# Characterization of the human fMLP receptor in neutrophils and in *Xenopus* oocytes

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**1**  N-formyl peptides (e.g. fMLP; N-formyl-L-methionyl-L-leucyl-phenylalanine) are potent mediators for inflammatory reactions. We report functional expression in *Xenopus* oocytes of human fMLP-R98 cDNA, without co-expression of the promiscuous G-protein subunit, Gα-16.

**2**  Stimulation of voltage-clamped oocytes ($-70$ mV) with fMLP produced a dose-dependent biphasic inward current with fast and slow components. Analysis using GTP-$\gamma$-S and cholera and pertussis toxins suggested these currents are mediated by an endogenous G-protein of the Gq family.

**3**  The fast current reversed at $-25$ mV and was blocked by SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid), suggesting the current is carried by $Cl^-$. The slow current showed weak inward rectification, was $Ca^{2+}$-dependent and blocked by $Cd^{2+}$, 4-AP (4-aminopyridine) and haloperidol, suggesting activation of a mixed population of cation channels.

**4**  Comparative experiments with human neutrophils using flow cytometric analysis showed that the proportion of neutrophils activated by fMLP was reduced in the presence of SITS, in the absence of external calcium and in the presence of $Cd^{2+}$, TEA (tetraethylammonium) and haloperidol but not 4-AP. In addition, the oxidative burst from activated neutrophils was reduced by SITS and by the absence of external calcium but not by $Cd^{2+}$, TEA, 4-AP or haloperidol.

**5**  We suggest that in human neutrophils activation by fMLP is dependent on store-operated calcium influx that appears to be regulated by $Cl^-$ channels and linked, in part, to non-selective cation channels.
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## Introduction

N-formylated peptides like fMLP (N-formyl-L-methionyl-L-leucyl-phenylalanine) play a major role as potent chemoattractants. They are believed to originate from either degraded bacterial or mitochondrial proteins (Carp, 1982; Marasco *et al.*, 1984). The N-formyl peptide receptor is G-protein coupled and mediates anti-inflammatory reactions in human neutrophils and other tissues (Becker *et al.*, 1998) such as the production of reactive oxygen derivatives (e.g. hydrogen peroxide) upon stimulation with fMLP.

First reports of the cloning and functional expression of the human fMLP receptor were first published in the early 1990s (Boulay *et al.*, 1990; Murphy & McDermott, 1991). Boulay *et al.* (1990) identified two variants of the fMLP receptor from a CDM8 expression library derived using mRNA from human myeloid cells differentiated to the granulocyte phenotype. Both clones (fMLP-R26 and -R98), expressed in COS-7 cells, specifically bound a highly efficient hydrophilic derivative of fMLP that could be removed using excess fMLP. Boulay *et al.* (1990) suggested

that these cDNAs represent allelic variations of the same gene.

The identity of the G-protein that couples the fMLP receptor to its intracellular responses has been investigated in a number of different cell types. Human leukaemia cells (HL-60) have been shown to express significant levels of pertussis toxin-insensitive Gα-16. However, this G-protein is down-regulated upon differentiation of HL-60 cells into neutrophils (Amatruda *et al.*, 1991; Klinker *et al.*, 1996). In human neutrophils, the intracellular responses to fMLP are affected to varying extents by pertussis toxin (reviewed in Amatruda *et al.*, 1991). It is suggested that in human neutrophils the signal transduction upon stimulation with fMLP is mainly mediated by pertussis toxin-sensitive G-proteins of the $G_i$ class (Bommakanti *et al.*, 1995). Oocytes of the frog *Xenopus laevis* have been used in several studies to express the human fMLP receptor (fMLP-R98) once the receptor was cloned (Burg *et al.*, 1995; Schultz *et al.*, 1992). However, in these studies the receptor was only functionally active when a promiscuous G-protein of the Gq-class, Gα-16, was co-expressed with the fMLP receptor.

In this study we report, in contrast to previous studies, functional expression of the human receptor for fMLP

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(fMLP-R98) in *Xenopus* oocytes, without the need for exogenous Gα-16. Stimulation of the oocytes with fMLP produced a biphasic inward current. GTP-γ-S was used to establish that this current is mediated by G-proteins. Cholera and pertussis toxins were used to analyse which G-protein is responsible for the current response. Current-voltage analysis and ion channel blocking drugs were used to characterize the ionic basis of the currents elicited by stimulation with fMLP.

Our purpose in exploring the response of oocytes expressing the human fMLP receptor was to assess this preparation as a model system with which to analyse the interaction of drugs, especially general anaesthetics, with this receptor and associated intracellular signalling pathways. Accordingly we were interested in the similarity, or otherwise, of the response of neutrophils to stimulation with fMLP in the presence of the various channel-blocking drugs and with reduced external calcium concentration.

# Methods

## Materials

Oocytes were harvested from extra-large mature female *Xenopus laevis* (Blades Biological, U.K.). W. Bautsch (Hannover Medical School) donated the cDNAs, in plasmid vector pCDM8, coding for the human R98 variant of the fMLP receptor and the Gα-16 subunit. Ultracompetent *E. coli* MC1061/p3 were obtained from Invitrogen (The Netherlands). Other materials were purchased from the following com-panies: N-formyl-L-methionyl-L-leucyl-phenylalanine (fMLP), tetraethyl-ammonium (TEA), 4 aminopyridine (4-AP), 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfphonic acid (SITS), cadmium chloride, barium chloride and guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S), cholera toxin and pertussis toxin from Sigma (Germany and U.K.); lidocaine from Braun (Germany), haloperidol from Janssen-Cilag (U.K.); Dihydrorhodamine 123 (DHR) and carboxy-seminaphthorhodafluor-l-acetoxymethylester (SNARF1/AM) from Molecular Probes (U.S.A.); Dulbecco's PBS from Life Technologies (Germany); propidium iodide (PI) from Serva (Germany)

## Characteristics of blood donors

This study was approved by the ethical committee of the University of Regensburg Medical School. After informed consent, venous blood was drawn from healthy donors with no history of infection 2 weeks prior to the experiments. Donors ($n = 6$) had a mean age of 32 with a range of 27 to 38 years. No donors had to be excluded because of abnormal blood or differential leukocyte counts.

## Leukocyte preparation and quantification of $H_2O_2$ production

Leukocyte isolation with Histopaque® 1.077 (Sigma, Germany) separation medium and labelling of the neutrophils with DHR, SNARF1/AM and PI was carried out as described in detail in previous publications (Frohlich *et al.*, 1996; Rothe & Valet, 1994). Cells were stimulated with fMLP for 15 min.

Analysis of neutrophil activation and oxidative burst was achieved using flow cytometry (FACScalibur, Becton Dickinson, U.S.A.) with argon ion laser excitation at 488 nm, measuring 10,000 cells of each stained sample. Data were acquired and processed using Cell Quest (Becton Dickinson, U.S.A.) and WinMDI 2.7 (Joseph Trotter, Scripps Institute, U.S.A.).

## Amplification and purification of the plasmid vector

Amplification and purification of the plasmid vector pCDM8 was performed using ultracompetent *E. coli* (MC 1061/p3) and the Wizard SV Miniprep DNA Purification System (Promega, U.K.).

## Preparation of cRNA

Plasmids containing the cDNA coding for the human fMLP receptor (R98) were linearized with HpaI and cRNA prepared using the mCAP mRNA capping kit (Stratagene, U.K.). The size of the cRNA was assessed using ethidium bromide stained agarose gel electrophoresis with RNA reference fragments of defined size.

## Preparation of oocytes and injection of cRNA

*Xenopus laevis* were killed by decapitation followed by destruction of the spinal cord after immobilization in ice water for 5 min and pieces of ovary tissue were removed. The oocytes were manually stripped from the ovarian lobes using a soft plastic strip. Stage V and VI oocytes were selected, injected with 35–45 nl cRNA using a hydraulic microinjector and placed into sterile pots containing modified Barth's solution (mM): NaCl 100, KCl 1, NaHCO$_3$ 2, MgSO$_4$ 0.82, Ca(NO$_3$)$_2$ 0.33, CaCl$_2$ 0.41, HEPES 10, pH 7.4) supplemen-ted with penicillin (100 u cm$^{-3}$) and streptomycin (100 μg cm$^{-3}$).

## Electrophysiological recording

Prior to use oocytes were defolliculated by incubation for 15 min in Ca$^{2+}$-free Barth's solution containing collagenase (50–100 u cm$^{-3}$) (Miledi & Woodward, 1989). Although not all somatic cells are removed by this method it is widely used (e.g. Kroll *et al.*, 1991). Manual removal of the vitelline envelope does remove all somatic cells but leaves the oocytes very fragile. Unless the somatic cells are contributing to the response (which is not the case in these experiments) or there is a need to improve the speed of access to the plasma membrane surface, then this additional step is unnecessary.

Electrophysiological recordings were made using two-electrode voltage-clamp (Biologic, France: VF 180 amplifiers and CA 100 clamp amplifier) at a holding potential of −70 mV and superfusion with ND96 (mM: NaCl 96, KCl 2, CaCl$_2$ 1.8, MgCl$_2$ 1, HEPES 5, pH 7.4 with NaOH).

## Experiments with ion channel blocking drugs and toxins

For all experiments with ion-channel blocking drugs on oocytes bath application of the drug (in ND96) was performed (with the oocyte at the holding potential of −70 mV) for 1.5 min before the oocyte was stimulated with

fMLP, in the presence of the channel-blocking drug. For experiments with pertussis (500 ng ml$^{-1}$) and cholera (25 $\mu$g ml$^{-1}$) toxins oocytes were incubated for 120 min (at normal resting membrane potential) with the toxin. Afterwards they were voltage-clamped at $-70$ mV again and stimulated twice with fMLP. Controls were incubated in parallel in ND96.

For the complementary experiments with neutrophils the leukocyte-rich plasma suspension was incubated with the different ion-channel blocking agents 10 min prior to stimulation with fMLP.

### Statistical analysis / data analysis

Current responses obtained from oocytes were recorded on a conventional potentiometric chart recorder and peak amplitudes were measured by hand. Currents recorded from individual oocytes were normalized, with respect to the appropriate control response from that oocyte, so that results could be pooled for analysis. Dose-response curves were constructed using non-linear curve fitting routines applied to a standard logistical equation (Origin V, Microcal Software Inc, U.S.A.).

Neutrophils were identified based on their typical forward (FSC) and side scatter (SSC) patterns and the SNARF1-related orange fluorescence (Figure 1). The SSC depends on the granularity of the cells, the FSC is related to their size. The rhodamine 123 fluorescence is proportional to the quantity of hydrogen peroxide produced by the cell. The percentage of the neutrophils activated after stimulation with fMLP, the mean fluorescence of the whole neutrophil population and the mean fluorescence of the activated cells were analysed. All experimental data obtained from neutrophil experiments are presented as non-normalized mean values with the standard error of the mean.
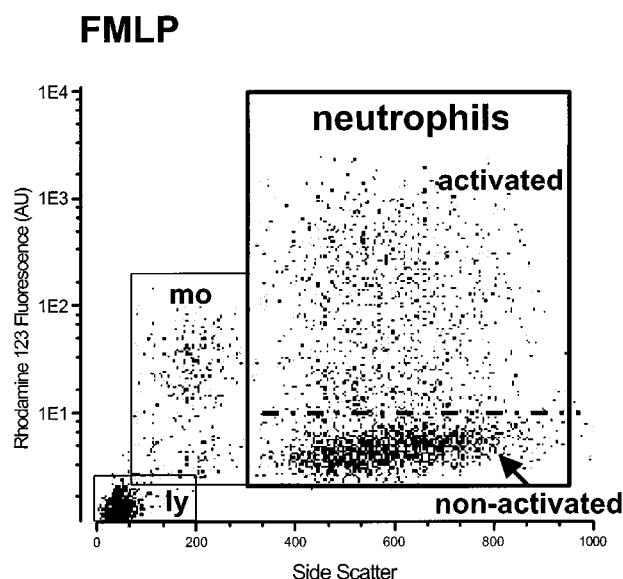
## FMLP



**Figure 1** Flow cytometric analysis of the oxidative response in human neutrophils following stimulation with fMLP (100 nM). The Y-axis represents the production of hydrogen peroxide (rhodamine fluorescence) in arbitrary units (AU); the X-axis indicates the side scatter (SSC) as a marker of cellular granularity. The figure shows activated and non-activated neutrophils as well as monocytes (mo) and lymphocytes (ly).

The statistical difference between treatment groups in neutrophil and oocyte experiments was tested, where appropriate, using Student's *t*-test.

## Results

### fMLP receptors expressed in oocytes after single injection of fMLP-cRNA

Receptors, expressed in *Xenopus* oocytes by injection of fMLP-R98 cRNA alone, were stimulated by bath application of fMLP (100 nM) for 30 s (holding potential $-70$ mV). This resulted in a biphasic inward current (Figure 2). Current amplitudes following stimulation with 100 nM fMLP were, typically, 200–300 nA. Control oocytes (no cRNA injection) did not show any current response upon fMLP stimulation. Current-voltage analysis (Figure 2) showed that the fast current had a reversal potential around $-25$ mV and, at holding potentials more negative than $-30$ mV, showed outward rectification. The slow current showed weak inward rectification and it was not possible to establish the reversal potential.

Analysis of the fMLP dose-response data from oocytes injected with fMLP-R98 cRNA alone gave EC$_{50}$ values of 4.5 and 1.9 nM, with Hill coefficients of 1.0 for both, for the fast and slow currents, respectively (Figure 3).

Injection of GTP-$\gamma$-S (40 nl at 200 $\mu$M) led to increases in the mean amplitudes of the fast and slow currents by approximately 30 fold ($31.2 \pm 11.5$; s.e.m., $n=8$) and 70 fold ($72.6 \pm 29.6$; s.e.m., $n=8$), respectively. To avoid an extended loss of holding potential, oocytes injected with GTP-$\gamma$-S were stimulated with fMLP using only 50 nM. Both the fast and slow currents remained unchanged on stimulation with fMLP (100 nM) after incubation of the oocytes with either cholera or pertussis toxin.

### fMLP receptors expressed in oocytes after co-injection of cRNA encoding G$\alpha$-16

The currents obtained in oocytes co-injected with cRNA encoding both the fMLP receptor and the G$\alpha$-16 G-protein subunit after stimulation with fMLP (100 nM) showed variable responses and, in general, current amplitudes that were smaller (less than 150 nA) compared to single injected cells. Occasionally, monophasic outward currents (Figure 4A) were observed following stimulation with fMLP (100 nM), which had a reversal potential around $-5$ mV (Figure 4C). A combination of an inward and an outward current was also observed (Figure 4B,D). However, the majority of oocytes showed the biphasic inward current response as described above for oocytes expressing fMLP receptors alone. Accordingly, the remaining experiments were conducted using oocytes expressing fMLP receptors alone.

### Pharmacological manipulation of the response of fMLP receptors

The amplitude of the fMLP-induced fast current (100 nM fMLP) was reduced by about 80% in the presence of SITS (1 mM) (Figure 5; $0.16 \pm 0.05$ (s.e.m., $n=4$); $P<0.01$).

Stimulation with fMLP (100 nM) whilst perfusing the oocytes (at a holding potential of $-70$ mV) with a Ca$^{2+}$-
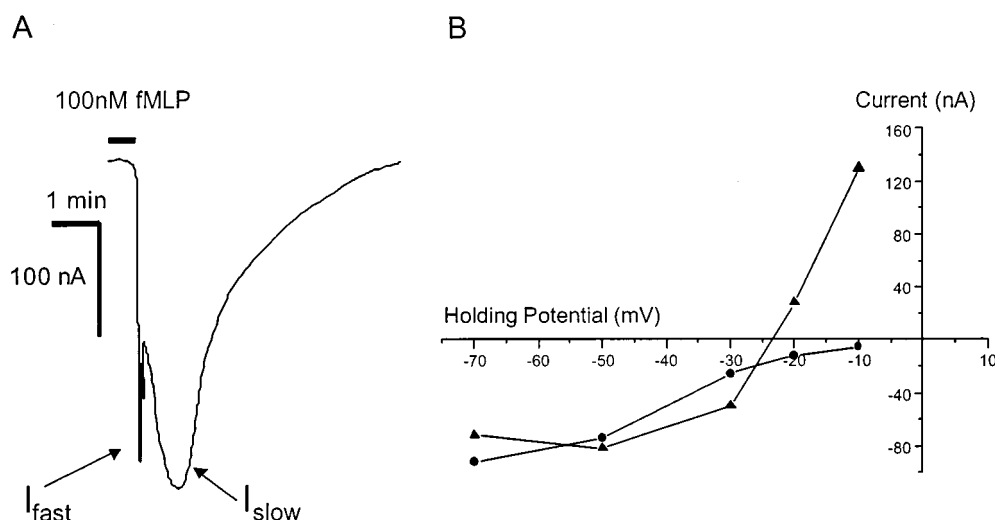
**Figure 2** (A) An example of the biphasic current induced by stimulation (30 s) with fMLP (100 nM) of *Xenopus* oocytes injected with cRNA encoding the human fMLP-R98 receptor. The fast component ($I_{fast}$) typically had rise times of 3 to 10 s and the slow component ($I_{slow}$) about 1 min. (B) An example of the current-voltage relationships for the fast current (solid triangles) and slow current (solid circles). The fast current exhibits a reversal potential at $-24$ mV and outward rectification at potentials less than $-30$ mV. The slow current exhibits weak inward rectification.

free ND96 resulted in a reduction in amplitude of the slow current by about 85% (Figure 5; $0.15 \pm 0.13$ (s.e.m., $n=3$); $P < 0.01$). When $Ca^{2+}$ was replaced with $Ba^{2+}$ the amplitude of the slow current was further reduced by some 10% (Figure 5; $0.042 \pm 0.0156$ (s.e.m., $n=5$); $P < 0.01$).

In the presence of $Cd^{2+}$ (5 mM) the amplitude of the slow current was reduced by over 85% (Figure 5; $0.13 \pm 0.1$ (s.e.m., $n=4$); $P < 0.01$). In the presence of 4-aminopyridine (4-AP; 1 mM) the amplitude of the slow current was reduced by some 70% (Figure 5; $0.28 \pm 0.17$ (s.e.m., $n=6$); $P < 0.01$). The extent of the channel blockade by 4-AP increased progressively from 0 to 70% following four sequential stimulations with fMLP, separated by 2 min. In the presence of haloperidol (13 $\mu$M) the amplitude of the slow current was reduced by some 35% (Figure 5; $0.64 \pm 0.06$ (s.e.m., $n=6$); $P < 0.01$). The amplitude of the slow current was unchanged in the presence of TEA (20 mM).

*Neutrophil responses*

When stimulated with 100 nM fMLP in standard PBS, $49.3 \pm 2.6\%$ (s.e.m., $n=23$) of the neutrophil population was activated, as measured by the production of hydrogen peroxide. The mean fluorescence of all neutrophils and of the activated neutrophils are given in Table 1.

In the absence of calcium the proportion of neutrophils activated was reduced by some 56% (Figure 6, $21.9 \pm 1.8\%$; s.e.m., $n=12$, $P < 0.01$). When calcium was replaced by barium (2 mM) the proportion of neutrophils activated was reduced by 87% (Figure 6, $6.3 \pm 0.3\%$; s.e.m., $n=12$, $P < 0.01$).

In the presence of the chloride channel-blocking drug SITS (1 mM) the proportion of neutrophils activated was reduced by 58% (Figure 6, $20.9 \pm 1.8\%$; s.e.m., $n=10$). Cadmium (5 mM), TEA (20 mM) and haloperidol (130 $\mu$M) reduced the proportion of neutrophils activated by 27, 37 and 98%, respectively (Figure 6, $35.8 \pm 2.5\%$, $n=18$, $P < 0.05$; $30.9 \pm 2.8$, $n=18$, $P < 0.05$; $1.64 \pm 0.51$, $n=12$, $P < 0.01$; all s.e.m.). 4-AP was without effect (Figure 6).

Treatment of neutrophils with SITS (1 mM) or replacement of $Ca^{2+}$ with $Ba^{2+}$ significantly reduced both the mean fluorescence of all neutrophils and the fluorescence of the activated neutrophils (see Table 1). Treatment with $Cd^{2+}$ (5 mM), TEA (20 mM) and 4-AP (1 mM) did not affect the mean fluorescence, either the total or that of the activated neutrophils (Table 1). In the presence of haloperidol (130 $\mu$M) the mean fluorescence of the activated neutrophils increased ($31 \pm 4.4$, s.e.m., $n=12$, $P < 0.01$).

## Discussion

We have demonstrated functional expression of human fMLP receptors (fMLP-R98) in oocytes after injection of the cRNA for that receptor alone. This is in marked contrast to previous studies that reported that co-expression of G$\alpha$-16 was necessary for functional expression (Burg *et al.*, 1995; Schultz *et al.*, 1992) although the same receptor variant was used.

The functional response (biphasic inward current) obtained in our experiments is specifically initiated by an agonist-receptor interaction because no response to fMLP could be induced in non-injected oocytes. The $EC_{50}$ for fMLP in oocytes expressing fMLP-R98 after single injection of the cRNA encoding this receptor (4.5 and 1.8 nM for the fast and slow currents, respectively) is comparable to the $EC_{50}$ obtained for the fMLP stimulated oxidative response in human neutrophils (13 nM, unpublished results). Earlier studies of fMLP binding to human neutrophils indicated the presence of both high and low affinity binding sites with Kd values of 2 and 180 nM, respectively (Atkinson *et al.*, 1988). However, the stimulation of chemotaxis in neutrophils is apparently more sensitive with an $EC_{50}$ of 0.07 nM (Prossnitz & Ye, 1997).

We conclude that the signal transduction in oocytes is mediated by an endogenous G-protein because application of GTP-$\gamma$-S resulted in a considerable increase in amplitude of

## (A) Fast conductance
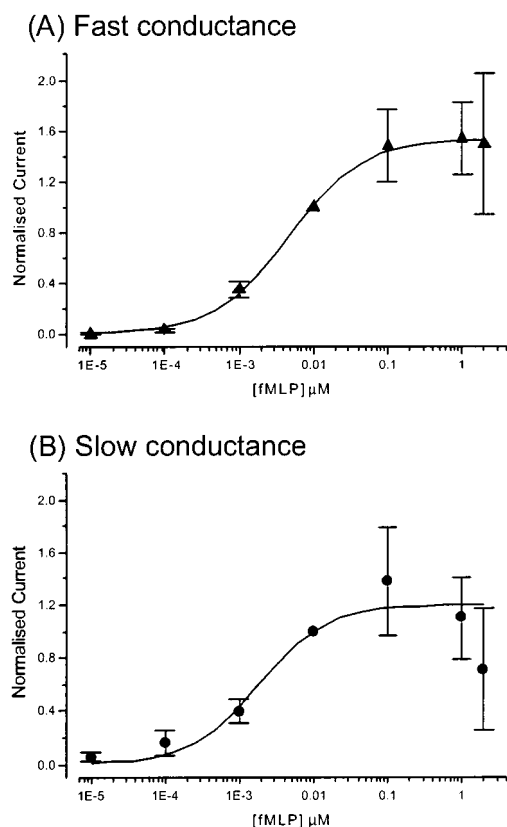


## (B) Slow conductance



**Figure 3** Dose-response curves for fMLP to elicit the fast (A) and slow (B) currents. In both cases, the ordinate shows the current elicited, normalized to that elicited by stimulation with 10 nM fMLP, and the abscissa the concentration of fMLP in $\mu$M. The curves are the best fit to the data on the basis of a standard logistic model (Origin V, Microcal Software Inc):

$$y = \frac{min - max}{1 + (x/EC_{50})^p} + max \qquad (1)$$

where: min was fixed at zero, max is the estimated maximum response, and $p$ is the Hill coefficient. In both cases, the symbols indicate the mean normalized current on the basis of data from eight different oocytes and the error bars the s.e.m. For the fast current ($\chi^2 = 0.0009$ for the fit), the maximum current was estimated at 1.53, the $EC_{50}$ at $4.54 \pm 0.35$ nM and the Hill coefficient at $0.88 \pm 0.05$. For the slow current ($\chi^2 = 0.06$ for the fit), the maximum current was estimated at 1.2, the $EC_{50}$ at $1.85 \pm 0.14$ nM and the Hill coefficient at $0.96 \pm 0.63$.

both the fast and slow currents following stimulation with fMLP. It is likely the G-protein is a member of the Gq-class because it is not susceptible to either pertussis or cholera toxin.

Direct comparison of our results with those reported previously (Burg *et al.*, 1995) is difficult. However, the shape of the current response described by Burg *et al.* (1995) is similar to those we obtained (see Figure 2). No assumption about the direction of the currents recorded by Burg *et al.* (1995) can be made from the orientation of their figure (no details are given in the text) because, in other publications from this research group, similar graphical presentation of currents have used both upward and downward orientations to illustrate currents obtained upon stimulation of C5a receptors, in equivalent experiments (Klos *et al.*, 1994; Kroll *et al.*, 1991).

We cannot explain why Burg *et al.* (1995) required the expression of the additional exogenous G-protein subunit to achieve functional expression. It is possible that diet or environmental factors in the maintenance of the *Xenopus laevis* play a part. However, conditions were standard; a 12-h light:dark cycle, a water temperature of 20°C and never more than six animals in each 80 litre tank. They were routinely fed on minced heart, although animals fed on *Xenopus* pellets also expressed functional receptors using the fMLP-R98 cRNA alone. It should be noted that in common with expression of different RNAs, both by us and by other groups (personal communication), functional expression during the months June to September was poor. The turnover of the endogenous G-protein that provides the coupling in our experiments seems complex. If oocytes were injected with fMLP-R98 cRNA more than 24 h after harvesting, functional expression was very erratic. This was observed even when oocytes from the same batch, which had been injected approximately 6 h after harvesting, had reliably expressed functional receptors. However, oocytes that reliably expressed functional receptors exhibited stable responses for up to 4 days. These observations may also contribute to the difference observed between the results we report and those of Burg *et al.* (1995). In summary, we found functional expression of fMLP receptors in oocytes most reliable without co-expression of the promiscuous G-protein.

The current-voltage analysis strongly suggests that the fast current is carried by Cl$^-$ because the chloride equilibrium potential in *Xenopus* oocytes is $-23$ mV (Faber & Korn, 1987). This is reinforced by the finding that the current is almost completely blocked by the chloride channel blocking drug SITS (Mitchell *et al.*, 1997). The rapid activation of this current suggests that the ion channel may be directly activated by a G-protein subunit following stimulation of the receptor. These features, together with its activation by GTP-$\gamma$-S, suggests the channel may resemble the Maxi Cl$^-$ channel (Sun *et al.*, 1992; Mitchell *et al.*, 1997).

The properties exhibited by the slow current, weak inward rectification, Ca$^{2+}$-sensitivity and blockade by Cd$^{2+}$, 4-AP and haloperidol, suggest the involvement of a cation channel but do not immediately suggest a single ion channel. The time course of the response suggests it may arise as a result of the activation of an intracellular signalling pathway that leads to channel opening perhaps by phosphorylation by protein kinases or by direct activation by Ca$^{2+}$. Oocytes are known to contain five channels that can pass K$^+$ ions, three that are highly selective for K$^+$ and two non-selective channels. The highly selective K$^+$ channels are a large-conductance Ca$^{2+}$-activated K$^+$ channel (Krause *et al.*, 1996), a slowly inactivating delayed rectifier K$^+$ channel (Lu *et al.*, 1990; Parker & Ivorra, 1990) and a Na$^+$-activated K$^+$ channel (Egan *et al.*, 1992). The non-selective channels are a stretch-activated channel (Lane *et al.*, 1993; Methfessel *et al.*, 1986) and a channel that is regulated by the external concentration of divalent cations (Arellano *et al.*, 1995), both of which are permeable to K$^+$ and Na$^+$. In addition, it is highly likely that *Xenopus* oocytes express Ca$^{2+}$-activated non-selective (CAN) channels permeable to Na$^+$ and K$^+$ since these are found widely expressed in numerous tissues (including oocytes) of many different species (Swandulla &
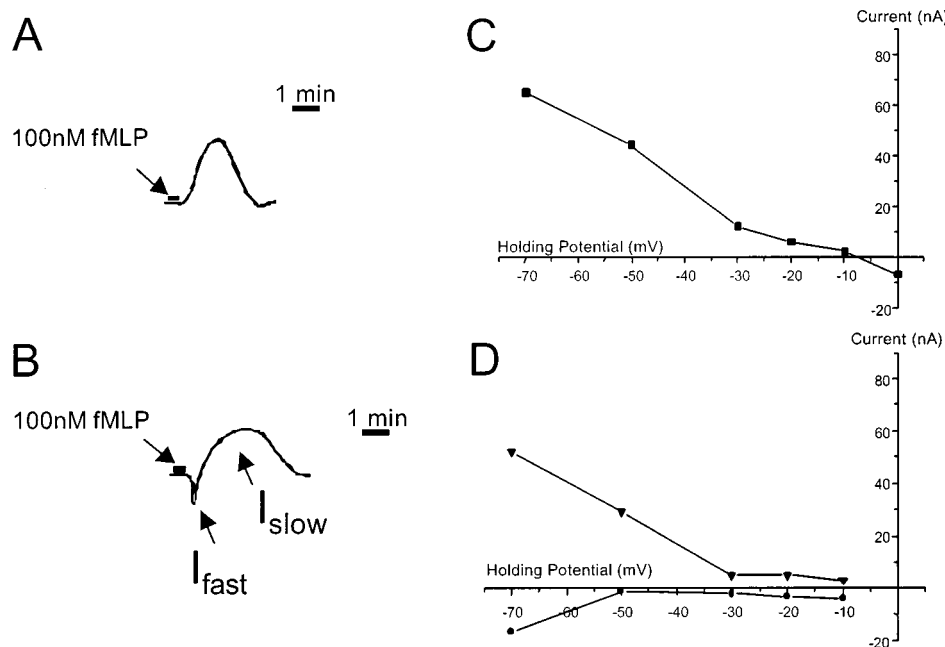
**Figure 4** Examples of the monophasic outward currents (A) and combined inward (fast) / outward (slow) currents (B) induced by stimulation (30 s) with fMLP (100 nM) of *Xenopus* oocytes co-injected with cRNAs encoding Gα-16 G-protein subunit and the human fMLP-R98 receptor. The currents had rise times comparable with those obtained from oocytes injected with cRNA encoding fMLP-R98 alone (see Figure 2). The associated current-voltage relationships are also shown for the monophasic outward current (C) and the combined inward and outward currents (D).
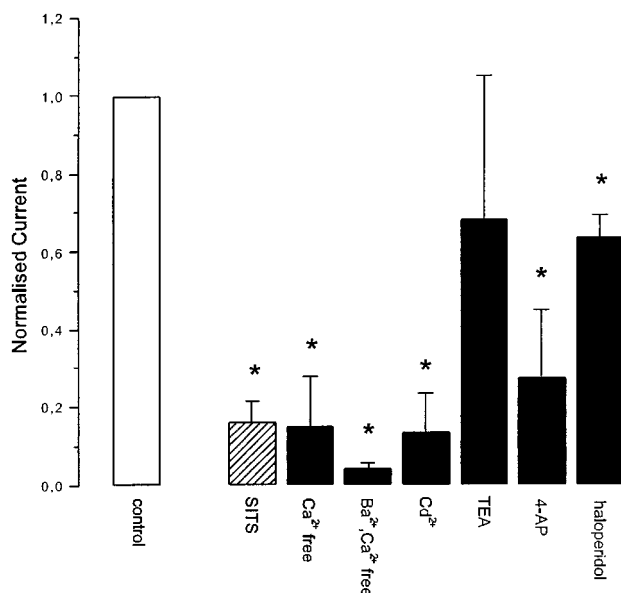


**Figure 5** The effect of a variety of channel-blocking drugs ((mM): SITS 1, $Ba^{2+}$ 2, $Cd^{2+}$ 5, TEA 20, 4-AP 1, haloperidol 130 $\mu$M) on the amplitude of the fast and slow currents elicited by stimulation with 100 nM fMLP. The bars represent the mean normalized current (normalized to the current elicited prior to application of the channel-blocking drug) and the error bars represent the s.e.m. The striped bar represents the fast current and the solid bars represent the slow current. Significant changes ($P < 0.05$, Student's *t*-test) in the amplitude of the currents are indicated by *. Detailed results are given in the text.

Partridge, 1990). In general, CAN channels show linear I–V relationships and slow activation. Our working hypothesis,

therefore, is that in the oocyte stimulation of the fMLP receptor activates an endogenous Gq-protein that in turn activates inositol 1,4,5-triphosphate to cause the release of intracellular $Ca^{2+}$. The released $Ca^{2+}$ is then free to activate one, or more, of the $Ca^{2+}$-activated cation channels.

One of the intracellular signalling pathways activated by stimulation of neutrophils with fMLP is the activation of PLC (phospholipase C) leading to the generation of IP3 and the release of intracellular calcium (Frohlich *et al.*, 1998). In turn, store-operated calcium influx (SOCI) has been associated with the activation of neutrophils by a variety of inflammatory mediators (Hauser *et al.*, 2001). Reducing $[Ca^{2+}]_o$ by replacing calcium in the bathing medium with barium almost abolished neutrophil activation by fMLP. It has also been suggested that a part of the final pathway for the production of the oxidative burst is the initiation of NADPH-oxidase (nicotinamide adenine dinucleotide phosphate) by PKC (protein kinase C) (Frohlich *et al.*, 1998). The activity of the PKC isoforms found in human neutrophils (α, β and γ) are calcium sensitive (Prossnitz & Ye, 1997). If SOCI is restricted then the activity of PKC would be reduced. This would be expected, in turn, to reduce the fluorescence of the activated neutrophils, as was observed. In human microglia SOCI is regulated by $Cl^-$ channels (McLarnon, *et al.*, 2000). If this mechanism operates in human neutrophils then, following the argument used above, it would explain the reduction in activation and reduced fluorescence of activated neutrophils in the presence of the $Cl^-$-channel blocking drug SITS.

Schumann *et al.* (1992) performed experiments using the whole-cell configuration of the patch-clamp technique to voltage-clamp single neutrophils. They showed that human neutrophils possess potassium and non-selective cationic

**Table 1** Flow cytometric analysis of the oxidative response in human neutrophils following stimulation with fMLP (100 nM) in the presence of a variety of ion-channel blocking drugs

| Buffer modification | Mean fluorescence of all neutrophils | Mean fluorescence of activated neutrophils | N |
|---|---|---|---|
| Control (PBS with $Ca^{2+}$, $Mg^{2+}$) | $9.3 \pm 0.5$ | $19.9 \pm 0.8$ | 23 |
| SITS 1 mM | $6.1 \pm 0.2$ | $13.6 \pm 0.7$ | 10 |
| $Ca^{2+}$ free | $5.8 \pm 0.7$ | $13.6 \pm 1.8$ | 12 |
| $Ba^{2+}$ 2 mM, $Ca^{2+}$ free | $3.3 \pm 0.3$ | $9.2 \pm 0.9$ | 12 |
| $Cd^{2+}$ | $8.1 \pm 0.5$ | $24.7 \pm 1.8$ | 18 |
| TEA 20 mM | $7.4 \pm 0.6$ | $18.3 \pm 1.7$ | 18 |
| 4-AP 1 mM | $10.9 \pm 0.5$ | $22.4 \pm 1.1$ | 10 |
| Haloperidol 130 $\mu$M | $1.64 \pm 0.51$ | $31.0 \pm 4.4$ | 12 |

((mM): SITS 1 $Ba^{2+}$ 2, $Cd^{2+}$ 5, TEA 20, 4-AP 1, haloperidol 130 $\mu$M). The table shows the mean fluorescence of all neutrophils and that of the neutrophils producing hydrogen peroxide (activated). Data are shown as mean values with s.e.m. Detailed results are given in the text.
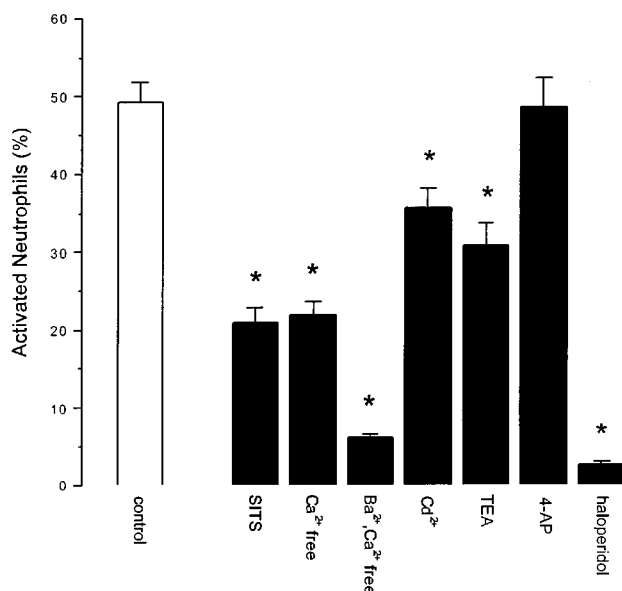


**Figure 6** The effect of a variety of channel-blocking drugs ((mM): SITS 1, $Ba^{2+}$ 2, $Cd^{2+}$ 5. TEA 20, 4AP 1, haloperidol 130 $\mu$M) on the proportion of neutrophils activated to produce hydrogen peroxide (neutrophil respiratory burst) following stimulation with 100 nM fMLP. The bars represent the mean percentage of neutrophils activated and the error bars represent the s.e.m. Significant changes ($P < 0.05$, Student's $t$-test) in the percentage activation are indicated by *. Detailed results are given in the text.

channels. Stimulation of the neutrophils with fMLP (100 nM, 30 s) activated cationic currents that showed voltage dependence and weak outward rectification. Activation of the non-selective cationic channels in human neutrophils was shown to involve both $Ca^{2+}$-dependent and -independent mechanisms. Von Tscharner *et al.* (1986) reported two separate voltage-dependent, non-selective cation channels in human neutrophils that were indirectly coupled to the fMLP

receptor and whose activation was $Ca^{2+}$-dependent. The varied effect of $Cd^{2+}$, TEA and haloperidol on the response of neutrophils to stimulation with fMLP may reflect varying effects on non-specific cation channels that are contributing to the calcium influx.

In summary, we have shown that functional expression of fMLP receptors in *Xenopus* oocytes can be achieved using fMLP-R98 cRNA alone. Once expressed, these receptors couple to an endogenous G-protein that mediates a biphasic inward current, comprising a fast current carried by $Cl^-$ and a slower current carried by cations. In human neutrophils activation by fMLP is dependent on store-operated calcium influx which appears to be regulated by $Cl^-$ channels and linked, in part, to non-selective cation channels.

*Xenopus* oocytes have been used extensively in experiments aimed at understanding the mechanisms underlying the effects of general anaesthetics. These experiments have included many directed at the effect of anaesthetics on G-protein coupled receptors (see for example, Durieux, 1995; Hollmann *et al.*, 2000). The attraction of the oocyte expression system as a model cell in which to study the effects of anaesthetics on the fMLP receptor is the potential to manipulate, independently, the receptor and the intracellular signalling pathway so as to dissect the separate effects of anaesthetics on these components. Hence our concern to characterize the nature of the response in detail. Whilst we accept that there are differences between the transduction of receptor binding to physiological effect in the oocyte and the neutrophil, we believe that a significant advance in our understanding of the interaction of anaesthetics and the fMLP receptor and its signalling pathway will result from experiments using the oocyte as a model system.

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