

Bi-component Staphylococcal Leukotoxins Induce Chloride Ions Fluxes in Human Neutrophils: Opening of Ca^{2+} -activated Cl^- Channels

Leïla Staali^{1,2,*}, Didier André Colin²

¹Department of Biotechnology, Natural and Life Sciences Faculty, Ahmed Ben Bella Oran1-University, Oran, Algeria

²Bacteriology Institute of Medical Faculty, Louis Pasteur University, Strasbourg, France

Email address:

lstaali1@yahoo.com (L. Staali)

*Corresponding author

To cite this article:

Leïla Staali, Didier André Colin. Bi-component Staphylococcal Leukotoxins Induce Chloride Ions Fluxes in Human Neutrophils: Opening of Ca^{2+} -activated Cl^- Channels. *International Journal of Microbiology and Biotechnology*. Vol. 5, No. 3, 2020, pp. 110-119.

doi: 10.11648/j.ijmb.20200503.16

Received: June 1, 2020; Accepted: June 15, 2020; Published: June 29, 2020

Abstract: The bi-component leukotoxins; γ -hemolysin and Panton and Valentin Leukocidin (PVL) from *Staphylococcus aureus* induce two independent cellular events 1) the formation of trans-membrane pores not permeable to chloride (Cl^-) ions and 2) the activation of at least, two modes of chloride fluxes (efflux/influx), including pre-existing Ca^{2+} -activated Cl^- channels (CaCC) in human polymorphonuclear neutrophils (PMNs). This was investigated by using spectrofluorometry techniques and the chloride-sensitive quencher fluorescent indicator, MQAE (N-(6-methoxyquinolyl) acetoacetyl ester). The ethidium bromide was used as an indicator for the trans-membrane pores formation by staphylococcal leukotoxins. In the absence of extracellular Ca^{2+} , HlgA/HlgB, HlgC/HlgB and LukS-PV/LukF-PV leukotoxins from *S. aureus* induced a massive efflux of chloride (Cl^-) ions. Interestingly, in the presence of extracellular Ca^{2+} , the HlgA/HlgB γ -hemolysin provoked a biphasic response of Cl^- movements (efflux/influx). Conversely to HlgA/HlgB and LukS-PV/LukF-PV, HlgC/HlgB leukotoxins did not induce any Cl^- movement under this condition (e.g. in the presence of extracellular Ca^{2+}). The potent Cl^- channel inhibitor, DIDS, did inhibit significantly the Cl^- fluxes caused by all pairs of staphylococcal leukotoxins tested in both conditions. In the present study, we found that the inhibitory effect of flufenamic acid, known as a Cl^- channel inhibitor, was restricted only to the Ca^{2+} -dependent Cl^- influx triggered only by HlgA/HlgB and LukS-PV/LukF-PV leukotoxins. These findings might suggest that, Cl^- fluxes in human neutrophils did involve at least, two different types of Cl^- pathways, depending on the absence or presence of extracellular Ca^{2+} . Both Cl^- channels blockers, DIDS and flufenamic acid did not alter the pores formation by staphylococcal leukotoxins. Furthermore, under conditions when the membrane pores formation was blocked by divalent ions (Ca^{2+} and/or Zn^{2+}), Cl^- ions movements were still observed. Taken together, our results strongly provide an evidence that: i) trans-membrane pores formed by staphylococcal leukotoxins: HlgA/HlgB, HlgC/HlgB (γ -hemolysin) and LukS-PV/LukF-PV (PVL) do not drive Cl^- ions fluxes ii) at least, two different types of Cl^- ions pathways are activated, depending on the absence or presence of extracellular Ca^{2+} , including Ca^{2+} -activated Cl^- channels (CaCC) and, iii) Ca^{2+} -activated Cl^- channels are mediated only by HlgA/HlgB and LukS-PV/LukF-PV leukotoxins.

Keywords: Pore-forming Toxin, *S. aureus*, Leukotoxin, Cl^- channels, γ -hemolysin, Panton-Valentin Leukocidin, Neutrophils, Spectrofluorometry

1. Introduction

S. aureus secretes a family of bi-component pore-forming leukotoxins whose components belong either to class S or class F [1]. The Pantone-Valentine Leukocidin (class S:

LukS-PV; class F: LukF-PV) is secreted by clinical strains associated with furuncles [2-3], and forms one toxic pair LukS-PV/LukF-PV. The γ -hemolysin (class S: HlgA, HlgC; class F: HlgB) which forms two toxic pairs HlgA/HlgB and HlgC/HlgB, is secreted by all clinical strains [4]. It has been

shown that, the class S component binding was a pre-requisite to binding of the class F component [5-6]. Both components (S/F) synergistically induce the opening of pre-existing Ca^{2+} channels [7] and then form pores through the membrane of human neutrophils [8]. These membrane pores result from oligomerization of both components (class S/class F) into the membrane and it was proposed from previous studies using synthetic lipidic vesicles that, these two components assembled as heterohexamers [9]. The heterohexamer structure was confirmed by stimulation of the pore construction after solving the crystal structure of LukF-PV [10]. Furthermore, it has been demonstrated that, these membrane pores were not permeable to divalent ions (Ca^{2+} , Mn^{2+} , Zn^{2+}) but might be pathways for Na^+ and K^+ ions [7, 11]. Again, we have previously reported [7, 12] that, bi-component staphylococcal leukotoxins did induce the opening of different types of Ca^{2+} channels, including Ca^{2+} -release activated Ca^{2+} (CRAC) channels, as results to a massive internal Ca^{2+} stores depletion. An increase in intracellular Ca^{2+} levels into PMNS might regulate different cellular events and the relevance of such a process, has not been elucidated and remained unclear in the activity of staphylococcal leukotoxins in human neutrophils. A previous study [13], did report that leukotoxins from *S. aureus* were able to control as an early intracellular event in signaling pathways, the activation of the respiratory burst as results to human neutrophils activation, during the immune response against staphylococcal infectious diseases. It is widely known that, chloride ions movements may represent an essential step in the cascade of early intracellular events leading to the human neutrophils pathophysiology. Nevertheless, the impact of staphylococcal leukotoxins on the Cl^- ions permeability in human PMNs has not been explored and a very little is known about the specificity of pores to Cl^- ions.

Chloride movements in human neutrophils may occur through diverse pathways. Thus, several types of Cl^- channels have been reported to be involved in human PMNs activation including, Ca^{2+} -activated Cl^- channels [14-15], voltage-dependent Cl^- channels [16], and swelling-activated Cl^- channels [17]. Furthermore, Gallin *et al.* [18] have previously demonstrated that, at least three different types of chloride channels might be activated and which could exhibit differences in their conductance (maxi, medium, small). In this context, attention has been focused in the present research to further investigate whether chloride ions movements through Cl^- channels could be a target during staphylococcal infections, which might probably contribute to cells damage before death.

The objectives of the present study were: 1) to determine whether the membrane pores formed by leukotoxins were Cl^- anions conductive pathways, 2) to investigate if pre-existing Cl^- ions pathways were involved in the leukotoxins activity, and 3) to compare the membrane activity (pores formation and Cl^- ions fluxes) caused by staphylococcal HlgA/HlgB, HlgC/HlgB and LukS-PV/LukF-PV leukotoxins. This was achieved in human neutrophils by using spectrofluorometry techniques and fluorescent probes. The chloride-sensitive

fluorescent indicator MQAE was a useful tool to determine the intracellular chloride activity. Interestingly, our data provided evidence that, *i)* membrane pores formed by staphylococcal leukotoxins did not drive Cl^- anions fluxes, *ii)* the time course of leukotoxins-stimulated Cl^- flux differed depending, on the leukotoxins pair tested and *iii)* in the absence of extracellular Ca^{2+} , both γ -hemolysin and Panton-Valentin Leukocidin (PVL) leukotoxins induced a massive Cl^- efflux through a specific Cl^- pathway sensitive only to DIDS. Whereas, in the presence of extracellular Ca^{2+} , only HlgA/HlgB and LukS-PV/LukF-PV leukotoxins induced the opening of another type of chloride channels, known as Ca^{2+} -activated Cl^- channels* sensitive to DIDS and flufenamic acid, potent blockers of pre-existing Cl^- channels.

2. Materials and Methods

2.1. Reagents

N-(6-methoxyquinolyl) acetoacetyl ester (MQAE) from Molecular Probes (Eugene, OR) was diluted in Me_2SO to 1 M. J. PREP was from Tech Gen International (Les Ulis, France). Flufenamic acid was from Parke-Davis (Detroit, MI). DIDS, 4, 4'-diisothiocyanato-dihydrostilbene-2, 2'-disulfonic acid and all other reagents were from SIGMA (L'Isle d'Abeau Chesnes, France).

2.2. Leukotoxins Purification

The 5 components of leukotoxins (LukS-PV, LukF-PV, HlgA, HlgC, HlgB) secreted from the *S. aureus* V8 strain (ATCC 49775) kindly provided by S. Thornley (Wellcome Laboratories, London, UK), were purified as described previously [1]. Briefly, they were stored at -80°C before use. In all experiments, class S components (HlgA, HlgC, LukS-PV) were injected at a final concentration 2.2 nM and class F (HlgB, LukF-PV) at 0.85 nM.

2.3. Polymorphonuclear Neutrophils Isolation

Human polymorphonuclear neutrophils (PMNs) were prepared from buffy-coat of healthy donors of either sex, kindly provided by the *Etablissement de Transfusion Sanguine (ETS) - Strasbourg, France*, as previously described [7]. Briefly, human PMNs enriched blood was centrifuged in J. Prep and the pellet was suspended in dextran for sedimentation. Contaminating erythrocytes were removed by an hypotonic lysis. After purification, the PMNs suspension was adjusted to 6.10^6 cells/ml in the assay medium containing (in mM): 140 NaCl, 5 KCl, 10 glucose, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 0.1 ethylene glycol-bis (b-aminoethyl ether)-N, N, N', N'-tetraacetic acid, and 3 Tris-base (pH 7.3). This method led to 98% viable cells as counted after May-Grunwald-Giemsa staining.

2.4. MQAE Loading

MQAE is a chloride ion (Cl^-)-quenched fluorescent

indicator considered as a useful tool to measure the dynamic changes in intracellular Cl^- levels [19-20]. It has been found to be a useful fluorescence dye for non-invasive measurements of the intracellular Cl^- concentration. In this study, to investigate the Cl^- movement in human neutrophils, PMNs were incubated with 10 μM MQAE during 60 min in an atmosphere of 95% air/5% CO_2 at 37°C in the assay medium. Then, loaded-PMNs were washed twice by centrifugations at 800xg for 10 min to remove the extracellular dye, and were re-suspended in the assay medium at 6×10^6 cells/ml.

2.5. Fluorescence Determination

Fluorescence intensity variations of chloride-sensitive indicator MQAE and ethidium bromide were simultaneously recorded with dual excitation and dual emission spectrofluorometer DeltaScan (Bio-Tek Kontron, PTI, St Quentin en Yvelines, France) with slit width set at 4 nm. For fluorescence measurements, one ml of PMNs suspension (6.10^6 cells/ml) was added to 1 ml of assay solution continuously stirred in a 4 ml quartz cuvette (1cm light path), thermostated at 37°C.

Relative fluorescence variations of quenched fluorescent indicator MQAE, recorded at $\lambda_{\text{EX}}=360$ nm, $\lambda_{\text{EM}}=460$ nm, were expressed in all results as arbitrary units (a.u.). An increase in MQAE fluorescence was relative to an efflux of Cl^- ions from human PMNs, whereas a decrease in the MQAE fluorescence intensity during time is proportional to the Cl^- ions influx, indicating an increase in intracellular Cl^- levels into neutrophils.

The fluorescence increase of the ethidium cation (100 μM ; $\lambda_{\text{EX}}=340$ nm, $\lambda_{\text{EM}}=600$ nm) through intercalation in nucleic acids subsequent to the cell penetration, was used as an indicator of the trans-membrane pores formation as described previously [7-8]. The fluorescence intensity obtained at the beginning of the recording was assigned to 0% of pore-formation and the fluorescence obtained by lysing PMNs by 0.2% (w/v) Triton X-100 corresponded to 100% of the pores formation.

For Ca^{2+} experiments, 1.1 mM CaCl_2 were added to the PMNs suspension 5 min before measurements in order to obtain 1 mM free Ca^{2+} . In some experiments, PMNs were pre-incubated with 500 μM DIDS or 50 μM flufenamic acid during 60 min then, rinsed to avoid fluorescence interferences from these compounds. The auto-fluorescence of PMNs was subtracted by the PTI software and the data extracted for transfert to SigmaPlot 4.1 (Jandel, Erkrath, Germany). The experiments described in figures are the most representative of four similar ones.

3. Results

3.1. HlgA/HlgB Induces Cl^- Fluxes

Previously, we have demonstrated that staphylococcal leukotoxins did induce the opening of pre-existing Ca^{2+} channels in human neutrophils [7]. Simultaneously, membrane pores were formed which were not permeable to

divalent ions. Recently, we have reported that, only in the presence of extracellular Ca^{2+} , all leukotoxins pairs tested (HlgA/HlgB, HlgC/HlgB, LukS-PV/LukF-PV) provoked the opening of Ca^{2+} -Release Activated Ca^{2+} (CRAC) channels, as results to the internal Ca^{2+} stores depletion [12]. In this context, we were interested in the present study to investigate an eventual activation of chloride channels by staphylococcal leukotoxins in human neutrophils, as it has previously demonstrated for other agonists [21-22]. In PMNs, the fluorescence intensity of the chloride-sensitive indicator MQAE decreased with increasing of the intracellular Cl^- ions by collisional quenching and was unaffected by pH variations [19, 23]. This property was used to determine the influence of the HlgA/HlgB leukotoxins addition on Cl^- movements in human neutrophils by recordings the fluorescence intensity variations of MQAE as shown in Figure 1.

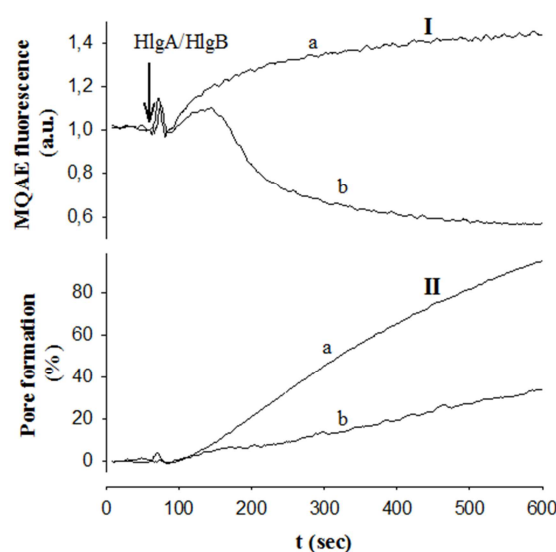


Figure 1. Effect of the HlgA/HlgB application on (I) MQAE fluorescence variations and (II) membrane pores formation in human PMNs, in the absence (a) or presence (b) of 1 mM extracellular Ca^{2+} .

In the absence of extracellular Ca^{2+} , the HlgA/HlgB addition induced a significant increase of the MQAE fluorescence represented as an arbitrary unit (a.u.) (Figure 1 Ia), due to a decrease in intracellular Cl^- level. This event was more likely due to an efflux of Cl^- ions from neutrophils. The simultaneous recording of the ethidium fluorescence intensity indicated an important concomitant pore-formation by leukotoxins (about 95%) (Figure 1IIa). The same experiment was carried out in the presence of 1 mM extracellular Ca^{2+} . Under these conditions, the HlgA/HlgB addition resulted in a bi-phasic response. First, an increase of MQAE fluorescence for about 70-80 seconds on average according to the donor, and then decreased during time (Figure 1Ib) as results of an increase in intracellular Cl^- level. This event indicated a dual effect of Cl^- movements, consisting of a small efflux followed by a marked influx of Cl^- ions. Thus, depending on the presence of extracellular Ca^{2+} , HlgA/HlgB provoked opposite Cl^- fluxes. In addition, a less important pore-formation (about 35-40%) was observed than, in the absence of extracellular

Ca^{2+} (Figure 1Ib) as described in previous studies [7-8]. However, although in the two Ca^{2+} situations, the pattern of the pore-formation was different, it could not be correlated with Cl^- movements suggesting that, Cl^- ions fluxes probably could involve another conductive chloride pathway.

3.2. Impermeability of Pores and Activation of Cl^- Channels

Previously, it has been confirmed that: *i*) trans-membrane pores formed by staphylococcal leukotoxins were not permeable to divalent ions (Ca^{2+} , Mn^{2+} , Zn^{2+}) and *ii*) divalent ions fluxes were driven by the opening of pre-existing Ca^{2+} channels [7]. Similarly, the question arising in the present research was to determine whether the pores formed by leukotoxins were permeable to Cl^- ions or, if Cl^- ions fluxes were driven through pre-existing chloride channels. To answer this question, we attempted to disturb the staphylococcal pore-formation by adding divalent ions (Ca^{2+} and/or Zn^{2+}) following the leukotoxins addition as previously described [7]. As shown in Figure 2, in the absence of extracellular Ca^{2+} , the HlgA/HlgB addition provoked an important pores formation (about 90%), this event persisted during time (Figure 2Ia).

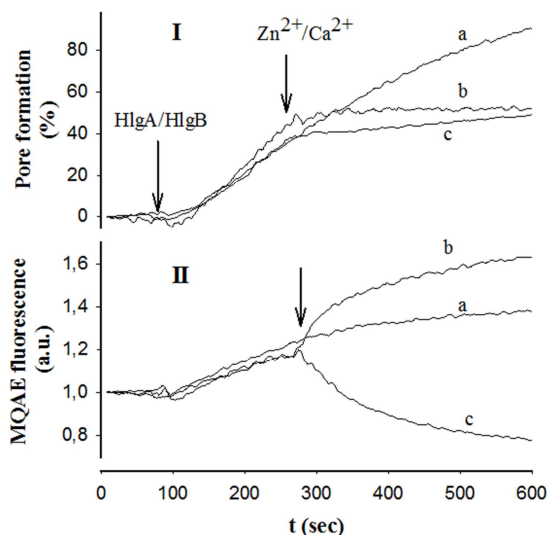


Figure 2. Effect of 2 mM Zn^{2+} (b) or 1 mM Ca^{2+} (c) addition on (I) the membrane pores formation and (II) MQAE fluorescence variations in human PMNs after the HlgA/HlgB application, in the absence (a) of extracellular Ca^{2+} .

Simultaneously, a significant increase of the MQAE fluorescence was shown (Figure 2IIa), which was likely associated to an efflux of Cl^- ions. Interestingly, the addition of 2 mM Zn^{2+} blocked totally the pore formation (Figure 2Ib) as it has previously shown [7-8], whereas variations in the MQAE fluorescence were still increased and sustained during time (Figure 2 IIb). These results suggested that, the Cl^- ions efflux was not going through the membrane pores, but might use another ions pathway of the PMNs membrane. Furthermore, in another set of experiment, when 1 mM Ca^{2+} was added in the cuvette after the HlgA/HlgB application, the functional pore formation was immediately and significantly reduced (Figure 2Ic). However, conversely to the Zn^{2+} effect,

an immediate decrease of MQAE fluorescence was observed (Figure 2IIc), likely due to an activation of new Cl^- ions pathways, which seemed to be a Ca^{2+} -dependent process. Our results strongly suggested that, membrane pores formed by HlgA/HlgB were not permeable to Cl^- ions. This was confirmed under conditions where the pores formation was totally abolished by adding 0.1 mM Ca^{2+} and 0.2 mM Zn^{2+} [7]. As shown in Figure 3, although the pores formation was completely inhibited, a significant movement of Cl^- ions was recorded after the ions blockers addition.

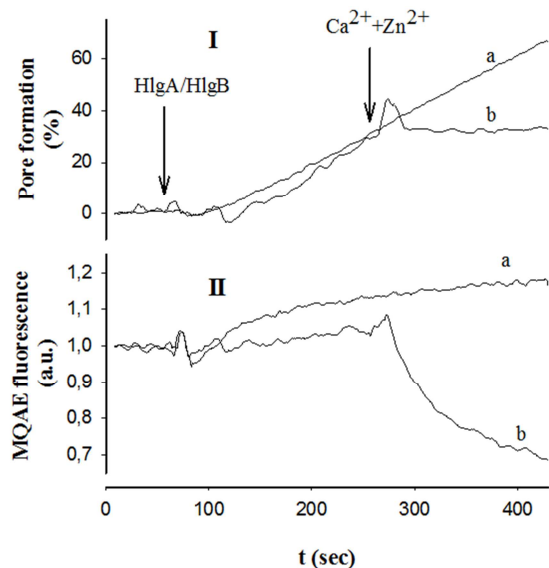


Figure 3. Effect of 0.1 mM Ca^{2+} and 0.2 mM Zn^{2+} addition (b) on (I) the membrane pores formation and (II) MQAE fluorescence variations in human PMNs after the HlgA/HlgB application, in the absence (a) of extracellular divalent ions.

These results did confirm two events: *i*) the impermeability of membrane pores to Cl^- ions and *ii*) the Cl^- movement (efflux/influx) did involve at least, two independent pathways, including a Ca^{2+} -dependent pathway. It has previously reported that a number of agonists such as ionomycin, could induce the activation of Ca^{2+} -dependent Cl^- channels [14, 24]. To further investigate this observation under both conditions, first, we tested the effect of the Ca^{2+} ionophore ionomycin on Cl^- movements in the absence of extracellular Ca^{2+} . As observed in Figure 4Ia, under this condition, no chloride movement was recorded, however, when 1 mM Ca^{2+} was injected, a significant decrease of MQAE fluorescence was observed. Thus, activation of PMNs with ionomycin led to an increase in a Cl^- channels activity likely due to a Ca^{2+} -dependent Cl^- pathway activation. This later event was abolished (Figure 1Ib) when PMNs were pretreated with a potent inhibitor of chloride channels, DIDS [25]. Similar experiments were performed to verify whether staphylococcal leukotoxins could induce the opening of Ca^{2+} -dependent Cl^- channels sensitive to DIDS. Figure 4IIa showed that, in the absence of extracellular Ca^{2+} , conversely to the ionomycin, HlgA/HlgB did induce an efflux of Cl^- ions, followed immediately by an influx of Cl^- ions when 1 mM Ca^{2+} was injected. The Cl^- fluxes

(efflux/influx) were totally inhibited when PMNs were pretreated with DIDS, in the absence as well as in the presence of extracellular Ca^{2+} (Figure 4IIb).

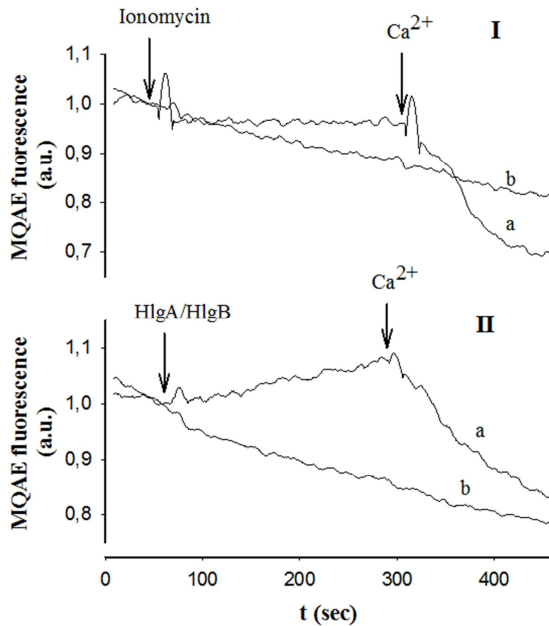


Figure 4. Effect of the 0.1 μM ionomycin (I) and HlgA/HlgB (II) application on MQAE fluorescence variations in human PMNs, before and after 1 mM Ca^{2+} addition in the absence (a) or presence (b) of DIDS (500 μM , 60 min).

Consequently, it seemed likely that, the Cl^- movement provoked by HlgA/HlgB in the presence of extracellular Ca^{2+} was likely due to the opening of Ca^{2+} -activated Cl^- channels (CaCC). A further evidence was given by Figure 5A, which did show that, in the absence of extracellular Ca^{2+} , the Cl^- efflux provoked by HlgA/HlgB was completely inhibited by DIDS but, not by flufenamic acid PMNs pre-treatment, a potent inhibitor of Ca^{2+} -activated Cl^- current [26] (Figure 5B) although, the pore-formation was not modified by both inhibitors, ruling out the role of pores in Cl^- ions fluxes.

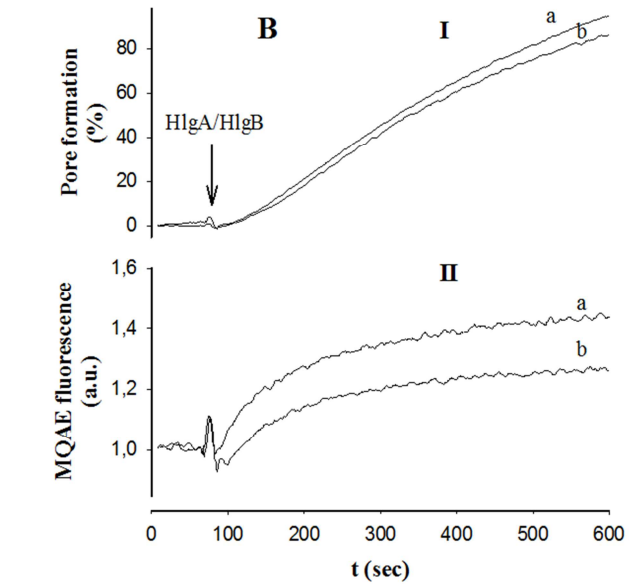
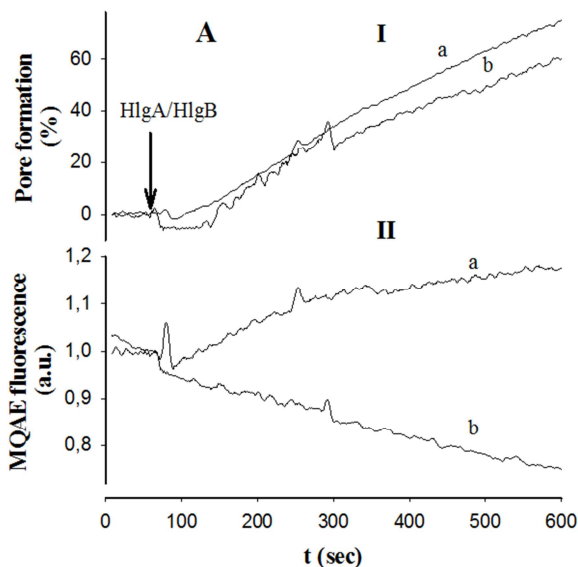


Figure 5. Effect of the HlgA/HlgB application on (I) the membrane pores formation and (II) MQAE fluorescence variations in human PMNs, in the absence (a) or presence (b) of 500 μM DIDS (A) or 50 μM flufenamic acid (B). [Ca^{2+}] = 0 mM.

Again, these observations were further confirmed when another set of experiments were performed on the same donor in the presence of extracellular Ca^{2+} . Conversely to in the absence of extracellular Ca^{2+} (Figure 6I), the Cl^- movement induced by HlgA/HlgB in the presence of extracellular Ca^{2+} , was completely inhibited by both blockers DIDS and flufenamic acid (Figure 6II). Again, the inhibitory effect of flufenamic acid was not observed in the absence of extracellular Ca^{2+} .

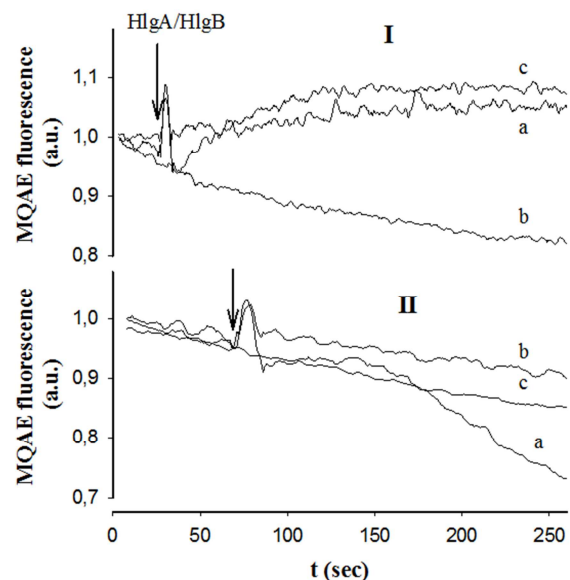


Figure 6. Effect of the HlgA/HlgB application on MQAE fluorescence variations in human PMNs, in the absence (a) or presence (b) of 500 μM DIDS (b) or 50 μM flufenamic acid (c). Experiments were performed in the absence (I) or presence (II) of 1 mM extracellular Ca^{2+} .

Furthermore, as shown in Figure 7, the Cl^- efflux induced by either LukS-PV/LukF-PV or HlgC/HlgB addition in the

absence of extracellular Ca^{2+} , was completely abolished when PMNS were pretreated with the potent Cl^- channels inhibitor, DIDS.

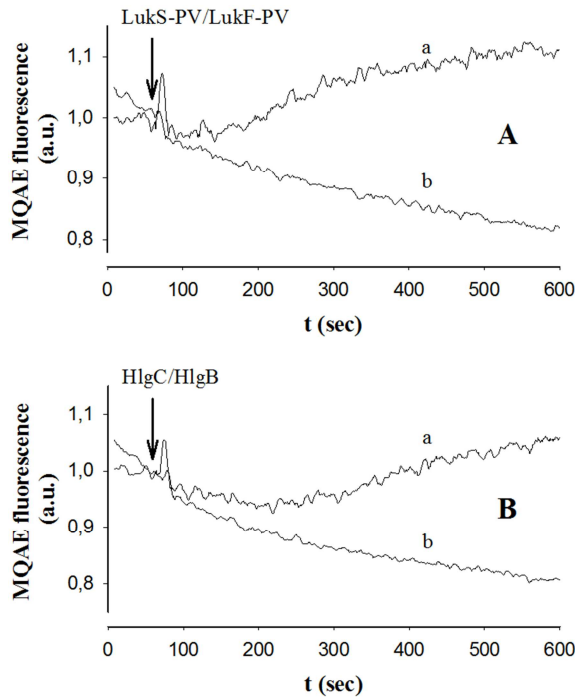


Figure 7. Effect of the LukS-PV/LukF-PV (A) or HlgC/HlgB (B) application on MQAE fluorescence variations in human PMNs, in the absence (a) or presence of 500 μM DIDS (b). $[\text{Ca}^{2+}] = 0 \text{ mM}$.

The present study strongly suggested that, intracellular variations of Cl^- ions fluxes (efflux/influx) did occur through pre-existing and specific chloride pathways and not through leukotoxins membrane pores. This new evidence was further confirmed by our results obtained with a particular donor in the presence of extracellular Ca^{2+} (Figure 8).

3.3. Comparison of HlgA/HlgB, HlgC/HlgB and LukS-PV/LukF-PV Effects

It was of interest to compare the effect of each pair of staphylococcal leukotoxins on the pore formation and Cl^- movements through the PMNs membrane. As obviously described, in the absence of extracellular Ca^{2+} , each pair tested HlgA/HlgB, HlgC/HlgB and LukS-PV/LukF-PV did induce the pore formation. Simultaneously, the same increase in the MQAE fluorescence intensity was recorded during the time, with differences in a time lag after the leukotoxins addition (Figure 7).

As previously confirmed, this event was due to a decrease in intracellular Cl^- level likely associated to an efflux of Cl^- ions from human PMNs. Thus, the three leukotoxins from *S. aureus* tested induced an efflux of Cl^- , with different potentialities, under the dependence on extracellular Ca^{2+} . In comparison experiments performed in the absence of extracellular Ca^{2+} as shown in Figure 8, either HlgA/HlgB (Figure 8A) or LukS-PV/LukF-PV (Figure 8B) did induce simultaneously, the pores formation and significant efflux of Cl^- ions.

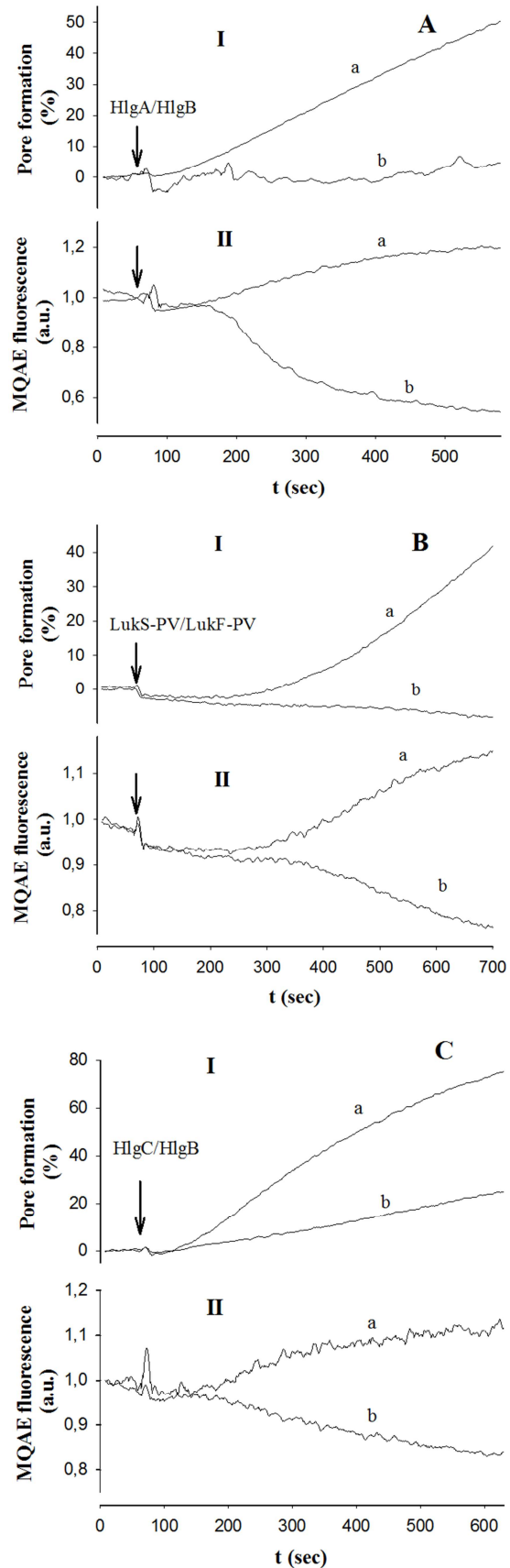


Figure 8. Effect of the HlgA/HlgB (A), LukS-PV/LukF-PV (B) or HlgC/HlgB (C) application on (I) the membrane pores formation and (II) MQAE fluorescence variations in human PMNs, in the absence (a) or presence (b) of 1 mM extracellular Ca^{2+} .

However, in the presence of extracellular Ca^{2+} , an important Cl^- movement was observed although the pores formation by both leukotoxins was abolished in this particular donor. These findings strongly confirmed that, both HlgA/HlgB γ -hemolysin and LukS-PV/LukF-PV leukotoxins did provoke simultaneously two independent cellular events: 1) the membrane pores formation not permeable to chloride ions and 2) the opening of specific Cl^- ions pathways, including Ca^{2+} -activated Cl^- channels sensitive to both DIDS and flufenamic acid. Nevertheless, we have observed that, in the presence of extracellular Ca^{2+} , the HlgC/HlgB combination did not induce an influx of Cl^- ions through Ca^{2+} -dependent Cl^- channels, as shown in Figure 8C.

4. Discussion

We have previously provided evidence that, bi-component staphylococcal leukotoxins did induce two independent cellular events in human neutrophils: 1) the opening of pre-existing Ca^{2+} channels and 2) the formation of trans-membrane pores not permeable to divalent ions (Ca^{2+} , Mn^{2+} , Zn^{2+} ...) [7]. These membrane pores determined by the ethidium influx seemed to be specific to monovalent ions (K^+ , Na^+) [11]. In a recent report, we have demonstrated that, these pore-forming leukotoxins from *S. aureus* were able to trigger a massive internal Ca^{2+} stores depletion followed by the opening of Ca^{2+} -release activated Ca^{2+} (CRAC) channels [12]. Interestingly, staphylococcal leukotoxins were considered as Ca^{2+} -channels agonists, they bound to specific membrane receptors [5-6] and as results they were able to activate neutrophils and induced consequently, the oxidative burst activation [13].

It has already been reported in a previous work that, a drastic decrease of intracellular Cl^- level might represent an essential step for the activation of the respiratory burst in human PMNs [22]. This event was thought to play a crucial role in the modulation of several critical neutrophil responses including, activation and up-regulation of adhesion molecules, cell attachment and spreading, cytoplasmic alkalization and activation of the oxidative burst [27]. Again, the Cl^- efflux has been shown to regulate β_2 integrin-mediated spreading and activation of the respiratory burst [28]. Since, the chloride ions efflux is an early event, that occurred following the human PMNs activation by several physiological soluble agonists such as, tumor necrosis factor- α (TNF- α) [15, 21] or non-physiological soluble agonists like fMLP, PMA [16], and ionomycin with the concomitant decrease of the intracellular chloride content, we were interested in the present study, to investigate the Cl^- ions movement in staphylococcal bi-component leukotoxins-activated human neutrophils.

A very little was known about an eventual involvement of Cl^- channels in chloride fluxes in the staphylococcal leukotoxins activity. This was explored by using the chloride-sensitive quencher fluorescent indicator MQAE, as a useful tool for determination of the intracellular chloride levels in human neutrophils [29]. First, we tested the effect of HlgA/HlgB γ -hemolysin on the membrane pores formation

simultaneously to Cl^- movements in neutrophils. Indeed, we found that, in the absence of extracellular Ca^{2+} , a significant increase in the MQAE fluorescence intensity was recorded as results to a sustained Cl^- efflux through the PMNs membrane. This event might lead to a marked decrease in intracellular chloride levels in these phagocytes. As it has previously described that, staphylococcal leukotoxins did not alter the intracellular Ca^{2+} level in the absence of extracellular Ca^{2+} [12], it was expected that in this condition, the Cl^- ions efflux provoked by HlgA/HlgB γ -hemolysin was independent of intracellular Ca^{2+} elevation. Nonetheless, in a previous work, it has been shown that, the Cl^- efflux appeared to be independent on either alterations of $[\text{Ca}^{2+}]_i$ or changes in the plasma membrane potential, and was independent on the protein tyrosine phosphorylation and decrease of cAMP levels [28]. Again, the Cl^- efflux triggered by the cross-linking of β_2 -integrin occurred in a Ca^{2+} -independent manner. A simultaneous cellular effect of HlgA/HlgB on PMNs was the trans-membrane pores formation (about 90%) which was significantly reduced (about 20%) in the presence of extracellular Ca^{2+} as previously reported [7-8]. However, under these conditions, we have observed a biphasic response of HlgA/HlgB, closely associated to an efflux of Cl^- followed by a sustained influx of chloride ions. This later event seemed to be a Ca^{2+} -dependent process. Consequently, we thought that Cl^- fluxes mediated by leukotoxins could be an independent event of the membrane pores formation. We further verified this hypothesis when the formation of pores was altered by adding the ions blockers ($\text{Ca}^{2+}/\text{Zn}^{2+}$) at a given concentration. Interestingly, under these conditions, an opposite Cl^- movement (efflux/influx) was observed, depending on the absence or presence of extracellular Ca^{2+} and conversely to Zn^{2+} ions, only the presence of Ca^{2+} could induce a massive influx of Cl^- ions. This strongly suggested that, membrane pores formed by staphylococcal leukotoxins were not permeable to Cl^- ions. This was again, confirmed by another set of experiment where the formation of membrane pores were completely abolished by 0.1 mM Ca^{2+} and 0.2 mM Zn^{2+} . As results, we provided the first evidence that, staphylococcal membrane pores did not drive Cl^- ions. Furthermore, it seemed that, another type of a Cl^- pathway was involved only in the presence of extracellular Ca^{2+} , which could be a Ca^{2+} -activated Cl^- pathway. Consequently, we proposed that bi-component staphylococcal leukotoxins could induce in addition to Ca^{2+} channels, the opening of specific Cl^- channels in their cellular membrane activity.

The intracellular chloride, as being a major anion in living cells, played a crucial role in maintaining cellular functions in many cell types and all these responses, were inhibited when PMNs were pre-treated with Cl^- channels blockers [25, 30]. For this reason, we tried to test the effect of known potent Cl^- channels inhibitors, DIDS and flufenamic acid on leukotoxin-activated Cl^- fluxes in both conditions. First, we attempted to verify whether the Ca^{2+} ionophore ionomycin could activate the Cl^- flux as it has previously reported [14, 24]. Thus, we found that only in the presence of extracellular Ca^{2+} , a significant movement of chloride ions was occurred which

was completely inhibited by the PMNs pretreatment with DIDS, used as a potent Cl^- channels blocker. This effect was compared to the HlgA/HlgB activity. Since both movements (efflux/influx) of chloride ions induced by HlgA/HlgB were totally abolished by DIDS, we proposed that γ -hemolysin was able to activate at least, two types of DIDS-sensitive Cl^- pathways, depending on the absence or presence of extracellular Ca^{2+} . Furthermore, these results strongly suggested the opening of Ca^{2+} -activated Cl^- channels by staphylococcal leukotoxins, as it has previously reported in neutrophils [14] and which were not found in macrophages.

It is unclear to date whether these pathways correspond to anion exchangers or to Cl^- channels, although the kinetics suggested the opening of Cl^- channels. Moreover, since the anions gradient was the same in every experiment, Cl^- fluxes through an anions exchanger/transporter would be always in the same direction whatever the Ca^{2+} situation. Furthermore, since the cell suspension was rinsed after pre-incubation with DIDS and before the leukotoxins addition, DIDS was only able to block irreversibly pre-existing membrane channels. Additionally, it has been shown that, DIDS was essentially inert towards anion exchange in PMNs and did not alter the Cl^- exchange activity [31-33]. Another type of chloride channels known as VRAC (*volume-regulated anion (Cl⁻) channel*)-mediated Cl^- currents has been proposed in β -cells [34]. Alternatively, changes in cell volume might be part of the response to the increase in intracellular $[\text{Ca}^{2+}]$ that could occur during cell activation. Thus, chloride movements across the cell plasma membrane might play a central role in cell volume and pH regulation and maintaining cellular functions in many cells types.

More interestingly, we have demonstrated in another set of experiments that, in the absence of extracellular Ca^{2+} , only DIDS could inhibit totally the Cl^- efflux, whereas it had no effect on the pores formation. The flufenamic acid inhibitor did not inhibit leukotoxins-induced Cl^- efflux under this condition. It has previously reported that, flufenamic acid was ineffective or slightly effective on the Cl^- efflux [24]. At a given concentration, the major route of Cl^- entry was inhibited by furosemide, another flufenamic acid analogue, in fibroblasts [35]. Again, it has been shown that, flufenamic acid did inhibit the increases in intracellular free calcium concentration in human neutrophils activated by fMLP. This inhibition was concluded to be due to the Ca^{2+} influx blocking in neutrophils [36]. Previously, it has also been shown [19] by using MQAE as a novel fluorescent indicator that, the initial Cl^- influx rate was reduced after exposure to furosemide in cultured aortic smooth muscle cells.

However, in the presence of extracellular Ca^{2+} , a rapid loss of Cl^- channels activity was observed by both inhibitors, DIDS and flufenamic acid, even no inhibitory effect was observed on the membrane pores formation. Our data suggested that, in addition to Ca^{2+} -release activated Ca^{2+} (CRAC) channels [12], bi-component leukotoxins from *S. aureus* did induce the opening of Ca^{2+} -activated Cl^- channels. These channels were indeed voltage-independent over the voltage range [14].

Previous studies have reported that, several neutrophils

agonists did stimulate a Cl^- out flux in human PMNs in the absence of extracellular Ca^{2+} [21]. Under this condition, although staphylococcal leukotoxins did not modify the intracellular Ca^{2+} level [7, 12], an important efflux of Cl^- ions was observed. Furthermore, Shimizu and coworkers [21] have demonstrated that, the intracellular Ca^{2+} elevation was not a pre-requisite for Cl^- fluxes in neutrophils, whereas Meneqazzi *et al.* [28] described Cl^- fluxes in human PMNs independent to the intracellular Ca^{2+} alteration.

Another important result of the present study was that, the combined actions of HlgA/HlgB and Ca^{2+} induced a consequent influx of Cl^- . Moreover, if the Cl^- channels opening was only due to the increase of intracellular Ca^{2+} , a Cl^- efflux instead of an influx would have been observed as already described [21]. One hypothesis would be that, this Cl^- influx is induced by a signaling transduction pathway activated by the leukotoxins binding and dependent on the extracellular Ca^{2+} since it has been shown that, leukotoxins induced an increase of intracellular Ca^{2+} by the opening of Ca^{2+} channels [7]. Thus, it was proposed that, this Cl^- conductive pathway corresponded to Ca^{2+} -activated Cl^- channels. This hypothesis was confirmed by *i)* the absence of the inhibitory effect of flufenamic acid in Ca^{2+} free medium, *ii)* the inhibitory effect of flufenamic acid on the Cl^- movement was only observed in the presence of Ca^{2+} . Previously, it has been reported that, an increase of the glucose concentration resulted in an accelerate entry/influx of Cl^- leading to an increase of Cl^- permeability into β -cells. [37]. Consequently, it has been proposed by these authors that, Ca^{2+} -activated Cl^- channels were required to a sustain glucose-stimulated membrane potential oscillations and insulin secretion in dispersed β -cells secretory responses. The oscillatory Ca^{2+} signaling event was thought to be critically dependent on trans-membrane Cl^- fluxes. Agonist-stimulated Cl^- movements seems to be a common phenomenon activated by several agonists, which act through different signal transduction pathways. Thus, the Ca^{2+} -activated Cl^- channels may play a key role in cell volume homeostasis and/or cell activation.

Finally, the comparative effect of bi-component staphylococcal leukotoxins on the PMNs membrane activity did show that: *i)* in the absence of extracellular Ca^{2+} , HlgA/HlgB; HlgC/HlgB and LukS-PV/LukF-PV did provoke a sustained efflux of chloride (Cl^-) anions, *ii)* the time course of an agonist-stimulated Cl^- efflux differed, depending on the leukotoxins pair tested and which, could act through different signal transduction pathways (unpublished data) and *iii)* in the presence of extracellular Ca^{2+} , only HlgA/HlgB and LukS-PV/LukF-PV were able to induce the opening of Ca^{2+} -activated Cl^- channels, allowing a massive influx of Cl^- ions. A previous work [13] did already report that, in contrast to HlgC/HlgB, only HlgA/HlgB and LukS-PV/LukF-PV combinations did induce the H_2O_2 production.

5. Conclusion

The present study seems to be the first one that analyses

chloride ion movements in human PMNs activated by bi-component leukotoxins from *S. aureus*. We have recently reported that, staphylococcal leukotoxins did trigger the opening of Ca^{2+} -release activated Ca^{2+} (CRAC) channels as results of a massive depletion of internal Ca^{2+} stores [12]. This may represent an important event in the signal transduction pathway in activated neutrophils by different combinations of staphylococcal pore-forming leukotoxins. Since the Cl^- efflux was a phenomenon that occurred early in PMNs activated with a variety of agonists, we were interested in the present research to investigate the Cl^- ions movement in leukotoxins-activated human neutrophils.

In this research, we have shown that, bi-component leukotoxins from *S. aureus* did exhibit a massive efflux of Cl^- ions in the absence of extracellular Ca^{2+} sensitive to DIDS, whereas in the presence of extracellular Ca^{2+} , an opposite Cl^- movement was recorded due to a sustained influx of Cl^- ions through the PMNs membrane. This was completely inhibited by either DIDS or flufenamic acid. Nevertheless, the role of Cl^- movements in the modulation of PMNs responses is still not yet define and a little is known about these ions channels in staphylococcal leukotoxins-activated PMNs. The mechanism of reuptake remains to be elucidated and, the question as to whether a relationship exists between Cl^- and Ca^{2+} movements deserves further investigations.

All together, these studies provide new insights into the role of Cl^- movements in neutrophils pathophysiology during staphylococcal infections. It has previously reported that, the most well-known serious human disease was cystic fibrosis, due to mutations in chloride channels [38]. In this context, this paper might contribute to highlight the role of Cl^- movements in signal transduction pathways that regulates PMNs responses (degranulation, oxidative burst) when activated by different staphylococcal pore-forming leukotoxins.

In conclusion, the trans-membrane pores formed by bi-component staphylococcal leukotoxins (HlgA/HlgB, HlgC/HlgB, LukS-PV/LukF-PV) do not drive Cl^- ions fluxes. In addition, it is proposed that these leukotoxins induce at least, the opening of two different types of Cl^- channels according to the leukotoxins pair considered. One of these two types seems to correspond to Ca^{2+} -activated Cl^- channels. These findings provide new evidence in favor of a close correlation between lowering of $[\text{Cl}^-]_i$ and activation of PMNs functions and are in agreement with previous studies. Further studies might be needed to better elucidate the connections between the different signaling pathways mediated by bi-component staphylococcal leukotoxins during infectious diseases.

Acknowledgements

The authors are very grateful to Mr Daniel Keller for leukotoxins purification. Dr. Didier André Colin was a researcher from the C. N. R. S. Dr. Leïla Staali was a recipient of a scholarship from the Algerian government (MESRS). This work was supported by a grant from *the Direction de la Recherche et des Etudes Doctorales* (UPRES-EA 1318).

The authors declare that they have no competing interests.

References

- [1] Prévost G, Cribier B, Couppié P, Petiau P, Supersac G, Finck-Barbançon V, Monteil H, Piémont Y (1995) Panton-Valentine leucocidin and gamma-hemolysin from *Staphylococcus aureus* ATCC49775 are encoded by distinct loci and have different biological activities. *Infect. Immun.* 63: 4121-4129.
- [2] Finck-Barbançon V, Prévost G, Piémont Y (1991) Improved purification of leukocidin from *Staphylococcus aureus* and toxin distribution among hospital strains. *Res. Microbiol.* 142: 75-85.
- [3] Cribier B, Prévost G, Couppié P, Finck-Barbançon V, Grosshans E, Piémont Y (1992) *Staphylococcus aureus* leukocidin: a new virulence factor in cutaneous infections? An epidemiological and experimental study. *Dermatology* 185: 175-180.
- [4] Couppié P, Cribier B, Prévost G, Grosshans E, Piémont Y (1994) Leukocidin from *Staphylococcus aureus* and cutaneous infections: an epidemiological study. *Arch. Dermatol.* (130): 1208-1209.
- [5] Colin DA, Mazurier I, Sire S, Finck-Barbançon V (1994) Interaction of the two components of leukocidin from *Staphylococcus aureus* with human polymorphonuclear leukocyte membranes: sequential binding and subsequent activation. *Infect. Immun.* 62: 3184-3188.
- [6] Guaduchon V, Werner S, Prévost G, Monteil H, Colin DA (2001) Flow cytometry determination of Panton-Valentine leukocidin S component binding. *Infect. Immun.* 69: 2390-2395.
- [7] Staali L, Monteil H, Colin DA (1998) The pore-forming leukotoxins from *Staphylococcus aureus* open Ca^{2+} channels in human polymorphonuclear neutrophils. *J. Membr. Biol.* 162: 209-216.
- [8] Finck-Barbançon V, Duportail G, Meunier O, Colin DA (1993) Pore formation by a two-component leukocidin from *Staphylococcus aureus* within the membrane of human polymorphonuclear leukocytes. *Biochim. Biophys. Acta.* 1128: 275-282.
- [9] Ferreras M, Hoper F, Dalla Serra M, Colin DA, Prévost G, Menestrina G (1998) The interaction of *Staphylococcus aureus* bi-component γ -hemolysin and leucocidins with cells and lipid membranes. *Biochim. Biophys. Acta* 1414: 108-126.
- [10] Pedelacq JD, Maveyraud L, Prevost G, Baba-Moussa L, Gonzalez A, Courcelle E, Shepard W, Monteil H, Samama JP, Mourey L (1999) The structure of *Staphylococcus aureus* leucocidin component (LukF-PV) reveals the fold of the water-soluble species of a family of transmembrane pore-forming toxins. *Structure* 7: 277-287.
- [11] Staali L, Monteil H, Colin DA (1998) Action mechanism of staphylococcal bi-component leucotoxins. *Toxicon* 36 (12): 1746.
- [12] Staali L, Colin DA (2020) The pore-forming leukotoxins from *S. aureus* involve Ca^{2+} -activated Ca^{2+} channels and other types of Ca^{2+} channels in Ca^{2+} entry into neutrophils. *International Journal of Microbiology and Biotechnology.* 5 (2): 55-68.

- [13] Colin DA, Monteil H (2003) Control of the oxidative burst of human neutrophils by staphylococcal leukotoxins. *Infection and Immunity*. 71 (7): 3724-3729.
- [14] Krause KH, Welsh MJ (1990) Voltage-dependent and Ca^{2+} -activated ion channels in human neutrophils. *J. Clin. Invest.* 85: 491-498.
- [15] Schumann L, Gardner P, Raffin TA (1993) Recombinant human tumor necrosis factor α induces calcium oscillation and calcium-activated chloride current in human neutrophils. *J. Biol. Chem.* 268: 2134-2140.
- [16] Schumann MA, Raffin TA (1994) Activation of voltage-dependent chloride current in human neutrophils by phorbol 12-myristate 13-acetate and formyl-methionyl-leucyl-phenylalanine. The role of protein kinase. *J. Biol. Chem.* 269: 2389-2398.
- [17] Simchowitz L, Textor J, Cragoe EJ (1993) Cell volume regulation in human neutrophils: 2-(aminoethyl) phenols as Cl^- channel inhibitors. *Am. J. Physiol.* 265: C143-155.
- [18] Gallin EK (1991) Ion channels in leukocytes. *Physiol. Rev.* 3: 775-811.
- [19] Koncz C, Daugirdas J (1994) Use of MQAE for measurement of intracellular $[\text{Cl}^-]$ in cultured aortic smooth muscle cells. *Am. J. Physiol.* 267: H2114-2123.
- [20] Ikeuchi Y, Kogiso H, Hosogi S, Tanaka S, Shimmamoto C, Inui T, Nakahara T, Marunaka Y (2018) Measurement of $[\text{Cl}^-]$ unaffected by the cell volume change using MQAE-based two-photon microscopy in airway ciliary cells of mice. *The Journal of Physiological Sciences* 68: 191-199.
- [21] Shimizu Y, Daniels RH, Elmore MA, Finnen MJ, Hill ME, Lackie JM (1993) Agonist-stimulated Cl^- efflux from human neutrophils: a common phenomenon during neutrophil activation. *Biochem. Pharmacol.* 45: 1743-1751.
- [22] Menegazzi R, Busetto S, Dri P, Cramer R, Patriarca P (1996) Chloride ion efflux regulates adherence, spreading, and respiratory burst of neutrophils stimulated by tumor necrosis factor- α (TNF) on biologic surfaces. *J. Cell Biol.* 135: 511-522.
- [23] Geddes CD, Apperson K, Karolin J, Birch DJ (2001) Chloride-sensitive fluorescent indicators. *Anal. Biochem.* 293 (1): 60-66.
- [24] West MR, Molloy CR (1996) A microplate assay measuring chloride ion channel activity. *Anal. Biochem.* 241: 51-58.
- [25] Phipps DJ, Branch DR, Schlichter LC (1996) Chloride channel blockers inhibit T lymphocytes activation and signaling. *Cell Signal* 8: 141-149.
- [26] Greenwood I, Large W (1995) Comparison of the effects of fenamates on Ca^{2+} -activated chloride and potassium currents in rabbit portal vein smooth muscle cells. *Br. J. Pharmacol.* 116: 2939-2948.
- [27] Busetto S, Trevisan E, Decleva E, Dri P, Menegazzi R (2007) Chloride movements in human neutrophils during phagocytosis: characterization and relationship to granule release. *The Journal of Immunology* 179: 4110-4124.
- [28] Menegazzi R, Busetto S, Decleva E, Cramer R, Dri P, Patriarca P (1999) Triggering of chloride ion efflux from human neutrophils as a novel function of leukocyte β_2 integrins: relationship with spreading and activation of the respiratory burst. *J. Immunol.* 162: 423-434.
- [29] Engblom AC, Akerman KE (1993) Determination of the intracellular free chloride concentration in rat brain synaptoneurosomes using a chloride-sensitive fluorescent indicator. *Biochem. Biophys. Acta* 1153 (2): 262-266.
- [30] Kanno T, Takishima T (1990) Chloride and potassium channel in U937 human monocytes. *J. Membr. Biol.* 116: 149-161.
- [31] Simchowitz L (1986) Chloride movements in human neutrophils: diffusion, exchange and active transport. *J. Gen. Phys.* 88: 167.
- [32] Simchowitz L (1990) Special topic: ion movements in leukocytes. *Annu. Rev. Physiol.* 52: 363-364.
- [33] Simchowitz L, Bibb JA (1990) Functional analysis of the modes of anion transport in neutrophils and HL-60 cells. *Annu. Rev. Physiol.* 52: 381-397.
- [34] Di Fulvio M, Aguilar-Bryan L (2019) Chloride transporters and channels in β -cell physiology: revisiting a 40-year-old model. *Biochemical Society Transactions* 47: 1843-1855.
- [35] Chao AC, Dix JA, Sellers MC, Verkman AS (1999) Fluorescence measurement of chloride transport in monolayer cultured cells. *Biophys. J.* 56: 1071-1081.
- [36] Kankaanranta H, Moilanen E (1995) Flufenamic and tolafenamic acids inhibit calcium influx in human polymorphonuclear leukocytes. *Mol. Pharmacol.* 47: 1006-1013.
- [37] Eberhardson M, Patterson S, Grapengiesser E (2000) Microfluorometric analysis of Cl^- permeability and its relation to oscillatory Ca^{2+} signaling in glucose-stimulated pancreatic β -cells. *Cellular Signalling* 12 (11-12): 781-786.
- [38] Andersson C, Roomans GM (2002) Determination of chloride efflux by X-ray microanalysis versus MQAE-fluorescence. *Microsc. Res. Tech.* 59 (6): 531-532.