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The non-steroidal anti-inflammatory drug piroxicam blocks ligand binding to the formyl peptide receptor but not the formyl peptide receptor like 1

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ABSTRACT

The anti-inflammatory drug piroxicam has been reported to affect the production of reactive oxygen species in phagocytes. This anti-inflammatory effect is thought to be mediated through inhibition of cyclooxygenase (COX), an enzyme important for prostaglandin synthesis. We have compared the effects of piroxicam on superoxide production mediated by two closely related G-protein coupled receptors expressed on neutrophils, the formyl peptide receptor (FPR) and the formyl peptide receptor like 1 (FPRL1). Neutrophils were stimulated with agonists that bind specifically to FPR (the peptide ligand *N*-formyl-Met-Leu-Phe, fMLF) or FPRL1 (the peptide ligand Trp-Lys-Tyr-Met-Val-L-Met-NH_2, WKYMVM) or both of these receptors (the peptide ligand Trp-Lys-Tyr-Met-Val-D-Met-NH_2, WKYMVm). Piroxicam reduced the neutrophil superoxide production induced by the FPR agonist but had no significant effect on the FPRL1 induced response. Neutrophil intracellular calcium changes induced by the agonist WKYMVm (that triggers both FPR and FPRL1) were only inhibited by piroxicam when the drug was combined with the FPRL1 specific antagonist, Trp-Arg-Trp-Trp-Trp-Trp (WRW_4), and this was true also for the inhibition of superoxide anion release. Receptor-binding analysis showed that the fluorescently labelled FPR specific ligand *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys (fNLFNYK), was competed for in a dose-dependent manner, by the FPR ligand fMLF and as well as by piroxicam.

We show that piroxicam inhibits the neutrophil responses triggered through FPR, but not through FPRL1 and this inhibition is due to a reduced binding of the activating ligand to its cell surface receptor.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are a common treatment for a number of disease states involving an inflammatory reaction. This group of drugs mediate their anti-inflammatory effects mainly by inhibition of cyclooxygenase (COX), an enzyme of prime importance for the synthesis of different prostaglandins [1,2]. A number of studies have demonstrated that neutrophils, important phagocytes of our innate immune system, have the capacity to synthesize and release prostaglandins via the COX pathway [3,4], suggesting that the function of activated neutrophils might be affected by

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NSAIDs. Accordingly, piroxicam, which belongs to the oxicam family of NSAIDs and is a non-selective COX-inhibitor, has been reported to affect neutrophil functions such as the production and release of superoxide anions generated by the activated NADPH-oxidase [5–7].

Neutrophilic granulocytes have the ability to migrate in response to gradients of chemoattractants, soluble molecules serving as ''danger signals''. In a number of inflammatory disorders the tissue damage is associated with the chemoattractant-guided accumulation of neutrophils and their subsequent release of reactive oxygen species and proteolytic enzymes. The list of structurally well-characterized leukocyte chemoattractants has steadily grown and the broad application of molecular biology techniques has led to identification of chemoattractant receptors. These receptors specifically recognize different chemoattractants, exhibit some sequence homologies and share structural features. They all belong to a pertussis toxin sensitive subfamily within the G protein-coupled receptor (GPCR) superfamily. The formyl peptide receptor (FPR) was the first neutrophil GPCR to be cloned and sequenced [8]. Soon after the FPR sequence was published, an orphan neutrophil FPR-like receptor, formyl peptide receptor like1 (FPRL1), was identified [8,9]. The FPR is a high affinity pattern recognition receptor with the ability to track bacteria releasing formylated peptides [10,11], supporting the idea that FPR may have a direct function in innate defence against bacterial infection. Following the discovery of FPRL1-specific ligands it has become obvious that this receptor possesses large functional similarities with the FPR [10,12]. Binding of ligands to FPR and FPRL1 thus induces a variety of leukocyte activities such as chemotactic movement, chemoattractant induced mobilization of granules, and superoxide anion production as a result of an activation of the NADPH-oxidase. The downstream signalling of FPR has been extensively studied as a model system and it was until recently, assumed that FPRL1 uses identical routes. However, lately differences in these receptors signalling pathways have been suggested [13,14].

In this study, we compare the effect of piroxicam on the neutrophil activity via the two neutrophil members of the formyl peptide family of receptors. We show that piroxicam reduces the activity triggered through FPR but has no effect on the FPRL1 and that the inhibitory effect is due to inhibition of ligand binding to FPR.

2. Materials and methods

2.1. Chemicals and reagents

Dextran and Ficoll-Paque were purchased from Amersham Pharmacia Biotec AB (Uppsala, Sweden) *N*-formyl-Met-Leu-Phe (fMLF), piroxicam, horseradish peroxidase (HRP), cytochalasin B (Cyt B) and complement factor 5a (C5a) were purchased from Sigma–Aldrich (Steinheim, Germany). The substances were dissolved to 10^{-2} M (fMLF), 10 mg/ml (Cyt B) resp. 10^{-1} M (piroxicam) in dimethyl sulphoxide (DMSO), C5a was dissolved to 1 mg/ml in distilled water and stored at −70 °C until used. The hexapeptides Trp-Lys-Tyr-Met-Val-L-Met-NH_2 and Trp-Lys-Tyr-Met-Val-D-Met-NH_2 (WKYMVM, WKYMVm) were synthesized and HPLC-purified by Alta Bioscience (University of Birmingham, UK). The peptides were dissolved in DMSO to 10^{-2} M and stored at −70 °C until used. The fluorescent molecules Fluo-3 and Fura-Red and the fluorescent-labelled *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys (fNLFNYK) were from Molecular Probes, Invitrogen detection technologies (Eugene, OR). The peptide fNLFNYK was dissolved in DMSO to 10^{-2} M and stored at −70 °C. The annexin 1 peptide ($Ac_{9–25}$, QAWFIENEEQEYVQTVK) was synthesized and HPLC-purified by Ross-Petersen ApS (Holte, Denmark). The peptide was dissolved in alkaline phosphate-buffered saline and stored at −70 °C. Cyclosporin H (CsH) was kindly provided by Novartis Pharma (Basel, Switzerland) and dissolved in DMSO to 10^{-2} M and stored at −70 °C. The FPRL1 antagonist Trp-Arg-Trp-Trp-Trp-Trp (WRW_4) was purchased from Genscript Corp. (Scotch Plains, NJ) and dissolved in DMSO to 10^{-2} M and stored at −70 °C.

2.2. Isolation of human neutrophils

Peripherial blood neutrophils were isolated from human buffy coats (The Blood Center, Sahlgrenska University Hospital, Göteborg) obtained from adult healthy blood donors. Erythrocytes were depleted by dextran (2%) sedimentation at $1 \times g$. Granulocytes, in the leukocyte rich supernatant, were separated from the mononuclear cells by gradient centrifugation on Ficoll-Paque. The remaining erythrocytes were eliminated by hypotonic lysis. Neutrophils were washed and re-suspended in Kreb's-Ringer glucose buffer (KRG, 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.7 mM KH_2PO_4, 8.3 mMNa_2HPO_4, 10 mM glucose, 1 mM $CaCl_2$, pH 7.3) and stored on ice until use.

2.3. Determination of superoxide anion release

The NADPH-oxidase dependent release of superoxide anion was measured with an isoluminol enhanced chemiluminescence (CL) system, using a six-channel Biolumat LB 9505 (Berthold Co. Wildbad, Germany). Disposable 4 ml polypropylen tubes containing 10^6 neutrophils and 900 μl reaction mixture with or without piroxicam and antagonists (WRW_4 2.5×10^{-6} M, Cyclosporin H (CsH) 10^{-6} M) diluted in KRG were equilibrated 5 min at 37 °C in the Biolumat before the addition of 100 μl of the appropriate stimulus (fMLF 10^{-6} M, Annexin-1 ($Ac_{9–25}$) 5×10^{-5} M, WKYMVM 10^{-6} M, WKYMVm 10^{-8} M and C5a 100 ng/ml diluted in KRG). The reaction mixture for measurement of reactive oxygen species (ROS) contained isoluminol (2×10^5 M, a cell-impermeable CL substrate) and horseradish peroxidase (HRP, 4U, a cell-impermeable peroxidase). The light emission was continuously recorded. Details about the technique are given in [15].

2.4. Intracellular calcium flux measurement

The calcium flux measurement was performed according to Partida-Sanchez et al. [16]. In brief, neutrophils were resuspended in cell loading medium (CLM, 1% fetal bovine serum in KRG with 1 mM Ca^{2+}) and loaded with Fluo-3 (4 μg/ml) and Fura-Red (10 μg/ml) at 37 °C for 30 min. Cells were washed twice in CLM and resuspended to a final concentration of 10^7/ml and put on ice. Neutrophils (10^5) and antagonists (CsH

10^{-6} M, WRW_4 10^{-6} M, and piroxicam 50 μM diluted in KRG) were mixed and the suspension was pre warmed at 37 °C for 5 min. The intracellular Ca^{2+} accumulation in individual cells was assessed by flow cytometry over 5 min. The fluorescence emission of Fluo-3 and Fura-Red was measured in the FL-1 and the FL-3 channel respectively. Data was analysed using FlowJo 5.7.1 (FlowJo, LLC, Ashland, OR) and the relative intracellular Ca^{2+} concentration was presented as the ratio between Fluo-3 and Fura-Red mean fluorescence intensity over time.

2.5. Competitive receptor-binding

Neutrophils (10^7/ml) were incubated at 15 °C for 10 min. To upregulate cellsurface receptors and prevent receptor internalization WKYMVM (10^{-7} M) and cytochalasin B (2,5 μg/ml) diluted in KRG were added and cells were incubated at 37 °C for 10 min. Unlabeled peptides were wash away with ice cold PBS. Cells were re-suspended in ice cold PBS and 10^5 cells were added to a tube containing the fluorescent labelled peptide, fNLFNYK (10^{-9} M final concentration diluted in PBS). This is a high affinity FPR ligand that possesses neutrophil activating capacities such as actin polymerization and superoxide production [17]. Binding was determined after 15 min by flow cytometry analysis and compared with binding to cells pre-treated (for 5 min) with different concentrations of non-labelled peptides or piroxicam diluted in PBS. Fluorescence data were analysed with Cell Quest (Becton Dickinson Immunocytometry System, San Jose, CA).

2.6. Statistics

Statistical analyses were performed with one-way ANOVA followed by Dunett's multiple comparison test using GraphPad Prism 2.0 software (GraphPad, Sandiego, CA). A P value less than 0.05 were considered as statistical significant.

3. Results

3.1. Piroxicam reduces neutrophil superoxide production triggered by FPR agonists

Piroxicam at high concentrations has earlier been shown to inhibit neutrophil superoxide production, but no specificity or selectivity with respect to the triggering agonist or receptor has been described [5,18]. We have determined the effects of piroxicam on neutrophils superoxide production/release induced through three different G-protein coupled receptors, the FPR and FPRL1 that belongs to the formyl peptide receptor family, and a receptor that not belongs to this receptor family, the complement fragment C5a receptor, C5aR. Signalling through these receptors was induced by the receptor-specific

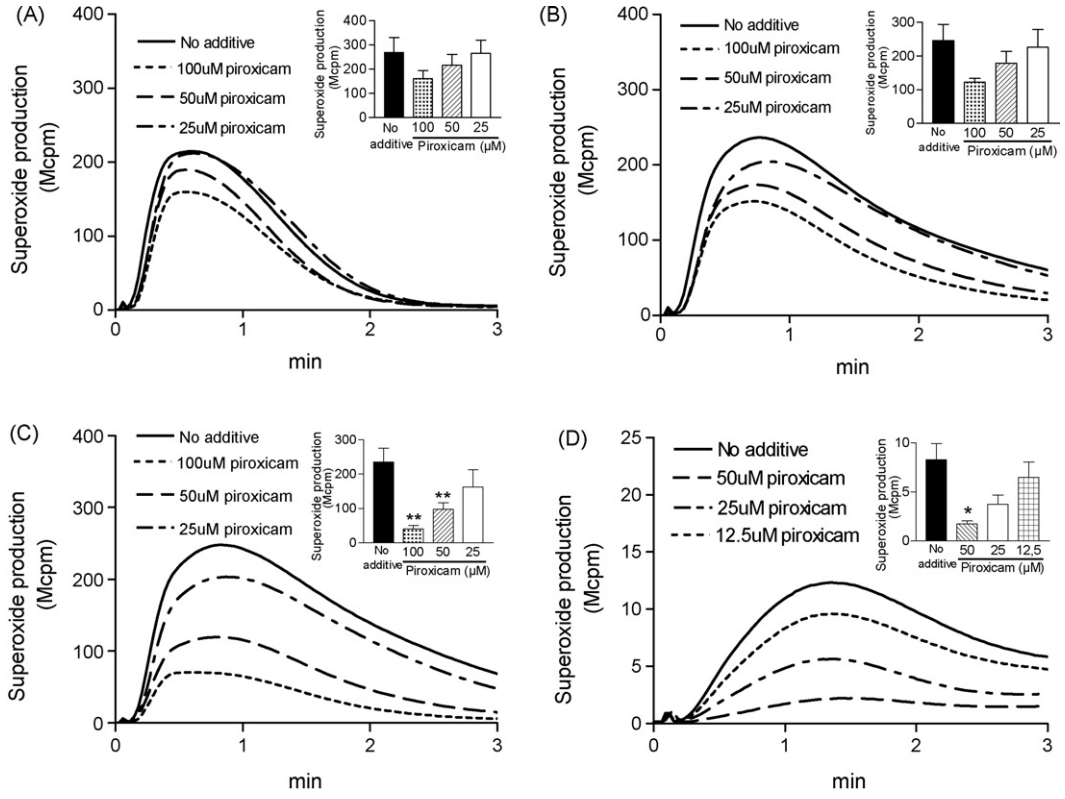

Fig. 1 – Neutrophil superoxide production and the effect of piroxicam. Neutrophils (10^6) were incubated with increasing concentrations of piroxicam (100, 50, 25, 12.5 μM) for 5 min at 37 °C and then stimulated with (A) the complement factor 5 fragment a, C5a (100 ng/ml), (B) the FPRL1 agonist WKYMVM (10^{-7} M), (C) the high affinity FPR agonist fMLF (10^{-7} M) or (D) the low affinity FPR agonist $Ac_{9–25}$ (1, 5×10^{-5} M). (A)–(D) shows one representative experiment out of six or three (D), means ± S.E.M. are shown in inset bar graphs. The induced superoxide production is presented as arbitrary units measured in 10^6 counts per minute (Mcpm). *P < 0.05, **P < 0.01.

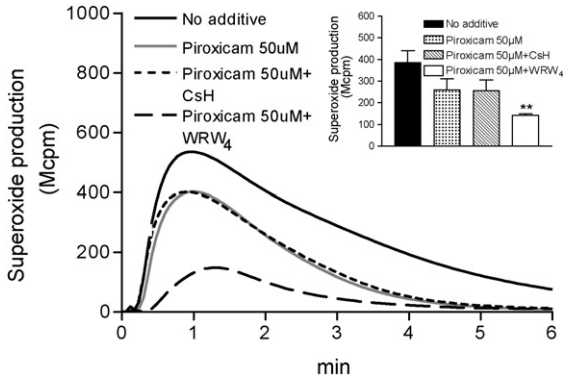

Fig. 2 – The effects of piroxicam on neutrophilic (10^6) superoxide production induced by WKYMVm (10^{-9} M). Piroxicams (50 μM) effect alone, in combination with cyclosporine H (CsH, 10^{-6} M) or in combination with WRWWWW (WRW_4, 5×10^{-6} M) are presented. The figure shows one representative experiment out of three, the mean ± S.E.M. is shown in an inset bar graph. The induced superoxide production is presented as arbitrary units measured in 10^6 counts per minute (Mcpm). $^{**}P < 0.01$.

agonists; fMLF (an FPR high- affinity agonist), $Ac_{9–25}$ (an FPR low-affinity agonist derived from annexin 1), the hexapeptide WKYMVM (an FPRL1 agonist), and complement split product C5a (a C5aR agonist). We found that the superoxide production elicited by C5a and WKYMVM was not significantly reduced ($P > 0.05$, $n = 6$) by either of the used concentrations 100, 50 and 25 μM of piroxicam (Fig. 1A and B). In contrast, the neutrophil responses induced by the FPR agonist fMLF was significantly reduced by 100 μM as well as 50 μM piroxicam ($P < 0.01$, $n = 6$) and the inhibition with the alternative FPR agonist $Ac_{9–25}$, was significantly reduced by 50 μM piroxicam ($P < 0.05$, $n = 3$; not tested with 100 μM, Fig. 1C and D). These data suggest a difference in sensitivity to piroxicam, between the two receptors of the FPR family and/or the agonists, fMLF/$Ac_{9–25}$ and WKYMVM.

3.2. *WKYMVm induced neutrophil superoxide release is inhibited by piroxicam when a receptor specific antagonist blocks FPRL1*

To determine if the effect of piroxicam was due to interaction with the agonist rather than the receptor we exchanged the two agonists with one, the WKYMVm peptide, which triggers a cellular response through both FPR and FPRL1 [19]. One of the receptors, FPR, is however not used unless signalling through the other receptor is blocked [20]. Piroxicam alone had a

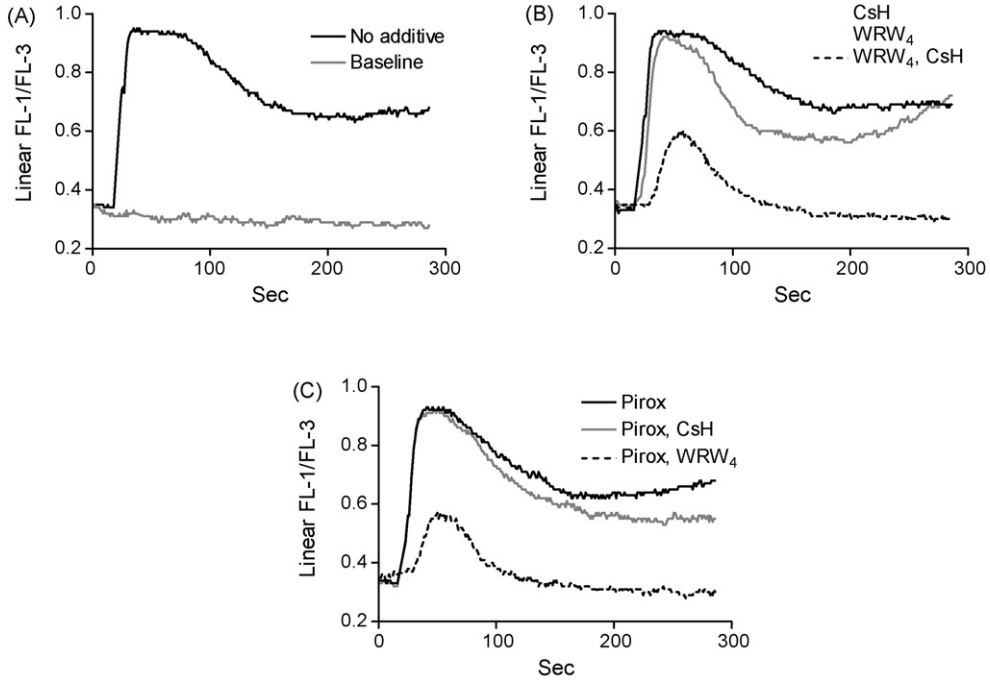

Fig. 3 – The change in intracellular calcium induced in neutrophils (5×10^{-5}) triggered with WKYMVm (10^{-9} M) using Fluo-3 and Fura-Red as calcium sensitive fluorescent markers. (A) The calcium transient induced by WKYMVm (black) is shown and the resting level without any stimulus is also included (grey). (B) The presence of the FPR antagonist cyclosporine H (CsH, 10^{-6} M, black) or the FPRL1 antagonist WRW_4 (10^{-6} M, grey) alone was without effect on the response, however, when these antagonists were combined (dotted line) the response was reduced. (C) No effects were seen with piroxicam (Pirox, 50 μM) alone (black) or in combination with the FPR antagonist CsH (grey), whereas a reduced calcium response was obtained when piroxicam was combined with WRW_4 (dotted line). The relative intracellular Ca^{2+} concentration is presented as the ratio in mean fluorescence ratio over time, between Fluo-3 and Fura-Red, and one representative experiment out of three is shown.

modest but not significant ($P > 0.05$, $n = 3$) inhibitory effect on the WKYMVm-induced response and there was no additional effect on the WKYMVm-induced neutrophil response when signalling trough FPR was blocked by the combination of piroxicam and the receptor specific antagonist cyclosporine H (Fig. 2). However, when piroxicam was used in combination with the FPRL1 antagonist WRW_4, the response was significantly inhibited ($P < 0.01$, $n = 3$, Fig. 2). These data show that the piroxicam-induced inhibition of oxidase activity is linked to the receptor rather than to the agonist, and the effect could be at the level of ligand binding or at the level of signalling.

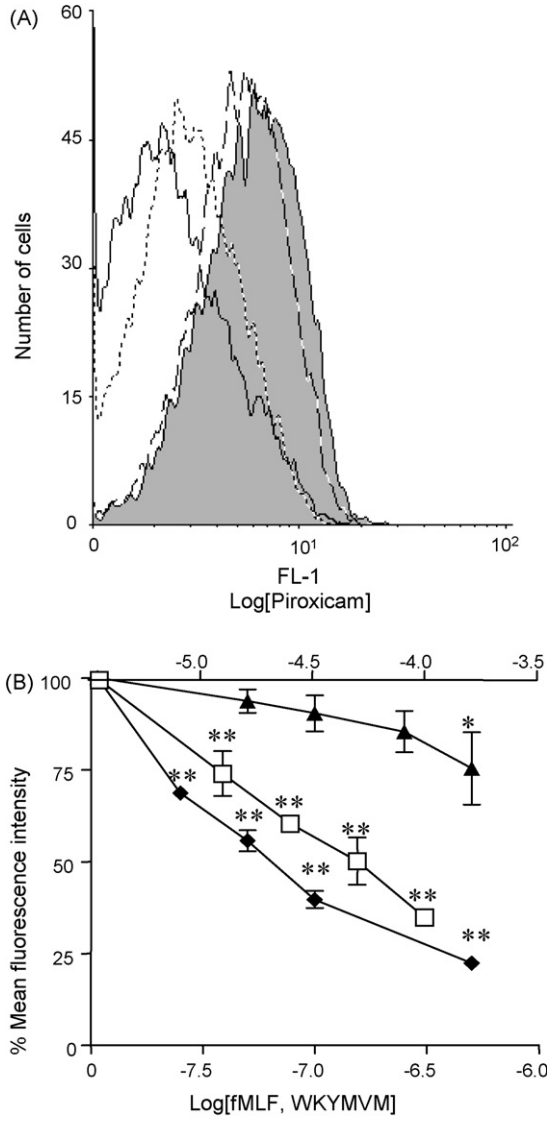

Fig. 4 – A competitive receptor-binding assay was performed by flow cytometry using the fluorescently labelled FPR agonist, fNLFNYK (10^{-9} M). (A) Piroxicam (50 μM, solid line) and fMLF (10^{-7} M, dotted line) competed with fNLFNYK (grey peak) for binding to FPR, while WKYMVM (10^{-7} M, semisolid line) had no effect. One representative experiment out of five is shown. (B) The statistical difference in mean percent fluorescence of fNLFNYK after addition of different concentrations of piroxicam ((□) upper *x*-axis), fMLF ((◆) lower *x*-axis) or WKYMVM ((▲) lower *x*-axis) is illustrated in a diagram. All data represent the mean ± S.E.M., $n = 3$. *$P < 0.05$, **$P < 0.01$.

3.3. *The WKYMVm induced calcium flux is inhibited by piroxicam only when a receptor specific antagonist blocks FPRL1*

In order to determine if piroxicam affects the receptor, we determined the transient increase in intracellular Ca^{2+}, since this is a signalling system very far ''up-stream'' (close to the receptor) in relation to the signalling cascade that ultimately leads to activation of the oxidase [21]. When the specific antagonist of FPR (CsH) or the specific antagonist of FPRL1 (WRW_4) was used alone there was no inhibitory effect on the intracellular calcium flux induced by WKYMVm ($P > 0.05$, $n = 3$, Fig. 3B). However, if the two antagonists were combined the transient increase in intracellular calcium was inhibited ($P < 0.01$, $n = 3$, Fig. 3B). Piroxicam had no inhibitory effect alone on the WKYMVm induced calcium response ($P > 0.05$, $n = 3$ Fig. 3C) neither had the combination of piroxicam with the FPR antagonist CsH ($P = >0.05$, $n = 3$). However, if piroxicam was combined with the FPRL1 antagonist WRW_4, the intracellular calcium response was inhibited ($P = 0.01$, $n = 3$, Fig. 3C). These data suggest that piroxicam affects the receptor or a very early event in FPR signalling.

3.4. *Piroxicam inhibits binding of the fluorescently labelled FPR agonist fNLFNYK*

Piroxicam's effect on agonist binding to FPR was determined by a competitive receptor-binding assay. Flow cytometry was used to measure binding of the fluorescently labelled FPR agonist fNLFNYK [22]. We found that fMLF (10^{-7} M) and piroxicam (50 μM) blocked the binding of fNLFNYK (10^{-9} M) to FPR, whereas the FPRL1 agonist WKYMVM (10^{-7} M) had no effect on fNLFNYK binding (Fig. 4A). Both fMLF and piroxicam significantly blocked the binding of fNLFNYK in a concentration-dependent manner whereas the FPRL1 agonist WKYMVM only marginally affected the binding of the fluorescent peptide even at a high concentration (5×10^{-7} M; Fig. 4B). We thus conclude that piroxicam specifically blocks binding of the fluorescent ligand to FPR.

4. Discussion

The non-steroidal family of anti-inflammatory drugs (NSAIDs), is a heterogenous group of chemical compounds that mainly mediate their effects by reducing prostaglandin synthesis. This is achieved through an inhibition of the key enzyme cyclooxygenase (COX) [1]. Although COX inhibition is the common mechanism for all NSAIDs some of them have been reported to have anti-inflammatory effects through mechanisms distinct from COX inhibition [5–7]. We show that the NSAID piroxicam, reduces the release of superoxide anions from neutrophils stimulated with specific agonists of the formyl peptide receptor (FPR) and that this inhibition is due to blocking of ligand binding. We also show that piroxicam, in order to inhibit the cellular response triggered by an agonist

that binds not only FPR but also the closely related formyl peptide receptor 1 (FPRL1), has to be combined with an antagonist for FPRL1.

FPR and FPRL1 possess a high degree of amino acid identity, but despite this, they bind different agonists and the second messenger triggering part of the receptors are somewhat different [19,23,24]. The formyl peptide receptor (FPR) is a high affinity pattern recognition receptor with abilities to bind bacterial derived/released formylated peptides [25]. Such peptides are recognized also by FPRL1 but binding occurs with a low affinity, but in addition this receptor recognizes a number of non-formylated peptides/proteins that also activate the receptor [24,26].

Piroxicam belongs to the oxicam family of NSAIDs and a special attention has been drawn to this drug because of its multifunctional potential [5,27,28]. Chemically, piroxicam (4-hydroxy-2-methyl-*N*-2-pyrimidyl-2*H*-1,2-benzothiazine-3-carboxyamide) is an enolic acid with a size comparable to that of fMLF. In polar solvents piroxicam exists as a zwitterion with a positively charged pyridinium and a negatively charged enolate [29]. It has earlier been reported that piroxicam inhibits neutrophil superoxide production in response to fMLF and that the drug binds directly to FPR [5,6,30]. To our knowledge, there is however no other studies that compares piroxicams ability to bind to the different receptors of the FPR family. It has also been suggested that piroxicam can interact with signaling intracellular G-protein complexes and inhibit the GTP/GDP exchange, a mechanism that should affect signaling through all G-protein coupled receptors [31]. Accordingly, the reduction of superoxide anion production mediated through FPR/FPRL1 and C5aR at high concentrations of piroxicam, may be an effect of several different inhibitory mechanisms working in parallel; a direct effect on the receptor, on the ligands, on COX, and on the signaling G-protein. The selectivity/specificity for FPR has not earlier been described, and based on the fact that the activity induced by the two structurally different FPR ligands (fMLF and Ac_{9-25}) it is more likely that the drug acts on the receptor rather then on the triggering ligand. The results obtained from experiments using WKYMVm strongly support this suggestion. In this peptide the L-methionyl group at the carboxyl end of WKYMVM has been replaced by the D-amino acid. WKYMVm binds and triggers a cellular response through both FPR and FPRL1 [19]. FPR, is however not used unless signalling through the other receptor is blocked. This is illustrated by the fact that blocking the receptors either with an antagonist against FPR (cyclosporine H) or FPRL1 (WRW_4) separately had no effect on the WKYMVm-triggered response but in combination, the two antagonists inhibited the cellular response [20]. Piroxicam alone or in combination with the FPR specific antagonist (cyclosporine H) had no effect on the neutrophil calcium flux but piroxicam together with the specific FPRL1 antagonist reduced the WKYMVm-triggered activity. We thus conclude that the selectivity for FPR mediated by piroxicam is achieved through an effect on the ability of the receptor to be triggered by the different ligands. Whether the selectivity for FPR is peculiar for NSAIDs within the oxicam family or a feature for FPR binding NSAIDs in general has not been investigate in this study.

No defined structure has been identified to be the determinant for agonist or antagonist binding to FPR. It has, however, been proposed that the first, second and as well as the third extracellular loops together with the transmembrane regions are important for formation of the ligand binding pocket [32,33]. This binding pocket of FPR is suggested to have a limited depth, accommodating as few as five amino acids [22], and piroxicam is a small molecule that theoretically could be able to fit in to this pocket. It is important to notice, that although piroxicam can bind to block agonist binding to FPR, it is not in itself able to induce a neutrophil response measured as superoxide anions production or a change in intracellular calcium (unpublished observations). This does, however, not exclude the possibility that piroxicam binds to the ligand pocket of FPR. To induce a cellular response, binding is not sufficient; an induction of a conformational change in the receptor is also required. With respect to the differences between FPR and FPRL1, the amino acids in the three extracellular domains but also the N-termini differ significantly, and no conclusions can thus be drawn, about the part of FPR that is of importance for ligand binding or effects of an inhibitor, from structural similarities/differences between the two receptors.

The knowledge that piroxicam competes with ligands that bind to FPR may be of importance for a deeper understanding of the anti-inflammatory effects possessed by this NSAIDs. It has recently been reported that not only phagocytes but also platelets express FPR on their cell surface and that these receptors are partly responsible for the migratory capacity of the platelet [34]. An impaired platelet migration due to NSAID medication might be one of several factors that affect blood clotting. To summarize the results in relation to neutrophil function, piroxicam inhibits the cellular response induced by FPR ligands, and the reduced response is due to a binding competition, to FPR, between piroxicam and the receptor specific ligands.

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