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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

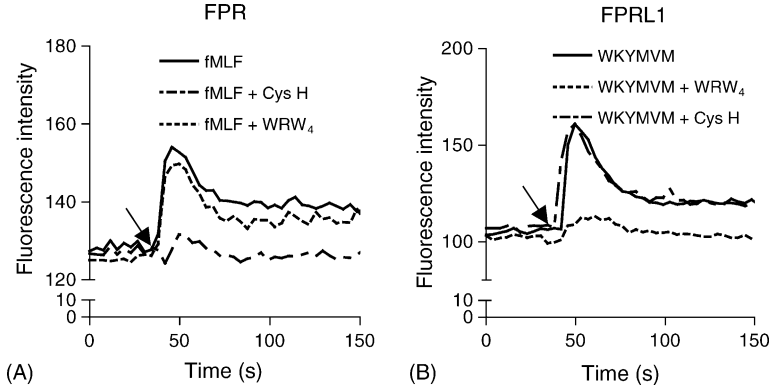

Fig. 1 – Effects of the receptor antagonists cyclosporine H (cys H; FPR specific) and WRWWWW (WRW_4; FPRL1 specific) on the intracellular Ca^{2+} transient in HL-60 cells expressing FPR or FPRL1. Stably transfected HL-60 cells expressing either FPR or FPRL1 were loaded with Fura-2 and incubated (at 37 °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.

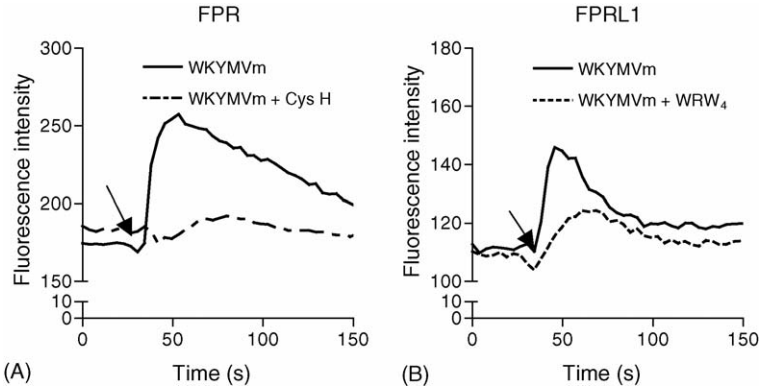

Fig. 2 – Effects of the receptor antagonists cyclosporine H (cys H; A) and WRWWWW (WRW_4; B) on the response in receptor expressing HL-60 using WKYMVm as triggering agonist. Stably transfected HL-60 cells expressing either FPR or FPRL1 were loaded with Fura-2 incubated (at 37 °C for 5 min) in the absence 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

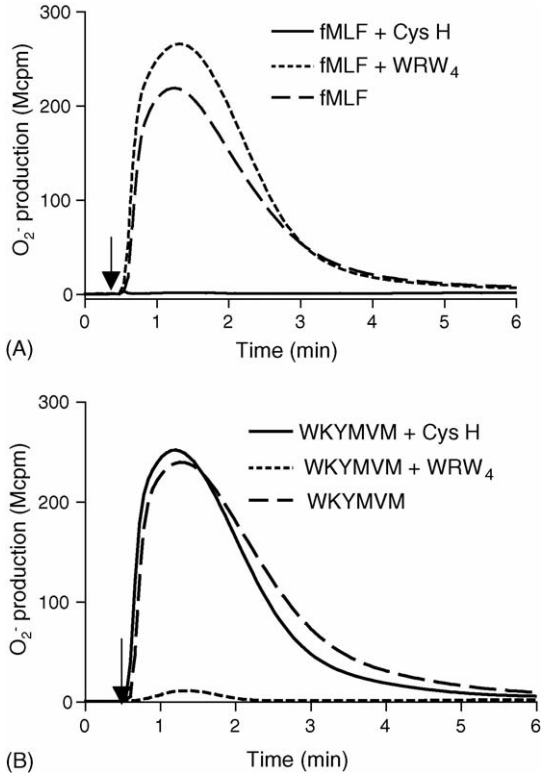

Fig. 3 – Superoxide production in neutrophils triggered with fMLF (A) or WKYMVM (B) and the effects of receptor specific antagonists. Neutrophils were pre-incubated at 37 °C for 5 min in the absence or presence of the antagonists, Cys H (1 μM) or WRW_4 (5 μM). The cells were then challenged with fMLF (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.

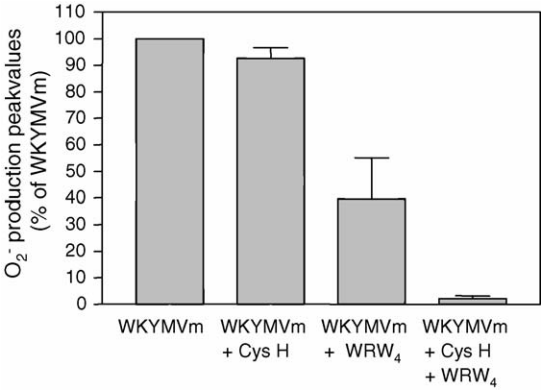

Fig. 4 – Superoxide production in neutrophils triggered with WKYMVm and the effects of receptor specific antagonists. Neutrophils were pre-incubated at 37 °C for 5 min in the absence or presence of the inhibitors, Cys H (1 μM), WRW_4 (5 μM) or a mixture of both. The cells were then challenged with WKYMVm (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

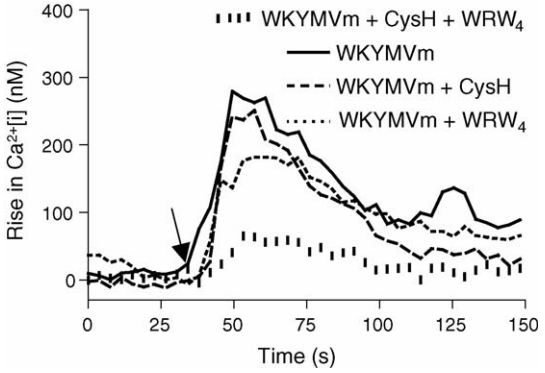

Fig. 5 – Effects of the receptor antagonists cyclosporine H (cys H; FPR specific) and WRWWWW (WRW_4; FPRL1 specific) on the intracellular Ca^{2+} transient in neutrophils. Cells were loaded with Fura-2 and incubated (at 37 °C for 5 min) in the absence or presence of the antagonists (1 μM cys H or 5 μM 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.

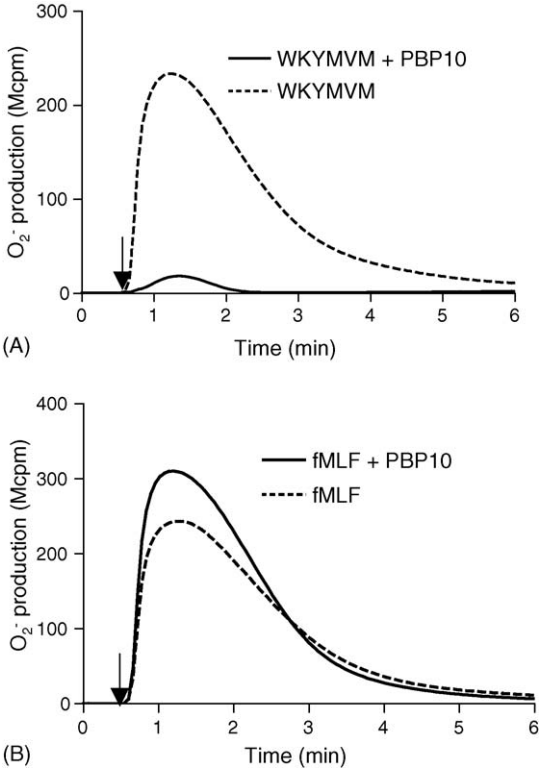

Fig. 6 – Effects of the PIP_2 binding peptide PBP10 on superoxide anion production in neutrophils triggered with WKYMVM (A) or fMLF (B). Neutrophils were pre-incubated at 37 °C for 5 min in the absence or presence of PBP10 (1 μM). The cells were then challenged with WKYMVM (A; 10^{-7} M) of fMLF (B; 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

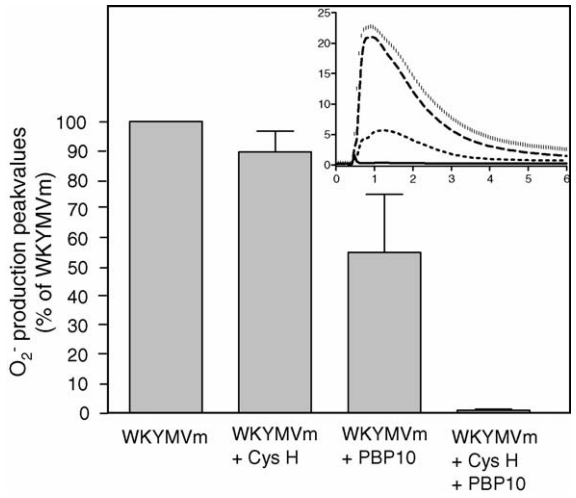

Fig. 7 – Effects of the PIP_2 binding peptide PBP10 and Cys H on superoxide anion production in neutrophils and differentiated HL-60 cells (inset) triggered with WKYMVm. The cells were pre-incubated at 37 °C for 5 min in the absence or presence of the inhibitors, PBP10 (1 μM), Cys H (1 μM) or a mixture 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 (——).

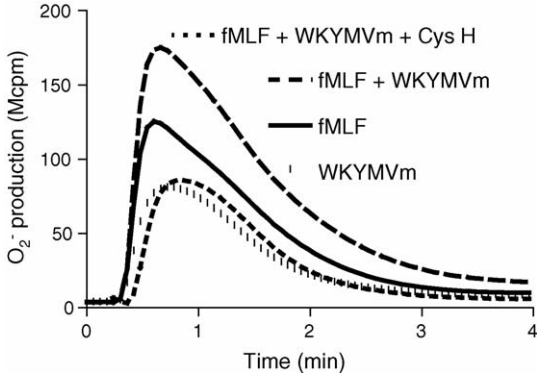

Fig. 8 – Effect of Cys H on neutrophil superoxide production in cells triggered simultaneously with WKYMVm and fMLF, two agonists with overlapping receptor specificities. Neutrophils were pre-incubated at 37 °C for 5 min in the absence or presence of Cys H (1 μM). The cells were then challenged with 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.

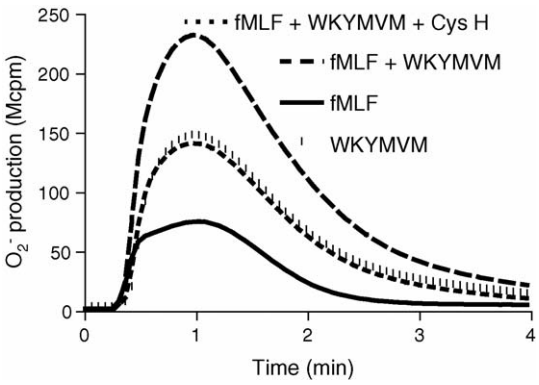

Fig. 9 – Effect of Cys H on neutrophil superoxide production in cells triggered simultaneously with WKYMVM and fMLF, two agonists with different receptor specificities. Neutrophils were pre-incubated at 37 °C for 5 min in the absence or presence of Cys H (1 μM). The cells were then challenged with WKYMVM (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.

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