

## Antimicrobial peptides from scorpion venom induce $\text{Ca}^{2+}$ signaling in HL-60 cells<sup>☆</sup>

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### Abstract

Parabutopeptin (PP) and opistopeptin 1 (OP1) are amphipathic  $\alpha$ -helical antimicrobial peptides that were recently isolated from scorpion venom. In assays in which single granulocyte-like HL-60 cells as well as cells in suspension were used, both peptides were able to induce a reversible  $\text{Ca}^{2+}$  release from intracellular stores and to increase  $\text{Ca}^{2+}$  influx. Both effects could be clearly differentiated for OP1, inducing  $\text{Ca}^{2+}$  release at lower concentrations. The  $\text{Ca}^{2+}$  release was pertussis toxin-sensitive indicating the involvement of G-proteins.  $\text{Ca}^{2+}$  release depended on the stage of differentiation of the cells with undifferentiated cells being the most sensitive. Desensitization occurred with OP1. No cross-desensitization occurred between OP1 and the bacterial chemoattractant fMLP indicating the involvement of different types of receptors.  $\text{Ca}^{2+}$  release by OP1 was found not to be mediated via interaction with the formyl peptide receptor-like 1. Although some of the results might favor a receptor-like interaction, the receptor involved could not be identified.

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Recently we isolated antimicrobial peptides (AMPs) from the venom of scorpions living in Southern Africa: parabutopeptin from *Parabuthus schlechteri* [1] and the opistopeptins from *Opisthophthalmus carinatus* [2]. They belong to a group of cationic,  $\alpha$ -helical, cysteine-free, amphipathic peptides. Members of this group are widely spread in nature and function as part of the innate defence mechanism against different kinds of pathogens [3]. Besides parabutopeptin and the opistopeptins, other peptides of this group have recently been isolated from scorpion venom: hadrurin [4], pandinins [5], and IsCTs [6,7]. Although the role of AMPs in the defence against microorganisms is well established, the mechanism of killing of bacteria, fungi, and other microorganisms is still a matter of discussion. Most likely, disintegration of

the cell membrane and its permeabilization for ions are the cause of cell death [3].

In addition to this function, a complex interaction of cationic AMPs with intracellular signaling has been described in vertebrates and humans. These effects have first been studied for mastoparan, a cysteine-free cationic peptide from wasp venom [8,9]. Further investigations revealed that these peptides are able to degranulate neutrophils and mast cells, to neutralize the action of endotoxins, and to promote chemotaxis of white blood cells (for reviews, see [10–14]). Concerning the mechanism of action of AMPs on intracellular signaling, it has been suggested that insertion of mastoparan into cell membranes directly affects G-proteins without the involvement of a receptor [15,16]. Recently, however, it has been shown that the bacterial peptide Hp(2–20) [17], the human peptide LL-37 [18], and a synthetic peptide domain derived from the V3 region of the HIV-1 gp120 [19], all three cysteine-free,  $\alpha$ -helical peptides, interact with the formyl peptide receptor FPRL1 influencing  $\text{Ca}^{2+}$  signaling in neutrophils. In addition to amphipathic peptides, LXA<sub>4</sub>, a lipid metabolite exhibiting

<sup>☆</sup> Abbreviations: AMP, antimicrobial peptide; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; FPR, formyl peptide receptor; FPRL1, formyl peptide receptor like 1; LXA<sub>4</sub>, lipoxin A<sub>4</sub>; PP, parabutopeptin; OP1, opistopeptin 1.

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anti-inflammatory properties, is able to increase  $[Ca^{2+}]_i$  in monocytes [20] and, to a lesser extent, in neutrophils [21] via interaction with the FPRL1 [22].

We found that fractions of scorpion venom containing antimicrobial peptides and synthetic PP and OP1 were able to degranulate human granulocytes and to stimulate chemotaxis [1,23]. Because degranulation of human granulocytes is known to be a  $Ca^{2+}$ -mediated process [24], we investigated the effects of PP and OP1 on intracellular  $Ca^{2+}$  in more detail and analyzed the type of receptor that eventually might be involved. Results were compared with the effects of melittin from bee venom and mastoparan. Additionally the action of the scorpion AMPs was compared with the effects of *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), a major leukocyte chemoattractant from *Escherichia coli* [25]. This compound releases  $Ca^{2+}$  from intracellular stores via an  $IP_3$ -mediated mechanism [26]. Finally, the effects of the scorpion peptides were compared to the actions of the FPRL1 ligands V3 peptide and LXA<sub>4</sub>.

Some of our findings fit with the interaction of PP and OP1 with a receptor but arguments will be presented to show that a non-receptor-mediated interaction cannot be excluded. In the latter case binding could result from interaction with specific lipid–protein clusters as has been hypothesized for some bacterial [27] and plant [28] toxins.

## Materials and methods

**Cell culture.** HL-60 cells were cultured in RPMI medium with L-glutamine (BioWhittaker Europe) supplemented with 20 vol% de-complemented calf serum (BioWhittaker Europe), 50 µg/ml gentamicin (Gibco), and 0.075 vol%  $NaHCO_3$  (Gibco). Granulocytic differentiation was induced by addition of dimethyl sulfoxide (DMSO), final concentration of 1.3 vol% for 1–4 days [29]. Cells were passaged three times a week, keeping the cell number below  $10^6$  cells/ml.

**Loading of cells with fura-2.** Fifty micrograms of fura-2 AM (Molecular Probes) was dissolved in 12.5 µl DMSO containing 0.5% pluronic acid (fura-2 AM concentration: 4 mM). The solution was sonicated. The loading medium was Tyrode solution (130 mM NaCl, 5.4 mM KCl, 1.2 mM  $MgSO_4 \cdot 7H_2O$ , 1 mM  $CaSO_4 \cdot 2H_2O$ , 10 mM Hepes, and 10 mM glucose, pH adjusted to 7.4 by addition of 1 M NaOH) with a final fura-2 AM concentration of 4 µM. About  $2 \times 10^5$  HL-60 cells for measurements on single cells or  $2 \times 10^7$  cells for measurements on cell suspensions were centrifuged (5 min, 50g) and resuspended in the loading medium. The loading was carried out for 45 min with continuous shaking at room temperature. Thereafter, the cells were resuspended in Tyrode solution.

**Fluorescence measurements in single HL-60 cells.** The suspension containing loaded cells was put on a glass coverslip coated with polylysine, was transferred to a perfusion bath, and was allowed to adhere to the glass for about 15 min during which fura-2 AM hydrolysis took place. For ratiometric fluorescence measurements in single cells, the PTI RF-D4012 fluorimeter model was used, as described previously [30]. The ratio of fura-2 fluorescence at 510 nm with excitation at 340 and 380 nm was indicative for  $[Ca^{2+}]_i$ . Cells were perfused continuously with Tyrode solution. Test solutions were applied to the cell under study via another perfusion system that was put close to the cell in order to obtain a fast solution exchange. Cationic,  $\alpha$ -helical peptides

were added until the maximum ratio of the fluorescence was obtained, unless otherwise stated. The experiments were carried out at room temperature. Parameters of the  $Ca^{2+}$  transients in single cells are given in the text as averages  $\pm$  SEM, *n* refers to the number of cells analyzed. Statistical analysis of these average values was performed by Student's *t* test with a significance level of 5%.

The relationship between the ratio of fura-2 fluorescence and  $[Ca^{2+}]_i$  was determined as described [31] and  $[Ca^{2+}]_i$  was calculated according to [31]

$$[Ca^{2+}]_i = K_d \times (S_{f2}/S_{b2}) \times ((R - R_{min})/(R_{max} - R))$$

with  $K_d$  236 nM [32].

**Measurements of intracellular  $Ca^{2+}$  concentration in cell suspensions.** After loading with fura-2, HL-60 cells were resuspended at a concentration of  $2 \times 10^7$ /ml. Measurements were carried out with an Aminco-Bowman fluorescence spectrophotometer. The sample was excited at 340 nm and the emitted light was measured at 510 nm. At the end of each experiment the maximal emission was determined by addition of 10 µM ionomycin (100% fluorescence) and the minimal fluorescence by subsequent addition of 10 mM EGTA (0% fluorescence).

**Products.** Parabutoxin (Swiss-Prot Accession No. P83312) and opisthotoxin 1 (Swiss-Prot Accession No. P83313) were chemically synthesized by Ansynth Service BV (The Netherlands) as described previously [2]. V3 peptide was a kind gift of Dr. Paul Proost (Rega Institute, Katholieke Universiteit Leuven, Belgium) and fMLP, pertussis toxin, and LXA<sub>4</sub> were purchased from Sigma.

## Results

### *Effects of fMLP and parabutoxin on $[Ca^{2+}]_i$ in HL-60 cells*

To analyze the potential interaction of antimicrobial scorpion toxins with the  $Ca^{2+}$  release mechanism in granulocyte-like HL-60 cells, the effects of synthetic PP and OP1 were investigated. In principle, a rise in  $[Ca^{2+}]_i$  can originate from an increased  $Ca^{2+}$  influx, e.g., via peptide-induced pores and/or by an intracellular release. To differentiate between both pathways,  $[Ca^{2+}]_i$  was determined by the  $Ca^{2+}$  sensitive probe fura-2 in the presence of scorpion peptides and in the absence ( $Ca^{2+}$ -free Tyrode solution containing 1 mM EGTA) and the presence of extracellular  $Ca^{2+}$ .

The ratio of fura-2 fluorescence in non-stimulated single granulocyte-like HL-60 cells was  $0.47 \pm 0.01$  ( $n = 23$ ) in the presence of extracellular  $Ca^{2+}$ . This corresponds to a  $[Ca^{2+}]_i$  of 35 nM. Upon stimulation with 0.1 µM fMLP, the ratio increased to a value of  $1.82 \pm 0.08$  (equivalent to a  $[Ca^{2+}]_i$  of 675 nM,  $n = 23$ ). Figs. 1A and B show examples of measurements on single cells. In the absence of extracellular  $Ca^{2+}$  the ratio of fura-2 fluorescence increased from  $0.46 \pm 0.02$  (30 nM,  $n = 7$ ) to  $1.77 \pm 0.11$  (640 nM,  $n = 7$ ) when 0.1 µM fMLP was added (not shown). No statistical difference exists between the maximal ratios of fura-2 fluorescence achieved in the presence and the absence of extracellular  $Ca^{2+}$ . In the 0  $Ca^{2+}$  experiments, the cells were superfused with EGTA-containing solution 3 min before the addition of fMLP. This period was kept short

to avoid a change in the  $\text{Ca}^{2+}$  content of intracellular stores. There was no statistical difference in amplitude of the  $\text{Ca}^{2+}$  transients induced by 0.1 and 1  $\mu\text{M}$  fMLP, indicating that the maximal release was induced by 0.1  $\mu\text{M}$  fMLP.

Similar  $\text{Ca}^{2+}$  transients were obtained with PP. Fig. 1A compares the effects of 0.5  $\mu\text{M}$  PP and 0.1  $\mu\text{M}$  fMLP on a single granulocyte-like HL-60 cell. From Fig. 1B it is obvious that the threshold concentration for PP ranged between 0.2 and 0.5  $\mu\text{M}$ . In contrast to fMLP, the amplitude of the  $\text{Ca}^{2+}$  transient in the presence of 0.5  $\mu\text{M}$  PP was significantly higher in the presence of external  $\text{Ca}^{2+}$ : the maximal ratio was  $2.03 \pm 0.09$  (820 nM,  $n = 6$ ) in the presence and  $1.66 \pm 0.09$  (580 nM,  $n = 10$ ) in the absence of extracellular  $\text{Ca}^{2+}$ , indicating a contribution of  $\text{Ca}^{2+}$  leak to the amplitude of the  $\text{Ca}^{2+}$  signal induced by 0.5  $\mu\text{M}$  PP. No statistical difference in the duration of the transient in the presence and the absence of extracellular  $\text{Ca}^{2+}$  could be observed with PP.

In order to investigate the contribution of G-proteins in the release of  $\text{Ca}^{2+}$  from intracellular stores induced by fMLP and PP, cells were pretreated with pertussis toxin. The release of intracellular  $\text{Ca}^{2+}$  induced by 0.5  $\mu\text{M}$  PP in the absence of extracellular  $\text{Ca}^{2+}$  could be inhibited by preincubating the cells with 500 ng/ml pertussis toxin for 2 h. From 26 single cells responding to PP, only 2 responded to PP after pertussis toxin treat-

ment while in control conditions 17 single cells out of 25 responded to a second application of PP. Analogous results were obtained with fMLP. The  $\text{Ca}^{2+}$  release after 2 h of incubation persisted in 63 single cells out of 75 tested in control conditions, while after pertussis toxin treatment only 5 single cells out of 57 responded to a second application of fMLP. The inhibition induced by pertussis toxin was statistically significant for both products. This finding indicates that a pertussis toxin-sensitive G-protein is involved in intracellular signaling by PP as is known for fMLP [26].

#### Effects of opistoprin 1 on $[\text{Ca}^{2+}]_i$ in HL-60 cells

As illustrated in Fig. 2A, a slightly higher concentration was needed to elicit a  $\text{Ca}^{2+}$  transient in single granulocyte-like HL-60 cells in response to OP1 compared to PP: a  $\text{Ca}^{2+}$  transient occurred at 0.8  $\mu\text{M}$  OP1 in the presence (ratio  $1.76 \pm 0.05$ , 635 nM,  $n = 16$ ) as well as in the absence (ratio  $1.55 \pm 0.12$ , 510 nM,  $n = 6$ ) of external  $\text{Ca}^{2+}$  (Fig. 2B). The amplitude was statistically the same as with fMLP and was independent of external  $\text{Ca}^{2+}$ . There was no difference in duration of the  $\text{Ca}^{2+}$  transient induced by OP1 in the presence and the absence of extracellular  $\text{Ca}^{2+}$ . These findings indicate that, at least at a concentration of 0.8  $\mu\text{M}$  OP1, an increased influx of  $\text{Ca}^{2+}$  does not contribute directly to the  $\text{Ca}^{2+}$  transient.

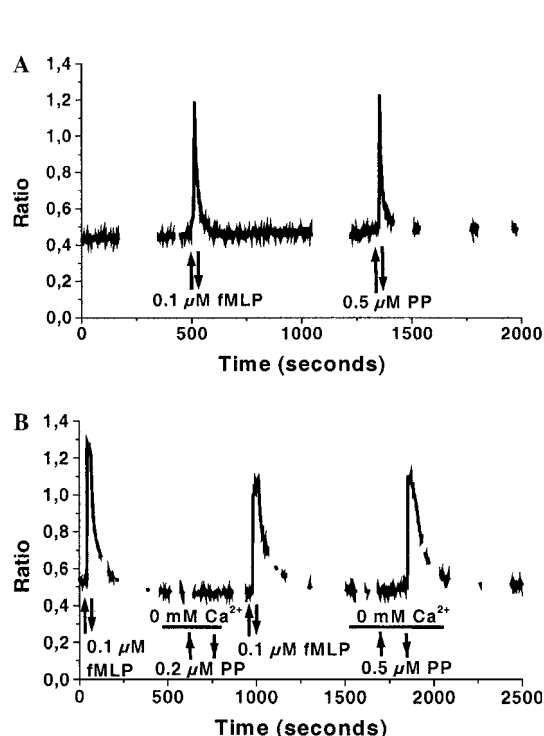


Fig. 1. Comparison of the effects of PP and 0.1  $\mu\text{M}$  fMLP on  $[\text{Ca}^{2+}]_i$  in single granulocyte-like HL-60 cells. (A) Effect of 0.5  $\mu\text{M}$  PP on  $[\text{Ca}^{2+}]_i$  in the presence of extracellular  $\text{Ca}^{2+}$ . (B) Effects of 0.2 and 0.5  $\mu\text{M}$  PP on  $[\text{Ca}^{2+}]_i$  in the absence of extracellular  $\text{Ca}^{2+}$ .

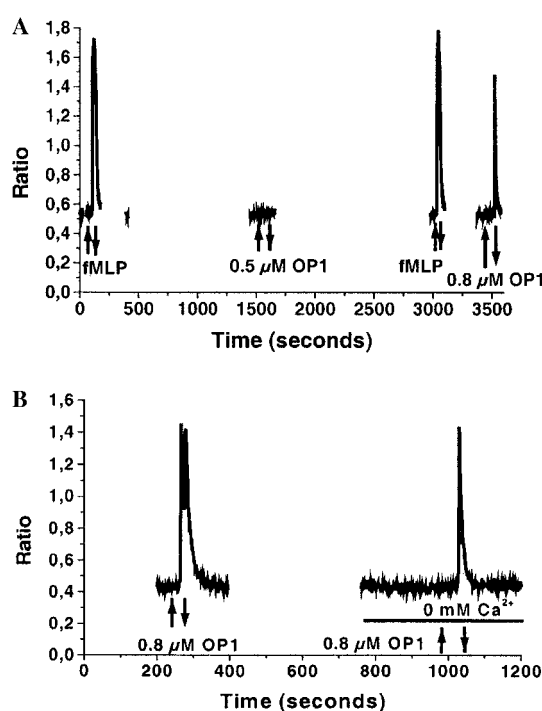


Fig. 2. Effects of opistoprin 1 on  $[\text{Ca}^{2+}]_i$  in single granulocyte-like HL-60 cells. (A) Effects of 0.5 and 0.8  $\mu\text{M}$  opistoprin 1 compared to the effect of 0.1  $\mu\text{M}$  fMLP,  $[\text{Ca}^{2+}]_o = 1 \text{ mM}$ . (B) Comparison of the effect of 0.8  $\mu\text{M}$  opistoprin 1 in the presence and the absence of 1 mM extracellular  $\text{Ca}^{2+}$ .

After a short application of 0.8  $\mu\text{M}$  OP1,  $[\text{Ca}^{2+}]_i$  returned to its initial value and the cells retained a low  $\text{Ca}^{2+}$  concentration (see Figs. 2A and B). This is different when higher peptide concentrations were used. At a concentration of 8  $\mu\text{M}$  OP1, the initial increase in  $[\text{Ca}^{2+}]_i$  was very similar to the response to 0.8  $\mu\text{M}$  (Figs. 3A and B). But, especially in the presence of external  $\text{Ca}^{2+}$ , the ratio oscillated between values of about 1 and 2.5 (240 and 1200 nM, respectively). Finally the fura-2 signal could not be further interpreted because the fluorescence signal at both excitation wavelengths drastically decreased, indicating that the probe leaked out of the cells. It is well known that  $\text{Ca}^{2+}$  overload itself may damage cell membranes and induce leak of intracellular contents. One could question therefore whether the increase of  $[\text{Ca}^{2+}]_i$  itself could be responsible for leakage of fura-2. Figs. 3B and D show that this was not the case for OP1 since in the absence of extracellular  $\text{Ca}^{2+}$ , the  $[\text{Ca}^{2+}]_i$  increased only to about 380 nM (ratio of 1.3) but leakage of fura-2 occurred after the same incubation time as in the presence of external  $\text{Ca}^{2+}$ . Similarly, PP was able to induce leakage of fura-2 but at a much lower concentration (1  $\mu\text{M}$ ).

#### Comparison with mastoparan and melittin

Similar results (not shown), but at different concentration ranges, were obtained with mastoparan and melittin. On single granulocyte-like HL-60 cells, melittin induced release of  $\text{Ca}^{2+}$  from intracellular stores at a

concentration of 1  $\mu\text{M}$  but leakage of  $\text{Ca}^{2+}$  already occurred at a concentration of 2  $\mu\text{M}$ . For mastoparan, effective concentrations were much higher: 20  $\mu\text{M}$  to elicit a  $\text{Ca}^{2+}$  transient in the absence of external  $\text{Ca}^{2+}$  and 50  $\mu\text{M}$  to induce  $\text{Ca}^{2+}$  leak.

In conclusion, concentrations of PP, melittin, and mastoparan that induce  $\text{Ca}^{2+}$  release and the ones that induce  $\text{Ca}^{2+}$  leak only differ by a factor of 1–2.5. On the other hand, both effects could be clearly separated in the presence of OP1 with a 10-fold higher concentration needed to induce  $\text{Ca}^{2+}$  leak. Therefore OP1 was further used to analyze the effects on  $\text{Ca}^{2+}$  release.

#### Release of intracellular $\text{Ca}^{2+}$ as a function of stage of differentiation of granulocyte-like HL-60 cells

To find out which type of receptor might eventually be involved in the  $\text{Ca}^{2+}$  release effect, HL-60 cells were studied on different stages of differentiation during which receptors were expressed to a variable extent [33]. For this purpose we investigated responses of single HL-60 cells to fMLP and to OP1 as a function of the time of differentiation from promyelocytes to granulocyte-like cells.

Eighty percent of the single HL-60 promyelocytes tested did respond to 0.8  $\mu\text{M}$  OP1 whereas only 20% of the cells responded to 0.1  $\mu\text{M}$  fMLP. After 1 day of differentiation, about 40% of the cells responded to 0.8  $\mu\text{M}$  OP1, after 2 days, the value further decreased to 9% and upon further differentiation, the cells were

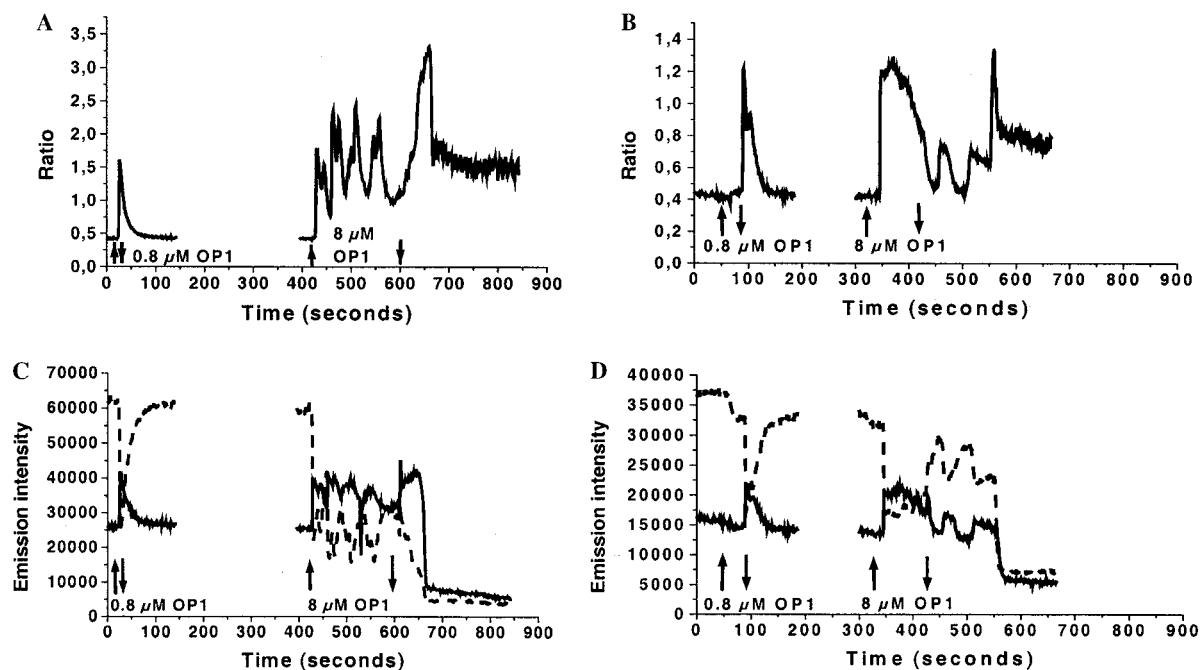


Fig. 3. Effects of 0.8 and 8  $\mu\text{M}$  opisthoporin 1 on  $[\text{Ca}^{2+}]_i$  in single granulocyte-like HL-60 cells in the presence (A,C) and the absence (B,D) of 1 mM extracellular  $\text{Ca}^{2+}$ . (A) and (B) represent the ratio of fura-2 fluorescence, (C) and (D) represent the emission at 505 nm at excitation wavelength of 340 (full line) and 380 (dashed line) nm of (A) and (B), respectively.

almost insensitive to  $0.8 \mu\text{M}$  OP1. A different picture was obtained with fMLP: the number of sensitive cells increased from 10% to 20% at days 0 and 1 to 100% after 4 days of differentiation. At 1–3 days of differentiation, 5–15% of the single cells responded to both agonists.

#### *Desensitization of the $\text{Ca}^{2+}$ transient induced by opistoprin 1*

To further explore the identity of a potential receptor for OP1, the occurrence of desensitization was studied in single cells and in cell suspensions. The action of  $0.1 \mu\text{M}$  fMLP is mediated via the formyl peptide receptor [34]. Because the experiments described above suggest that at least one component of the signaling cascade induced by fMLP and OP1 is different, we investigated whether cross-desensitization occurred between both mechanisms. If this occurs, it might indicate occupancy of the same receptor. It has been shown that the FPR desensitizes when occupied by fMLP and this is caused by phosphorylation of the FPR [35]. Cells were differentiated for 1 day to a stage that responsiveness to both peptides was present.

Fig. 4A shows a  $\text{Ca}^{2+}$  transient elicited by addition of fMLP to a suspension of granulocyte-like HL-60 cells. The rise of  $\text{Ca}^{2+}$  is transient even in the continuous presence of fMLP. The fall in  $[\text{Ca}^{2+}]_i$  indicates that the released  $\text{Ca}^{2+}$  is either transferred back to the intracellular stores or to the extracellular environment. A fur-

ther increase of the fMLP concentration had no effect on  $[\text{Ca}^{2+}]_i$ . This might indicate that internal stores become progressively emptied or that the ligand–receptor complex progressively desensitizes in the presence of a filled  $\text{Ca}^{2+}$  store. These experiments were repeated on single cell level. FMLP was added to single cells for the same period of time as in the experiments on cell suspensions. Thereafter, the fMLP was washed out for about 10 s. Similar to the experiments on cell suspensions, the single cells did not respond to a second application of fMLP. Responsiveness progressively returned as a function of washing time between both applications. When experiments were started with OP1 and subsequently followed by a further increase in OP1 concentration (in cell suspension, Fig. 4B) or a second application of OP1 (on single cells, not shown) similar results were obtained.

Figs. 4C and D show that after addition of  $0.8 \mu\text{M}$  OP1, the cells still responded to  $0.1 \mu\text{M}$  fMLP and vice versa, indicating that the intracellular  $\text{Ca}^{2+}$  stores were not empty after addition of the first agonist. Thus, a response to OP1 desensitizes the cells making them unresponsive to a subsequent addition of the same peptide. Because of the absence of cross-desensitization between both compounds on single cells, the absence of cross-desensitization in cell suspensions cannot be explained by the presence of cell populations that react differently to fMLP and OP1. In conclusion, all these results indicate that most probably the receptor for fMLP is different from a potential receptor for OP1.

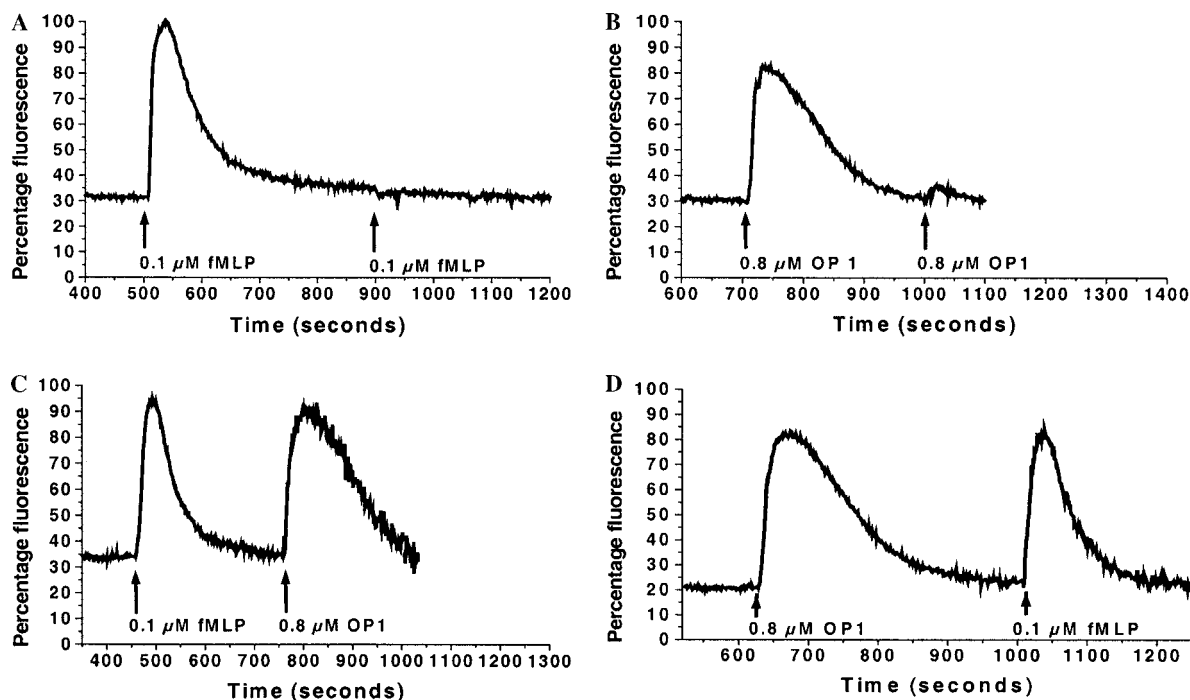


Fig. 4. Measurements of changes in  $[\text{Ca}^{2+}]_i$  induced by  $0.1 \mu\text{M}$  fMLP and  $0.8 \mu\text{M}$  opistoprin 1 in suspensions of granulocyte-like HL-60 cells.  $[\text{Ca}^{2+}]_o = 1 \text{ mM}$ . (A) Desensitization of fMLP-induced  $\text{Ca}^{2+}$  signaling. (B) Desensitization of OP1-induced  $\text{Ca}^{2+}$  signaling. (C,D) No cross-desensitization occurs between  $\text{Ca}^{2+}$  signaling induced by  $0.1 \mu\text{M}$  fMLP and  $0.8 \mu\text{M}$  OP1.

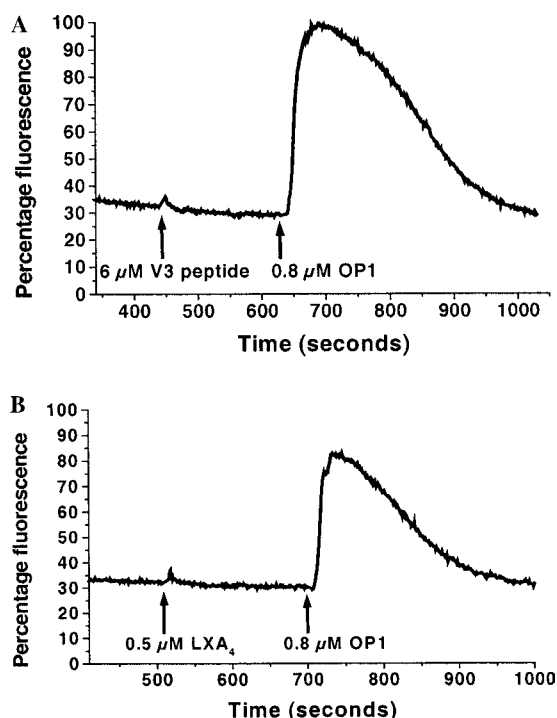


Fig. 5. Measurements of changes in  $[\text{Ca}^{2+}]_i$  induced by 6  $\mu\text{M}$  V3 peptide (A), 0.5  $\mu\text{M}$  LXA<sub>4</sub> (B), and 0.8  $\mu\text{M}$  opisthoporin 1 in suspensions of granulocyte-like HL-60 cells.  $[\text{Ca}^{2+}]_o = 1 \text{ mM}$ .

#### Comparison of the effects of opisthoporin 1 with FPRL1 ligands

Because from previous findings the FPR can be excluded as the potential target of OP1, other potential receptors that have been described in granulocytes were investigated. Fig. 5 illustrates the effects of V3 peptide, LXA<sub>4</sub>, and OP1 in granulocyte-like HL-60 cells in suspension. For V3 peptide and LXA<sub>4</sub> concentrations were used that have been described to release  $\text{Ca}^{2+}$  from intracellular stores in neutrophils [19,21]. Only a very small increase in  $[\text{Ca}^{2+}]_i$  upon addition of 3–6  $\mu\text{M}$  V3 peptide (Fig. 5A) or 0.5  $\mu\text{M}$  LXA<sub>4</sub> (Fig. 5B) could be observed. Fig. 5 also shows that the HL-60 cells that were unresponsive to V3 peptide and LXA<sub>4</sub> responded to OP1 in the same way as described in Figs. 4B and D. These experiments indicate that OP1 is able to induce  $\text{Ca}^{2+}$  release in cells that are insensitive to agonists interacting with the FPRL1 receptor.

#### Discussion

We found that cationic, antibacterial peptides isolated from the venom of the South-African scorpions *P. schlechteri* and *O. carinatus* increased intracellular  $\text{Ca}^{2+}$ . Comparable results were obtained for melittin and mastoparan. The rise in  $\text{Ca}^{2+}$  originated from two sources: a release from intracellular  $\text{Ca}^{2+}$  stores and an

increased  $\text{Ca}^{2+}$  leak from the extracellular space. Both effects were found to occur in the micromolar range with the action on  $\text{Ca}^{2+}$  release being the most sensitive effect. The order of potency was PP > melittin > OP1 > mastoparan. This order of potency is different from their antibacterial activity: PP and OP1 are by far the most potent peptides on Gram-negative bacteria, while melittin and mastoparan are most potent on Gram-positive bacteria [2]. For PP and melittin the concentrations influencing  $\text{Ca}^{2+}$  release and  $\text{Ca}^{2+}$  leak overlapped, while for OP1  $\text{Ca}^{2+}$  release occurred at concentrations that did not induce  $\text{Ca}^{2+}$  leak.

The time course of the  $\text{Ca}^{2+}$  release induced by the amphipathic peptides was very similar to the receptor (FPR)-dependent response to fMLP: in the absence of external  $\text{Ca}^{2+}$ , the amplitudes of the  $\text{Ca}^{2+}$  releases were not statistically different for fMLP, PP, and OP1. In addition, the fact that the response to 0.1  $\mu\text{M}$  fMLP did not depend on extracellular  $\text{Ca}^{2+}$  and that its maximal effect was observed at this concentration indicated that the same fraction of intracellular  $\text{Ca}^{2+}$  was released by the different agonists.

The pertussis toxin-sensitivity of the  $\text{Ca}^{2+}$  release points to the involvement of G-proteins in the  $\text{Ca}^{2+}$  release action. Also for mastoparan [8] and dermaseptin [36] the involvement of a G<sub>i</sub>-protein has been suggested. Similar dual actions on  $\text{Ca}^{2+}$  release and  $\text{Ca}^{2+}$  permeability of the surface membrane have been reported for the bacterial pore-forming peptides aerolysin, staphylococcal  $\alpha$ -toxin, and streptolysin O. They induce changes in  $[\text{Ca}^{2+}]_i$  in granulocyte-like HL-60 cells that are partly pertussis toxin-sensitive [37].

We found that  $\text{Ca}^{2+}$  release elicited by scorpion peptides could not be explained by an interaction with FPR or FPRL1. Although the dependency of the OP1 action on the stage of differentiation as well as the desensitization experiments are in agreement with a receptor-mediated action, the question remains open as long as the receptor has not been identified. Pertussis toxin-inhibitable G<sub>i</sub>-proteins are present in HL-60 promyelocytes and differentiation of HL-60 cells is accompanied by enhanced expression of G<sub>i</sub>-proteins [33]. In the case of a direct receptor-independent interaction with G-proteins, a decreasing response to OP1 as a function of differentiation of HL-60 cells is rather unexpected. Arguments that may favor a non-receptor-dependent mechanism are: the rather low affinity of the binding site and the close proximity of concentrations that affect signaling and disrupt membranes. The latter finding indicates that insertion of the scorpion peptides into the membrane with effects on membrane-associated transduction proteins and pore formation is part of the same effect. Suppression of superoxide production in human neutrophils has been explained by a direct interaction of scorpion toxins with the NADPH oxidase complex (unpublished observation). This occurs in the

same concentration range as needed for  $\text{Ca}^{2+}$  release. These findings indicate that insertion of these molecules into the membrane may activate (G-proteins) and inhibit (NADPH oxidase) subunits of complex membrane-bound protein systems. For both systems it has been shown that amphipathic molecules may interact with isolated subunits in an acellular system (for G-proteins see above; for NADPH oxidase see [38]).

For aerolysin, differences in  $\text{Ca}^{2+}$  transients have been reported as a function of differentiation of granulocyte-like HL-60 cells. But in contrast to our results, sensitivity towards aerolysin increased as a function of differentiation [37]. Specificity of aerolysin action has been explained by the binding of proaerolysin to GPI (glycosylphosphatidylinositol) anchored proteins [27], which are concentrated in lipid rafts, sphingolipid- and cholesterol-rich microdomains in the cell membrane [39]. Lipid rafts have been described to influence intracellular signaling. They may contain some of the components of a signaling pathway that only becomes activated when a particular molecule inserts in the raft [40]. Therefore, interaction of scorpion peptides with these microdomains could lead to modulation of intracellular signaling and at (slightly) higher concentrations to pore formation. Further research, however, is needed to confirm this hypothesis. If a classical high affinity receptor is not the intermediate in toxin–G-protein interaction, the observed desensitization is most probably related to modulation at the level of the G-protein itself. It is not known whether regulators of G-protein signaling (RGS proteins) play any role in this action.

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