ORIGINAL RESEARCH PAPER

VIP modulates human macrophages phenotype via FPRL1 via activation of RhoA-GTPase and PLC pathways

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Abstract

Objective and design This study is aimed at uncovering the signaling pathways activated by vasoactive intestinal peptide in human macrophages

Materials Human peripheral blood mononuclear cell-derived macrophages were used for the in vitro investigation of the VIP-activated signaling pathways.

Methods and treatment Time-course and dose–response experiments and siRNA were used in human macrophages co-challenged with various concentrations of VIP and different MAPK pharmacologic inhibitors to investigate signaling pathways activated by VIP. Flow analysis was performed to assess the levels of CD11b, CD35 and CD66. Luminescence spectrometry was used to measure the levels of the released hydrogen peroxide and the intracellular calcium levels in the media.

Results Macrophages incubated with VIP showed increased phospho-AKT and phospho-ERK1/2 levels in a GTP-RhoA-GTPase-dependent manner. Similarly, VIP increased intracellular release of H_2O_2 and calcium via PLC and GTP-RhoA-GTPase, in addition to inducing the expression of CD11b, CD35, CD66 and MMP9. Furthermore, VIP activated P38 MAPK through the cAMP/PKA pathway but was independent of both PLC and RhoA signaling. The above-mentioned VIP effects were mediated via activation of the FPRL1 receptor.

Conclusion VIP/FPRL1/VPAC/GTP-RhoA-GTPase signaling modulated macrophages phenotype through activation of multiple signaling pathways including ERK1/2, AKT, P38, ROS, cAMP and calcium.

Keywords VIP · FPRL1 · Rho kinase · Macrophage · MAPK

Introduction

Macrophages are mononuclear cells that play prominent roles in immune responses. They exhibit their defensive roles mainly through phagocytosis. In addition, they regulate lymphocytes' activation and proliferation, and they

have crucial role in the activation of T and B-lymphocytes by acting as antigen presenting cells [1]. They promote homeostasis by responding to internal and external changes within the body, not only as phagocytes, but also through trophic, regulatory, and repair functions [2]. Vasoactive intestinal peptide (VIP) is a 28-amino acids peptide that was initially detected in the intestine [3] and was then identified as a neuropeptide located both in the central and peripheral nervous system [4]. It plays multiple physiological functions, including neurotransmission, immune regulation, vasodilation and secretagogue. It is released by both neurons and immune cells, and its receptors are expressed by various immune cells [5]. Three types of VIP receptors were identified: VPAC1, VPAC2 and PAC1 [6]. Of these three receptors, VPAC1 is mainly expressed in immune cells, and is considered the major mediator of the immunomodulatory effects of VIP. Different studies focusing on the expression of the three types of receptors showed that VPAC1 was constitutively expressed in

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lymphocytes, macrophages, monocytes, dendritic cells and mast cells [5].

VIP was reported to inhibit the production of TNF, IL-6, IL10 and IL12, but to induce the production of iNOS in LPS-stimulated macrophages in a VPAC1-dependent manner [7]. Moreover, other reports indicated that VIP inhibited COX-2 expression in activated macrophages, downregulated macrophage-derived high mobility group box-1 (HMGB1), and suppressed the inflammatory response of microglia in a model of Parkinson's disease [7–10]. VIP was also reported to inhibit the expression of multiple chemokines, including CXCL1/KC, CXCL2, CCL2, CCL3, CCL4 and CCL5 in murine macrophages [11–13]. Recently, VIP was shown to suppress NLRP3 inflammasome activation in LPS-activated macrophages [14]. Additionally, its immunomodulatory responses improved myocarditis and atherosclerosis [15]. To these stated roles, VIP was suggested to regulate oxidative stress in some types of cells. In this context, Fujimori et al. (2011) have reported that VIP reduced oxidative stress through inhibiting NADPH oxidase in pancreatic acinar cells during pancreatic damage [16]. In the same context, VIP reduced ROS production and inflammation induced by the formyl peptide, fMLF, in primary human phagocytes. They also showed that the action of VIP was mediated through its VPAC1 receptor, and that VIP inhibited fMLF-induced phosphorylation of ERK1/2 and p38 MAPKs [17]. However, the roles of VIP in the regulation of ROS generation in macrophages specifically, along with the relationship between VIP and formyl peptides receptors, are still uncovered.

Previous studies reported that VIP regulated the expression of several transcription factors including AP-1, NFκB, CREB and IRF-1 [7, 11, 18–21]. Also VIP inhibited MEKK1/MEK4/JNK signaling pathway in LPS-stimulated macrophages, specifically through the VPAC1/cAMP/PKA pathway [19]. Additionally, another study showed that VIP suppressed macrophages-mediated inflammation by downregulating IL-17 expression in a PKC and PKA-dependent manner [22]. In addition to its above-mentioned signaling effects in macrophages, VIP was reported to control N-methyl-D-aspartic acid-induced motility in dendritic cells through modifying Rho-GTPase and PI3K activities [23]. However, the relationship between VIP and Rho-GTPase and the activated downstream signaling pathways, in macrophages specifically, are not well understood. Rho-GTPases are signaling G-proteins that control numerous signaling transduction pathways in different types of cells [24]. They are principally known for their important role in regulating the actin cytoskeleton [24]. In macrophages, Rho-GTPases and PI3K were reported to be required for the phagocytosis of apoptotic cells [25], but no previous papers have investigated if Rho-GTPases are involved in the signal transduction events mediated by VIP.

In the current study, we investigated the signaling transduction pathways of VIP in monocytes-derived macrophages. We have shown that VIP activated Akt, ERK1/2 and P38 kinases in a RhoA-GTPase-dependent manner. This function of VIP seems to be dependent on the FPRL2 receptor. Furthermore, VIP provoked the intracellular release of H_2O_2 and calcium in a mechanism that also involves PLC, in addition to inducing the expression of CD11b, CD35, CD66 and MMP9.

Materials and methods

Isolation of human monocytes

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a Ficoll-Metrizoate density gradient (Lymphoprep; Lucron Bioproducts, Axis-Shield, Norway). Monocytes were further purified by adherence for 90 min to gelatin-coated plastic flasks previously incubated with FBS. PBMC were cultured for 5 days to acquire macrophage phenotype in RPMI 1640 medium (Biowhitteker Europe) containing 10% heat-inactivated fetal bovine serum (NV Life Technologie, Rockville, MD), 100 U/ml penicillin, and 100 Ag/ml of streptomycin. Differential cell counts revealed that these preparations were > 95% monocytes and > 99% viable. Prior to stimulation cells were first washed with warm sterile PBS (1X) and incubated overnight in a serum free medium. The next day the culture media was replaced with a new serum free medium and cells were then stimulated with various activators/inhibitors at the indicated concentrations, or with media as control vehicle for the specified time as indicated. The study was approved by the IRB of the Lebanese University and was conducted according to the declaration of Helsinki.

Reagents

WRW4 was obtained from Calbiochem (La Jolla, CA, USA). VIP, C3a and fMLP, PMA and U73122 were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Western blotting

Macrophages (10^7 cells) were suspended in RPMI 1640 medium and stimulated at 37 °C for the desired durations of time. Total cell lysates were obtained in 50 mM HEPES [pH 7.5], 250 mM sodium chloride (NaCl), 1 mM EDTA, 0.5% Triton X-100, 0.5 mM DTT, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM PMSF, 20 mM Pefabloc, and 2 Ag/mL aprotinin, antipain, leupeptin, and

pepstatin. Proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred to PVDF membranes (Amersham Biosciences) at 125 V for 45 min. Membranes were blocked with TBST (TBS containing 0.05% Tween 20) containing 5% bovine serum albumin (BSA) overnight at 4 °C. The blots were then rinsed with TBST (3 times) for 5 min at 25 °C and incubated with primary antibodies diluted in TBST for 1 h at 25 °C. The blots were then rinsed with TBST (3 times) for 5 min at 25 °C and incubated in the appropriate secondary antibody diluted in TBST for 1 h at 25 °C. The blots were rinsed another 3 times for 5 min with TBST before detection by enhanced chemiluminescence (ECL) (Amersham Biosciences). Blots were stripped by incubation in 62.5 mM Tris–HCl, 2% SDS, 100 mM 2-mercaptoethanol (pH 6.7) for 30 min at 50 °C. Stripped blots were then rinsed extensively with TBST and reproved as described above. Detection of p38 (Tyr182), phospho-ERK (Tyr 204), phospho-AKT (Ser 473), GTP-RhoA-GTPase and anti-FPRL2 was done using specific antibodies at a dilution of 1:1000 raised against each protein (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The total Protein-A peroxidase antibody was used at dilution of 1:10 000, and was obtained from Sigma. The rabbit anti-VPAC-1 polyclonal Ab (AS69-Santa Cruz Biotechnology, Santa Cruz, CA, USA) was kindly provided as a gift from K. Freson and C. Van Geet (KUL, Leuven, Belgium).

RNA isolation and RT-PCR

Total RNA from macrophages was isolated by the use of TRIzol reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer protocol. The RNAs were cleaned by treatment with RNase-free DNase I (Stratagene, La Jolla, CA, USA). 100 ng of total RNA was used in the RT-PCR. RT-PCR was performed as previously reported using specific primers set for FPRL2 and VPAC2 [24–27]. Cells previously reported to express these receptors were used as positive controls. PCR products were identified on 1–2% agarose gel after ethidium bromide staining.

SiRNA of RhoA

Macrophages were plated at 2×10^5/ml in 12-wells plates the day before siRNA transfection in DMEM cell growth media, depleted from antibiotics. ON-TARGET plus SMART pool siRNAs targeting the human RhoA or non-targeting siRNA used as a negative control were purchased from Dharmacon. Each siRNA was used at a concentration of 20 nmol/l and transfected into cells using the lipofectamine reagent according the manufacturer's protocol. Effects of siRNA on mRNA and protein expression were assessed by qRT-PCR and flow cytometry at 24 and 96 h post-transfection, respectively. The day before transfection, cells were plated in 6-well dishes

at the density of 3×10^5. Transfection was carried out by using 100 nmol/l of SMARTpool and 6 μl of DharmaFECT (Dharmacon). Cells were harvested 48 h after transfection and analyzed by FACS. The sequences selected for the sense and antisense strands are for anti-RhoA siRNA, sense 5′-GACAUGCUUGCUCAUAGUCTT-3′, antisense 3′-TTCUGUACGAACGAG-UAUCAG-5′; for the control siRNA, sense 5′-CAGU-CAGGAGGAUCCAAAGTG-3′, antisense 3′-TTGUCAGUCCUCCUAGGUUUC-5′.

Flow cytometry

The expression of CD11b (Mac-1), CD35 (CR1) and CD66 was analyzed on a flow cytometer (FACSort; Becton Dickinson), as previously described [26] and results were reported as mean fluorescence TSEM. Antibodies CD11b, CD35 and CD66 were purchased from Becton Dickinson (San Jose, CA). Control of isotype-matched Ab was assayed in parallel.

Measurement of intracellular calcium concentration

Free intracellular calcium was determined in purified macrophages loaded with the calcium-sensitive dye Fluo-3/AM and calcium concentration was calculated as previously described [26, 27].

## Assessment of H_2O_2 and matrixmetalloproteinase-9 (MMP-9) production

Intracellular production of H_2O_2 was analyzed on a Perkin Elmer LS50B luminescence spectrometer (Fluometer). Macrophages (106/ml) were incubated at 37 °C for 20 min with 20 mM 2,7-dichlorofluorescein(DCF) diacetate (Molecular Probes, Eugene, OR, USA). After labeling, cells were treated, and the production of H_2O_2 was then monitored every 10 min by measuring DCF emission at 525 nm; results were expressed as relative fluorescence intensity SEM. MMP-9 production was determined by a gelatin assay as previously described [28, 29].

Measurement of cAMP levels

Macrophages were plated at 2×10^5/ml in 12-wells in DMEM cell growth media, depleted from antibiotics and serum. cAMP assay kit was achieved using standard kit from R and D according to the manufacturer protocol.

Statistical analysis

Statistical differences were determined with Student's t-test or Wilcoxon matched pairs test using Prism 3.0 statistical software (Graphpad, San Diego, CA). In experiment where
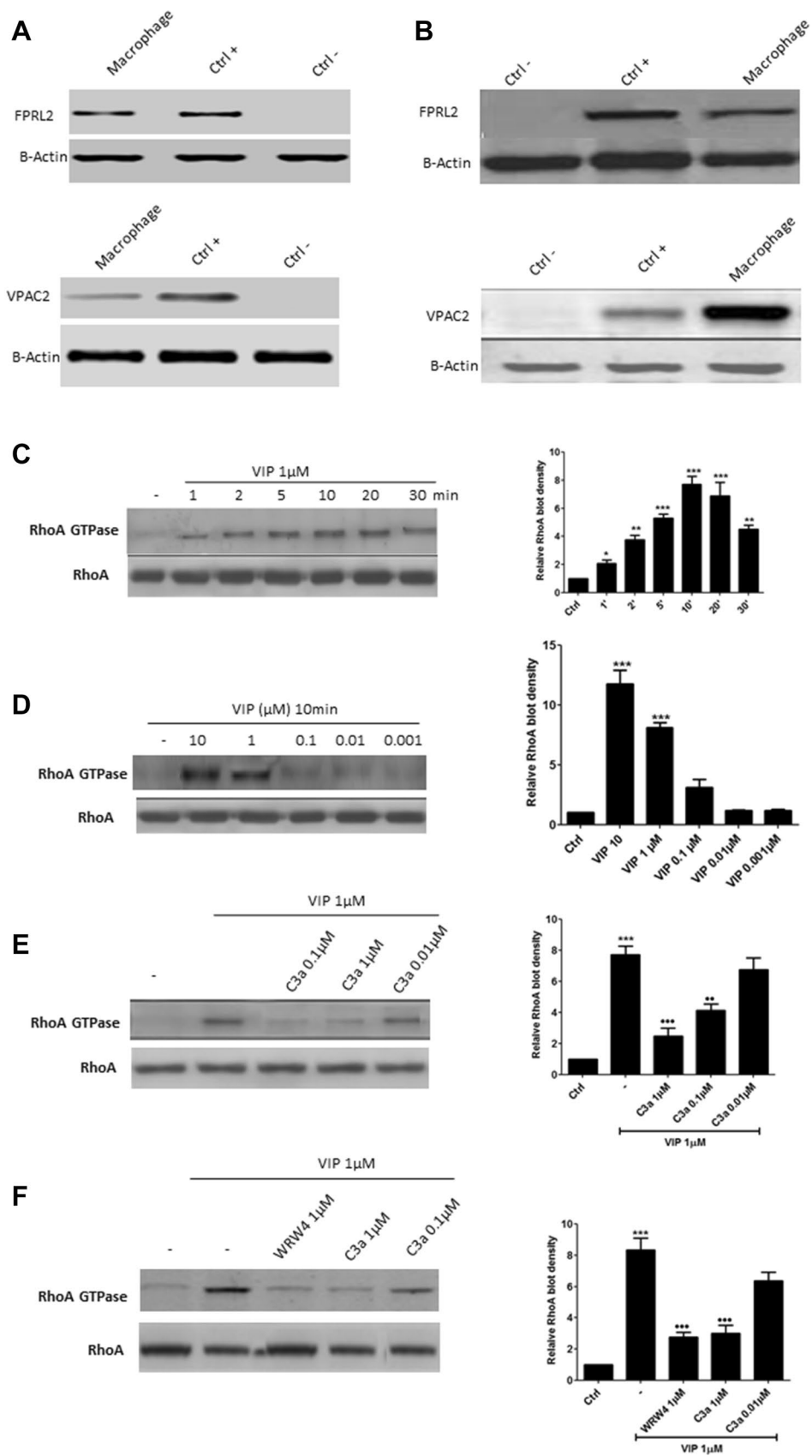
VIP induces GTP Rho-GTPase in macrophages, in an FPRL2-dependent manner. In **a** RNA was isolated from macrophages and control cells as indicated. Positive control (Ctlr+) was done using HCT116 colon cancer line cDNA positive for FPRL2 expression. The negative control (Ctrl−) was done using CHO cell lines negative for FPRL2 expression. Positive controls (Ctlr+) was done using SUP T1 cell cDNA (VPAC-2 receptor). The negative control (Ctrl−) was done using CHO cell lines negative for VPAC-2 expression. The expression levels were normalized to beta-actin. In **b** proteins were extracted from macrophages and control cells as indicated. Western blotting was used to assess the expression levels of both FPRL2 and VPAC proteins. The expression levels were normalized to beta-actin protein content. In **c** macrophages were incubated with either vehicle or with 1 μM of VIP for various time (1–30 min) as indicated. Cell lysates were then analyzed by western blot using antibody raised against GTP-RhoA-GTPase. The expression levels were normalized to RhoA protein content and signal intensity was quantified by densitometry ($n = 3$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. vehicle control. In **d** macrophages were incubated with either vehicle or with increasing concentrations of VIP (0.001–10 μM) for 10 min Cell lysates were then analyzed by western blot using antibody raised against GTP-RhoA-GTPase. The expression levels were normalized to RhoA protein content and signal intensity was quantified by densitometry ($n = 3$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. vehicle control. In **e** macrophages were incubated for 10 min with either vehicle, VIP (1 μM) alone, or VIP (1 μM)+C3a (0.01–1 μM). The cells lysates were then analyzed by western blot using antibody raised against GTP-RhoA-GTPase. Expression levels were normalized to RhoA protein content and signal intensity was quantified by densitometry ($n = 3$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. vehicle control. In **f** macrophages were incubated for 10 min with either vehicle, VIP (1 μM) alone, VIP (1 μM)+WRW4 (1 μM), or VIP (1 μM)+C3a (0.1 or 1 μM). The cells lysates were then analyzed by western blot using antibody raised against GTP-RhoA-GTPase. Expression levels were normalized to RhoA protein content and signal intensity quantified by densitometry ($n = 3$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. vehicle control

cells were exposed to VIP and inhibitors, differences were regarded as significant when *($p < 0.05$), **($p < 0.01$), or ***($p < 0.001$), versus control untreated cells.

Results

VIP induced RhoA-GTPase in monocytes-derived macrophages, in an FPRL2-dependent manner

The presence of FPRL2 and VPAC2 in macrophages was validated on the protein and gene expression levels by Western blotting and PCR, respectively. The results confirmed that both FPRL2 and VPAC2 (Fig. 1a, b) are indeed expressed in macrophages.

In order to investigate the effect of VIP on RhoA-GTPase activation in monocytes-derived macrophages, the cells were incubated with various concentrations of VIP for different durations of time. The time-course experiments showed that RhoA-GTPase was induced shortly after one minute of treating the cells with 1 μM of VIP (Fig. 1c). Additionally, VIP dose-dependently induced RhoA-GTPase, with a

maximum induction level at a dose of 10 μM (Fig. 1d). The co-treatment of cells with VIP and a specific RhoA-GTPase inhibitor, C3a transferase (C3a), reversed the observed effect of VIP on RhoA-GTPase (Fig. 1e). Similarly, the selective FPRL2 inhibitor, WRW4, also reversed the VIP-induced RhoA-GTPase induction (Fig. 1f). These results indicated that VIP activates RhoA-GTPase, and the mechanism of activation potentially involves FPRL2 receptor.

VIP induced the expression of CD11b, CD35, CD66 in a GTP-RhoA-GTPase-dependent manner

We further investigated the effect of VIP on the expression of levels of CD11b, CD35 and CD66 markers by flow cytometry. Our results indicated that VIP significantly induced CD11b expression in a dose-dependent manner, with a maximum expression levels induced at 10^{-5} M of VIP (Fig. 2b). Additionally, the time-course experiments showed that the significant increase of CD11b expression was observed within 15 min of VIP treatment and reached a maximum expression after 90 min of treatment (Fig. 2a). Interestingly, the effect of VIP on CD11b expression was significantly inhibited by the GTP-RhoA-GTPase inhibitor, C3a (1 μM), and by the phospholipase C inhibitor, U73122 (10 μM). However, the inhibition of protein kinase A (PKA) by H89 had no significant effect on the activity of VIP (Fig. 2c). Similarly, the exposure of macrophages to VIP induced a significant increase in the expression of CD35 and CD66, reaching a maximum expression level at a dose of 10^{-5} M VIP. This effect was also inhibited by the GTP-RhoA-GTPase inhibitor C3a (1 μM) and the phospholipase C inhibitor U73122 (10 μM), but not by the PKA inhibitor H89 (10 μM) (Fig. 2d–i). Furthermore, VIP-induced CD11b expression in a RhoA dependent manner, since, RhoA knockdown significantly reversed VIP effect (Fig. 2j). Collectively, our data indicated that VIP induced the expression of CD11b, CD35 and CD66 in macrophages in a GTP-RhoA-GTPase and PLC-dependent but PKA independent manner.

VIP activates ERK1/2, Akt and P38 signaling, and it induces MMP9 expression

To further investigate the signaling pathways activated downstream VIP and GTP-RhoA-GTPase, we conducted time-course response and dose–response experiments. Treatment of macrophages with 1 μM of VIP induced a significant increase in phopsho-ERK1/2, phospho-Akt and phosphp-P38 levels shortly after 1 min of stimulation. This observed effect of VIP on ERK 1/2 and Akt induction was reversed by the GTP-RhoA-GTPase inhibitor C3a, but was not affected by H89 or U73122. Interestingly, only inhibition of PKA with H89 was able to significantly reduce
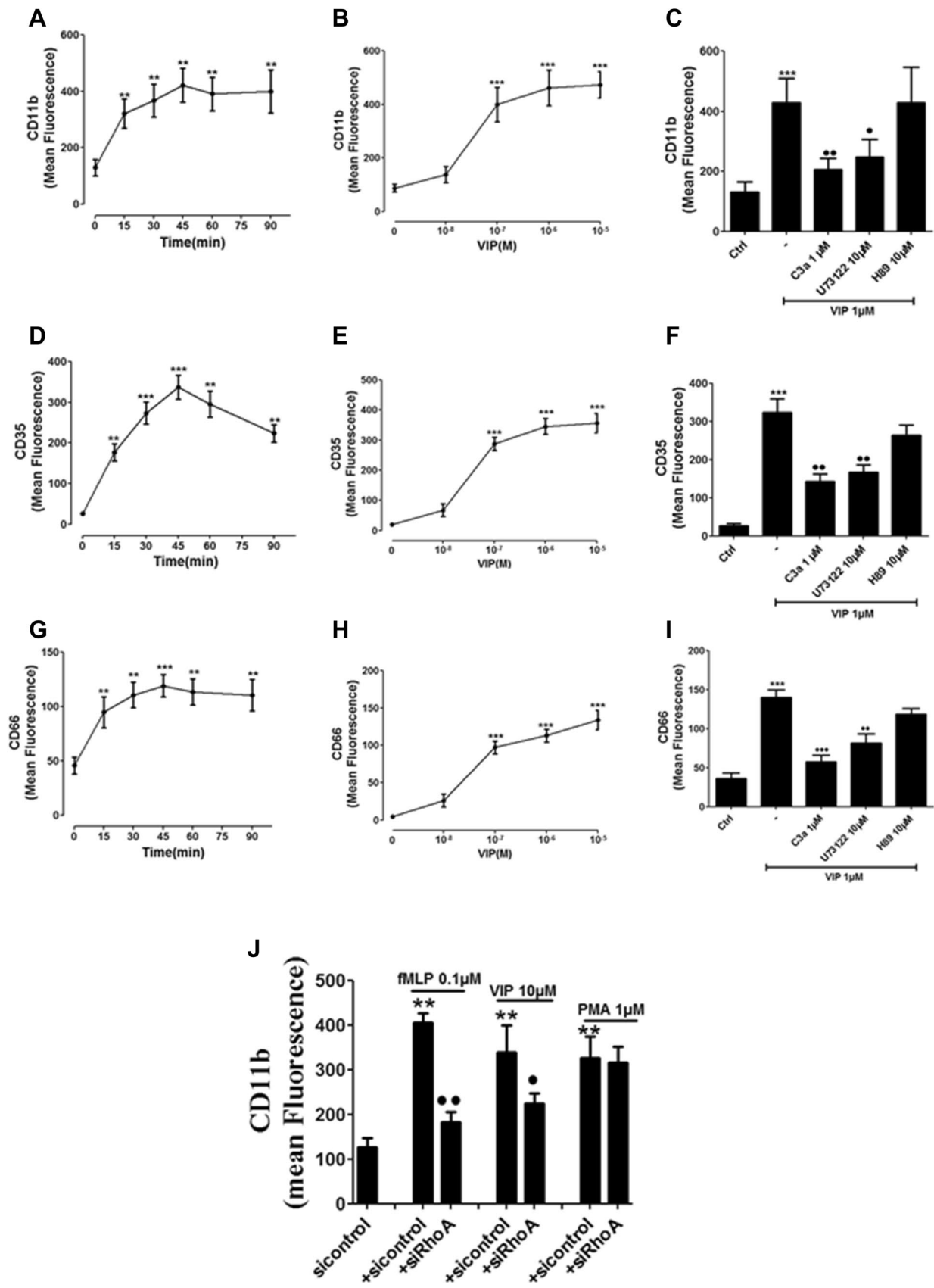
◄**Fig. 2** VIP induces the expression of CD11b, CD35, CD66 in an RhoA-GTPase-dependent manner. In **a** time-course expression levels of CD11b measured by flow cytometry in macrophages treated for up to 90 min with VIP (1 μM) as indicated. In **b** CD11b expression levels were measured by flow cytometry in macrophages treated with increasing doses (10^{-8} to 10^{-5} μM) of VIP as indicated. In **c** CD11b expression levels were measured in macrophages incubated with either vehicle, VIP (1 μM) alone, VIP (1 μM)+C3a (1 μM), VIP (1 μM)+U73122 (10 μM) or VIP (1 μM)+H89 (10 μM) ($n = 3$), **$p < 0.01$) ***$p < 0.001$ vs. VIP alone. In **d** the time-course expression levels of CD35 measured by flow cytometry in macrophages treated for up to 90 min with VIP (1 μM) *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. vehicle control. In **e** the expression levels of CD35 measured by flow cytometry in macrophages treated with different doses (10^{-8} to 10^{-5} μM) of VIP. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. vehicle control. In **f** the expression levels of CD35 were measured in macrophages incubated with either vehicle, VIP (1 μM) alone, VIP (1 μM)+C3a (1 μM), VIP (1 μM)+U73122 (10 μM) or VIP (1 μM)+H89 (10 μM). ($n = 3$) ***$p < 0.001$ vs. vehicle control.·*$p < 0.01$ vs. VIP alone. In **g** the time-course expression levels of CD11b measured by flow cytometry in macrophages treated for up to 90 min with VIP (1 μM). ($n = 3$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. vehicle control. In **h** the expression levels of CD11b measured by flow cytometry in macrophages treated with different doses (10^{-8} to 10^{-5} μM) of VIP. *$p < 0.01$, ***$p < 0.001$ vs. dose 0. In **i** the expression levels of CD11b were measured in macrophages incubated with either vehicle, VIP (1 μM) alone, VIP (1 μM)+C3a (1 μM), VIP (1 μM)+U73122 (10 μM) or VIP (1 μM)+H89 (10 μM). ($n = 3$) ***$p < 0.001$ vs. vehicle control.·$p < 0.01$ vs. VIP alone. In **j** macrophages were incubated with either RhoA siRNA or with control fMLP (0.1 μM), VIP (1 μM) or PMA (1 μM) for 10 min. The expression levels of CD11b were then measured under the different studied conditions. ($n = 3$) ***$p < 0.001$, **$p < 0.01$ vs. vehicle control. $p < 0.005$ and·$p < 0.01$ vs. treated siControl

phospho-P38 levels induced by VIP (Fig. 3a–d). These data suggested that VIP activated ERK1/2 and Akt in a GTP-RhoA-GTPase dependent manner, but p38 MAPK in a PKA-dependent manner**.** As shown in Fig. 3e, knock down of RhoA with specific siRNA significantly decreased the ability of fMLP and VIP to induce Akt phosphorylation. However, RhoA knockdown was without effect phopsho-AKT levels induced by the phorbol ester PMA (Fig. 3f). These results indicated that both VIP and fMLP activated Akt pathway activation through GTP-RhoA-GTPase pathway activation.

We further assessed the effect of VIP on pro-MMP9 expression levels. Time-course experiments showed that VIP induced pro-MMP9 expression after 5 min of stimulation, with a maximum expression level reached after 10 min of stimulation (Fig. 3g). VIP induced expression of pro-MMP9 was inhibited by H89 but not C3a (Fig. 3h). The latter suggests that VIP mediated pro-MMP9 expression via the PKA pathway, but independently of GTP-RhoA-GTPase.

### VIP induces H₂O₂ and intracellular calcium release in macrophages, in a GTP-RhoA-GTPase dependent manner

We finally investigated the effect of VIP on the intracellular calcium signaling in macrophages. Accordingly, treatment

of macrophages with 0.1 μM of VIP induced a significant rise in intracellular calcium, peaking after 100 s of stimulation. This effect was significantly abrogated by either C3a or U73122 treatment (Fig. 4a, b). Similarly, treatment of macrophages with 1 μM of VIP induced a significant increase in H_2O_2 levels reaching a maximum after 20 min of treatment. H_2O_2 levels induced by VIP were significantly abrogated by C3a and U73122, but were not affected by H89 (Fig. 4c, d). Furthermore, GTP-RhoA-GTPase knockdown by siRNA technology inhibited H_2O_2 levels induced by fMLP, VIP or PMA respectively (Fig. 4e). Finally, we investigated the levels of intracellular cAMP induced in monocytes/macrophages challenged with VIP. Accordingly, VIP induced intracellular concentration of cAMP in a timely manner that peaked after 3 minutes of stimulation (Fig. 4f). Inhibition of RhoA with C3a (1 μM) was without effect in this regard (Fig. 4g).

Collectively, our data suggest that VIP regulated the release of H_2O_2 and calcium in macrophages through activation of GTP-RhoA-GTPase and PLC. Additionally, fMLP regulated H_2O_2 release in macrophages through GTP-RhoA-GTPase. Also, VIP also regulated increased intracellular cAMP in macrophages through the GTP-RhoA-GTPase pathway.

Discussion

Inflammation is the defense mechanism that is irreplaceably vital to health [30]. During an inflammatory response, monocytes selectively traffic to the sites of inflammation, where they produce inflammatory cytokines and differentiate into inflammatory macrophages [31, 32]. The infiltration of monocytes into the inflammation site involves a crucial step called diapedesis, which is the extravasation of cells through the endothelium [33]. The retraction of endothelial cells is one of the mechanisms that increase the endothelial permeability to allow the infiltration of monocytes [34]. The phosphorylation of the myosin like chain and the reorganization of the cytoskeleton are two important molecular mechanisms for increasing the endothelial permeability and for facilitating immune cells infiltration [35–38]. The reorganization of F-actin during the organization of the cytoskeleton is considered a key step for the retraction of both endothelial and immune cells [39]. The molecular events regulating the cytoskeleton reorganization in immune cells were previously reported to be highly regulated by the Rho family of GTPases [40]. Additionally, Rho GTPases were also reported to regulate the migration, polarization and adhesion of neutrophils [41]. In macrophages, Rho-GTPases were suggested to be required for the phagocytosis of apoptotic cells [25]. Moreover, Rho GTPases were shown to be required for the CSF-1-induced migration of macrophages to
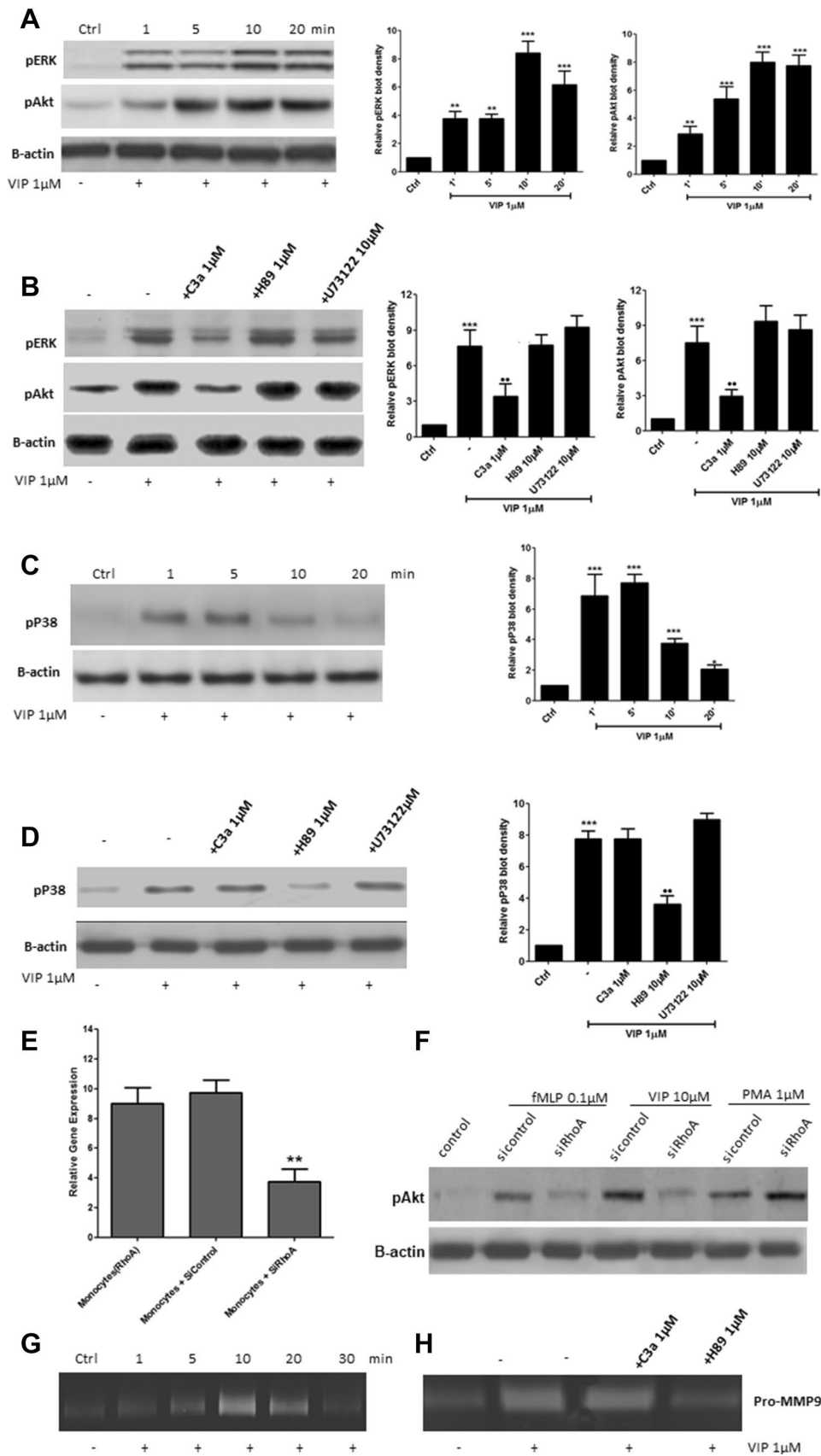
◄**Fig. 3** VIP activates ERK1/2, Akt and P38 and induces MMP9 expression in monocytes-derived macrophages. In **a** macrophages were incubated with either vehicle or with VIP (1 μM) for various durations of time (1–20 min). Cell lysates were then analyzed by Western blot using antibodies raised against phospho-ERK1/2 and phospho-AKT. The expression levels were normalized to beta-actin protein content and the signal intensity was quantified by densitometry ($n=3$), $*p<0.05$, $**p<0.01$, $***p<0.001$ vs. vehicle control. In **b** macrophages were incubated with either vehicle, VIP (1 μM) alone, VIP (1 μM)+C3a (1 μM), VIP (1 μM)+U73122 (10 μM) or VIP (1 μM)+H89 (10 μM). Cell lysates were then analyzed by western blot using antibodies raised against phospho-ERK1/2 and phospho-AKT. The expression levels were normalized to beta-actin protein content and the signal intensity was quantified by densitometry ($n=3$), $*p<0.05$, $**p<0.01$, $***p<0.001$ vs. vehicle control. In **c** macrophages were incubated with either vehicle or with VIP (1 μM) for various durations of time (1–20 min). Cell lysates were then analyzed by western blot using antibody raised against phospho-P38. The expression levels were normalized to beta-actin protein content and the signal intensity was quantified by densitometry ($n=3$), $*p<0.05$, $**p<0.01$, $***p<0.001$ vs. vehicle control. In **d** macrophages were incubated with either vehicle, VIP (1 μM) alone, VIP (1 μM)+C3a (1 μM), VIP (1 μM)+U73122 (10 μM) or VIP (1 μM)+H89 (10 μM). Cell lysates were then analyzed by western blot using antibodies against phospho-p38. The expression levels were normalized to beta-actin protein content and the signal intensity was quantified by densitometry ($n=3$), $*p<0.05$, $**p<0.01$, $***p<0.001$ vs. vehicle control. In **e** macrophages were incubated with either vehicle, RhoA siRNA or with control siRNA for 48 h prior to stimulation. Then, 5 μg of the total isolated RNA were used to assess the expression level and the relative gene expression level of GTP-RhoA-GTPases by RT-PCR. In **f** macrophages were incubated with either vehicle, RhoA siRNA or with control siRNA for 48 h prior to stimulation. The cells were then incubated with vehicle, fMLP (0.1 μM), VIP (10 μM) or PMA (1 μM). Cell lysates were then analyzed by western blot using antibodies against phospho-AKT. The expression levels were normalized to beta-actin protein content and the signal intensity was quantified by densitometry ($n=3$), $*p<0.05$, $**p<0.01$, $***p<0.001$ vs. vehicle control. In **g** pro-MMP9 expression levels in macrophages incubated with either vehicle or VIP (1 μM) for up to 30 min. In **h** pro-MMP9 expression levels of in macrophages incubated with either vehicle, VIP (1 μM), VIP (1 μM)+C3a (1 μM) or VIP (1 μM)+H89 (1 μM). ($n=3$), $*p<0.05$, $**p<0.01$, $***p<0.001$ vs. vehicle control

the inflammation site [42]. Although the prominent roles of Rho GTPases are well described in macrophages and other immune cells, the detailed signaling pathways activated downstream them are not well characterized yet. Having in mind that VIP has different important VPAC1-dependent functions in macrophages, investigating VIP-induced Rho activation and possible downstream signaling pathways represents a critical key step to help understand the behavior of macrophages during inflammation. In the current study, we reassured the presence of VPAC1 in our model of monocytes-derived macrophages, and we showed that VIP induces the expression of RhoA GTPases through the VPAC1 receptor. Interestingly, we have also shown that the VIP-induced RhoA GTPase activation was not only mediated through VPAC1, but also partially depended on FPRL2 receptor. FPRL2 is the receptor of fMLP, which is a strong

leucocyte chemoattractant [43]. In a previous paper for our team, we've shown that fMLP activated Akt and ERK1/2 in an RhoA-GTPase-dependent manner [44]. This provoked us to investigate VIP signaling in macrophages using the same approaches and additionally assessing any cross link between VIP and fMLP. The additional knowledge we provide not only helps in understanding downstream signaling pathways of VIP, but also guides the deciphering of complex interlinks between VIP and fMLP signaling pathways.

Downstream RhoA GTPase activation, VIP further induced the activation of Akt and ERK 1/2. However, the activation of these kinases was independent of PKA and PLC. In macrophages, the PI3K/Akt/mTOR pathway mediates signals from different receptors including cytokine receptors, insulin receptors and pathogen-associated molecular pattern receptors [45]. The Akt pathway converges inflammatory and metabolic signals to regulate macrophage responses and modulates the phenotype activation. It also regulates the M1/M2 polarization of macrophages. Identifying VIP as an activator of Akt pathway points toward possible functional desired effects for VIP in macrophages, which shall be investigated following the current investigation of the signaling pathways. Moving to the MAPKs, p38 and ERK1/2, a wide array of functions has been reported in literature. These functions include macrophages development [46], cytokines and cox-2 secretion [47, 48] and LPS-induced cellular activation [49]. Besides, VIP was reported to inhibit the production of TNF, IL-6, IL10 and IL12 in LPS-stimulated macrophages, in addition to inhibiting COX2 expression in activated macrophages [7–10]. These functions for VIP in macrophages were not investigated in terms of signaling. We hereby suggest that the above stated roles for VIP in macrophages could most probably have been played through the MAPKs—dependent signaling. The fact that VIP is able to activate these kinases again suggests prominent expected functions for VIP in macrophages, which should be further investigated also.

In addition to these results, we have also shown that VIP induces intracellular calcium and H_2O_2 release in macrophages in an RhoA- and PLC-dependent manner. However, this activity was independent of PKA. This suggests that the VIP-Rho-GTPases axis is involved in controlling the response of macrophages to stress signals through regulating ROS and calcium production. The signaling pathways linking VIP/RhoA-GTPase to the release of ROS and calcium, should still be further investigated.

CD11b is major molecule that controls activation and migration of macrophages into the inflammatory sites. Interestingly, CD11b negatively regulated the immune system and abrogated the autoreactive B-cell response in systemic lupus erythematous [50]. Furthermore, its activation impaired the accumulation of intracellular lipid droplet and thus impeded IL-13—induced differentiation of
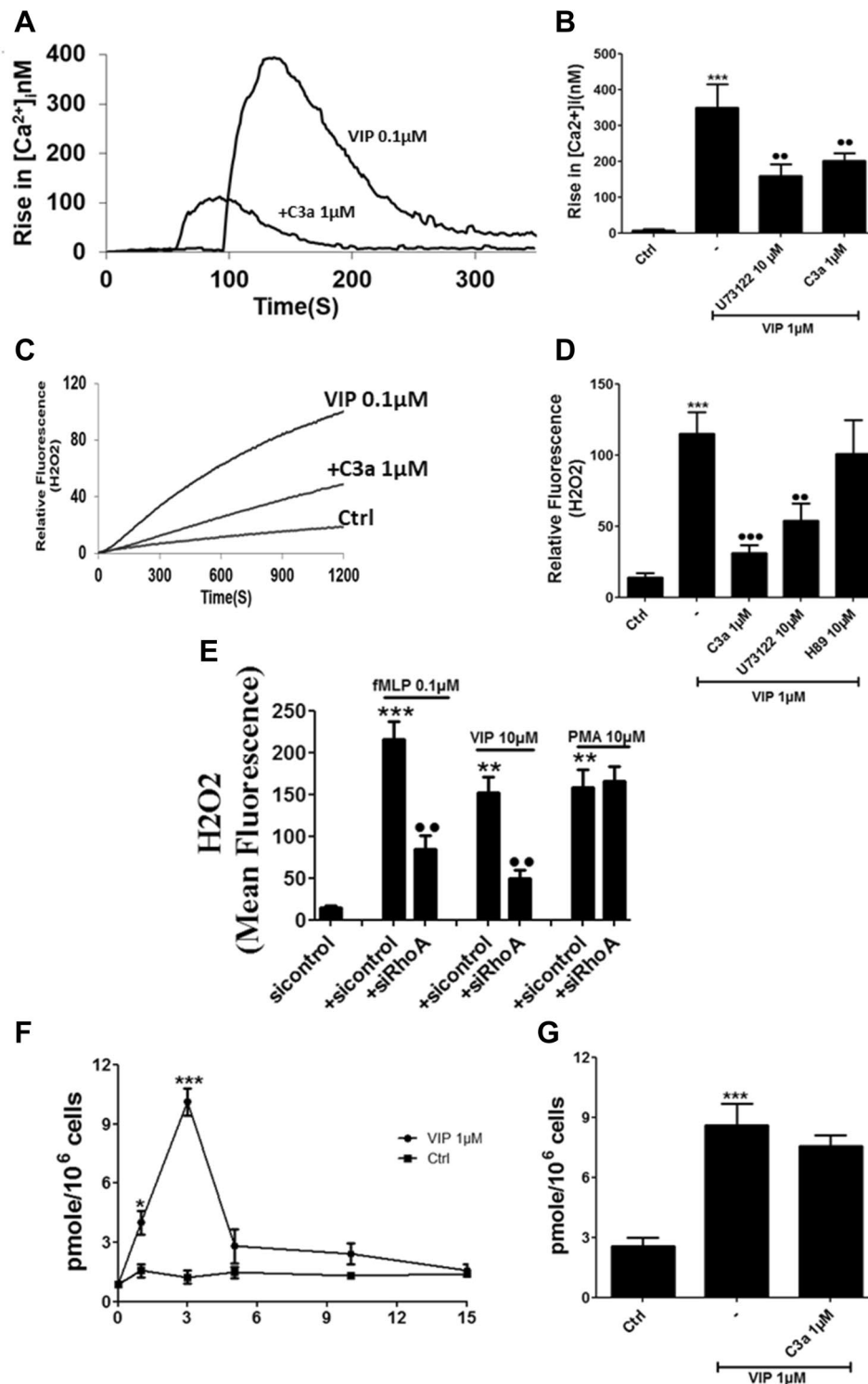
macrophages into foam cells [51]. Finally, CD11b protected animals from endotoxic chock by inhibiting Toll-like receptor signalling in macrophages [52]. Therefore, assessing the expression level of CD11b in VIP-treated macrophages is of considerable importance. Furthermore, we previously showed that upregulation of the complement receptor 1

(CD35) and MMP9 are associated with proinflammatory exocytosis mechanism in macrophage induced by VIP [53]. Finally, CD66 is marker of macrophages adhesion [54].

We previously showed that VIP mediated its proinflammatory effect through specific G protein-coupled receptor VIP/pituitary adenylate cyclase-activating protein

◄**Fig. 4** VIP induces H_2O_2 and calcium release in macrophages, in an RhoA-GTPase dependent manner. In **a** intracellular calcium concentrations were measured by luminescence spectrometry in macrophages incubated with VIP (1 μM) or C3a (1 μM). In **b** intracellular calcium concentrations were measured by luminescence spectrometry in macrophages incubated with vehicle, VIP (1 μM) alone, VIP (1 μM)+C3a (1 μM) or VIP (1 μM)+U73122 (10 μM). ($n=3$) ***$p<0.001$ vs. vehicle control. $p<0.01$ vs. VIP alone. In (**c**), H_2O_2 levels of were measured in macrophages incubated with vehicle, VIP (1 μM) alone or VIP (1 μM)+C3a (1 μM). In **d** H_2O_2 levels were measured in macrophages incubated with vehicle, VIP (1 μM) alone or VIP (1 μM)+C3a (1 μM), VIP (1 μM)+U73122 (10 μM), VIP (1 μM)+H89 (10 μM). (**$n=3$**) ***$p<0.001$ vs. vehicle control. $p<0.01$ vs. VIP alone. In **e** macrophages were incubated with either vehicle, RhoA siRNA or with control siRNA for 48 h prior to stimulation. The cells were then incubated with vehicle, fMLP (0.1 μM), VIP (10 μM) or PMA (1 μM). The levels of H_2O_2 were then measured under the different studied conditions. ($n=3$), ***$p<0.001$, **$p<0.005$ vs. vehicle control. $p<0.005$ vs. treated siControl. In **f** intracellular cAMP levels were measured in macrophages incubated with either vehicle or with VIP (1 μM) for up to 15 min. ($n=3$), *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. vehicle control. In **g** intracellular cAMP concentrations were measured in macrophages incubated for three minutes with either vehicle (name), VIP (1 μM) or VIP (1 μM)+C3a (1 μM). ($n=3$), *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. vehicle control

(VPAC1) receptor, and via FPRL1. VIP/VPAC1 activated cAMP/P38MAPK pathway that modulated the expression of CD11b, CD35 and MMP9. Furthermore, VIP/VPAC1 interaction activated cAMP/EPAC/PI-3 K/ERK that modulated CD11b expression [53]. Furthermore, we showed that fMLP induced AKT and ERK activation through ROS/RhoA pathway in cultured macrophages [44]. Interestingly, our results showed that VIP increases the expression of the complement receptors CD11b, CD35 and CD66, all of control important macrophage functions. These data indicated that VIP play a crucial role in regulating important functions in macrophages.

In our study, we used pharmacologic inhibitors to assess some of the signaling kinases involved in VIP effect. Although, these inhibitors are known to be specific, we cannot fully rule out potential off target effects. While in vitro cell culture systems represent the mainstay biologic models, it will not replace in vivo environment offered by in vivo rodent model. In order to minimize such effect, we used human monocytes instead of cell lines to mimic at best in vivo environment.

In conclusion, our current work reassures VIP as a key player in macrophages signaling and functions. We present VIP RhoA GTPases-Akt/ERK axes as prominent pathways in the regulation of macrophages functions. We showed that VIP induced the above signaling pathways downstream RhoA-GTPase through its VPAC1 receptor and dependently on FPRL2 receptor. It regulates the expression of the three compliments CD11b, CD35 and CD66, in addition to provoking the release of intracellular H_2O_2 and calcium.

Compliance with ethical standards

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