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# Indolicidin action on membrane permeability: Carrier mechanism versus pore formation

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ABSTRACT

Indolicidin, a 13-residue cationic peptide with extremely high tryptophan content, exhibits broad-spectrum antimicrobial as well as hemolytic activity. To gain insight into the mechanism of indolicidin action on membrane permeability, liposome leakage induced by this peptide was studied by using various probes with vesicles of different lipid compositions. In liposomes containing negatively charged lipids, indolicidin induced rather unselective permeabilization. By contrast, the peptide appeared to be selective in provoking leakage of neutral, egg phosphatidylcholine (PC) liposomes: it effectively induced the release of negatively charged fluorescent dyes, carboxyfluorescein (CF), calcein and sulforhodamine B, but was unable to induce the leakage of a neutral compound, glucose, and that of positively charged doxorubicin. Moreover, organic anions, such as fatty acids, were found to suppress the indolicidin-induced CF leakage of egg PC liposomes. Based on these results, we concluded that indolicidin facilitates the dye release from uncharged lipid vesicles not by formation of membrane pores as it is generally accepted for the majority of antimicrobial peptides but rather via translocation of dye molecules across the membrane in the form of dye–peptide complexes, i.e. indolicidin operates as an organic anion carrier. This conclusion was supported by observing the formation of complexes between indolicidin and pyrenebutyrate in solution. The indolicidin analog having only one arginine was ineffective in pyrenebutyrate binding and CF transport. The mode of action proposed here for indolicidin can be related to that previously postulated for oligoarginine derivatives which are able to carry organic anions across liposomal and bulk phase membranes [Sakai N. & Matile S. J. Am. Chem. Soc. 2003, 125:14348–14356]. The newly identified mechanism of peptide ionophoric activity in uncharged lipid membranes may be involved in hemolytic action of indolicidin via induction of plasma membrane permeability for important anionic metabolites which disturbs regulation of osmotic balance ultimately leading to erythrocyte membrane rupture.



## 1. Introduction

Interaction of cationic peptides with lipid membranes usually perturbs membrane permeability barrier which may lead to membrane disruption [1]. This mode of interaction is inherent to many antimicrobial peptides which exert their action through permeabilization of cellular membranes [2–4]. Depending on the type of peptide and membrane lipid composition, the mechanism of aqueous pore formation by these peptides corresponds to toroidal pore [5,6], sinking raft model [7], barrel-stave pore [8,9], carpet model [10], electroporation [11,12], formation of reversed micelles [13], and induced lateral phase separation of membrane lipids [14].

On the other hand, arginine-containing peptides and peptide mimics were shown to exhibit qualitatively different mechanisms of membrane permeabilization by forming complexes with hydrophilic anions and transporting them across membranes [15,16]. These findings are closely related to observations of translocation of several polycationic peptides across artificial lipid bilayers [5,17,18] and eukaryotic cell membranes [19] that facilitates transport of proteins and other cargo molecules [20,21]. The ability of polycationic peptides to permeate across membranes was confirmed by NMR data on the interaction of arginine residues with phosphate groups of phospholipids located at the opposite side of the membrane [22].

The present study deals with the mechanism of lipid membrane permeabilization by indolicidin. This short peptide (Ile-Leu-Pro-Trp-Lys-Trp-Pro-Trp-Trp-Pro-Trp-Arg-Arg-$NH_2$) was isolated from cytoplasmic granules of bovine neutrophils [23]. It is active against both gram-positive and gram-negative bacteria [24], and also against fungi [25,26], and HIV-1 [27], but unfortunately also displays hemolytic activity [25].

Mechanisms of both antimicrobial and hemolytic action of indolicidin remain poorly understood. Some investigations have pointed to

*Abbreviations:* BLM, bilayer lipid membrane; CF, carboxyfluorescein; SRB, sulforhodamine B; DPhPC, diphytanoylphosphatidylcholine; DPhPG, diphytanoylphosphatidylglycerol; egg PC, egg yolk phosphatidylcholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); HK, Hexokinase; G6PDH, Glucose-6-phosphate dehydrogenase

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cellular membrane permeabilization by indolicidin as a predominant mode of its membrane activity [28–31]. Falla et al. [28] detected indolicidin-induced pores in experiments on planar bilayer lipid membranes at relatively high voltages (>70 mV).

Studies on liposomes have revealed indolicidin-induced efflux of liposome-entrapped fluorescent probes: carboxyfluorescein [32], calcein [17,33–37] and ANTS/DPX [29,38]. In the present work, indolicidin-induced liposomal release of different markers was examined in order to elucidate the mechanism of the leakage process. For egg PC vesicles, our data favored the carrier-type mechanism of the efflux rather than formation of aqueous pores. We surmise that by inducing efflux of essential anionic metabolites, the carrier-type mechanism may be involved in hemolytic action of indolicidin.

## 2. Materials and methods

The following peptides were studied in the present work:

Indolicidin, Ile-Leu-Pro-Trp-**Lys**-Trp-Pro-Trp-Trp-Pro-Trp-**Arg**-**Arg-**$NH_2$
Ind7, Ile-**Lys**-Pro-Trp-**Lys**-Trp-Pro-Trp-**Lys**-Pro-Trp-Ala-**Arg-**$NH_2$
Ind8, Ile-**Lys**-Pro-Trp-**Lys**-Trp-Pro-Trp-**Lys**-Pro-Trp-**Arg-Arg-**$NH_2$.

The synthesis of the peptides was described in details in [39]. Briefly, they were prepared by solid-phase Nα-Boc methodology on the 4-methylbenzhydrylamine resin using the 1-hydroxybenzotriazole esters and neutralization *in situ*. HPLC-purification of the samples gave pure peptides (purity >95%). The fidelity of the peptides was confirmed by MALDI-TOF MS. The expected/measured molecular masses (Da) were: 1906.33/1908.31 (Indolicidin), 1805.27/1806.76 (Ind7), and 1863.31/1864.06 (Ind8).

Egg yolk phosphatidylcholine (egg PC), diphytanoylphosphatidylcholine (DPhPC), diphytanoylphosphatidylglycerol (DPhPG), 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (DOPG) were from Avanti Polar Lipids (Alabaster, AL, USA). 9,10-Dibromo-palmitoylphosphatidylcholine (9,10-BrPC) was a generous gift from Stanislav Zakharov (Purdue University). Other chemicals including 5,6-carboxyfluorescein (CF), calcein, sulforhodamine B (SRB) and doxorubicin were from Sigma.

Liposomes loaded with CF were prepared by extrusion through 100-nm filter (Avanti Mini-Extruder) from appropriate phosphatidylcholine in a solution containing 100 mM CF titrated with Tris–base. A mixture of egg PC and cholesterol (63:37 mol%) or a mixture of egg PC, DOPG and cholesterol (45:18:37 mol%) was used in some experiments. The unloaded CF was then removed by passage through a Sephadex G-50 coarse column using eluting buffer (10 mM Tris, 100 mM KCl, pH = 7.4, unless otherwise indicated in legends to figures). Calcein-loaded liposomes and SRB-loaded liposomes were prepared in solutions containing 100 mM calcein or 50 mM SRB, respectively, and passed through the column. Doxorubicin-loaded liposomes from egg PC were prepared in solution containing 23 mM doxorubicin and passed through the column. To load negatively charged liposomes with doxorubicin, we used an alternative method via preparation of liposomes having pH gradient (pH 3 inside and pH 7 outside) and their subsequent incubation with 0.1 mM doxorubicin [40].

To measure dye efflux, liposomes were diluted to the final concentration indicated in figure legends and the dye fluorescence was monitored with a Panorama Fluorat 02 spectrofluorimeter (Lumex, Russia). Excitation and emission wavelengths were 490 and 520 nm for CF, 493 and 530 nm for calcein, 560 and 590 nm for SRB, and 490 and 590 nm for doxorubicin, respectively. The extent of the dye leakage was calculated as follows: $Y(\%) = 100\ (F_t - F_0)/(F_{100} - F_0)$, where $F_0$ and $F_t$ represent the initial and the final (steady-state) levels of fluorescence before and after the peptide addition, respectively, and $F_{100}$ is the fluorescence level after complete disruption of liposomes by the addition of the detergent, Triton-X100 (0.1%).

Liposomes loaded with glucose were prepared according to Haxby [41] from a mixture of egg PC and cholesterol (63:37 mol%) or a mixture of egg PC, DOPG and cholesterol (45:18:37 mol%) to prevent intrinsic glucose leakage. Dried lipid mixture was hydrated in a solution containing 300 mM glucose and the mixture was vortexed, passed through a cycle of freezing and thawing, extruded through 0.1-μm pore size polycarbonate membranes, and passed through the column using 100 mM Tris, 130 mM NaCl, 3.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 2 mM ATP, 1 mM NADP, pH = 8.5 as an eluting buffer. Measurements were carried out in the same buffer in the presence of 10 μg/ml Hexokinase (HK) and 100 μg/ml Glucose-6-phosphate dehydrogenase (G6PDH) at t = 37 °C. The appearance of glucose was recorded via an increase in NADPH fluorescence at 460 nm (excitation at 345 nm), according to coupled reactions:

$$\text{Glucose} + \text{ATP} \rightarrow \text{Glucose-6-phosphate} + \text{ADP}\ (\text{HK})$$
$$\text{G6P} + \text{NADP} \rightarrow \text{6-phosphogluconate} + \text{NADPH}\ (\text{G6PDH}).$$

### 2.1. Indolicidin binding assay

Binding of indolicidin to membranes was monitored using liposomes containing brominated lipids known to quench tryptophan fluorescence [42]. Liposomes with high 9,10-dibromo-palmitoylphosphatidylcholine (9,10-BrPC) content were made from a 2% solution of 9,10-BrPC/egg PC (50:50 mol%) in chloroform. The buffer solution used for preparation of the brominated liposomes contained 10 mM MES, 10 mM Tris, 10 mM β-alanine, 0.1 M KCl, pH 7.0. The same buffer was used in experiments on tryptophan fluorescence quenching by the brominated liposomes. Tryptophan fluorescence was excited at 280 nm.

### 2.2. Bilayer lipid membrane measurements

Bilayer lipid membranes (BLMs) were formed from a 2% solution of DPhPC or a mixture of DPhPC/DPhPG (80%/20%) in *n*-decane on a hole in a Teflon partition separating two compartments of a chamber containing aqueous buffer solutions. A chamber with 0.55-mm diameter hole was used in the experiments. Indolicidin was added from an aqueous stock solution to the *cis* compartment (the *trans* compartment served as virtual ground) under constant stirring conditions. The electric currents (I) were recorded under voltage-clamp conditions. Voltage was applied to BLMs with Ag-AgCl electrodes placed directly into the experimental chamber. The current measured by means of a patch-clamp amplifier (OES-2, OPUS, Moscow, Russia) was digitized using an NI-DAQmx (National Instruments, Austin, TX) and analyzed with a personal computer, using WinWCP Strathclyde Electrophysiology Software designed by J. Dempster (University of Strathclyde, UK).

All experiments were performed at room temperature (23 °C) in the buffer solution containing 10 mM Tris, 100 mM KCl, pH = 7.4, unless otherwise indicated in figure legends.

## 3. Results

The addition of indolicidin to CF-loaded liposomes formed of egg PC and cholesterol (63:37 mol%) resulted in rapid efflux of the dye as evidenced by an increase in CF fluorescence (Fig. 1A, curve 1). The addition of membrane-disrupting agent Triton-X100 led to only 20% additional increase in CF fluorescence. In contrast to CF efflux, indolicidin induced neither glucose (Fig. 1A, curve 2) nor doxorubicin (Fig. 1A, curve 3) efflux from liposomes of the same composition. Quite different patterns of the liposomal release (Fig. 1B) were observed with vesicles formed of egg PC, DOPG and cholesterol (45:18:37 mol%): in the presence of negatively charged lipids, indolicidin provoked both glucose (curve 2) and doxorubicin (curve 3) efflux comparable to that of CF (curve 1). These results support the idea of different mechanisms of indolicidin interaction with neutral

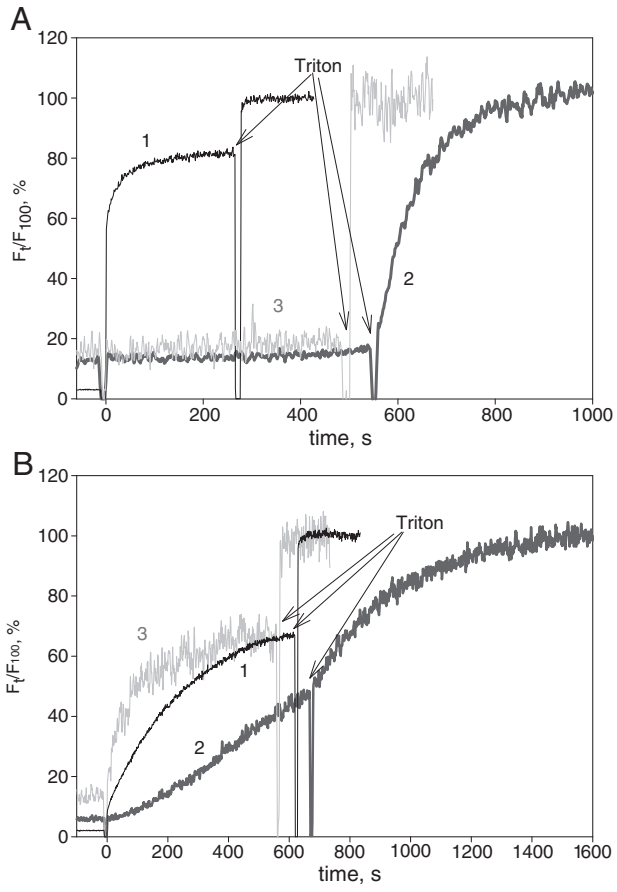


**Fig. 1.** Time courses of $F_t/F_{100}$ after the addition of indolicidin (1 μM, curve 1 or 10 μM, curve 2, 3 μM curve 3) at t = 0 and subsequent addition of Triton-X100 (0.1%) reflecting efflux of CF (curve 1), glucose (curve 2) and doxorubicin (curve 3) from liposomes. A. Liposomes were from egg PC and cholesterol (63:37 mol%), final lipid concentration was 7 μM (curve 1), 70 μM (curve 2) or 21 μM (curve 3). In curve 1 the buffer was 10 mM Tris, 100 mM KCl, pH = 8.5. In curve 2 the buffer was 100 mM Tris, 130 mM NaCl, 3.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, pH = 8.5. In curve 3 the buffer was 10 mM Tris, 100 mM KCl pH = 7.4. B. Liposomes were from egg PC, DOPG and cholesterol (45:18:37 mol%), final lipid concentration was 7 μM (curve 1), 70 μM (curve 2) or 21 μM (curve 3). In curves 1 and 2 the buffer was 100 mM Tris, 130 mM NaCl, 3.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, pH = 8.5. In curve 3 the buffer was 10 mM Hepes, 0.45 M sucrose pH = 7.0.

and negatively charged membranes [29,38,43]. Moreover, the failure of indolicidin to induce glucose leakage from egg PC liposomes is incompatible with aqueous pore formation under these conditions, as the molecular radius of glucose is smaller than that of CF. Noteworthy, the indolicidin-induced release of CF from DOPG-containing liposomes was substantially lower than that from neutral liposomes, in line with the recent data on oligoarginine-induced liposomal calcein release [44] and earlier data on CF release induced by guanidinium-containing synthetic polymers [16].

To further test the dependence of leakage activity of indolicidin on the charge of penetrants, we measured dye efflux from egg PC liposomes with another negatively charged fluorescent marker. As seen from Fig. 2A, indolicidin also induced efflux of anionic SRB (curve 2) and calcein (curve 3) even with more potency than that of CF (curve 1). Thus, the liposome leakage elicited by indolicidin proved to be anion-selective.

To probe the possibility of competition between CF and other organic anions for indolicidin as a carrier, we studied their influence on the CF efflux from egg PC liposomes. The experiments were carried out at non-saturating concentrations of indolicidin (0.1 μM) which led to about 30% efflux during 300 s of recording (Fig. 3A, curve 1). 10 μM palmitate blocked the CF efflux (Fig. 3A, curve 2), while 1 μM was substantially less effective (data not shown). We tested also the effect of oleate, dodecylsulfate, and pyrenebutyrate (Fig. 3A, curves 3, 4, and 5, respectively). All the anions studied decreased the rate of the CF efflux, although to different extents, pyrenebutyrate being the less effective. Remarkably, increasing concentration of pyrenebutyrate up to 50 μM led to marked stimulation of the indolicidin-induced CF efflux (Fig. 3A, curve 6), in agreement with data on activation of polyarginine-mediated CF efflux from liposomes by pyrenebutyrate [15,45,46].

With liposomes containing DOPG, the effect of palmitate on the CF efflux became opposite to that observed with neutral liposomes: the leakage induced by 0.5 μM indolicidin (Fig. 3B, curve 1) was substantially accelerated in the presence of 5 μM palmitate (Fig. 3B, curve 2), which also favored the different nature of indolicidin activity in negatively charged membranes.

The indolicidin-induced CF and SRB efflux from egg PC liposomes exhibited strong pH dependence with the highest activity at alkaline pH (Fig. 4). It should be noted that the well-known pH dependence of CF fluorescence in solution [47] complicated the measurements. However, SRB fluorescence is constant in a broad range of pH and the consistency of pH dependences measured for CF and SRB confirmed the notion that the observed pH dependence was an intrinsic property of indolicidin. The peptide has one lysine and an open N-terminus which carries a protonatable amino group with pKs in the alkaline pH range [48]. It can be proposed that after the transfer of a peptide–CF complex across the membrane, free peptide molecules can be back-

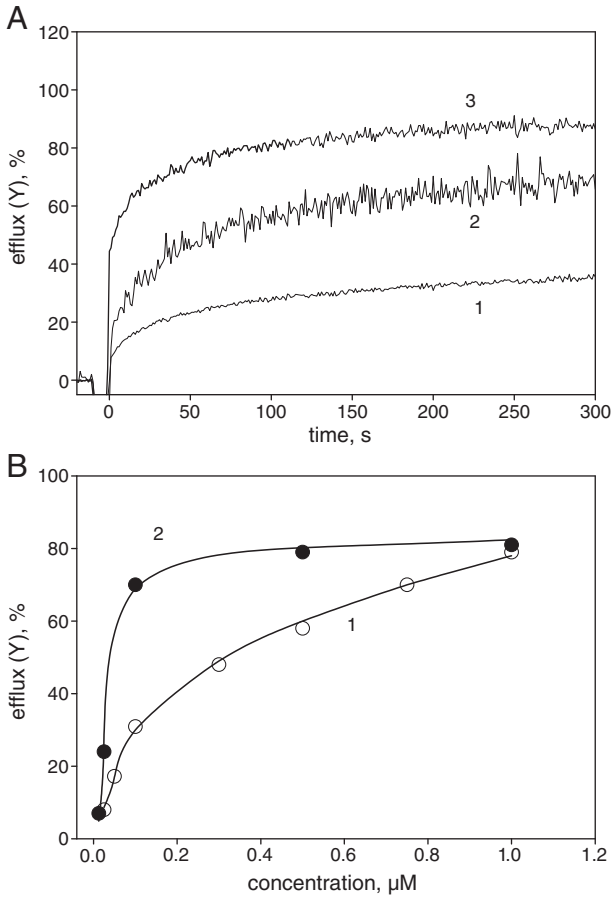


**Fig. 2.** A. Time courses of CF (curve 1), SRB (curve 2) and calcein (curve 3) efflux from liposomes made from egg PC (7 μM). Concentrations of indolicidin were 0.1 μM (curves 1 and 3) and 25 nM (curve 2). B. Dependence of CF (open circles, curve 1) and SRB (closed circles, curve 2) efflux from liposomes made from egg PC (7 μM) on the concentration of indolicidin. The magnitude of the dye release was recorded 200 s (CF) and 10 s (SRB) after the addition of indolicidin.

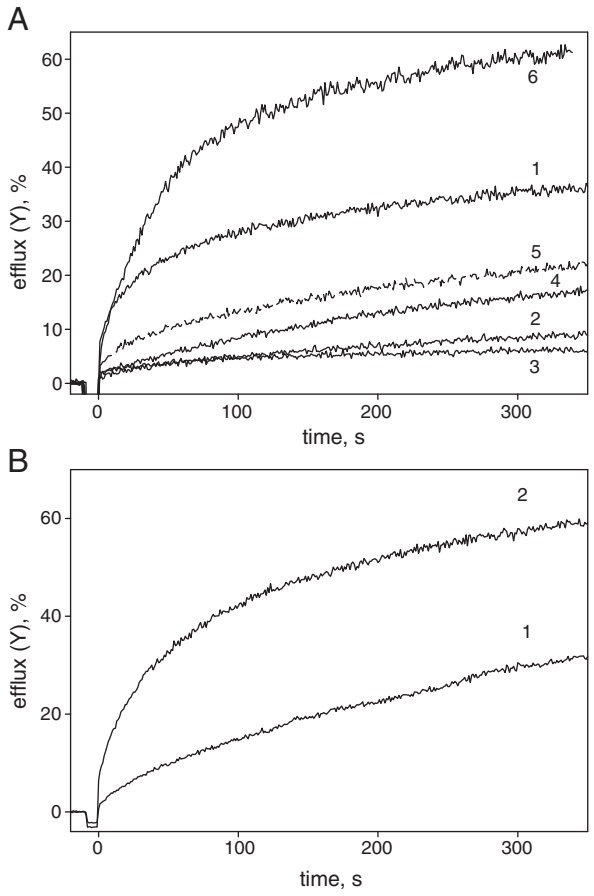


**Fig. 3.** A. Effect of oleate (10 μM, curve 2), palmitate (10 μM, curve 3), lauryl sulfate (20 μM, curve 4), pyrenebutyrate (10 μM, curve 5, or 50 μM, curve 6) on the CF efflux induced by 0.1 μM indolicidin (curve 1), liposomes were from egg PC (7 μM). B. Effect of palmitate (5 μM, curve 2) on the CF efflux induced by 0.5 μM indolicidin (curve 1), liposomes were from egg PC, DOPG and cholesterol (45:18:37 mol%) with final lipid concentration of 7 μM.

transferred in a deprotonated form (or as a complex with hydroxide), thereby explaining the observed pH dependence.

Fig. 5 shows the interaction of indolicidin, Ind7, and Ind8 (0.7 μM) with liposomes containing brominated lipids. Here the tryptophan fluorescence intensity of the peptides is plotted versus lipid

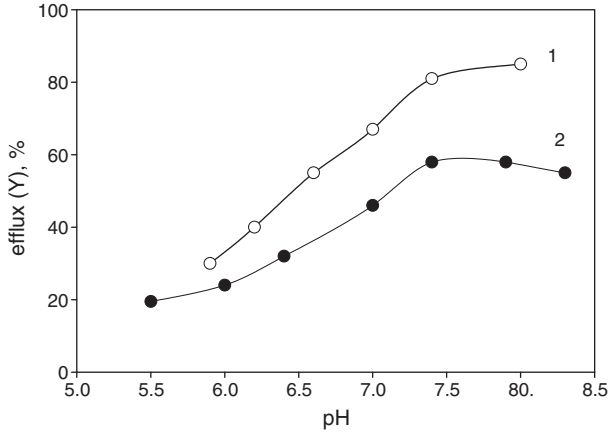


**Fig. 4.** The pH dependence of CF (curve 1) and SRB (curve 2) efflux from egg PC (7 μM) liposomes induced by indolicidin. The extent of the dye release was measured 200 s after the addition of 1 μM indolicidin (hollow circles, curve 1), and 20 s after the addition of 0.1 μM indolicidin (filled circles, curve 2). The solution was 10 mM Tris, 10 mM Bis–tris, 100 mM KCl (curve 1) or 10 mM Tris, 10 mM Mes, 100 mM KCl (curve 2).

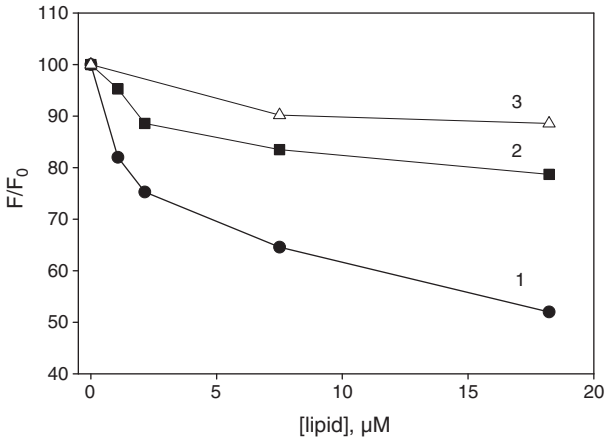


**Fig. 5.** Binding of indolicidin (circles, curve 1), Ind7 (squares, curve 2) and Ind8 (triangles, curve 3) to 9,10-BrPC/egg PC (50:50 mol%) liposomes. The extent of quenching of indolicidin fluorescence and its analogs by the brominated liposomes is presented as a function of lipid concentration.

concentration. The brominated liposomes produced higher extent of quenching in the case of indolicidin, as compared to the mutants, presumably due to deeper insertion of its tryptophans into the membrane. In fact, the tryptophan emission bands of indolicidin, Ind7, and Ind8, which peaked at 347 nm, 354 nm, and 354 nm in the aqueous solution without liposomes, shifted their maxima to 337 nm, 349 nm, and 346 nm, respectively, upon addition of egg PC liposomes (data not shown). The fluorescence quenching resulting from the peptide binding to liposomes tended to saturation at a lipid to peptide ratio less than 10:1. These data can be compared with the dose dependence of CF and SRB leakage induced by indolicidin (Fig. 2B) which showed tendency to saturation at about the same lipid to peptide ratio. Therefore, the concentration dependence of the CF and SRB efflux shown in Fig. 2B was partly accounted for by the peptide binding to liposomes. The difference between CF and SRB efflux could be attributed to a difference in the rate of translocation of the complexes across the membrane or a difference in the dye binding to peptide.

Carrier-type mechanism implies the formation of complexes between a cationic peptide and an organic anion. Pyrenebutyrate is a convenient reporter of formation of complexes that leads to its aggregation and appearance of excimer emission at 470 nm [15]. Fig. 6 shows a series of emission spectra of pyrenebutyrate at different indolicidin concentrations in solution. The peptide induced the formation of pyrene excimers in a dose-dependent manner (Fig. 6,

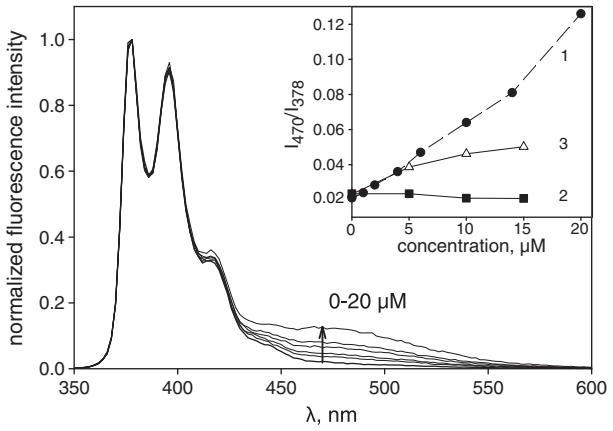


**Fig. 6.** Fluorescence emission spectra of pyrenebutyrate (100 μM) in the aqueous buffer solution in the presence of indolicidin (0–20 μM), excitation at 340 nm. Inset: the ratio of excimer and monomer emission intensities ($I_{470}/I_{378}$) as a function of indolicidin (circles, curve 1), Ind7 (squares, curve 2), and Ind8 (triangles, curve 3) concentrations.

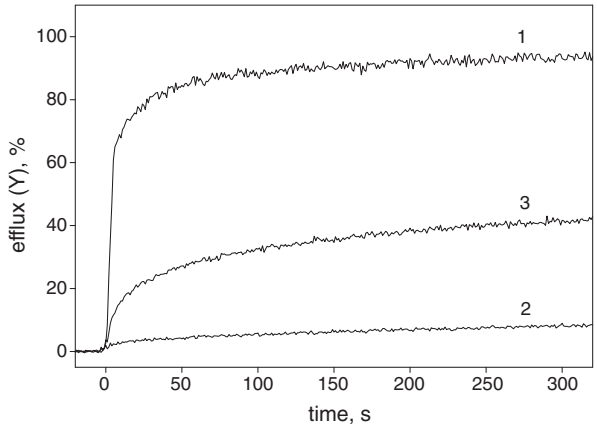


**Fig. 7.** CF efflux from egg PC (7 μM) liposomes induced by 1 μM indolicidin (curve 1), 1 μM Ind7 (curve 2), and 1 μM Ind8 (curve 3).

curve 1 of inset). Formation of excimers can be suppressed by the addition of non-fluorescent organic anions, for example, palmitate. In fact, while the $I_{470}/I_{378}$ value (the ratio of excimer and monomer emission intensities) was 0.126 in the presence of 100 μM pyrenebutyrate and 20 μM indolicidin, the $I_{470}/I_{378}$ value diminished to 0.043 after the addition of 40 μM palmitate. Pyrene excimer formation can be tentatively ascribed to the presence of two neighboring arginines in the indolicin sequence, because according to [15], binding of pyrenebutyrate to oligoarginines leads to excimer formation. In line with this consideration, the Ind7 analog having only one Arg did not form complexes with pyrenebutyrate, while Ind8 with two arginines showed formation of the complexes (Fig. 6, curves 2 and 3 of inset).

We also tested the potency of indolicidin analogs Ind7 and Ind8 to induce the CF efflux (Fig. 7). In agreement with the inability of Ind7 to bind pyrenebutyrate (Fig. 6, inset), the peptide was inactive in the induction of CF efflux (Fig. 7, curve 2). Importantly, Ind7 having only one arginine in contrast to indolicidin and Ind8 containing a couple of arginines displayed the complete loss of the hemolytic activity [39]. Ind8 had intermediate leakage activity between indolicidin and Ind7 (Fig. 7, curve 3) which correlated with the weaker ability of Ind8 than that of wild-type indolicidin to form complexes with pyrenebutyrate as shown by the pyrenebutyrate fluorescence assay (Fig. 6 inset). These data favor the essential role of a peptide–CF complex formation in the leakage activity of indolicidin.

It was previously shown that indolicidin induced ion conductance in planar lipid bilayers containing negatively charged lipids via formation of ion channels [28]. We performed experiments with indolicidin using planar bilayers formed from neutral DPhPC (Fig. 8A) or a mixture of DPhPC/DPhPG, 80%/20% (Fig. 8B). In zwitterionic membranes (Fig. 8A), indolicidin induced fluctuations of the current at high voltages (+100 mV) suggesting formation of some kind of ion channels, however, no channel activity was detected at voltages less than 50 mV. The conductance of the neutral membrane also did not increase if 5 μM dodecylsulfate was added in the presence of indolicidin (data not shown). In agreement with the previous finding [28], in membranes containing negatively charged lipids (Fig. 8B), the addition of indolicidin provoked an increase in the transmembrane current even at low voltages (25 mV) and current fluctuations were more pronounced. Taking into account that liposomal membranes used in our experiments did not have transmembrane potential, it follows from Fig. 8A that indolicidin can hardly induce channel formation in zwitterionic membranes.

According to [49], leakage of CF from CF-loaded liposomes can result from osmotic swelling of liposomes induced by channel former providing permeation of inorganic ions but not of CF. This type of leakage is sensitive to external sucrose which neutralizes osmotic disbalance [49]. Control experiments showed that the addition of 200 mM sucrose did not prevent the indolicidin-mediated CF efflux pointing to another mechanism of CF efflux in our system (data not shown).

## 4. Discussion

Monitoring liposome leakage has long been a very popular assay of the effect of different treatments on membranes, in particular, the action of membrane-active peptides. The present study highlights the importance of testing different types of encapsulated molecules of comparable molecular weight other than routinely used fluorescence dye release techniques as indicators for peptide activities. Our data on the release of different probes from zwitterionic liposomes induced by indolicidin are not consistent with the pore-type mechanism of transport as noted above but can be readily explained by the carrier-type mechanism similarly to that suggested by Matile and colleagues [15,45,46] for oligoarginines. The following results evidenced in favor

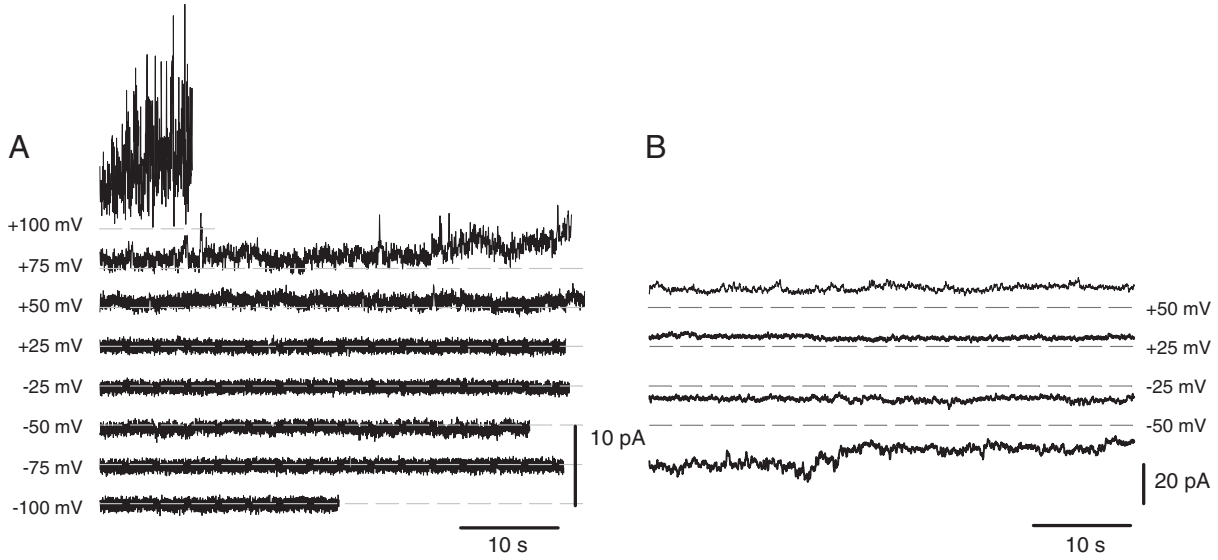


**Fig. 8.** Records of indolicidin-induced current across a planar bilayer lipid membrane made from DPhPC (A) or a mixture of DPhPC/DPhPG, 80%/20% (B) at different voltages. 1 μM of indolicidin was added at the *cis* side, the *trans* side was virtual ground. For convenience, grey dashed lines corresponding to zero current level were drawn.

of the carrier type of mechanism of indolicidin action on neutral membranes: i) Indolicidin transported organic anions (CF, calcein, SRB) across egg PC membranes while it was inactive in the case of neutral glucose or cationic doxorubicin (Fig. 1A). ii) Indolicidin formed complexes with pyrenebutyrate anions in a manner sensitive to the presence of other organic anions (Fig. 6). iii) There was a competition between several organic anions (dodecylsulfate, palmitate) and CF for the indolicidin-mediated transport across egg PC membranes (Fig. 3). iv) Indolicidin did not induce ion channel activity at low voltages in planar bilayer lipid membranes formed of zwitterionic lipids (Fig. 8A).

Importantly, indolicidin did not induce an increase in conductance of a planar membrane even in the presence of organic anions. This result showed that the peptide transported CF and other anions in an electrically neutral form, i.e. the transport was non-electrogenic. The carrier type of mechanism of non-electrogenic nature implied also that indolicidin moved backward across the membrane without CF in a neutral form as well. This conclusion suggested that the backward transfer can proceed in a complex with hydroxide. In fact, CF efflux increased at neutral and alkaline pH (Fig. 4) presumably due to facilitation of indolicidin–hydroxide complex formation. A similar model was proposed for CF transport mediated by guanidinium-containing polymers [16].

Several studies [39,50–55] considered the effect of sequence variation of indolicidin on its antimicrobial and hemolytic activity. The data on indolicidin mutants differing in the arginine content [39,50,54] support the essential role of arginine residues in the peptide. The present results on the carrier-type activity of indolicidin associated with the presence of a double-arginine motif in the peptide sequence in combination with the previous data on the loss of hemolytic activity of indolicidin in its mutant lacking one arginine [39] may suggest the involvement of the carrier-type mechanism in the peptide hemolytic activity. Importantly, the newly described mode of indolicidin effect on membrane permeability is observed in zwitterionic bilayer lipid membranes generally considered as a model of eukaryotic cell membranes. It can be speculated that indolicidin provokes erythrocyte lysis by disturbing metabolic regulation of osmotic balance (an example of such a disturbance is represented by an increase in host erythrocyte permeability after plasmodia infection [56]) which results from induction of either anionic metabolite efflux or influx of the peptide itself into the cytoplasm.

## Acknowledgements

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