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Membrane permeabilization by α-helical peptides: a flow cytometry study

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Abstract

The permeabilization by α-helical peptides of nucleated mammalian cells can be monitored by flow cytometry. Ethidium bromide, a non fluorescent and poorly membrane permeant molecule, becomes strongly fluorescent only upon binding to DNA. On this basis, the permeabilization of the plasma membrane of HL60 promyelocytic cells induced by α-helical peptides such as melittin, succinylated melittin and anionic peptides derived from the N-terminus of HA2 subunit of the influenza virus hemagglutinin, was measured. Melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2) caused a rapid (< 5 min) and dose-dependent (ED_{50} = 0.5 μM) permeabilization of HL60 cells at neutral pH, whereas the succinylated derivative induced cell permeabilization only at pH below 4.5 with an ED_{50} = 18 μM. The permeabilization by the anionic E5CA peptide (GLFEAIAEFIEGGWEGLIEGCA) containing 5 glutamic residues occurred (ED_{50} = 11 μM) at pH ranging from 6.5 to 6.0; replacing the tryptophan residue in position 14 by a phenylalanine residue decreased by about 1 unit the pH at which membrane permeabilization was effective. The membrane permeabilization activity of the E5CA peptide was reversibly abolished when the peptide was linked to a protein carrier. These results show that α-helical peptide-induced membrane permeabilization can be easily monitored by using flow cytometry in the presence of a non permeant dye. This method allows a rapid screening and an efficient mean of selection of peptides suitable to induce membrane permeabilization.

Keywords: Ethidium bromide; Melittin; Fusogenic peptide; HA2 influenza virus hemagglutinin

1. Introduction

Small peptides having an α-helix structure such as melittin, staphylococcal δ-toxin, mastoparan or bombolitins (for a review see [1]), or contained in virus fusion proteins (for reviews see [2,3]), or having a β-sheet structure such as defensins (for a review see [4]), have membrane disrupting activity including fusion and lysis. Extensive studies of fusion and lytic activities of these peptides were mainly approached by using membrane models such as lipid bilayer membranes, egg phosphatidylcholine (PC) vesicles, dioleoylphosphatidylcholine (DOPC) vesicles, or hemolysis of erythrocytes. Because the effects and the data obtained by using membrane models cannot be directly transposable to cellular membranes, fluorescence methods were developed to evaluate the membrane lesions of eukaryotic cells. The rationale of this approach is based on the use of nonpermeant fluorescent dyes which enter the cells only upon plasma membrane damaging.

In the present work, the permeabilization of the plasma membrane of HL60 promyelocytic cells was measured by flow cytometry using ethidium bromide, a nonfluorescent and poorly membrane-permeant molecule, which becomes strongly fluorescent upon binding to DNA. Several peptides were tested including melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2), a basic 26 amino acid peptide-amide from the venom of the honey bee (*Apis mellifera*) having membrane fusion activity and a powerful hemolytic activity in a pH independent manner (for a review see [5]), succinylated melittin having membrane fusion activity at acidic pH [6], and analogues of E5 peptide (GLFEAIAEFIEGGWEGLIEG), an anionic 20 amino acid peptide derived from the N-terminal segment of HA2 subunit of the influenza virus hemagglutinin [7–9] able to induce liposome fusion and hemolysis of erythrocytes at acidic pH.

Abbreviations: BET, ethidium bromide; BSA, bovine serum albumin; DTT, dithiothreitol; Lact, lactosyl residue; SPy, thiopyridinyl residue; TCEP, tris(carboxyethyl)phosphine.

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2. Material and methods

2.1. E5 peptide derivatives

GLFEAIAEFIEGGWEGLIEGCA (E5CA) and GLFEAIAEFIEGGFEGLIEG (E5-W14F) [10] peptides were synthesized by using Boc protected amino acids and a phenylacetamido-methyl polystyrene resin (Neosystem, Strasbourg, France). The peptide was cleaved from resin and deprotected with the low-high HF method in the presence of *p*-cresol according to [11]. The peptide was washed with dichloromethane, solubilized in distilled water by addition of 1 N NaOH and precipitated by acidification with 1 N HCl. The thiol group of the cysteinyl residue of the E5CA peptide (11 mg; 4.6 μmol) was protected by reaction with dithiodipyridine (5 mg; 23 μmol) (Aldrich, Strasbourg, France) in a H_2O/methanol (50:50; v/v) mixture. The solution was neutralized by addition of 1 N NaOH and stirred for 2 h at 20°C to yield the E5CA-SPy peptide. The peptides were desalted by gel filtration on Bio-gel P-2 (Bio-Rad) in water and freezed-dried. The peptide concentration was determined by using ϵ_{280nm} = 5600 M l^{-1} cm^{-1} for E5CA and 10480 M l^{-1} cm^{-1} for E5CA-SPy.

Succinylated melittin

Melittin (60 mg, 21 μmol) (Sigma, St. Louis, MO, USA), dissolved in 4 ml of 9.1 M sodium acetate at pH 9.4, was reacted for 2 h at 20°C with succinic anhydride (100 mg, 0.96 mmol) (Aldrich). The pH was maintained at pH 9.4 by adding 1 N NaOH; then, 100 mg succinic anhydride was added, and the solution stirred for 4 h. The succinylation of the three lysine ϵ-amino groups and of the N-terminal amino group of melittin was monitored by staining spotted aliquots on a silicagel plate with ninhydrin reagent. When no staining was observed, succinylated melittin was precipitated at 4°C for 20 h upon acidification with 1 N HCl. The precipitate was collected by microfuge; the pellet was washed twice with 1 N HCl, and the pellet was solubilized in distilled water upon neutralization with 1 N NaOH. The number of remaining amino groups on succinylated melittin was determined by using fluorescamine: the fluorescence intensity obtained with succinylated melittin was less than 2% of that obtained with the same concentration of unmodified melittin, indicating that succinylation was roughly complete. The melittin concentration was determined by using ϵ_{280nm} = 5570 M l^{-1} cm^{-1} [6].

2.2. Coupling of E5CA to lactosylated bovine serum albumin via a disulfide bridge

E5CA-SPy (5.8 mg; 2.5 μmol) was treated for 30 min at 20°C in 1 ml 0.1 M Hepes buffer, pH 7.4 in the presence of 25 mM DTT (Serva, Heidelberg, Germany). In order to remove the excess of DTT and pyridine-2-thione, the peptide was precipitated five times by acidification with 6 N HCl after solubilization in distilled water and neutralization with 1 N NaOH. The reduced peptide dissolved in 1 ml of 0.1 M Hepes buffer, pH 7.4 and neutralized with 1 N NaOH, was reacted for 15 h at 20°C with lactosylated bovine serum albumin (Lact-BSA) (19 mg; 0.25 μmol) in 1 ml of 0.1 M Hepes buffer, pH 7.4 (BSA substituted with lactosyl residues as previously described [12] was further substituted with 11 dithiopyridinyl groups upon reaction with *N*-succinimidyl 3-(2-pyridyldithio)propionate [13,14]). Then the solution was dialyzed twice for 24 h at 4°C against 2 l H_2O and freeze-dried. The number of E5CA peptides bound per Lact-BSA molecule was determined from the amino acid composition obtained after 5.6 N HCl hydrolysis at 106°C for 72 h and analysis by reverse phase HPLC chromatography on C_{18} column of the phenylthiohydantoin derivatives. Five E5CA peptides, in average, were bound per Lact-BSA molecule. The homogeneity of the conjugate was checked by SDS/PAGE electrophoresis. Under nonreducing conditions, the conjugate migrated as a single band with an apparent molecular mass higher (M_r about 90000) than that of unconjugated Lact-BSA (M_r about 80000); under reducing conditions and cleavage of the disulfide bridges, the conjugate migrated as the unconjugated Lact-BSA.

2.3. Cells

HL60 promyelocytic cells were grown in RPMI 1640 medium, supplemented with 10% fetal bovine serum (Gibco, Renfrewshire, UK), 2 mM L-glutamine (Merck, Darmstadt, Germany), 100 units/ml of penicillin and 0.1 mg/ml of streptomycin (Eurobio, Paris, France) at 37°C in a humidified atmosphere (5% CO_2-95% air).

2.4. Flow cytometry analysis

HL60 cells growing in RPMI complete medium were collected in exponential-growth phase, washed and suspended ($1 \cdot 10^6$ cells/ml) in sheath fluid (134 mM NaCl, 3.75 mM KCl, 15.24 mM NaF, 1.9 mM KH_2PO_4, 16.53 mM Na_2HPO_4, 0.2% 2-phenoxyethanol, pH 7.4). HL60 cells ($5 \cdot 10^5$ cells) in 0.9 ml of sheath fluid were mixed at 20°C with 12.5 μM ethidium bromide (BET, Molecular Probes, La Jolla, CA, USA) (5 μl of a 1 mg/ml stock solution in H_2O) in the absence or in the presence of peptides (100 μl of a solution containing various peptide concentrations). The pH of the cell suspension was adjusted from 7.4 to 4.5 by adding 10 to 70 μl of 0.3 N HCl. The cell fluorescence intensity was measured by using a FACS Analyzer (Becton Dickinson, Sunnyvale, CA, USA) equipped with a FACSlite unit (Becton Dickinson). A 488 nm excitation wavelength was produced by a 15 mW cold air argon laser; a 520 ± 10 nm interference filter was installed to collect the emitted green fluorescence; a 550 nm dichroic filter and a 580 long-pass filter were used to

collect the emitted red fluorescence; 90° light scatter, electric volume and red fluorescence were simultaneously recorded in list mode on a minimum of 5000 cells at 200–300 events per second. The data were analyzed by using the Consort 30 device (Hewlett Packard).

3. Results

Ethidium bromide (BET) is a small non fluorescent, poor membrane permeant molecule which emits a red fluorescence when it is bound to DNA. BET can, therefore, be used to study cell permeabilization by flow cytometry: when the plasma membrane is permeabilized, BET enters the cells, reaches the nucleus and binds to DNA, and the cells emit a red fluorescence. Therefore, permeabilized cells become fluorescent in the presence of BET whereas non permeabilized cells do not. When HL60 cells were incubated in the presence of 12.5 μM BET, they were poorly labeled (Fig. 1a). The cell fluorescence intensity remained very low for up to 30 min ($I_F = 5$) at 20°C and then slightly increased ($I_F = 13$ after 40 min incubation) due to a slow diffusion of BET across the plasma membrane. In the presence of 0.01% saponin, a non ionic permeabilizing agent, HL60 cells were strongly labeled ($I_F = 358$) as soon as upon 5 min incubation with 12.5 μM BET (Fig. 1b). As shown in Fig. 2, the cell fluorescence intensity was maximum with 12.5 μM BET in the presence of 0.01% saponin indicating that the concentration of BET was high enough to allow BET to react within few minutes with the major part of HL60 cell DNA. The membrane permeabilization activity of several amphipathic peptides on living cells was studied.

3.1. Melittin-induced membrane permeabilization

Melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2) is a basic peptide contained in the venom of the european honey bee *Apis mellifera* which has a membrane

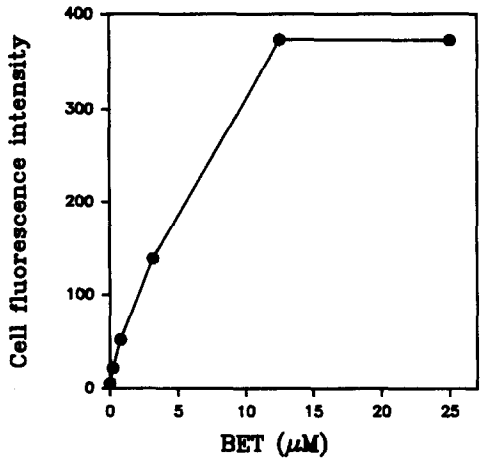

Fig. 2. BET concentration dependence. HL60 cells ($5 \cdot 10^5$ cells) were incubated at 20°C in the presence of 0.01% saponin and BET. After 5 min, the red cell fluorescence intensity was measured by flow cytometry.

fusion activity and a powerful hemolytic activity. In the presence of 0.66 μM melittin at pH 7.4, 66% of HL60 cells mixed with 12.5 μM BET became highly fluorescent within 5 min (Fig. 3A). The fluorescence intensity of permeabilized cells was as great as that obtained upon permeabilization with saponin (Fig. 1b). Under these conditions, melittin did not induce cell lysis as shown by the absence of broken cells in flow cytometry. The permeabilization activity of the plasma membrane was dependent upon the melittin concentration (Fig. 3B): 10% and 85% of the cells were labeled in the presence of 0.25 μM and 1 μM melittin, respectively. The concentration of melittin required to label 50% of the cells was 0.5 μM.

3.2. Succinylated melittin-induced membrane permeabilization

Succinylated melittin, in which the four amino groups (one from the N-terminal amino acid and three from the lysine residues) were succinylated, induced a rapid fusion

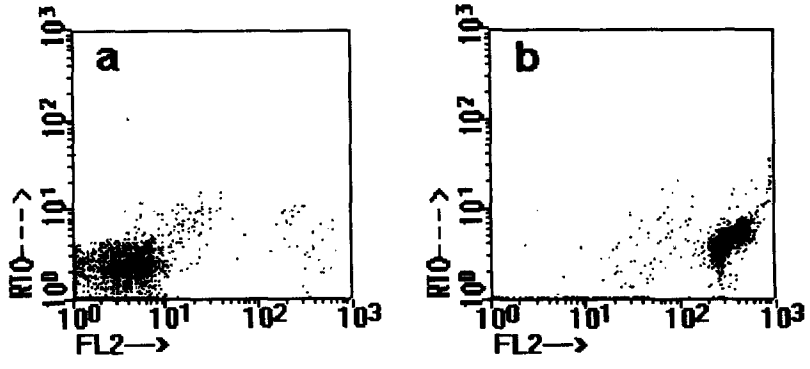

Fig. 1. Flow cytometry measurements of membrane permeabilization. HL60 cells ($5 \cdot 10^5$ cells) in 0.9 ml of sheath fluid were incubated at 20°C with 12.5 μM BET (5 μl of a 1 mg/ml stock solution in H_2O) in the absence (a) or in the presence (b) of 0.01% saponin (100 μl of a 0.1% stock solution in sheath fluid). After 5 min, the red cell fluorescence intensity was measured by flow cytometry and the percentage of red labeled cells was determined. RTO is the right angle light scatter. FL2 is the red fluorescence intensity of cells.

of egg phosphatidylcholine vesicles only at pH lower than 5.2 with a maximum at pH 5.1, due to protonation of the succinyl carboxyl groups in the hydrophobic segment of the peptide [6]. Permeabilization of the plasma membrane in the presence of succinylated melittin was induced at a lower pH than that found to induce the fusion of egg PC vesicles (Fig. 4). HL60 cells incubated in the presence of both 12.5 μM BET and succinylated melittin for 15 min became fluorescent at pH lower than 4.5. Cells had to be incubated at pH as low as 4.2 to obtain 50% of the labeled cells in the presence of 30 μM succinylated melittin for 15 min (Fig. 4). The fluorescence intensity of permeabilized cells was as great as that obtained upon permeabilization with saponin (Fig. 1b). Under such low pH conditions, broken cells were not detected in flow cytometry and in control experiments in the absence of succinylated melittin the fluorescence intensity of HL60 cells incubated in the presence of 12.5 μM BET for 15 min remained as low as cells incubated at neutral pH. Succinylated melittin which like melittin at neutral pH, induces fusion at low pH, has a less efficient membrane permeabilization activity: at pH 4.0, in the presence of succinylated melittin, the ED_{50} was 18 μM and 3.7 μM after 7 min and 15 min incubation, respectively whereas, in the presence of non succinylated melittin, it was 0.5 μM after 5 min incubation (Fig. 4, inset).

3.3. E5 anionic peptide-induced membrane permeabilization

The anionic E5 peptide (GLFEAIAEFIEGGWEGLIEG) in which 4 glutamic acid residues replaced G-4, G-8, T-15 and D-19 of the native peptide sequence of the N-terminal segment (GLFGAIAGFIEGGWTGMIDG) of the HA2

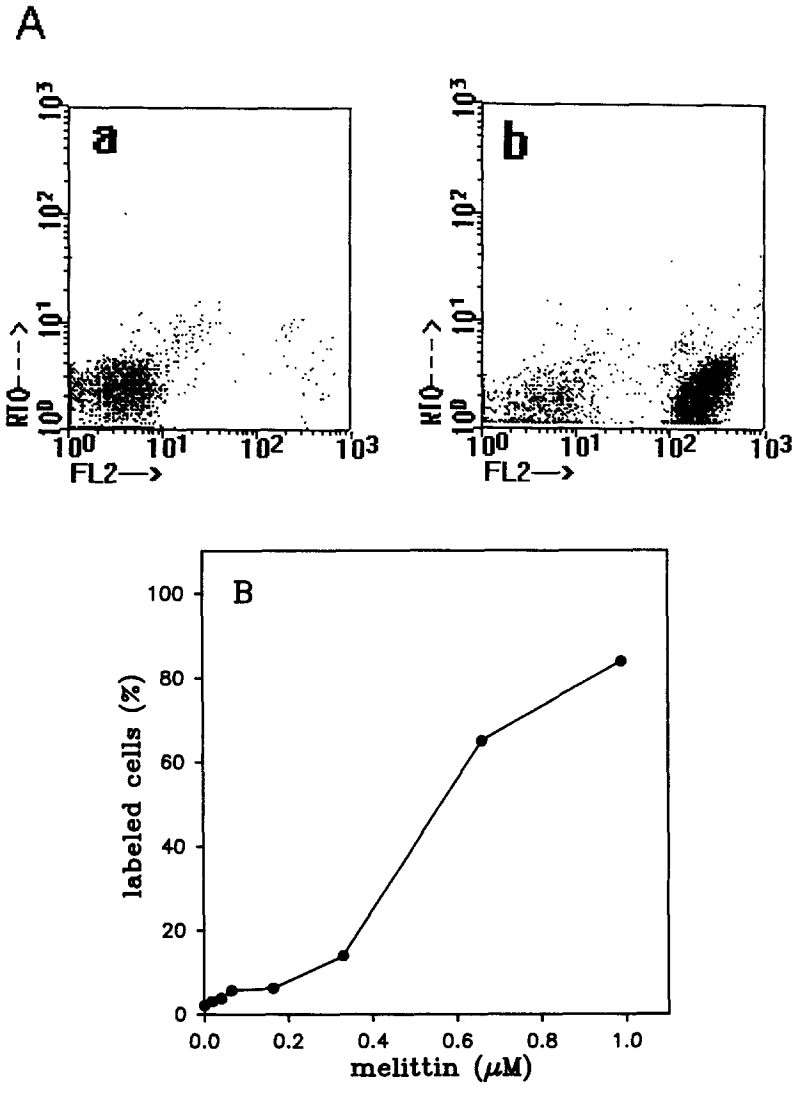

Fig. 3. (A) Melittin-induced membrane permeabilization. HL60 cells ($5 \cdot 10^5$ cells) in 1 ml of sheath fluid were incubated at 20°C with 12.5 μM BET in the absence (a) or in the presence (b) of 0.66 μM (2 μg/ml) melittin. (B) Concentration-dependence of melittin-induced membrane permeabilization. After 5 min, the red cell fluorescence intensity was measured by flow cytometry and the percentage of red labeled cells was determined.

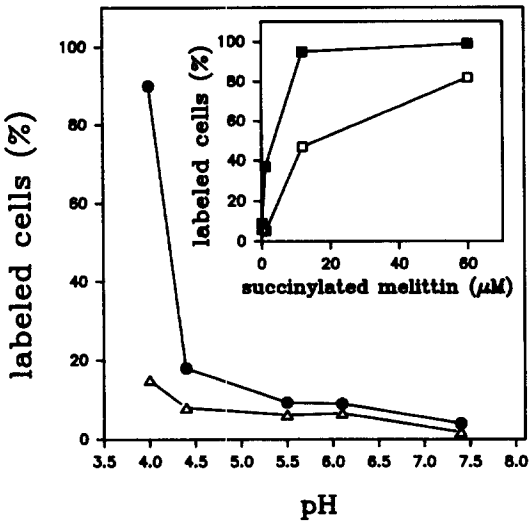

Fig. 4. pH-dependence of succinylated melittin-induced membrane permeabilization. HL60 cells ($5 \cdot 10^5$ cells) in 1 ml of sheath fluid were incubated at 20°C with 12.5 μM BET in the absence (△-△) or in the presence (●-●) of 30 μM (100 μg/ml) succinylated melittin. The pH of the cell suspension was lowered by addition of aliquots (10 to 70 μl) of a 0.3 N HCl solution. After 5 min, the red cell fluorescence intensity was measured by flow cytometry and the percentage of red labeled cells was determined. Inset: concentration-dependence of succinylated melittin-induced membrane permeabilization. HL60 cells ($5 \cdot 10^5$ cells) in 1 ml of sheath fluid were mixed at 20°C with 12.5 μM BET in the presence of succinylated melittin. The pH of the cell suspension was lowered to pH 4.0 by addition of aliquots 70 μl of a 0.3 N HCl solution. The red cell fluorescence intensity was measured either 7 (□-□) or 15 min (■-■) later by flow cytometry and the percentage of red labeled cells was determined.

subunit of the hemagglutinin of A/PR/8/34 influenza virus strain, was found to mimic the fusogenic activity of the influenza virus HA2 hemagglutinin [7–9]. This peptide has a random coil structure at neutral pH and an α-helix structure at pH lower than 6.0; it induces the fusion and the leakage of liposomes and the hemolysis of erythrocytes at pH between 6.0 and 5.0. The membrane permeabilization activity of a E5-analogue peptide (E5CA) containing an additional cysteinyl and one alanyl residues in position 21 and 22, respectively, was assessed as described for melittin and succinylated melittin. The E5CA peptide has a pH- and concentration-dependent membrane permeabilization activity (Figs. 5 and 6). HL60 cells incubated in the presence of both 12.5 μM BET and E5CA peptide became strongly labeled at low pH (Fig. 5): in the presence of 36 μM E5CA peptide, within 5 min, all the cells were labeled at pH 6.0, 50% at pH 6.4 and no cells at pH 7.4 (Fig. 6); the ED_{50} was 3 μM and 11 μM at pH 5.6 and pH 6.1 upon 5 min incubation, respectively (Fig. 7). The fluorescence intensity of permeabilized cells was as great as that obtained upon permeabilization with saponin (Fig. 1b).

The pH of the membrane permeabilization efficiency depended on the amino acid sequence of the peptide and in particular it depended on the presence of a tryptophan residue in position 14. Changing the tryptophan residue by a phenylalanine residue (E5-W14F), resulted in a peptide with an optimal activity at a lower pH, about 1 unit below the optimal pH at which membrane permeabilization occurred with E5 or E5CA: 50% of the cells were labeled at pH 5.6 in the presence of 36 μM E5-W14F peptide whereas 50% of the cells were labeled at pH 6.4 with a peptide containing the tryptophan residue (E5CA) (Fig. 6).

3.4. Membrane permeabilization activity of E5CA peptide bound to Lact-BSA

The efficiency of the membrane permeabilization of HL60 cells was drastically modified when E5CA peptide

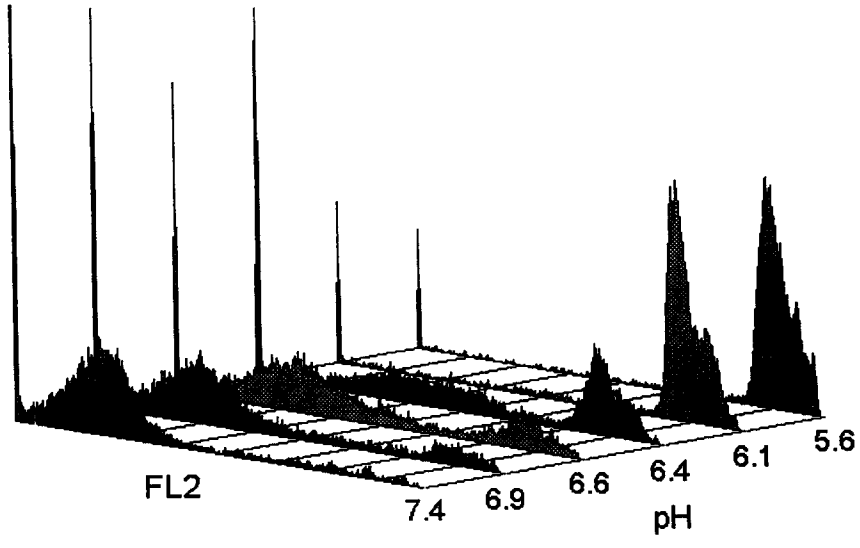

Fig. 5. pH-dependence of E5CA anionic peptide-induced membrane permeabilization. HL60 cells ($5 \cdot 10^5$ cells) in 1 ml of sheath fluid were incubated at 20°C with 12.5 μM BET in the presence of 36 μM (90 μg/ml) E5CA peptide. The pH of the cell suspension was lowered by addition of aliquots (10 to 70 μl) of a 0.3 N HCl solution. After 5 min, the red cell fluorescence intensity was measured by flow cytometry.

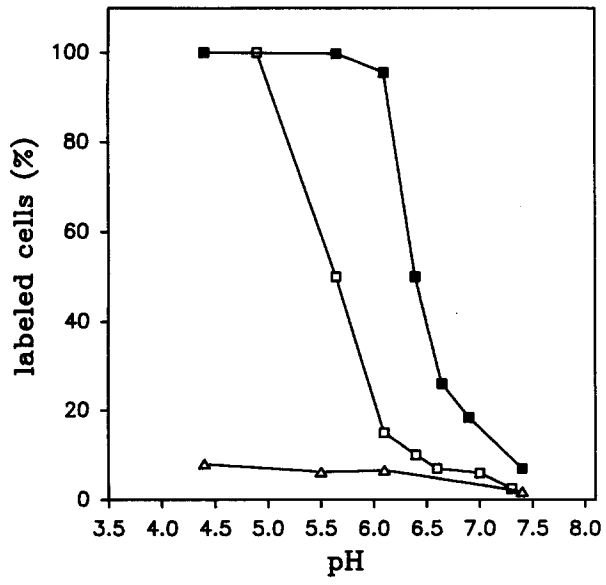

Fig. 6. pH-dependence of E5CA and E5-W14F anionic peptide-induced membrane permeabilization. HL60 cells ($5 \cdot 10^5$ cells) in 1 ml of sheath fluid were incubated at 20°C with 12.5 μM BET in the absence (△-△) or in the presence of 36 μM (90 μg/ml) of either (■-■) E5CA or (□-□) E5-W14F anionic peptides. The pH of the cell suspension was lowered by addition of aliquots (10 to 70 μl) of a 0.3 N HCl solution. After 5 min, the red cell fluorescence intensity was measured by flow cytometry and the percentage of red labeled cells was determined.

was linked to a protein. Five molecules of E5CA peptide containing a cysteinyl residue were linked via a disulfide bridge onto bovine serum albumin substituted with lactosyl

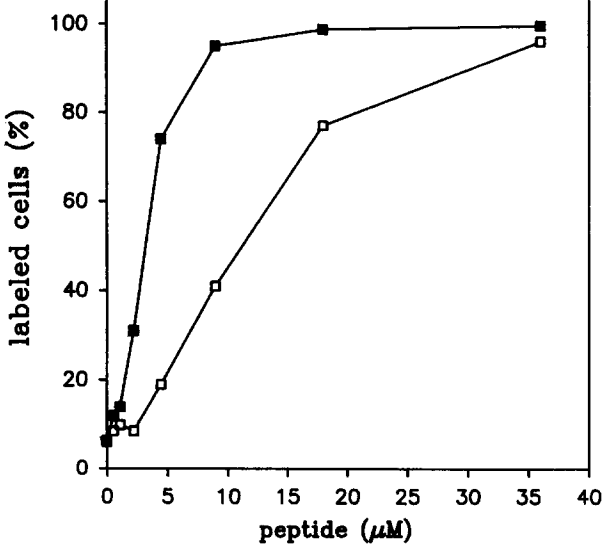

Fig. 7. Concentration-dependence of E5CA anionic peptide-induced membrane permeabilization. HL60 cells ($5 \cdot 10^5$ cells) in 1 ml of sheath fluid were incubated at 20°C with 12.5 μM BET in the absence or in the presence of various concentration of E5CA peptide. The pH of the cell suspension was lowered to either (□-□) pH 6.6 or (■-■) pH 6.1 by addition of 40 and 50 μl of a 0.3 N HCl solution, respectively. After 5 min, the red cell fluorescence intensity was measured by flow cytometry and the percentage of red labeled cells was determined.

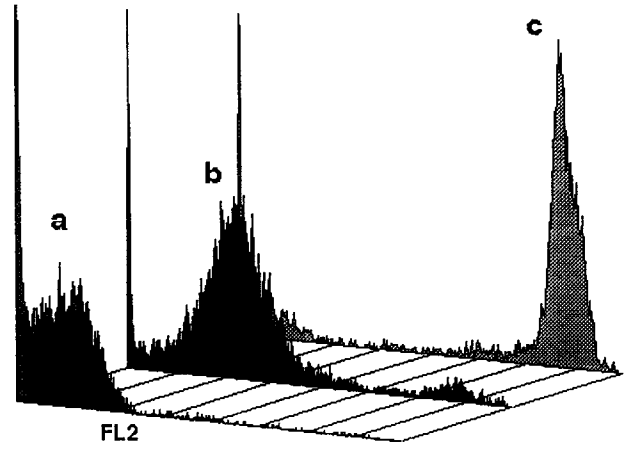

Fig. 8. Permeabilization efficiency of E5CA peptide linked to Lact-BSA. HL60 cells ($5 \cdot 10^5$ cells) in 1 ml of sheath fluid were incubated at 20°C with 12.5 μM BET in the presence of 37 μM E5CA peptide linked to Lact-BSA (E5CA-,Lact-BSA) either at pH 7.4 (a) or at pH 5.6 (b). (c) HL60 cells were incubated at pH 5.6 with 12.5 μM BET in the presence of E5CA-,Lact-BSA pre-treated with TCEP (5.2 mM, 15 min, 20°C, pH 7.4). After 10 min, the red cell fluorescence intensity was measured by flow cytometry and the percentage of red labeled cells was determined.

residues (Lact-BSA) and the membrane permeabilization activity of the conjugate was measured and compared to that of free E5CA peptide. In the presence of 37 μM E5CA peptide bound to Lact-BSA, 5% of the cells were labeled within 10 min incubation at pH 5.6 (Fig. 8b). When the conjugate E5CA-,Lact-BSA was treated with TCEP (tris(carboxyethyl)phosphine, Molecular Probes, La Jolla, CA, USA) in order to cleave the disulfide bridge between E5CA peptide and Lact-BSA and then incubated at pH 5.6 in the presence of 12.5 μM BET, 90% of HL60 cells were labeled within 10 min (Fig. 8c). Therefore, the membrane permeabilization activity of E5CA peptide linked to a protein such as Lact-BSA was drastically reduced.

4. Discussion

In the present work, the membrane permeabilization activity of several α-helical peptides was studied on living cells by flow cytometry with ethidium bromide (BET), a poorly membrane permeant molecule which is not fluorescent as long as it is free but becomes fluorescent upon binding to nucleic acids. HL60 promyelocytic leukemic cells permeabilized with 0.01% saponin in the presence of 12.5 μM BET, became strongly fluorescent within 5 min. Because BET diffused slowly into HL60 cells, its use is limited to short experiments, less than one hour; for longer incubation periods, a less permeant dye such as ethidium homodimer-1 could be used [15]. Alternatively, propidium iodide, an other poorly membrane permeant dye which also fluoresces upon binding to nucleic acids could also be used; this dye was used for instance to assess tumor cell

permeabilization by a membrane-active antitumor agent (mouse mammary tumor and HL60 cells) [16] and by tumor necrosis factor (murine fibrosarcoma L929 cells and WEHI cells) [17].

The permeabilization properties of several α-helical peptides were assessed. Melittin, an extensively studied amphipathic peptide was found to permeabilize $5 \cdot 10^5$ HL60 cells in a concentration dependent manner with an $ED_{50} = 0.5$ μM. This value is close to that of the concentration inducing hemolysis: indeed, 0.3 μM melittin induced hemolysis of human erythrocytes within 10 min [5]. The membrane destabilization induced by melittin is probably due to the formation of pores induced by a cluster of four aggregated amphipathic α-helices in the plasma membrane [5]. In the presence of melittin (and also of the other α-helical peptides), HL60 cells were rapidly labeled by BET and the percentage of highly fluorescent cells increased when the concentration of melittin increased. Conversely, the spontaneous diffusion of BET through the intact plasma membrane, as expected, increased slowly.

Peptides containing acid residues increase their hydrophobicity when the pH becomes acidic, form amphipathic α-helices and may interact with phospholipid membranes. This is the case of the succinyl derivative of melittin which induces fusion of phospholipid vesicles upon protonation of its carboxylic groups at low pH [6]. The concentration of succinylated melittin required to permeabilize HL60 cells was much higher ($ED_{50} = 18$ μM at pH below 4.0) than that of melittin. The inefficiency of succinylated melittin at neutral pH could be related to its amphiionic character : indeed, succinylated melittin possesses two positive charges and 4 negative charges while melittin contains 5 positive charges and no negative charges. The efficiency of succinylated melittin at acidic pH related to the lost of the negative charges, is however low because there is only two positive charges instead of five.

The anionic E5CA peptide permeabilized HL60 at pH ranging from 6.5 to 6.0 ($ED_{50} = 11$ μM at pH 6.1). This efficiency was close to that required to induce hemolysis of human erythrocytes: 5 μM E5-analogue peptides induced 100% hemolysis at pH 5.5, 50% at pH 6.0 and no hemolysis at pH 6.5 [9]. The E5 anionic peptide forms an α-helix upon protonation of the glutamic residues by lowering the pH down to 5.0 [18] and does not bind to the cell surface due to the negatively charged side chains of the five glutamic residues, but interacts with the plasma membrane upon lowering the pH medium close to 5.0. The formation of clusters of several E5CA α-helical monomers in the lipid membrane could induce membrane destabilization as does GALA, another anionic peptide, which was shown to destabilize egg phosphatidylcholine large unilamellar vesicles in acidic medium by forming pores containing 8 to 12 GALA monomers within the bilayer structure [19].

When the tryptophan residue at position 14 was replaced by a phenylalanine residue, the E5-W14F peptide required a lower pH (about 1 unit) to permeabilize HL60 cells. This result is in agreement with the findings that: (i) melittin, upon photooxidative destruction of its tryptophan, as well as a tryptophan-free melittin analogue were less active [20], and (ii) peptides, analogous to the N-terminal segment of the HA2 subunit of the influenza virus hemagglutinin, had a lytic activity 1000-fold lower when the tryptophanyl and the tyrosyl residues at positions 21 and 22, respectively, were replaced by glycyl residues [21].

Recently, peptides analogous to the N-terminal segment of the HA2 subunit of the influenza virus hemagglutinin [21,22], the E5CA peptide [10] and the GALA peptide [23] were used to enhance the gene transfer efficiency by plasmid/polylysine-ligand complexes through receptor-mediated endocytosis. These permeabilizing peptides are supposed to increase the transmembrane passage of plasmids into the cytosol. The mechanism involved in this process is not yet understood but it seems to require membrane disruption of the endocytotic vesicles. Succinylated melittin and the E5-W14F peptide which require a pH (4.0 and 5.0, respectively) lower than that required by the E5CA peptide to induce membrane permeabilization were not able to enhance the transfection efficiency of HepG2 cells by using DNA/lactosylated polylysine complexes (Midoux, P., et al., unpublished results). The lumen of the early endosomic vesicles which is slightly acidic (pH close to 6.0) and which has a low degradation activity, could be efficiently destabilized by the E5CA peptide; in contrast, with succinylated melittin and the E5-W14F peptide, the membrane destabilization did not occur in early vesicles because the pH is too high and could occur in more acidic compartments such as late endosomes and lysosomes, but the efficiency is lost because such compartments are very efficient in degrading the endocytosed material. In order to increase their cellular specificity and their intracellular concentration upon endocytosis, fusogenic peptides can be coupled to a carrier. However, we found that the efficiency of the membrane permeabilization activity of the E5CA peptide was drastically reduced upon its linkage via a disulfide bridge to lactosylated bovine serum albumin; but its membrane permeabilization activity was completely recovered upon reduction of the disulfide bridge. This suggests that E5CA peptide must be released from the carrier inside endocytotic vesicles in order to recover its capacity to destabilize endosomal or lysosomal membranes.

In conclusion, the flow cytometry method described in this paper allows a rapid screening and is an efficient mean to select peptides suitable to induce membrane permeabilization.

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