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The Pore-forming Leukotoxins from *S. aureus* Involve Ca^{2+} Release-Activated Ca^{2+} Channels and Other Types of Ca^{2+} Channels in Ca^{2+} Entry into Neutrophils

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Abstract: The pore-forming bi-component leukotoxins from *Staphylococcus aureus* induce two independent cellular events *1)* the formation of trans-membrane pores not permeable to divalent ions and *2)* the opening of pre-existing Ca^{2+} channels in human polymorphonuclear neutrophils (PMNs). The influx of Ca^{2+} and Mn^{2+} (Mn^{2+} was used as a Ca^{2+} surrogate) in Fura2-loaded human PMNs was determined by spectrofluorometry techniques. The present study showed that, in the presence of extracellular Ca^{2+}, the staphylococcal HlgA/HlgB γ-hemolysin induced a rapid Ca^{2+} release from internal Ca^{2+} stores before the onset of a Mn^{2+} (Ca^{2+}) influx. The sustained increase of Ca^{2+} and Mn^{2+} influx was partially inhibited by the ionic blockers of Ca^{2+} Release-Activated Ca^{2+} (CRAC) channels, La^{3+} and Ni^{2+}. Furthermore, the incubation of human PMNs with either TMB8 or thapsigargin did inhibit significantly the Ca^{2+} release mediated by leukotoxins simultanously to a clear decrease of Ca^{2+} and Mn^{2+} influx. The internal Ca^{2+} release induced by γ-hemolysin was also inhibited by PMNs pretreatment with a pertussis toxin, NaF, caffeine, ryanodine, cinnarizine and flunarizine and consequently, the Mn^{2+} (Ca^{2+}) influx was significantly reduced. Moreover, different Ca^{2+} signaling pathways blockers such as U73122, staurosporine, thyrphostin A9 and okadaic acid were tested on the leukotoxins activity. Taken together, this work provided evidence that, in the presence of extracellular Ca^{2+}, bi-component staphylococcal leukotoxins provoked in human PMNs after a specific binding to their membrane receptors, a rapid depletion of internal Ca^{2+} stores mediating a CRAC channels activation. This Ca^{2+}-dependent mechanism seems likely to be associated to the heterotrimeric G-proteins activation. Interestingly, in the absence of extracellular Ca^{2+}, the staphylococcal leukotoxins tested induced the opening of an important divalent ions (Ca^{2+}, Mn^{2+}, Ni^{2+}) pathway not sensitive to CRAC channels blockers. Consequently, we strongly suggested that other types of Ca^{2+} channels might be involved in bi-component leukotoxins activity, including Ca^{2+} channels dependent on the protein kinase C activation.

Keywords: Pore-forming Toxin, Leukotoxin, γ-hemolysin, Ca^{2+} Channels, *S. aureus*, Neutrophils, CRAC Channels, Spectrofluorometry

1. Introduction

Pore-forming toxins (PFTs) are key virulence factors produced by a variety of important human bacterial pathogens, including *Staphylococcus aureus (S. aureus)*, Group A and B streptococci, *Streptococcus pneumoniae*, *Vibrio cholerae*, *Clostridium septicum*, *Escherichia coli* and *Mycobacterium tuberculosis* [1-2]. For numerous human pathogenic bacteria, PFTs expressed during infections contributed significantly to the virulence *in vivo*. Among these cytotoxic proteins, the bi-component γ-hemolysin from *S. aureus* was first described by Smith & Price in 1938 [3] who isolated it from α, β, δ hemolysins and was purified during the 70s by different authors [4-5]. Later, it has been shown to be produced by 99%

of randomly selected clinical strains [6]. Currently, it is well known that γ-hemolysin is composed of three proteins: HlgA, HlgB and HlgC [7-9] which form two active pairs HlgA/HlgB and HlgC/HlgB constituting with the Panton-Valentin leukocidin (PVL) [10] a family of leukotoxins presenting comparable activities [11]. It was first, suggested that the PVL created aspecific pores through the membrane of human polymorphonuclear neutrophils (PMNs) whose conformation did change into ion-sized pores in the presence of extracellular Ca^{2+} [12]. These ion-sized pores were responsible of an important Ca^{2+} influx inducing the exocytosis of the granular content of PMNs [13-14]. Later, we have clearly demonstrated by the use of ionic specific fluorescent probes and Ca^{2+} channels blockers that, staphylococcal leukotoxins did induce the opening of pre-existing Ca^{2+} channels belonging to the PMNs membrane in the absence and the presence of extracellular Ca^{2+} [15]. Consequently, it was concluded that leukotoxins from *S. aureus* after a specific binding to their membrane receptors, mediated two independent types of activity: *i*) they are Ca^{2+} channels agonists, *ii*) after the membrane insertion and oligomerization using a complex multistep process, they form specific pores comparable to those formed by α-toxin [16-17] dramatically disturbing cellular homeostasis.

It is well established that, non-excitable human neutrophils do not possess voltage-operated Ca^{2+} channels (VOCC) [18-21], whereas several types of receptor operated Ca^{2+} channels (ROCC) are involved in mediating Ca^{2+} (Mn^{2+}) entry through the plasma membrane of PMNs. In general, calcium signaling in non-excitable cells is primary initiated by the activation of surface membrane receptors coupled to phospholipase C (PLC) and stimulates as results, a calcium signaling process [22]. The PLC activation by membrane receptors leads to a breakdown of phosphatidylinositol 4, 5-bipohosphate in the plasma membrane and production of diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP_3) [23]. These membrane receptors are functionally, linked to heterotrimeric G proteins [24] or are closely associated to cytosolic tyrosine kinase [25]. However, Putney and Bird [26, 27] proposed that, the Ca^{2+} influx pathway could be mediated by Ca^{2+} stores depletion known as a capacitative Ca^{2+} entry (CCE). In a large number of non-excitable cells such as human neutrophils, this entry results from the opening of a class of channels known as store-operated calcium entry (SOCE) [28-31], although another receptor-operated Ca^{2+} pathway can be activated by *N*-formyl-methionyl-leucyl-phenylalanine (f-MLP) in differenciated HL60 cells [32].

In general, Ca^{2+} is an essential second messenger activating several cellular signaling pathways and a sustained increase in intracellular [Ca^{2+}] could modify the host response leading to cell death [33]. In addition, a number of Ca^{2+}-dependent effects have been reported in immune cells inducing the activation of several intracellular signaling molecules such as phospholipase A_2 (PLA_2) [34].

A better understanding of the receptor-regulated calcium signaling triggered by the bi-component leukotoxins from *S. aureus*, was the purpose of the present study. In this context, we have attempted to identify and discern specific calcium signaling processes and mechanisms involved by staphylococcal leukotoxins following their specific binding to their PMNs membrane receptors. First, by fluorescence-based technique using calcium-sensitive probes, we tried to investigate an eventual activation of Ca^{2+} Release-Activated Ca^{2+} (CRAC) channels as results of Ca^{2+} signaling pathways induced by each pair tested from leukotoxins: HlgA/HlgB, HlgC/HgB (γ-hemolysin) and LukS-PV/LukF-PV (Panton and Valentin Leukocidin) in human neutrophils. Interestingly, in the present study, our results are representative only for the HlgA/HlgB γ-hemolysin pair and clearly demonstrated that, in the presence of extracellular Ca^{2+}, staphylococcal leukotoxins induced a biphasic process of calcium mobilization. The later was composed of the Ca^{2+} release from internal Ca^{2+} stores which was coupled to, and activated the Ca^{2+} (Mn^{2+}) influx through the opening of Ca^{2+}-release activated Ca^{2+} (CRAC) channels as described previously [35-36]. Additionally, we provided an evidence of the opening of other types of receptors operated Ca^{2+} channels (ROCC) that did not involve store-operated Ca^{2+} entry [37-38], comprising Ca^{2+} channels which might be a PKC-dependent process since, PLC-coupled receptors may activate pathways that were unrelated with store-operated Ca^{2+} channels.

2. Materials and Methods

2.1. Reagents

Fura2 and Fura2/AM were obrained from Molecular Probes (Eugene, OR). J. Prep was purchased from Tech Gen International (Les Ulis, France). All other chemicals, including Dextran, TMB8, thapsigargin, staurosporine, ryanodine, caffeine and EGTA were from Sigma (L'Isle-d'Abeau Chesnes, France). Pertussis toxin, NaF, tyrphostin A9, cinnarizine, flunarizine and U73122 were from Calbiochem (Meudon, France).

2.2. Toxin Production

The different proteins HlgA (32 kDa), HlgB (35 kDa), HlgC (32 kDa), LukS-PV (38 kDa) and LukF-PV (32 kDa) were prepared from *S. aureus* strain ATCC 49775 by chromatography on a cation exchange column and hydrophobic interactions as described previously [11]. The purified components were stored at -80°C until utilization at a final concentration of 2.2 nM (HlgA) and 0.85 nM (HlgB) presented in this study.

2.3. Cell Isolation and Fura2 Loading

Human polymorphonuclear neutrophils (PMNs) were obtained from buffy coats of healthy volounteers of either sex, kindly provided by the Centre de Transfusion Sanguine (Strasbourg, France). They were prepared as described previously [15]. Briefly, human PMNs enriched blood was centrifuged in J. Prep and the pellet was resuspended in Dextran for sedimendation. Contaminating erythrocytes were

removed by an hypotonic lysis. 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 x10^6 cells/ml. This method led to 98% viable PMNs as counted after May-Grunwald-Giemsa staining.

PMNs were incubated with 2 µM Fura2/AM for 45 min at a room temperature in the assay medium then, washed twice by centrifugation at 800xg for 10 min to remove extracellular dye, and were resuspended in the assay medium at 6x10^6 cells/ml. For Ca^{2+} experiments, 1.1 mM $CaCl_2$ was added to the PMNs suspension 5 min before measurements in order to obtain 1 mM extracellular free Ca^{2+}.

2.4. Spectrofluorometric Measurements

Variations in fluorescence intensity were recorded using a spectrofluorometer DeltaScan (Kontron, PTI, Montigny-le-Bretonneux, France) equipped with dual excitation and dual emission monochromators (slit width=4 nm). The fluorescence of Fura2 was recorded at λ_{EM}=510 nm in one emission monochromator after excitation by the two excitation monochromators at wavelengths λ_{EX}=340 nm and λ_{EX}=360 nm. The measurement of Mn^{2+} permeability by the quenching of Fura2 fluorescence at the isosbestic wavelength λ_{EX}=360 nm, was used as a surrogate for a Ca^{2+} permeability [39, 15], since it has been shown to be a good tracer in human PMNs for the opening of Ca^{2+} channels [29]. This presents identical kinetics of activation for both divalent ions (Ca^{2+} and Mn^{2+}) [30-31]. At a given Ca^{2+} concentration, Mn^{2+} decreased the fluorescence of Fura2 without modifying the shape of the excitation spectrum as shown in Figure 1A. Conversly, at a given Mn^{2+} concentration, the addition of increasing Ca^{2+} concentrations increased the maximum intensity of fluorescence and shifted it to the left (Figure 1B).

In the intracellular medium, the fluorescence intensity recorded at λ_{EX}=340 nm was given by the unchelated Fura2 molecules, those whose fluorescence was increased by intracellular Ca^{2+} and those whose fluorescence was decreased by chelation of intracellular Mn^{2+}. The fluorescence recorded at λ_{EX}=360 nm was given by the unchelated Fura2 molecules, those whose fluorescence was not modified by Ca^{2+} chelating and those whose fluorescence was decreased by Mn^{2+} chelating. Consequently, since Ca^{2+} or Mn^{2+} bound to the same site of Fura2 molecule and since both fluorescence intensities were recorded from the same PMNs, an increase of fluorescence observed at λ_{EX}=360 nm even when Mn^{2+} had already penetrated into PMNs, was likely due to the Ca^{2+} influx into PMNs or released from intracellular stores and competing with Mn^{2+} which cannot be extruded from PMNs.

Fluorescence variations were expressed in arbitrary units (a. u.) when registered at λ_{EX}=340 nm and in % of Fura2 quenching when registered at λ_{EX}=360 nm (%: beginning of the registration; 100%: Triton X100 addition).

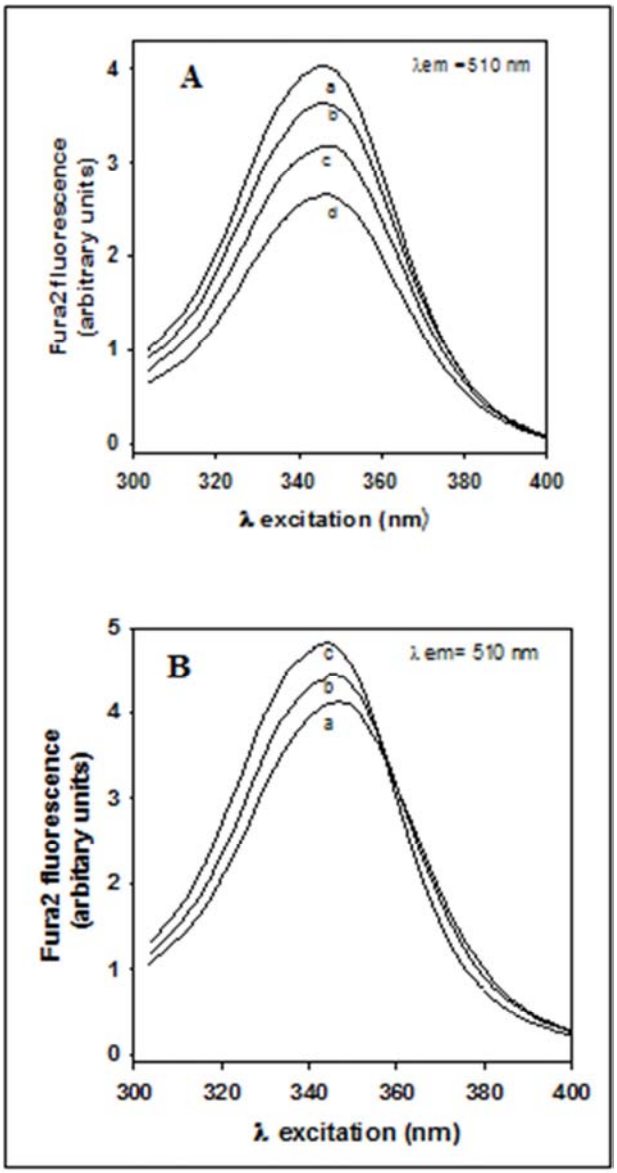

Figure 1. *Excitation spectra of Fura2 in the presence of varying concentrations of Ca^{2+} and Mn^{2+}. A: [Ca^{2+}]=1 mM, [Mn^{2+}] in µM 0 (a), 100 (b), 200 (c), 400 (d). B: [Mn^{2+}]=100 µM, [Ca^{2+}] in µM: 1 (a), 100 (b), 500 (c).*

When ethidium bromide (100 µM) was added in order to determine the formation of trans-membrane pores as described previously [12], the excitation monochromator set at λ_{EX}=340 nm was used with the second emission monochromator set at λ_{EM}=600 nm. The ethidium influx was expressed in % (0%: beginning of the registration; 100%: maximum of ethidium intercalated in nucleic acids after Triton X100 addition).

For fluorescence measurements, 1 ml of PMNs suspension (6x10^6 cells/ml) was added to 1 ml of assay solution continuously stirred in a 4 ml quartz cuvette (1 cm light path) thermostated at 37°C. The autofluorescence of PMNs was substracted by the PTI software and the data extracted for transfer to SigmaPlot 4.1 (Jandel, Erckrath, Germany). The experiments described in figures are the most representative of four similar ones.

3. Results

Previously, we have reported that the HlgA/HlgB γ-hemolysin pair did provoke, in the absence or in the presence of 1 mM extracellular free Ca^{2+}, the opening of pre-existing Ca^{2+} channels involving divalent ions influx through the plasma membrane of human PMN [15]. In the absence of extracellular Ca^{2+}, this was demonstrated by using 0.1 mM Mn^{2+} as a Ca^{2+} surrogate and by recording the fluorescence of Fura2 at λ_{EX}=360 and λ_{EM}=510 nm (Figure 2).

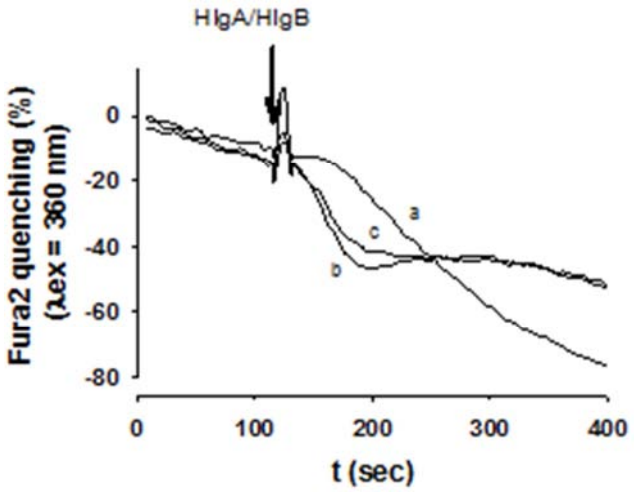

Figure 2. *Effect of the HlgA/HlgB application on Mn^{2+} influx in human PMNs in the presence of different Ca^{2+} concentration. [Ca^{2+}] in mM: 0 (a), 0.1 (b), 1 (c); [Mn^{2+}]=0.1 mM.*

Under these conditions, the leukotoxin induced a decrease in fluorescence intensity of Fura2-loaded neutrophils indicating an influx of Mn^{2+} into PMNs (Figure 2a). Although the Ca^{2+} binding does not modify the fluorescence of Fura2 at λ_{EX}=360 nm, the recordings obtained in the presence of 0.1 and 1 mM Ca^{2+} presented different time lags and courses (Figure 2b, c). The entry of Mn^{2+} appeared sooner and was more rapid in the presence of extracellular Ca^{2+}.

The simultaneous influence of 1 mM Ca^{2+} and 0.1 mM Mn^{2+} on Fura2-fluorescence intensity was further, studied as described in Figure 3. At λ_{EX}=340 nm, the addition of HlgA/HlgB induced a four phases response. The first two phases (Figure 3. 1; 2) consisted of a significant increase and decrease in Fura2 fluorescence, respectively. Then, during the third and fourth phase (Figure 3. 3; 4), the same evolution was recorded but over a longer period of time. The first phase was absent when recording at λ_{EX}=360 nm. Interestingly, the insert presented in Figure 3 showed that, the increase in fluorescence of Fura2 at λ_{EX}=340 nm preceded by about 10 sec the decrease of fluorescence observed at λ_{EX}=360 nm. Since this decrease, caused by an influx of Mn^{2+} was likely due to the opening of Ca^{2+} channels, the preceding increase of fluorescence recorded at λ_{EX}=340 nm might be due to the depletion of the internal Ca^{2+} stores. This time lag difference was observed for each population of PMNs activated by leukotoxins and, lasted 5 to 15 sec depending on the donor.

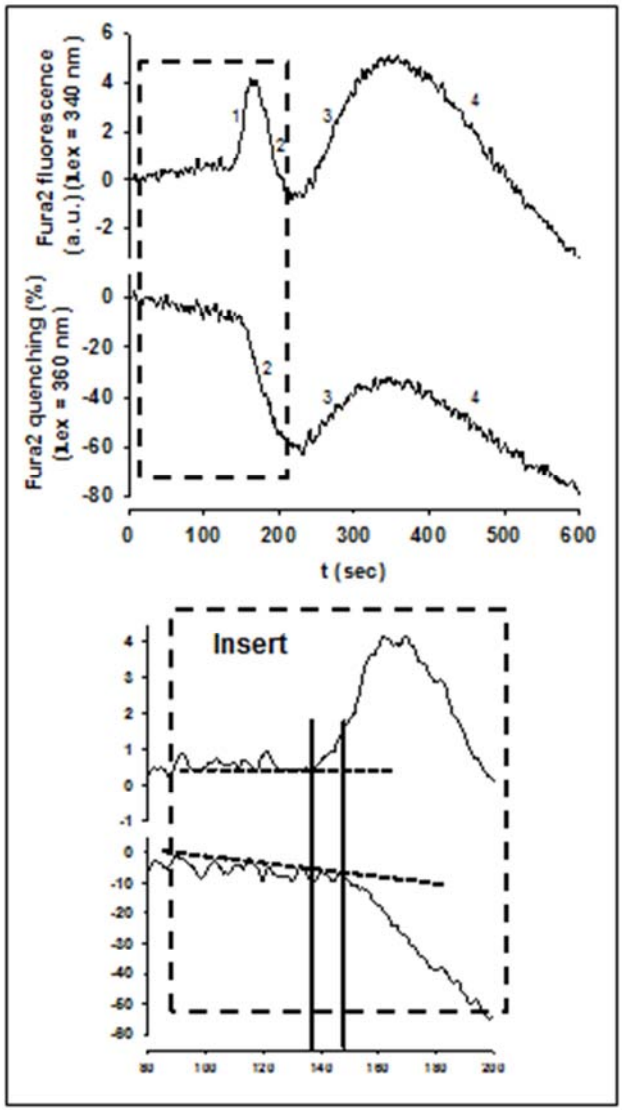

Figure 3. *Effect of the HlgA/HlgB application on Ca^{2+} and Mn^{2+} influx in human PMNs in the presence of 1 mM Ca^{2+} and 0.1 mM Mn^{2+}. The different phases 1, 2, 3 and 4 are indicated. The insert describes the different time lags obtained at the two excitation wavelengths (340 nm and 360 nm).*

Consequently, it was proposed that staphylococcal HlgA/HlgB leukotoxins might induce, in the presence of extracellular Ca^{2+}, the opening of Ca^{2+}-released activated Ca^{2+} (CRAC) channels mediating Ca^{2+} and Mn^{2+} influxes as was demonstrated for agonists such as LTB4, PAF [40] and fMLP [19, 32]. Similar results were obtained with both HlgC/HlgB and LukS-PV/LukF-PV leukotoxins used at the same concentration under the same conditions (1 mM Ca^{2+} and 0.1 mM Mn^{2+}) (unpublished data). During the third phase, the increase in fluorescence intensity of Fura2 at both excitation wavelengths (λ_{EX}=340 nm and λ_{EM}=360 nm) was indicative of an increase in the intracellular Ca^{2+} competing with the Mn^{2+} chelated by Fura2 (see *spectrofluorometric measurements*). During the last phase, Mn^{2+} was still penetrating the cells.

The purpose of this research was to investigate the intracellular molecular mechanisms involved in the opening of Ca^{2+} channels including the CRAC channels by leukotoxins. It is well known that, thapsigargin provoked a rapid depletion

of internal calciosomes by inhibiting the sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity [41], inducing the opening of CRAC channels as being inhibited by La^{3+} [42]. This was verified under the experimental conditions used in this study, as shown in Figure 4a.

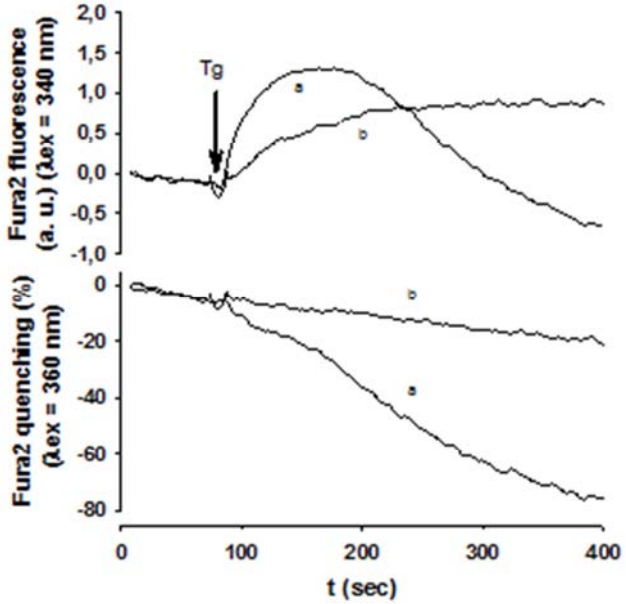

Figure 4. *Effect of La^{3+} on Ca^{2+} and Mn^{2+} influx induced by thapsigargin. [Ca^{2+}]=1 mM, [Mn^{2+}]=0.1 mM, [La^{3+}]=0 mM (a) and 0.1 mM (b), [Tg]=0.1 mM.*

However, in the presence of 0.1 mM La^{3+}, most widely used for blocking the store-operated Ca^{2+} entry [43], the treatment with 0.1 mM thapsigargin (Tg) caused an increase of intracellular Ca^{2+} (λ_{EX}=340 nm), consecutive to an IP_3-sensitive Ca^{2+} stores depletion [44-45]. Whereas, the subsequent influx of Mn^{2+} and Ca^{2+} (λ_{EM}=360 nm), via the store-operated Ca^{2+} entry, was completely inhibited showing that, no CRAC channels were opened (Figure 4b).

When the same experiment was performed with HlgA/HlgB in the presence of 1 mM Ca^{2+} and 0.1 mM Mn^{2+}, the Ca^{2+} stores depletion was still observed in the presence of 0.1 mM La^{3+} but, conversly to thapsigargin, the Mn^{2+} influx was partially inhibited (Figure 5I). Similar results were obtained in the presence of 1 mM Ni^{2+} (Figure 5II), another known blocker of CRAC channels [20].

Since La^{3+} and Ni^{2+} did inhibit partially the Mn^{2+} (Ca^{2+}) influx, we suggested that another type of Ca^{2+} (non-CRAC) channels could be involved in the signaling pathway induced by staphylococcal bi-component leukotoxins. Moreover, in the absence of extracellular Ca^{2+} and Mn^{2+}, HlgA/HlgB did not alter the fluorescence of Fura2 (λ_{EX}=340 nm) indicating that it did not provoke the Ca^{2+} release from the internal Ca^{2+} stores in Ca^{2+} free medium (unpublished data) as did 1 nM ionomycin, known to open CRAC channels [28, 46]. This effect did not impair the pore-formation capabilities of the leukotoxin [15]. In the only presence of 0.1 mM Mn^{2+}, the addition of 0.1 mM La^{3+} was without influence on the Mn^{2+} influx (unpublished data) showing that, another type of ionic channels not sensitive to La^{3+}, was opened by leukotoxins under these conditions. Simultaneously, no increase of ethidium fluorescence was observed. This later experiment also indicated that Fura2 quenching by Mn^{2+} was conclusively due to an entry of Mn^{2+} into PMNs and not to a leak of Fura2 through the pores formed by the leukotoxin since they were closed by La^{3+}.

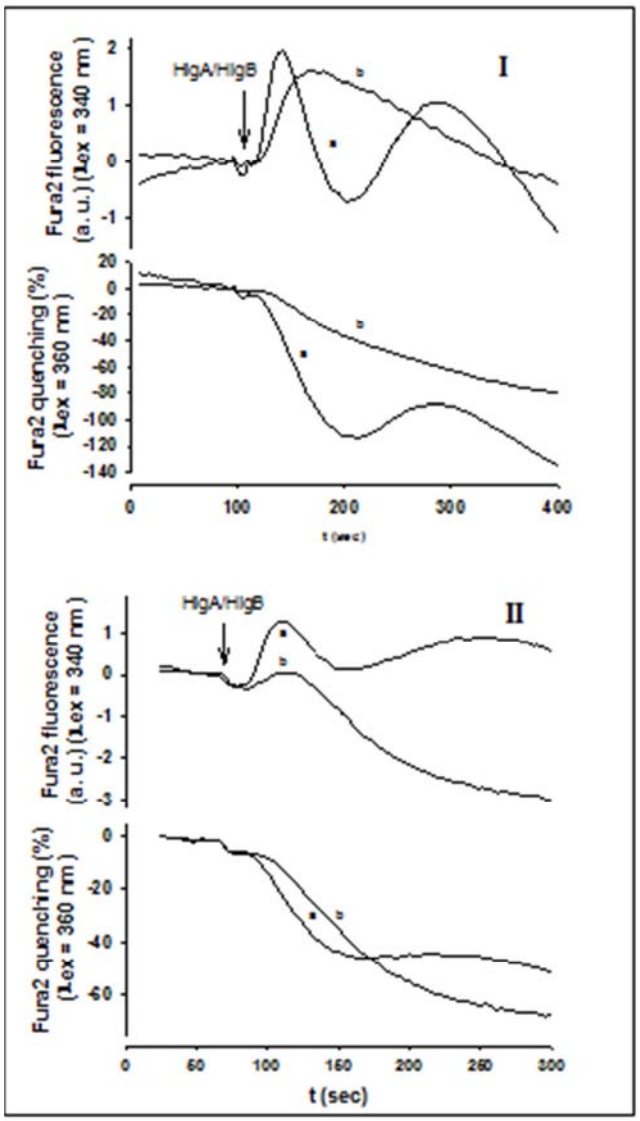

Figure 5. *Effect of La^{3+} on Ca^{2+} and Mn^{2+} influx induced by the HlgA/HlgB application in human PMNs. PMNs were incubated (I) during 2 min in the absence (a) or presence (b) of 0.1 mM La^{3+}; (II) during 2 min in the absence (a) or presence (b) of 1 mM Ni^{2+}. [Ca^{2+}]=1 mM, [Mn^{2+}]=0.1 mM.*

Another experiment designed on the PMNs of a particular donor, showed that, in the absence of extracellular Ca^{2+}, although an influx of ethidium was observed, no variation of Fura2 fluorescence intensity consecutive to a Mn^{2+} influx was recorded (Figure 6). This confirmed that, trans-membrane pores formed by staphylococcal leukotoxins were not permeable to divalent ions (Mn^{2+}, Ca^{2+}).

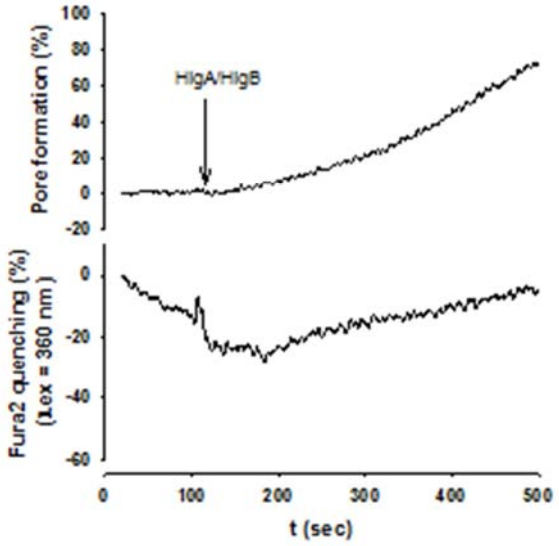

Figure 6. *Effect of the HlgA/HlgB application on Mn^{2+} influx and pore formation in human PMNs in the absence of extracellular Ca^{2+}. $[Mn^{2+}]$=0.1 mM, [Bet]=100 µM.*

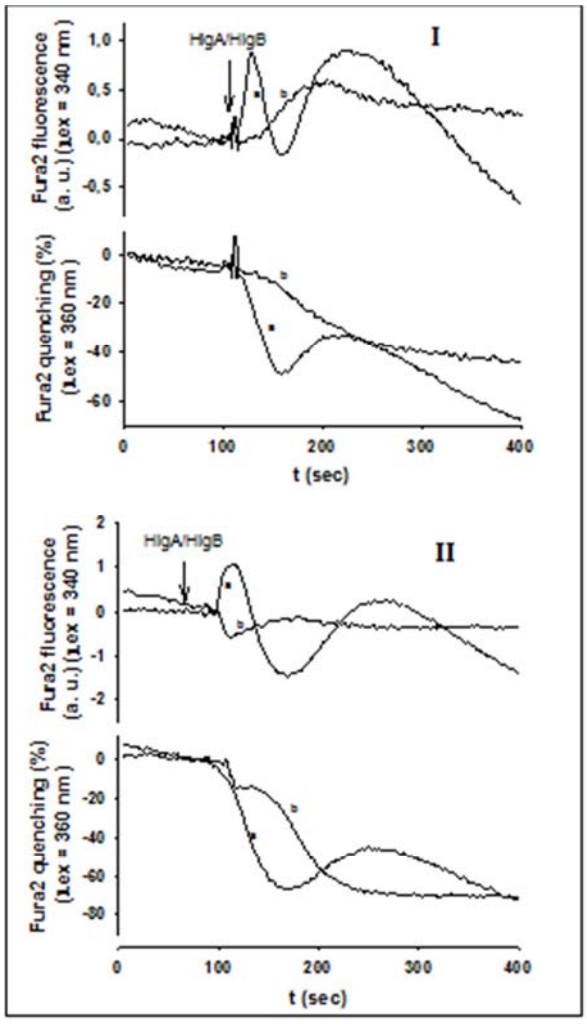

Figure 7. *Effect of thapsigargin and TMB8 on Ca^{2+} and Mn^{2+} influx induced by the HlgA/HlgB application in human PMNs in the presence of extracellular Ca^{2+}. PMNs were incubated (I) during 60 min in the absence (a) or presence (b) of 0.1 mM thapsigargin; (II) during 90 min in the absence (a) or presence (b) of 0.5 mM TMB8. $[Ca^{2+}]$=1 mM, $[Mn^{2+}]$=0.1 mM.*

The implication of CRAC channels in the mechanism of HlgA/HlgB activity was further, demonstrated by modifying the availability of Ca^{2+} stores. In one set of experiments, PMNs were pre-incubated with 0.1 mM thapsigargin before the leukotoxins application. Under these conditions, the Ca^{2+} stores were depleted and their refilling was inhibited [47-48]. Later following a 60 min incubation, the CRAC channels were again inactivated [49]. Under these conditions, Figure 7I did show that, the initial rise in intracellular Ca^{2+} was not observed after the HlgA/HlgB application. Again, the Ca^{2+} and Mn^{2+} influx was considerably, decreased and delayed. Another set of experiments was designed in which PMNs were pre-treated during 90 min with 0.5 mM TMB8, a potent inhibitor of Ca^{2+} release from internal Ca^{2+} stores [50-51]. Therefore, in the absence of extracellular Ca^{2+}, TMB8 did not affect fluorescence variations at λ_{EX}=360 nm (unpublished data) and, consequently, did not modify the Mn^{2+} influx linked to the Ca^{2+} channels opening. However, in the presence of 1 mM Ca^{2+} and 0.1 mM Mn^{2+}, the initial rise of intracellular Ca^{2+} was not observed and consequently, the influx of divalent ions (Mn^{2+} and Ca^{2+}) was significantly decreased (Figure 7II) as already shown after the thapsigargin pre-treatment.

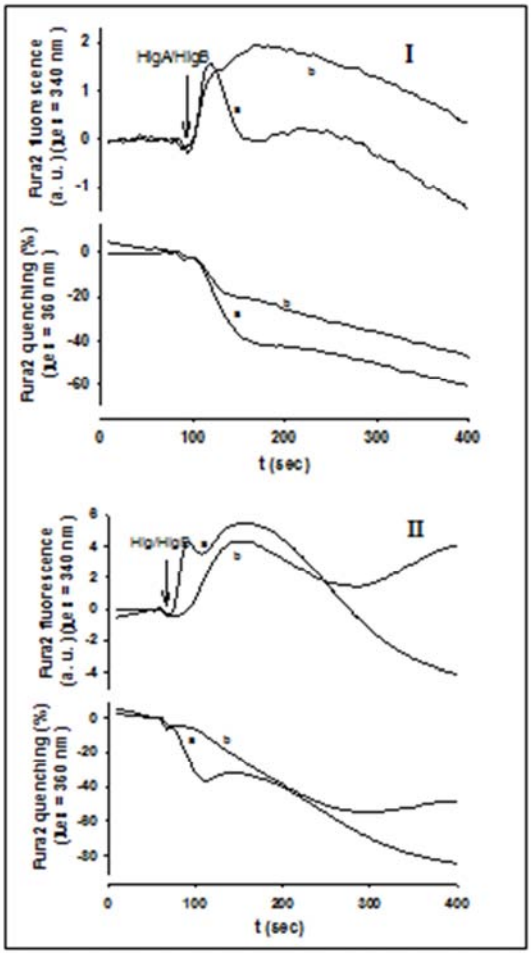

Figure 8. *Effect of tyrphostin A9 and okadaic acid on Ca^{2+} and Mn^{2+} influx induced by the HlgA/HlgB application in human PMNs in the presence of extracellular Ca^{2+}. PMNs were incubated (I) during 5 min in the absence (a) or presence (b) of 5 µM tyrphostin A9; (II) during 60 min in the absence (a) or presence (b) of 1µM okadaic acid. $[Ca^{2+}]$=1 mM, $[Mn^{2+}]$=0.1 mM.*

It has previously reported [52-53] that, the CRAC channels activation could be associated to the Ca^{2+} influx factor (CIF), which seemed to be regulated by the Ca^{2+}-stores-associated enzyme suggesting that, the activity of CRAC channels is a mechanism dependent on phosphorylation-dephosphorylation process [53-55].

Furthermore, Marhaba *et al*. [56] showed that tyrphostin A9 did inhibit the Ca^{2+} release-dependent phosphorylation and the Ca^{2+} influx through the opening of CRAC channels in Jurkat cells. The effect of this inhibitor was tested on the CRAC channels activation by leukotoxins. As results, in the presence of 5 µM tyrphostin A9, the initial rise recorded at λ_{EX}=340 nm and which was linked to a Ca^{2+} release after leukotoxins addition was slightly inhibited, and Mn^{2+} influx was partially reduced. Interestingly, only two phases were observed at λ_{EX}=340 nm (Figure 8I), suggesting that the opening of CRAC channels was blocked whereas another type of Ca^{2+} channels triggered by leukotoxins was not affected. Moreover, when PMNs from the same donor were pre-treated with 1 µM okadaïc acid, a potent inhibitor of phosphatase, a significant increase in intracellular Ca^{2+} levels was observed whereas the CRAC channels activation was blocked (Figure 8II) as it has previously reported [57].

These authors suggested that okadaic acid by inhibition of CIF degradation in lymphocytes, might induce a sustained increase in Ca^{2+} levels. We further investigated whether the phospholipase C activity was involved in the action mechanism of leucotoxins. For this, 20 µM U-73122, a potent phospholipase C (PLC) inhibitor [58-59], was added just before the leukotoxins application. Consequently, although IP_3 production in response to leukotoxins was partially, inhibited by U-73122, inducing a slight inhibition of Ca^{2+} release and CRAC channels activation, the third and fourth phases were completely suppressed (Figure 9I).

These results could be interpreted as staphylococcal leukotoxins might induce a Ca^{2+} release by another pathway involved in addition to an IP_3-induced Ca^{2+} release (IP_3-ICR) and, another type of Ca^{2+} channels different from CRAC channels could be activated. This hypothesis was verified in the present study. Again, the pre-incubation of PMNs during 60 min with 1 µM staurosporine, a potent protein kinase C (PKC) inhibitor [60-61], gave similar results as observed with U73122 during the third and fourth phases (Figure 9II) suggesting that, in the presence of extracellular Ca^{2+}, the Ca^{2+} influx induced by leukotoxins addition during the third phase could likely be a PKC-dependent process. This was a further evidence that, HlgA/HlgB might induce the opening of another type of Ca^{2+} channels different from CRAC channels. It is noteworthy that, in the absence of extracellular Ca^{2+}, La^{3+}, TMB8, U-73122, tyrphostinA9, okadaïc acid and staurosporine did not modify the Mn^{2+} influx induced by leukotoxins (unpublished data).

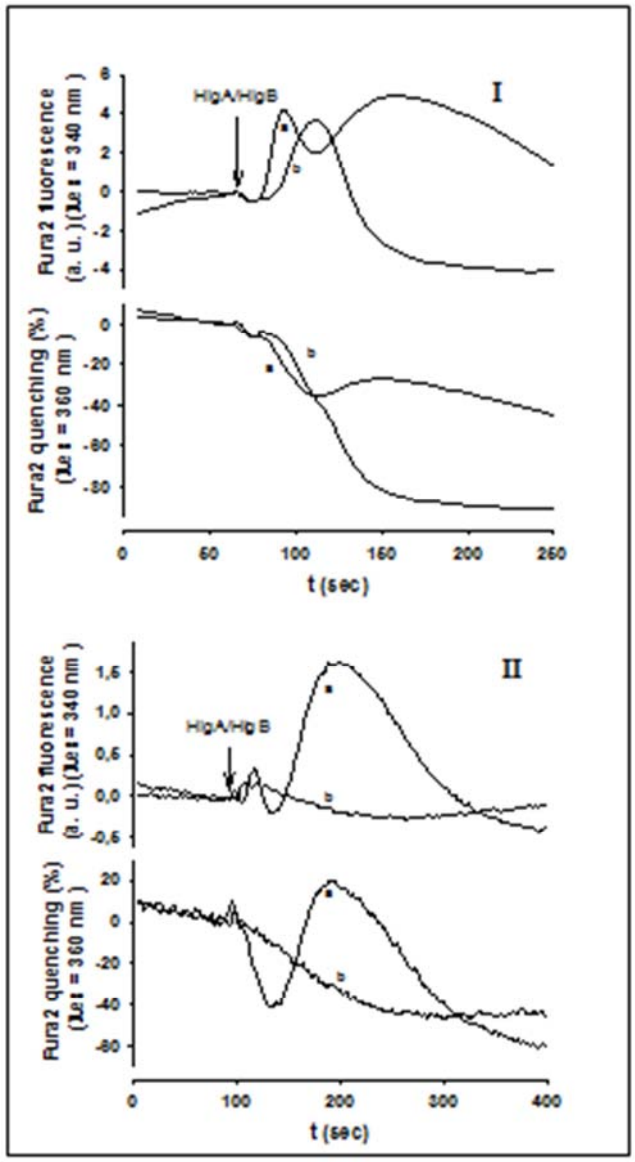

Figure 9. *Effect of U73122 and staurosporine on Ca^{2+} and Mn^{2+} influx induced by the HlgA/HlgB application in human PMNs in the presence of extracellular Ca^{2+}. PMNs were incubated (I) during 5 min in the absence (a) or presence (b) of 20 µM U73122; (II) during 60 min in the absence (a) or presence (b) of 1µM staurosporine. [Ca^{2+}]=1 mM, [Mn^{2+}]=0.1 mM.*

It has been reported that, at least three different types of internal Ca^{2+} stores could be involved in the increase of cytosolic $[Ca^{2+}]_i$ after cells activation [62-63]. This was verified in the present work by using specific direct- and indirect-inhibitors for internal Ca^{2+} stores depletion as shown in Figure 10.

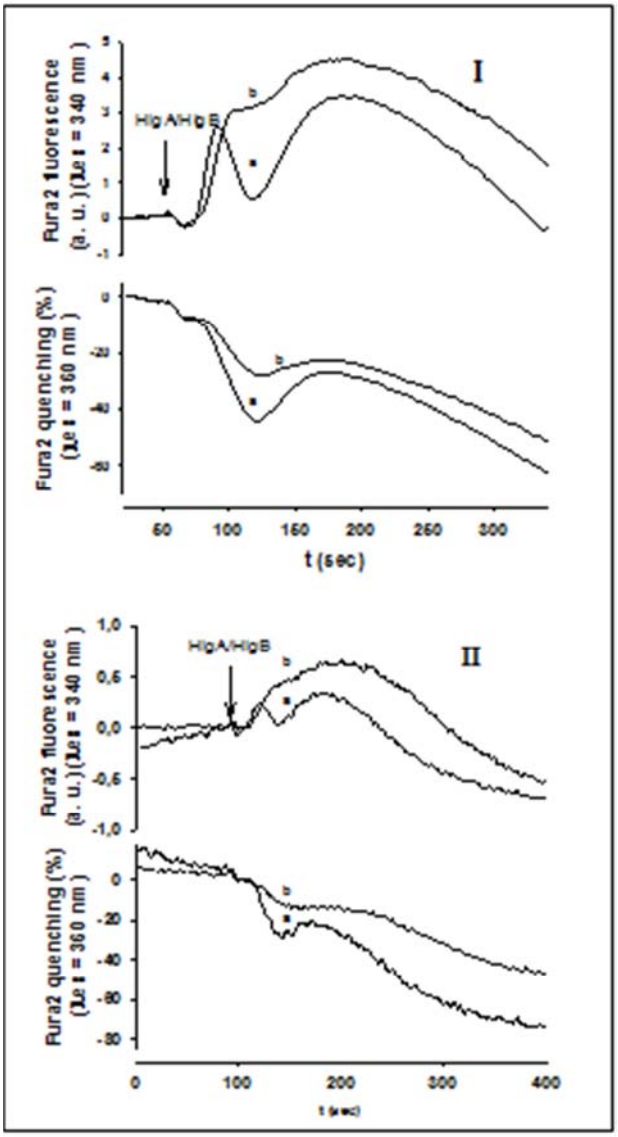

Figure 10. *Effect of cinnarizine and flunarizine on Ca^{2+} and Mn^{2+} influx induced by the HlgA/HlgB application in human PMNs in the presence of extracellular Ca^{2+}. PMNs were incubated (I) during 60 min in the absence (a) or presence (b) of 100 μM cinnarizine; (II) during 60 min in the absence (a) or presence (b) of 100 μM flunarizine. [Ca^{2+}]=1 mM, [Mn^{2+}]=0.1 mM.*

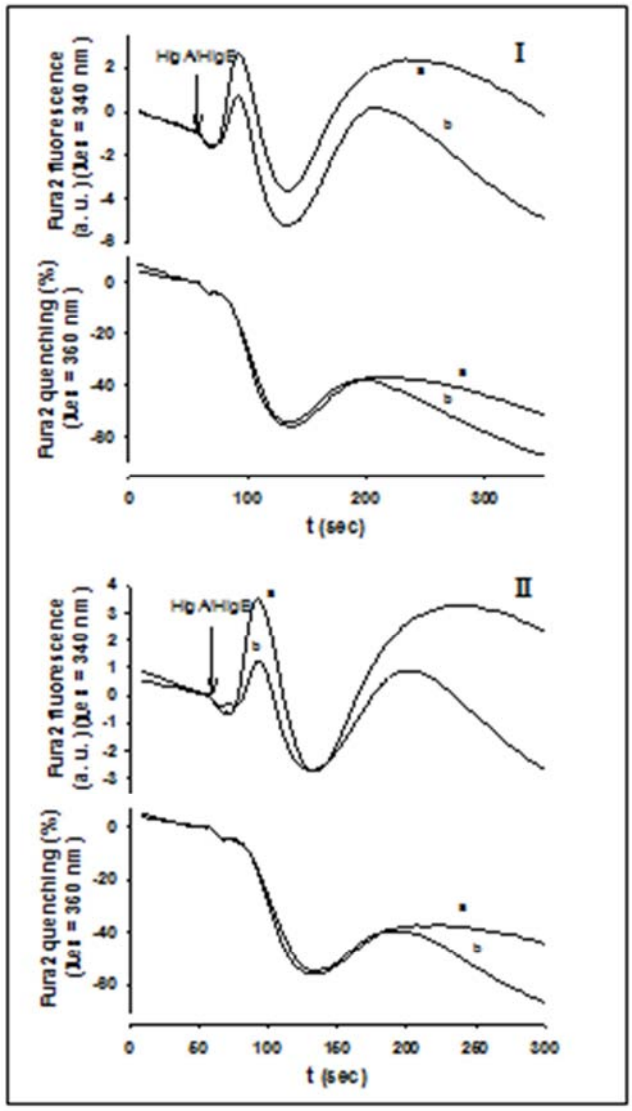

Figure 11. *Effect of ryanodine and caffeine on Ca^{2+} and Mn^{2+} influx induced by the HlgA/HlgB application in human PMNs in the presence of extracellular Ca^{2+}. PMNs were incubated (I) during 60 min in the absence (a) or presence (b) of 5 μM ryanodine; (II) during 60 min in the absence (a) or presence (b) of 100 μM caffeine. [Ca^{2+}]=1 mM, [Mn^{2+}]=0.1 mM.*

First, we tested the effect of cinnarizine and flunarizine, known as potent inhibitors of IP_3-Induced Ca^{2+} Release (IP_3-ICR) [64-65], on the Ca^{2+} release and CRAC channels activation after the leukotoxins application. As observed in Figure 10, both 100 μM cinnarizine (Figure 10I) and 100 μM flunarizine (Figure 10II) induced a significant inhibition of CRAC channels opening consequently, to the Ca^{2+} release inhibition. These results suggested that HlgA/HlgB might trigger the Ca^{2+} release from the IP_3-sensitive Ca^{2+} stores (IP_3-ICR). Furthermore, the hypothesis of the implication of another trigger than phospholipase C and subsequent PtdIns*P3* formation in Ca^{2+} stores depletion was tested.

Again, since the Ca^{2+}-Induced Ca^{2+} Release (CICR) is sensitive to caffeine and ryanodine [66], both inhibitors were tested on the HlgA/HlgB activity as represented in Figure 11.

The pre-treatment of human PMNs during 60 min with either 5 μM ryanodine (Figure 11I) or 100 μM caffeine (Figure 11II) induced a significant reduction of about half of intracellular Ca^{2+} levels mediated by the leukotoxins addition during the first phase, although Mn^{2+} influx was not significantly modified. Taken together, our funding suggested that the HlgA/HlgB γ-hemolysin might trigger the Ca^{2+} release from at least, two different types of internal Ca^{2+} stores: IP_3-ICR and CICR.

Moreover, it is widely known that the CRAC channels activation is associated to the binding of an agonist to heterotrimeric G-proteins-associated receptors [52]. The implication of G-proteins in the Ca^{2+} signaling mediated by the HlgA/HlgB application was verified by using, specific blockers of G-proteins activity such as NaF [67] and pertussis toxin [68-69] as shown in Figure 12.

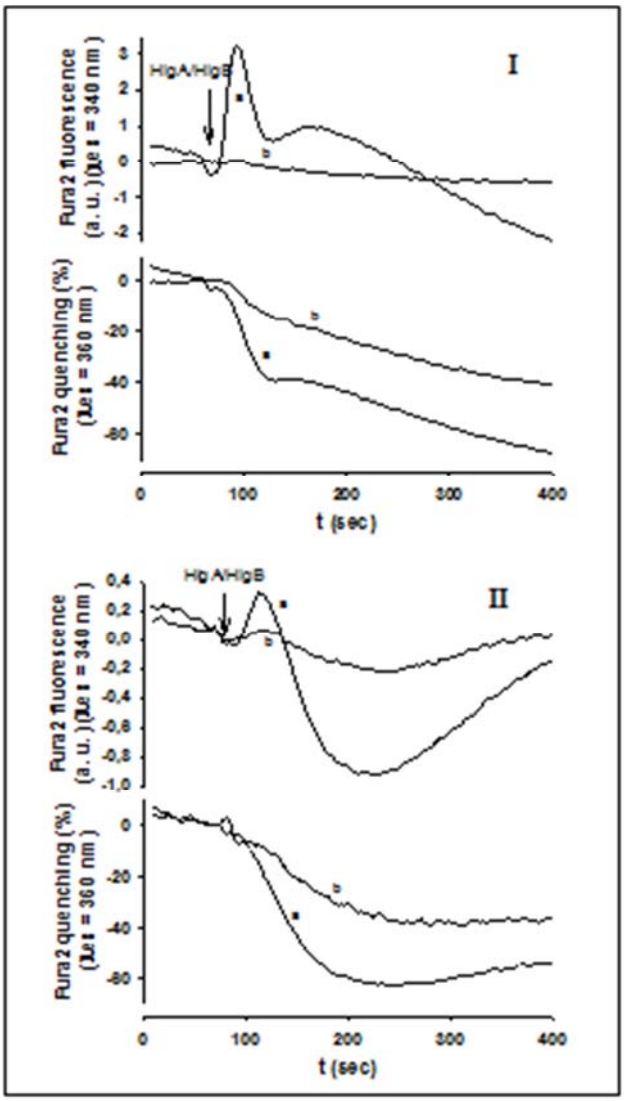

Figure 12. *Effect of NaF and pertussis toxin on Ca^{2+} and Mn^{2+} influx induced by the HlgA/HlgB application in human PMNs in the presence of extracellular Ca^{2+}. PMNs were incubated (I) during 60 min in the absence (a) or presence (b) of 20 mM NaF; (II) during 120 min in the absence (a) or presence (b) of 500 ng/ml pertussis toxin. [Ca^{2+}]=1 mM, [Mn^{2+}]=0.1 mM.*

Interestingly, the pre-treatment of human PMNs with 20 mM NaF during 60 min (Figure 12I) or with 500 ng/ml pertussis toxin during 120 min (Figure 12II) abolished significantly the Ca^{2+} release from internal stores and as results the influx of divalent ions (Ca^{2+} and Mn^{2+}) was significantly inhibited. Whereas, in the absence of extracellular Ca^{2+}, both inhibitors did not modify the Mn^{2+} influx provoked by HlgA/HlgB (unpublished data), confirming our hypothesis that, staphylococcal leukotoxins might induce the opening of at least, two different types of Ca^{2+} channels.

4. Discussion

Previously, it has been demonstrated that in addition to their pore-forming activity [12], the bi-component leukotoxins from *S. aureus* were potent agonists for Ca^{2+} channels in human PMNs [15]. In that study, it was also shown that pores formed by these leukotoxins were not inhibited by Ca^{2+} channels blockers which, totally abolished Ca^{2+} and Mn^{2+} influxes. This led to the suggestion that, pores were not permeable to divalent ions. This hypothesis was confirmed by the present study where it was shown in a specific donor that, Mn^{2+} did not penetrate into PMNs although a large influx of ethidium through trans-membrane pores was observed after the leukotoxins application (Figure 6). Furthermore, in the presence of 0.1 mM La^{3+}, no pores were opened, although Mn^{2+} still penetrated into PMNs (unpublished data). These observations ruled out the implication of pores in divalent ions (Ca^{2+}, Mn^{2+}, Ni^{2+}) permeability and allowed to investigate the opening of pre-existing Ca^{2+} channels by recording the fluorescence of Fura2-loaded human neutrophils without systematically checking the pore-formation.

The use of two excitation wavelengths, 340 nm and 360 nm for which the fluorescence of Fura2 was insensitive to Ca^{2+} variations, gave indications of Ca^{2+} and Mn^{2+} fluxes closely associated to the Ca^{2+} channels opening, and the Ca^{2+} release from internal Ca^{2+} stores. Although Fura2 fluorescence was insensitive to Ca^{2+} variations at λ_{EX}=360 nm, this study showed that, in the presence of Mn^{2+}, significant Ca^{2+} variations may have an influence on the fluorescence by competition between the two divalent ions at the chelating site of Fura2. This characteristic allowed to determine that, divalent ions (Ca^{2+} and Mn^{2+}) influx when occurring during time, presented several phases after the HlgA/HlgB application. Similar results were obtained when LukS-PV/LukF-PV or HlgC/HlgB were tested for their activity (unpublished data). Actually, divalent ions also compete for the same sites on the Ca^{2+} channels and it has been shown that, Mn^{2+} at higher concentrations (10 mM), could be a blocking agent [46]. However, since the extracellular concentration of divalent ions was not modified during the experiment, the fluorescence variations observed in this study reflected the selectivity of the different activated channels.

It is widely known that, the release of Ca^{2+} from internal Ca^{2+} stores provokes the opening of CRAC channels responsible for a Ca^{2+} influx in human neutrophils [29-30]. An activation of CRAC channels by HlgA/HlgB was demonstrated in the presence of extracellular Ca^{2+}, by recording Fura2 fluorescence variations at both excitation wavelengths 340 and 360 nm. A similar observation was made by Montero *et al*. [29], after stimulation of human neutrophils with the ionomycin, known as a Ca^{2+} ionophore. A series of experiments was realized in order to determine whether increases of intracellular Ca^{2+} levels induced by HlgA/HlgB in human PMNs were due to the Ca^{2+} stores depletion and, which mediated consequently, the opening of CRAC channels. Previous studies [70-71, 19] have reported that, the intracellular application of inositol-1, 4, 5-triphosphate (IP_3) and high concentrations of Ca^{2+} buffer resulted in the activation of CRAC channels. Interestingly, our results clearly demonstrated that, *i*) the intracellular Ca^{2+} level was rapidly increased by the leukotoxins addition before, the onset of a

Mn^{2+} (Ca^{2+}) influx associated to the opening of Ca^{2+} channels; *ii*) the previous Ca^{2+} release from internal Ca^{2+} stores by thapsigargin, an inhibitor of a SERCA Ca^{2+}-ATPase activity, did suppress the fast initial rise of intracellular Ca^{2+} induced by the leukotoxins addition, then, delayed and inhibited significantly the divalent ions influx; and, *iii*) the incubation of PMNs with TMB8 had a comparable effect, as thapsigargin.

Although TMB8 is a potent blocker of an IP_3-induced Ca^{2+} release [64, 50-51], it can be a weak inhibitor of Ca^{2+} channels and protein kinase C (PKC) activity. Interestingly, in the present study, we evidenced that protein kinase C was involved at different levels in the action mechanism of leukotoxins, in particular, during the phase 3 and 4 as it has been demonstrated by a clear inhibition of a Ca^{2+} influx when PMNs were pretreated with staurosporine, a potent inhibitor of PKC activity [60-61]. Furthermore, in the absence of extracellular Ca^{2+}, TMB8 did not modify the Mn^{2+} influx (unpublished data). Other experiments were performed with potent inhibitors of CRAC channels opening [42-43, 20] in order to verify that the initial increase of intracellular [Ca^{2+}] was independent of the opening of Ca^{2+} channels. Indeed, La^{3+} and Ni^{2+}, used as potent blockers of CRAC channels [20, 42], did not modify the initial intracellular Ca^{2+} rise but reduced significantly the Fura2 quenching by Mn^{2+} at λ_{EX}=360 nm. La^{3+} may inhibit the opening of CRAC channels but not those activated in the absence of extracellular Ca^{2+} (unpublished data).

Furthermore, it has been reported by Marhaba [56] that, tyrphostin A9 could affect the CRAC channels activation in Jurkat cells by blocking proteins phosphorylation occurring after the internal Ca^{2+} stores release. In human PMNs, tyrphostin A9 decreased the Mn^{2+} influx after the HlgA/HlgB addition and seemed to attenuate but not significantly the Ca^{2+} release. Further evidence that, an eventual involvement of a phosphorylation/dephosphorylation-dependent mechanism in CRAC channels activity [57, 72-73] by the γ-hemolysin leukotoxin, was confirmed when the okadaic acid was tested. Indeed, our results were supported by previous reports. In this study, we also attempted to identify the different types of internal Ca^{2+} stores, triggered by staphylococcal leukotoxins. This was confirmed by using potent blockers of the internal Ca^{2+} release, cinnarizine and flunarizine for the inositol-1,4,5-triphosphate (IP_3)-induced Ca^{2+} release (IP_3-ICR) [64-65], and caffeine or ryanodine for the Ca^{2+}-induced Ca^{2+} release (CICR) [74]. As results, we have suggested that at a least, two different types of Ca^{2+} stores including IP_3-ICR and CICR [75] were involved in the HlgA/HlgB activity.

Another argument of the CRAC channels opening by leukotoxins through a G-proteins effect, was given by the fact that, the initial rise in intracellular Ca^{2+} and Mn^{2+} influx were inhibited by either NaF [67] or pertussis toxin [76]. These results strongly suggested the involvement of heterotrimeric G-proteins in the CRAC channels activation by leukotoxins, as already proposed for other agonists [52]. Thus, the opening of CRAC channels through a mechanism involving the G-proteins activation, has already been described in previous reports [76-77]. Taken together, all these arguments provided evidence for the downstream effect of the specific binding of the bi-component HlgA/HlgB γ-hemolysin to its receptors in human neutrophils, leading to the CRAC channels activation via a G-proteins-dependent process.

The binding site of the HlgA/HlgB pair has not yet been determined but it has already been described that, LukS-PV, another member of the staphylococcal leukotoxin family which was also an inducer of the opening of Ca^{2+} channels [15], bound to a unique class of receptors belonging to the PMNs membrane with Kd=6 nM [13]. The HlgA protein presented also, a concentration-dependent binding but did not share the same receptor with LukS-PV since HlgA did not compete with LukS-PV for binding [78]. The identification of staphylococcal bi-component leukotoxins receptors in human target cells [79-81] has revealed new insights into how leukotoxins modulate immune cells functions.

In contrast to other CRAC channels activators [20, 28], the γ-hemolysin was unable, in the absence of extracellular Ca^{2+}, to induce the depletion of internal Ca^{2+} stores. Several hypothesis could explain this discrepancy. First, some authors failed to use EGTA or another Ca^{2+} chelator in their *free Ca^{2+} medium* and the several μM of Ca^{2+} present in ionic solutions as impurities, could have been sufficient for activation. Second, the absence of extracellular Ca^{2+} might modify the complex *leukotoxin-receptor* in a conformation unable to activate the stores cascade including PtdIns*P*3 formation. Third, the leukotoxin could involve different signaling pathways depending on the presence of extracellular Ca^{2+}.

This later hypothesis was strengthened by two observations: *i*) U-73122 did inhibit the first two phases suggesting that, the Ca^{2+} release triggered by leukotoxins, could be dependent in a part, to the PtdIns*P*3 formation; this result might be considered cautiously since, although U-73122, a potent inhibitor of phospholipase C, completely abolished the depletion of Ca^{2+} stores induced by fMLP [62], it has been shown, to directly evoked a Ca^{2+} influx in MDCK cells [59]. Thus, both effects could occur in the present experiments, and overlap an inhibition of the initial rise provoked by HlgA/HlgB; and *ii*) the incubation with caffeine and ryanodine partially inhibited the initial rise in intracellular Ca^{2+}, suggesting the implication of the Ca^{2+}-Induced Ca^{2+} Release (CICR), as previously described in macrophages [82]. In addition, the existence of at least, two types of Ca^{2+} stores in human PMNs has been strongly demonstrated [83]. Thus, we have proposed that HlgA/HlgB might likely induce Ca^{2+}-Induced Ca^{2+} Release (CICR) and Ca^{2+}-Induced PtdIns*P*3 Release (IP_3-ICR) in their action mechanism on target PMNs.

Previous studies have reported that, CRAC channels being impermeable to La^{3+}, Ni^{2+} and Co^{2+} [84], are more permeable to Ca^{2+} than Mn^{2+} [20, 72] although the Mn^{2+} influx was sufficient to decrease the fluorescence intensity of Fura2 at both wavelengths during the second phase. During the third phase, since the Ca^{2+} stores were already been depleted, the increase of the Fura2 fluorescence could only be explained by the opening of an additional more selective Ca^{2+} entry

pathway. As the opening of this pathway was not observed in the absence of Ca^{2+} and, was completely inhibited by U-73122 and staurosporine, it was more likely that, this pathway might be Ca^{2+}- and PKC-dependent Ca^{2+} channels. However, in the absence of extracellular Ca^{2+}, γ-hemolysin leukotoxins did induce a consequent Mn^{2+} influx which was not subsequent to Ca^{2+} stores depletion and was not affected by all inhibitors tested. Putney & Bird [26] have already shown that, in the mouse lacrimal acinar cell in the absence of extracellular Ca^{2+}, the plasma membrane entry pathway could be activated by thapsigargin without prior transient elevation of intracellular Ca^{2+}, but they did not check the type of channel opened.

In the present study, the divalent ions (Mn^{2+}) pathway activated in Ca^{2+} free medium was not sensitive to La^{3+}, TMB8, U-73122, tyrphostin A9, staurosporine and pertussis toxin (unpublished data). Consequently, it seemed likely that in the absence of extracellular Ca^{2+}, the Ca^{2+} channels opening mediated by staphylococcal leukotoxins were different from those activated by leukotoxins in the presence of Ca^{2+}, which were dependent on the internal Ca^{2+} stores depletion (CRAC channels) or by protein kinase C (PKC) dependent-signaling and which were also independent on a pertussis toxin-sensitive G-proteins action. Previous studies have suggested that, the eventual regulation of Ca^{2+} signaling pathways mediated by staphylococcal leukocidin could be enhanced, through G-proteins [85] or not [86-87] according to the specificity of receptors characterized for the PFTs from *S. aureus*. Further investigations are necessary to conclude the involvement of these later channels when Ca^{2+} is present in the external medium, even if their opening could be suspected in some recordings according to the donor.

5. Conclusion

In this research, we have proposed that, bi-component staphylococcal leukotoxins after their specific binding to their membrane receptors, might enhance the opening of at least, three different types of Ca^{2+} channels: *i*) store-operated Ca^{2+} influx termed Ca^{2+}-release activated Ca^{2+} (CRAC) channels [88] involving trimeric G-proteins, in the presence of extracellular Ca^{2+}; *ii*) non-store operated Ca^{2+} channels protein kinase C-dependent, which exhibit a very low permeability to Mn^{2+} in the presence of extracellular Ca^{2+}; and *iii*) non-store-operated Ca^{2+} channels mediated independently to PKC- and pertussis toxin sensitive-G proteins effect, as clearly evidenced in the absence of extracellular Ca^{2+}.

The activation of at least, three different types of Ca^{2+} channels by staphylococcal leukotoxins would be modulated by the absence or presence of the extracellular Ca^{2+} and by the donor. Although, Demaurex *et al*. [20] proposed that the store-operated Ca^{2+} pathway was the main pathway of Ca^{2+} entry in human neutrophils, our results reinforce the hypothesis of the presence of non-store-operated channels in PMNs. In fact, Montero *et al*. [32] suggested that, another receptor-operated Ca^{2+} pathway blocked by Ni^{2+} could be activated in HL60 cells differenciated by dimethyl sulfoxide. Again, Wenzel-Seifert *et al*. [89] hypothesized that PMNs were equipped with multiple subtypes of non-selective ions channels but both the current and preceding study [15] showed that, human PMNs seemed to possess several types of divalent ions channels. Staphylococcal leukotoxins appear to be a convenient tool to study the different types of pre-existing Ca^{2+} channels expressed in human neutrophils and the mechanism involved in their activation.

In conclusion, the present study brings new insights concerning the mode of action of staphylococcal bi-component leukotoxins. Until now, the conclusions presented in the literature were controversial and originated from two laboratories: Woodin [90] proposed a hypothetical model for leukocidin in which the main determinant was an interaction with membrane phospholipids to form membrane pores and activated membrane bound adenylate cyclase [91]. Later, Noda *et al*. [92] proposed that, leukocidin activated an endogenous membrane bound phospholipase A_2 and stimulated the ouabain-insensitive Na^+, K^+-ATPase. Interestingly, the present work has clearly demonstrated that, leukotoxins from *S. aureus* might induce two independent events at the membrane level: *i*) a sustained Ca^{2+} influx into human PMNs through the opening of different types of Ca^{2+} channels, including Ca^{2+}-released activated Ca^{2+} (CRAC) channels which, induce a non-competent immunoreaction of human professional phagocytes and *ii*) the formation of trans-membrane pores not permeable to divalent ions, leading to the cells lysis that, precedes the cells death. These two events may be of fundamental and represent an important step in developing several staphylococcal infectious diseases worldwide. Our findings might indicate that, the modulation of host cells signaling cascades, rather than the host cell lysis, could be the major physiological role enhanced by staphylococcal leukotoxins during the course of infection and, which could likely modulate disease severity.

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The authors declare that they have no competing interests.

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