

Antimicrobial and immunostimulatory peptide, KLK, induces an increase in cytosolic Ca^{2+} concentration by mobilizing Ca^{2+} from intracellular stores

Julian Weghuber^{1*}, Anna M. Lipp[†], Jacqueline Stadlbauer^{*}, Michael C. Aichinger[‡], Verena Ruprecht^{*}, Alois Sonnleitner[†], Gerhard J. Schütz^{*} and Tamás Henics[‡]

^{*} Biophysics Institute, Johannes Kepler University Linz, Altenbergerstr. 69, 4040 Linz, Austria

[†] Center for Advanced Bioanalysis, Scharitzerstraße 6–8, 4020 Linz, Austria

[‡] Department of Genetics, Max F. Perutz Laboratories, 1030 Vienna, Austria

Abstract

The cationic antimicrobial immunomodulatory peptide, KLK (KLKL₅KLK), exerts profound membrane interacting properties, impacting on ultrastructure and fluidity. KLK–membrane interactions that lead to these alterations require the ability of the peptide to move into an α -helical conformation. We show that KLK induces an increase of the intracellular Ca^{2+} concentration in human T24 cells. The effect of KLK is buffer-sensitive, as it is detected when HBSS buffer is used, but not with PBS. This, together with the lack of effect of the middle leucine-to-proline-substituted peptide derivative [KPK (KLKLLPLLKLK)], indicates that it is the conformational propensity rather than the net positive charge that contributes to the effect of KLK on intracellular Ca^{2+} level of T24 cells. We show that, although KLK slightly stimulates Ca^{2+} influx into the cell, the bulk increase of Ca^{2+} levels is due to KLK-induced depletion of intracellular Ca^{2+} stores. Finally, we demonstrate a KLK-induced switch of PS (phosphatidylserine) from the inner to the outer plasma membrane leaflet that contributes to the onset of early apoptotic changes in these cells.

Keywords: apoptosis; Ca^{2+} ; influx; KLK; store depletion

1. Introduction

KLK (KLKL₅KLK), the positively charged, amphipathic antimicrobial peptide has been studied in detail to uncover mechanisms about its interaction with membranes. We have demonstrated that, while KLK appears as a non-pore-forming peptide, it readily interacts with vesicle and cell membrane, profoundly altering their ultrastructure and fluidity (Aichinger et al., 2008). Depending on the composition of a membrane-mimicking environment, KLK can undergo multiple conformational transitions (Aichinger et al., 2008).

The second messenger Ca^{2+} influences a broad range of cellular and physiological processes. Changes in membrane fluidity and ultrastructure are often linked to perturbations of intracellular Ca^{2+} homeostasis. For example, membrane injury caused by silica nanoparticles in macrophages is accompanied by increases of reactive oxygen species and intracellular Ca^{2+} concentration (Yang et al., 2009). Furthermore, increasing evidence points to the involvement of Ca^{2+} (namely, exocytotic insertion of vesicular Ca^{2+} -conducting proteins into the plasma membrane) as a mechanism of coupling exocytotic activity to compensatory endocytosis, a fundamental process in maintaining cell surface area (Vogel, 2009; Usmani et al., 2010; Watanabe et al., 2010). Thus, we examined whether membrane-interacting effects of KLK were linked to changes in intracellular Ca^{2+} levels. We show that KLK induces an increase of the intracellular Ca^{2+} concentration in human T24 cells. This effect of KLK is buffer

sensitive, since it is detected when HBSS (Hanks buffered saline solution) is used but is absent in PBS. An immunologically inert variant of KLK with a middle leucine to proline mutation (KLKLLPLLKLK, KPK) (Aichinger et al., 2008) lacks this effect, indicating that the increase of intracellular Ca^{2+} levels depends on the conformation of the peptide rather than its net positive charge. KLK slightly stimulates Ca^{2+} influx, but it also leads to a depletion of intracellular Ca^{2+} stores. Finally, we show that KLK induces a switch of PS (phosphatidylserine) from the inner to the outer plasma membrane leaflet, a characteristic feature of early apoptotic stages in eukaryotic cells.

2. Materials and methods

2.1. Reagents

KLK and KPK were provided by Intercell AG. HBSS with/without Ca^{2+} and PBS with Ca^{2+} were from PAA Laboratories. BAPTA-AM was purchased from Invitrogen.

2.2. DNA constructs

pEGFP-C1-Lact-C2 [GFP (green fluorescent protein) fused with C2 domain of bovine lactadherin] was purchased from Haematologic Technologies Inc.).

¹ To whom correspondence should be addressed (email julian.weghuber@jku.at).

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Abbreviations: FBS, fetal bovine serum; KLK, KLKL₅KLK; KPK, KLKLLPLLKLK; GFP, green fluorescent protein; HBSS, Hanks buffered saline solution; Lact-C2-GFP, lactadherin fused to GFP; PS, phosphatidylserine.

2.3. Cell culture

Human T24 cells were from American Type Culture Collection. FBS (fetal bovine serum), media, antibiotics, trypsin and Geneticin (G418 sulfate) were purchased from PAA Laboratories GmbH. Culture plates were from Greiner Bio One International. A Gene Pulser electroporation unit (X-cell) and electroporation cuvettes were from Bio-Rad. T24 cells were cultured in RPMI medium supplemented with 10% FBS and grown at 37°C in a humidified incubator ($\geq 95\%$) with 5% CO₂ in air. For stable expression, 70% confluent cells were harvested and transfected with 10 μg of plasmid DNA using the X-cell electroporator with the following electroporation conditions: 240 V, 950 μF , unlimited resistance, 4 mm gap cuvettes and RPMI16 without FBS as the electroporation buffer. Cells were plated into 100 mm culture dishes and grown for 48 h. The medium was removed and replaced with fresh medium supplemented with 400 $\mu\text{g}/\text{ml}$ G418. Medium was changed every 3 days, and 15–20 days later, individual neomycin-resistant colonies were selected for propagation and analysis. Transfected cells were grown on 3-cm glass coverslips and analysed 48 h after seeding.

2.4. Ca²⁺ measurements

T24 cells were loaded with Indo1-AM (3 μM , Invitrogen) in complete RPMI-1640 medium at room temperature for 30 min and washed twice with Ca²⁺-containing HBSS buffer. This cell permeable ratiometric dye allows for monitoring changes in free cytoplasmic Ca²⁺ levels (Grynkiewicz et al., 1985). All fluorescence measurements were performed with a self-developed microscope device (CytoScout[®]) (Paar et al., 2007). As a light source, a mercury lamp (HBO100, Zeiss) was used, and fluorescence was excited and collected via a $\times 10$ Fluar objective (Zeiss). All bandpass filters and dichroic mirrors were from AHF Analysentechnik. Cells were illuminated at 333 nm (333/30), and fluorescence emission at 405 nm (405/20) and 485 nm (485/25) was detected simultaneously by 2 CoolSnap HQ[™] CCD cameras (Photometrics). First, cells were acquired for 20 s without stimulation to monitor baseline activity, then KLK, or KPK, in their respective buffers, were added, and cells were acquired for another 3–4 min. Image processing and analysis was performed by ImageJ and MATLAB[®] (MathWorks[™], Inc.).

2.5. Annexin V staining

Human T24 cells were grown on 3-cm glass coverslips to 70% confluency and incubated with 10 nmol/ml KLK for 15 or 30 min, respectively, followed by fixation with 4% paraformaldehyde for 30 min at room temperature. After washing with HBSS buffer, Annexin V-Cy5 staining was performed following the manufacturers instructions (Apoptosis Detection Kit, BioVision).

2.6. Confocal microscopy

LSM images were taken by means of a LSM 510 Meta confocal laser scanning microscope using a $\times 40$ 1.2NA water immersion objective (Zeiss).

3. Results

3.1. KLK but not KPK increases intracellular Ca²⁺ concentration

To study the impact of KLK on the intracellular Ca²⁺ concentration, we used the fluorescent Ca²⁺ indicator Indo1-AM. Human T24 cells were loaded with 3 μM Indo1-AM, and the ratio of fluorescence detected at 405 and 485 nm was determined for all cells in a selected area at given time-points (Figure 1A). Measurements were done in HBSS buffer containing Ca²⁺. After 20 s, cells were treated with 100 nmol/ml KLK, and Ca²⁺ levels were subsequently monitored for 4 min. A remarkable increase in intracellular Ca²⁺ concentration occurred 20 s after KLK addition (Figure 1B). The increase reached a plateau within 60 s and remained constant for 4 min (Figure 1B). When the middle leucine-to-proline-substituted and immunologically inert derivate of KLK, termed KPK (Aichinger et al., 2008), was tested in the same system, no increase in the intracellular Ca²⁺ concentration occurred (Figure 1C). Moreover, the presence of KPK in the incubation buffer did not prevent the effect of KLK on the Ca²⁺ level induced by subsequent administration of KLK to the cells (Figure 1C).

3.2. KLK-mediated changes of intracellular Ca²⁺ concentration are dependent on buffer conditions

To obtain more clues about the effects of KLK, the intracellular Ca²⁺ concentration of T24 cells in Ca²⁺-containing PBS buffer in the presence of 100 nmol/ml KLK was monitored. Interestingly, in contrast to our results obtained in HBSS including Ca²⁺, no

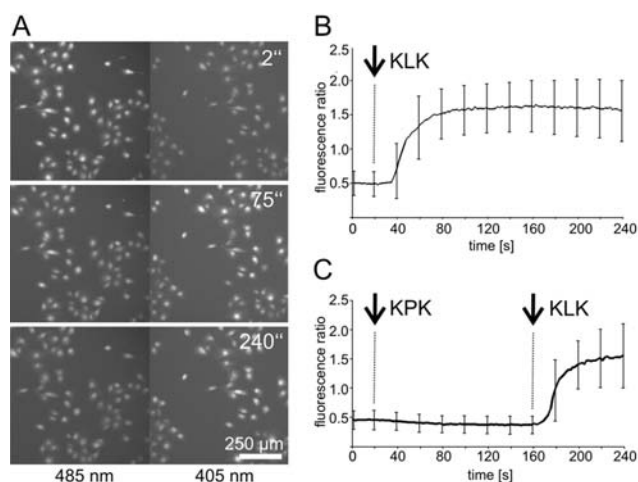


Figure 1 KLK increases intracellular Ca²⁺ concentration in human T24 cells (A) Emission at 485 nm (left) and 405 nm (right) of Indo1-AM-labelled T24 cells. Representative images taken after 2, 75 and 240 s, respectively (top, middle and lower pictures, respectively). (B) Ca²⁺ influx measurements of T24 cells showing elevation of intracellular Ca²⁺ levels upon addition of 100 nmol/ml KLK after 20 s (average of 125 analysed cells; for better illustration, error bars are only shown for every 20th data point). (C) Same settings as in (B), but incubation with 100 nmol/ml KPK after 20 s and additionally 100 nmol/ml KLK after 160 s (average of 70 analysed cells). Measurements in (B) and (C) were performed in HBSS buffer with Ca²⁺.

effect of KLK on intracellular Ca^{2+} levels was observed under these conditions (Figure 2A). Since it was the α -helical conformation that could not be induced in sodium phosphate buffer in our previous measurements (Aichinger et al., 2008), it could be assumed that the presence of a high phosphate concentration prevented the correct folding of KLK into its α -helical form. This conformational hindrance might inhibit the interaction of KLK with the T24 cell membrane that is necessary for the peptide to induce ultrastructural and/or fluidity changes responsible for an increase in cytoplasmic Ca^{2+} levels.

3.3. KLK induces depletion of intracellular Ca^{2+} stores

Intracellular Ca^{2+} concentration was therefore measured after incubation of T24 cells with 100 nmol/ml KLK in nominally Ca^{2+} -free HBSS buffer. The Ca^{2+} level was also increased under these conditions (Figure 2B), but with a lower magnitude compared with measurements performed in HBSS buffer containing Ca^{2+} .

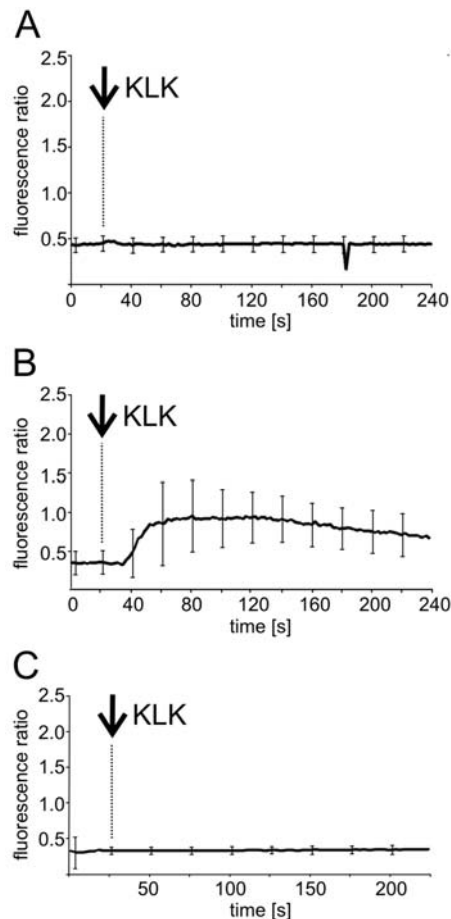


Figure 2 KLK induces depletion of intracellular Ca^{2+} stores

(A) Ca^{2+} influx measurements of T24 cells labelled with Indo1-AM in PBS buffer containing Ca^{2+} (average of 42 analysed cells). Addition of 100 nmol KLK after 20 s. (B) Same measurements in HBSS buffer without Ca^{2+} (average of 67 analysed cells). (C) Ca^{2+} influx measurements of Indo1-AM-loaded T24 cells incubated with the membrane-permeable Ca^{2+} chelator BAPTA-AM (10 μM). Addition of 100 nmol/ml KLK after 20 s; average of 48 analysed cells.

Additionally, the Ca^{2+} concentration was slightly decreased within 4 min, a phenomenon not seen when extracellular Ca^{2+} was present (Figure 2B). We conclude that KLK induces a fast depletion of intracellular Ca^{2+} stores and also promotes the influx of Ca^{2+} from the extracellular buffer. To check this assumption, we used the membrane-permeable Ca^{2+} chelator, BAPTA-AM [1, 2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester)]. T24 cells loaded with 3 μM Indo1-AM were incubated with 10 μM BAPTA-AM in Ca^{2+} -free HBSS buffer for 20 min at 37°C in the dark. Addition of KLK did not result in an increase in the intracellular Ca^{2+} concentration (Figure 2C). These experiments clearly show that KLK addition leads to a fast elevation of the cytoplasmic Ca^{2+} level by depleting Ca^{2+} stores, probably in the endoplasmic reticulum. This depletion may induce further entry of Ca^{2+} from extracellular sources via, for example, activation of SOCs (store-operated channels) (Csutura et al., 2006; Salido et al., 2009).

3.4. KLK mediates a switch of PS from the inner to the outer plasma membrane leaflet

Increased intracellular Ca^{2+} concentration can result in early apoptotic signalling (Mattson and Chan, 2003). The switch of PS from the inner to the outer plasma membrane leaflet is a characteristic event of cells undergoing the early stages of apoptosis (Li et al., 2003). Therefore, to investigate this possibility in KLK-treated T24 cells, two different methods were applied to follow the interleaflet switch of PS. First, fluorescently labelled Annexin V, which binds PS on the outer leaflet, was used. No fluorescent signal was observed in cells exposed to KLK for 15 min and subsequently stained with Annexin V (Figure 3A), but staining of some cells occurred 30 min post-KLK treatment (Figure 3B). Secondly, we used another fluorescent probe, which targets PS. The C2 domain of Lact-C2-GFP (lactadherin fused to GFP) specifically binds PS in the inner plasma membrane leaflet (Yeung et al., 2008). This probe in T24 cells was stably expressed (Figure 3C) and monitored a potential effect of KLK on its localization. Addition of 100 nmol/ml KLK resulted in a remarkable loss of the plasma membrane localization of Lact-C2-GFP and an increased staining of intracellular organelles by the probe within 30 min (Figure 3D). These data indicate that a PS switch occurs upon KLK treatment, and cells undergo early stages of apoptosis within 30 min of peptide addition.

4. Discussion

Variations of intracellular Ca^{2+} concentrations are essential for numerous cellular processes transducing extracellular signals into living cells (Clapham, 1995). Once it enters the cytoplasm, Ca^{2+} exerts regulatory effects on different enzymes and proteins, playing a pivotal role in a wide range of cellular processes (Uhlen and Fritz, 2010).

We have described an easily detectable induction of intracellular Ca^{2+} levels by the cationic antimicrobial peptide, KLK, when human T24 cells are exposed to this peptide. This effect does not solely depend on the positive charge of the peptide and requires

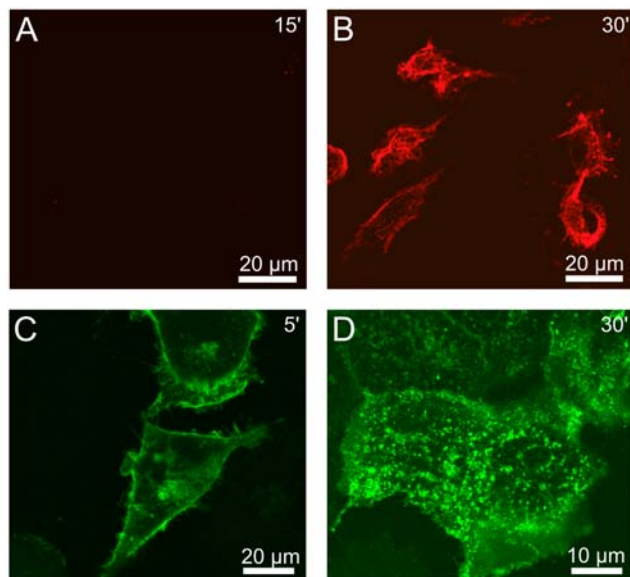


Figure 3 KLK induces a switch of PS from the inner to the outer plasma membrane leaflet

(A) and (B) Confocal images of T24 cells treated with 100 nmol/ml KLK for 15 (A) or 30 (B) min. Subsequently, cells were fixed in paraformaldehyde and stained with Annexin V-Cy5. (C) and (D) Confocal images of T24 cells expressing the PS-binding Lact-C2-GFP probe incubated with 100 nmol KLK for 5 (C) or 30 (D) min. Cells were fixed in paraformaldehyde.

the α -helical conformation of KLK, no similar effect being detected by KPK and no KLK effect observed in PBS (Aichinger et al., 2008). KPK completely loses its propensity to aggregate into intermolecular β -sheeted structures as well as into α -helical structures upon appropriate environmental conditions (Aichinger et al., 2008). Circular dichroism spectra in water showed random coil conformation for both KLK and KPK. In Na-phosphate buffer, KLK, but not KPK, aggregated into β -sheeted complexes, while the lipid-mimicking environment rendered KLK into α -helical structure, a conformational transition, which was not observed with KPK (Aichinger et al., 2008). Because KLK and KPK carry the same net positive charge, we assume that the observed effect of KLK on the intracellular Ca²⁺ levels requires the onset of conformational transitions of the peptide. Although, KLK weakly promotes Ca²⁺ influx into T24 cells, its effect of increasing cytoplasmic Ca²⁺ levels is linked to depletion of intracellular Ca²⁺ stores, most likely the ER (endoplasmic reticulum). Finally, this phenomenon could be linked to early apoptotic signalling in T24 cells, demonstrated by a PS switch between the plasma membrane leaflets, a known sign of early apoptotic activity.

The mechanism(s) by which KLK elicits an increase of cytoplasmic Ca²⁺ concentration remain(s) elusive. We speculate that electrostatic interaction of KLK with certain surface receptors may contribute to the activation of Ca²⁺ signalling via different pathways, e.g. the phospholipase C-dependent pathway, which leads to the release of Ca²⁺ from the endoplasmic reticulum (Berridge, 2009).

Author contribution

Julian Weghuber designed and performed experiments and wrote the manuscript. Anna Lipp and Jacqueline Stadlbauer performed the Ca influx measurements. Michael Aichinger designed the experiments. Verena Ruprecht assisted in the digital data processing and evaluation. Alois Sonnleitner assisted in the Ca influx measurements. Gerhard Schütz designed the experiments. Tamás Henics wrote the manuscript.

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References

- Aichinger MC, Ortbauer M, Reipert S, Zauner W, Bogner P, Froschauer E, Nowikovsky K, Lingnau K, Von GA, Schweyen R, Henics T. Unique membrane-interacting properties of the immunostimulatory cationic peptide KLKL(5)KLK (KLK). *Cell Biol Int* 2008;32:1449–58.
- Berridge MJ. Inositol trisphosphate and calcium signalling mechanisms. *Biochim Biophys Acta* 2009;1793:933–40.
- Clapham DE. Calcium signaling. *Cell* 1995;80:259–68.
- Csutora P, Zarayskiy V, Peter K, Monje F, Smani T, Zakharov SI, Litvinov D, Bolotina VM. Activation mechanism for CRAC current and store-operated Ca²⁺ entry: calcium influx factor and Ca²⁺-independent phospholipase A2beta-mediated pathway. *J Biol Chem* 2006;281:34926–35.
- Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* 1985;260:3440–50.
- Li MO, Sarkisian MR, Mehal WZ, Rakic P, Flavell RA. Phosphatidylserine receptor is required for clearance of apoptotic cells. *Science* 2003;302:1560–3.
- Mattson MP, Chan SL. Calcium orchestrates apoptosis. *Nat Cell Biol* 2003;5:1041–3.
- Paar C, Wurm S, Pfarr W, Sonnleitner A, Wechselberger C. Prion protein resides in membrane microclusters of the immunological synapse during lymphocyte activation. *Eur J Cell Biol* 2007;86:253–64.
- Salido GM, Sage SO, Rosado JA. Biochemical and functional properties of the store-operated Ca²⁺ channels. *Cell Signal* 2009;21:457–61.
- Uhlen P, Fritz N. Biochemistry of calcium oscillations. *Biochem Biophys Res Commun* 2010;396:28–32.
- Usmani SM, Fois G, Albrecht S, Von As, Dietl P, Wittekindt OH. 2-APB and capsaizepine-induced Ca²⁺ influx stimulates clathrin-dependent endocytosis in alveolar epithelial cells. *Cell Physiol Biochem* 2010;25:91–102.
- Vogel SS. Channeling calcium: a shared mechanism for exocytosis–endocytosis coupling. *Sci Signal* 2009;2:e80.
- Watanabe H, Yamashita T, Saitoh N, Kiyonaka S, Iwamatsu A, Campbell KP, Mori Y, Takahashi T. Involvement of Ca²⁺ channel synprint site in synaptic vesicle endocytosis. *J Neurosci* 2010;30:655–60.
- Yang H, Wu Q, Tang M, Kong L, Lu Z. Cell membrane injury induced by silica nanoparticles in mouse macrophage. *J Biomed Nanotechnol* 2009;5:528–535.
- Yeung T, Gilbert GE, Shi J, Silvius J, Kapus A, Grinstein S. Membrane phosphatidylserine regulates surface charge and protein localization. *Science* 2008;319:210–3.

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