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The Staphylococcal Pore-forming Leukotoxins Open Ca^{2+} Channels in the Membrane of Human Polymorphonuclear Neutrophils

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Abstract. The ability of leukotoxins secreted by *Staphylococcus aureus* to modify the permeability of the membrane of human polymorphonuclear neutrophils has been studied by spectrofluorometry and appropriate fluorescent probes. This family of bicomponent leukotoxins is constituted by, at least, three pairs of proteins: LukS-PV/LukF-PV, HlgA/HlgB, HlgC/HlgB. After binding of both components to the membrane, each pair induces influxes of divalent cations and ethidium in polymorphonuclear neutrophils, although with different intensities. The influx of divalent cations appears sooner than the influx of ethidium. The pathway for divalent cations is not permeable to monovalent cations (Na^+, K^+, ethidium$^+$) and is blocked by Ca^{2+} channel inhibitors that do not block the fluxes of ethidium and monovalent cations. It is concluded that the leukotoxins bind to a receptor linked to a divalent cation-selective channel or to the channel itself which is activated. Then, the leukotoxins open a second pathway by insertion into the membrane and subsequent formation of aspecific pores allowing an influx of ethidium.

Key words: Human polymorphonuclear neutrophil—Calcium channel—Leukotoxin—Pore-forming toxin—*Staphylococcus aureus*—Spectrofluorometry

Introduction

A leukotoxic activity produced by *Staphylococcus aureus* (*S. aureus*) was observed by Van der Velde as early as 1894 [32]. Later, Panton and Valentine [22] differentiated the so-called Panton and Valentine Leukocidin (PVL) from hemolysins in the strain V8 isolated from a case of chronic furunculosis. PVL was characterized by Woodin [34, 35] as being composed of two proteins named, on the basis of their elution by chromatography, F (fast eluted, 32 kDa) and S (slow eluted, 38 kDa). PVL induces edema, erythema and necrosis in the skin of the rabbit [6] and PVL-producing strains which represent 2% of clinical strains of *S. aureus* are strongly associated with furuncles [24]. The leukotoxic activity was described to arise from the formation of ion-sized and aspecific pores through the membrane inducing dose-dependent divalent cations and ethidium fluxes into human polymorphonuclear neutrophils (PMNs) [10]. After the binding of S to a unique receptor [4] which appears with the metamyelocyte stage during the differentiation of the target cells [19], the subsequent binding of F initiates the formation of the pores. This is followed, in the presence of Ca^{2+}, by the liberation of the granule contents of PMNs [4, 19] and the production of inflammation mediators [14, 17]. Another two-component toxin inducing hemolysis, called γ-hemolysin, was isolated from *S. aureus* [13, 30] and was shown to be produced by 99% of randomly selected clinical strains of *S. aureus*. Sequencing and cloning of all genes encoding these toxins have revealed that γ-hemolysin was constituted by three proteins [5, 26, 27, 28] sharing also leukotoxic activity and making a family of leukotoxins with PVL [25]. The five proteins can be classified in class S components: S (LukS-PV), HlgA (32 kDa), HlgC (32 kDa) with 63 to 75% identity, and class F components: F (LukF-PV), HlgB (34 kDa) with 70% identity.

The aim of this study was, first, to compare the pore-forming abilities of these leukotoxins presenting strong identities between them, and, second, to analyze the specificity of the ion-sized pores opened through the

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membrane. The results show that the ion-sized pores are specific for divalent cations and sensitive to Ca^{2+} channel blockers although the aspecific pores are not. It is suggested that the staphylococcal leukotoxins are calcium channel agonists and pore-forming toxins. Preliminary results were presented at the Third International Workshop on Pore-forming Toxins in Mainz (Germany, 26–28 September, 1996).

Materials and Methods

Reagents

Fura2-AM, fluo3-AM, potassium binding fluorescent indicator-acetoxymethyl ester (PBFI-AM), Na-green tetraacetate and Pluronic F-127 from Molecular probes (Eugene, OR) were diluted in Me_2SO to 1 mM. JPREP was from TechGen International (Les Ulis, France). All other reagents were from Sigma (L'isle d'Abeau Chesnes, France).

Leukotoxin Preparation

The 5 components of the staphylococcal leukotoxins were prepared according to a recently reported method [25]. Briefly, the *S. aureus* V8 strain (ATCC 49775) kindly provided by S. Thornley (Wellcome Laboratories, London, UK) was grown to the stationary growth phase with vigorous rotary shaking in CCY-modified medium at 37°C. Proteins were precipitated from the supernatant by 80% ammonium sulfate (wt/vol) resuspended and subjected to a first chromatography on a Sepharose SP Fast Flow column (Pharmacia, Uppsala, Sweden). Fractions of the isocratic solution and eluted fractions were separately subjected to cation-exchange MonoS® Fast Performance Liquid Chromatography (FPLC, Pharmacia) and proteins were further purified on an Alkyl-Superose® FPLC (Pharmacia) and stored at −80°C until utilization.

The purity of the different components of leukotoxins was determined by SDS/PAGE analysis and immunoblotting on PHAST® System (Pharmacia) with affinity-purified LukS-PV or LukF-PV rabbit polyclonal antibodies [11] according to Towbin, Staehelin & Gordon [31]. Characterization of the purified proteins was performed by determining their N-terminal peptide sequences.

Polymorphonuclear Neutrophils Isolation

Human polymorphonuclear neutrophils (PMNs) were prepared from buffy-coat of healthy donors of either sex, kindly provided by the Centre Régional de Transfusion Sanguine (Strasbourg, France). White cells-enriched blood (30 ml) diluted with 0.9% (w/v) NaCl (1/3) were layered on 12 ml JPREP and centrifuged for 20 mn at 800 × *g*. The pellet resuspended in 30 ml of 0.9% NaCl was added to 10 ml of 6% (w/v) dextran for sedimentation during 30 min. Contaminating erythrocytes were removed by 40-sec hypotonic lysis and subsequent washes in the assay solution containing (in mM): 140 NaCl, 5 KCl, 10 glucose, 10 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 0.1 ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, and 3 Tris-base (pH 7.3). The suspension was adjusted to 7.10^6 PMNs/ml. More than 98% PMNs were obtained, as counted after May-Grünwald-Giemsa staining.

Loading PMNs with Fluorescent Probes

Variations in intracellular free Ca^{2+}, Zn^{2+}, Mn^{2+}, Na^+ and K^+ were determined by recording the fluorescence variations of fluorescent dyes loaded in PMNs. Loading of PMNs was carried out with 2 μM Fura2-AM for 45 min or 2 μM Fluo3-AM with 0.1% Pluronic F-127 for 60 min at room temperature for Ca^{2+} measurements, and with 5 μM Na-green tetraacetate or PBFI-AM, for Na^+ and K^+ respectively, with 0.1% (w/v) Pluronic F-127 for 90 min in an atmosphere of 95% air/5% CO_2 at 37°C. After the incubation period, the cells were washed twice by 800 × *g* centrifugations for 10 min.

Fluorescence Determination

Variations in fluorescence intensities were recorded using a dual excitation and dual emission spectrofluorometer Deltascan (Kontron, PTI, Montigny-le-Bretonneux, France) with slit widths set on 4 nm which allowed simultaneous registration of, at least, 2 fluorescent probes. One ml of PMNs (7.10^6 cells/ml) was added to 1 ml of the assay solution under constant stirring in a 4-ml quartz cuvette (1-cm light path) thermostated at 37°C. The increase in fluorescence of ethidium cation added in bromide form at 100 μM was used as an indicator of aspecific pores formation as already described [10]. The fluorescence obtained by lysing the PMNs in the cuvette by 0.2% (w/v) Triton X-100 addition was considered as an activity of 100%. The fluorescence data from loaded probes were standardized as following: when arbitrary units (a. u.) were used, the saturation value (Fura2, Fluo3, Na-green) or the minimal fluorescence (PBFI) was obtained by Triton X-100 addition. For measurements of Fura2 fluorescence in the presence of Mn^{2+}, the data were expressed in % of Fura2 quench (0%: beginning of the registration; 100% : Triton X-100 addition). The S and F components were always injected at 2.2 nM and 0.85 nM respectively. All the data were plotted with the PTI software and then extracted for transfer to SigmaPlot 4.1 (Jandel, Erckrath, Germany) and graph significative response curves. Each experiment was performed at least four times, and the most representative studies are shown in figures.

Results

Comparison of Leukotoxins Effects

As shown in Fig. 1, HlgA, a S-component of γ-hemolysin was without effect on fluorescent probes (similar data were obtained with HlgC). In contrast, the addition of the F-component, HlgB, provoked significant modifications of fluorescence intensities. The increase in the fluorescence of ethidium was greater in the absence of Ca^{2+} (Fig. 1*B*) than in its presence (Fig. 1*A*). This difference is probably due to the protection of the membrane damage by divalent cations, a common feature of pore-forming agents [18, 20]. In the presence of

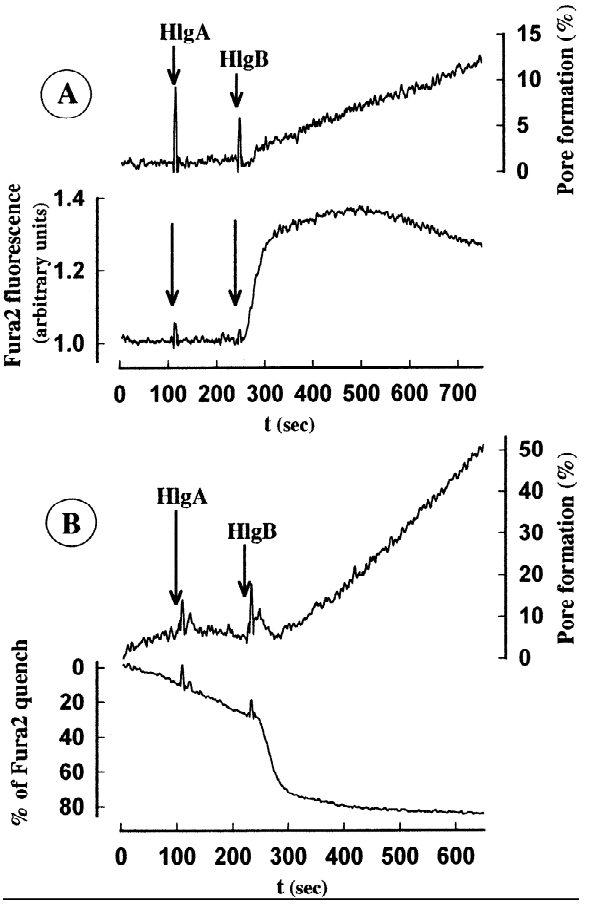

Fig. 1. Effect of the leukotoxin components HlgA and HlgB on the pore formation through the membrane of human PMNs and the influx of divalent cations. PMNs were loaded with Fura2 and the variations of fluorescence intensity were recorded, (A) in the presence of 1-mM free Ca^{2+} (λ_{EX} = 340 nm, λ_{EM} = 510 nm; Fura2 saturation: 1.5 a. u.) and, (B) in the presence of 100 μM Mn^{2+} (λ_{EX} = 360 nm, λ_{EM} = 510 nm). The pore formation was determined from the variations of fluorescence intensity of ethidium (100 μM, λ_{EX} = 340 nm, λ_{EM} = 600 nm) previously added in the cuvette. HlgA (2.2 nM) and HlgB (0.85 nM) were successively injected as indicated by the arrows.

Ca^{2+}, the fluorescence of Fura2 loaded in PMNs was increased after leukotoxin injection. In the absence of Ca^{2+}, it has been previously described that intracellular free Ca^{2+} was not increased by the LukS-PV/LukF-PV pair [10] and, thus, no Ca^{2+} was released from the calciosomes. However, the ability of the leukotoxins to form ion-sized pores in the absence of Ca^{2+} was determined by using Mn^{2+} as a surrogate for Ca^{2+} [21]. Indeed, the chelation of Mn^{2+} by Fura2 induced a decrease of its fluorescence which indicated the penetration of divalent cations in PMNs as shown in Fig. 1*B.* An influx of Mn^{2+} was also obtained in the presence of Ca^{2+} (*data not shown*), indicating that the increase in intracellular Ca^{2+} concentration was linked to a Ca^{2+} influx.

The activity of the three pairs of leukotoxin from *S.*

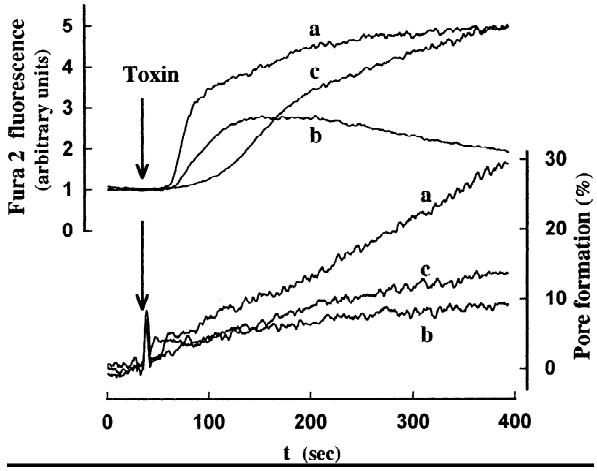

Fig. 2. Comparison of the effect of the three pairs of leukotoxins on pore formation and Ca^{2+} influx in the presence of 1-mM free Ca^{2+} in human PMNs. PMNs were loaded with Fura2 (λ_{EX} = 340 nm, 380 nm, λ_{EM} = 510 nm; Fura2 fluorescence is expressed by the ratio of the fluorescence intensities obtained at the two excitation wavelengths; saturation value : 5.5). The pore formation was determined as in Fig. 1. The two components of leukotoxins HlgA and HlgB (*a*), HlgC and HlgB (*b*), LukS-PV and LukF-PV (*c*) were simultaneously added as indicated by the arrows.

aureus HlgA/HlgB, HlgC/HlgB and LukS-PV/LukF-PV, was tested in the presence of 1 mM free Ca^{2+} as shown in Fig. 2. The two pairs HlgA/HlgB and HlgC/HlgB shared the same biological activity as was already demonstrated for the LukS-PV/LukF-PV pair [10], i.e., an increase in intracellular free Ca^{2+} concentration (ion-sized pore formation) and an influx of ethidium (aspecific pore formation). Nevertheless, some differences appeared in the duration of the time lags and in the amplitude of the effects between the three pairs, the HlgA/HlgB pair being the most potent. Variable differences between the time lags of the influxes of Ca^{2+} and ethidium were also observed depending on the donors. An example of the determination of the time lags presented by the PMNs from a particular donor is given by the Fig. 3. In this example, the influx of Mn^{2+} was initiated more than 100 sec before the influx of ethidium took place. Such a difference obtained with LukS-PV/LukF-PV in the absence of Ca^{2+} was also obtained, although with variable lengths of time, with the two other pairs of leukotoxins. The variability of the differences observed between the lag times implies that they originate from a biological rather than a technical cause. As a matter of fact, if only one sort of pore was formed by the toxin and if the time lags had for origin different thresholds of detection for ethidium and Fura2 or different speed of diffusion through the pore, they would be constant from one stock to another stock of PMNs.

In the following of this paper, only data from the HlgA/HlgB pair are presented although similar results

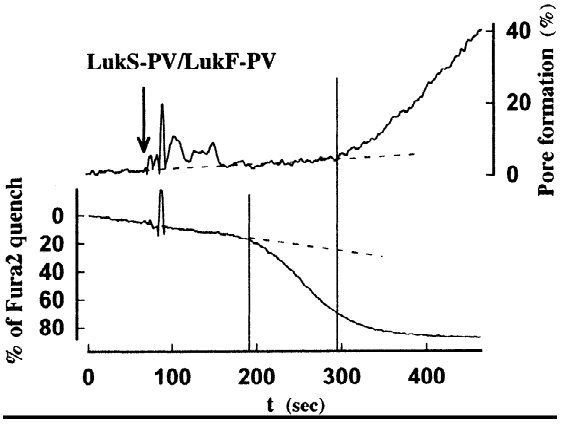

Fig. 3. Comparison of the time lags preceding the PVL-mediated influx of Mn^{2+} and pore formation in human PMNs showing the uncoupling of both events. PMNs were loaded and the fluorescence intensities determined as described in Fig. 1, B. PVL (LukS-PV/LukF-PV) was added as indicated by the arrow.

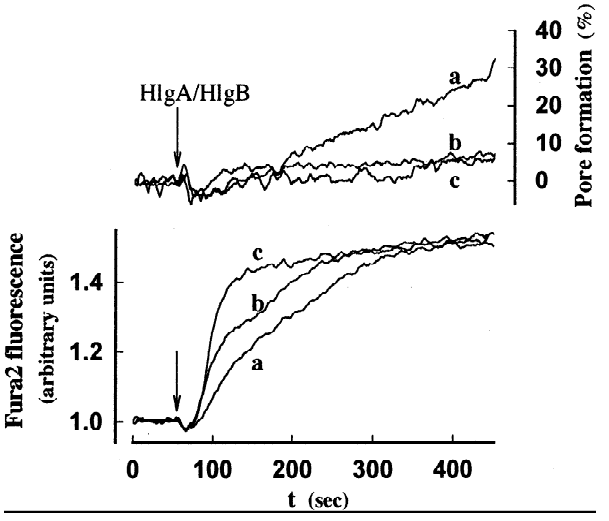

Fig. 4. Effect of different concentrations of divalent cations on the HlgA/HlgB-mediated pore formation and influx of divalent cations in human PMNs. PMNs were loaded and the fluorescence intensities determined as described in Fig. 1*A* (Fura2 saturation : 1.8 a. u.) in the presence of 0.2 mM Zn^{2+} and a : 0, b : 0.1, c : 1 mM Ca^{2+}. HlgA/HlgB was added as indicated by the arrow.

were obtained with the two other pairs LukS-PV/LukF-PV and HlgC/HlgB.

Cationic Specificity

It has been previously shown [10] that ion-sized pores formed by LukS-PV/LukF-PV were open to divalent cations Ca^{2+}, Mg^{2+}, Zn^{2+}, and Mn^{2+}. The ion-sized pores formed by the two other pairs induced the same specificity for divalent cations (*data not shown*). To determine the specificity of the ion-sized pores to monovalent cations Na^+ and K^+, conditions were determined where the ion-sized pores were open but the aspecific pores were closed. Thus, the influence of different concentrations of Ca^{2+} in the presence of 0.2 mM Zn^{2+} was tested on the influxes of divalent cations and ethidium provoked by the leukotoxins. As shown by Fig. 4*a*, in the sole presence of 0.2 mM Zn^{2+}, an increase of Fura2 and ethidium fluorescence was observed, indicating an influx of Zn^{2+} and ethidium. When 0.1 mM Ca^{2+} was added with Zn^{2+} the increase of Fura2 fluorescence was greater indicating an influx of Ca^{2+} but the influx of ethidium was completely inhibited (Fig. 4*b*). A further increase of Ca^{2+} concentration to 1 mM involved a higher increase of Fura2 fluorescence, indicating a higher increase of influx of Ca^{2+} and the ethidium fluorescence was still unchanged (Fig. 4*c*). This experiment clearly shows that the data obtained do not result from a competition between the three cations through a unique type of pores and that, in the presence of 0.1 mM Ca^{2+} and 0.2 mM Zn^{2+}, the aspecific pores were in a closed state although the ion-sized pores remained in a functional state. In the same conditions, the fluorescence of Na-green and PBFI loaded in PMNs were not modified after the application of the leukotoxin although they increased and decreased respectively in the absence of divalent cations (Fig. 5). These findings were verified by using PMNs loaded simultaneously with Na-green and Fura2 or with PBFI and Fluo3. Thus, the ion-sized pores do not conduct Na^+ and K^+.

Effect of Ca^{2+} Channel Blockers

The specificity of the ion-sized pores towards divalent cations prompted us to reconsider how they were formed. Were they formed by the insertion of the toxin itself or were they preexisting Ca^{2+} channels belonging to the plasma membrane activated by the leukotoxins? The question was answered using several well-known blockers of Ca^{2+} channels on PMNs loaded with Fluo3 or Fura2. They were incubated 120 min with 400 μM verapamil, 400 μM D600, a verapamil analogue, 400 μM nifedipine, a dihydropyridine inhibitor, or 400 μM adenosine, an inhibitor of flunarizine-sensitive Ca^{2+} channels [23] and 2 min with econazole or miconazole [2.5 μM] prior application of leukotoxins. In the presence of Ca^{2+} (Fig. 6), D-600 (as verapamil) or adenosine inhibited partially the increase of the intracellular Ca^{2+} concentration which was completely abolished in the presence of econazole (or miconazole). Nifedipine had no significant action on the effect of leukotoxins (*data not shown*). In the absence of Ca^{2+}, D-600 (as verapamil) and econazole (as miconazole) induced an inhibition of the Mn^{2+} influx (Fig. 7).

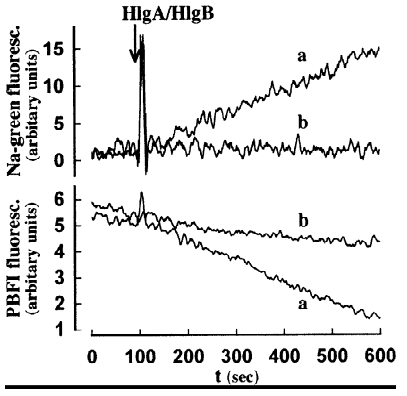

Fig. 5. Effect of divalent cations on the HlgA/HlgB-mediated influx of Na^+ and efflux of K^+ in human PMNs. PMNs were loaded with Na-green (λ_{EX} = 488 nm, λ_{EM} = 540 nm, Na-green saturation = 20 a. u.) and PBFI (λ_{EX} = 360 nm, λ_{EM} = 500 nm, minimal fluorescence of PBFI in the presence of Triton = 0 a. u.) and fluorescence variations were recorded in the absence (*a*) and in the presence (*b*) of 0.1 mM Ca^{2+} and 0.2 mM Zn^{2+} HlgA/HlgB was added as indicated by the arrow.

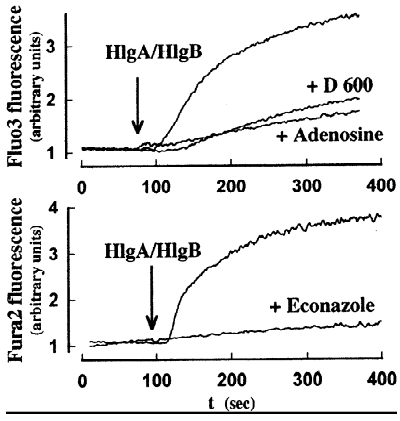

Fig. 6. Effect of Ca^{2+} channel blocking agents on the HlgA/HlgB-mediated Ca^{2+} increase in PMNs. PMNs were loaded with Fluo3 (λ_{EX} = 488 nm, λ_{EM} = 530 nm, Fluo3 saturation = 4.5 a. u.) or Fura2 (wavelengths settings as in Fig. 2) and the fluorescence variations recorded in the presence of 1 mM free Ca^{2+}. PMNs were incubated 2 h with 0.4 mM D-600 or 0.4 mM adenosine and 2 min with 2.5 μM econazole as indicated. HlgA/HlgB was added as indicated by the arrow.

In the presence (Fig. 8*A*) as in the absence of Ca^{2+} (Fig. 8*B*) the ethidium influx was neither inhibited by D-600 nor by adenosine nor by econazole or miconazole (*not shown*). Furthermore, it was verified that D-600, in the absence of Ca^{2+}, was also unable to inhibit Na^+ and K^+ fluxes (Fig. 9) as ethidium fluxes. Verapamil, D-600, nifedipine, econazole and miconazole were without toxicity when applied alone. However, the fluorescence variations recorded in the presence of econazole (or miconazole) were carried out for short periods of time because of their proper toxicity against PMNs particularly in the absence of Ca^{2+} after leukotoxin application.

At the lower concentration of 40 μM for which verapamil and D-600 are known to be specific voltage dependent Ca^{2+} channel blockers, neither inhibition of the increase of intracellular Ca^{2+} concentration, in the presence of Ca^{2+}, nor inhibition of the influx of Mn^{2+}, in the absence of Ca^{2+}, could be observed (*data not shown*). These observations and the absence of inhibition by nifedipine are consistent with the absence of voltage dependent Ca^{2+} channels in PMNs [1, 33].

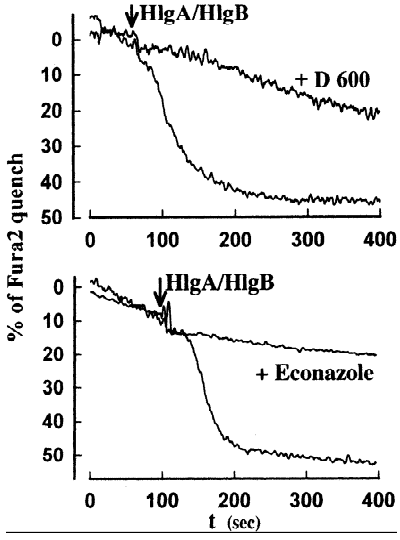

Fig. 7. Effect of Ca^{2+} channel blocking agents on the HlgA/HlgB-mediated Mn^{2+} influx in human PMNs in the absence of Ca^{2+}. PMNs were loaded with Fura2 and the fluorescence variations (λ_{EX} = 360 nm, λ_{EM} = 510 nm) were recorded in the presence of 100 μM Mn^{2+}. PMNs were incubated with D-600 or econazole as described in Fig. 6. HlgA/HlgB was added as indicated by the arrows.

Discussion

This study reports a common mechanism of action for the leukotoxins from *S. aureus* on the membrane of human PMNs, i.e., after the binding of both components, an increase of the intracellular free Ca^{2+} concentration followed by an influx of ethidium. A possible explanation of these two occurrences could be that the leukotoxins open pores for Ca^{2+} and ethidium comparable to those activated in lymphocytes by purinoceptors [2]. This proposal can be rejected since the Ca^{2+} influx does not appear simultaneously to the ethidium entry. Another argument for the formation of pores by the leukotoxin proteins is that the pair HlgA/HlgB forms pores in black lipid membrane by direct insertion of the two components in the lipid bilayer (G. Menestrina, *personal communication*). Moreover, this conclusion is reinforced by the structural homologies described between leukotoxins

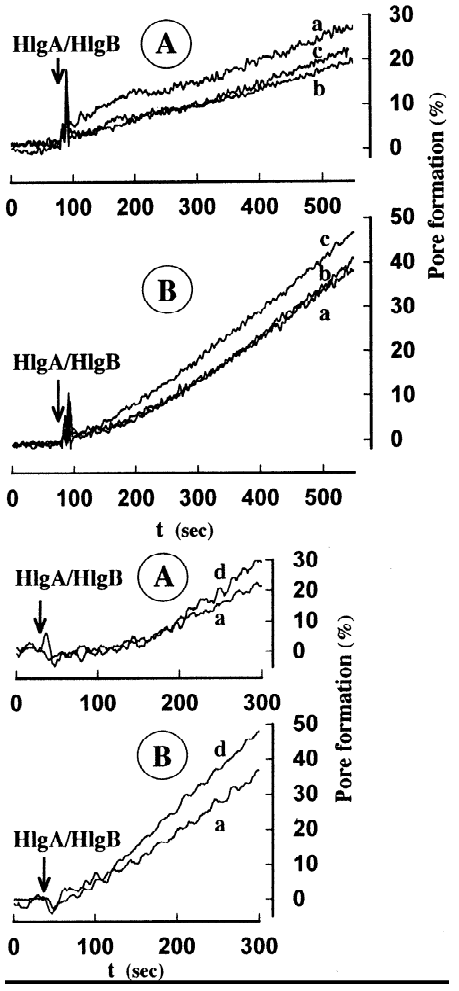

Fig. 8. Effect of Ca^{2+} channel blocking agents on the HlgA/HlgB-mediated pore formation in human PMNs. The variations of fluorescence of ethidium (100 μM, λ_{EX} = 340 nm, λ_{EM} = 600 nm) were recorded in the presence (*A*) and in the absence (*B*) of 1 mM Ca^{2+}. PMNs were incubated in the absence (*a*) or the presence of D600 (*b*), adenosine (*c*), or econazole (*d*) as described in Fig. 6. HlgA/HlgB was added as indicated by the arrows.

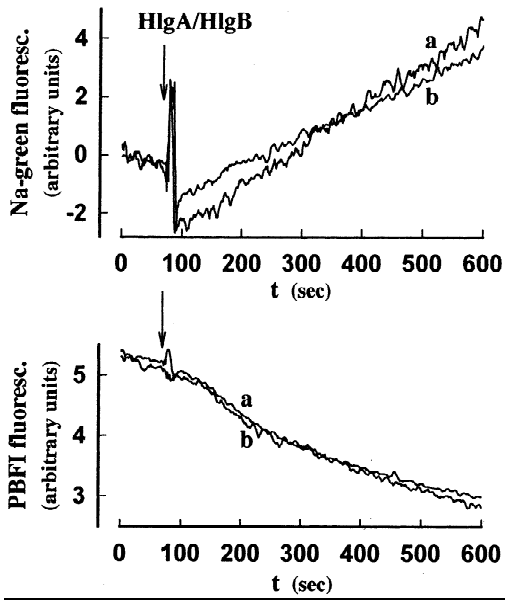

Fig. 9. Effect of D-600 on the HlgA/HlgB-mediated monovalent cations fluxes in human PMNs. PMNs were loaded with Na-green or PBFI as described in Fig. 5, in the absence (*a*) or the presence (*b*) of 0.4 mM D-600.

and α-toxin from *S. aureus* [16] which form pores through the plasma membrane by insertions as heptamers [29]. Previous experiments showed that the increase of intracellular Ca^{2+} concentration was generated by a Ca^{2+} influx through ion-sized pores [10]. The authors suggested that PVL formed aspecific pores through the membrane whose conformation could be modified by Ca^{2+} to ion-sized pores since, in the presence of Ca^{2+}, the ethidium influx and, consequently, the number of aspecific pores, was decreased. The results obtained in this study are not consistent with such a hypothesis: (i) ion-sized pores are open even in the absence of Ca^{2+}, (ii) the opening of the aspecific pores is delayed after ion-sized pores opening, (iii) ion-sized pores are not permeable to Na^+ and K^+, (iv) divalent cations influxes are inhibited by Ca^{2+} channel blockers in the presence as in the absence of Ca^{2+}, and, (v) the influx of ethidium and the fluxes of monovalent cations are not sensitive to the presence of the blockers. All these arguments, taken together, strongly suggest the involvement of a Ca^{2+} channel in the mechanism of action of the staphylococcal leukotoxins. The heat stable enterotoxin B from *Escherichia coli* was shown to open a receptor-operated Ca^{2+} channel in epithelial cells [9]. Further experiments are necessary to determine whether the binding site of leukotoxins is a channel itself or a receptor linked to a Ca^{2+} channel. This latter hypothesis, if verified, could imply that the GTP-binding proteins which have been involved in the activity of PVL [15] are linked to the receptor part of the Ca^{2+} channel-receptor complex activated by the leukotoxins.

These experiments also bring out a particular feature of the aspecific pores. First, because different types of Ca^{2+} channel blockers inhibit divalent cations influx but not ethidium influx, and second, because a low level of Ca^{2+} and Zn^{2+} inhibits the ethidium influx but enters in the PMNs, it can be concluded that the aspecific pores are not permeable to divalent cations. Other evidence of the partitioning of the leukotoxin activity between an intrinsic channel and an extrinsic pore was given by the observation that, in the Fura2-loaded PMNs of two particular donors, the influx of ethidium was the only ion flux observed after leukotoxin injection in the absence of Ca^{2+} despite the presence of Mn^{2+} (*unpublished results*).

The three leukotoxins present similar activities on

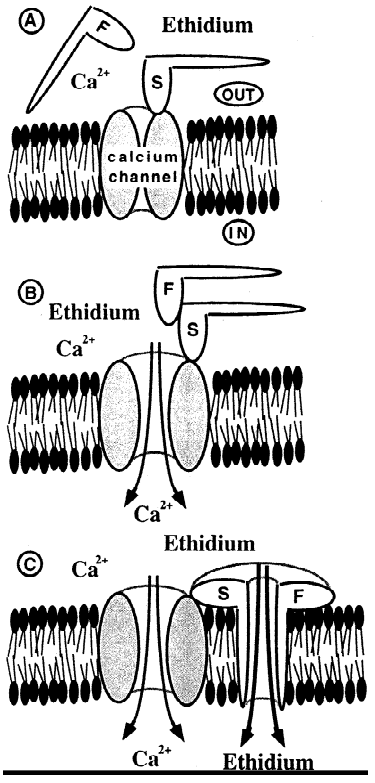

Fig. 10. Schematic representation of the activity of the leukotoxins in the membrane of PMNs. The forms of the S and F components are purely fictitious but inspired by the mushroom-shaped complex described for α-toxin (27). (*A*) binding of the S component to the Ca^{2+} channel; (*B*) binding of the F component to the S component-Ca^{2+} channel complex and opening of the channel; (*C*) insertion of the leukotoxin through the membrane and opening of the aspecific pore.

PMNs, although with different intensities. This could be an indication of the involvement of the same Ca^{2+} channel by the three leukotoxins or of the same receptor-Ca^{2+} channel complex. The differences in the toxic intensities could be, then, the result of different affinities of the leukotoxins for their binding site. Nevertheless, leukotoxins do not present the same scope of target cells since, in contrast to HlgA/HlgB, PVL is not hemolytic in man. Consequently, the S-components might possess different receptors belonging to the same family, or they could bind to the same receptor and the selectivity would be the consequence of different interrelationships between the F-components and the membrane environment. Determination of the binding of the different components is currently in progress to answer these questions.

It has been shown that human PMNs do not possess voltage-operated Ca^{2+} channels [1, 8, 33], but receptor-mediated and store-regulated Ca^{2+} channels [3, 7]. In the state of the experiments, it is not possible, in the presence of Ca^{2+}, to distinguish between these two possibilities. In the absence of Ca^{2+}, neither PVL [10] nor the two other leukotoxins induced a mobilization of the Ca^{2+} stores which indicates, at least in this condition, a direct action of leukotoxins on the channel complex instead of an indirect activation through the depletion of intracellular Ca^{2+} stores. The type of Ca^{2+} channel activated by leukotoxins remains to be determined, but, a nonselective Ca^{2+}-activated cation channel conducting Ca^{2+} [12] cannot be implicated since Na^+ and K^+ fluxes can be inhibited independently of divalent cations influxes. Conversely, a nonselective divalent cation (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}) channel is likely to be involved by leukotoxins.

It is proposed that the activity of the leukotoxins can be schemed as in Fig. 10. When the leukotoxins are in solution with the target cells, the S-component binds to a receptor [4] linked to a Ca^{2+} channel or to a Ca^{2+} channel itself (Fig. 10*A*). Then, the secondary binding of the F-component induces the opening of the Ca^{2+} channel involving a Ca^{2+} influx (Fig. 10*B*). The consecutive insertion of the leukotoxin components in the membrane forms a pore large enough to involve an ethidium influx (Fig. 10*C*).

The characterization of the receptors and the determination of the type of Ca^{2+} channel activated by the leukotoxins as well as the mechanisms involved in the opening of the Ca^{2+} channel by the different leukotoxins may present fundamental implications in the physiopathology of *S. aureus* infections.

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