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Regulation of Human Polymorphonuclear Leukocytes Functions by the Neuropeptide Pituitary Adenylate Cyclase-Activating Polypeptide after Activation of MAPKs

Issam Harfi,* Stéphane D’Hondt,† Francis Corazza,‡ and Eric Sariban*2

Anti-inflammatory activities of pituitary adenylate cyclase-activating protein (PACAP) are mediated in part through specific effects on lymphocytes and macrophages. This study shows that in human polymorphonuclear neutrophils (PMNs), PACAP acts as a proinflammatory molecule. In PMNs, vaso-intestinal peptide/PACAP receptor 1 (VPAC-1) was the only receptor found to be expressed by RT-PCR. Using VPAC-1 Ab, we found that VPAC-1 mRNA was translated into proteins. In PMNs, PACAP increases cAMP, inositol triphosphate metabolites, and calcium. It activates two of the three members of the MAPK superfamily, the ERK and the stress-activated MAPK p38. U73122, an inhibitor of phospholipase C (PLC), inhibits PACAP-induced ERK activation, whereas p38 MAPK phosphorylation was unaffected. Using specific pharmacological inhibitors of ERK (PD098059) and p38 MAPK (SB203580), we found that PACAP-mediated calcium increase was ERK and PLC dependent and p38 independent. PACAP primes fMLP-associated calcium increase; it also primes fMLP activation of the respiratory burst as well as elastase release, these last two processes being ERK and PLC dependent and p38 MAPK independent. PACAP also increases membrane expression of CD11b and release of lactoferrin and metallo proteinase-9 (MMP-9). These effects were PLC dependent (CD11b, lactoferrin, MMP-9), ERK dependent (CD 11b, lactoferrin, MMP-9), and p38 dependent (CD11b, lactoferrin). We conclude that PACAP is a direct PMN activator as well as an effective PMN priming agent that requires PLC, ERK, and p38 MAPK activities. The Journal of Immunology, 2004, 173: 4154–4163.

Pituitary adenylate cyclase-activating protein (PACAP) shares 65% homology with vaso-intestinal peptide (VIP) and belongs to the VIP-glucagon growth hormone-releasing factor secretin of structurally related peptides. The effects of PACAP are exerted through a family of three VIP/PACAP (VPAC) receptors that belong to the secretin G-protein-coupled receptor. VPAC-1 and VPAC-2 receptors exhibit similar affinities for the two neuropeptides, whereas PACAP receptor (PAC-1) exhibits a 300- to 1000-fold higher affinity for PACAP than for VIP (1).

All three VPAC receptors couple to stimulation of adenyl cyclase via the heterotrimeric Gs protein and activation of phospholipase C (PLC) via the heterotrimeric Gq protein. PACAP also increases intracytoplasmic calcium levels through mobilization of intracellular calcium stores and/or activation of calcium influx (2–5).

In rat, PACAP is widely distributed in the brain, with the highest concentration occurring in the hypothalamic area. PACAP released by hypothalamic nerve terminals is transported to the pituitary, where it acts on all endocrine cells, releasing a wide variety of hormones (1). The isolation of tunicate PACAP cDNA was the first report of any superfamily member to be isolated from an invertebrate. Studies of PACAP peptides isolated from different species indicate that amino acids and nucleotides that encode PACAP have been maintained for over 600 million years, a degree of identity unknown for any hormone of comparable size. This indicates that PACAP is involved in the regulation of important biological functions. Because tunicates lack a pituitary gland and do not have pituitary-like hormones, the ancestral role of PACAP must be distinct from that of releasing hormones (6, 7). In fact, like the other hypothalamic neurohormones, PACAP is also distributed in a number of peripheral organs including the endocrine pancreas, gonads, and respiratory and uro-genital tracts. It has been implicated in a broad range of biological processes including reproduction; development; growth; cardiovascular, respiratory, and digestive functions; and immune responses (1).

Within hemopoietic tissues, VPAC-1 receptor has been consistently found to be expressed in human peripheral blood lymphocytes, human resting monocytes, murine lymphocytes, and macrophages, whereas VPAC-2 receptor is inducible in these cells. PAC-1 receptor is constitutively expressed in rat peritoneal macrophages and in the human THP-1 monocytic cell line (8, 9). Both VIP and PACAP peptides have been found to be synthesized in murine thymocytes, T subsets, and B cells from the spleen and lymph nodes. PACAP mRNA has not been detected in hemopoietic cells (10). Biologically, PACAP has been found to have both anti-inflammatory (9–11) and proinflammatory (12–15) effects.

We have recently reported that human myeloid cells express a functional VPAC-1 receptor with an increase in cAMP and calcium level after PACAP exposure (5, 16). Because these second messengers are playing a key role in polymorphonuclear neutrophils (PMNs),...
we investigated in this study the eventual role of PACAP in PMN physiology.

Materials and Methods

Cells and reagents

Human PMNs were isolated from citrated or heparinized venous blood from healthy volunteers, using a one-step separation on Polymorphprep (Axis-Shield, Oslo, Norway). Highly purified human PACAP27 was obtained from Neosystem (Strasbourg, France). fMLP, TNF-α, PMA, the MEK inhibitor PD98059, the p38 MAPK inhibitor SB203580, and the PLC inhibitor U73122 were purchased from Sigma-Aldrich (St. Louis, MO). Polyclonal Ab against ERK2, mAb against phosphorylated ERK1/2 (Tyr204), and polyclonal Ab against p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal Ab against phosphorylated JNK (Thr183/Tyr185) and polyclonal Ab against phosphorylated JNK/stress-activated protein kinase (Thr183/Tyr185) were purchased from Upstate (Lake Placid, NY). RPMI 1640 was purchased from BioWhitaker (Verviers, Belgium). HBSS was obtained from Invitrogen (Paisley, U.K.). PR3 primers were obtained from Eurogentec (Seraing, Belgium) (17). RPMI 1640 was purchased from BioWhitaker (Beverly, MA). The rabbit anti-VPAC-1 polyclonal (Tyr204), and polyclonal Ab against p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal Ab against phosphorylated p38 MAPK (Thr180/Tyr182) and polyclonal Ab against phosphorylated JNK/stress-activated protein kinase (Thr183/Tyr185) were purchased from New England Biolabs (Beverly, MA). The rabbit anti-VAPC-1 polyclonal Ab was a kind gift of K. Fresco and C. Van Geet (Katholieke Universiteit Leuven, Leuven, Belgium) (17). RPMI 1640 was purchased from BioWhitaker (Verviers, Belgium). HBSS was obtained from Invitrogen (Paisley, U.K.). PR3 primers were obtained from Eurogentec (Seraing, Belgium).

Measurement of intracellular cAMP, cytosolic calcium, and inositol 1,4,5-trisphosphate (IP3)

For measurement of cAMP, PMNs were isolated and resuspended at 5 × 10⁶ cells/ml in the culture medium (HBSS) for 5–10 min in a shaking incubator. The buffer was replaced by 200 μl of HBSS containing 400 μM isobutylmethylxanthine (IBMX), a cAMP phosphodiesterase inhibitor, and the incubation was continued for 5 min. Then PACAP was added. The reactions were terminated by placing the samples on ice. Cyclic AMP levels were determined by using a cAMP Enzymeimmunoassay Biotrak system (dual range) kit (Amersham, Buckinghamshire, U.K.) according to the manufacturer’s instructions. Free intracellular calcium was determined with an LSS500 Fluorescence Photometer (PerkinElmer, Wellesley, MA) as reported (5). For IP₃, measurements, PMNs were suspended in HBSS containing 20 mM LiCl, 2 mM HEPES, and 0.1% BSA. Cells (2 × 10⁶/ml to 1 × 10⁷/ml) were preincubated at 37°C for 5 min and then incubated with various concentrations of PACAP for specific times. The total volume of the reaction mixture was 200 μl. The amount of IP₃ was determined with a cGMP-inositol 1,4,5-trisphosphate [³H] assay system (Amersham) as suggested by the manufacturer.

RNA isolation and RT-PCR

RNA isolation and PCR were performed as previously reported using specific primers set for PACAP, VIP, PAC-1, VPAC-1, and VPAC-2 (5).

Western blot analysis and determination of ERK, p38, and JNK phosphorylation

PMNs suspended in complete RPMI 1640 medium (10% FBS, 1000 μ/ml penicillin, 1000 μ/ml streptomycin, and 20 mM glucose) were preincubated for 5 min at 37°C. After stimulation, as indicated, the cells were placed on ice and harvested by centrifugation. Cells were lysed in ice-cold 1% Brij97 lysis buffer (200 mM boric acid, 150 mM NaCl (pH 8)) containing 1 mMol/L sodium orthovanadate, 1 mM EDTA, and 1 mM PMSF. After centrifugation (13,000 rpm for 15 min), the supernatant was resolved on 10% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Hybond C; Amersham). Membranes were probed with Abs against VPAC-1, ERK2, p38, phosphorylated ERK1/2 (Tyr204), phosphorylated p38 (Thr180/Thr182), and phosphorylated JNK (Thr183/Thr185). Proteins were detected by ECL (Amersham).

Chemiluminescence measurement of respiratory burst

The respiratory burst of neutrophils was measured by a chemiluminescence method previously described (18). Cells were preincubated with cytochalasin B (5 μg/ml) and with PACAP at 37°C for 5 min before addition of FMLP (0.5 μM). When indicated, cells were pretreated with inhibitors of PLC and MAPKs pathways.

Measurement of elastase and lactoferrin release

For elastase measurement, cells (6 × 10⁶/ml) were resuspended at 37°C in 400 μl of medium containing 5 μg/ml cytochalasin B for 5 min before addition of PACAP at the indicated concentration and time. Then, FMLP was added for another 10 min (priming experiment). When indicated, cells were pretreated with inhibitors of PLC and MAPKs. Released elastase activity in the supernatants was measured as follows: 25 μl of supernatant was mixed with 125 μl of a buffer consisting of 33.3 mM HEPES and 0.17 M NaCl (pH 7.4) containing the elastase-specific chromogenic substrate N-methoxyxysuccinyl-(t-Ala-Lys)-t-propyl-valine-nitroanilide (MeosucAAPV-pNa; Sigma-Aldrich) at the final concentration of 0.33 mM. After 30 min at room temperature, change in OD was measured at a wavelength of 405 nm.

For lactoferrin measurement, PMNs (3 × 10⁶/ml) were resuspended at 37°C in 400 μl of medium containing 5 μg/ml cytochalasin B for 5 min. After stimulation with PACAP at the indicated concentration and time, cells were sedimented by centrifugation at 400 × g for 10 min. Release of the specific granule marker lactoferrin in supernatants was determined by the use of a sandwich ELISA system (Biosyntech, Portland, OR).

Surface expression measurement of CD11b

Purified PMNs (250 μl; 1 × 10⁶ cells/ml) in HBSS containing Ca²⁺ and Mg²⁺ were incubated with agonists for indicated periods at 37°C. The cells were mixed with PE-conjugated mAb against CD11b (BD Biosciences, San Jose, CA) for 20 min at 4°C; control of isotype-matched Ab was assayed in parallel. Samples were assayed as described (18).

Gelatin zymography

Metallo proteinase-9 (MMP-9) was determined by gelatin zymography as described (19). Conditioned serum-free medium from PMA-activated PBMCs was used as a positive control for zymographic analysis. As negative control, medium alone was used.

Statistical analysis

Results are expressed as mean ± SEM of n independent experiments, each experiment using cells from separate donors and performed in duplicate. Data were analyzed as appropriate either by Wilcoxon matched pairs test or by ANOVA using Prism 3.0 statistical software (GraphPad, San Diego, CA). Differences were considered statistically significant when p < 0.05.

Results

PACAP increases cAMP, IP₃, and calcium accumulation in PMNs

Because VPAC-1 receptors have been linked to stimulation of adenyl cyclase, activation of PLC, and calcium increase, changes in the second messengers cAMP, IP₃, and calcium were evaluated in PACAP-treated PMNs (Fig. 1). In the presence of IBMX, PACAP (10 μM) produces a 12-fold increase in cAMP levels from resting levels of ~385 fmol/10⁶ cells to peak values of ~4700 fmol/10⁶ cells (Fig. 1A). This cAMP increase is observed within 5 min of PACAP (10 μM) exposure with a peak 10 min after treatment. Thereafter, cAMP level returns to basal values. Significant increase in cAMP is observed at a concentration of 10 μM (Fig. 1B).

PACAP also affects the phosphoinositide signal transduction pathway. As shown in Fig. 1C, the production of IP₃ upon stimulation with 10 μM PACAP is greatly enhanced. This effect is very rapid, with a maximum of twofold IP₃ increase observed 5 s after peptide exposure. This effect is dose dependent with significant IP₃ accumulation at 10 μM (Fig. 1D).

Stimulation of fluo-3-acetoxyethyl ester containing PMN with 1 μM PACAP causes a rapid rise in cytosolic calcium from a resting level of ~100 nM to transient peak values in excess of 300 nM. Peak values are observed between 10 and 20 s, with a return to basal levels by 90 s (Fig. 1E). This effect is dose dependent with significant increase at 1 μM and further increase at 10 μM (Fig. 1F).

From these data, we conclude that in human PMNs, PACAP modulates in a time- and dose-dependent manner the accumulation of the second messengers cAMP, IP₃, and calcium.
PMNs express the VPAC-1 receptor

To identify the nature of the receptor involved, we performed RT-PCR on RNA derived from human neutrophils using specific primers from the three different receptors. We also looked at the expression of VIP and/or PACAP in these cells. Gel electrophoresis of the PCR product showed that VPAC-1 is the only receptor found to be expressed in human resting neutrophils. In addition, there is no expression of PACAP or VIP peptides (Fig. 2A). Immunoblotting using VPAC-1 Ab confirmed that mRNA VPAC-1 detected by RT-PCR was translated into proteins (Fig. 2B). Neutrophils exposed to PACAP did not change the level of expression of the VPAC-1 receptor protein (Fig. 2B).

Activation of the ERK and p38 MAPK pathways in PACAP-treated PMNs

We investigated whether MAPK modules could be a downstream target of PACAP (Fig. 3). As shown in Fig. 3A, stimulation of PMNs with PACAP results in a rapid phosphorylation of ERK at 15 s, which becomes maximal at 1 min, with a sharp decrease at 5 min and an undetectable level at 20 min. The effect of PACAP on ERK activation is concentration dependent, with significant activation at 10 μM (Fig. 3B). To test the specificity of ERK activation, we tested the ability of the specific ERK inhibitor PD98059 to interfere with the effect of PACAP. As shown in Fig. 3C, PD98059 induces a concentration-dependent inhibition of ERK that is detectable at 0.5 μM, clearly visible at 5 μM, and maximal at 50 μM. To establish whether the activation of ERK is mediated through PLC, we preincubated PMNs with U73122, an irreversible inhibitor of PLC. As shown in Fig. 3D, U73122 inhibits PACAP-induced ERK phosphorylation with an effect already detected at 10 μM, a complete inhibition is observed at 100 μM U73122.

Activation of p38 MAPK by PACAP was also evaluated. As shown in Fig. 3E, in PMNs stimulated with 10 μM PACAP, there is a marked and transient activation of p38 MAPK with a maximal effect at 1 min; p38 phosphorylation in response to PACAP occurs in a concentration-dependent fashion, with detectable phosphorylation observed at 1 μM and maximal effect at 10 μM (Fig. 3F).
The use of the pharmacological inhibitor SB203580, which is specific to p38 MAPK activity, results in an inhibition of PACAP activation of p38 MAPK. This effect is dose-dependent with a maximal inhibition at 10 μM (Fig. 3G). To examine the involvement of PLC in the activation of p38 MAPK by PACAP, PMNs were preincubated with U73122. As shown in Fig. 3H, PACAP-associated p38 MAPK phosphorylation is unaffected by U73122.

As shown in Fig. 3I, there is no effect of PACAP on JNK phosphorylation, whereas TNF-α-treated PMNs present a strong JNK phosphorylation signal.

We conclude that PACAP, like other PMN proinflammatory stimuli, activates ERK and p38 and that ERK activation, in contrast with p38, is PLC dependent.

**PACAP-mediated calcium increase is PLC and ERK dependent and p38 MAPK independent**

Because we have shown that PACAP stimulates inositol phosphate turnover and increases the PMN intracellular calcium content, we investigated whether IP₃ could be a second messenger for calcium increase in these cells by using the PLC inhibitor U73122 and its inactive analog U73343.

As shown in Fig. 4, A and B, U73122 inhibits in a dose-dependent manner PACAP increase in calcium with complete inhibition at 10 μM. The inactive analog U73343 does not have any effect on calcium mobilization (Fig. 4, C and D). These data suggest that PACAP-induced calcium increase is mediated at least by a Gq-PLC-IP₃ pathway.

Because we have shown that PLC modulates ERK and not p38 activities, we investigated the effect of direct inhibition of ERK and p38 on calcium mobilization in PMNs exposed to PACAP. As shown in Fig. 4, E and F, PD98059 produces a dose-dependent inhibition of calcium increase in PACAP-treated PMNs, whereas SB203580 (Fig. 4, G and H) was without effect.

We conclude that in PMNs, IP₃ and ERK phosphorylation are relevant intracellular messengers in PACAP-induced calcium signals.
PACAP primes fMLP-associated calcium increase

Because a number of molecules are known to prime PMNs for the effects of fMLP (20), we asked whether PACAP at $10^{-8}$ M, $10^{-7}$ M, and $10^{-6}$ M could prime the increase in cytoplasmic calcium in PMNs exposed to $10^{-10}$ M, $10^{-9}$ M, and $10^{-8}$ M fMLP.

The low PACAP concentration of $10^{-8}$ M did not have any effect on calcium mobilization by fMLP (data not shown). In contrast, PACAP $10^{-7}$ M and $10^{-6}$ M significantly primes the fMLP-associated calcium increase. This was observed at all three concentrations of fMLP (Table I).

PACAP primes fMLP increase in both respiratory burst and elastase release: this is PLC and ERK dependent and p38 MAPK independent

To evaluate whether the signaling network activated by PACAP in PMNs was relevant to PMN physiology, several PMN activities were studied. We investigated the ability of PACAP to prime fMLP-associated respiratory burst. As shown in Fig. 5A, pretreatment with 1 $\mu$M or 10 $\mu$M PACAP significantly increases the respiratory burst in fMLP-treated (0.5 $\mu$M) PMNs. The priming effect is inhibited by U73122 and PD98059 but not by SB203580 (Fig. 5, B–D). PACAP alone was not able to activate the oxidase (data not shown).

Elastase release by PMNs is a marker of degranulation of azurophil/primary granules that occurred during PMN activation (21). fMLP-stimulated elastase release was not affected by preincubation with either U73122 or by the MEK inhibitors PD98059 or SB203580. Thus, these inhibitors could be used to assess the role of PLC and MAPK pathways in the priming effect of PACAP. As shown in Fig. 6E, PACAP primes fMLP-induced elastase release with significant activity observed at 0.1 $\mu$M, a lower concentration than the one observed for oxidative burst priming. As shown in Fig. 5, F–H, this effect is PLC and ERK dependent and p38 MAPK independent.

PACAP increases the release of lactoferrin: this is PLC, ERK, and p38 dependent

Lactoferrin has been considered as a specific marker of secondary granules (21). As shown in Fig. 6, PACAP induces a rapid release of lactoferrin already detected after 1 min of peptide exposure (Fig. 6A). This effect is dose dependent, with an increase of 3.2-fold in lactoferrin levels after 10 $\mu$M PACAP, from resting level of ~400 ng/ml to a peak value of ~1300 ng/ml (Fig. 6B). The release of lactoferrin by PACAP is sensitive to the inhibition of PLC, ERK, and p38 MAPK (Fig. 6C). We conclude that secondary granule exocytosis is dependent on both PLC and MAPK pathways.

PACAP increases PMN membrane expression of the integrin CD11b: this is PLC, ERK, and p38 MAPK dependent

Adhesion, a pivotal functional action in leukocytes, is mediated largely by the leukocyte-specific integrin CD11b, which in PMNs is contained in secretory vesicles (21). We also found that PACAP (10 $\mu$M) increases the membrane expression of CD11b, with maximal effect observed at 60 min of peptide exposure (Fig. 6D). Expression of CD11b was also evaluated without neutrophil purification. Whole blood samples were incubated with PACAP, erythrocytes were lysed, and the samples were analyzed by flow cytometry with gating on optical scatter parameters to select neutrophils as shown by the dot plot in Fig. 6D, inset. Incubation of whole blood with PACAP also enhanced the cell surface expression of CD11b, confirming the result obtained with purified neutrophils.

Table I. PACAP primes fMLP-induced rise in $[Ca^{2+}]_{i}$

<table>
<thead>
<tr>
<th></th>
<th>fMLP (0 nM)</th>
<th>fMLP (0.1 nM)</th>
<th>fMLP (1 nM)</th>
<th>fMLP (10 nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PACAP (0 $\mu$M)</td>
<td>28.03 ± 5.42</td>
<td>29.91 ± 7.80</td>
<td>199.99 ± 17.93</td>
<td>411.52 ± 61.72</td>
</tr>
<tr>
<td>PACAP (0.1 $\mu$M)</td>
<td>14.98 ± 4</td>
<td>150.99 ± 37.08</td>
<td>61.72 ± 61.72</td>
<td>457.9 ± 101.35</td>
</tr>
<tr>
<td>PACAP (1 $\mu$M)</td>
<td>140.26 ± 12.17</td>
<td>150.99 ± 37.08</td>
<td>261.63 ± 16.40</td>
<td>457.9 ± 101.35</td>
</tr>
</tbody>
</table>

* $p < 0.001$; $a$ $p < 0.01$; $b$ $p < 0.05$. Significantly different from fMLP-stimulated rise in $[Ca^{2+}]_{i}$.
PACAP increases MMP-9 secretion: this is PLC and ERK dependent and p38 MAPK independent

In PMNs, MMP-9 protein is contained in the gelatinase/tertiary granules and is secreted during cell activation (21).

To evaluate whether PACAP mobilizes tertiary granules, increase in MMP-9 secretion was evaluated by zymography. As shown in Fig. 7A, after 1 min of peptide exposure, there is already a massive secretion of MMP-9. This effect is dose dependent, with consistent increase in MMP-9 secretion observed at 0.1 μM PACAP (Fig. 7B). MMP-9 secretion is PLC dependent (Fig. 7D), ERK dependent, and p38 MAPK independent (Fig. 7C).

Discussion

VIP and PACAP binding sites and expression of VPAC-1 and/or PAC-1 receptor by RT-PCR have been reported in monocytic cells of both human and murine origin (5, 8, 16, 22). VPAC-1 receptor mRNA and protein has also been found in human bone marrow cells and in human megakaryoblasts (23). In addition, we have recently reported that among five human myeloid leukemic cell lines analyzed, four were expressing VPAC-1 by RT-PCR, one was also expressing VPAC-2, and none of these cell lines were expressing PAC-1 receptor (5).

In monocytes or macrophages, VIP/PACAP suppress the production of several proinflammatory factors, including TNF-α, IL-6, IL-12, NO, and IFN-γ, and stimulate the production of anti-inflammatory cytokines such as IL-10 (11, 24). In vitro and in vivo studies using VIP agonists and antagonist have indicated that VPAC-1 is the main mediator of the biological activities of the neuropeptide (11, 25).

Aside from these anti-inflammatory effects, there are reports on monocyes and neutrophils describing proinflammatory activity of PACAP. Thus, in monocytes/macrophages, PACAP increases cell motility, is chemoattractant, and increases in a dose-dependent manner phagocytosis and production of superoxide anion. These effects were mediated by the PAC-1 receptor (12, 13). PACAP also increases IL-6 production in resting monocytes as well as in monocytes exposed to low doses of LPS (14). This stimulatory effect was exerted primarily through the VAPC-1 receptor. In human neutrophils, PACAP, in contrast with VIP, enhances the expression of the β2 integrin CD11b together with expression of CD66b and CD63, markers of secondary and azurophil granules, respectively, that are mobilized during cellular activation (15). In this work, we confirm and extend data concerning the proinflammatory effects of PACAP on human neutrophils.

Cyclic AMP, metabolites of phosphoinositides, and calcium are three second messengers playing key roles in neutrophil physiology. All three signal transduction pathways were found to be activated in neutrophils exposed to PACAP. By RT-PCR, only VPAC-1 receptor expression was detected in PMNs. Using VPAC-1 Ab, we showed that VPAC-1 mRNA was translated into proteins. We have also found (our unpublished observations) that VIP at a concentration similar to that of PACAP increases cAMP in neutrophils. Because VPAC-1, in contrast with PAC-1, displays similar affinity for VIP and PACAP and is coupled to both Gαs and Gq, this suggests that VPAC-1 receptor simulation mediates the activation of the different metabolic pathways reported in our study. However, we cannot exclude that another member of the VIP/PACAP receptor family yet to be defined might also be involved.
Downstream signaling in stimulated neutrophils includes activation of the MAPKs (26). In PMNs, ERK and p38 are activated by cytokines (26–29), chemoattractant (26, 29), lipoxygenase metabolites (30), bacterial LPS (31), P2Y (32), or CD11b (33) receptor occupancy. Phosphorylation of JNK has also been reported (34) but is not a constant feature in activated PMNs (35). In cells of neuronal origin, PACAP activates both ERK and p38 signaling pathways (36–39). In PMNs, we also found that PACAP was activating these pathways without affecting JNK. It is of interest that inhibition of PLC inhibits both ERK phosphorylation and calcium mobilization without affecting p38. Similar results have been found in primary endothelial cells exposed to VEGF, where inhibition of PLC resulted in a complete inhibition of ERK without having any effect on p38 activity (40). This indicates that, in PMNs, ERK and p38 are differentially sensitive to PLC activation after PACAP exposure and that changes in cytosolic calcium are not mandatory for p38 activation. In human neutrophils, PLC inhibitor inhibits the arachidonic acid-stimulated respiratory burst.

**FIGURE 6.** Lactoferrin release and CD-11b up-regulation in PACAP-treated PMNs. Time course of PACAP-activated lactoferrin release (A), dose-dependent effect (B), and effects of inhibitors of the PLC and MAPK pathway (C). Neutrophils were treated for the indicated times with 10 μM PACAP (A), incubated for 10 min at 37°C with PACAP at various concentrations (B), or preincubated for 10 min with 10 μM U73122 or for 30 min with 50 μM PD98059 or 10 μM SB203580, followed by stimulation with PACAP (10 μM) for 10 min at 37°C (C). Cells receiving stimulus alone and left without inhibitor served as controls. Lactoferrin release was determined in neutrophil supernatants using a sandwich ELISA system (**p < 0.01; *p < 0.05; as compared with buffer-treated neutrophils). Significant reductions of the lactoferrin release by PD98059, SB203580, and U73122 are indicated by # (p < 0.001). PACAP induces CD-11b up-regulation in PMNs. D, Kinetics: PMNs (1 × 10^6 cells/ml) were incubated with PACAP (10 μM) or with medium (negative control) at 37°C. Cells were removed at various time periods, and then were stained and fixed. CD11b expression was measured by means of PE-labeled anti-CD11b and was analyzed by flow cytometry. The results are expressed as a percentage ± SEM of HBSS-treated controls from four different experiments; mean fluorescence intensity for control samples is 243.9 ± 23.4. E, Concentration-dependent effect: PMNs (1 × 10^6 cells/ml) incubated for 60 min at 37°C with PACAP at various concentrations were evaluated by flow cytometry as described in D for CD11b expression. The results are expressed as a percentage ± SEM of HBSS-treated controls from four different experiments. **p < 0.001; *p < 0.01 vs vehicle treatment. F–H, CD11b up-regulation is PLC (F), ERK (G), and p38 (H) dependent. PMNs were preincubated with the MEK inhibitor PD98059, the p38 kinase inhibitor SB203580 for 30 min, or the PLC inhibitor U73122 for 10 min, and then they were challenged with PACAP (10 μM) for 60 min. Expression of CD11b was evaluated by flow cytometry as described above. Results are expressed as percentage ± SEM of HBSS-treated controls from four different experiments. *p < 0.01 vs PACAP alone. I, Representative histogram of CD11b expression after PACAP treatment is shown. Neutrophils were gated out as shown by the dot plot in the inset.
SDS was removed after electrophoresis, and the medium was subjected to electrophoresis on 10% SDS-polyacrylamide gels containing 1 mg/ml gelatin. Three others.

Response to PMA exposure. Data from one experiment, representative of positive control indicating the amount of MMP-9 secreted by PBMCs in preincubated with PD98059 (50, 5, and 0.5 \( \mu M \)) for 5 min (\( B \)). At the end of the treatment, a total of 15 \( \mu l \) of conditioned medium was subjected to electrophoresis on 10% SDS-polyacrylamide gels containing 1 mg/ml gelatin. SDS was removed after electrophoresis, and gels were incubated to restore gelatinase activity. Lane 1 (CTR +) is a positive control indicating the amount of MMP-9 secreted by PBMCs in response to PMA exposure. Data from one experiment, representative of three others.

C and D. Effects of MAPK inhibitors on PACAP-induced increase of MMP-9 activity in PMNs. Cells resuspended in HBSS were preincubated with PD98059 (50, 5, and 0.5 \( \mu M \)) or with SB203580 (10, 1, and 0.1 \( \mu M \)) for 30 min at 37°C before stimulation with 10 \( \mu M \) PACAP. The conditioned medium, obtained after 5 min, was subjected to gelatin zymography. E. Effects of the PLC inhibitor on PACAP-induced increase of MMP-9 activity in PMNs. PMNs were preincubated with U73122 (10, 1, and 0.1 \( \mu M \)) for 10 min at 37°C and then challenged with PACAP (10 \( \mu M \)); MMP-9 expression was conducted as described above.

FIGURE 7. PACAP increased PMN secretion of MMP-9. Time course of MMP-9 release by neutrophils in response to PACAP stimulation (A) and dose-response effect of PACAP on MMP-9 secretion (B). PMNs (5 \times 10^5/ml in HBSS) were treated with 10 \( \mu M \) PACAP (A) at 37°C for the indicated periods (0–10 min) or with increasing concentration of PACAP for 5 min (\( B \)). At the end of the treatment, a total of 15 \( \mu l \) of conditioned medium was subjected to electrophoresis on 10% SDS-polyacrylamide gels containing 1 mg/ml gelatin. SDS was removed after electrophoresis, and gels were incubated to restore gelatinase activity. Lane 1 (CTR +) is a positive control indicating the amount of MMP-9 secreted by PBMCs in response to PMA exposure. Data from one experiment, representative of three others. C and D. Effects of MAPK inhibitors on PACAP-induced increase of MMP-9 activity in PMNs. Cells resuspended in HBSS were preincubated with PD98059 (50, 5, and 0.5 \( \mu M \)) or with SB203580 (10, 1, and 0.1 \( \mu M \)) for 30 min at 37°C before stimulation with 10 \( \mu M \) PACAP. The conditioned medium, obtained after 5 min, was subjected to gelatin zymography. E. Effects of the PLC inhibitor on PACAP-induced increase of MMP-9 activity in PMNs. PMNs were preincubated with U73122 (10, 1, and 0.1 \( \mu M \)) for 10 min at 37°C and then challenged with PACAP (10 \( \mu M \)); MMP-9 expression was conducted as described above.

and calcium release, but not the activity of the PI3 kinase, indicating the independence of PI3K signaling and PLC activation (41). Because we show in this study that p38 activity is PLC independent, it is possible that in PMN, p38 phosphorylation is more tightly associated with PI3K than with PLC activation. In this regard, a PI3K/p38 MAPK pathway activation of the NADPH oxidase has been reported in bovine neutrophils (42).

Calcium mobilization (43) and MAPK are associated with the generation of reactive oxygen species (44, 45) and with granule storage exocytosis (46). PACAP alone was not effective in increasing respiratory burst; however, it greatly primes such activity in fMLP-exposed PMNs. This priming was PLC and ERK dependent and p38 independent, indicating that calcium mobilization plays a major role in PACAP-mediated increase in reactive oxygen species. In this regard, significant PACAP priming of fMLP-associated calcium increase was observed at different concentrations of PACAP as well as different concentrations of fMLP. Major differences exist between the different granule subsets regarding the extent to which they are mobilized (21, 33). We found that PACAP increases the release of lactoferrin and MMP-9, indicating that it can mobilize specific secondary granules (lactoferrin) and gelatinase granules. In addition, it primes fMLP release of elastase contained in the azurophilic granules, the less readily exocytosed granules. Thus, all granule subsets present in PMNs are sensitive to the effect of PACAP. Integrin participates in multiple cellular signaling events and transmits signals to the cells from the surrounding environment. These outside-in signals play an important role in the development of an inflammatory response in cells of both lymphoid and myeloid origins. CD11b membrane expression, which is regulated by intracellular calcium mobilization, and generation of reactive oxygen species were also found to be positively modulated by PACAP. Thus, in PACAP-treated PMNs, mobilization of secretory vesicles transforms a passive cell to a highly responsive \( \beta_1 \) integrin cell primed for phagocytosis and migration into tissues.

Signal transduction pathways leading to vesicle and granule exocytosis have not been well documented; ERK, p38, Src kinases, PI3K, PLC, and focal adhesion kinase have been involved in recent studies (32, 33, 46–50). In our work, PACAP-associated exocytosis of secondary, tertiary granules as well as secretory vesicles was found to be PLC, ERK, and/or p38 dependent; MMP-9 secretion was the only process to be p38 independent. Our study also indicates that, according to the triggering agent, PMNs use different pathways to stimulate degranulation. Thus, similar to our result indicating that CD11b up-regulation is p38 dependent, it has been reported that clustering L-selectins use p38 MAPK to increase CD11b membrane expression (33). In contrast, fMLP or soluble fibrinogen-induced exocytosis of secretory vesicles was found to be p38 independent (47). In addition, whereas we found that PACAP-associated secondary granule exocytosis is ERK dependent, fMLP-associated granular exocytic responses have been reported to be ERK independent (44, 46). Similarly, priming and activation of phospholipase A\( _2 \) and NADPH oxidase are differentially dependent on both p38 and ERK pathways according to the nature of the triggering agent (51).

Mammalian ovulatory process involves the remodeling by the matrix metallo-proteinases of extracellular matrix triggered by the gonadotropines. PACAP has been reported to act synergistically with relaxin to increase the secretions of MMP-2 in rat theca-interstitial cells (52). Thus, PACAP serves as an ovarian physiologic mediator of gonadotropin functions. MMP-9 has been involved in the regulation of hematopoiesis: MMP-9 causes shedding of soluble kit ligand and enables hemopoietic progenitor cells to translocate from a quiescent to a proliferative niche (53). It also induces proteolytic cleavage of IL-8/CXCL8, potentiating the proinflammatory activities of this chemokine (54). Because we found that PACAP in PMNs increases the release of MMP-9 and regulates MAPK, a pathway involved in cell migration and cell activation, it is possible that PACAP delivered by peptidergic nerve fibers within the bone marrow is a physiological regulator of hemopoietic precursor cells as well as an activator of mature myeloid cells. The recent report that Ikaros, a selective hemopoietic transcription factor, regulates early neutrophil differentiation (55) and that VPAC-1 is a target for Ikaros (56) provides additional reasons to think that PACAP might play a role in early myeloid cell physiology.
It is of interest that significant changes in PMN functions were already observed at 10^{-7} M PACAP, a concentration similar to the one reported to modulate neuroendocrine functions (57, 58).

Altogether, our results indicate that PACAP acts as a proinflammatory agent in human PMNs. This is in contrast to the in vitro anti-inflammatory effect and in vivo protective role of PACAP in endotoxic shock (59). Similar results have been observed with catecholamines, mediators of the sympathetic nerve system; they inhibit the production of proinflammatory cytokines by TH1 cells, stimulate the production of type 2 anti-inflammatory cytokines, and boost regional immune responses through induction of IL-1, TNF-α, and IL-8 production, to restrict locally the inflammatory process (60). Thus, as described for catecholamines, the apparent discrepancy between pro- and anti-inflammatory activities of PACAP might be the result of a systemic anti-inflammatory effect to protect the organism from the detrimental consequences of proinflammatory cytokines and a local peptidase-associated proinflammatory effect induced by the neuropeptide necessary for controlling the infections. In this regard, the effect of PACAP on neutrophil proinflammatory cytokine production, apoptosis, and NF-κB activity is under investigation in our laboratory.

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