Pituitary Adenylate Cyclase-Activating Polypeptide 27 Is a Functional Ligand for Formyl Peptide Receptor-Like 1[1]

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Although the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) has been implicated in the regulation of several immune responses, its target receptors and signaling mechanisms have yet to be fully elucidated in immune cells. In this study, we found that PACAP27, but not PACAP38, specifically stimulated intracellular calcium mobilization and ERK phosphorylation in human neutrophils. Moreover, formyl peptide receptor-like 1 (FPRL1) was identified as a PACAP27 receptor, and PACAP27 was found to selectively stimulate intracellular calcium increase in FPRL1-transfected rat basophile leukocytes-2H3 cell lines. In addition, PACAP27-induced calcium increase and ERK phosphorylation were specifically inhibited by an FPRL1 antagonist, Trp-Arg-Trp-Trp-Trp-Trp (WRW4), thus supporting the notion that PACAP27 acts on FPRL1. In terms of the functional role of PACAP27, we found that the peptide stimulated CD11b surface up-regulation and neutrophil chemotactic migration, and that these responses were completely inhibited by WRW4. The interaction between PACAP27 and FPRL1 was analyzed further using truncated PACAPs and chimeric PACAPs using vasoactive intestinal peptide, and the C-terminal region of PACAP27 was found to perform a vital function in the activation of FPRL1. Taken together, our study suggests that PACAP27 activates phagocytes via FPRL1 activation, and that this results in proinflammatory behavior, involving chemotaxis and the up-regulation of CD11b. *The Journal of Immunology,* **2006, 176: 2969–2975.**

The two pituitary adenylate cyclase-activating polypeptides (PACAPs),[3] PACAP27 and PACAP38, are neuropeptides that belong to the secretin/glucagon/vasoactive intestinal peptide (VIP) family (1). PACAPs are multifunctional peptide hormones that influence diverse biological functions, e.g., the cell cycle, smooth muscle and cardiac muscle relaxation, bone metabolism, and endocrine/paracrine function (2). In addition, during recent years, the effects of PACAPs on the immune system have been partially elucidated. In this context, PACAPs both suppress and activate inflammation by regulating the interleukins, IL-1β, IL-6, and IL-10 (3–8).

Three distinct G protein-coupled receptors (GPCR) of PACAPs have been identified (PAC1, VPAC1, and VPAC2) (9–12). PAC1 can be activated by PACAPs, but not by VIP, whereas VPAC1 and VPAC2 are activated by both. Therefore, we undertook to elucidate PACAP-mediated immune cell functions by investigating the receptor expression pattern. PAC1 has been reported to inhibit IL-6 production in stimulated macrophages, despite its up-regulation of IL-6 secretion in unstimulated macrophages (3, 4). VPAC1 has also been reported to participate in PACAP-induced IL-6 secretion in unstimulated macrophages (4). However, the specific nature of the involvement of PACAP receptors in immune-related functions has yet to be adequately demonstrated.

In this study, we attempted to characterize the functional roles of PACAP in human neutrophils, a type of phagocytic leukocyte, and to identify the cell surface receptors involved in these processes. Interestingly, we found that PACAP27 exerts a stimulatory effect on an important chemoattractant receptor, formyl peptide receptor (FPR)-like 1 (FPRL1). In addition, we conducted an analysis of the region of PACAP27 found crucial for the binding and activation of FPRL1, its specific receptor.

Materials and Methods

Materials

PACAP27, PACAP38, and VIP were obtained from Phoenix Pharmaceuticals. Truncated PACAPs (tPACAPs) were synthesized by the Peptide Library Support Facility (Pohang, Korea). Chimeric PACAPs (cPACAPs) were purchased from GenScript, radioiodinated PACAP27 (^{125}I-labeled) were obtained from PerkinElmer, and PBMC separation medium (Histopaque-1077) was obtained from Sigma-Aldrich. RPMI 1640 medium and high glucose DMEM were obtained from Invitrogen Life Technologies; dialyzed FBS was obtained from HyClone. Fura-2 penta-acetoxymethyl ester (fura 2-AM) was obtained from Molecular Probes; anti-phospho-ERK Abs and anti-ERK2 Abs were obtained from Cell Signaling Technology. PE-labeled human CD11b Abs were obtained from BD Pharmingen, *Limulus* Amebocyte Lysates assay (QCL-1000) was obtained from Cambrex Bioscience, and polymyxin b was obtained from Sigma-Aldrich. Chemotaxis multiwell chambers were obtained from Neuroprobe.

Cell culture

FPRL1-expressing rat basophile leukemia (RBL)-2H3 (FPRL1/RBL), FPR-expressing RBL-2H3 (FPR/RBL), and vector-transfected RBL-2H3 (vector/RBL) were donated by Dr. R. D. Ye (University of Illinois, Chicago, IL). FPRL1/RBL, FPR/RBL, and vector/RBL were maintained at

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[3] Abbreviations used in this paper: PACAP, pituitary adenylate cyclase-activating polypeptide; VIP, vasoactive intestinal peptide; GPCR, G protein-coupled receptor; FPR, formyl peptide receptor; FPRL1, FPR-like 1; RBL, rat basophile leukemia; tPACAP, truncated PACAP; cPACAP, chimeric PACAP; $[Ca^{2+}]_i$, intracellular calcium concentration; PTX, pertussis toxin.

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37°C in a humidified 5% CO_2 atmosphere in high glucose DMEM supplemented with 20% (v/v) heat-inactivated FCS and G418 (500 μg/ml). FPRL1/RBL, FPR/RBL, and vector/RBL were subcultured every 3 days.

Preparation of neutrophils and monocytes

Peripheral blood was collected from healthy donors. Human neutrophils were isolated by dextran sedimentation followed by hypotonic erythrocyte lysis and lymphocyte separation medium gradient, as previously described (13). Isolated human neutrophils were used promptly. PBMCs were separated on a Histopaque-1077 gradient. After twice washing with HBSS without Ca^{2+} and Mg^{2+}, the PBMCs were then suspended in 10% FBS containing RPMI 1640 medium and incubated for 60 min at 37°C to let the monocytes attach to the culture dish. The cells were washed five times with warmed RPMI 1640 medium to wash out lymphocytes and then the attached monocytes were collected as described before (13).

Intracellular calcium mobilization measurements

Intracellular calcium concentrations ($[Ca^{2+}]_i$) were determined using Grynkiewicz's method with fura 2-AM (14). Briefly, prepared cells were incubated with 3 μM fura 2-AM at 37°C for 50 min in fresh serum-free RPMI 1640 medium with continuous stirring. Cells (2×10^6) were aliquoted for each assay in Ca^{2+}-free Locke's solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 5 mM HEPES (pH 7.3), 10 mM glucose, and 0.2 mM EGTA). Fluorescence changes at 340 and 380 nm using a common emission wavelength of 500 nm were measured, and fluorescence ratios were converted to $[Ca^{2+}]_i$ (14).

Intracellular cyclic AMP measurements

Briefly, neutrophils were isolated and resuspended at 5×10^6 cells/ml in HBSS for 5–10 min in a shaking incubator. The HBSS was then replaced with 100 μl of HBSS containing 500 μM isobutylmethylxanthine (a cAMP phosphodiesterase inhibitor) for 5 min, and then cells were stimulated for 10 min, the reaction was terminated by adding 1 ml of ethanol, and cAMP levels were determined by using cAMP measuring kit (Neuronex) according to the manufacturer's instructions.

Western blot analysis for ERK phosphorylation

ERK phosphorylation levels were measured by Western blotting, as described previously (13). Cells (2×10^6/assay) were stimulated with the indicated concentration of agonist for 5 min, then washed with serum-free RPMI 1640 medium, and lysed in lysis buffer (20 mM HEPES (pH 7.2), 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 50 mM NaF, and 1 mM Na_3VO_4). Detergent-insoluble materials were pelleted by centrifugation (12,000 $\times$ *g*, 15 min, 4°C), and the soluble supernatant fraction was removed and either stored at −80°C or used immediately. Laemmli sample buffer was added to these fractions and boiled (5 min). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell Microscience; BA85). Blocking was performed using TBS buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20) containing 5% nonfat dry milk. Membranes were probed with a phospho-ERK-specific primary Ab or ERK2 Ab for 3 h at room temperature. Subsequently, immunoblots were washed and incubated with a HRP-linked secondary Ab (Kirkegaard & Perry Laboratories) for 1 h at room temperature, rinsed four times in TBS buffer, and then developed with HRP-dependent chemiluminescence reagents (Amersham Biosciences).

FACS analysis

Cells (2×10^5/assay) were washed with FACS buffer (PBS containing 1% BSA and 0.1% sodium azide), incubated with human AB type serum for 10 min on ice, and stained with PE-labeled human CD11b Ab. They were then analyzed using a FACSCalibur system (BD Biosciences), as described previously (15).

Chemotaxis assays

Chemotaxis assays were performed using multiwell chambers (Neuroprobe) (13). Prepared human neutrophils were suspended in RPMI 1640 medium at a 1×10^6 cells/ml, and 25 μl of this suspension was placed into the upper well of a chamber separated from the lower chamber, which was filled with testing solutions, by a 3-μm filter (not coated with polyvinylpyrrolidone). After incubating for 2 h at 37°C, nonmigrated cells were removed by scraping, and cells that had migrated across the filter were dehydrated, fixed, and stained with hematoxylin (Sigma-Aldrich). Stained cells in five randomly chosen high-power fields (×400) were then counted.

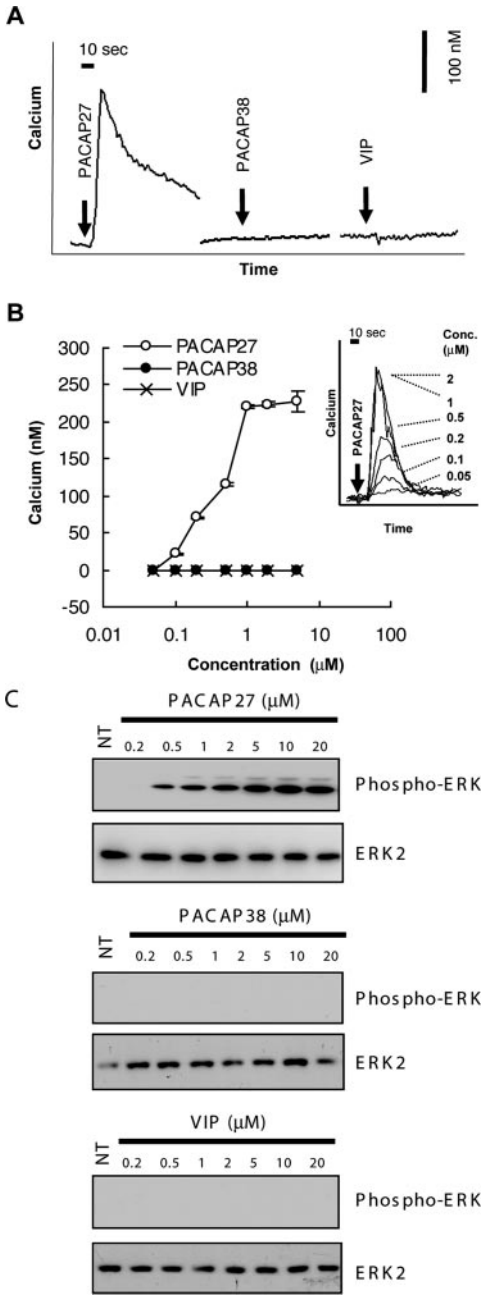

FIGURE 1. PACAP27 selectively induces intracellular signaling in human neutrophils. *A* and *B*, Fura 2-loaded human neutrophils were treated with agonist peptides (PACAP27, PACAP38, or VIP). Changes at 340 and 380 nm were monitored and fluorescence ratios were converted to $[Ca^{2+}]_i$. Neutrophils were stimulated with 1 μM PACAP27, PACAP38, or VIP (*A*). Dose dependency was tested at various concentrations (50 nM-5 μM) of PACAP27, PACAP38, or VIP in human neutrophils (*B*). Data are presented as means ± SE of four independent experiments, each of which was performed in triplicate (*B*). *C*, ERK phosphorylation was assessed by Western blotting, using a phospho-ERK-specific Ab. Neutrophils were incubated with various concentrations (200 nM–20 μM) of PACAP27, PACAP38, or VIP. The results shown are representative of four independent experiments, each performed in duplicate.

Ligand-binding analysis

Ligand-binding analysis was performed as previously described (16). Briefly, FPRL1/RBL cells were seeded at 1×10^5 cells/well onto a 24-well plate and cultured overnight. After blocking them with blocking buffer (33 mM HEPES (pH 7.5), 0.1% BSA in RPMI 1640 medium) for 2 h, 50 pM of ^{125}I-labeled PACAP27 (PerkinElmer) was added to the cells in binding

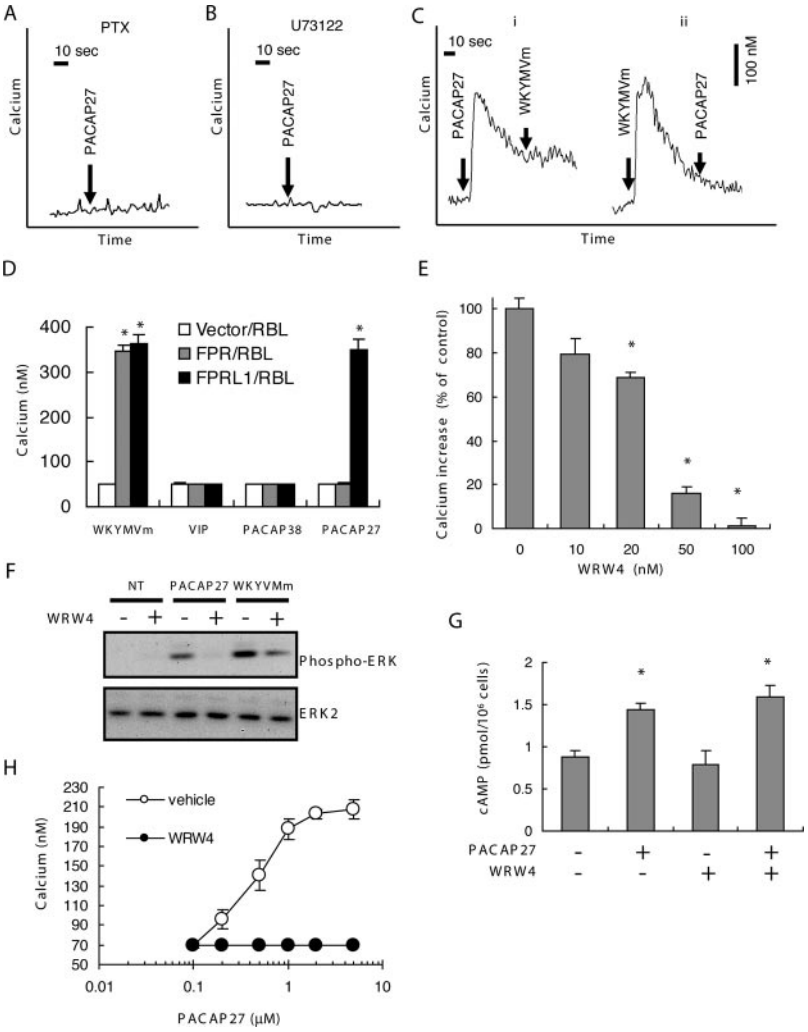

FIGURE 2. PACAP27 activates human neutrophils via FPRL1. *A–E* and *H*, Fura 2-loaded human neutrophils or RBL-2H3 cells were treated with agonist peptides. Changes at 340 and 380 nm were monitored and fluorescence ratios were converted to $[Ca^{2+}]_i$. Neutrophils were treated with 2 μg/ml PTX (*A*) or 2 μM U73122 (*B*) before being stimulated with PACAP27, for 3 h or 30 min, respectively. C, Neutrophils were stimulated with 1 μM PACAP27 and this was followed by adding 10 nM WKYMVm (*i*), or 10 nM WKYMVm and then 1 μM of PACAP27 (*ii*). *D*, FPRL1/RBL, FPR/RBL, and vector/RBL cells were stimulated with 1 μM PACAP27, PACAP38, or VIP. *E*, Neutrophils were stimulated with vehicle or with various WRW4 concentrations for 30 s before the addition of 1 μM PACAP27. The results shown are representative of four independent experiments performed in duplicate. *F*, ERK phosphorylation was assessed by Western blotting using phospho-ERK-specific Ab. Neutrophils were incubated with either vehicle or 1 μM WRW4 for 30 s and then treated for 5 min with 10 nM WKYMVm or 1 μM PACAP27. Data present the means ± SE of four independent experiments performed in triplicate (*D* and *E*). *G*, cAMP elevation was measured, as described in *Materials and Methods*. Neutrophils were stimulated with vehicle or with 1 μM WRW4 for 30 s and then treated with 10 μM PACAP27 for 10 min. *H*, Human monocytes were treated with vehicle or with 1 μM WRW4 for 30 s before the addition of various PACAP27 concentrations. The results shown are representative of four independent experiments performed in duplicate. ∗, $p <$ 0.01 vs vehicle treatment.

buffer (PBS containing 0.1% BSA), in the presence of the test peptides (cold PACAP27, tPACAPs, and cPACAPs), and then incubated for 3 h at 4°C with continuous shaking. The cells were then washed five times with ice-cold binding buffer, and 200 μl of lysis buffer (20 mM Tris (pH 7.5), 1% Triton X-100) was added to each well for 20 min at room temperature. Lysates were then collected and counted using a gamma-ray counter.

Statistics

Results are expressed as means ± SE. In the figure legends, ∗ indicates $p <$ 0.01 vs the appropriate vehicle-treated control.

Results

PACAP27 specifically stimulates intracellular signaling in human neutrophils

The expressions of PACAP receptors in immune cells have been reported by several groups (3, 4), but their functions are unclear. Here, we found that the stimulation of human neutrophils with 1 μM PACAP27 profoundly increased $[Ca^{2+}]_i$ (Fig. 1*A*). However, neither PACAP38 nor VIP increased $[Ca^{2+}]_i$. To confirm this PACAP27-specific activation, we examine the dose dependencies of PACAP27, PACAP38, or VIP and found that only PACAP27 increased $[Ca^{2+}]_i$ (Fig. 1*B*). At 100 nM, PACAP27 induced a significant $[Ca^{2+}]_i$ increase (Fig. 1*B*, *inset*). PACAP27-induced signaling was also observed to be associated with the dose-dependent phosphorylation of ERK (Fig. 1*C*). These data suggest that PACAP27 specifically stimulates human neutrophils. In view of the fact that VPAC1 can be stimulated by VIP or PACAP38, as well as by PACAP27, our results were not consistent with those of a previous report which suggested that VPAC1 functions as a PACAP receptor in neutrophils (15). These data suggest that another receptor may be involved in the process of PACAP27-induced intracellular signaling in human neutrophils.

PACAP27 uses FPRL1 as a specific receptor

To determine the characteristic properties of the PACAP27-specific receptor in human neutrophils, we assessed the effects of pertussis toxin (PTX) or U73122 (a specific phospholipase C inhibitor) on PACAP27-mediated calcium signaling (Fig. 2, *A* and *B*). Several chemoattractant receptors have been reported to exert stimulatory effects on neutrophils via PTX-sensitive GPCRs and by the activation of phospholipase C (13). To determine whether PACAP27 can stimulate known chemoattractant receptors in human neutrophils, we analyzed calcium signaling in response to sequential stimulation using PACAP27 and the known chemoattractants, fMLP, WKYMVm, or C5a (Fig. 2*C* and data not shown). Treatment with 1 μM PACAP27 and 10 nM WKYMVm resulted in bidirectional desensitization, suggesting that both ligands share the same receptor (Fig. 2*C*). Because WKYMVm stimulates members of the FPR family, particularly FPRL1 at low nanomolar concentrations (17), we examined the effects of PACAP27 on calcium signaling in RBL-2H3 cells expressing either FPR or FPRL1 (FPR/RBL or FPRL1/RBL). PACAP27 was found to exert a profound stimulatory effect on FPRL1/RBL cells, but not on vector/RBL or FPR/RBL cells (Fig. 2*D*). We also examined the effects of the FPRL1-selective antagonist, Trp-Arg-Trp-Trp-Trp-Trp

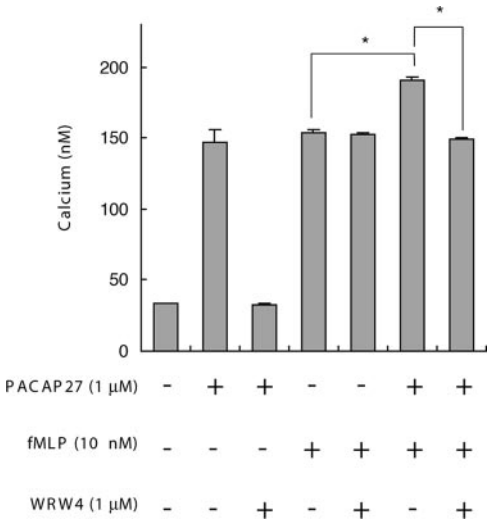

FIGURE 3. PACAP27 primes fMLP-induced calcium signaling via FPRL1. Changes at 340 and 380 nm were monitored and fluorescence ratios were converted to $[Ca^{2+}]_i$. Neutrophils were treated with vehicle or 1 μM WRW4 for 30 s, before being stimulated with vehicle, 1 μM PACAP27, 10 nM fMLP, or both. The results shown are representative of two independent experiments performed in duplicate. *, $p < 0.01$ vs control.

(WRW4) (15), on PACAP27-induced signaling in human neutrophils. WRW4 successfully inhibited PACAP27-induced $[Ca^{2+}]_i$ up-regulation (Fig. 2*E*), but failed to inhibit PACAP27-induced cAMP elevation (Fig. 2*G*), indicating that WRW4 does not affect VPAC1, which has been reported to be expressed in human neutrophils (15). ERK phosphorylation was also completely inhibited by pretreating with WRW4, indicating that this ERK phosphorylation is also a part of the FPRL1-dependent signaling cascade (Fig. 2*F*). Because monocytes were reported to express FPRL1 (17), we also examined the effects of WRW4 on PACAP27-induced calcium signaling in human monocytes. WRW4 successfully inhibited PACAP27-induced calcium signaling in human monocytes (Fig. 2*H*).

PACAP27 primes fMLP-induced calcium signaling in a FPRL1-dependent manner

Previously, PACAP27 has been reported to prime fMLP-induced calcium signaling (15). To determine the FPRL1 dependency, we examined the effect of PACAP27 on fMLP-induced calcium signaling with or without WRW4. As shown in Fig. 3, fMLP-induced calcium signaling was not affected by WRW4, indicating that fMLP acts on FPR. PACAP27 notably enhanced fMLP-induced calcium signaling, and this event was abolished by WRW4 treatment, indicating the priming effect was FPRL1-dependent.

PACAP27 induces CD11b up-regulation in neutrophils in an FPRL1-dependent manner

PACAP27 has been found to stimulate the surface expression of CD11b, a known mediator of neutrophil adhesion and mobilization (15). Purified neutrophils were incubated with PACAP27, and analyzed by flow cytometry, as shown by the dot plots in Fig. 4*A*. We observed that PACAP27 up-regulated CD11b, maximally at 10 μM (Fig. 4*B*). Moreover, CD11b up-regulation was inhibited completely by the FPRL1 antagonist, WRW4, indicating that it is a FPRL1-dependent process (Fig. 4*C*). To abolish the possibility of endotoxin contamination of PACAP27, we measured endotoxin content in the PACAP27 sample via *Limulus* Amebocyte Lysates assay (QCL-1000; Cambrex Bioscience), and endotoxin was not detected (much <0.1 endotoxin units/mg, data not shown). We also tested heat inactivation and polymyxin b (Sigma-Aldrich) treatment on PACAP27-induced CD11b up-regulation. There is no difference among PACAP27, boiled PACAP27, and polymyxin b-treated PACAP27, indicating that the synthetic PACAP27 is endotoxin-free.

PACAP27 induces the chemotactic migration of neutrophils in an FPRL1-dependent manner

As FPRL1 participates in leukocyte migration in concert with several specific ligands, we examined whether PACAP27 induces neutrophil chemotaxis. It was found that it elicited the chemotactic migration of neutrophils dose-dependently with maximal activity at 10 μM (Fig. 5*A* and Table I). The involvement of FPRL1 in

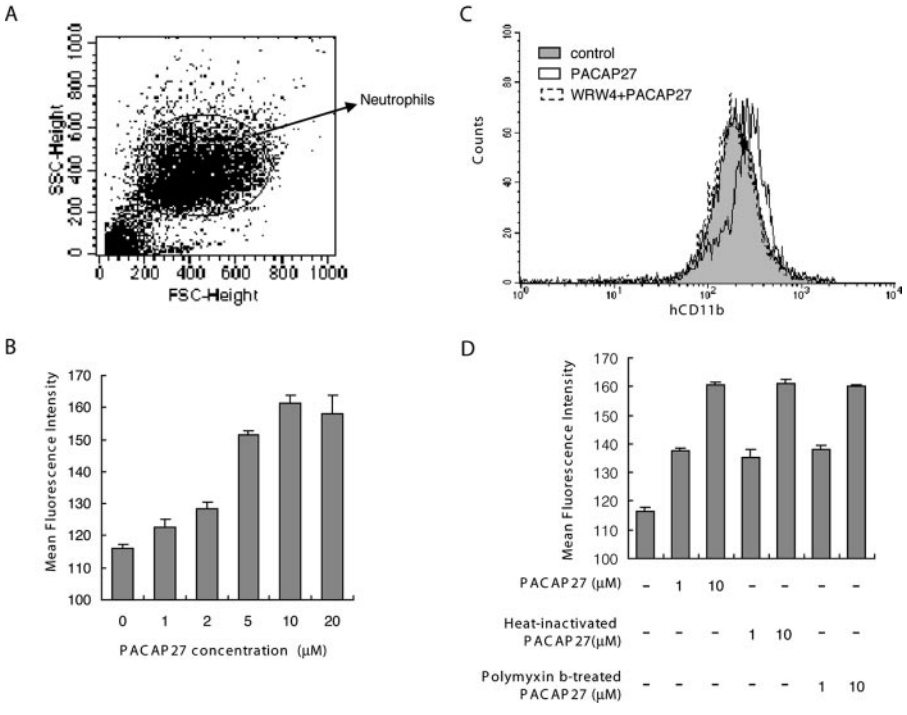

FIGURE 4. PACAP27 induces the up-regulation of CD11b in neutrophils via FPRL1. Surface CD11b expression was determined via FACS analysis. Neutrophils were gated out (*A*); CD11b levels are represented by mean fluorescence intensity (*B*) or histograms (*C*). Purified neutrophils were incubated with various concentrations of PACAP27 for 1 h (B) or with vehicle or 1 μM WRW4 for 30 s before being treated with 10 μM PACAP27 for 1 h (*C*). *D*, Purified neutrophils were incubated with 1 or 10 μM of PACAP27, heat-inactivated PACAP27, or polymyxin b-treated PACAP27 for 1 h. Heat inactivation was performed for 10 min in boiling water. PACAP27 was pretreated with 5 μM polymyxin b for 1 h in 37°C. The results shown are representative of four independent experiments performed in duplicate.

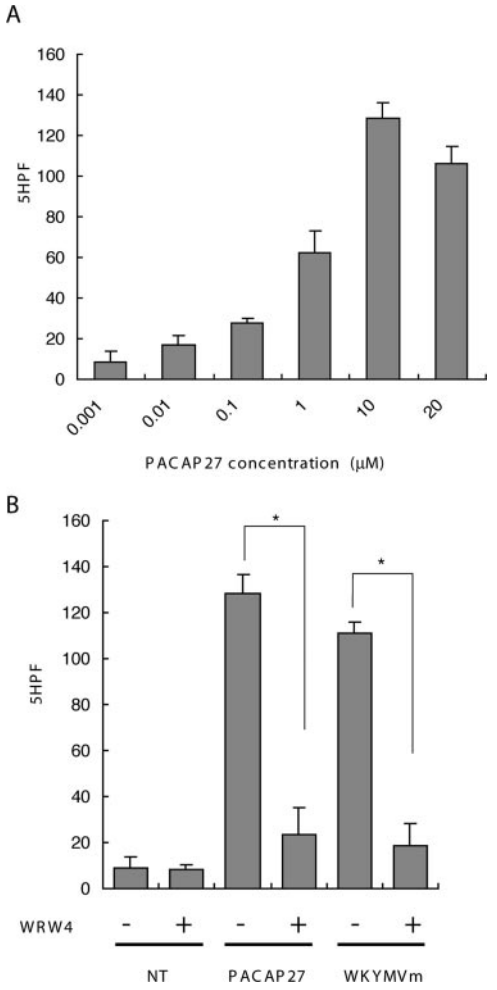

FIGURE 5. PACAP27 induces neutrophil chemotaxis via FPRL1. Chemotaxis assays were conducted using a modified Boyden chamber assay, as described in *Materials and Methods*. Neutrophil chemotaxis was examined using various concentrations of PACAP27 (*A*). Neutrophils were tested using vehicle, 10 nM WKYMVm, or 1 μM PACAP27 in the absence and presence of 1 μM WRW4 (*B*). Data are presented as the means ± SE for migrated neutrophils per field were counted in triplicate of four independent experiments. *, $p < 0.01$ vs vehicle treatment.

PACAP27-induced neutrophil chemotaxis was examined using the FPRL1 antagonist, WRW4. As shown in Fig. 5*B*, PACAP27-induced neutrophil chemotaxis was completely inhibited by WRW4, indicating that this process requires FPRL1 (Fig. 5*B*).

The C-terminal region of PACAP27 is important for its interaction with FPRL1

To characterize the interaction between PACAP27 and FPRL1, a number of tPACAPs were synthesized by deleting the N- or C-terminal sequences of PACAP27 (Fig. 6*A*). EC_{50} values with respect to $[Ca^{2+}]_i$ increases in FPRL1/RBL cells were then calculated. Sequential N-terminal truncations resulted in progressively lower efficacies, which suggest that this region contributes only partially to FPRL1 activation. However, none of the C-terminal-tPACAPs exhibited activity, indicating that the C-terminal sequences are critical for the activation of FPRL1. Interestingly, tPACAP9-27 was shown to partially activate FPRL1, despite the inability of tPACAP9-27 to activate PAC1 (18, 19), indicating that PACAP27 stimulates these FPRL1 and PAC1 in different ways. The binding affinity of tPACAPs to FPRL1 was also measured. As shown in Fig. 6*B*, this binding normally correlates with calcium-increasing activity, but tPACAP9-27 exhibited

Table I. *Checkboard analysis of neutrophil after treatment with PACAP27*[a]

Above / Below	Medium	PACAP27 (μM) 0.1	1	10
Medium	0 ± 0	0 ± 0	0 ± 0	0 ± 0
PACAP27 (μM)				
0.1	34.7 ± 2.5	19.3 ± 3.1	16.0 ± 5.7	16.9 ± 6.2
1	59.3 ± 1.0	33.7 ± 1.4	21.3 ± 3.4	20.7 ± 8.4
10	162.3 ± 31.0	147.7 ± 28.8	72.7 ± 11.5	73.7 ± 6.9

[a] Neutrophil migration was analyzed for 2 h across a polycarbonate membrane. Various concentrations of PACAP27 were placed in the upper and lower compartments of the chambers. Data are presented as means ± SE for migrated neutrophils cells per field counted in triplicate of two independent experiments.

almost the same binding affinity as PACAP27 ($K_d = 52.3 \pm 1.6$ nM). These results suggest that the N-terminal region (first to eighth) of PACAP27 is not associated with binding affinity, but rather that it contributes to full activation.

Based on an analysis of the VIP sequence, which is similar to that of PACAP27, though it does not interact with FPRL1, we designed several cPACAPs by substituting VIP amino acid residues (Fig. 6*C*). Substitutions of the 24th or the 25th amino acids resulted in a pronounced loss of activity (Fig. 6*D*), and of binding affinity ($K_d = 2.1 \pm 0.13$ μM, $K_d = 2.0 \pm 0.17$ μM, respectively), whereas substitutions of the 13th or 9th had no effect on binding affinity ($K_d = 51.2 \pm 3.3$ nM) (Fig. 6*E*). cPACAP24,25VIP had lowest binding affinity ($K_d = 8.7 \pm 0.75$ μM). Thus, it appears that C-terminal amino acid residues from 22 to 27 are primary contributors to binding and subsequent receptor activation, and that the 24th and 25th hydrophobic amino acid residues are major determinants. The central region from 9 to 21 seems to contribute only marginally to receptor binding and activation, and that the N-terminal region from 1 to 8 is required for full activation.

Discussion

In this study, we observed PACAP27-specific signaling in human neutrophils and its relations with calcium and ERK signaling, the up-regulation of CD11b, and with chemotactic migration. Previously known receptors like PAC1, VPAC1, and VPAC2 were found to be unhelpful in terms of explaining these PACAP27-specific activities, and thus, we hypothesized that another receptor is involved in this process. The present study reveals that this receptor is FPRL1.

To prove the hypothesis that another receptor is involved in the PACAP27-specific activities, we showed the cross-desensitization between PACAP27 and WKYMVm (Fig. 2*B*). Cross-desensitization provides a straightforward and powerful means of illustrating receptor sharing. However, some GPCR groups do codesensitize via single receptor activation for reasons, like receptor oligomerization, sequestration, and others (20). To solve this problem, we used the antagonizing peptide, WRW4, which does not activate but does bind FPRL1 (16). Desensitization events between two GPCRs usually occur via agonist-induced receptor activation (20). In addition, we show that the FPRL1-specific antagonist peptide, WRW4, can inhibit PACAP27-induced calcium signaling. FPRL1-expressing RBL2H3 cells were used to confirm this effect, and it was found that PACAP27-specific signaling only occurred on FPRL1-expressing cells (Fig. 2*D*). These findings indicated that PACAP27 specifically activates human neutrophils by activating FPRL1.

Previously, it has been reported that PACAP27 primes neutrophil response to the fMLP (15). Bacterial fMLP can activate FPR and FPRL1 at high concentrations, but fMLP activates only FPR at low

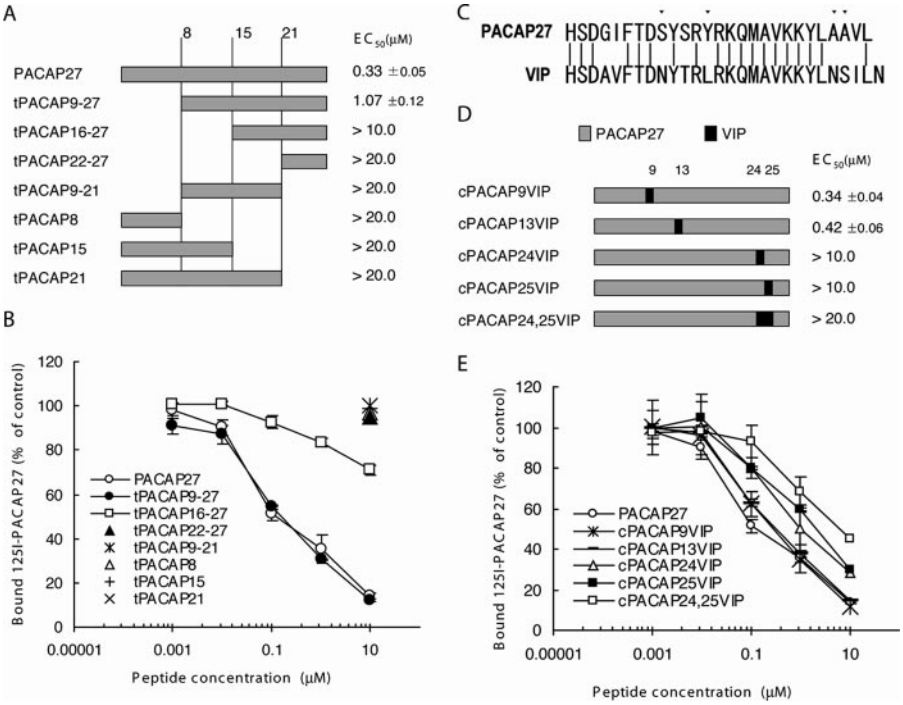

FIGURE 6. The FPRL1-PACAP27 interaction is mediated predominantly by the C-terminal region of PACAP27. tPACAPs and cPACAP were tested using FPRL1-expressing RBL-2H3 cells. EC_{50} values were obtained by measuring increases in $[Ca^{2+}]_i$ activity (*A*). FPRL1/RBL cells (1×10^5 cells/200 μl) were used for the binding assay (*B*). FPRL1/RBL cells were pretreated with various concentrations of unlabeled PACAP27 or tPACAPs before being treated with ^{125}I-labeled PACAP27 (50 pM). Controls were prepared by pretreating with vehicle before ^{125}I-labeled PACAP27 treatment (*B*). The amino acid sequences of PACAP27 and VIP were compared, and four residues were selected (▼) for the construction of chimeras on the basis of their chemical properties (*C*). The EC_{50} values of cPACAPs with respect to increasing $[Ca^{2+}]_i$ activity were measured (*D*). The receptor binding affinities of the cPACAPs were determined in a manner identical to that used for tPACAPs (*E*). Data are presented as means ± SE of four independent experiments performed in triplicate (*A* and *B*, *D* and *E*).

concentrations (21). Therefore, we hypothesized that the PACAP27-induced priming event on fMLP signaling is a result of the combined activation of these two receptors, FPRL1 and FPR. To prove this hypothesis, we measured the effect of WRW4 on the priming event (Fig. 3), and found that this event is FPRL1-dependent.

The regulation of the immune system by PACAP is likely to occur in a complex manner, as reflected by the inflammatory cytokine secretions of several immune cells. In monocytes and macrophages, PACAP molecules suppress the production of the proinflammatory cytokines, TNF-α, IL-6, and IL-12 (6–8). In contrast, in unstimulated macrophages and astrocytes, PACAP molecules initiate the IL-6 secretion, which induces a proinflammatory response (3–5). Chemotactic migration events also show this degree of complexity. PACAPs have a stimulatory effect on macrophage chemotaxis (22), but an inhibitory effect on lymphocyte chemotaxis (22, 23), suggesting that PACAP can both promote and inhibit immune response. Although PACAP functioning has been examined by analyzing the expression patterns of various specific receptors (e.g., PAC1, VPAC1, and VPAC2), no evidence sufficiently explains this complexity. However, the present study shows for the first time, that FPRL1 is a PACAP27-specific receptor which mediates the up-regulation of CD11b and chemotactic migration, like other FPRL1 agonists, e.g., WKYMVm, LL-37, and LXA4 (21). Furthermore, FPRL1 mediates the PACAP27-induced calcium signaling in human monocytes (Fig. 2*H*) and U937 monocytic cell lines (data not shown). Taken together, we could suggest that FPRL1 mediates the inflammatory activity of PACAP27, a finding that should help elucidate the complicated interactions of PACAP and immune cells.

Previously, Cardell and colleagues (24) demonstrated that PACAP38 or VIP inhibit fMLP-induced neutrophil chemotaxis. Because the present study shows that PACAP27-induced neutrophil chemotaxis and FPRL1 are required for this process (Fig. 5), it is interesting to recall that the two different PACAPs have different effects on neutrophil chemotaxis. Although, we did not elucidate the inhibitory effects of PACAP38 and VIP on chemotaxis, we speculate that VPAC1 might mediate an inhibitory effect, because it is expressed in neutrophils (15). It would be interesting to know the physiological relevance for the opposing role of PACAP38 and PACAP27 on the regulation of neutrophil chemotaxis.

Although no report has mentioned the pathophysiological relevance of the relation between PACAP molecules and neutrophils, some evidence is available in the literature. In particular, in the nasal cavity, PACAP molecules are known to affect glandular secretion (25). Interestingly, neutrophils are found in nasal cavity (26), and have been reported to play a major role in inflammatory disease in the nasal cavity (26). Therefore, it is possible that the local concentration of PACAP is markedly elevated in the nasal cavity under some conditions. However, no report is available on PACAP level changes with respect to the pathologic condition of the nasal cavity, and studies on disease-related PACAP27 changes are required to reveal the physiological role of PACAP27 with respect to the control of neutrophil behavior.

Recently, structurally important motifs were identified to participate in the interaction between PAC1 and PACAP (18). Specifically, the N-terminal region of PACAP is critical for receptor activation, and the C-terminal region for binding affinity (18). Therefore, PAC1 shows a similar affinities and sensitivities to PACAP27 and PACAP38. The present study demonstrates the interaction between FPRL1 and PACAP27 through the use of tPACAP or cPACAP analogs (Fig. 6). The C-terminal region of PACAP27 is crucial for both binding and activation, and the 11

additional residues in PACAP38 might hinder its binding to FPRL1 and thus facilitate PACAP27 selectivity. Although, this is the first report of PACAP27-selective behavior in immune cells, similar activity has been reported in rat smooth muscle cells (27, 28), and though FPRL1 has not been described in smooth muscle cells, one group reported that fMLP, an agonist of FPR and FPRL1, induces transient coronary arterial muscle contraction (29). Therefore, it is important that the direct relation between FPRL1 and the PACAP27-selective response in smooth muscle cell should be understood.

GPCRs are classified into subfamilies according to their amino acid and nucleotide sequences. In general, GPCR subfamilies have similar ligands and binding motifs (30). For example, although sphingosine-1-phosphate is able to activate several receptors, these belong to the same rhodopsin-like GPCR subfamily (31, 32). Opioid receptors, also members of the rhodopsin-like GPCR family, are activated by multiple opioid peptides and share binding motif sequences (33). Interestingly, FPRL1 and the original PACAP receptors PAC1, VPAC1, and VPAC2 belong to different subfamilies, i.e., PAC1, VPAC1, and VPAC2 are members of the secretin-like GPCR subfamily, whereas FPRL1 is a rhodopsin-like GPCR (34). Furthermore, FPRL1 and PAC1 use different motifs to bind PACAP27. Taken together, we suggest that PACAP27-FPRL1 coupling presents a novel model of GPCR-ligand interaction.

The present study demonstrates that FPRL1 is a PACAP27-specific receptor, and suggests that PACAP27 activates phagocytes via FPRL1 activation. Although PACAP27 signaling requires FPRL1, PACAP27 also clearly retains the ability to stimulate VPAC1, which exists in neutrophils. Therefore, further studies of interactions between these two receptors and PACAP27 are required if we are to fully understand the function of PACAP in immune cells.

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Disclosures

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References

1. Hoyle, C. H. 1998. Neuropeptide families: evolutionary perspectives. *Regul. Pept.* 73: 1–33.
2. Sherwood, N. M., S. L. Krueckl, and J. E. McRory. 2000. The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagons superfamily. *Endocr. Rev.* 21: 619–670.
3. Martinez, C. M., M. Delgado, D. Pozo, J. Leceta, J. R. Calvo, D. Ganea, and R. P. Gomariz. 1998. Vasoactive intestinal peptide and pituitary adenlyate cyclase-activating polypeptide modulate endotoxin-induced IL-6 production by murine peritoneal macrophages. *J. Leukocyte Biol.* 63: 591–601.
4. Martinez, C., M. Delgado, D. Pozo, J. Leceta, J. R. Calvo, D. Ganea, and R. P. Gomariz. 1998. VIP and PACAP enhance IL-6 release and mRNA levels in resting peritoneal macrophages: in vitro and in vivo studies. *J. Neuroimmunol.* 85: 155–167.
5. Gottschall, P. E., I. Tatsuno, and A. Arimura. 1994. Regulation of interleukin-6 (IL-6) secretion in primary cultured rat astrocytes: synergism of interleukin (IL-1) and pituitary adenylate cyclase activating polypeptide (PACAP). *Brain Res.* 637: 197–203.
6. Delgado, M., J. Leceta, and D. Ganea. 2003. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit the production of inflammatory mediators by activated microglia. *J. Leukocyte Biol.* 73: 155–164.
7. Delgado, M., C. Martinez, D. Pozo, J. R. Calvo, J. Laceta, D. Ganea, and R. P. Gomariz. 1999. Vasoactive intestinal peptide (VIP) and pituitary adenlyate cyclase-activation polypeptide (PACAP) protect mice from lethal endotoxemia through the inhibition of TNF-α and IL-6. *J. Immunol.* 162: 1200–1205.
8. Delgado, M., and D. Ganea. 1999. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit interleukin-12 transcription by regulating nuclear factor κB and Ets activation. *J. Biol. Chem.* 274: 31930–31940.
9. Sheward, W. J., E. M. Lutz, A. J. Copp, and A. J. Harmar. 1998. Expression of PACAP, and PACAP type 1 (PAC1) receptor mRNA during development of the mouse embryo. *Brain Res. Dev. Brain Res.* 109: 245–253.
10. Couvineau, A., C. Rouyer-Fessard, D. Darmoul, J. J. Maoret, I. Carrero, E. Ogier-Denis, and M. Laburthe. 1994. Human intestinal VIP receptor: cloning and functional expression of two cDNA encoding proteins with different N-terminal domains. *Biochem. Biophys. Res. Commun.* 200: 769–776.
11. Laburthe, M., A. Couvineau, P. Gaudin, J. J. Maoret, C. Rouyer-Fessard, and P. Nicole. 1996. Receptors for VIP, PACAP, secretin, GRF, glucagon, GLP-1, and other members of their new family of G protein-linked receptors: structure-function relationship with special reference to the human VIP-1 receptor. *Ann. NY Acad. Sci.* 805: 94–109.
12. Schafer, H., J. Zheng, C. Morys-Wortmann, U. R. Folsch, and W. E. Schmidt. 1999. Structural motifs of pituitary adenylate cyclase-activating polypeptide (PACAP) defining PAC1-receptor selectivity. *Regul. Pept.* 79: 83–92.
13. Bae, Y. S., H. Bae, Y. Kim, T. G. Lee, P. G. Suh, and S. H. Ryu. 2001. Identification of novel chemoattractant peptides for human leukocytes. *Blood* 97: 2854–2862.
14. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260: 3440–3450.
15. Harfi, I., S. D'Hondt, F. Corazza, and E. Sariban. 2004. Regulation of human polymorphonuclear leukocytes functions by the neuropeptide pituitary adenylate cyclase-activating polypeptide after activation of MAPKs. *J. Immunol.* 173: 4154–4163.
16. Bae, Y. S., H. Y. Lee, E. J. Jo, J. I. Kim, H. K. Kang, R. D. Ye, J. Y. Kwak, and S. H. Ryu. 2004. Identification of peptides that antagonize formyl peptide receptor-like 1-mediated signaling. *J. Immunol.* 173: 607–614.
17. Le, Y., W. Gong, B. Li, N. M. Dunlop, W. Shen, S.B. Su, R. D. Ye, and J. M. Wang. 1999. Utilization of two seven-transmembrane, G protein-coupled receptors, formyl peptide receptor-like 1 and formyl peptide receptor, by the synthetic hexapeptide WKYMVm for human phagocyte activation. *J. Immunol.* 163: 6777–6784.
18. Inooka, H., T. Ohtaki, O. Kitahara, T. Ikegami, S. Endo, C. Kitada, K. Ogi, H. Onda, M. Fujino, and M. Shirakawa. 2001. Conformation of peptide ligand bound to its G-protein coupled receptor. *Nat. Struct. Biol.* 8: 161–165.
19. Zawilska, J. B., P. Niewiadomski, and J. Z. Nowak. 2003. PAC1 receptors in chick cerebral cortex: characterization by binding of pituitary adenylate cyclase-activating polypeptide, [^{125}I]-PACAP27. *Neurosci. Lett.* 338: 155–158.
20. Werry, T. D., G. F. Wilkinson, and G. B. Willars. 2003. Mechanisms of cross-talk between G-protein-coupled receptors resulting in enhanced release of intracellular Ca^{2+}. *Biochem. J.* 374: 281–296.
21. Le, Y., P. M. Murphy, and J. M. Wang. 2002. Formyl-peptide receptors revisited. *Trends Immonol.* 23: 541–548.
22. Garrido, E., M. Delgado, C. Martinez, R. P. Gomariz, and M. De la Fuente. 1996. Pituitary adenylate cyclase-activating polypeptide (PACAP38) modulates lymphocyte and macrophage functions: stimulation of adherence and opposite effect on mobility. *Neuropeptides* 30: 583–595.
23. Delgado, M., M. De la Fuente, C. Martinez, and R. P. Gomariz. 1995. Pituitary adenylate cyclase-activating polypeptide (PACAP27 and PACAP38) inhibit the mobility of murine thymocytes and splenic lymphocytes: comparison with VIP and implication of cAMP. *J. Neuroimmunol.* 62: 137–146.
24. Kinhult, J., R. Uddman, M. Laan, A. Linden, and L. O. Cardell. 2001. Pituitary adenlyate cyclase-activating peptide inhibits neutrophil chemotaxis. *Peptides* 22: 2151–2154.
25. Hegg, C. C., E. Au, A. J. Roskams, and M. T. Lucero. 2003. PACAP is present in the olfactory system and evokes calcium transients in olfactory receptor neurons. *J. Neurophysiol.* 90: 2711–2719.
26. Nagakura, T., T. Onda, Y. Iikura, T. Masaki, H. Nagakura, and T. Endo. 1989. High molecular weight-neutrophil chemotactic activity in nasal allergy. *Allergy Proc.* 10: 233–235.
27. Cox, H. M. 1992. Pituitary adenylate cyclase activating polypeptides. PACAP27 and PACAP38: stimulators of electrogenic ion secretion in the rat small intestine. *Br. J. Pharmacol.* 106: 498–502.
28. Ekblad, E., and F. Sundler. 1997. Distinct receptors mediate pituitary adenylate cyclase-activating peptide- and vasoactive intestinal peptide-induced relaxation of rat ileal longitudinal muscle. *Eur. J. Pharmacol.* 334: 61–66.
29. Keitoku, M., M. Kohzuki, H. Katoh, M. Funakoshi, S. Suzuki, M. Takeuchi, A. Karibe, S. Horiguchi, J. Watanabe, S. Satoh, et al. 1997. FMLP actions and its binding sites in isolated human coronary arteries. *J. Mol. Cell Cardiol.* 29: 881–894.
30. Kristiansen, K. 2004. Molecular mechanism of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function. *Pharmacol. Ther.* 103: 21–81.
31. Rosen, H., and J. Liao. 2003. Sphingosine 1-phosphate pathway therapeutics: a lipid ligand-receptor paradigm. *Curr. Opin. Chem. Biol.* 7: 461–468.
32. Kostenis, E. 2004. A glance at G-protein-coupled receptors for lipid mediators: a growing receptor family with remarkably diverse ligands. *Pharmacol. Ther.* 102: 243–257.
33. Waldhoer, M., S. E. Bartlett, and J. L. Whistler. 2004. Opioid receptors. *Ann. Rev. Biochem.* 73: 953–990.
34. Murphy, P. M., T. Ozcelik, R. T. Kenney, H. L. Tiffany, D. McDermott, and U. Francke. 1992. A structural homologue of the *N*-formyl peptide receptor: characterization and chromosome mapping of a peptide chemoattractant receptor family. *J. Biol. Chem.* 267: 7637–7643.