and an L_{233} corresponding to K_{231} and M_{233} in FPR2. Although very similar to F2Pal$_{16}$ in amino acids, the FPR1-pepducin Pal-KIHKQGLIKSSRPLRV (F1Pal$_{16}$) was inactive on primary neutrophils as no cell activity was induced by this peptide (Fig 1B). This shows a clear difference between FPR1 and FPR2 in that only the latter receptor is susceptible to

activation by a pepducin corresponding to its third intracellular loop.

To investigate the specific influence of the two differing amino acids, we synthesized two FPR2-FPR1 "chimeric" pepducins, $F2Pal_{16K5\rightarrow Q}$ (the FPR2 specific K_{231} was replaced with the FPR1 specific Q_{231}) and $F2Pal_{16M7\rightarrow L}$ (the FPR2 specific M_{233} was replaced with the FPR1 specific L_{233}). Interestingly, the $F2Pal_{16M7\rightarrow L}$ was as active as the $F2Pal_{16}$ peptide in inducing ROS release from neutorphils, while no activation was induced by the $F2Pal_{16K5\rightarrow Q}$ (Fig 1C).

3.2. $F2Pal_{16}$ activates human neutrophils specifically through FPR2

Pepducins have been suggested to target the intracellular part of the receptor from which its sequence is derived. Based on the fact that FPR2 shares a large sequence homology with FPR1 in intracellular signaling domains, we next evaluated the receptor preference for $F2Pal_{16}$ using several different approaches. All results point to one conclusion – FPR2 is the targeted receptor. Performing receptor desensitization experiments using known FPR1 and FPR2 agonists we found that neutrophils first activated with $F2Pal_{16}$ and then with the FPR1 selective agonist fMLF were fully responsive to the second challenge with fMLF (Fig 2A). In contrast, $F2Pal_{16}$ stimulated cells were desensitized, i.e. non-responsive to the FPR2 selective agonist WKYMVM (Fig 2B). Thus, $F2Pal_{16}$ desensitizes the cells to stimulation with FPR2 agonist, but not to an FPR1 specific agonist. A preference for FPR2 by $F2Pal_{16}$ was confirmed by the observation that a specific FPR2 antagonist, the WRW_4 peptide [27], inhibited the $F2Pal_{16}$-induced activity (Fig 3). Inhibition was also demonstrated for FLIPr, an FPR2 specific antagonist from *S.aureus* [28] as well as the membrane permeable gelsolin-derived peptide PBP10 (Fig 3). No inhibitory effect was, however, obtained with

cyclosporin H (Fig 3), a potent FPR1 antagonist [27].

The receptor preference of $F2Pal_{16}$ for FPR2 was further confirmed using HL-60 cells stably expressing either FPR1 or FPR2. Stimulation of cells over-expressing FPR2 with $F2Pal_{16}$ induced a robust and transient increase in intracellular Ca^{2+} with a potency and a time-course of the response similar to that induced by the specific FPR2 agonist WKYMVM (Fig 4A). No Ca^{2+} response was observed when FPR1 over-expressing cells were stimulated with $F2Pal_{16}$, while these cells responded well to the FPR1 specific agonist fMLF (Fig 4B). Taken together, our data conclusively show that the $F2Pal_{16}$ is a potent FPR2-specific neutrophil activator and that the signals generated by this pepducin are similar to those generated by conventional, extracellular FPR2 agonists.

3.3. Length, charge, and the presence of the palmitoyl group are important determinants for FPR2 pepducin activity

Pepducin variants (listed in Table 1) with progressive C-terminal truncations of $F2Pal_{16}$ were designed, synthesized and, examined for agonistic effect. There was an inverse relationship between pepducin length and the activity down to 10 amino acids. Removal of one more amino acid from $F2Pal_{10}$, generating $F2Pal_9$, resulted in a 50% loss of potency (Fig 5A). After removal of yet another amino acid, the C-terminal lysine, generating $F2Pal_8$, there was a complete loss of activity (Fig 5A). The $F2Pal_{10}$ peptide was the most potent FPR2 pepducins examined with an EC_{50} value of 100 nM for activation of the neutrophil NADPH-oxidase (Fig 5 B).

$F2Pal_{10}$ peptides in which K_5 (corresponding to K_{231} in the third intracellular loop of FPR2) was exchanged for an N, A, or Q were inactive as illustrated by their inability to trigger

superoxide release from primary human neutrophils (Fig 6A) and the lack of Ca^{2+} responses in FPR2 expressing HL-60 cells (shown for $F2Pal_{10K5\rightarrow N}$ in Fig 6B). These results are in total agreement with those described above for the longer $F2Pal_{16}$ pepducin (Fig 1C). The peptide $F2Pal_{10K5\rightarrow R}$ in which the K in position 5 corresponding to K_{231} in FPR2 was replaced with the positively charged R retained the activity of $F2Pal_{10}$ pepducin (Fig 6). The importance of positive charge in position 5 in mediating pepducin activity was further illustrated by the fact that an FPR1-like pepducin (Pal-KIHKRGLIKS, $F1Pal_{10Q5\rightarrow R}$) in which the Q_5 was substituted with the positively charged R, was as active as the $F2Pal_{10}$ peptide. It is also worth noting that, although the amino acid sequence of $F1Pal_{10Q5\rightarrow R}$ differs from that of the third intracellular loop of FPR2 in two positions and from that of FPR1 in only one position, the activity was mediated exclusively through FPR2 (data not shown). In comparison to the K in position 5, the K in position 4 is less important for activity, as a pepducin ($F2Pal_{10K4\rightarrow Q}$) in which K_4 was replaced by a Q, was still active, although with a reduced potency (data not shown).

In accordance with the suggested mode of action of pepducins, removal of the N-terminal palmitoyl group from $F2Pal_{10}$ resulted in a complete loss of activity even up to concentrations of 100 μM (shown for 10 μM in Fig 7A). An $F2Lau_{10}$ peptide in which a lauryl moiety (12 carbon atoms long) replaced the palmitoyl moiety (16 carbon atoms long) of $F2Pal_{10}$, retained a dose-dependent activity but was not as potent as the $F2Pal_{10}$ pepducin (Fig 7B).

In summary, these data suggest that the potent agonistic effect of the F2Pal pepducin relies on the length of the fatty acid as well as the length and charge of the peptide.

3.4. $F2Pal_{10}$ inhibits binding of the FPR2 agonist Cy5-WKYMVM to neutrophils

In order to study the effect of $F2Pal_{10}$ on agonist binding to FPR2, we utilized the fluorescently labeled agonist, Cy5-WKYMVM and quantified binding/inhibition of binding to neutrophils by FACS-analysis. To evaluate the binding of the fluorescent Cy5-conjugated WKYMVM, Cy5-WKYMVM was allowed to bind in the absence or presence of an excess (100 times) of non-labeled WKYMVM. The non-labeled WKYMVM largely inhibited the binding of the fluorescent probe (Fig 8A). The binding of Cy5-WKYMVM was inhibited also by $F2Pal_{10}$ in a dose-dependent manner (Fig 8B). The level of inhibition obtained with 1 μM of $F2Pal_{10}$ was in the same range as that obtained with 100 nM of WKYMVM (Fig 8B inset).

Using a replacement assay in which the Cy5-WKYMVM peptide was allowed to interact with neutrophils for 45 min on ice prior to the addition of non-labeled peptides, we could show that binding was only modestly affected by an addition of excess of peptide ($F2Pal_{10}$ or WKYMVM) that was allowed to compete with the Cy5-WKYMVM peptide (Fig 8C).

3.5. $F2Pal_{10}$ activates a mutated FPR2 ($FPR2_{K231M233\rightarrow QL}$)

The difference in primary sequence between the FPR1 and the FPR2 in the third intracellular loop is limited to two amino acids in positions 231 and 233. These two amino acids were mutated to give a FPR2 mutant ($FPR2_{mut}$) that included the two FPR1-specific amino acids. When expressed in HL-60 cells, this $FPR2_{mut}$ still recognized and responded to the FPR2 specific agonist WKYMVM (not shown and [6]). F2Pal pepducin $F2Pal_{10}$ triggered a response through $FPR2_{mut}$ (Fig 9). The relative potency of the different F2Pal peptides did not differ between the $FPR2_{mut}$ and the wild type FPR2 and no activity was

induced in these cells by the FPR1 pepducin F1Pal$_{16}$ (data not shown). Taken together, these data suggest that at the receptor level, sequences/regions apart from those in the third intracellular loop may be involved in pepducin sensitivity.

4. DISCUSSION

Having the capacity to permeate cell membranes, pepducins interact with and control FPR2 mediated activities, thereby providing unique tools for the regulation of innate immune related activities in human neutrophils. The neutrophil activating pepducins described here have amino acid sequences originating from the third intracellular loop of FPR2 as a common denominator [6, 7]. A pepducin with an amino acid sequence corresponding to the same region of the closely related receptor FPR1 lacked all activity ([6] and this study). A number of pepducins targeting different GPCRs have been described. In contrast to conventional agonists for GPCRs, cell-permeable pepducins exert their receptor-mediated effects from inside the plasma membrane, but the mechanism by which such peptides transduce intracellular signals in a receptor selective/specific manner has not been clarified in detail [1]. It is assumed that all pepducins act on the intracellular region of the specifically targeted GPCR and induce their effects through a so-called allosteric modulation of intracellular receptor domains that couple to G-proteins and other signaling/regulating proteins [29]. Our data indicate that there might be different, and possibly unique, mode of action related to each individual pepducin/receptor pair.

It is believed that pepducins incorporate their lipid tail into the phospholipid bilayer, enabling the peptide part of the construct to flip over the membrane and become exposed on the cytosolic side of the membrane. This process has been termed ‘insertion and inversion’ [29]. Even though the precise mechanism has not been worked out at the molecular level, the membrane binding and transport steps of the pepducin technology are fairly easy to explain in physico-chemical terms. It has been shown experimentally that pepducins may enter intact cells [3], but since these experiments were performed at 37^{o}C on functionally

intact cells it is hard to determine if the intracellularly localized peptides have just passed the membrane or are there as a result of an endocytic uptake of receptor-ligand complexes. In order to be active, the pepducins must have the ability to pass the plasma membrane, an ability linked to the presence of the hydrophobic moiety (the palmitoyl group). However, this does not conclusively prove that they initiate signaling from the cell interior. To definitely prove that a pepducin triggers its target receptor from the cytosolic side is a much more challenging task than to merely show that the peptide can pass the membrane. Basically, several criteria should be fulfilled for the pepducin concept to be valid, and one such criterion is that there should be a difference in activity between the fatty acid-linked and the non-lipidated peptide. This criterion is fulfilled by the neutrophil F2Pal pepducins examined here. Another criterion is that the pepducin should be able to trigger a response in cells expressing the targeted receptor alone. This criterion is also met for the activating F2Pal series of pepducins described here. It should be noticed, however, that this criterion is valid also for classical extracellular receptor specific agonists and is by no means a unique property for pepducins.

With respect to the F2Pal series examined in this study, one criterion usually fulfilled for other pepducins is not met; namely an insensitivity to extracellular receptor antagonists. We determined the effects of three different FPR2 selective antagonists, the WRW_4 hexapeptide, the bacterial peptide FLIPr, as well as the membrane-permeable PBP10 peptide [27, 30]. Our results conclusively demonstrate that F2Pal-induced neutrophil activity induced by the FPR2 pepducins is blocked by the antagonists. Since no detailed information regarding the pharmacological characteristics of the FPR2 specific inhibitory peptides is available, the mechanism can at this point not be clarified. Our results may however be explained without

the necessity to abandon the pepducin concept, if future studies show that the antagonists used have the same properties as earlier described FPR1 antagonists. These are either inverse agonists rather than competitive neutral antagonists [31, 32], or they trigger a change of the receptor that affect the affinity state and this change is achieved without any G-protein coupling [33]. Binding to FPRs of agonists as well as antagonist induces an affinity change of the occupied receptor that is transferred from a low- to a high-affinity state, and interestingly enough this process occurs also at low temperatures (4^{o}C) [33, 34, 35]. Induction of such an affinity-switch could possibly explain our binding data showing that F2Pal$_{10}$ and WKYMVM inhibit binding when added together with Cy5-WKYMVM but that these two peptides basically lack effects when added to the fluorescent probe occupied receptors. These data highlights the notion that the activation/inhibition and modulation processes that regulate FPRs are very complex, and it is intriguing that the inhibitory effects of an antagonist or an allosteric modulator might require some type of cell/receptor activation that is uncoupled from the G protein mediated signal transduction pathway [33]. The precise mechanism of action of a given GPCR is determined not only by the nature of the specific receptor but is also largely influenced by the type of ligand examined, e.g., agonist, antagonist or pepducins. Future studies should aim to clarify not only the precise site (extracellularly or intracellularly) of action of FPR2 pepducins but also whether binding of the known receptor antagonists induces this type of G-protein independent receptor activation.

Inside the cell, the generally accepted model for how pepducins activate signaling states that the peptide moiety of the molecule interacts with the corresponding regions of the specifically targeted receptor exposed on the inner leaflet of the membrane. This interaction

is suggested to lead to a stabilization of the receptor in an 'activated' state. Our data on neutrophil activation by the F2Pal pepducins, indicate that allosteric interaction with cytosolic receptor domains triggers the same type of receptor dynamics and signaling as when the receptor is activated by a conventional agonist that binds from the extracellular side. In both cases signaling starts very rapidly after addition of the activating compound, the peak of the response is reached at similar times (after around a minute in terms of O_2^- production) and once signaling has terminated the receptor is homologously desensitized.

The GPCR family members all have a similar seven transmembrane structure. Even though the initial pepducin strategy was to use peptide sequences identical to the third intracellular loop of the receptor to target this domain specifically, later work has shown that pepducins can successfully be generated with amino acid sequences corresponding to any of the cytoplasmic receptor domains [36]. The prevalent view regarding the mechanism for how a particular pepducin activates its target receptor is believed to be the stabilization of the target GPCR in a particular conformational/signaling state that involves an allosteric binding/modulation of that particular part of the receptor from which the peptide sequence of the pepducin is derived. Accordingly, the activation induced by a pepducin with an amino acid sequence corresponding to the third intracellular loop of the targeted receptor should involve that particular part of that receptor [1]. However, how such activation is achieved is still unclear.

Although the two neutrophil receptors FPR1 and FPR2 are very similar at the amino acid level, particularly in the cytoplasmic parts of the receptors, no activity was induced by the F1Pal peptide, differing from the activating F2Pal molecule in only two amino acids. The difference with respect to activity is actually related to one single amino acid, corresponding

to K_{231} of the receptor. The presence of this FPR2-specific K in the pepducins appears to be critical for pepducin activity. Pepducins in which K_5 (corresponding to K_{231} in the receptor) was replaced with a Q (the corresponding one in FPR1), an A or an N do not activate FPR2, but when replaced by another positively charged amino acid R the activity was retained. It is obvious that the presence of a charged amino acid in position five of FPR2 pepducins is of major importance in receptor activation. Accordingly, we found that when the non-charged Q_5 in the F1Pal pepducin was replaced with an R, this FPR1 like pepducin activated neutrophils, but the receptor responsible for activation was FPR2, suggesting a fundamental difference between the two receptors. Our earlier study using PBP10 have clearly demonstrated that the two FPRs, although eliciting very similar cellular responses, differ when it comes to signaling [6]. It is important to notice that the FPR2 selective cell permeable inhibitor PBP10 inhibits also the activity induced by F2Pal pepducins. When the impact of the charge was further evaluated on the receptor level, we found that the response induced by WKYMVM, in cells expressing the $FPR2_{K231M233\rightarrow QL}$ was intact, suggesting that the charge alone does not change FPR2 signaling. Moreover, this FPR1-like receptor mutant $FPR2_{K231M233\rightarrow QL}$ could still be activated by $F2Pal_{10}$, showing that there is no direct link between the amino acid sequence in the activating pepducin and that of the receptor mediating the response. It remains to be determined if the explanation to this at a molecular level is, that the third intracellular loop is not directly involved in mediating the pepducin effect or if a co-operation between different regions of the receptor is required. Such a co-operation has been suggested to be of importance for the activity of pepducins originating from protease activated receptors [37].

The two human FPRs enable neutrophils to detect diverse agonists derived from microbes or

mitochondria, are structurally very similar in their cytoplasmic signaling domains [9, 16, 38]. The third intracellular loop has been suggested to have an important role in signaling transmitted by GPCRs including adrenergic and muscarinic receptors [39]. No studies have been performed to identify the signaling regions of FPR2, but with respect to FPR1, the use of receptor-mimetic peptides have shown that a peptide spanning the third intracellular loop, i.e., (K_{227} to P_{239}) has no effect on binding to the G-protein, suggesting that the third intracellular loop of alone is not important for mediating G-protein coupling in FPR1 [40]. The data suggest instead that membrane spanning extensions on both sides of the third intracellular loop of are of importance G-protein binding [40]. These regions that are very similar in FPR1 and FPR2, but even though nothing is known about the structure-function relationship for FPR2, it is reasonable to assume that intracellular signals generated by the two FPRs rely on very similar structural changes when activated. The fact that FPR2 but not FPR1 is susceptible to activation by pepducins corresponding to the third cytoplasmic loop clearly implies that there are significant differences between these two closely related receptors. We have earlier disclosed one additional difference, namely that a rhodamine-linked and membrane-permeable peptide inhibitor (PBP10) selectively targets signaling from FPR2 [18, 19]. This peptide inhibits the F2Pal-induced neutrophil activation ([6] and this study) suggesting that the two compounds (PBP10 and the F2Pal pepducin) target the same signaling structure. We note that the peptide sequence in PBP10 has no sequence similarity with any regions in FPR2. Whether PBP10 inhibits FPR2 signaling by competing with the pepducin-like region for G-protein coupling or by the interaction with other portions of the receptor remains to be determined.

Taken together the data presented here clearly show that the same functional response and

signaling pathways are activated when the signaling cascade is initiated through agonists binding to the extracellular domains of FPR2 (outside-in signaling), or when signaling starts through an allosteric modulation of the receptor parts exposed on the cytosolic side of the membrane (inside-in signaling). Our results with receptor antagonists are not in agreement with the proposed allosteric modulation mechanism for pepducins and the molecular background underlying the antagonist sensitivity of F2Pal-induced activity has to be further investigated. Irrespective of the precise mechanism of pepducin activation, we can now add one more fundamental difference to those earlier described [16], between the two very closely related pattern recognition receptors FPR1 and FPR2.

5. ACKNOWLEDGEMENTS

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Figure legends

Fig 1. The pepducin F2Pal$_{16}$ induces activation of the neutrophil superoxide generating NADPH-oxidase

A): The mode of action of a pepducin, e.g., F2Pal$_{16}$ (left) and a conventional receptor agonist, e.g., WKYMVM (right) interacting with a G-protein coupled receptor that is transferred from a resting to a signaling state upon agonist binding. The activated receptor forms a signaling complex by association with the heterotrimeric G-protein. **B):** Primary human neutrophils were activated by addition of an FPR2-derived pepducin F2Pal$_{16}$ (solid line; 5 μM final concentration) or an FPR1-derived F1Pal$_{16}$ (dashed line; 5 μM final concentration). The release of superoxide anions was used as the read out system and the activity was recorded continuously. For comparison, the neutrophil response induced by WKYMVM (100 nM final concentration) is shown in the inset. The time point for addition of the agonists is indicated by arrows and a representative experiment out of more than five is shown. **C):** Primary human neutrophils were activated by addition (indicated by arrow) of the pepducin F2Pal$_{16}$ (solid line, 5 μM) and the F2Pal$_{16}$ mutants F2Pal$_{16K5\rightarrow Q}$ (5 μM, dashed line), and F2Pal$_{16M7\rightarrow L}$ (dotted line, 5 μM). The release of superoxide anions was recorded continuously. One representative experiment out of five is shown. Abscissa, time of study (min); ordinate, superoxide production in CPMx10^{-6} (arbitrary units).

Fig 2. The pepducin F2Pal$_{16}$ desensitizes neutrophils to the FPR2 selective agonist WKYMVM, but not to the FPR1 selective agonist fMLF

A): Primary human neutrophils were first activated by addition of the pepducin F2Pal$_{16}$ (final concentration 5 μM, added at the long arrow) and when the response induced had declined, a second stimulation with FPR1 specific agonist fMLF (10 nM final concentration, added at the short arrow) was received by the same batch of cells and the release of superoxide anions was continuously measured. The control neutrophils received fMLF only and the response induced is shown (dashed

line). **B):** Primary human neutrophils were first activated by addition of the pepducin F2Pal$_{16}$ (final concentration 5 µM, added at the long arrow) and when the response induced had declined, a second stimulation with FPR2 specific agonist WKYMVM (10 nM final concentration, indicated by a short arrow) was received by the same batch of cells and the release of superoxide anions was continuously measured. The control neutrophils received WKYMVM only and the response induced is shown (dashed line). A representative experiment out of more than five is shown. Abscissa, time of study (min); ordinate, superoxide production in CPMx10^{-6} (arbitrary units).

Fig 3. The pepducin F2Pal$_{16}$-induced response in primary neutrophils is inhibited by FPR2 selective antagonists

Primary human neutrophils were activated by addition of the pepducin F2Pal$_{16}$ (final concentration 5 µM) in the presence of the FPR2 antagonists PBP10 (1µM), FLIPr (2.5 µg/ml) and WRW$_4$ (5 µM) or the FPR1 antagonist CysH (1 µM). The antagonists were allowed to interact with the cells for 5 min before addition of F2Pal$_{16}$ and the release of superoxide anions was continuously measured. Data are expressed as percent superoxide production (peak values) compared to the F2Pal$_{16}$ induced in the absence of any antagonist (mean ± SEM, n=3). The inset shows a representative experiment out of five demonstrating a complete inhibition by the FPR2 antagonist PBP10 (1 µM, dashed line) and lack of inhibition by the FPR1 antagonist CysH (1 µM, dotted line). Control neutrophils (solid line) were incubated in the absence of antagonist. The time point for addition of F2Pal$_{16}$ is indicated by an arrow. Abscissa, time of study (min); ordinate, superoxide production in CPMx10^{-6} (arbitrary units). ***p<0.001; n.s. not significant.

Fig 4. The F2Pal$_{16}$ pepducin induces a transient change in cytosolic calcium in cells over-expressing FPR2

A): HL-60 cells over-expressing FPR2 were loaded with Fura-2 and the F2Pal$_{16}$ peptide (0.25 µM final concentration, solid line) was added to the cells and the change of free cytosolic Ca^{2+} was

monitored. The time point for addition of $F2Pal_{16}$ is indicated by an arrow. The Ca^{2+} response induced by the FPR2 specific agonist WKYMVM (10 nM, dashed line) is included as a positive control. **B):** HL-60 cells over-expressing FPR1 were loaded with Fura-2 and the $F2Pal_{16}$ peptide (0.25 μM, solid line) was added to the cells and the change of free cytosolic Ca^{2+} was monitored. The time point for addition of $F2Pal_{16}$ is indicated by an arrow. For comparison, the Ca^{2+} response induced by the FPR1 specific agonist fMLF (10 nM, dashed line) is included as a positive control. Traces of representative calcium responses are shown and the experiments have been performed at least three times. Abscissa, time of study (sec); ordinate, fluorescence (arbitrary units).

Fig 5. The $F2Pal_{10}$ and $F2Pal_9$ pepducins trigger superoxide production from human neutrophils

A): Neutrophils were activated with 0.6 μM (final concentration) of $F2Pal_{10}$ and the C-terminal truncated peptides $F2Pal_9$ and $F2Pal_8$, and the release of superoxide anions was continuously recorded. The time point for addition of the pepducin is indicated by an arrow. A representative experiment out of more than three is shown. Abscissa, time of study (min); ordinate, superoxide production in $CPMx10^{-6}$ (arbitrary units). The inset shows the relative activity of $F2Pal_9$ and $F2Pal_8$ expressed in percent of the peak value obtained by $F2Pal_{10}$ (mean ± SEM, n=3). ***$p<0.001$

B): Primary human neutrophils were activated by different concentrations of $F2Pal_{10}$ and the production of superoxide recorded continuously is used as the read out system. The concentration dependency is shown and the peak values were used to calculate the percent of maximum release (100%) induced by $F2Pal_{10}$ at concentrations ≥0.3 μM. Abscissa, concentration of the pepducin; ordinate, peak value of superoxide production (percent of max). The inset shows representative responses from the maximum release induced by 0.6 μM and a low response induced by 0.04 μM $F2Pal_{10}$. Inset; abscissa, time of study (min); ordinate, superoxide production in $CPMx10^{-6}$ (arbitrary units).

Fig 6. The $F2Pal_{10K5->R}$ pepducin induces superoxide production in primary neutrophils and a transient change in cytosolic calcium in cell over-expressing FPR2

A): Superoxide production induced by the pepducin $F2Pal_{10}$ mutants from primary human neutrophils stimulated with $F2Pal_{10}$ and $F2Pal_{10}$ mutants $F2Pal_{10K5\rightarrow R}$, $F2Pal_{10K5\rightarrow N}$, $F2Pal_{10K5\rightarrow A}$, and $F2Pal_{10K5\rightarrow Q}$ (0.6 μM final concentration for all peptides) and the release of superoxide was recorded continuously. Data were presented as relative superoxide production comparing the peak values of the responses (percent of the response induced by $F2Pal_{10}$) (mean ± SEM, n=3). **B):** Ca^{2+} responses induced by pepducin $F2Pal_{10}$ and peptide mutants in Fura-2 loaded HL-60 cells over-expressing FPR2. The pepducin $F2Pal_{10}$ (0.05 μM), $F2Pal_{10K5\rightarrow R}$ (0.05μM) and $F2Pal_{10K5\rightarrow N}$ (1 μM) were added (indicated by an arrow) and the change of free cytosolic Ca^{2+} was monitored by the Fura-2 fluorescence. Traces of representative calcium responses are shown and the experiments have been performed at least three times. Abscissa, time of study (sec); ordinate, fluorescence (arbitrary units).

Fig 7. The palmitoyl group is of importance for the $F2Pal_{10}$ induced activity

A): The superoxide production/release by primary human neutrophils following addition of the pepducin $F2Pal_{10}$ (0.3 μM final concentration) or a non-palmitoylated variant with the same peptide sequence $F2_{10}$ (10 μM final concentration). The release of superoxide anions was used as the read out system and the activity was recorded continuously. The time point for addition of the peptides is indicated by an arrow and a representative experiment out of more than three is shown. Abscissa, time of study (min); ordinate, superoxide production in $CPMx10^{-6}$ (arbitrary units). **B):** HL-60 cells over-expressing FPR2 were activated by different concentrations (final concentrations from 0.05 to 1 μM) of the $F2Lau_{10}$ pepducin containing a lauryl group as the hydrophobic anchor but with the same peptide sequence as $F2Pal_{10}$. The change in intracellular Ca^{2+} was monitored by the Fura-2 fluorescence. $F2Lau_{10}$ was added at the time point indicated by an arrow. Traces of representative calcium responses are shown and the experiments have been performed at least three times. Abscissa, time of study (sec); ordinate, fluorescence (arbitrary units).

Fig 8. The $F2Pal_{10}$ pepducin inhibits binding of the Cy5-WKYMVM peptide to neutrophils

A): The binding of Cy5-WKYMVM (1 nM final concentration) to primary human neutrophils was determined by flow cytometry. Cells were incubated on melting ice with the fluorescently labeled Cy5-WKYMVM peptide in the absence (filled grey) or in the presence of an excess of non-labeled WKYMVM (100 nM final concentration, solid line). The non-fluorescent WKYMVM reduced binding and these values were defined as 100% inhibition. One representative histogram out of four is shown. **B):** The binding of Cy5-WKYMVM (1 nM final concentration) to primary human neutrophils was determined by flow cytometry. Cells were incubated on melting ice with the fluorescent labeled Cy5-WKYMVM peptide in the absence (filled grey) or in the presence of different concentrations of $F2Pal_{10}$ (final concentrations 1 μM (solid line), 0.1 μM (dotted line) and 0.01 μM (dashed line), respectively). At the highest concentration of the $F2Pal_{10}$ pepducin, binding of Cy5-WKYMVM was reduced to the same level as by WKYMVM. The figure shows FACS curves from one representative experiment. Inset: Inhibition of Cy5-WKYMVM binding to human neutrophils by 1 μM and 0.1 μM $F2Pal_{10.}$ Data are expressed as percent of the maximal inhibition obtained with WKYMVM (100 nM final concentration; mean ± SEM, n=4). **C):** Cells were incubated on ice for 45 min with Cy5-WKYMVM (1nM final concentration) prior to the addition of WKYMVM (100 nM, grey bars) or $F2Pal_{10}$ (1 μM, black bars) and the cell associated fluorescence was determined over time as indicated. Data are expressed as percent of control (fluorescence obtained in the absence of inhibitory peptides at indicated time points) (mean ± SEM, n=3).

Fig 9. The $F2Pal_{10}$ pepducin activates HL-60 cells over-expressing an FPR2 mutant ($FPR2_{mut}$)

HL-60 cells stably expressing $FPR2_{mut}$ ($FPR2_{K231M233\rightarrow Q231L233}$) were loaded with Fura-2 and different concentrations (0.5 μM, solid line and 0.05 μM, dashed line) of the $F2Pal_{10}$ peptide was added to the cells and the change of free cytosolic Ca^{2+} was monitored. The time point for addition of $F2Pal_{16}$ is indicated by an arrow. The third intracellular loop of the mutated FPR2 is identical to that of FPR1

and this was achieved through an exchange of the two amino acids in the third intracellular loop that differ between the two receptors. Traces of representative calcium responses are shown and the experiments have been performed at least three times. Abscissa, time of study (sec); ordinate, fluorescence (arbitrary units).

Fig 1. Forsman *et al*

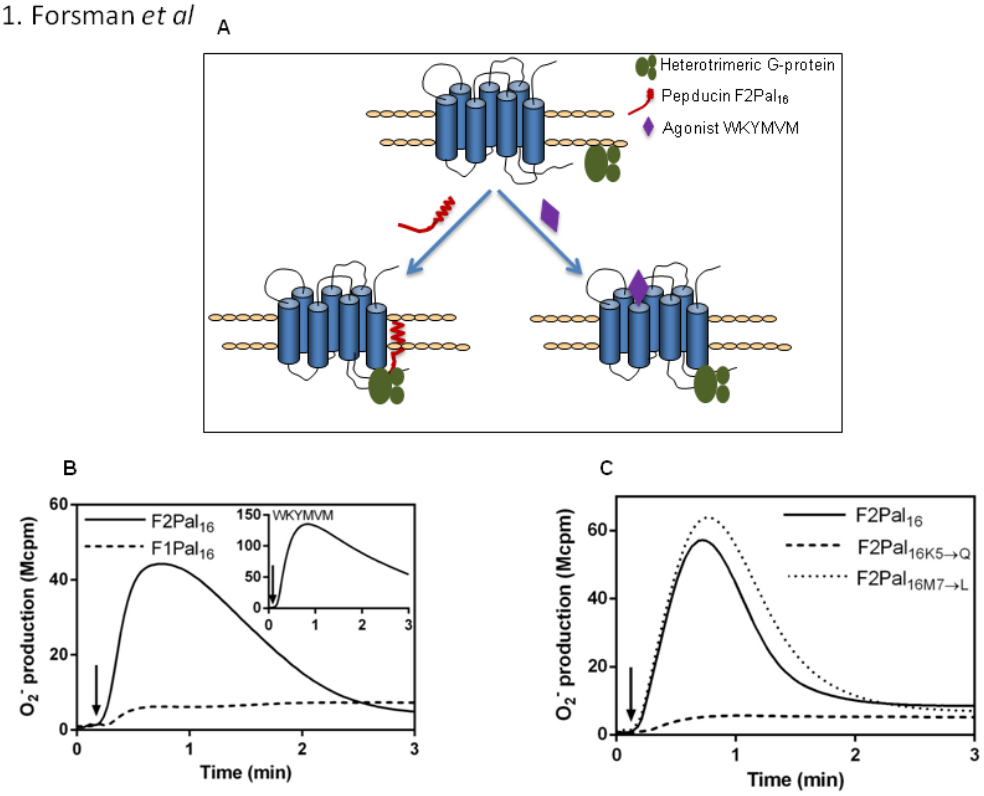

Fig 2. Forsman *et al*

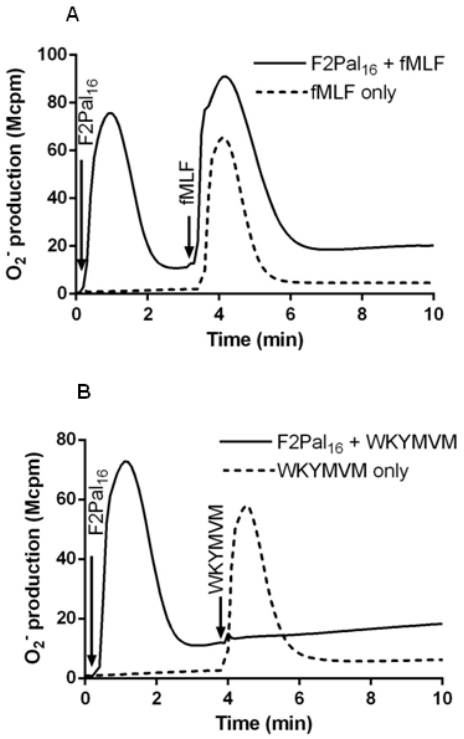

Fig 3. Forsman *et al*

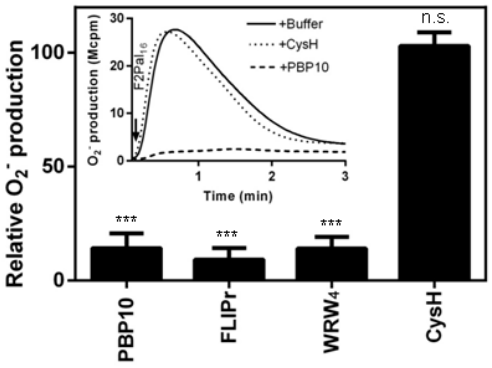

Fig 4. Forsman *et al*

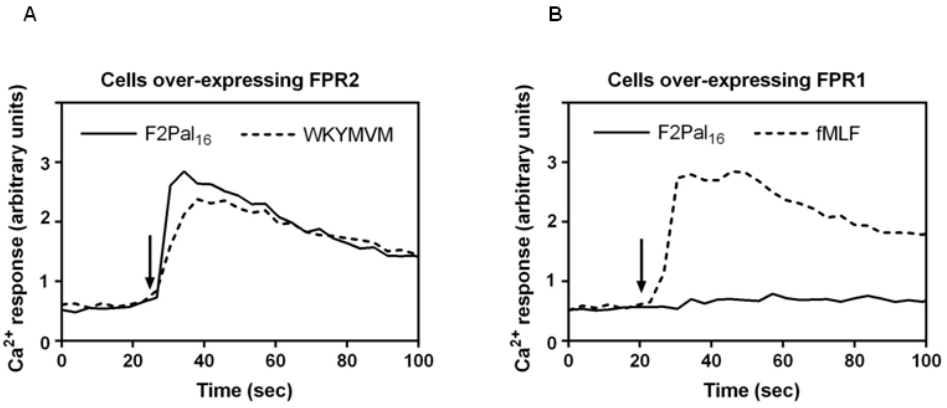

Fig 5. Forsman *et al*

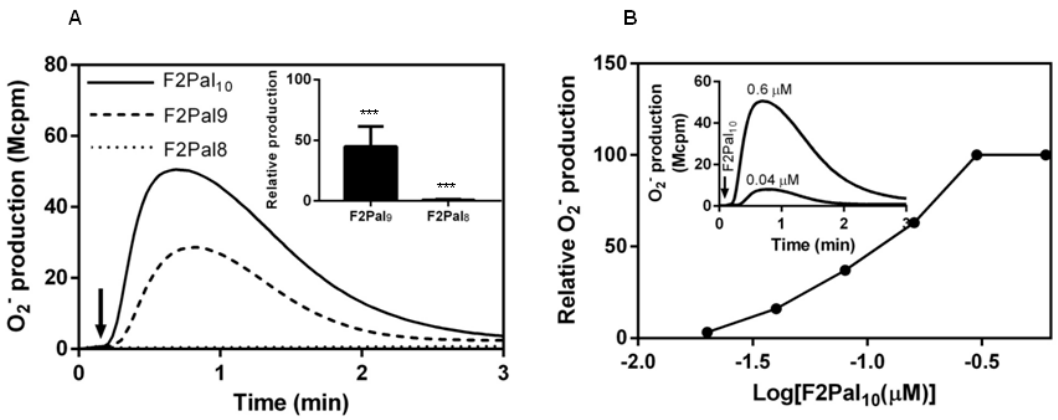

Fig 6. Forsman *et al*

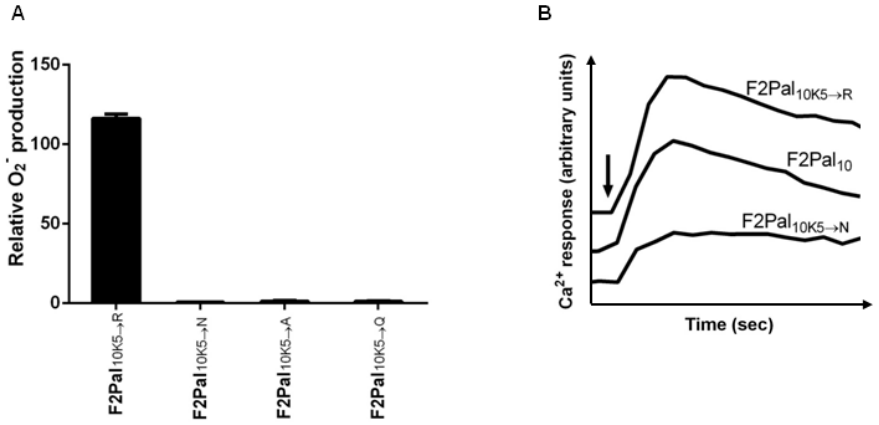

Fig 7. Forsman *et al*

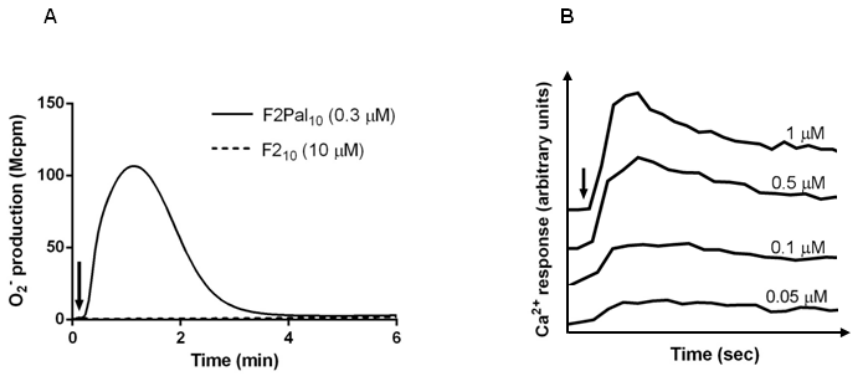

Fig 8. Forsman *et al*

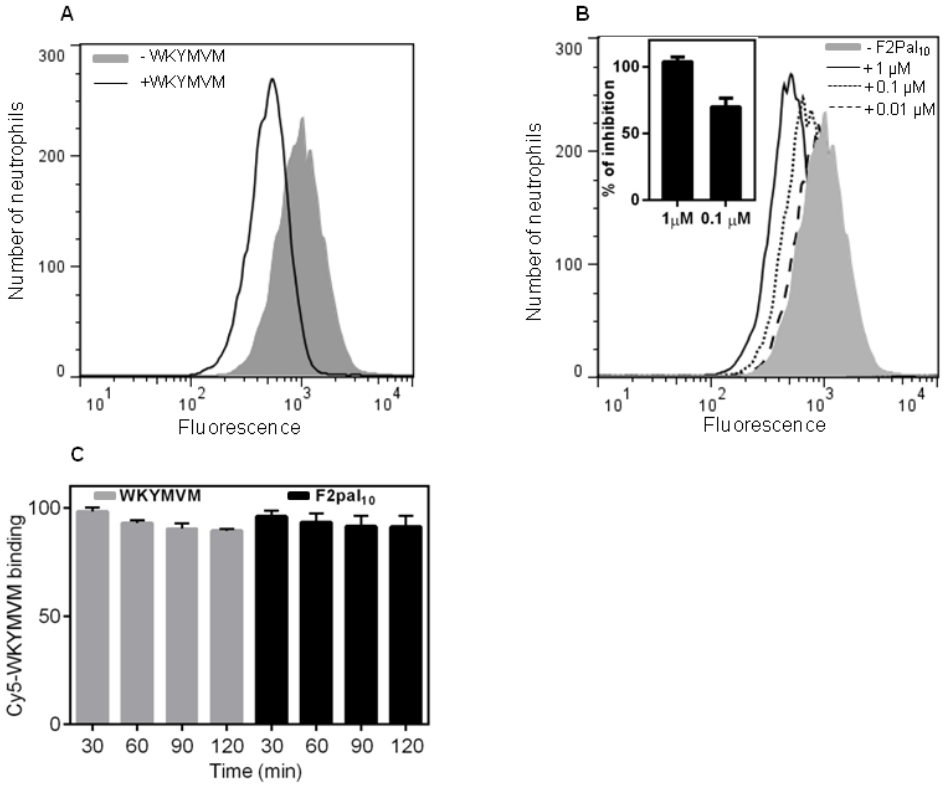

Fig 9. Forsman *et al*

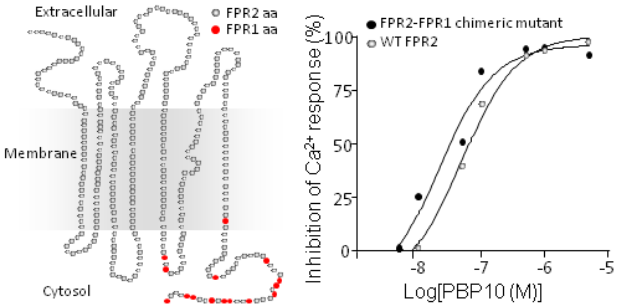

Fig 3. Schematic drawing of FPR2 (grey) and the amino acids differing between FPR2 and FPR1 in the third intracellular loop and cytoplasmic tail are marked in red (Left). The inhibitory effect of PBP10 on Ca^{2+} signalling in FPR2 and a chimeric FPR2-FPR1 in which the cytoplasmic tail was replaced by the corresponding one in FPR1 (right).