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Differential Calcium Regulation of Proinflammatory Activities in Human Neutrophils Exposed to the Neuropeptide Pituitary Adenylate Cyclase-Activating Protein

Issam Harfi,* Francis Corazza,† Stéphanie D’Hondt,* and Eric Sariban2*

The neuropeptide pituitary adenylate cyclase-activating protein (PACAP) acts via the G protein-coupled receptor vasoactive intestinal peptide/PACAP receptor-1 to induce phospholipase C/calcium and MAPK-dependent proinflammatory activities in human polymorphonuclear neutrophils (PMNs). In this study, we evaluate other mechanisms that regulate PACAP-evoked calcium transients, the nature of the calcium sources, and the role of calcium in proinflammatory activities. Reduction in the activity of PMNs to respond to PACAP was observed after cell exposure to inhibitors of the cAMP/protein kinase A, protein kinase C, and PI3K pathways, to pertussis toxin, genistein, and after chelation of intracellular calcium or after extracellular calcium depletion. Mobilization of intracellular calcium stores was based on the fact that PACAP-associated calcium transient was decreased after exposure to 1) thapsigargin, 2) Xestospongin C, and 3) the protonophore carbonyl cyanide 4-(trifluoromethoxy) phenyl hydrazone; inhibition of calcium increase by calcium channel blockers, by nifedipine and verapamil, indicated that PACAP was also acting on calcium influx. Such mobilization was not dependent on a functional actin cytoskeleton. Homologous desensitization with nanomoles of PACAP concentration and heterologous receptors desensibilization by G protein-coupled receptor agonists were observed. Intracellular calcium depletion modulated PACAP-associated ERK but not p38 phosphorylation; in contrast, extracellular calcium depletion modulated PACAP-associated p38 but not ERK phosphorylation. In PACAP-treated PMNs, reactive oxygen species production and CD11b membrane up-regulation in contrast to lactoferrin release were dependent on both intracellular and extracellular calcium, whereas matrix metalloproteinase-9 release was unaffected by extracellular calcium depletion. These data indicate that both extracellular and intracellular calcium play key roles in PACAP proinflammatory activities. The Journal of Immunology, 2005, 175: 4091–4102.

Calcium ions are universal secondary messengers that are key players in many cellular signal transduction pathways, which regulate diverse functions such as secretion, cell motility, proliferation, and cell death. Cytosolic calcium concentration is tightly regulated. Ligand-stimulated increases in intracellular calcium concentration come mainly from two sources: internal stores that release calcium and channels in the plasma membrane that open to allow external calcium to flow into the cell. In a number of cells, the release of intracellular-stored calcium following receptor activation acts as a trigger for longer calcium signals derived from calcium influx through membrane calcium channels (1, 2). In polymorphonuclear neutrophils (PMNs),3 changes in cytosolic free calcium concentration have been consid-

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3 Abbreviations used in this paper: PMN, polymorphonuclear neutrophil; GPCR, G protein-coupled receptor; PACAP, pituitary adenylate cyclase-activating protein; VIP, vasoactive intestinal peptide; VPAC, VIP/PACAP receptor; PKA, protein kinase A; PLC, phospholipase C; IP3, inositol 1,4,5-triphosphate; PTX, pertussis toxin; PKC, protein kinase C; ROS, reactive oxygen species; MMP-9, matrix metalloproteinase-9; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; PAF, platelet-activating factor; Tg, thapsigargin; NPY, neuropeptide Y; SP, sub-

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subsequent increase in inositol 1,4,5-triphosphate (IP3) and calcium (4). In addition, VPAC1-mediated calcium increase is partially blocked by pertussis toxin (PTX), indicating that for this receptor, Gqi is involved (5). In nonhemopoietic cells, PACAP-evoked calcium transient has been reported to be dependent on the activation of cAMP/PKA, PLC-β, and protein kinase C (PKC), or to be PKA and PKC independent (5). These mechanisms act differentially on the mobilization of intracytoplasmic calcium from IP3-sensitive and -insensitive stores and on the modulation of multiple calcium channels or specific L-type calcium channel present on the plasma membrane (4).

Of interest, the GC-rich 5′ flanking region of the VPAC1 receptor contains potential binding sites for several nuclear factors such as PU-1, NF-IL6, NF-κB, and IFN regulatory factor 1, known to be important for myeloid lineage differentiation and myeloid regulated functions (6), and we have recently shown that PACAP, through the GPCR VPAC1, acts as a proinflammatory molecule in PMNs (7). The wall of blood vessels are richly innervated by PACAP-containing fibers, which are also present in a number of extracerebral organs (4). Thus, it is possible that interaction between these PACAP peptidergic-containing nerve fibers and VPAC1-presenting PMNs play a role in local inflammation. Changes in pro- and anticoagulant activities are associated with systemic as well as specific inflammations (8). In this regard, it is of interest to note that circulating PACAP has been recently identified as a new physiological inhibitor of platelet activation (9). We have shown that secondary messengers activated by PACAP in neutrophils include CAMP, IP3, and calcium with downstream activation of the MAPK ERK and p38. The inflammatory activities stimulated by PACAP in these cells included the production of reactive oxygen species (ROS), up-regulation in the membrane expression of the integrin CD11b, and release of secondary (lactoferrin) and tertiary (matrix metalloproteinase-9; MMP-9) granules (7).

In this study, we investigate the intracellular mechanisms following PACAP receptor activation that leads to calcium release in human neutrophils. We evaluate the contribution of the extracellular and the intracellular calcium as well as the different sources of the intracellular calcium stores sensitive to PACAP exposure. We investigate whether the calcium signal is consistent with G protein-dependent pathways and calcium response in neutrophils regulated functions (6), and we have recently shown that PACAP, through the GPCR VPAC1, acts as a proinflammatory molecule in PMNs (7). The wall of blood vessels are richly innervated by PACAP-containing fibers, which are also present in a number of extracerebral organs (4). Thus, it is possible that interaction between these PACAP peptidergic-containing nerve fibers and VPAC1-presenting PMNs play a role in local inflammation. Changes in pro- and anticoagulant activities are associated with systemic as well as specific inflammations (8). In this regard, it is of interest to note that circulating PACAP has been recently identified as a new physiological inhibitor of platelet activation (9). We have shown that secondary messengers activated by PACAP in neutrophils include CAMP, IP3, and calcium with downstream activation of the MAPK ERK and p38. The inflammatory activities stimulated by PACAP in these cells included the production of reactive oxygen species (ROS), up-regulation in the membrane expression of the integrin CD11b, and release of secondary (lactoferrin) and tertiary (matrix metalloproteinase-9; MMP-9) granules (7).

In this study, we investigate the intracellular mechanisms following PACAP receptor activation that leads to calcium release in human neutrophils. We evaluate the contribution of the extracellular and the intracellular calcium as well as the different sources of the intracellular calcium stores sensitive to PACAP exposure. We investigate whether the calcium signal is consistent with G protein mediation by manifesting the phenomenon of homologous and heterologous desensitization. Finally, we evaluate how calcium regulates proinflammatory activities including ROS production, CD11b up-regulation, lactoferrin, and MMP-9 extracellular release.

Materials and Methods

Cells and reagents

Human PMNs were isolated as described previously (5). Highly purified human PACAP was obtained from Neosystem. FMLP, Xestospongin C, GF109203X, the mitochondrial protonophore carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), H-89, SKF96365, verapamil, nifedipine, genistein, LY292002, wortmannin, CaCl2, 2′,5′-dideoxyadenosine, platelet-activating factor (PAF), and cytochalasin B were purchased from Sigma-Aldrich. Intracellular calcium chelator (10, 11), calcium mobilization inhibitors (11), and inhibitors of signaling pathways (12–15) were used at concentrations lower or similar to the ones reported in other studies on neutrophils (10–15). Fluoc/AM, PTX, BAPTA-AM, EGTA, thapsigargin (Tg), neuropeptide Y (NPY), and substance P (SP) were purchased from VWR. The rabbit anti-VPAC1 polyclonal Ab was a gift of K. Freson and C. Van Geet (Katholieke Universiteit Leuven, Leuven, Belgium) (9). RPMI 1640 was purchased from BioWhittaker. FBS, HBSS, and PBS were purchased from Invitrogen Life Technologies. Unless otherwise noted, treatment was performed using PMNs, 1 × 106 cells/ml, exposed to 1 μM PACAP.

VPAC1 cell surface expression

Expression of VPAC1 receptor in PMNs was investigated by immunocytochemistry, immunofluorescence, and flow cytometry. VPAC1 staining for immunocytochemistry was performed by the alkaline phosphatase anti-alkaline phosphatase (AAPA) method. PMNs cytopsins were fixed in PBS-buffered 4% paraformaldehyde for 20 min at room temperature after washing in TBS (pH 7.6) for 5 min. Slides were then incubated with anti-VPAC1 receptor or isotype-matched IgG1 in TBS for 1 h at room temperature. The AAPA method was then performed with a commercial kit (DakoCytomation) followed by hematoxylin counter staining. For immunofluorescence, PMNs were fixed in methanol for 3 min and stained with specific Ab to VPAC1 receptor or control rabbit IgG. Primary Ab was detected by FITC-conjugated Ab. For flow cytometry, samples (2.5 × 105 PMNs) were permeabilized (BD Biosciences FACS Permeabilizing Solution 2) and incubated for 45 min with the anti-VPAC1 polyclonal Ab or IgG1 control in the presence of human IgG to block nonspecific binding. Cells were incubated with FITC-conjugated goat anti-rabbit IgG (1:100) in the dark for 45 min on ice and analyzed on FACSscan.

Intracellular calcium measurement and Western blot

Free intracellular calcium was determined with an LS50B Fluorescence Photometer (PerkinElmer) as reported (16). Intracellular calcium concentration was calculated as described previously (17); peak values and plateau phase were recorded, this last data being calculated as the area under the curve starting at the peak, up to 200 s after, and expressed as arbitrary unit.

ERK and p38 MAPK phosphorylation was determined as reported (7), using Abs against p38 and phosphorylated p38 (Thr180/Thr182) from New England Biolabs and phosphorylated ERK1/2 (Ty202/Thr204) from Santa Cruz Biotechnology.

Oxidative burst and degranulation of neutrophils

Intracellular oxidative burst was evaluated by flow cytometry using 2′,7′-dichlorofluorescein (DCFH). PMNs were preincubated for 30 min with 100 μg/ml DCFH-diacetate in a water bath at 37°C with horizontal agitation. DCFH-diacetate diffuses into cells and is hydrolyzed into nonfluorescent DCFH. For each sample, half of the PMNs were used as controls, and the other half was then stimulated with PACAP for 20 min. H2O2 produced during the PMN oxidative response oxidized the nonfluorescent intracellular DCFH into highly fluorescent dichlorofluorescein. Dichlorofluorescein fluorescence emission was assayed at 530 nm after excitation cells at 488 nm. The expression of the surface marker CD11b (Mac-1) on the neutrophil cell surface was used as an indicator of degranulation of secretory vesicles. Quantification of lactoferrin and MMP-9 release was used as an indication of degranulation of specific granules and tertiary granules, respectively. CD11b up-regulation, lactoferrin, and MMP-9 releases were evaluated as described previously (7).

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). Differences were considered statistically significant when p < 0.05.

Results

Membrane expression of VPAC1 on the surface of PMN

Immunohistochemical staining technique using the peroxidase/antiperoxidase enzyme procedure, immunofluorescence, and flow cytometry studies (Fig. 1) demonstrated the presence of VPAC1 receptor protein detected previously by Western blotting (7).

G protein-dependent pathways and calcium response in PACAP-activated PMNs

In neutrophils, PACAP through the GPCR VPAC1 dose-dependently increases CAMP, IP3, and calcium (7). We already published that PLC inhibition by U73122 inhibits calcium release in PACAP-treated neutrophils, whereas the inactive analog U73343 does not have any effect indicating the presence of a Gqi/GaQL-PLC-IP3 pathway (7).
In PMNs, most of the intracellular releasing calcium is stored in the IP3-sensitive endoplasmic reticulum (3). Entry of calcium in IP3-sensitive organelles is regulated by a calcium-dependent ATPase. Tg, by inhibiting the re-uptake of calcium through calcium-dependent ATPase, depletes intracellular calcium in neutrophils. PACAP applied after Tg (2 μM) exposure was unable to induce a calcium signal, indicating that PACAP-induced calcium signals were associated with calcium release from the endoplasmic reticulum stores (Fig. 3A).

According to the nature of the stimulus, this initial rise in calcium due to mobilization of intracellular stores may or may not activate calcium influx across the plasma membrane to amplify and sustain the overall increase in intracytoplasmic calcium. To evaluate the role of external calcium influx in PACAP signaling, experiments were performed in calcium-free medium (0 mM calcium, 2 mM EGTA). Application of PACAP in such a media induced an increase in calcium (Fig. 3B) significantly lower from the one recorded in the presence of calcium (Fig. 3B). Further confirmation of the role of extracellular calcium was provided by experiments, where the addition of calcium (2 mM) back to the calcium-free medium resulted in an increase in intracytoplasmic calcium in PACAP-treated PMNs (Fig. 3B). This supports the fact that PACAP also causes mobilization of extracellular stores. Indeed, the application of SKF96365, a specific calcium channel blocker, induced a lower calcium increase in PACAP-treated cells: SKF96365 (10 μM) reduced by 73 ± 7% the calcium response to PACAP (Fig. 3, C and D). The participation of verapamil-sensitive calcium channels was demonstrated using specific inhibitors such as verapamil (10 μM) (Fig. 3, E and F) and nifedipine (10 μM) (Fig. 3, G and H), which decreased by more than 70 ± 11% the effect of PACAP. In contrast, inhibitors of N-type (ω-conotoxin GVIA, 10 μM) and P/Q-type channels (ω agatoxin IVA, 10 μM) were without any effect (data not shown). The results suggest that subsequent to PACAP-induced calcium mobilization from intracellular stores, a sustained influx of calcium through the plasma membrane takes place with participation of verapamil-sensitive calcium plasma membrane channels.

Nature of the intracellular calcium pool
Activation of PLC leads to the generation of IP3 and diacylglycerol. In neutrophils, calcium release from the endoplasmic reticulum into the cytosolic compartment occurs through the opening of the universal IP3 receptor channels, which is modulated by IP3. PMNs exposure to Xestospongin C (10 μM), a specific blocker of the IP3 receptor, resulted in a decrease by 92 ± 7% of the calcium response to PACAP, indicating that IP3-sensitive calcium pools are involved in PACAP-evoked calcium increase (Fig. 4, A and B).

PLC-generated diacylglycerol activates PKC. In PMNs, PKC is implicated in neutrophil oxidase activation, in regulation of membrane-cytoskeleton interaction, and in the modulation of FMLP-induced calcium increase. Inhibition of PKC by the nonselective PKC inhibitor GF109203X (10 μM) resulted in a 81 ± 5% decrease in PACAP-induced calcium signal, indicating that members of the PKC family modulate also PACAP-induced calcium increase (Fig. 4, C and D). The presence of IP3-insensitive stores such a mitochondria have been described in neutrophils (20). The use of the protonophore FCCP (5 μM), which causes depletion of mitochondrial calcium, significantly decreases by 59 ± 14% the PACAP-associated calcium increase, indicating that mitochondria contribute also to the PACAP-associated calcium signal (Fig. 4, E and F).

Role of PI3K and tyrosine phosphorylation
PI3K activity is important in several key signaling pathways that govern PMNs functions (21). In addition, PI3K has been reported to modulate GPCR- and non-GPCR-mediated calcium increase (22, 23). We thus investigated whether PACAP-induced calcium signal occurs downstream of PI3K activity by using the PI3K inhibitors LY29402 and wortmannin. As shown in Fig. 5, A and B, LY29402 (20 μM) partially decreased by 32 ± 7% PACAP-induced calcium transient. Similar results were observed with wortmannin (100 nM; data not shown). Thus, PI3K is required for full activation of the calcium signaling pathway in neutrophils.
Tyrosine phosphorylation events are associated to early intracellular signals initiated by a number of agonists. Genistein, a general tyrosine kinase inhibitor, has been reported to decrease calcium mobilization in fMLP-treated PMNs (24), whereas no effect was observed by others (25). To determine whether a genistein-sensitive step exists in PACAP-treated PMNs, cells were exposed to genistein (10 μM) before PACAP exposure. Pretreatment with genistein for 10 min did not inhibit the calcium rise to a peak because there was no significant difference of average peak of calcium between control and genistein-treated cells (Fig. 5, C and D). In contrast, the plateau level was decreased by 65 ± 8% after genistein treatment (Fig. 5E). This effect was specific to genistein-mediated tyrosine kinase inhibition because the dose of genistein used was well beyond the dose of 300 μM associated with nonspecific-serine/threonine kinase inhibition (26). This is consistent with previous results, which indicate that kinetics of calcium increase in PMNs consists in two closely coupled events, with a rapid and transient GPCR-dependent release of intracytoplasmic-stored calcium followed by a slow entry of extracellular calcium, which may be dependent on tyrosine kinase activation (27).

Actin cytoskeleton and PACAP
Actin disruption has been reported to modulate calcium entry in PMNs activated by fMLP (28) or by receptor cross-linking (22). To determine whether PACAP signaling pathway in PMNs was dependent on the actin cytoskeleton, cells were treated with cytochalasin B (5 μg/ml), which disrupts actin fibers, and then stimulated with PACAP. As shown in Fig. 5F, PACAP-dependent increase in calcium was not affected by the absence of a functional actin cytoskeleton.

Homologous and heterologous desensitization
Prolonged stimulation of GPCRs results in desensitization, which can be homologous and heterologous (29). Homologous desensitization is specific for receptor and its agonist (30). We then examined whether the response of PMNs to PACAP manifests homologous desensitization. As shown in Fig. 6, A and C, 5-min neutrophils pre-exposure to PACAP decreased the capacity by 75 ± 9% of the neuropeptide to mobilize intracellular-free calcium. Homologous desensitization was less pronounced after 15
min, 60 ± 13%, and was not statistically different from PACAP alone (Fig. 6, B and C); it was not associated to a cAMP/PKA-mediated effect because pretreatment with forskolin, a cAMP-elevating agent, had no effect on the PACAP-mediated calcium peak (data not shown). We then took advantage of this biological effect of PACAP to evaluate the minimal PACAP concentration necessary to desensitize the VPAC1 receptor for calcium mobilization. As shown in Fig. 6, D–F, desensitization was still observed at 50 nM PACAP exposure and disappeared at 10 nM peptide treatment. Heterologous desensitization refers to a process whereby activation of one type of receptor results in the desensitization of different receptors (30). Cross-desensitization studies with the GPCR agonists SP, NPY, and fMLP, using calcium mobilization as a measurement of receptor activation, were performed. As shown in Fig. 7, A–C, pretreatment (5 min) with SP (30 µM) or NPY (1 µM) was associated with partial desensitization, 60 ± 7% and 62 ± 2%, respectively, whereas fMLP (0.1 µM) completely abrogated the PACAP-induced calcium signal in neutrophils (Fig. 7D). Kinetic studies indicate that profound cross-desensitization effects were still present after 30 min (Fig. 7D) and 60 min (data not shown) of

**FIGURE 3.** PACAP-induced intra- and extracellular calcium mobilization. A, PACAP does not induce calcium signals in PMNs preincubated with Tg. Twelve minutes after addition of 2 µM Tg, 1 µM PACAP was added in fluo-3-loaded cell suspension (1 × 10⁶ cells/ml, RPMI 1640 containing 5 mM calcium; pH 7.2). B, PACAP stimulates a transient calcium signal in PMNs that depends on the presence of extracellular calcium. Fluo-3-loaded PMNs were stimulated with 1 µM PACAP in calcium-free PBS containing 2 mM EGTA; at the time indicated, 2 mM CaCl₂ was added to calcium-depleted PMNs, and changes in cytoplasmic calcium concentration were monitored. C–H, Effect of calcium channel blockers on entry of calcium into PMNs. Inhibitory effect of SKF96365 (SKF) (C and D), verapamil (E and F), and nifedipine (G and H) on the mobilization of intracellular calcium in PMNs exposed to PACAP are presented. Preincubation time before PACAP exposure was 10 min for SKF96365 and verapamil and 30 min for nifedipine. Data from a single experiment representative of a minimum of four independent experiments are shown (C, E, and G). Peak values are recorded after PACAP treatment and are reported as the mean ± SEM of a minimum of four different experiments (D, F, and H). *, p < 0.05 vs PACAP alone.
fMLP exposure at a time where complete recovery from homologous desensitization was observed. However, reciprocal desensitization was not observed: thus, cells pretreated with PACAP (1 μM) were still fully sensitive to the calcium-rising effect of fMLP (0.1 μM) (Fig. 7E).

Roles of intra- and extracellular calcium mobilization in PACAP-induced PMN activation

In PMNs, calcium-dependent and -independent pathways are activated by proinflammatory molecules. In addition, according to the nature of the stimulus, proinflammatory activities are dependent on intra- and/or extracellular calcium mobilization. We have previously shown that PACAP activates multiple PMN proinflammatory functions (7), including exocytosis of secondary and tertiary granules and priming of fMLP-induced exocytosis of primary granules and respiratory burst; PACAP alone also increases ROS production in neutrophils (our unpublished observation). To evaluate the respective role of intra- and extracellular calcium mobilization in PACAP-associated PMN activation, we used PMNs where intracellular calcium was depleted by cell exposure to BAPTA, an intracellular calcium chelator (10, 11), and PMNs where extracellular calcium was depleted by adding in the calcium-free medium the calcium chelator EGTA. As shown in Fig. 8A, BAPTA (10 μM) completely blocked calcium mobilization, whereas at 0.1 μM the inhibition was incomplete. Thus, additional experiments were performed using the higher concentration. Cell exposure to BAPTA (10 μM) inhibited by 40 ± 7% and 46 ± 3%, respectively, the PACAP-associated increase in ROS production and CD11b membrane up-regulation (Fig. 8C–D); and cell exposure to EGTA (2.5 mM) inhibited by 36 ± 11% and 35 ± 1%, respectively, the PACAP-associated increase in ROS production and CD11b membrane up-regulation (Fig. 8C–D). In contrast, lactoferrin secretion was not affected by intra- or extracellular calcium depletion, indicating in addition that such treatment did not affect PMN functions (Fig. 8E). Finally, PACAP-associated MMP-9 secretion was dependent on the presence of intracytoplasmic calcium but was not sensitive to extracellular calcium depletion (Fig. 8F).
Calcium mobilization and MAPK activation

In neutrophils, ERK, and p38 MAPK are activated by a number of proinflammatory molecules. We have reported previously that in neutrophils, PACAP phosphorylates both ERK and p38 MAPK. In addition, in these cells, calcium increase and stimulation of proinflammatory functions are differentially sensitive to ERK and p38 phosphorylation (7). As shown in Fig. 9, ERK and p38 MAPK phosphorylation were also differentially sensitive to calcium depletion. Thus, in PACAP (10 μM)-exposed neutrophils, BAPTA prevented ERK but not p38 phosphorylation; in contrast, EGTA or cell exposure to SKF96365 prevented p38 but not ERK phosphorylation. These data indicate that extracellular calcium influx is needed for p38 activation.

Discussion

PACAP is a neurotrophic, neuroprotective peptide, which displays numerous functions in both cells of neuronal and nonneuronal origin (4). After interaction with one of the three specific GPCR/VPAC receptors, PACAP induces the formation of second messengers including cAMP, IP3, and calcium (4). Inflammatory process expose PMNs to a wide variety of agonists that can attract, prime, or activate PMNs via GPCRs, which typically signal by increasing calcium in addition to activating other second messengers. We have recently reported that PACAP in human neutrophils activates multiple proinflammatory functions through PLC/ERK and p38 MAPK activation. In these cells, PACAP also increases intracytoplasmic calcium through mechanisms, which are PLC/ERK dependent but p38 independent (7). In this current work, we have investigated the following: 1) the contribution of intra- and extracellular calcium in PACAP-evoked calcium transient; 2) the relationships between calcium and activation of downstream signaling pathways such as those mediated by ERK and p38 MAPK, known to be activated by proinflammatory molecules; and 3) the association between calcium mobilization and activation of neutrophil functions such as ROS and exocytosis of vesicles and secondary and tertiary granules.

Within human hemopoietic tissues, cell surface VPAC1 receptor expression has been reported in megakaryoblast platelets, (9, 31) T cells, and monocytes (32, 33). We have previously reported the presence of VPAC1 proteins in human PMN (7). We extend these

FIGURE 5. PACAP-induced intracellular calcium mobilization is PI3K, tyrosine kinase dependent and cytoskeleton independent. Inhibitory effects of LY29402 (LY) (A and B), genistein (C-E), and cytochalasin B (cyt. B) (F) on the mobilization of intracellular calcium in PMNs exposed to PACAP are presented. Preincubation time before PACAP exposure was 30 min for LY29402, 10 min for genistein, and 5 min for cytochalasin B. Data from a single experiment representative of a minimum of four independent experiments are shown (A, C, and F). Peak values are recorded after PACAP treatment and are reported as the mean ± SEM of a minimum of four different experiments (B and D). *, p < 0.05 vs PACAP alone.
data by demonstrating the presence of membrane expression of VPAC1 receptor in PMNs using immunohistochemistry, immunofluorescence, and flow cytometry studies.

GPCR-mediated calcium transients are known to be G\textsubscript{q/11} and G\textsubscript{i/11} regulated. This calcium signal is biphasic, with an early peak followed by a plateau phase. The early peak is associated with mobilization of intracellular calcium from IP3 receptor and/or ryanodine receptor-gated calcium stores and/or mitochondria (20). This leads to activation of calcium release-activated calcium influx through multiple mechanisms, some of them being dependent on tyrosine kinase activation and/or cyclic ADP ribose production (34–36). This secondary event is important for the initiation of well-defined PMN functions (35, 37–38). Thus, fMLP-activated PMN chemotaxis is absolutely dependent upon extracellular calcium influx, whereas chemotaxis for other stimuli like IL-8 does not require calcium influx (35).

In nonhemopoietic cells, PACAP mobilizes calcium from both intra- and extracellular sources through multiple pathways, according to cell types and nature of the VPAC receptors present on the cell surfaces. In these cells, calcium mobilization has been linked to mechanisms, which are cAMP/PKA and PLC/PKC dependent, cAMP/PKA and PLC independent, and ryanodine/caffeine dependent (4). Calcium influx through L-, N-, and Q-type calcium channels has also been reported (39). In hemopoietic cells, PACAP has been reported to have both pro- and antiinflammatory properties (40). Although there are numerous reports on PACAP-associated

![Figure 6](http://www.jimmunol.org/)
PACAP elicits a calcium signal in PMNs that undergoes homologous desensitization. PMNs loaded with fluo-3 were sequentially stimulated with PACAP. PACAP (1 μM) is added at time 0 and again at 5 min (A) or after 15 min (B). PMNs were stimulated with PACAP (1 μM) at time 0 (control peak), whereas an aliquot is preincubated with 50 nM PACAP (D) or 10 nM PACAP (E) for 5 min before 1 μM PACAP exposure. Data from a single experiment representative of a minimum of four independent experiments are shown (A, B, D, and E). Peak values are recorded after PACAP treatment and are reported as the mean ± SEM of a minimum of four different experiments (C and F). * p < 0.05 vs PACAP alone.

![Figure 7](http://www.jimmunol.org/)
PACAP elicits a calcium signal in PMNs that undergoes reciprocal heterologous desensitization. PMNs loaded with fluo-3 were sequentially stimulated with agonists. Time points at which the agonists were added and nature of the agonists are noted as indicated. PMNs were stimulated with PACAP (1 μm) at time 0 (control peak), whereas an aliquot is preincubated with 30 nM SP (A), 1 μM NPY (B), or 0.1 μM fMLP (D) for 5 min before PACAP exposure. In D, the effect of 30-min preincubation with fMLP before PACAP exposure is also shown. The reciprocal experiments where fMLP is added after 5 min of PACAP exposure are shown in E. Data from a single experiment representative of a minimum of four independent experiments are shown (A, B, D, and E). Area under the curve is recorded after PACAP treatment and are reported as the mean ± SEM of a minimum of three different experiments (C and F). * p < 0.05 vs PACAP alone.
calcium mobilization in nonhemopoietic cells (4), there have been no studies except from our laboratory on such effect in hemopoietic cells (7, 16, 33). Similarly to what has been described with other GPCRs, we found that PACAP-mediated calcium signals in PMNs is PLC/IP3 and PTX dependent as well as cAMP/PKA dependent, indicating the participation of Gaq and Gs proteins. In these cells, IP3-containing calciosomes and mitochondria are sources of intracellular calcium. The role of extracellular calcium is suggested by the fact that PACAP-mediated calcium signal is abolished in cells cultured in calcium-free medium. Furthermore, the addition of calcium in calcium-free medium restores in PACAP-treated cell the calcium signal, which was previously abolished, and PMNs preincubated with the cation channel entry inhibitor SKF96365 or the L-channel calcium blockers verapamil and nifedipine demonstrate a markedly diminished calcium response to PACAP. Altogether, these results indicate in PACAP-treated cells the presence of significant calcium influx mechanisms most likely activated by depletion of intracellular calcium stores.

Tyrosine kinase inhibitors, in addition to blocking a number of functions in activated PMNs, inhibit also FMLP and FcγRIIIa-induced calcium transients but do not inhibit intracytoplasmic calcium storage mobilization by FcγRIIIb (24). In our study, genistein inhibits the plateau phase of the PACAP-evoked calcium transient characterized by the sustained elevation of calcium via calcium influx without affecting the initial peak, which results from internal mobilization. This suggests that calcium influx induced by depletion of IP3-dependent and -independent intracellular calcium stores by PACAP is regulated via tyrosine kinase pathways. Similar involvement of tyrosine kinase activity in the regulation of calcium entry has been reported in other cell types (34). In addition to this pathway, we cannot rule out the existence of other PACAP-stimulated calcium entries, which contribute to the plateau phase but are not genistein sensitive (41).

In a number of cells of nonhemopoietic origin, PACAP modulates calcium influx through L-type calcium channel (4). In Drosophila, mutations in the amnesiac gene, which encodes a peptide homologous to human PACAP, reduce the L-type current in larval muscles (42). Store-operated calcium influx channels in human myeloid cells resemble L-type calcium channel lacking a voltage sensor (43). Among the different calcium channels, the presence of verapamil- and nifedipine-sensitive calcium channels (44, 45) or L-type calcium channel (11) has been reported on the PMN plasma membrane. We also found verapamil- and nifedipine-sensitive calcium influx in PACAP-treated cells, whereas inhibitors of other membrane channel types were not effective. Because PMNs do not express voltage-gated calcium channels, our results indicate that opening of unknown verapamil-sensitive plasma calcium membrane receptor channel as well as the activation of tyrosine kinase pathway participate in calcium influx across the plasma membrane in PACAP-treated PMNs.

PI3K activation plays a pivotal role in PMN stimulation (13). Neutrophils posses two types of class 1 PI3K: the ubiquitous class
l_{1\alpha} PI3K is activated through receptors that have intrinsic tyrosine kinase activity or are coupled to src-like kinase, and the more specific class l_{1\beta} PI3K is activated via the βγ subunits of G proteins. In nonhemopoietic cells, PI3K has been reported to modulate both intracellular and extracellular calcium mobilization. In PMNs, PI3K-activated calcium transient has been reported to occur in non-GPCR-activated neutrophils (46). We also found that such pathways participate in PACAP-mediated calcium increase, although complete abrogation of calcium mobilization using inhibitors of the PI3K pathway was never obtained, indicating that multiple regulatory pathways are involved in calcium mobilization. Cross-linking of receptors for the Fc portion of the IgG molecules present on the surface of PMNs triggers a number of responses including calcium mobilization and phagocytosis, which require a functional actin cytoskeleton (22). In addition, actin disruption increases calcium entry in fMLP-activated PMNs (28). In contrast, in our study we found that PACAP-mediated calcium increase was independent on a functional actin cytoskeleton.

Chemoattractant-mediated inflammatory responses are regulated by desensitization whereby prolonged stimulation with appropriate agonist results in the decrease in chemoattractant receptor-mediated signals. Human neutrophils are known to undergo rapid homologous chemoattractant receptor desensitization, whereas cross-desensitization among these receptors also occurs (30). We also found that PACAP-treated PMNs display both homologous desensitization as well as GPCR/agonists-induced cross-desensitization of calcium mobilization. Within the agonists, cross-desensitization with fMLP was much more pronounced than with NPY or SP. Of interest, whereas fMLP completely abrogated the calcium mobilization effect of PACAP, the reciprocal effect was not observed: thus, PACAP treatment did not have any statistical effect on the magnitude or duration of subsequent fMLP response. These results agree with previous data indicating that among GPCR/agonists fMLP receptor is resistant to cross-desensitization by other peptide chemoattractants (30). Thus, predominance of the fMLP-mediated cross-desensitization indicates that fMLP released by invasive bacteria and lysed cells plays a major role in orchestrating the host innate immune responses overriding host-derived recruitment through PACAP and other chemokine receptors. Of interest, down-regulation of VPAC1 receptor was also observed in stimulated human T lymphocytes and might represent a mechanism for restricting bystander activation of T cells (32). Affinity of the VPAC1 receptor is in the nanomole range. Investigating PACAP homologous desensitization mechanism, we demonstrated that PACAP at nanomole concentration was effective to desensitize VPAC1 receptor for calcium mobilization, and experiments are in progress in our laboratory to evaluate how this desensitization translates into decrease in PMN activity. According to the nature of the stimulus, NADPH oxidase activation has been found to be calcium dependent or independent (27, 47). In PMNs, changes in intracellular calcium levels are required to mediate fMLP oxidase activation. In these cells, depletion of intracytoplasmic calcium mobilization has been reported to affect the oxidase (25), whereas the role of extracellular calcium is controversial (25, 37). In our study, we found that both intracellular and extracellular calcium mobilization were necessary for PACAP-mediated oxidase activation. Because both pools cooperate, it is possible that PACAP-induced intracellular calcium release is initially necessary to activate subsequently calcium influx through the plasma membrane, which will constitute the main source of calcium needed to release H_{2}O_{2}.

Both phagolysosomal fusions, which consist in the fusions of phagocytic vesicles with granules, and degranulation have been reported to have an absolute dependency on calcium changes (3). In contrast, the associated biochemical events for neutrophil shape change, actin polymerization, and phagocytosis do not require a rise in cytosolic free calcium concentration (3). PMN peroxidase-negative granules include the lactoferrine secondary (specific) granules, the gelatinase tertiary granules, and secretory vesicles These organelles display specific functions according to the type of proteolytic enzymes, bacterial proteins, and membrane proteins present on the granule membranes. Investigation of calcium-induced exocytosis in PMNs led to the recognition of granules with high-affinity exocytosis, with a well-defined hierarchy of granule mobilization: CD11b-containing secretory vesicles are the most rapidly mobilizable organelles, followed by gelatinase/tertiary granules, whereas the liberation of specific granules occurs considerably slower (48, 49). Of interest, we observed similar hierarchy in calcium-depleted mediated inhibition of granular exocytosis in PACAP-treated PMNs. Thus, we found that PACAP-mediated CD11b vesicle exocytosis was dependent on both intra- and extracellular calcium, whereas gelatinase/tertiary granules exocytosis was only dependent on intracellular calcium. In addition, intra- or extracellular calcium depletion did not prevent PACAP to release the content of lactoferrin/specific granules. Thus, the kinetics of granules exocytosis inhibition in calcium-depleted PACAP-treated PMNs are consistent with the hierarchy of their mobilization observed in cells challenged with increasing calcium.

The respective role of ERK and p38 MAPK phosphorylation in granular exocytosis is complex and seems to be stimulus and granules specific. Thus, ERK plays a role in CD11b up-regulation in soluble fibrinogen-activated PMNs but not in IL-1β-stimulated neutrophils (50, 51). Similarly, ERK plays no role in fMLP-stimulated secondary granules exocytosis (52), whereas ERK inhibition decreases the exocytosis of lactoferrin in PACAP-treated PMNs.
PMNs (7). Of interest, we found a close relationship between calcium depletion and ERK and p38 phosphorylation. Thus, ERK could not be phosphorylated in cells exposed to the intracellular calcium chelator BAPTA, whereas p38 could not be activated in cells where extracellular calcium was chelated with EGTA. Calcium entry channels might be modulated directly either from information derived from the activated VPAC1 receptor or through direct conformational coupling processes between the endoplasmic reticulum and plasma entry channels without important change in cytoplasmic calcium concentration (4). This might explain why p38 could still be phosphorylated despite cell exposure to the cytoplasmic calcium chelator BAPTA.

We conclude that human PMNs possess functional VPAC1 receptors, which mediate after PACAP interaction, calcium-dependent proinflammatory activities through the activation of multiple regulatory pathways. Both intra- and extracellular calcium are necessary for full cell activation and participate in distinctive calcium-dependent/MAPK pathways, including an intracellular calcium/ERK pathway, which regulates gelatinase/tertiary granules release, and an extracellular calcium/p38 pathway, which may be involved in ROS production; both pathways are necessary for vesicle exocytosis.

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Disclosures

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References


