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# Staphylococcal Pore-forming Leukotoxins: Opening of Ca<sup>2+</sup>-activated K<sup>+</sup> Channels and Specificity of Membrane Pores in Human Neutrophils

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**Abstract:** Pore-forming toxins are key virulence determinants produced by human bacterial pathogens *Staphylococcus aureus*, inducing two independent cellular events in neutrophils. Upon a specific binding to membrane receptors, both Panton and Valentin Leukocidin and  $\gamma$ -hemolysin induced an increase of Na<sup>+</sup> and K<sup>+</sup> fluxes, likely associated to the activation of preexisting ionic channels or to the membrane pores formation. This was investigated by using, spectrofluorometry techniques and, specific molecular probes in human neutrophils. Interestingly, we found that, in the absence of extracellular Ca<sup>2+</sup>, leukotoxins did form membrane pores, which were large enough to allow a massive entry of ethidium into neutrophils. Simultaneously, sustained Na<sup>+</sup> influx and K<sup>+</sup> efflux were observed. Another set of experiments carried out in the presence of extracellular Ca<sup>2+</sup> did show that, the percentage of pores formed by leukotoxins was significantly, reduced due to the Ca<sup>2+</sup> effect to eventually protect cells from lysis. The simultaneous recording of Na<sup>+</sup> and K<sup>+</sup> movements showed a significant increase of the K<sup>+</sup> efflux although, the Na<sup>+</sup> influx was reduced. By using potassium channels blockers, we found that, the potassium efflux enhanced by the presence of extracellular Ca<sup>2+</sup>, was markedly, inhibited in apamin-, charybdotoxin-, tetrodotoxin-, and quinine-pretreatment neutrophils. We also found that, the increase of the K<sup>+</sup> efflux was reduced by either, thapsigargin or TMB8, potent blockers of the internal Ca<sup>2+</sup> stores depletion. Consequently, we proposed that, the activation of another potassium pathway by leukotoxins, known as Ca<sup>2+</sup>-activated K<sup>+</sup> channels following the Ca<sup>2+</sup> stores depletion. Furthermore, potassium channels blockers did not affect ethidium, Na<sup>+</sup> and K<sup>+</sup> movements, in the absence of extracellular Ca<sup>2+</sup>. Moreover, in this condition, no monovalent ions movement was recorded, when the pores formation was altered by tetra-ethyl-ammonium. In the present study, we further highlighted the specificity of membrane pores to Na<sup>+</sup> and K<sup>+</sup> ions when, the pores formation was completely blocked by divalent ions blockers (Ca<sup>2+</sup> and Zn<sup>2+</sup>). Under these conditions, no monovalent ions movement, was recorded although, a significant influx of Ca<sup>2+</sup> and Zn<sup>2+</sup> was observed after the leukotoxins application. In conclusion, our data provided an evidence that, staphylococcal leukotoxins induced in human neutrophils: 1) the opening of Ca<sup>2+</sup>-activated K<sup>+</sup> channels, only in the presence of 1 mM extracellular Ca<sup>2+</sup>; 2) the formation of membrane pores, which exhibited a high specificity to monovalent cations and, 3) an influx of sodium, through a tetrodotoxin not-sensitive pathway ruling out the hypothesis that, Na<sup>+</sup> channels could be activated by leukotoxins.

**Keywords:** Pore-forming Toxin, Panton and Valentin Leukocidin,  $\gamma$ -hemolysin, *S. aureus*, Spectrofluorometry, Neutrophils, Ca<sup>2+</sup>-activated K<sup>+</sup> Channels, Na<sup>+</sup> Channels

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## 1. Introduction

The Gram-positive bacterium *Staphylococcus aureus* is one

of the most important and common human pathogens that causes severe invasive infections, from mild skin and soft tissue infections to sepsis, and potentially lethal necrotizing

fasciitis [1-3]. The multidrug-resistant *S. aureus* is a major cause of morbidity and mortality worldwide and, is estimated to cause over 80,000 cases of invasive diseases annually, in the United States [4-5]. During infection, *S. aureus* can induce cell apoptosis through various pathways [6]. Thus, the main critical virulence factor of the clinical strains that might increase the potential of diseases is the bi-component leukotoxins known as pore-forming toxins (PFTs). These cytotoxins expressed *in vivo* during infections consist of two separate components: class S (slow eluted) and class F (fast eluted) named on the basis of their elution by chromatography with the ability to act in synergy and disrupt the host cells membrane by forming membrane pores, which are large to allow an influx of ethidium into human neutrophils [7-8]. The ability to produce pore-forming toxins is already, reported for number of Gram-positive bacterial species such as *S. aureus* or *S. pyogenes*, being able to secrete cytotoxins such as  $\alpha$ -toxin [9-10] or streptolysin O [9, 11], respectively, that could have broad effects on the host immune response. Moreover, it is well known that, a number of pathogenic Gram-negative bacteria are also able to secrete toxins known as \*pore-forming toxins\* such as aerolysin, an hemolysin from *Aeromonas hydrophila* [12-13] or  $\alpha$ -toxin from *Escherichia coli* [10]. It is generally, accepted that, pore-forming toxins secreted by various bacterial secretion systems, need a specific cellular receptor to bind to the host cell and form membrane pores [14].

Among leukotoxins from *S. aureus*, bi-component pore-forming leukotoxins:  $\gamma$ -hemolysin (HlgA/HlgB, HlgC/HlgB) and Panton and Valentin Leukocidin (PVL; LukS-PV/LukF-PV) which, provoked two independent cellular events on the membrane of human neutrophils (PMNs). First, upon their specific binding to membrane receptors, they induced the opening of pre-existing  $\text{Ca}^{2+}$  channels [8] including,  $\text{Ca}^{2+}$ -release activated  $\text{Ca}^{2+}$  (CRAC) channels [15]. Then, after insertion and, oligomerization into the membrane, they formed large pores into host cells membrane not permeable to divalent ions ( $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mn}^{2+}$ ) [8, 15], leading to immune cells lysis before death. Since the underlying mechanism was still obscure, recently, it has been questioned whether these membrane pores were involved in  $\text{Cl}^-$  ions movements (efflux/influx) through the PMNs membrane. Indeed, we found that, *i)* the PMNs activation by staphylococcal leukotoxins did induce the opening of pre-existing  $\text{Cl}^-$  channels to allow  $\text{Cl}^-$  ions fluxes and *ii)* staphylococcal membrane pores did not drive chloride ions [16]. The transport of various ions through the plasma membrane of cells, was thought to play a central role in immune cells responses during infectious diseases. Previous studies have already suggested that, the  $\text{Na}^+$  influx was involved in the activation of the  $\text{O}_2^-$  production in human neutrophils [17]. Later, Krautwurst *et al.* have proposed the stimulatory role for the  $\text{Na}^+$  influx in the activation of both  $\beta$ -glucuronidase release and  $\text{O}_2^-$  production. According to these authors,  $\text{Na}^+$  ions might directly affect the exocytosis and, enhance as well the G-proteins activation, leading to the signal amplification [18]. Furthermore, the  $\text{K}^+$  efflux is well

known to induce several host cells alterations, including, the activation of inflammasome and p38 MAP kinase. Again, the  $\text{K}^+$  ions efflux promoted stress-activated and mitogen-activated protein kinase (MAP kinase) pathways that could protect against the PFTs activity *in vitro* and *in vivo* [19-21], but the decrease in cytosolic potassium levels also altered the cellular metabolic state, triggered innate immune signaling and might cause pro-inflammatory cells death [22-23].

The membrane permeability of human neutrophils to monovalent ions ( $\text{Na}^+$ ,  $\text{K}^+$ ), has already been reported in previous studies [24]. However, the mechanism pathway involved in both  $\text{Na}^+$  and  $\text{K}^+$  fluxes was still not well defined. In a previous work [8, 25], we have reported that, bi-component leukotoxins from *S. aureus* did induce both  $\text{Na}^+$  and  $\text{K}^+$  fluxes which, seemed to be occurred through membrane pores, in the absence of extracellular  $\text{Ca}^{2+}$ . To date, little is known about the specificity of staphylococcal membrane pores to monovalent ions ( $\text{Na}^+$ ,  $\text{K}^+$ ) or whether, pre-existing potassium and sodium channels are involved when PMNs are activated either by  $\gamma$ -hemolysin (HlgA/HlgB, HlgC/HlgB) or Panton and Valentin Leukocidin (LukS-PV/LukF-PV) from *S. aureus*. In this regards, the aim of this study is first, to arise the question whether, specific ionic (sodium and potassium) channels are involved in the staphylococcal leukotoxins activity as, we have previously demonstrated that, these leukotoxins induced the opening of pre-existing  $\text{Ca}^{2+}$  [8, 15], and  $\text{Cl}^-$  channels [16], upon their specific binding to membrane receptors. Secondly, to verify whether, monovalent ions ( $\text{Na}^+$ ,  $\text{K}^+$ ) fluxes would occur through membrane pores, formed by leukotoxins, since it has previously described that, membrane staphylococcal pores were not permeable to both, divalent cations ( $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mn}^{2+}$ ), and monovalent anions ( $\text{Cl}^-$ ). The present research is investigated in human neutrophils by spectrofluorometry techniques using specific probes in order to verify an eventual activation of  $\text{K}^+$  and/or  $\text{Na}^+$  channels by staphylococcal pore-forming leukotoxins and, to carefully clarify the membrane pores specificity towards  $\text{Na}^+$  and  $\text{K}^+$  cations.

## 2. Materials and Methods

### 2.1. Reagents

The specific probes for  $\text{Ca}^{2+}$  (Fura2/AM, Fluo3),  $\text{K}^+$  (PBFI) and  $\text{Na}^+$  ( $\text{Na}^+$ -green) were obtained from Molecular Probes (Eugene, OR). J. Prep was purchased from Tech Gen International (Les Ulis, France). All other chemicals, including Dextran, ionomycin, thapsigargin, and EGTA were from Sigma (L'Isle-d'Abeau Chesnes, France) except charybdotoxin, tetrodotoxin, apamin and quinine, which were from Calbiochem (Meudon, France).

### 2.2. Leukotoxins Production

The different components of leukotoxins: HlgA, HlgC, HlgB ( $\gamma$ -hemolysin) and LukS-PV, LukF-PV (Panton and Valentin Leukocidin; PVL) were prepared from *S. aureus*

strain ATCC 49775 by chromatography on a cation exchange column and hydrophobic interactions as described previously [26]. The purified components were stored at -80°C before use in all experiments at a final concentration 2.2 nM (class S components; HlgA, HlgC, LukS) and, 0.85 nM (class F components; HlgB, LukF), respectively.

### 2.3. Polymorphonuclear Neutrophils Isolation

Human polymorphonuclear neutrophils (PMNs) were obtained from buffy coats of healthy volunteers of either sex, kindly provided by the \*Centre de Transfusion Sanguine (Strasbourg, France)\*. They were prepared as previously described [8]. Briefly, the human PMNs enriched blood was centrifuged in J. Prep and, the pellet was re-suspended in Dextran for sedimentation. Contaminating erythrocytes were removed, by hypotonic lysis. Finally, the purified PMNs were suspended in the assay medium containing, 140 mM NaCl, 5 mM KCl, 10 mM glucose, 0.1 mM EGTA, 10 mM Hepes and 3 mM Tris base (pH 7.3) at 6 x10<sup>6</sup> cells/ml. This method led to 98% viable PMNs as counted after May-Grunwald-Giemsa staining.

### 2.4. Fura2, Fluo3, PBFI and Na<sup>+</sup>-green Loading

For Ca<sup>2+</sup> measurements, human neutrophils were loaded either, with 2 μM Fura2-AM or 2 μM Fluo3-AM with 0.1% Pluronic F-127 (Molecular Probes) for 45 min at 37 °C in the assay medium as previously described [8]. When experiments were carried out to determine Na<sup>+</sup> and K<sup>+</sup> fluxes, human PMNs were incubated respectively, either with 5 μM Na<sup>+</sup>-green tetra-acetate [8, 27] or 5 μM PBFI-AM (potassium binding benzo-furan isophosphate) [8, 28] with 0.1% Pluronic F-127 during 90 min in an atmosphere of 95% air/5% CO<sub>2</sub> at 37°C in the assay medium. After the incubation period, loaded-PMNs were washed twice by 800xg centrifugations, for 10 min to remove the extracellular dye then, re-suspended in the assay medium at 6x10<sup>6</sup> cells/ml.

### 2.5. Fluorescence Determination

Fluorescence intensity variations of potassium-sensitive indicator PBFI or sodium-sensitive indicator Na<sup>+</sup>-green and ethidium bromide were simultaneously recorded, with a dual excitation and dual emission a spectrofluorometer DeltaScan (Bio-Tek Kontron, PTI, Montigny-le-Bretonneux, France) with slit width set 4 nm.

The different molecular probes used in this study were chosen carefully (Fura2/Na<sup>+</sup>-green) and (Fluo3/PBFI) to allow a simultaneous recording of Ca<sup>2+</sup>/Na<sup>+</sup> and Ca<sup>2+</sup>/K<sup>+</sup> ions fluxes. Relative fluorescence variations registered respectively, at λ<sub>EX</sub>=360 nm; λ<sub>EM</sub>=500 nm (PBFI), λ<sub>EX</sub>=488 nm; λ<sub>EM</sub>=540 nm (Na<sup>+</sup>-green), λ<sub>EX</sub>=340 nm; λ<sub>EM</sub>=510 nm (Fura2) and λ<sub>EX</sub>=488 nm; λ<sub>EM</sub>=530 nm (Fluo3), were expressed in all results as arbitrary units (a.u.). An increase in Na<sup>+</sup>-green, Fluo3 or Fura2 fluorescence intensity was relative to an influx of Na<sup>+</sup> or Ca<sup>2+</sup> ions, respectively, whereas a decrease in the PBFI fluorescence intensity during time is proportional to an efflux of K<sup>+</sup> ions.

The fluorescence increase of the ethidium bromide (100 μM; λ<sub>EX</sub>=360 nm and λ<sub>EM</sub>=600 nm) was used to determine the membrane pores formation, as described previously [7-8]. The ethidium influx was expressed in % (0%: beginning of the registration; 100%: maximum of the intercalated ethidium, in nucleic acids after the Triton X100 addition).

For fluorescence measurements, 1 ml of PMNs suspension (6x10<sup>6</sup> cells/ml), was added to 1 ml of the assay solution, continuously stirred in a 4 ml quartz cuvette (1 cm light path) thermostated at 37°C. For Ca<sup>2+</sup> experiments, 1.1 mM CaCl<sub>2</sub> were added to the PMNs suspension 5 min before measurements, in order to obtain 1 mM extracellular free Ca<sup>2+</sup>. The PMNs auto-fluorescence was subtracted using, the PTI software and, data were extracted for the transfer to SigmaPlot 4.1 (Jandel, Erckrath, Germany). The experiments described in figures are the most representative of four similar ones.

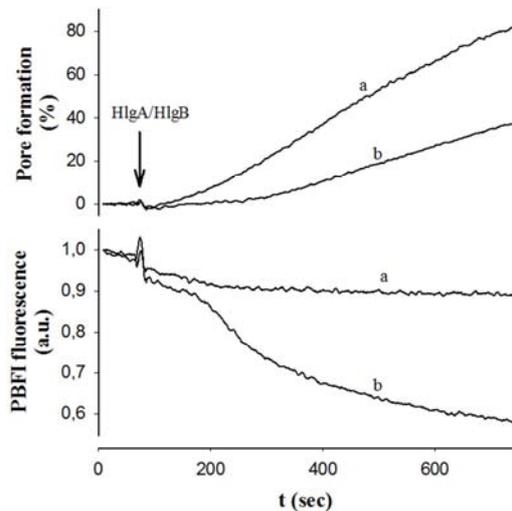
## 3. Results

### 3.1. Na<sup>+</sup>, K<sup>+</sup>, and Ethidium Fluxes Provoked by HlgA/HlgB

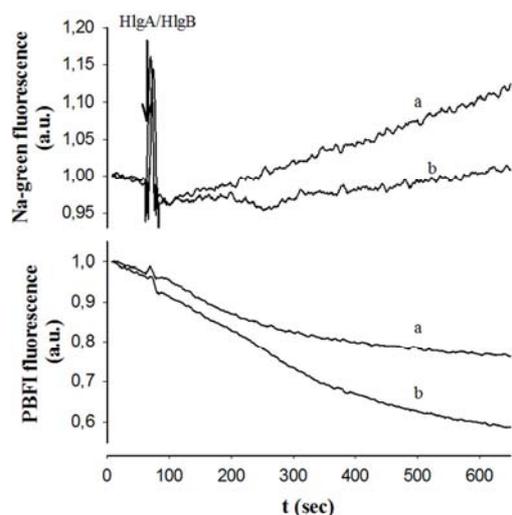
We have previously reported that, in the absence of extracellular Ca<sup>2+</sup>, staphylococcal leukotoxins did provoke the formation of membrane pores, which seemed to be permeable to Na<sup>+</sup>, K<sup>+</sup> and ethidium cations [8, 25]. Furthermore, we demonstrated that both, γ-hemolysin and Panton and Valentin Leukocidin (PVL) from *S. aureus* were able to induce the opening of pre-existing Ca<sup>2+</sup> [8, 15] and, Cl<sup>-</sup> channels in human neutrophils [16]. Despite the role of Ca<sup>2+</sup> to protect PMNs from lysis by reducing the number (%) of membrane pores formed by leukotoxins [7-8], it had been shown later that, an increase of the intracellular level of Ca<sup>2+</sup> ions did play a key role in activating CRAC (Ca<sup>2+</sup>-release activated Ca<sup>2+</sup>) and CaCC (Ca<sup>2+</sup>-activated Cl<sup>-</sup>) channels [15-16]. In this context, we were interested in the present research, to investigate first, an eventual activation of potassium (K<sup>+</sup>) or/and sodium (Na<sup>+</sup>) channels by staphylococcal leukotoxins as it has previously reported for others agonists [29-30]. Secondly, to confirm our hypothesis that, membrane staphylococcal pores were specific only to monovalent cations. The first set of experiments was performed to determine the influence of the HlgA/HlgB γ-hemolysin addition on both ethidium, K<sup>+</sup> and Na<sup>+</sup> movements in human neutrophils by recordings the fluorescence intensity variations of PBFI (K<sup>+</sup>) and Na<sup>+</sup>-green (Na<sup>+</sup>), in the absence and, the presence of 1 mM Ca<sup>2+</sup>. As obviously indicated, the ethidium bromide was used as, an indicator for the pores formation by leukotoxins as shown in Figure 1.

In the absence of extracellular Ca<sup>2+</sup> (Figure 1a), the HlgA/HlgB addition provoked the pores formation (about 80%) determined by an influx of the ethidium and, which was significantly reduced (about 30%) in the presence of 1 mM extracellular Ca<sup>2+</sup> (Figure 1b). The simultaneous recording of PBFI fluorescence variations in the absence of extracellular Ca<sup>2+</sup>, did show a decrease of the fluorescence intensity represented as an arbitrary unit (a.u.) (Figure 1a), likely due to an efflux of K<sup>+</sup> ions. However, when the same experiment was

carried out in the presence of 1 mM extracellular  $\text{Ca}^{2+}$ , an important efflux of potassium ions was observed (Figure 1b) suggesting that, another  $\text{K}^+$  ions pathway could be activated and seemed to be a  $\text{Ca}^{2+}$ -dependent process. Again, similar experiments were performed to follow simultaneously, the PMNs membrane permeability to  $\text{Na}^+$  and  $\text{K}^+$  ions. Indeed, in the absence of extracellular  $\text{Ca}^{2+}$ , as shown in Figure 2a, the HlgA/HlgB addition induced simultaneously an increase in  $\text{Na}^+$ -green and a small decrease of PBF1 fluorescence intensities, likely associated to an influx of  $\text{Na}^+$  and an efflux of  $\text{K}^+$ , respectively. However, in the presence of 1 mM extracellular  $\text{Ca}^{2+}$ , conversely to the  $\text{Na}^+$  influx which was reduced in these condition, the  $\text{K}^+$  efflux was markedly enhanced (Figure 2b) as previously shown.



**Figure 1.** Effect of the HlgA/HlgB application on the membrane pores formation (%) and PBF1 fluorescence variations (a.u.) in human PMNs, in the absence (a) or presence (b) of 1 mM extracellular  $\text{Ca}^{2+}$ .



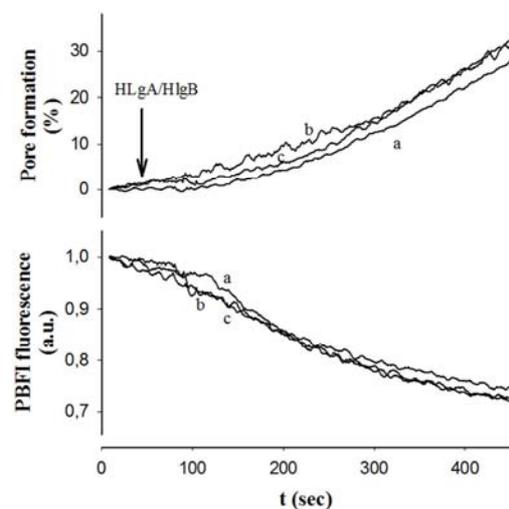
**Figure 2.** Effect of the HlgA/HlgB application on Na-green and PBF1 fluorescence variations (a.u.) in human PMNs, in the absence (a) or presence (b) of 1 mM extracellular  $\text{Ca}^{2+}$ .

Consequently, we proposed that, in the absence of extracellular  $\text{Ca}^{2+}$ , membrane pores formed by staphylococcal leukotoxins were involved in both  $\text{Na}^+$ ,  $\text{K}^+$  and ethidium fluxes.

Whereas, in the presence of 1 mM extracellular  $\text{Ca}^{2+}$ , when the percentage (%) of membrane pores was reduced, a significant decrease of both ethidium and  $\text{Na}^+$  influx was observed, but the  $\text{K}^+$  efflux was significantly increased. These data strongly suggested that, a novel  $\text{K}^+$  pathway  $\text{Ca}^{2+}$ -dependent not permeable to sodium might be involved in the leukotoxins activity, likely associated to the opening of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels. This hypothesis was further explored since, we have previously demonstrated that, leukotoxins from *S. aureus* could induce the opening of both  $\text{Ca}^{2+}$ -released activated  $\text{Ca}^{2+}$  channels [15] and  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels [16] through the PMNs membrane.

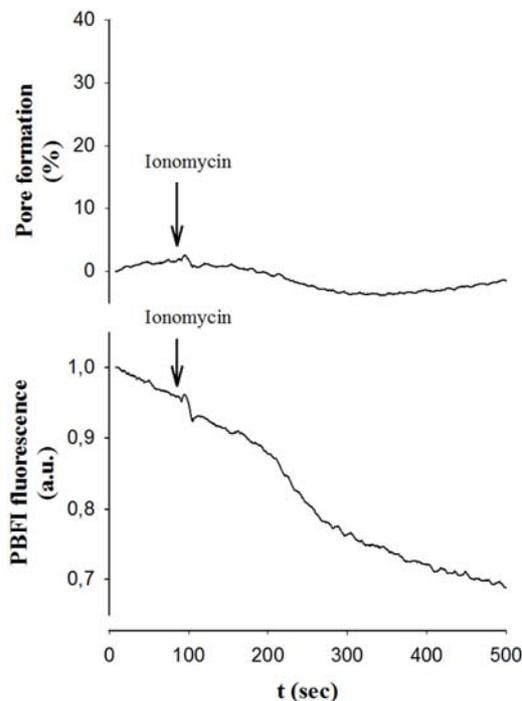
### 3.2. Activation of $\text{Ca}^{2+}$ -activated $\text{K}^+$ Channels by Staphylococcal Leukotoxins

It has been demonstrated in a previous study [31] that, the activation of human neutrophils by a number of agonists, such as fMLP or ionomycin, could induce the opening of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels as results of the internal  $\text{Ca}^{2+}$  stores depletion. Since, we have recently reported that, both Panton and Valentin Leukocidin (PVL) and,  $\gamma$ -hemolysin were able to release the calcium from internal  $\text{Ca}^{2+}$  stores, only in the presence of extracellular  $\text{Ca}^{2+}$  [15] and, the efflux of  $\text{K}^+$  ions was enhanced, only by the presence of extracellular  $\text{Ca}^{2+}$ , we tried, first, to verify whether in the absence of extracellular  $\text{Ca}^{2+}$ , the efflux of  $\text{K}^+$  was mediated through, pre-existing  $\text{K}^+$  channels or through, staphylococcal membrane pores. This hypothesis was verified using potent blockers of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels, charybdotoxin [32-34] and apamin [35-36]. As shown in Figure 3a, in the absence of extracellular  $\text{Ca}^{2+}$ , the HlgA/HlgB addition provoked an influx of ethidium through membrane pores simultaneously to a sustained efflux of  $\text{K}^+$  ions. Both events were not altered during time when, human PMNs were pretreated during 60 min, with either 20  $\mu\text{M}$  charybdotoxin (Figure 3b) or 10  $\mu\text{M}$  apamin (Figure 3c).



**Figure 3.** Effect of the HlgA/HlgB application on the membrane pores formation (%) and PBF1 fluorescence variations (a.u.) in human PMNs, in the absence (a) or presence of 20  $\mu\text{M}$  charybdotoxin (b) and 10  $\mu\text{M}$  apamin (c). [ $\text{Ca}^{2+}$ ]<sub>e</sub> = 0 mM.

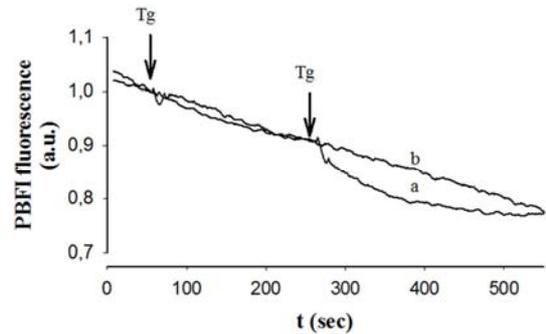
Again, when 2 mM  $\text{Ba}^{2+}$ , known as a potent inhibitor of both voltage-dependent and  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels [36-39] was added after the leukotoxins application in the absence of extracellular  $\text{Ca}^{2+}$ , no inhibition of both events (ethidium influx and  $\text{K}^+$  efflux) was observed (unpublished data). Interestingly, these results strongly suggested that, in the absence of extracellular  $\text{Ca}^{2+}$ , HgA/HlgB leukotoxins did not involve the  $\text{K}^+$  channels activation in the potassium ions movement and, in this condition, the  $\text{K}^+$  efflux would be mediated through, staphylococcal membrane pores. Furthermore, in the absence of extracellular  $\text{Ca}^{2+}$ , the addition of 1  $\mu\text{M}$  ionomycin did induce a significant efflux of  $\text{K}^+$  ions without modifying the ethidium fluorescence intensity (Figure 4). This confirmed that, the use of ionomycin at a given concentration [40] would induce as results to the internal  $\text{Ca}^{2+}$  release, the activation of potassium channels in neutrophils [31, 41], which were not permeable to ethidium cations.



**Figure 4.** Effect of the 1  $\mu\text{M}$  ionomycin application on the membrane pores formation (%) and PBF1 fluorescence variations (a.u.) in human PMNs, in the absence of extracellular  $\text{Ca}^{2+}$ .

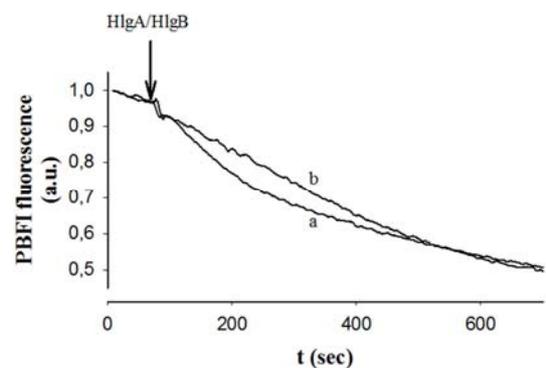
Thus, we conclude that, the HlgA/HlgB  $\gamma$ -hemolysin did not induce the opening of  $\text{K}^+$  channels in the absence of extracellular  $\text{Ca}^{2+}$  and, in this condition, membrane pores through human PMNs were involved in the permeability of  $\text{K}^+$  ions. It is well known that, thapsigargin provoked a rapid depletion of internal  $\text{Ca}^{2+}$  stores by inhibiting the sarcoplasmic-endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) activity [42], inducing as results the opening of CRAC channels. In the present study, we tried to arise the question whether thapsigargin could activate  $\text{K}^+$  channels after the internal  $\text{Ca}^{2+}$  stores depletion as previously demonstrated [43]. Indeed, as shown in Figure 5, when human PMNs were activated by thapsigargin at a given concentration (100  $\mu\text{M}$ ),

an efflux of  $\text{K}^+$  ions was clearly observed and which was totally inhibited by a potent blocker of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels, apamin (10  $\mu\text{M}$ ). As, it has already been shown for ionomycin, no ethidium entry was observed through potassium channels activated by thapsigargin (unpublished data). These results suggested that, an increase of the cytosolic  $\text{Ca}^{2+}$  level could activate  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels in human neutrophils as previously described [29, 30, 44].



**Figure 5.** Effect of thapsigargin (20  $\mu\text{M}$  followed by 100  $\mu\text{M}$ ) application on PBF1 fluorescence variations (a.u.) in human PMNs, in the absence (a) or presence (b) of 10  $\mu\text{M}$  apamin (60 min).  $[\text{Ca}^{2+}]_i = 0 \text{ mM}$ .

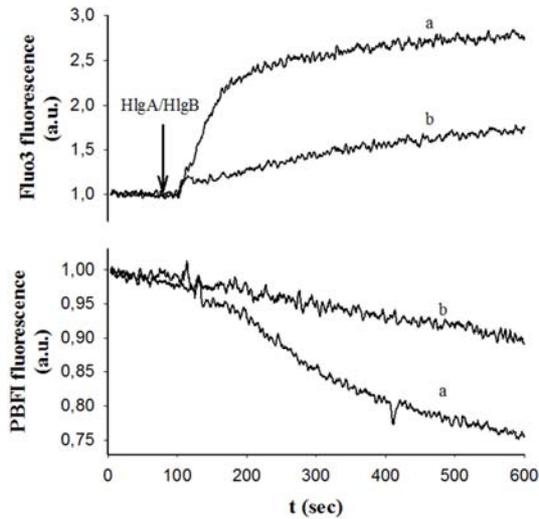
This was further investigated when another set of experiments was performed with HlgA/HlgB in the presence of 1 mM extracellular  $\text{Ca}^{2+}$ . When PMNs were pre-incubated with 100  $\mu\text{M}$  thapsigargin for 60 min, before the leukotoxins application, the  $\text{K}^+$  efflux was reduced (Figure 6). Under these conditions, the  $\text{Ca}^{2+}$  stores were depleted and their refilling was inhibited [42, 45].



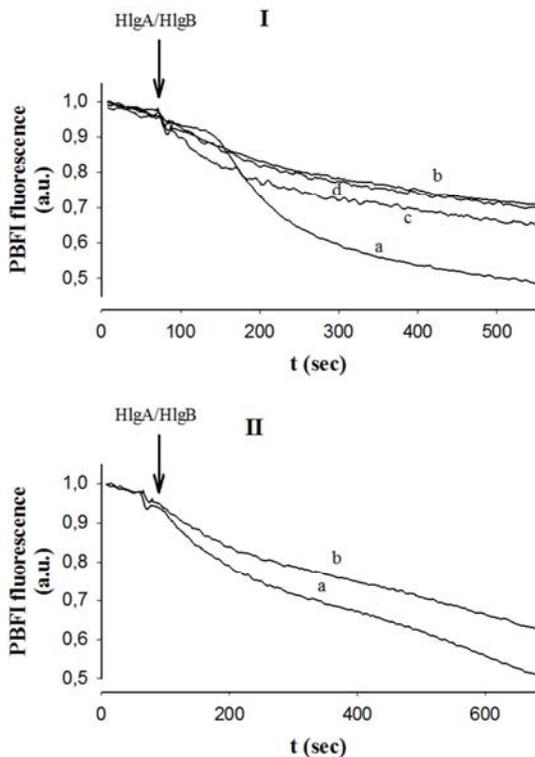
**Figure 6.** Effect of the HlgA/HlgB application on PBF1 fluorescence variations (a.u.) in human PMNs, in the absence (a) or presence (b) of 100  $\mu\text{M}$  thapsigargin (60 min).  $[\text{Ca}^{2+}]_e = 1 \text{ mM}$ .

Another set of experiments was designed in which, PMNs were pre-treated during 90 min with 500  $\mu\text{M}$  TMB8, a potent inhibitor of the  $\text{Ca}^{2+}$  release from internal  $\text{Ca}^{2+}$  stores [46-47]. Therefore, in the presence of 1 mM extracellular  $\text{Ca}^{2+}$ , the sustained increase of Fluo3 fluorescence variations, linked the increase of the intracellular  $\text{Ca}^{2+}$  level, was significantly reduced as shown in Figure 7. This was likely associated to the inhibition of the  $\text{Ca}^{2+}$  influx through  $\text{Ca}^{2+}$ -release activated  $\text{Ca}^{2+}$  (CRAC) channels as results to the inhibition of  $\text{Ca}^{2+}$  stores depletion by TMB8 [15]. Simultaneously, a significant inhibition of the  $\text{K}^+$  efflux through a  $\text{Ca}^{2+}$ -dependent process,

was observed when, neutrophils were pre-treated with TMB8. These results strongly suggested an eventual opening of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels by staphylococcal leukotoxins.



**Figure 7.** Effect of the HlgA/HlgB application on Fluo3 and PBFI fluorescence variations (a.u.) in human PMNs, in the absence (a) or presence (b) of 500  $\mu\text{M}$  TMB8 (120 min).  $[\text{Ca}^{2+}] = 1 \text{ mM}$ .

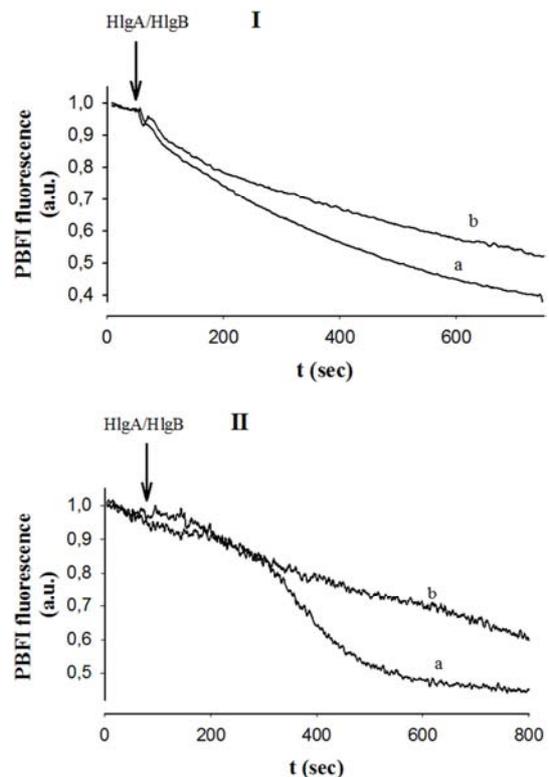


**Figure 8.** Effect of the HlgA/HlgB application on PBFI fluorescence variations (a.u.) in human PMNs, in the absence (a) or presence of 20  $\mu\text{M}$  tetrodotoxin (90 min) (Ib), 10  $\mu\text{M}$  apamin (60 min) (Ic), 20  $\mu\text{M}$  charybdotoxin (60 min) (Id) or 100  $\mu\text{M}$  quinine (15 min) (IIb).  $[\text{Ca}^{2+}] = 1 \text{ mM}$ .

A further evidence was given by Figure 8I which did show that, in the presence of 1 mM extracellular  $\text{Ca}^{2+}$ , the  $\text{K}^+$  efflux provoked by the HlgA/HlgB addition was completely inhibited by 10  $\mu\text{M}$  apamin [36, 48, 49], 20  $\mu\text{M}$  charybdotoxin [32, 50, 51] and 20  $\mu\text{M}$  tetrodotoxin [52] used

as specific and potent blockers of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels. A similar inhibitory effect was observed when, PMNs were pre-treated with 100  $\mu\text{M}$  quinine, another potent inhibitor for  $\text{K}_{\text{Ca}^{2+}}$  channels [51, 53, 54], as shown in Figure 8II.

Previous studies [55, 56] did report that, the opening of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels by several agonists in different types of cells might involve the activity of protein kinase in their signaling pathways. This was verified when the effect of H-89, a specific inhibitor of protein kinase A (PKA) [57] was tested on the activity of HlgA/HlgB-activated  $\text{K}^+$  channels. Indeed, as shown in Figure 9I, a significant inhibition of the  $\text{K}^+$  ions efflux was recorded when PMNs were pre-incubated with 5  $\mu\text{M}$  H-89 during 45 min. A similar inhibition was also observed (Figure 9II) when PMNs were pre-treated with 20  $\mu\text{M}$  PMA used as an activator of protein kinase C (PKC) [58]. Taken together, these results suggested an eventual involvement of protein kinase A/C in the activity of staphylococcal leukotoxins to induce the opening of pre-existing  $\text{K}^+$  channels.



**Figure 9.** Effect of the HlgA/HlgB application on PBFI fluorescence variations (a.u.) in human PMNs, in the absence (a) or presence of 5  $\mu\text{M}$  H-89 (45 min) (Ib) and 20  $\mu\text{M}$  PMA (90 min) (IIb).  $[\text{Ca}^{2+}] = 1 \text{ mM}$ .

Again, the involvement of sodium ( $\text{Na}^+$ ) channels in the activity of staphylococcal leukotoxins, was later explored in order to determine the specificity of membrane pores towards monovalent ions. For this, the tetrodotoxin as a potent inhibitor for sodium channels [59], was tested on the  $\text{Na}^+$  influx induced by staphylococcal leukotoxins. Interestingly, under these conditions, the  $\text{Na}^+$  movement was not modified by the tetrodotoxin (unpublished data), although the  $\text{K}^+$  efflux was markedly reduced as obviously described. Based on these

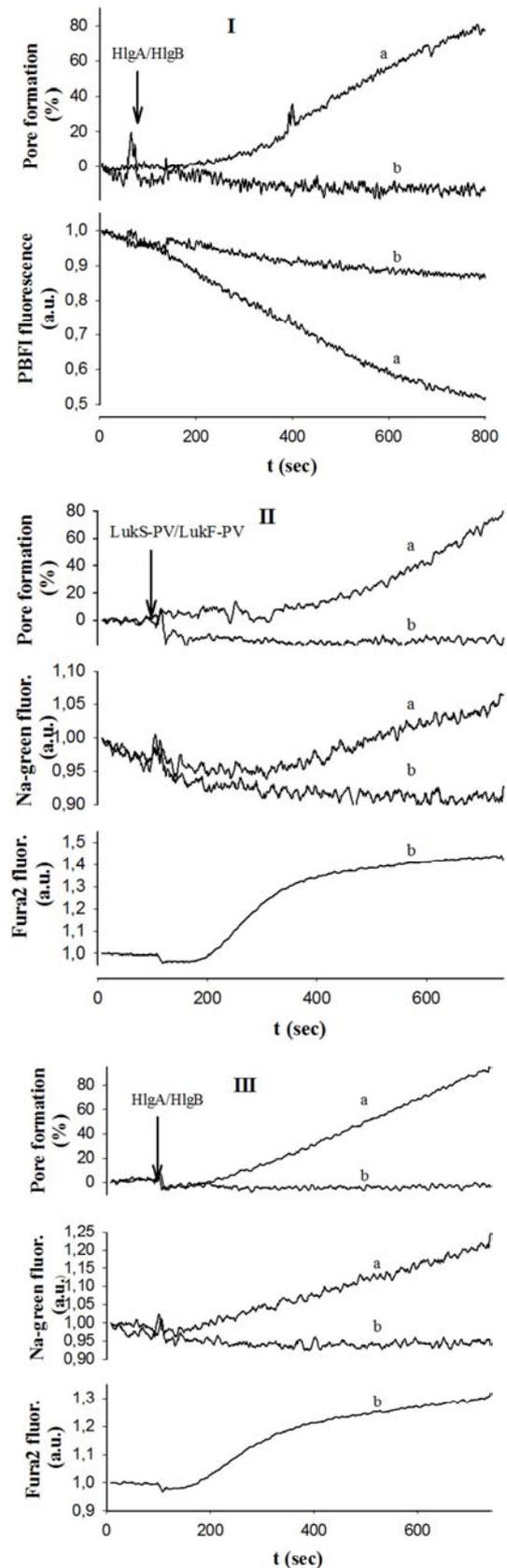
observations, we thought that,  $\text{Na}^+$  channels were not involved in the leukotoxins activity and consequently,  $\text{Na}^+$  ions might penetrate human neutrophils through membrane pores. Thus, the specificity of pores formed by leukotoxins from *S. aureus*, was further elucidated.

### 3.3. Specificity of Membrane Pores to Monovalent Ions ( $\text{Na}^+$ , $\text{K}^+$ )

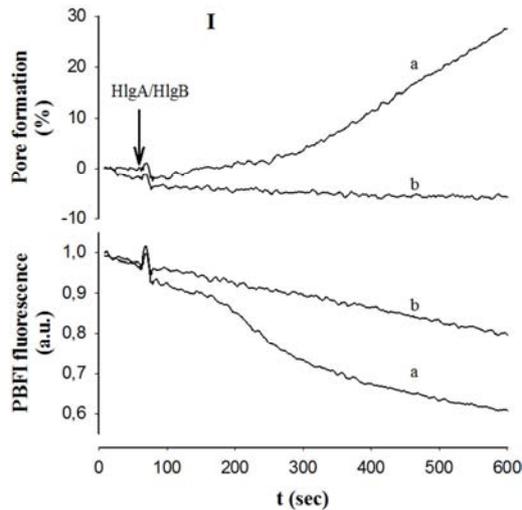
It has previously demonstrated that, bi-component staphylococcal leukotoxins did provoke the membrane pores formation in human neutrophils that, allowed the ethidium entry into target cells [7]. According to these authors, these pores could change their conformation in the presence of ions blockers such as  $\text{Zn}^{2+}$  or  $\text{Ca}^{2+}$  at higher concentration, to allow the divalent ions influx through ionic pores. Later, we provided an evidence that, staphylococcal leukotoxins  $\gamma$ -hemolysin and, Panton and Valentin Leukocidin (PVL) were able to induce two independent cellular events 1) the opening of pre-existing  $\text{Ca}^{2+}$  channels and 2) the formation of membrane pores which, seemed to be specific to monovalent cations ( $\text{Na}^+$ ,  $\text{K}^+$ , ethidium) [8] [25]. At a given concentration, the addition of  $\text{Ca}^{2+}$  and/or  $\text{Zn}^{2+}$  could reduce or completely inhibited the pores formation [8] [15]. Since, we [16] recently reported that, staphylococcal membrane pores did not drive  $\text{Cl}^-$  ions and divalent ions ( $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ ) [8] [15], we aimed in the present work to further elucidate the specificity of these trans-membrane pores towards monovalent ions such as  $\text{Na}^+$  and  $\text{K}^+$ . As shown in Figure 10Ia, in the absence of extracellular divalent ions blockers ( $\text{Ca}^{2+}$  and  $\text{Zn}^{2+}$ ), the application of HlgA/HlgB  $\gamma$ -hemolysin provoked simultaneously, significant movements of ethidium (influx) and  $\text{K}^+$  (efflux) ions, likely through PMNs membrane pores.

Interestingly, both movements were completely blocked, in the presence of both extracellular  $\text{Ca}^{2+}$  and  $\text{Zn}^{2+}$  used at given concentrations 0.1 mM and 0.2 mM, respectively (Figure 10Ib). These observations confirmed our previous hypothesis that, both ethidium and  $\text{K}^+$  ions movements could involve the same pathway under specific conditions [8]. Similar experiments were performed to further confirm the specificity of membrane pores towards monovalent ions,  $\text{Na}^+$ . As shown in Figure 10II (LukS-PV/LukF-PV) and, Figure 10III (HlgA/HlgB), no monovalent cations (ethidium and  $\text{Na}^+$ ) influx, was observed when the pores formation was completely inhibited by divalent ions blockers, 0.1 mM  $\text{Ca}^{2+}$  and 0.2 mM  $\text{Zn}^{2+}$ . Under these conditions, an important influx of divalent ions ( $\text{Ca}^{2+}$  and  $\text{Zn}^{2+}$ ), was sustained after the leukotoxins application.

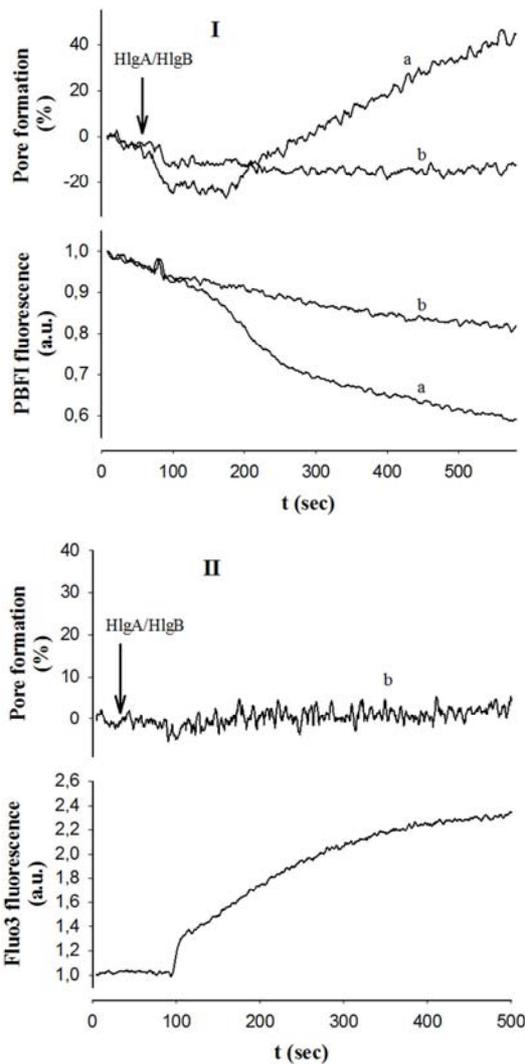
All together, these results strongly suggested that, the influx of  $\text{Na}^+$  ions was mediated through membrane pores whereas, the divalent ions ( $\text{Ca}^{2+}$  and  $\text{Zn}^{2+}$ ) influx was induced independently, through leukotoxins-activated  $\text{Ca}^{2+}$  channels. Again, similar results were obtained for both, ethidium and  $\text{K}^+$  movements when the membrane pores formation by HlgA/HlgB was blocked by 0.1 mM  $\text{La}^{3+}$  in the absence of extracellular  $\text{Ca}^{2+}$ , as shown in Figure 11.



**Figure 10.** Effect of the HlgA/HlgB (I, III) and LukS-PV/LukF-PV (II) application on the membrane pores formation (%) (I, II, III), PBF1 (I), Na-green (II, III) and Fura2 (II, III) fluorescence variations (a.u.) in human PMNs, in the absence (a) or presence (b) of 0.1 mM  $\text{Ca}^{2+}$  and 0.2 mM  $\text{Zn}^{2+}$ .



**Figure 11.** Effect of the HlgA/HlgB application on the membrane pores formation (%) and PBF1 fluorescence variations (a.u.) in human PMNs, in the absence (a) or presence (b) of 0.1 mM  $La^{3+}$ .  $[Ca^{2+}]_e = 0$  mM.



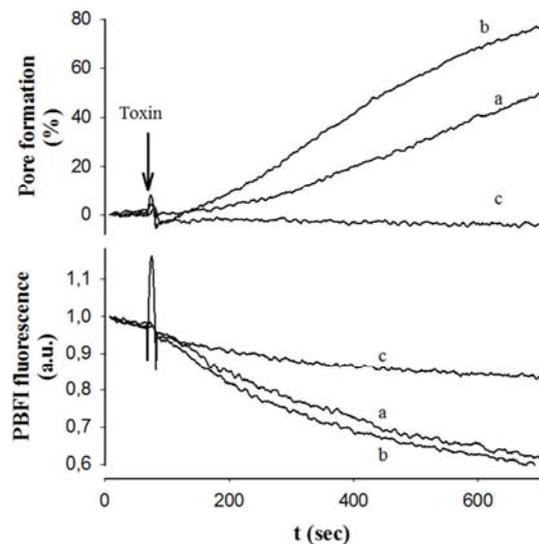
**Figure 12.** Effect of the HlgA/HlgB application on the membrane pores formation (%) (I, II), PBF1 (I) and Fluo3 (II) fluorescence variations (a.u.) in human PMNs, in the absence (a) or presence (b) of 10 mM TEA. (I)  $[Ca^{2+}]_e = 0$  mM, (II)  $[Ca^{2+}]_e = 1$  mM.

A further confirmation for the specificity of membrane pores to monovalent ions was given by, another set of experiments carried out in the absence of extracellular divalent ions ( $Ca^{2+}$  and/or  $Zn^{2+}$ ) and, the presence of 10 mM TEA, known as an inhibitor of potassium channels [37-38] [60]. Under these conditions, the membrane pores formation was totally inhibited and as results, no  $K^+$  efflux was recorded after the leukotoxins application (Figure 12I). An inhibitory effect was also, shown on the  $Na^+$  influx under this condition (unpublished data). Again, when the staphylococcal membrane pores formation was blocked by 10 mM TEA, in the presence of extracellular  $Ca^{2+}$ , an important influx of  $Ca^{2+}$  was recorded and which was sustained after the leukotoxins addition (Figure 12II).

This was a further confirmation that, divalent ions did enter human PMNs through specific pre-existing  $Ca^{2+}$  channels, which were not permeable to ethidium cations [8, 15]. These authors did also reported that, membrane pores provoked by staphylococcal leukotoxins were not permeable to divalent ions ( $Ca^{2+}$ ,  $Zn^{2+}$ ,  $Ni^{2+}$ ,  $Mn^{2+}$ ).

#### 3.4. Comparative Effects of HlgA/HlgB, HlgC/HlgB and LukS-PV/LukF-PV

In the present study, we have attempted to compare the cellular effect of different combinations of staphylococcal leukotoxins: HlgA/HlgB, HlgC/HlgB and LukS-PV/LukF-PV on the membrane of human neutrophils. First, in the absence of extracellular  $Ca^{2+}$ , both HlgA/HlgB (Figure 13a) and, HlgC/HlgB (Figure 13b) leukotoxins provoked the formation of membrane pores with different potentialities, allowing an important influx of ethidium into target cells simultaneously, to an efflux of  $K^+$  ions. Interestingly, as shown in Figure 13c with this particular donor, LukS-PV/LukF-PV was unable to induce both cellular events (ethidium influx and  $K^+$  efflux).

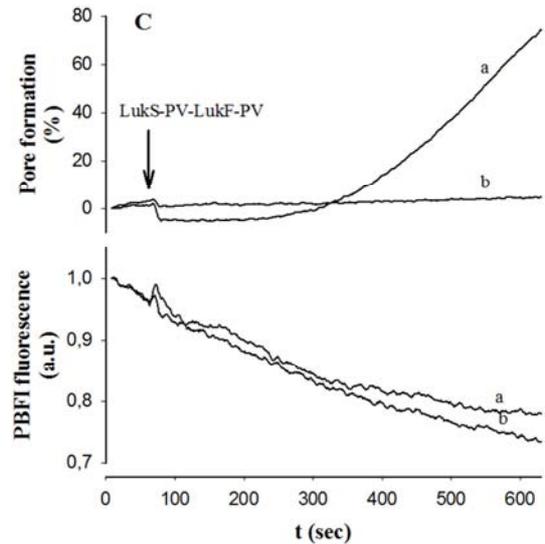


**Figure 13.** Effect of the HlgA/HlgB (a), HlgC/HlgB (b) and LukS-PV/LukF-PV (c) application on the membrane pores formation (%) and PBF1 fluorescence variations (a.u.) in human PMNs.  $[Ca^{2+}]_e = 0$  mM.

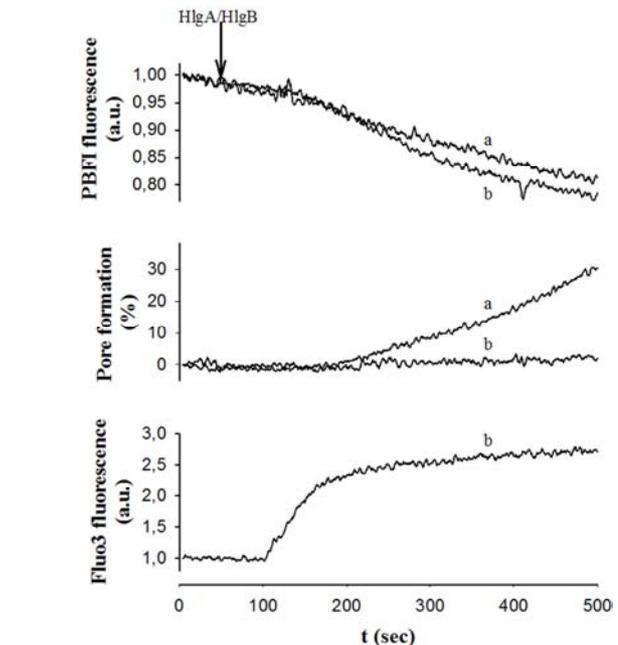
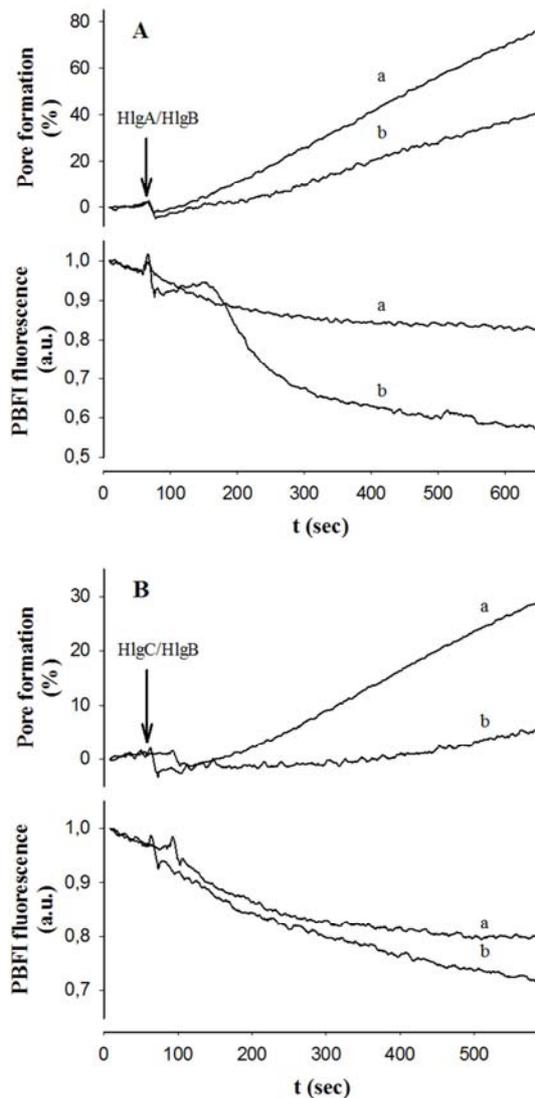
These results did strongly confirmed that, in the absence of

extracellular  $\text{Ca}^{2+}$ , the  $\text{K}^+$  efflux was mediated through membrane pores and no  $\text{K}^+$  channels were activated in this condition. Furthermore, comparative effects of different pairs of leukotoxins, tested on human PMNs isolated from the same donor, were presented in Figure 14.

Thus, in the absence of extracellular  $\text{Ca}^{2+}$ , all leukotoxins pairs tested were able to induce simultaneously an influx of ethidium and, an efflux of  $\text{K}^+$  ions through the membrane pores (Figure 14a). Nonetheless, in the presence of 1 mM extracellular  $\text{Ca}^{2+}$ , we found that, conversely to HlgA/HlgB and, HlgC/HlgB leukotoxins, LukS-PV/LukF-PV was unable to form pores that allowed the ethidium influx, whereas an efflux of  $\text{K}^+$  ions was observed, strongly associated to the opening of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels by leukotoxins (Figure 14b). Taken together, our data confirmed the hypothesis that, the potassium efflux was mediated by, two independent pathways depending on the absence, or presence of extracellular  $\text{Ca}^{2+}$ . This was strongly, confirmed as shown in Figure 15, when the HlgA/HlgB effect was tested on neutrophils obtained from another particular donor.



**Figure 14.** Effect of the HlgA/HlgB (A), HlgC/HlgB (B) and LukS-PV/LukF-PV (C) application on the membrane pores formation (%) and PBFI fluorescence variations (a.u.) in human PMNs, in the absence (a) or presence (b) of 1 mM extracellular  $\text{Ca}^{2+}$ .



**Figure 15.** Effect of the HlgA/HlgB application on the membrane pores formation (%), PBFI and Fluo3 fluorescence variations (a.u.) in human PMNs in the absence (a) and presence (b) of 1 mM extracellular  $\text{Ca}^{2+}$ .

Interestingly, although the membrane pores formation was completely blocked by 1 mM extracellular  $\text{Ca}^{2+}$ , an efflux of potassium ions through  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels was recorded simultaneously, to an influx of  $\text{Ca}^{2+}$  ions through  $\text{Ca}^{2+}$  channels after the leukotoxins application. To conclude, our data provided evidence that, the  $\text{K}^+$  efflux induced by leukotoxins was mediated by both pathways;  $\text{K}^+$  channels and staphylococcal membrane pores, depending on the absence and presence of extracellular  $\text{Ca}^{2+}$ . Again, these membrane pores were permeable with a high specificity to monovalent ions,  $\text{Na}^+$  and  $\text{K}^+$ .

## 4. Discussion

*Staphylococcus aureus* are invasive human pathogenic bacteria, which provoke lesions in target cells by modifying their membrane permeability to different ions. The ability of pathogenic clinical isolates of *S. aureus* to cause diseases, is conditioned by their ability to express specific determinants i.e. virulence factors such as pore-forming toxins (PFTs) during infection. Among these, bi-component leukotoxins such as Panton and Valentin Leukocidin (PVL) and,  $\gamma$ -hemolysin (HlgA/HlgB, HlgC/HlgB) are able to form pores inserted into PMNs membrane, which are large enough to allow an entry of ethidium, as previously described [7-8], leading to cell lysis. In previous studies [8, 15], we have been investigating the cellular events triggered by staphylococcal leukotoxins. Thus, we provided evidence that, Panton and Valentin Leukocidin (PVL) and,  $\gamma$ -hemolysin did provoke once bound to their target receptors, in the absence as in the presence of 1 mM extracellular  $\text{Ca}^{2+}$ , two independent cellular effects: 1) the opening of pre-existing  $\text{Ca}^{2+}$  channels and 2) the formation of pores inserted into the PMNs plasma membrane using a complex multistep process. Interestingly, these membrane pores have been shown to be not permeable to divalent ions ( $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ni}^{2+}$ ) although, they seemed to be involved in the monovalent ions ( $\text{Na}^+$  and  $\text{K}^+$ ) fluxes [8] [25].

Furthermore, we have recently highlighted the specificity of staphylococcal trans-membrane pores to monovalent anions ( $\text{Cl}^-$ ) and demonstrated again that, these pores were not involved in chloride ions movements (efflux/influx) through the PMNs membrane [16]. This was strongly, confirmed by a further evidence that, PFTs from *S. aureus* did induce the opening of  $\text{Cl}^-$  channels including  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels involved in  $\text{Cl}^-$  fluxes. Further investigations have already shown by simultaneous recordings of monovalent cations ethidium,  $\text{Na}^+$  and  $\text{K}^+$  movements that, bi-component staphylococcal leukotoxins could increase the membrane permeability to  $\text{Na}^+$  and  $\text{K}^+$  in the absence of extracellular  $\text{Ca}^{2+}$  [8] [25]. This was appeared to occur through, membrane pores that, allowed the ethidium entry into human neutrophils.

It is well known that, a large family of pore-forming toxins (PFTs) is produced by several Gram-positive bacteria, such as, *Listeria monocytogenes*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Clostridium perfringens* and *Bacillus anthracis*. These toxins which, include listeriolysin O (LLO), perfringolysin O, pneumolysin and streptolysin O (SLO), are responsible for a broad range of host responses [61] following their binding on the target membrane. Hence, they provoke the trans-membrane pores formation, which could exhibit an ion selection. An important consequence of pores formation by PFTs is the efflux of cellular  $\text{K}^+$ , which does occur at a negative membrane potential [62] as it has been shown, for *Listeria* Listeriolysin O (LLO), from *L.monocytogenes*. The  $\text{K}^+$  efflux subsequent to cell perforation by PFTs, is known to induce not only the lipid metabolic pathway [63] but, also to be responsible for the activation of caspase-1, histone modification and,

inflammasome activation [64]. Moreover, it has been shown that, the  $\text{K}^+$  efflux is required for the PFTs-induced autophagy and, may regulate the uptake of the pathogen as described by Vadia *et al.* [65]. In this context, we have attempted to further explore the leukotoxins from *S. aureus* activity on the membrane permeability towards  $\text{K}^+$  ions and, verify an eventual activation of  $\text{K}^+$  channels by  $\gamma$ -hemolysin and, PVL leukotoxins. Thus, in the absence of extracellular  $\text{Ca}^{2+}$ , staphylococcal leukotoxins induced a significant and sustained  $\text{Na}^+$ ,  $\text{K}^+$  and ethidium fluxes, as obviously described [8]. However, in the presence of 1 mM extracellular  $\text{Ca}^{2+}$ , we have observed that, although the influx of both ethidium and,  $\text{Na}^+$ , was significantly reduced, likely due to the protective effect of  $\text{Ca}^{2+}$  ions to prevent cells from lysis/death, the efflux of  $\text{K}^+$  was markedly increased in this condition, suggesting the activation of a novel  $\text{Ca}^{2+}$ -dependent pathway mediated in the potassium efflux.

It is well established that, number of ions such as calcium, potassium and chloride have important functional consequences during infectious diseases. Moreover, both  $\text{K}^+$  and,  $\text{Cl}^-$  channels had already been described to play a key role in the TNF-mediated liver cells death [39]. In this context, we have tried to verify the hypothesis that,  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels would be involved in the leukotoxins activity, upon their specific membrane binding as, we have recently reported that, staphylococcal leukotoxins did induce the opening of  $\text{Ca}^{2+}$ -release activated  $\text{Ca}^{2+}$  (CRAC) [15] and,  $\text{Cl}^-$  channels [16]. The  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels may play a key role in cell volume homeostasis and/or cellular activation [29] although, they are not well studied in neutrophils, as those in other types cells such as T and B cells [66-67]. The most comprehensive study of  $\text{K}^+$  channels in human neutrophils, was published, in 1990 by Krause and Welsh [29]. To explore the membrane permeability of human neutrophils to potassium ions, we first tested the effect of ionomycin, used as at a given concentration ( $\leq 1 \mu\text{M}$ ) to activate  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels as it has previously described [31, 41, 40]. Our data did show a sustained efflux of  $\text{K}^+$  ions following the PMNs activation by ionomycin, whereas no ethidium influx was observed during time suggesting, an activation of  $\text{K}^+$  channels  $\text{Ca}^{2+}$ -dependent, which were not permeable to ethidium cations. Similar results were obtained when, neutrophils were stimulated with thapsigargin, an activator of  $\text{K}^+_{\text{Ca}^{2+}}$  channels [43]. Furthermore, we found that  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels activated by thapsigargin, were totally inhibited by apamin [68].

Indeed, the ability of bi-component leukotoxins from *S. aureus* (HlgA/HlgB, HlgC/HlgB, LukS-PV/LukF-PV) to induce the opening of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels in human PMNs was strongly confirmed when the action of different inhibitors of the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels were tested for their ability to inhibit the  $\text{K}^+$  efflux. These agents such as, apamin, quinine [69] and charybdotoxin [50] were tested to assess the membrane permeability to potassium and sodium. A previous study [51] had reported that, the quinine effect was persisted comparing to charybdotoxin on  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels. It seems that, quinine is a wide spectrum channel

blocker affecting voltage-sensitive  $\text{K}^+$  channels as well as  $\text{Ca}^{2+}$ -activated ones [70, 71]. Nowadays, apamin is the most specific but not a universal blocker of the  $\text{Ca}^{2+}$ -activated potassium conductance. The  $\text{K}^+$  efflux enhanced by the presence of 1 mM extracellular  $\text{Ca}^{2+}$  was significantly inhibited by the different blockers tested although, the inhibitory effect of these compounds on the pore formation was not observed when the experiments were carried out in the absence of extracellular  $\text{Ca}^{2+}$ . In this condition, since the ethidium influx and,  $\text{K}^+$  efflux were not affected by all these blockers, we strongly suggested an eventual activation of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels by leukotoxins, only in the presence of extracellular  $\text{Ca}^{2+}$ . It has previously shown that, an increase in the cytosolic concentration of  $\text{Ca}^{2+}$  caused the erythrocytes of most mammalian species to become much more permeable to  $\text{K}^+$  ions as first described by Gardos *et al.* [72]. These channels were sensitive to quinine [73] and, charybdotoxin [34, 74]. Again, this was confirmed when potent inhibitors for the internal  $\text{Ca}^{2+}$  stores depletion, TMB8 [46, 47] and, thapsigargin [42, 45] were tested on the leukotoxins-activated  $\text{K}^+$  channels in the presence of 1 mM extracellular  $\text{Ca}^{2+}$ . Consequently, the  $\text{K}^+$  efflux induced by leukotoxins was markedly, reduced in both situations. This was strongly associated to the opening of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels by staphylococcal leukotoxins. These findings were supported by the previous study [75] which suggested that,  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels were in correlation to the opening of  $\text{Ca}^{2+}$ -release activated  $\text{Ca}^{2+}$  (CRAC) channels which, were induced as results to the internal  $\text{Ca}^{2+}$  stores depletion. This later event was already reported in a recent work when, human neutrophils were activated by staphylococcal leukotoxins [15].

Furthermore, it is well established that, membrane ion channels selective for  $\text{K}^+$  ions, vary greatly in regards to their unitary conductance, voltage- and  $\text{Ca}^{2+}$ -dependence, and affinity for blocking agents [76]. Likewise, the  $\text{K}^+$  permeability specifically activated by an increase in the intracellular  $\text{Ca}^{2+}$  concentration, was first, identified in red blood cells by Gardos *et al.* [72] then, characterized in the cell membrane of a wide variety of excitable (central and peripheral neurons) and non-excitable (erythrocytes) cells [77]. Moreover, there is evidence for subtypes of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels in different tissues. In fact, three main types ( $\text{BK}_{\text{Ca}}$ ,  $\text{IK}_{\text{Ca}}$  and  $\text{SK}_{\text{Ca}}$ ), have been identified and, each has now been cloned [78]. Earlier functional, binding and structural studies suggested that, there are several kinds of  $\text{SK}_{\text{Ca}}$  channels. The intermediate ( $\text{IK}_{\text{Ca}}$ ) and small ( $\text{SK}_{\text{Ca}}$ ) conductance  $\text{Ca}^{2+}$ -sensitive  $\text{K}^+$  channels have already been described in endothelial cells [79]. As results, it was suggested a novel negative feedback mechanism in endothelium by which, intracellular  $\text{Ca}^{2+}$  would have an opposing effect (activation and blocking) on  $\text{SK}_{\text{Ca}}$  and  $\text{IK}_{\text{Ca}}$  channels, depending on local  $\text{Ca}^{2+}$  levels [79]. Again, Benton *et al.* [80] found differences in the effects of Gardos Channel [72] blockers that are likely to reflect different modes of interaction with the channel. Finally, to conclude, our data proposed a novel type of ionic channels namely  $\text{Ca}^{2+}$ -activated  $\text{K}^+$

channels\* which, was involved in the bi-component staphylococcal leukotoxins (HlgA/HlgB, HlgC/HlgB, LukS-PV/LukF-PV) activity, only in the presence of extracellular  $\text{Ca}^{2+}$ .

Further experiments were carried out to better, understand the intracellular mechanism involved in the leukotoxins activity to induce  $\text{K}^+_{\text{Ca}^{2+}}$  channels in human neutrophils. Number of studies [81, 82] have already suggested the role of protein kinase A (PKA) in the opening of potassium channels  $\text{Ca}^{2+}$ -dependent. Interestingly, these reports did support our results obtained when, the effect of H-89, a potent inhibitor of PKA, was tested on leukotoxins-activated  $\text{K}^+$  channels. The inhibitory effect of H-89 on the  $\text{K}^+$  efflux suggested that, a phosphorylation- dependent process was required for the  $\text{K}^+$  channels activation. Based on this observation, we have advanced the concept that, number of intracellular  $\text{Ca}^{2+}$  signaling pathways were involved during the ion channels activation by staphylococcal leukotoxins. On the other hand, we found that, PMA could block completely the  $\text{K}^+$  efflux triggered by leukotoxins in the presence of extracellular  $\text{Ca}^{2+}$ . In a previous work, Alhuwalia *et al.* [55] found that, the phorbol ester phorbol 12-myristate 13-acetate (PMA), a protein kinase C-dependent NADPH oxidase activator [56], significantly increased the intracellular  $\text{Ca}^{2+}$  in neutrophils and activated consequently,  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  currents. In contrast, Peers *et al.* [83] have already described in a previous study, the inhibitory effect of protein kinase C (PKC) on  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels in rat carotid body type I cells. Based on this report, we thought that, the opening of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels by leukotoxins could involve the PKC activity. Moreover, Peppelenbosch *et al.* [84] have already demonstrated that, the PKC activator, TPA (12-O-teradecanoylphorbol-13-acetate), had an inhibitory effect on the  $\text{K}^+$  efflux induced by the epidermal growth factor (EGF). Again, a previous study [85] did show that, the toxicity effect of Panton and Valentin Leukocidin (PVL) was significantly, reduced on PMA-pretreated HL-60 cells and, both phospholipase  $\text{A}_2$  (PLA2) and C (PLC) were involved in this process. According Marunaka *et al.* [54], the activation of quinine-sensitive  $\text{K}^+$  channels was mediated through, the protein tyrosine kinase (PTK) activity in a cytosolic  $\text{Ca}^{2+}$ -dependent manner. In conclusion, we suggested that, both PKA and PKC might be involved to regulate leukotoxins-activated  $\text{K}^+$  channels whereas, the molecular mechanisms by which staphylococcal leukotoxins involved the  $\text{K}^+$  channels in their activity, remained incompletely understood and, further studies would be needed to better clarify this intracellular process.

Nevertheless, in the present research, we found that in the absence of extracellular  $\text{Ca}^{2+}$ , the  $\text{K}^+$  efflux was mediated through, membrane pores formed by leukotoxins. These findings were also supported when the inhibitory effect of  $\text{Ba}^{2+}$ , a potent blocker for voltage- and  $\text{K}^+_{\text{Ca}^{2+}}$  channels [36], was not observed in the absence of extracellular  $\text{Ca}^{2+}$  (unpublished data). Moreover, the  $\text{Na}^+$  influx seemed to be mediated through membrane pores, since no inhibitory effect was recorded when, neutrophils were pre-treated with

tetrodotoxin, a potent blocker for  $\text{Na}^+$  channels [59] (unpublished data). Whereas, the  $\text{K}^+$  efflux induced by leukotoxins in the presence of extracellular  $\text{Ca}^{2+}$ , was inhibited by tetrodotoxin as it was previously reported [52].

Another important result of the present study was the specificity of membrane pores formed by staphylococcal leukotoxins to monovalent ions  $\text{Na}^+$  and  $\text{K}^+$ . We have previously demonstrated that, both  $\gamma$ -hemolysin and, PVL leukotoxins provoked in the absence of extracellular  $\text{Ca}^{2+}$ , the membrane pores formation in PMNs which allowed simultaneous  $\text{Na}^+$ ,  $\text{K}^+$  and ethidium fluxes [8, 25]. This was further confirmed when the pores formation was blocked in the presence of both divalent ions blockers 0.1 mM  $\text{Ca}^{2+}$  and 0.2 mM  $\text{Zn}^{2+}$ . In this condition, no  $\text{Na}^+$  and  $\text{K}^+$  movement was observed although, a sustained influx of  $\text{Ca}^{2+}$  and  $\text{Zn}^{2+}$  was recorded. This was likely due to the protective effect of  $\text{Ca}^{2+}$  [86] and  $\text{Zn}^{2+}$  [87] at a given concentration. Moreover, a similar inhibitory effect was observed on monovalent ions movements when, 0.1 mM  $\text{La}^{3+}$  was used to block the pore formation preventing cells from lysis, as previously described [88-89]. Finally, blocking of the pores formation by ionic blockers ( $\text{Ca}^{2+}$  and/or  $\text{Zn}^{2+}$ ) and,  $\text{La}^{3+}$ , at a given concentration, abrogated the  $\text{K}^+$  efflux and  $\text{Na}^+$  influx. This consequently, confirmed that, membrane pores formed by leukotoxins did exhibit a high specificity to  $\text{Na}^+$  and  $\text{K}^+$  ions, as it has already described for number of PFTs, which were able to induce an efflux of  $\text{K}^+$  through membrane pores formed on target cells [64]. In contrast to  $\text{Na}^+$  influx, the  $\text{K}^+$  efflux seemed to be a potent signal induced by different bacteria during infection, which might trigger at least, two separate pathways [64], depending on the absence or presence of extracellular  $\text{Ca}^{2+}$ . Moreover, previous studies [22] did report that, the NLRP3 inflammasome activation by bacterial toxins was correlated with an efflux of  $\text{K}^+$  and influx of  $\text{Na}^+$  but not, with permeation of  $\text{Cl}^-$  ions. Again the  $\text{Na}^+$  influx was not an absolute requirement for NLRP3 activation conversely to the  $\text{K}^+$  efflux which was shown to activate not only multiple protein kinases [90] but, to induce p38-MAP kinase signaling in lung epithelial cells, likely associated with inflammatory or immune signaling responses [91].

To better, evaluate the specificity of membrane pores to different ions we further investigated the blocking action of TEA on the leukotoxins activity. Thus, in the absence of extracellular  $\text{Ca}^{2+}$ , the inhibitory effect on the pores formation did prevent  $\text{Na}^+$  and  $\text{K}^+$  fluxes (influx/efflux), whereas in another set of experiments,  $\text{Ca}^{2+}$  channels were remained activated when TEA was tested on the  $\text{Ca}^{2+}$  influx in the presence of 1 mM extracellular  $\text{Ca}^{2+}$ . In conclusion, our data all together provided a strong support for the notion that, in the absence of extracellular  $\text{Ca}^{2+}$ , staphylococcal membrane pores were involved in the monovalent cations ( $\text{Na}^+$ ,  $\text{K}^+$ ) movements since under these condition,  $\text{K}^+$  channels were not activated. Nevertheless, in the presence of extracellular  $\text{Ca}^{2+}$ ,  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels were stimulated to mediate  $\text{K}^+$  ions efflux, upon a specific binding of leukotoxins to their membrane receptors. More interestingly, our results ruled out the hypothesis that,  $\text{Na}^+$  channels would be activated, by

staphylococcal leukotoxins in both situations, and the sodium influx might occur only through membrane pores. Finally, a further evidence to confirm the specificity of pores towards monovalent ions, was given by the result obtained with a specific donor, in a comparative study performed with HlgA/HlgB, HlgC/HlgB and, LukS-PV/LukF-PV leukotoxins. First, in the absence of extracellular  $\text{Ca}^{2+}$ , LukS-PV/LukF-PV was unable to provoke the pores formation and, as results, no  $\text{K}^+$  efflux was observed, conversely to HlgA/HlgB and, HlgC/HlgB, which were able to induce simultaneously, ethidium and  $\text{K}^+$  fluxes through membranes pores. In this condition,  $\text{K}^+$  channels were not activated by, staphylococcal leukotoxins. Secondly, in the presence of 1 mM extracellular  $\text{Ca}^{2+}$ , we found that, although LukS-PV/LukF-PV was able to induce an efflux of  $\text{K}^+$  through  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels, no membrane pores were formed by this leukotoxin. These results strongly confirmed our evidence that, staphylococcal leukotoxins may induce two independent cellular events involved in the  $\text{K}^+$  efflux (membrane pores and  $\text{K}^+$  channels).

## 5. Conclusion

Bacterial infectious diseases are a leading cause of death by producing a number of virulence factors such as pore-forming toxins (PFTs), which disrupt the plasma membrane of host cells provoking cell lysis. These PFTs are produced by, many bacteria, predominately by Gram-positive bacterial pathogens such as, *S. aureus*. It has been described that, pore-forming toxins namely membrane-damaging toxins including,  $\gamma$ -hemolysin (HlgA/HlgB, HlgC/HlgB) and, LukS-PV/LukF-PV (PVL) from *S. aureus* can promote and activate apoptotic cascades at sub-lytic concentrations through the intracellular pathway, destroying mitochondrial membrane and releasing of cytochrome c [92] to promote cells death. Additionally, a number of  $\text{Ca}^{2+}$ -depending events have been reported during immune cells responses against staphylococcal infections, including degranulation/secretion of granules contents of PMNs which occurred via the exocytosis, a  $\text{Ca}^{2+}$ -dependent process [93] and burst oxidative and ROS production [94]. Recently, we have described the mechanism involved in the activity of staphylococcal leukotoxins to induce, two independent cellular events, in human neutrophils: *i*) the opening of  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  channels including,  $\text{Ca}^{2+}$ -release activated  $\text{Ca}^{2+}$  (CRAC) channels and,  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels as results, to the internal  $\text{Ca}^{2+}$  stores depletion, and *ii*) upon their insertion and oligomerization, they formed membrane pores, leading to cell lysis [15-16]. In general, intracellular  $\text{Ca}^{2+}$ , as being an important second messenger that activates several signaling pathways, we thought that, bi-component leukotoxins from *S. aureus* were potent regulators of intracellular signaling and immunity, involving different intracellular effectors depending on the leukotoxins combination tested (unpublished data).

Of great interest, our recent findings [15-16] together with the current study are the first to predominantly, shed light on the mechanism of the bi-component staphylococcal

leukotoxins activity. We highlighted two independent cellular events induced by these pore-forming leukotoxins: 1) the opening of pre-existing ions channels including, Ca<sup>2+</sup>, Cl<sup>-</sup> and K<sup>+</sup> channels, and 2) the formation of trans-membrane pores impermeable to divalent ions (Ca<sup>2+</sup>, Zn<sup>2+</sup>, Mn<sup>2+</sup>, Ni<sup>2+</sup>) and monovalent anions (Cl<sup>-</sup>) despite, their high specificity to monovalent ions (Na<sup>+</sup>, K<sup>+</sup>). Furthermore, it has been argued in the present research that, the K<sup>+</sup> efflux is mediated through two independent pathways (pores and Ca<sup>2+</sup>-activated K<sup>+</sup> channels), depending, on the absence (pores) and presence of extracellular Ca<sup>2+</sup>. These data suggested a novel mechanism by which staphylococcal leukotoxins and, other PFTs could modulate host cell survival and inflammatory pathways during the course of a bacterial infection.

Hence, Ca<sup>2+</sup>-activated K<sup>+</sup> channels have been described to be involved, in autoimmunity, as well as cardiovascular diseases [95]. It has also, been reported that, ion channels are key regulators of innate and, adaptive immune responses. For instance, K<sup>+</sup> channels have been implicated in a number of human pathologies, such as, asthma [96], T-cell mediated autoimmune disease [97-98], immune response to infection and inflammation [99]. For this, these channels are currently, used as clinical targets and, this would be essential on the context of developing potential therapeutic interventions for severe bacterial infections.

More interestingly, it has long been described that, K<sup>+</sup> efflux would be required for the PFT-induced autophagy [21] [100] and, to mediate p38 MAPK activation by *S. aureus* alpha-toxin, *Vibrio cholerae* cytolysin (VCC), Streptolysin O (SLO) and *E. coli* hemolysin A (HlyA) [20]. PFTs were also been described to be able to trigger apoptosis as reported for  $\alpha$ -toxin from *S. aureus* [6, 101]. Thus, both ROS (reactive oxygen species) and K<sup>+</sup> efflux likely associated to the stimulation of K<sup>+</sup> channels, were considered as an intracellular signal essential for triggering inflammasome activation, during various patho-physiological conditions. Again, previous studies revealed that, PFTs might induce an efflux of K<sup>+</sup> from target cells, leading to various outcomes ranging from inflammasome to pyroptosis to autophagy, depending on the cell type and as expected, inhibition of PFTs-mediated K<sup>+</sup> efflux did prevent the recruitment and activation of caspase-2, as an initiator caspase to mediate apoptotic cell death [102]. This would prevent cell death. Moreover, the death-inducing role of Panton and Valentin Leukocidin (PVL) from *S. aureus* in neutrophils has been previously described [92, 103, 04].

In conclusion, our research provided new insights into the complexity of cellular responses against staphylococcal infections. We highlighted in the present study, a novel signaling cascade triggered by staphylococcal leukotoxins (Panton and Valentin Leukocidin and  $\gamma$ -hemolysin) upon their specific binding to membrane receptors. They do induce the opening of Ca<sup>2+</sup>-activated K<sup>+</sup> channels as results of the internal Ca<sup>2+</sup> stores depletion. To our knowledge, it is becoming evident that, Ca<sup>2+</sup> plays a key role and, is involved in a wide range of host cell responses against leukotoxins. Further investigations would be needed to elucidate how, these leukotoxins could modulate host signaling cascades

generated from multiple signals that, are mobilized by ion fluxes to develop new means to fight pathogens during infections.

## Conflict of Interest

The authors declare they have no competing interests.

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