Biochemical Pharmacology 80 (2010) 1012–1020

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Amiloride derivatives modulate PS externalization in neutrophil-like PLB-985 cells

Mickael Bourge [a,b], Asma Tlili [a,b], Sophie Dupré-Crochet [a,b], Oliver Nüβe [a,b], Jean-Claude Sulpice [a,b,*]

[a] Univ Paris-Sud, Orsay F-91405, France
[b] INSERM, U757, Orsay F-91405, France

ARTICLE INFO

Article history:
Received 22 April 2010
Accepted 9 June 2010

Keywords:
Amiloride derivative
Phosphatidylserine
ER-stress
Ischemia/reperfusion
Phagocyte

ABSTRACT

During brain or cardiac ischemia/reperfusion neutrophils are recruited and activated contributing to inflammation and tissue damage. Neutrophils are removed from inflamed tissues by phosphatidylserine-dependent phagocytosis. Production of reactive oxygen species by the neutrophil NADPH-oxidase is known to affect phosphatidylserine externalization. Amiloride derivatives are inhibitors of the sodium–proton exchanger providing substantial protection in animal models of brain and cardiac ischemia/reperfusion injury; however their effects on neutrophils remain incompletely known. We investigated the effect of 5-(N,N-hexomethylene)amiloride (HMA) on phosphatidylserine externalization in wild type and NADPH-oxidase deficient PLB-985 cells differentiated into neutrophils. We show that HMA had a dual effect: (1) 60 μM HMA induced phosphatidylserine externalization in at least 40% of the cells; (2) 20 μM HMA had no direct effect but enhanced phosphatidylserine externalization induced by cell activation with PMA or calcium ionophore A23187. Both effects were independent of the NADPH-oxidase and were not due to changes in intracellular pH. 60 μM HMA induced a capacitative calcium entry which was necessary for phosphatidylserine externalization. The HMA-induced PS externalization was inhibited by salubrinal, an inhibitor of ER-stress-linked apoptosis. Lower HMA concentration enhanced PMA or A23187 effects through PKC and calcium dependent pathways. The caspase inhibitor Z-VAD-FMK weakly diminished phosphatidylserine externalization, suggesting that activation of caspases 7, 8, 9 and 3 was not involved. Increasing phosphatidylserine externalization by low concentrations of HMA improved the engulfment of PMA-activated PLB-985 cells by macrophages, providing a novel therapeutic strategy to limit the accumulation of neutrophils in injured tissues.

1. Introduction

Ischemia/reperfusion injury plays a central role in the development of tissue injury in cardiovascular, kidney and central nervous system diseases, including acute stroke. Neutrophil recruitment and activation are major components of ischemia/reperfusion pathophysiology [1,2] and may be a target for therapeutic intervention [3]. Sodium–proton exchanger (NHE) inhibitors have emerged as important therapeutic options for the attenuation and treatment of organ failures during ischemia/reperfusion [4–6]. Amiloride derivatives are efficient NHE-1 inhibitors and depending on their concentration have been also implicated in the induction of apoptosis or growth inhibition [7,8]. However the physiopathologic and therapeutic effects of amiloride derivatives on neutrophils have been poorly investigated. Neutrophil apoptosis induced by amiloride derivatives has been mainly attributed to cytosolic acidification consecutive to the NHE inhibition [9,10]. In endothelial cells, amiloride derivatives induce endoplasmic reticulum (ER) calcium depletion and subsequent ER-stress leading to apoptosis independent of NHE inhibition and acidification [7]. ER-stress response acts to restore normal ER homeostasis and is therefore cytoprotective. However, if ER-stress is too strong or persistent, programmed cell death can be engaged, a process that is implicated in hypoxia, ischemia/reperfusion injury, neurodegeneration, heart disease, and diabetes [11].

IRE1, ATF6 and PERK are the three main ER-stress transducers. They are inactive due to their binding to the ER chaperone BiP/Grp78, which is a key regulator of the ER-stress response. When

Abbreviations: APLT, aminophospholipid translocase; Carboxy-H_2DCFDA-AM, carboxy-H_2-dichloro-dihydrofluorescein-diacetate-diacetoxy methylester; CGD, chronic granulomatous disease; EIPA, 5-(N-ethyl-N-isopropyl)amiloride; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; FCS, fetal calf serum; HMA, 5-(N,N-hexamethylene)amiloride; KO, knockout; NADPH, nicotinamide adenine dinucleotide phosphate-oxidase (reduced form); NHE, Na^+/H^+ exchanger; PI, propidium iodide; PMA, phorbol-12-myristate-13-acetate; PS, phosphatidylserine; ROS, reactive oxygen species.

* Corresponding author at: INSERM, U757, University Paris-Sud 11, Building 443, 15 rue Georges Clémenceau, F-91405 Orsay, France. Tel.: +33 01 69 15 76 44; fax: +33 01 69 15 58 93.
E-mail address: jean-claude.sulpice@u-psud.fr (J.-C. Sulpice).

0006-2952/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2010.06.016

proteins misfold in the ER lumen, BiP dissociates from the ER-stress transducers, leading to their activation. PERK activation induces eIF2α phosphorylation protecting against cell death. Conversely, the GADD34/PP1 phosphatase complex leads to eIF2α-P dephosphorylation and overexpression of GADD34 correlates with apoptosis. Inhibition of the GADD34/PP1 phosphatase complex (e.g. by salubrinal), enforcing eIF2α phosphorylation, protects cells against ER-stress-induced apoptosis. IRE1 association with TRAF2 activates cJUN NH_2-terminal kinase (JNK) or caspase cascade *via* caspase 12 (rodent) -4 (humans) cleavage, both pathways leading to cell death. Bcl-2 family members also reside in the ER membrane and influence apoptosis induced by ER-stress. The control of apoptotic pathways activated downstream of ER-stress is not fully known and how these processes might be manipulated to limit tissue injury during inflammation has not been extensively investigated [12,13].

Recognition and removal of apoptotic cells imply cell surface modifications such as externalization of phosphatidylserine (PS). Furthermore, PS-dependent recognition of dying cells by macrophages regulates the release of pro- or anti-inflammatory cytokines *in vitro* and *in vivo* [14].

In quiescent cells, the distribution of major phospholipids in the membrane bilayer is asymmetric. PS and phosphatidylethanolamine are sequestered in the inner leaflet whereas phosphatidylcholine and sphingomyelin are mainly in the outer leaflet. Phospholipid asymmetry is maintained by an ATP-dependent enzyme named aminophospholipid translocase (APLT) [15]. In various physiological conditions (apoptosis, cell activation), phospholipid asymmetry is lost and PS is exposed at the cell surface [16]. Mechanisms driving PS externalization are not yet entirely elucidated and multiple pathways can be implicated depending on the various physiopathologic conditions. Generally, PS externalization slightly precedes apoptosis, however there is growing evidence that PS externalization, which triggers cell recognition and engulfment by macrophages, can be induced independently of classical signs of apoptosis such as caspase activation and/or DNA damages [17–25]. This potentially avoids loss of cell membrane integrity and tissue damage. During acute and chronic inflammation, large numbers of neutrophils become activated and infiltrate the inflamed tissue. Most of these cells will die rapidly and have to be removed to end the inflammatory reaction.

In neutrophils, the production of reactive oxygen species (ROS) through NADPH-oxidase activation is classically implicated in PS externalization in neutrophils death process [26], also participating in cell removal by macrophages [27]. The NAPDH oxidase also liberates large amounts of protons in the cytosol and the NHE has a major role in the control of neutrophil cytosolic pH.

The human promyelocytic leukaemia cell line PLB-985 can be differentiated to acquire major characteristics of mature neutrophils [28–30] in particular the capacity to produce large quantities of ROS through NADPH-oxidase activation [28]. We have investigated the impact of amiloride derivative treatment on PS externalization and cell engulfment by macrophages in the differentiated wild type PLB-985 or CGD-like (chronic granulomatous disease) cell line in which the gene encoding for the NADPH-oxidase subunit $gp91^{phox}$ was disrupted by homologous recombination [31].

In differentiated wild type or $gp91^{phox}$ knockout PLB-985 cells, HMA induced in a dose-dependent manner a rapid PS externalization. Remarkably, HMA at a dose that did not induce a direct effect, drastically enhanced PS externalization induced by cell activation with PMA or calcium ionophore A23187 in both types of cells, suggesting a process independent of oxidative processes consecutive to NADPH-oxidase activation. Consequently, we have investigated the mechanisms of PS externalization specific to amiloride derivatives in $gp91^{phox}$ knockout cells.

2. Materials and methods

2.1. Materials

Phorbol-12-myristate-13-acetate (PMA), 5-(N-ethyl-N-isopropyl)amiloride (EIPA), 5-(N,N-hexomethylene)amiloride (HMA), BAPTA-AM, calcium ionophore (A23187), ionomycin, thapsigargin, nigericin and propidium iodide (PI) were from Sigma (Saint Quentin, France). PKC inhibitor 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide, GF109203X, salubrinal, JNK inhibitor SP600125 and general caspase inhibitor Z-VAD-FMK were obtained from Calbiochem (VWR International, Val de Fontenay, France). Calcium, ROS and pH probes, respectively Fluo4-AM, carboxy-H_2DCFDA-AM and BCECF-AM were from Molecular Probes (Invitrogen, Cergy Pontoise, France). All other chemical were purchased from PROLABO (VWR International, Val de Fontenay, France).

2.2. Cell culture and differentiation of PLB-985 and $gp91^{phox}$ knockout PLB-985 cells

Both human myeloid leukaemia cell lines PLB-985 (a generous gift from Dr. Stasia from Grenoble with the authorization of Dr. Dinauer from Indiana) were cultured and differentiated as previously described [28]. Efficiency of differentiation was routinely estimated by measurement of the cell surface expression of CD11b, CD32, and CD64 by using specific fluorescent antibodies (ImmunoTools, Friesoythe, Germany) and flow cytometry.

In the various experimental conditions, differentiated cells (2×10^6/ml) were incubated at 37 °C in saline medium containing 140 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM Hepes (pH 7.4) and 5% de-complemented fetal calf serum. In calcium free medium, $CaCl_2$ was substituted by 500 μM of EGTA. To impose intracellular pH cells were pre-incubated 30 min in the saline media adjusted at various pH (6.25–8) [32].

2.3. Flow cytometry analysis

For all experiments, fluorescent probes were excited at 488 nm with an argon laser source, and emission was analyzed at 530 nm (FL1) for FITC, carboxy-H_2DCFDA, Fluo4- or BCECF; at 575 nm (FL2) for phycoerythrine, at 630 nm (FL3) for propidium iodide. The cytometer was a FACScan (Becton-Dickinson) driven by the CellQuest software. All the measurements were made with a constant photomultiplier gain and the percentage of gated-events or geometric mean fluorescence was used to quantify the responses.

2.4. Endogenous PS externalization

PS exposure was essayed with the binding of FITC–annexin V (Bender MedSystems, Vienna, Austria) in the presence of PI (1 μg/ml), a marker of plasma membrane integrity. When cells were incubated in calcium free medium, 2 mM calcium was added just before FITC–annexin V and cells were incubated 10 min at room temperature. This subsequent addition of calcium did not influence PS externalization as shown by the absence of FITC–annexin V binding in cells treated with the calcium ionophore A23187 in calcium free medium (see Section 3).

2.5. Cytosolic pH (pH_i) and ROS measurement

Measurement of pH_i was performed by using BCECF-AM and flow cytometry as previously described [33]. PLB-985 cells (2×10^6/ml in saline media) were loaded with 2.7 μM BCECF-AM (30 min at 37 °C). The response of the probe was calibrated by

incubating the loaded cells in calibration media constituted with high-potassium buffers at pH 6.5–8 in the presence of 2 μM nigericin, which exchanged protons against potassium ions and allowed to equilibrate extra- and intracellular pH.

ROS production was estimated by flow cytometry by using the cell-permeant dye carboxy-H_2DCFDA-AM. Cells (2×10^6/ml) were loaded for 30 min at room temperature with 250 nM of carboxy-H_2DCFDA-AM. Hydrogen peroxide (H_2O_2) produced in the extracellular medium by dismutation of superoxide anion can reach the cytosol through the plasma membrane and oxidizes carboxy-H_2DCFDA to a fluorescent compound (DCF, emission at 530 nm). Presence of ROS inside the cells was quantified by the geometric mean of the fluorescence histogram.

2.6. Kinetic of cytosolic calcium variations

Cells were loaded with 2 μM of Fluo4-AM during 30 min at 37 °C, centrifuged and washed in saline medium containing 2 mM calcium or 500 μM EGTA. After various times of incubation, calcium and/or agonists were added and the fluorescence of the cells was measured at 530 nm (FL1 channel) by flow cytometry in time-lapse mode. Necrotic cells were identified by labelling with PI and discarded by gating on the FL3 channel. The variations in $[Ca^{2+}]_i$ were calibrated from the equation: $[Ca^{2+}]_i = K_d[(F - F_{min})/(F_{max} - F)]$, where K_d is the dissociation constant of the Ca^{2+}–Fluo4 complex (345 nM), F_{max} the maximum fluorescence (obtained after addition of 2 μM ionomycin in Ca medium), and F_{min} the minimum fluorescence (obtained in cells loaded with 50 μM of BAPTA).

2.7. Phagocytosis of activated PLB-985 cells by cultured RAW 264.7 macrophages

RAW 264.7 macrophages were seeded onto coverslips in 24 well plates (5×10^4 cells/well) and cultured overnight in RPMI 1640 medium containing 10% FCS. Resting or activated PLB-985 cells were labeled with 6 μM Hoechst 33342 for 15 min at 37 °C. Cells were then washed twice and co-cultured at 2×10^6 cells/ml with macrophages for 1 h at 37 °C. After incubation, unbound cells were washed three times with RPMI 1640 medium and three times with PBS. Adherent cells were fixed with 1% formaldehyde. The cells were examined under a Zeiss fluorescence microscope. A minimum of 100 macrophages were analyzed per experimental condition. To confirm a role of externalized PS in PLB-985 phagocytosis, activated PLB-985 cells were incubated 10 min at 37 °C with 2.5 μg of annexin V before the co-culture with macrophages. Results are expressed as the number of phagocytozed PLB-985 cells per 100 macrophages.

2.8. Data analysis

Data are means ± SE of a minimum of three independent experiments. Differences between means were evaluated by Student's *t*-test with $p < 0.05$ being taken as the level of significance. * means $p < 0.05$.

3. Results

3.1. HMA induced phosphatidylserine externalization independent of NADPH-oxidase in differenciated-PLB-985

To study the mechanisms of PS externalization induced by amiloride derivatives, FITC–annexin V binding and membrane permeability were assessed by flow cytometry. In the wild type (Fig. 1A) or gp91^{phox} knockout PLB-985 cells (Fig. 1B), after 3 h of incubation, HMA induced PS externalization in a dose-dependent manner without loss of membrane integrity (PS+/PI− cells). PS

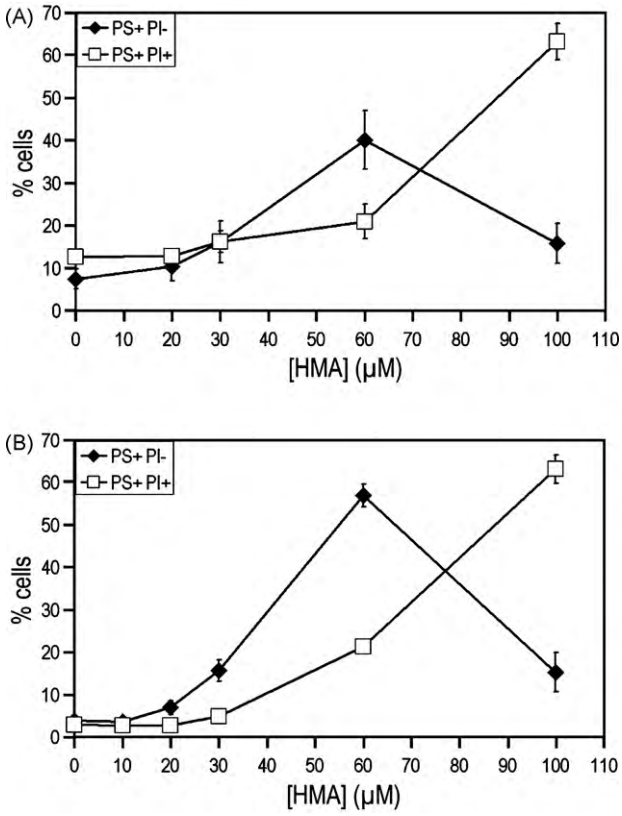

Fig. 1. Dose-dependent effect of amiloride derivative (HMA) on PS externalization in wild type (A) or gp91^{phox} knockout PLB-985 (B) cells. Cells were incubated 3 h at 37 °C in presence of increasing concentration of 5-(N,N-hexomethylene)amiloride (HMA). PS externalization (% of PS+ PI− cells) and necrosis (% of PS+ PI+ cells) were estimated by flow cytometry after binding of FITC–annexin V and addition of propidium iodide (PI). Results are means ± SE, $n \geq 3$.

externalization was not detectable at low concentrations (10 μM or 20 μM) and became significant at 60 μM and remarkably, 100 μM HMA already induced signs of necrosis in a majority of cells (PS+/PI+ cells). HMA at low or highest concentration did not cause detectable ROS production as measured by flow cytometry with the DCF probe (data not shown). In the same condition, 200 nM of PMA, which are able to fully activate NADPH-oxidase [26], induced massive ROS production (basal fluorescence was multiplied by 50) (data not shown).

HMA induced PS externalization in the same manner in wild type or gp91^{phox} knockout PLB-985 cells suggesting that the HMA effect was independent of NADPH-oxidase activation.

3.2. At low concentrations, HMA enhanced phosphatidylserine externalization induced by PMA or A23187 calcium ionophore

During ischemia/reperfusion, many neutrophils are recruited as well as activated in affected tissues. So, we have studied the effect of HMA on PS externalization in PLB-985 cells activated by PMA or the calcium ionophore A23187.

In our conditions, after 3 h of incubation, 200 nM PMA induced PS externalization in 20% (PS+/PI− cells) of the wild type cells (Fig. 2A), whereas in the gp91^{phox} knockout PLB-985 cells PS externalization was limited to about 5% of cells but not totally suppressed (Fig. 2B). Remarkably, in presence of 20 μM HMA, the effect of PMA was multiplied by 2.9 in the wild type cells and by 5.6 in the gp91^{phox} knockout PLB-985 cells (Fig. 2A and B).

This effect of HMA was also reproduced by other amiloride derivatives such as EIPA or DMA (data not shown). Interestingly, the effect of HMA was independent of NADPH-oxidase activation.

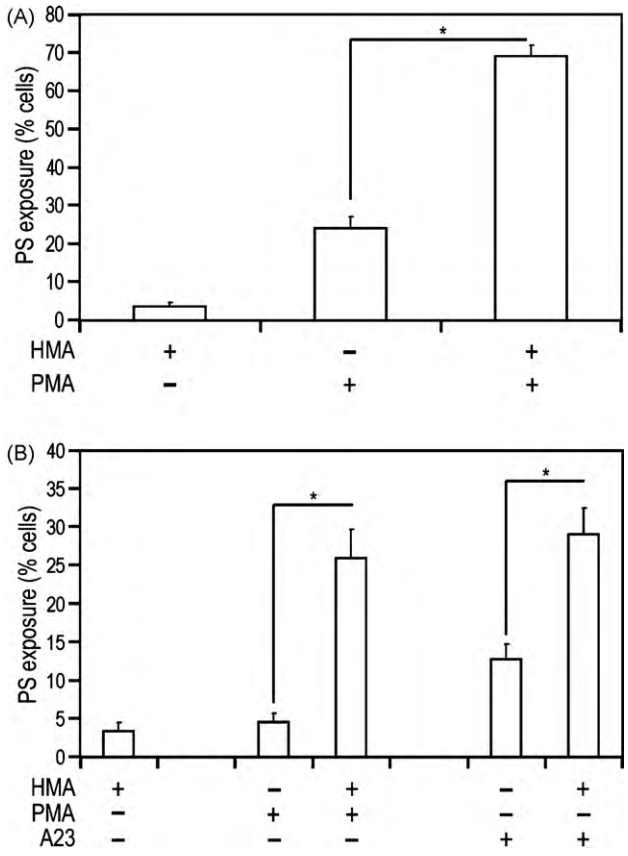

Fig. 2. HMA amplifies PS externalization in cells activated by PMA or calcium ionophore (A23187). Wild type PLB-985 cells (A) were incubated in presence of 200 nM of PMA with or without 20 μM of HMA and gp91^{phox} knockout PLB-985 cells (B) were incubated in presence of 200 nM of PMA or 3 μM of A23187 with or without 20 μM of HMA. PS externalization (% of PS+ PI− cells) was estimated as described in the legend of Fig. 1, and percentages of cells were subtracted from their respective controls. Results are means ± SE, $n \geq 3$.

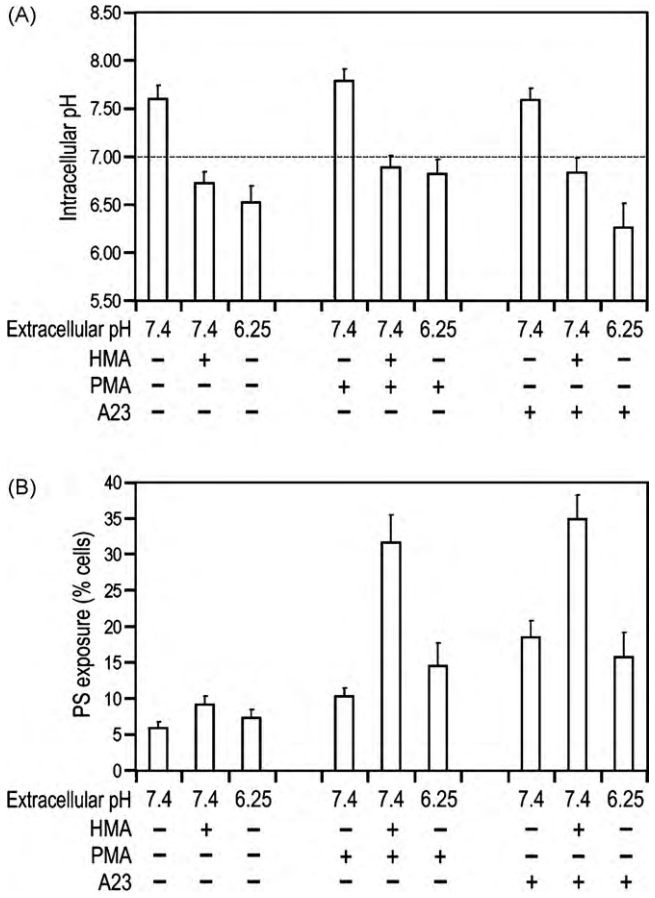

Fig. 3. Effect of imposed cytosolic acidification on PS externalization in gp91^{phox} knockout PLB-985 cells activated or not by PMA or A23187. Cytosolic acidification was imposed by pre-incubation of gp91^{phox} knockout PLB-985 cells 30 min at 37 °C in acidified medium (pH 6.25), and cells were incubated without stimulation or with 200 nM of PMA or 3 μM of A23187, with or without 20 μM HMA. (A) In the various experimental conditions, cytosolic pH (pH_i) was measured after 120 min of incubation by using BCECF-AM loaded cells and flow cytometry and comparison of the fluorescence with that of a calibration range (pH 6.5–8). (B) In similar experimental conditions, PS externalization (% of PS+ PI− cells) was estimated as described in the legend of Fig. 1. Results are means ± SE, $n \geq 3$.

As PS externalization classically implicates an increase in the concentration of the cytosolic calcium [16], we first investigated whether HMA influences the effect of a calcium ionophore on PS externalization. Treatment of gp91^{phox} knockout PLB-985 cells with 3 μM A23187, a concentration able to induce full phospholipid scrambling [34], induced PS externalization in 10% of cells and this effect was multiplied by 2.3 in presence of 20 μM of HMA (Fig. 2B). Thus, low concentrations of HMA increased PS externalization due to at least two modes of cell activation.

3.3. HMA acted on PS externalization independently of its effect on the intracellular pH

The sodium–proton exchanger (NHE) is known to be crucial for the maintenance of cytosolic pH and amiloride derivatives are commonly used as NHE inhibitors. In gp91^{phox} knockout PLB-985 cells, inhibition of NHE with 20 μM HMA induced significant acidification likely due to basal metabolism (Fig. 3A). To investigate the role of intracellular acidification in PS externalization, we have reproduced the effect of HMA on intracellular pH in resting or activated cells, by manipulating the pH of the incubation medium [32]. Incubation of gp91^{phox} knockout PLB-985 cells in medium buffered at a pH of 6.25 resulted in a stabilization of the intracellular pH at about 6.5, a value comparable to the one obtained with 20 μM HMA (Fig. 3A). Activation of cells with PMA or A23187 alone did not significantly influence intracellular pH. After incubation with both agonists in the presence of 20 μM HMA or in pH 6.25 medium, the intracellular pH decreased to 6.8, reaching even 6.30 when the cells were activated by A23187 in pH 6.25 medium. Thus, incubation of cells in medium buffered at pH 6.25 mimicked the effect of HMA on the intracellular pH in resting or activated cells.

As we were able to decrease the intracellular pH in a HMA independent way, we looked at the consequences of the cytosolic acidification on PS externalization. Fig. 3A and B show that diminution of intracellular pH did not induce PS externalization by itself and did not influence the effect of PMA or A23187 suggesting that the acidification induced by HMA was not involved in PS externalization.

Consequently, HMA triggered PS externalization by pathways that are not directly dependent on the intracellular acidification induced by NHE inhibition.

3.4. HMA triggered PS externalization through pathways dependent on calcium influx

In endothelial cells, HMA induces an increase in calcium concentration by capacitative calcium entry [7]. Here, we have investigated the effects of HMA on the calcium homeostasis in the resting or activated gp91^{phox} knockout PLB-985 cells as a model of phagocytes.

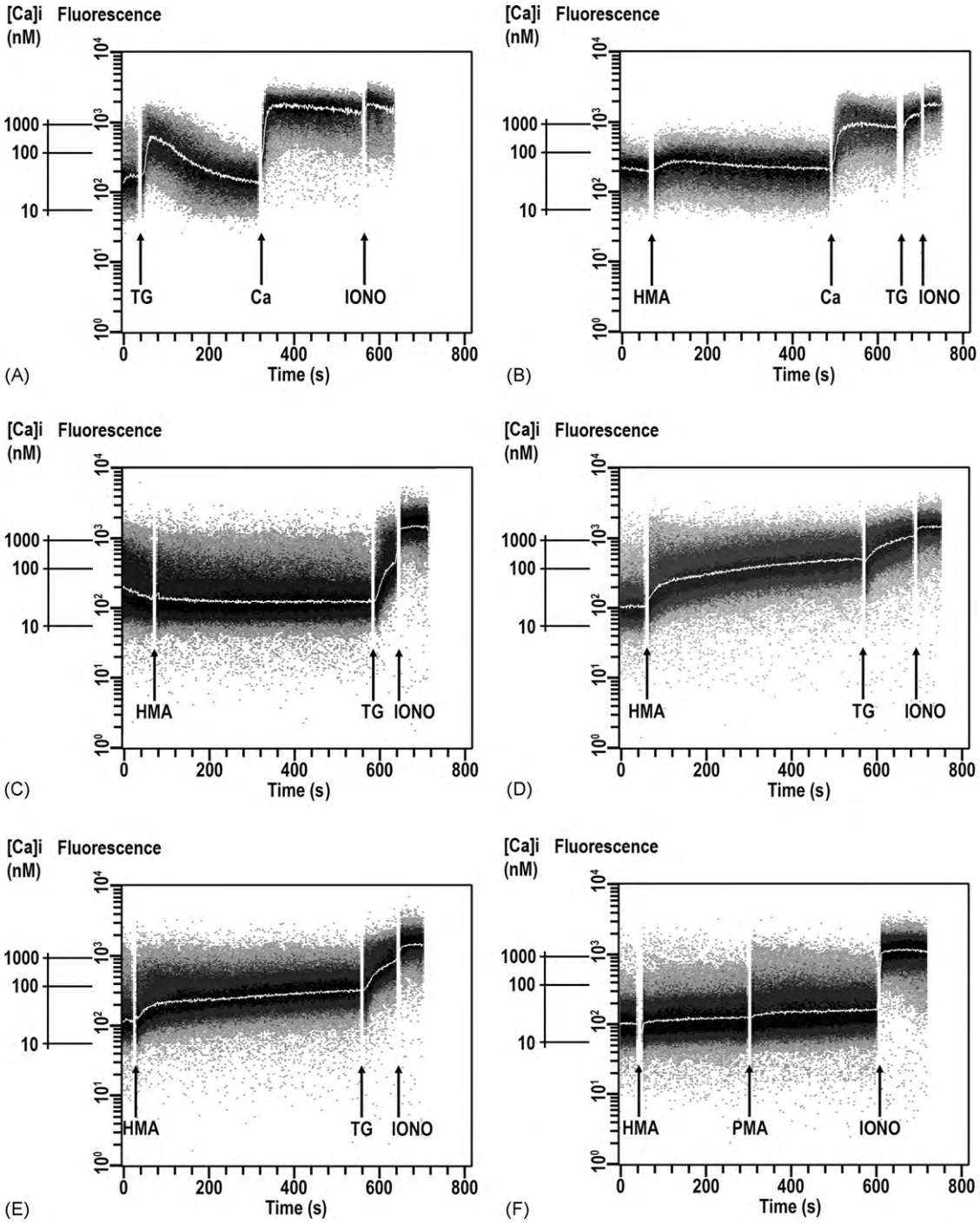

Fig. 4. Effect of HMA on the cytosolic calcium concentrations. Kinetics of variations of cytosolic calcium concentration were estimated by fluorescence measurement of gp91^{phox} knockout PLB-985 cells loaded with Fluo4-AM by flow cytometry. The white line in each panel represents the geometric mean of Fluo4 fluorescence for each kinetic point, calculated with WinMDI Freeware. Capacitative entry of calcium was characterized in cells incubated in calcium free medium by addition of 0.5 μM of thapsigargin (A) or 30 μM of HMA (B) and subsequent addition of 2 mM calcium. Kinetics of calcium influx were measured in cells incubated in calcium medium and addition of HMA 20 μM (C), 30 μM (D), 60 μM (E) or HMA 20 μM followed by 200 nM PMA (F). In each experimental condition, maximal fluorescence was estimated by addition of 2 μM ionomycine at the end of the kinetic. In abscissa, time was indicated in seconds.

Kinetics of the variations of cytosolic calcium concentration induced by HMA were evaluated by time-lapse measurement of fluorescence of Fluo4-loaded PLB-985 cells by flow cytometry [35,36]. For all the experimental conditions, aliquots of cells were from a unique batch of Fluo4-loaded cells. In Fig. 4, the level of minimal fluorescence was measured in presence of BAPTA (F_{min} = 20) and the maximal response was obtained by final addition of 2 μM ionomycin (F_{max} = 1256 ± 55.9). The observation of the fluorescence kinetics allows comparison of the cell response between different experimental conditions. As a positive control, capacitative calcium entry was triggered with 500 nM thapsigargin, an inhibitor of ER calcium pumps. In calcium-deprived medium, thapsigargin induced a transient calcium release from internal calcium stores. Subsequent addition of 2 mM calcium induced an increase in fluorescence level due to a calcium influx characteristic of capacitative calcium entry (Fig. 4A). In comparison to thapsigargin, 30 μM HMA induced a smaller but more prolonged increase in fluorescence due to calcium release, and addition of calcium induced

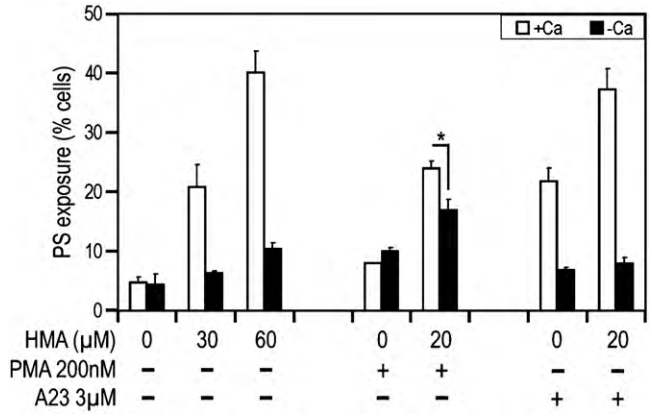

Fig. 5. Effect of calcium influx on PS externalization. gp91^{phox} knockout PLB-985 cells were incubated during 3 h at 37 °C in medium containing 2 mM calcium (+Ca) or 500 μM EGTA (−Ca). PS externalization (% of PS+ PI− cells) was estimated as described in the legend of Fig. 1. Results are means ± SE, $n \geq 3$.

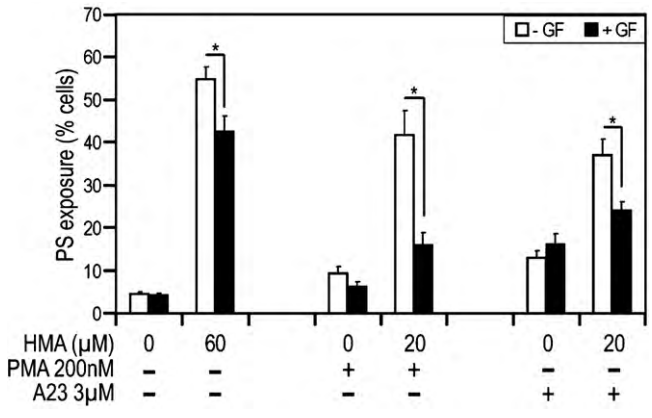

Fig. 6. Role of PKCs in PS externalization. gp91^{phox} knockout PLB-985 cells pretreated (+GF) or not (−GF) with 500 nM of GF109203X were incubated at 37 °C for 3 h, with 60 μM of HMA, 200 nM PMA or 3 μM A23187 with or without 20 μM HMA. PS externalization (% of PS+ PI− cells) was estimated as described in the legend of Fig. 1. Results are means ± SE, $n \geq 3$.

capacitative calcium influx (Fig. 4B). In calcium containing medium, 20 μM HMA induced a small calcium increase and did not prevent subsequent triggering by thapsigargin (Fig. 4C). However, 30 μM and 60 μM HMA induced a slow and sustained rise of the intracellular calcium concentration (Fig. 4D and E). Interestingly, the very weak increase in fluorescence induced by 20 μM HMA was only slightly enhanced by a subsequent addition of 200 nM of PMA (Fig. 4F).

In parallel to the observation of the Fluo4 fluorescence attributable to the variations of cytosolic calcium concentrations, we studied the impact of calcium influx induced by HMA on PS externalization by incubating the cells in calcium free medium.

Suppression of calcium influx drastically inhibited PS externalization induced by HMA (30 μM or 60 μM) (Fig. 5). In cells loaded with the calcium chelator BAPTA, the effect of 60 μM HMA was also suppressed, even in the presence of extracellular calcium (preliminary data, not shown). The effect of PMA enhanced by 20 μM HMA, was only partially reduced by the absence of calcium influx (Fig. 5), in correlation with the weak effect of HMA plus PMA on cytosolic calcium increase shown above (Fig. 4C and F). Finally, as expected, suppression of calcium in the medium suppressed PS externalization induced by A23187, but also the co-stimulatory effect of 20 μM HMA.

The data suggest that PS externalization stimulated by HMA alone was strongly calcium dependent whereas the co-stimulatory effect of HMA on PMA activation had a major calcium independent component.

3.5. Role of PKC in PS externalization induced or enhanced by HMA

Since 20 μM HMA enhanced the effect of PMA even in the absence of calcium, it could have an additional role beyond the stimulation of a calcium signal. We therefore investigated the implication of PKCs in PS externalization by using a general PKC inhibitor GF109203X [37]. Pre-incubation of the gp91^{phox} knockout PLB-985 cells with 500 nM GF109203X, a concentration sufficient to completely inhibit ROS production induced by 200 nM PMA in PLB-985 cells (data not shown), partially reduced (22.4% of inhibition) PS externalization induced by 60 μM HMA (Fig. 6). The combined effect of PMA plus 20 μM HMA was substantially reduced (61.4% of inhibition) by GF109203X. The effect of A23187 was not influenced by the PKC inhibitor, whereas the combined effect of A23187 plus 20 μM HMA was significantly inhibited by 35% (Fig. 6). Altogether these data suggest that PKCs do not play a major role in PS externalization by HMA alone. HMA may enhance PS externalization either directly through the modulation of PKCs activated by PMA or calcium ionophore or through regulation of a pathway downstream of PKC activation.

3.6. elF2α phosphorylation but not cJUN NH_2-terminal kinase (JNK) was involved in PS externalization downstream of HMA treatment

We have investigated the implication of ER-stress, potentially induced by HMA, in PS externalization, by using 30 μM of salubrinal or 5 μM of SP600125, which inhibit respectively elF2α-P dephosphorylation [12,38] or JNK activation [39].

Pre-incubation of gp91^{phox} knockout PLB-985 cells with 30 μM of salubrinal drastically inhibited PS externalization induced by 60 μM HMA (Fig. 7A) whereas the combined effect on PS externalization induced by 20 μM HMA plus PMA or A23187 was not modified. Fig. 7B shows that the JNK inhibitor (SP600125) did not influence PS externalization by HMA alone or in combination with PMA or A23187. These data suggest that high concentrations of HMA induced PS externalization *via* ER-stress pathways implicating PERK activation and elF2α phosphorylation, but independently of JNK activation. However, low concentrations of HMA in addition to PMA or A23187 seem to act independently of ER-stress.

3.7. Z-VAD-FMK did not inhibit PS externalization

As PS externalization can be linked to caspase activation associated or not with apoptosis, we have investigated the role of caspases in the effect of HMA on PS externalization. In gp91^{phox} knockout PLB-985 cells, the general caspase inhibitor Z-VAD-FMK at 10 μM, a concentration generally sufficient to specifically inhibit caspases [40,41], had no inhibitory effect on PS externalization (Fig. 8). However, at a 10-fold higher concentration, Z-VAD-FMK partially inhibited PS externalization induced by 60 μM HMA and the co-stimulatory effect of 20 μM HMA together with A23187. This result may indicate that caspases less sensitive to Z-VAD-FMK could be involved, such as caspase 4 [12]. Z-VAD-FMK slightly increased the effect of 20 μM HMA and PMA.

3.8. Externalized PS increased the engulfment of PLB-985 cells by RAW 264.7 macrophages

As PS plays an important role in the elimination of apoptotic cells by macrophages, participating in the regulation of inflammation, we investigated whether HMA increased the capacity of macrophages to engulf PLB-985 cells. RAW 264.7 macrophages

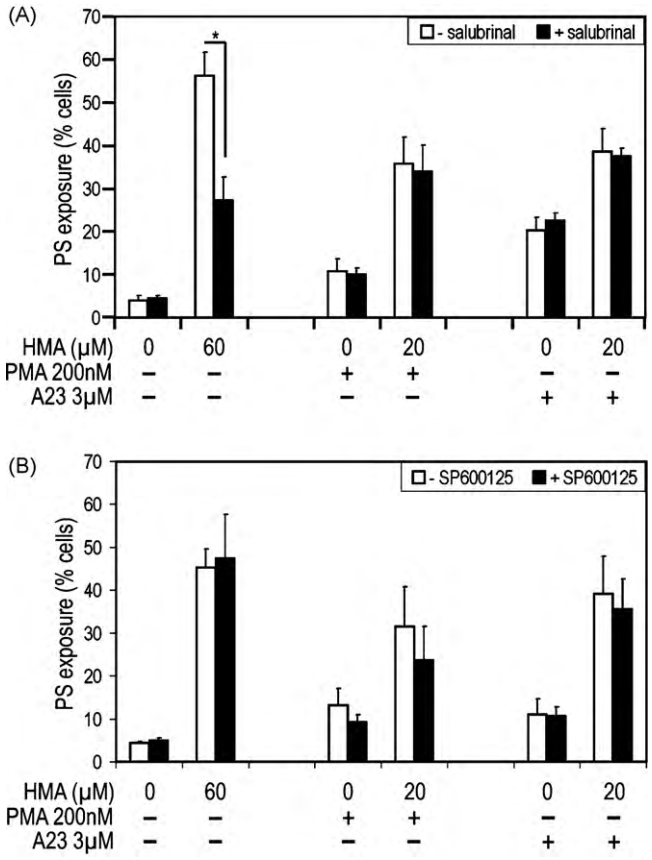

Fig. 7. Effect of inhibitors of elF2α dephosphorylation or JNK dependent apoptotic pathways downstream of ER-stress on PS externalization. gp91^{phox} knockout PLB-985 cells pretreated or not with 30 μM of salubrinal (A) or 5 μM SP600125 (B) were incubated at 37 °C for 3 h, with 60 μM of HMA, 200 nM PMA or 3 μM A23187 with or without 20 μM HMA. PS externalization (% of PS+ PI− cells) was estimated as described in the legend of Fig. 1. Results are means ± SE, $n \geq 3$.

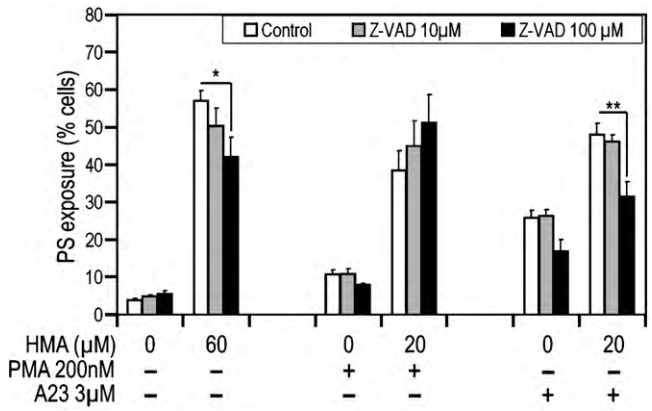

Fig. 8. Effect of caspase inhibition on PS externalization. gp91^{phox} knockout PLB-985 cells pretreated or not with 10 μM or 100 μM of Z-VAD-FMK were incubated at 37 °C for 3 h, with 60 μM of HMA, 200 nM PMA or 3 μM A23187 with or without 20 μM HMA. PS externalization (% of PS+ PI− cells) was estimated as described in the legend of Fig. 1. Results are means ± SE, $n \geq 3$.

engulfed significantly more wild type and gp91^{phox} knockout PLB-985 cells activated by PMA than unstimulated PLB-985 cells (Table 1). This uptake was further enhanced when PS externalization was increased by a treatment with HMA. The key role of PS in the engulfment process was shown by the capacity of annexin V to bind to externalized PS and largely decrease uptake. Interestingly, HMA treatment favoured similarly the engulfment of wild type and CGD-like cells such as gp91^{phox} knockout PLB-985.

4. Discussion

Amiloride derivatives are NHE inhibitors and potential therapeutic agents in ischemia/reperfusion injury. However, depending on their concentration they can induce cell death in various types of tissues [7,9,10,42]. PS externalization plays an essential role in reconnaissance and removal of undesirable cells, a process designed as efferocytosis [14,27]. This phenomenon is particularly important to remove large numbers of neutrophils from blood circulation and infected or inflamed tissues. We therefore investigated the effect of HMA in a useful range of concentrations [5,7,9] on PS externalization in neutrophil-like human PLB-985 cells. We identified a dual concentration-dependent effect of HMA. (i) 60 μM HMA stimulated PS externalization and (ii) 20 μM HMA acted as co-stimulator for PMA- or A23187-induced PS externalization. These effects appeared rapidly within 3 h of treatment. This stimulatory effect of an amiloride derivative on PS externalization was efficient to increase the phagocytosis of PLB-985 cells by macrophages. Thus HMA may be useful to accelerate neutrophil elimination from an inflammatory environment.

HMA specifically inhibits the sodium–proton exchanger NHE [5,8] inducing cytosolic acidification. Although acidification generally accompanies apoptosis, the role attributed to pH_i in apoptosis is controversial, the mechanisms are not yet elucidated and the impact of pH_i on PS externalization is rarely reported [43,44] in leukocytes and other cells. Modulation of cytosolic acidification due to the activity of NHE, has been implicated in stimulation [9,33] or inhibition of apoptosis [42]. In leukaemic cells, a constitutive cytosolic alkalinization was correlated with tumorigenicity and inhibition of NHE restored apoptosis [10]. Our data show that cytosolic acidification, induced by cell activation and modulated by HMA, positively correlated with PS exposure induced by PMA or A23187 in wild type (not shown) or gp91^{phox} knockout cells. However, as shown in gp91^{phox} knockout cells, cytosolic acidification imposed by NH_4Cl treatment (data not shown) or by pre-incubation in pH 6.25 medium (Fig. 3) did not by itself trigger PS externalization in resting cells and did not mimic the effect of HMA in cells activated by PMA or A23187. These data,

Table 1
Effect of HMA on PS externalization and cell engulfment by cultured macrophages. Wild type (WT) or gp91^{phox} knockout PLB-985 cells (KO) were treated for 3 h at 37 °C with PMA, in the presence or not of 20 μM of HMA, stained with Hoechst 33342 and co-cultured with RAW 264.7 macrophages. A minimum of 100 macrophages per condition were counted and the results are expressed as the number of phagocytozed PLB-985 cells per 100 macrophages. To confirm the role of PS, PLB-985 cells were pre-incubated with annexin V (A5) before co-incubation with macrophages. Results are means ± SE, $n \geq 3$. nd, no determined.

	Control	HMA	PMA	PMA + HMA	PMA + HMA + A5
Engulfed PLB-985 cells per 100 macrophages					
Wt	7.89 ± 2.98	7.12 ± 3.62	20.48 ± 3.24*	30.65 ± 4.08§	13.30 ± 4.27#
KO	9.00 ± 2.18	12.45 ± 1.94*	13.56 ± 3.47*	17.35 ± 3.05§	nd

* Significantly different from control.
§ Significantly different from PMA-stimulated cells.
# Significantly different from (PMA + HMA)-stimulated cells.

in agreement with recent work on endothelial cells [7], suggest that HMA could influence PS externalization by pathways other than NHE inhibition and cell acidification.

Park and colleagues recently showed that HMA liberates calcium from the endoplasmic reticulum and subsequently initiates store operated calcium influx in endothelial cells. HMA targets the same Ca^{2+} pool as thapsigargin and Ca^{2+} depletion was induced rapidly after HMA addition. At maximal concentration, 100 μM HMA, the amplitude and the kinetics were similar to those induced by thapsigargin [7]. Our data show that HMA had a similar dose-dependent effect on calcium signals in PLB-985 cells. Furthermore, PS externalization induced by HMA 60 μM was drastically diminished by suppression of Ca influx.

Calcium depletion from ER is a major cause of ER-stress and HMA has been shown to cause calcium release from the ER and increased transcription of the ER-stress genes [7]. In PLB-985 cells, PS externalization induced by 60 μM HMA was inhibited by calcium deprivation and by salubrinal, an inhibitor of an ER-stress dependent apoptosis pathway, suggesting that calcium and ER-stress were implicated. The HMA effect was superior to that of calcium ionophore, despite the much higher calcium increase caused by the ionophores. Thus ER-stress and calcium acted together on PS externalization.

However, the presence of 20 μM HMA amplified the effect of A23187 leading to PS externalization in 30–40% of cells entirely dependent on Ca influx but not inhibited by salubrinal. Consequently, the amplification of the A23187 effect by 20 μM HMA could be explained by an increased sensitivity for calcium-induced PS externalization.

PS externalization has been previously described as dependent on phosphorylation of scramblase by PKCδ [45] and during spontaneous apoptosis of neutrophils, inhibition of PKCs abolished PS externalization [46]. We have investigated the implication of PKC in PS externalization in the various conditions by using a general PKC inhibitor, GF109203X. Our data suggest that activation of PKCs by HMA alone contributed moderately to PS externalization. GF109203X substantially inhibited the amplifier effect of HMA on PS externalization in cells activated by PMA or A23187. This suggests that HMA acted either directly through the modulation of PKC activation or through a regulation of pathways downstream of PKC activation.

We investigated the potential role of caspases in PS externalization by using the general caspase inhibitor Z-VAD-FMK. At a standard concentration of 10 μM, Z-VAD-FMK had no effect on PS externalization in PLB-985 cells. These data suggest that PS externalization was not linked to a caspase dependent apoptosis pathway. Only at 10-fold higher concentration Z-VAD-FMK partly inhibited PS externalization induced by 60 μM HMA and the amplifier effect of 20 μM HMA with A23187, whereas surprisingly, the effect of PMA together with HMA was slightly increased. Caspase 12 in rodents and caspase 4 in humans are potentially activated by a calcium-activated calpain downstream of ER-stress [12]. These caspases being less sensitive to inhibition by Z-VAD-FMK, the data suggest that caspase 4 could contribute to PS externalization.

Our data are in agreement with previous reports of apoptotic cell death and PS externalization without caspase activity [19]. Furthermore, in several types of cells, PS externalization was shown to be independent of apoptosis [21,22]. There is growing evidence that non-apoptotic processes also contribute to regulate neutrophil homeostasis. Whereas neutrophils undergo rapid spontaneous apoptosis *in vitro*, the exact role of apoptosis in their elimination remains unclear [22] and their clearance after pathogen phagocytosis is reported as caspase-independent [18,20,23,25].

In conclusion, treatment of neutrophil-like PLB-985 cells with 60 μM of the amiloride derivative HMA stimulated rapid externalization of PS. The stimulation did not require NADPH-oxidase activation and could not be explained by cytosolic acidification. Our results suggest that HMA liberates calcium from the ER, causes store operated calcium influx as well as ER-stress. Calcium and ER-stress together appear to promote PS externalization partially dependent on PKC activation. At a threefold lower concentration, HMA did not directly stimulate PS externalization but enhanced substantially PS externalization in cells activated by either PMA or calcium ionophores. This is the first description of a co-stimulatory activity of 20 μM HMA that apparently did not require calcium release or induction of ER-stress. However, it required PKC activity.

Treatment with HMA amplified PS externalization and enhanced engulfment by macrophages of wild type and CGD-like PLB-985 cells. Amiloride derivatives have been used in preclinical trials for treatment of ischemia–reperfusion injury, we show that they could have previously unexpected effects on leukocytes. We propose that amiloride derivatives could provide a novel therapeutic strategy in inflammatory diseases to favour removal of activated neutrophils from injured tissues.

Acknowledgements

We greatly appreciate the gift of wild type and CGD-like PLB-985 cells from Dr. Marie-José Stasia (Laboratoire Enzymologie, CHU Grenoble, France) and Dr. Mary Dinauer (Indiana). We would like to give our thanks to Michel Hours for technical assistance. This work was supported by grants from the French Ministry of Science (PhD fellowship for AT and program ACI DRAB), INSERM and the University Paris-Sud (BQR).

References

[1] Black SC. In vivo models of myocardial ischemia and reperfusion injury: application to drug discovery and evaluation. J Pharmacol Toxicol Methods 2000;43:153–67.
[2] Linfert D, Chowdhry T, Rabb H. Lymphocytes and ischemia–reperfusion injury. Transplant Rev (Orlando) 2009;23:1–10.
[3] Suzuki Y, Matsumoto Y, Ikeda Y, Kondo K, Ohashi N, Umemura K. SM-20220, a Na(+)/H(+) exchanger inhibitor: effects on ischemic brain damage through edema and neutrophil accumulation in a rat middle cerebral artery occlusion model. Brain Res 2002;945:242–8.
[4] Fliegel L. Regulation of the Na(+)/H(+) exchanger in the healthy and diseased myocardium. Expert Opin Ther Targets 2009;13:55–68.
[5] Masereel B, Pochet L, Laeckmann D. An overview of inhibitors of Na(+)/H(+) exchanger. Eur J Med Chem 2003;38:547–54.
[6] Mentzer Jr RM, Bartels C, Bolli R, Boyce S, Buckberg GD, Chaitman B, et al. Sodium–hydrogen exchange inhibition by cariporide to reduce the risk of ischemic cardiac events in patients undergoing coronary artery bypass grafting: results of the EXPEDITION study. Ann Thorac Surg 2008;85:1261–70.
[7] Park KS, Poburko D, Wollheim CB, Demaurex N. Amiloride derivatives induce apoptosis by depleting ER Ca(2+) stores in vascular endothelial cells. Br J Pharmacol 2009;156:1296–304.
[8] Suzuki T, Tsukamoto I. Apoptosis induced by 5-(N,N-hexamethylene)-amiloride in regenerating liver after partial hepatectomy. Eur J Pharmacol 2004;503:1–7.
[9] Coakley RJ, Taggart C, McElvaney NG, O'Neill SJ. Cytosolic pH and the inflammatory microenvironment modulate cell death in human neutrophils after phagocytosis. Blood 2002;100:3383–91.
[10] Rich IN, Worthington-White D, Garden OA, Musk P. Apoptosis of leukemic cells accompanies reduction in intracellular pH after targeted inhibition of the Na(+)/H(+) exchanger. Blood 2000;95:1427–34.
[11] Xu C, Bailly-Maitre B, Reed JC. Endoplasmic reticulum stress: cell life and death decisions. J Clin Invest 2005;115:2656–64.
[12] Boyce M, Yuan J. Cellular response to endoplasmic reticulum stress: a matter of life or death. Cell Death Differ 2006;13:363–73.
[13] Szegezdi E, Logue SE, Gorman AM, Samali A. Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Rep 2006;7:880–5.
[14] Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta PGE2, and PAF. J Clin Invest 1998;101:890–8.
[15] Devaux PF. Static and dynamic lipid asymmetry in cell membranes. Biochemistry 1991;30:1163–73.
[16] Zwaal RF, Schroit AJ. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. Blood 1997;89:1121–32.

[17] Balasubramanian K, Mirnikjoo B, Schroit AJ. Regulated externalization of phosphatidylserine at the cell surface: implications for apoptosis. J Biol Chem 2007;282:18357–64.
[18] Lagasse E, Weissman IL. bcl-2 inhibits apoptosis of neutrophils but not their engulfment by macrophages. J Exp Med 1994;179:1047–52.
[19] Leist M, Jaattela M. Four deaths and a funeral: from caspases to alternative mechanisms. Nat Rev Mol Cell Biol 2001;2:589–98.
[20] Shi J, Gilbert GE, Kokubo Y, Ohashi T. Role of the liver in regulating numbers of circulating neutrophils. Blood 2001;98:1226–30.
[21] Smrz D, Draberova L, Draber P. Non-apoptotic phosphatidylserine externalization induced by engagement of glycosylphosphatidylinositol-anchored proteins. J Biol Chem 2007;282:10487–9.
[22] Stowell SR, Karmakar S, Stowell CJ, Dias-Baruffi M, McEver RP, Cummings RD. Human galectin-1, -2, and -4 induce surface exposure of phosphatidylserine in activated human neutrophils but not in activated T cells. Blood 2007;109:219–27.
[23] Tyurina YY, Basova LV, Konduru NV, Tyurin VA, Potapovich AI, Cai P, et al. Nitrosative stress inhibits the aminophospholipid translocase resulting in phosphatidylserine externalization and macrophage engulfment: implications for the resolution of inflammation. J Biol Chem 2007;282:8498–509.
[24] van Delft MF, Smith DP, Lahoud MH, Huang DC, Adams JM. Apoptosis and non-inflammatory phagocytosis can be induced by mitochondrial damage without caspases. Cell Death Differ 2010;17:821–32.
[25] Wilkie RP, Vissers MC, Dragunow M, Hampton MB. A functional NADPH oxidase prevents caspase involvement in the clearance of phagocytic neutrophils. Infect Immun 2007;75:3256–63.
[26] Sanmun D, Witasp E, Jitkaew S, Tyurina YY, Kagan VE, Ahlin A, et al. Involvement of a functional NADPH oxidase in neutrophils and macrophages during programmed cell clearance: implications for chronic granulomatous disease. Am J Physiol Cell Physiol 2009;297:C621–31.
[27] Fernandez-Boyanapalli RF, Frasch SC, McPhillips K, Vandivier RW, Harry BL, Riches DW, et al. Impaired apoptotic cell clearance in CGD due to altered macrophage programming is reversed by phosphatidylserine-dependent production of IL-4. Blood 2009;113:2047–55.
[28] Pedruzzi E, Fay M, Elbim C, Gaudry M, Gougerot-Pocidalo MA. Differentiation of PLB-985 myeloid cells into mature neutrophils, shown by degranulation of terminally differentiated compartments in response to N-formyl peptide and priming of superoxide anion production by granulocyte-macrophage colony-stimulating factor. Br J Haematol 2002;117:719–26.
[29] Ratthe C, Girard D. Investigation of the interleukin (IL)-4/IL-4 receptor system in promyelocytic leukaemia PLB-985 cells during differentiation toward neutrophil-like phenotype: mechanism involved in IL-4-induced SOCS3 protein expression. Br J Haematol 2008;140:59–70.
[30] Simons MP, Leidal KG, Nauseef WM, Griffith TS. TNF-related apoptosis-inducing ligand (TRAIL) is expressed throughout myeloid development, resulting in a broad distribution among neutrophil granules. J Leukoc Biol 2008;83:621–9.
[31] Zhen L, King AA, Xiao Y, Chanock SJ, Orkin SH, Dinauer MC. Gene targeting of X chromosome-linked chronic granulomatous disease locus in a human myeloid leukemia cell line and rescue by expression of recombinant gp91phox. Proc Natl Acad Sci USA 1993;90:9832–6.
[32] Suzuki K, Namiki H. Cytoplasmic pH-dependent spreading of polymorphonuclear leukocytes: regulation by pH of PKC subcellular distribution and F-actin assembly. Cell Biol Int 2007;31:279–88.
[33] Gottlieb RA, Giesing HA, Zhu JY, Engler RL, Babior BM. Cell acidification in apoptosis: granulocyte colony-stimulating factor delays programmed cell death in neutrophils by up-regulating the vacuolar H(+)-ATPase. Proc Natl Acad Sci USA 1995;92:5965–8.
[34] Sulpice JC, Zachowski A, Devaux PF, Giraud F. Requirement for phosphatidylinositol 4,5-bisphosphate in the Ca(2+)-induced phospholipid redistribution in the human erythrocyte membrane. J Biol Chem 1994;269:6347–54.
[35] Courageot MP, Lepine S, Hours M, Giraud F, Sulpice JC. Involvement of sodium in early phosphatidylserine exposure and phospholipid scrambling induced by P2X7 purinoceptor activation in thymocytes. J Biol Chem 2004;279:21815–23.
[36] Kunzelmann-Marche C, Freyssinet JM, Martinez MC. Regulation of phosphatidylserine transbilayer redistribution by store-operated Ca^{2+} entry: role of actin cytoskeleton. J Biol Chem 2001;276:5134–9.
[37] Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, et al. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J Biol Chem 1991;266:15771–8.
[38] Lopez JJ, Redondo PC, Salido GM, Pariente JA, Rosado JA. N,N,N′N′-tetrakis(2-pyridylmethyl)ethylenediamine induces apoptosis through the activation of caspases-3 and -8 in human platelets. A role for endoplasmic reticulum stress. J Thromb Haemost 2009;7:992–9.
[39] Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, et al. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci USA 2001;98:13681–6.
[40] Lepine S, Lakatos B, Courageot MP, Le Stunff H, Sulpice JC, Giraud F. Sphingosine contributes to glucocorticoid-induced apoptosis of thymocytes independently of the mitochondrial pathway. J Immunol 2004;173:3783–90.
[41] Park HY, Song MG, Lee JS, Kim JW, Jin JO, Park JI, et al. Apoptosis of human neutrophils induced by protein phosphatase 1/2A inhibition is caspase-independent and serine protease-dependent. J Cell Physiol 2007;212:450–62.
[42] Cobo JM, Garcia-Canero R, Valdez JG, Barrasso AM, Sailer BL, Crissman HA. Attenuation of apoptotic DNA fragmentation by amiloride. J Cell Physiol 1998;175:59–67.
[43] Lagadic-Gossmann D, Huc L, Lecureur V. Alterations of intracellular pH homeostasis in apoptosis: origins and roles. Cell Death Differ 2004;11:953–61.
[44] Matsuyama S, Llopis J, Deveraux QL, Tsien RY, Reed JC. Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. Nat Cell Biol 2000;2:318–25.
[45] Frasch SC, Henson PM, Kailey JM, Richter DA, Janes MS, Fadok VA, et al. Regulation of phospholipid scramblase activity during apoptosis and cell activation by protein kinase Cdelta. J Biol Chem 2000;275:23065–73.
[46] Nusbaum P, Laine C, Bouaouina M, Seveau S, Cramer EM, Masse JM, et al. Distinct signaling pathways are involved in leukosialin (CD43) down-regulation, membrane blebbing, and phospholipid scrambling during neutrophil apoptosis. J Biol Chem 2005;280:5843–53.