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Synthesis and Characterization of a New Biotinylated Gramicidin

EMMANUELLE SUAREZ[a], EMMANUELLE DE[b], GERARD MOLLE[b], RENÉ LAZARO[a,*] and PHILIPPE VIALLEFONT[a]

[a] Laboratoire des Aminoacides, Peptides et Protéines, UPRESA CNRS 5075, Universités Montpellier I, 34095 Montpellier Cedex 5, France
[b] IFRMP 23, Polymères, Biopolymères, Membranes, UMR CNRS 6522, Université de Rouen, 76821 Mont Saint-Aignan Cedex, France

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Abstract: A new linear gramicidin analog bearing a biotinyl group grafted on C-terminal part was designed to study ligand–receptor interactions. The C-terminal alcohol in the native peptide was first replaced by an amino group. Then the peptide was synthesized on a polystyrene resin functionalized by the 2-chlorotrityl chloride following a biotinylation performed in solution. This new *N'*-biotinyl-(EDA)^{15}-Gramicidin A was reconstituted in planar lipid bilayers and exhibited channel activities similar to those of natural gramicidin, with unitary conductance value about 30 ps in 1 M KCl. Furthermore this ionophore activity was quenched by addition of streptavidin in the surrounding medium. Our system is an outstanding tool for monitoring ligand–receptor interactions and could be used for designing a new biosensor. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: biotinylgramicidin; peptide synthesis; planar lipid bilayers; ion channel; biotin–avidin interactions

INTRODUCTION

Gramicidin A is a hydrophobic, natural pentadecapeptide (Figure 1, **1a**) exhibiting strong conductance property when inserted in lipid bilayers. The pore channel resulting from the so-called head-to-head molecule dimerization [1] is one of the simplest, well known and most efficient ionophore peptide acting at a molecular level. It is noteworthy that the conductance of only one single active channel made of two monomers can easily be detected. This characteristic makes the gramicidin a good base from which to consider the detection of the ligand–receptor interactions at the molecular level. Among the different ligand–receptor models, the avidin–biotin is one of the most sensitive systems. Previous study showed that the grafting of biotin (receptor) on C-terminal part of gramicidin A did not influence the ionophore properties of the gramicidin channel [2]. Other studies [3,4] have also shown that a number of modifications at the peptide C-terminal part, i.e. close to the pore entrance, had no influence on the conductance value. Biotinyl-gramicidin-based biosensor was reconstituted in black

RVal-Gly-Ala-leu-Ala-val-Val-val-Trp-leu-Trp-leu-Trp-leu-Trp-NHC_2H_4**R'**

1a) **R** = HCO-, **R'** = -OH: natural gramicidin A.

1b) **R** = Fmoc-, **R'** = $-NH_2$: N-Fmoc, $(EDA)^{15}$ gramicidin A.

1c) **R** = Fmoc-, **R'** = -NH-(+)-Biotin: N-Fmoc, *N'*-biotinyl-$(EDA)^{15}$ gramicidin A

1d) **R** = H-, **R'** = -NH-(+)-Biotin: desformyl, *N'*-biotinyl-$(EDA)^{15}$ gramicidin A

1e) **R** = HCO-, **R'** = -NH-(+)-Biotin: *N'*-biotinyl-$(EDA)^{15}$ gramicidin A.

Figure 1 Structure of the new gramicidin-A analogs.

Abbreviations: EDA, ethylenediamine; DIEA, diisopropylethylamine; HATU, *O*-(7azabenzotriazol-1-yl)-1,1-3,3-tetramethyluroniumhexafluorophosphate; TFE, trifluoroethanol; DPhPC, diphytanoylphosphatidylcholine.

* Correspondence to: Laboratoire des Aminoacides, Peptides et Protéines, UPRESA CNRS 5075, Universités Montpellier I, Montpellier II, Place E. Bataillon, 34095 Montpellier Cedex 5, France.

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lipid bilayers and impedance spectroscopy experiments showed a decrease of the membrane conductance when streptavidin was added in the measurement compartment [2,5].

However, in this system, the linkage between biotin and ethanolamine moiety of the gramicidin is made via a spacer (hydroxycarbon chain) by an esterification reaction. Other studies based on native gramicidin modified by esterification [6] or urethane formation [7] were also reported. These linkages exhibit a weak chemical stability, however. Thus, we consider replacing them by a more convenient amide bound, requiring an NH_2-terminal

Several attempts were carried out unsuccessfully to replace the OH-terminal part by an amine in the natural gramicidin (unpublished data). Therefore we investigated a total synthesis of a new $(EDA)^{15}$-gramicidin A (**1b**) by solid phase peptide synthesis from a chlorotrityl resin followed by a biotinylation. We show in our study that the new analog *N′*-biotinyl-$(EDA)^{15}$-gramicidin A (**1e**) is functionally active in planar lipid bilayers. In addition, ligand–receptors interactions were controlled by reconstitution system using avidin and biotinylated-gramicidin.

MATERIALS AND METHODS

General Procedures

Solvents and reagents were of analytical grade, ethylene diamine (EDA) was redistilled over KOH before use. For thin layer chromatography, silica gel glass plates (Merck analytic or preparative 60F254) were used with $CHCl_3$/MeOH/AcOH/H_2O: 400/60/1/4 in volume as eluent. For semi-preparative HPLC purification, a nucleosil C-18 column Macherey-Nagel, 10 μm porosity 250×10 mm was used whereas for analytical purposes, a narrower column (250×4.6 mm) containing the same solid phase was used. ^{1}H NMR spectra were run with a Brucker AC-250 spectrometer and a JEOL JMS DX 400 was used for FAB^+ Mass spectrometry (Glycerol/Thioglycerol matrix).

Solid Phase Peptide Synthesis

A sample containing 1 g (1.6 meq) of 2-chlorotrityl chloride copolystyrenic resin (Novabiochem, 200–400 mesh, 1% DVB) was added to DCM (15 ml) containing EDA in 5.6 M excess (0.6 ml, 9 mmol) under [N_2] and gently stirred overnight. After successive washings with DCM and ethyl ether, the resin was dried under vacuum and kept at 5°C. An aliquot (0.1 g) was subjected to amine determination by the Charpentier–Volhard method giving 0.9 meq/g of free amine functionalization. A small fraction (0.125 g, 0.11 mmol) of this resin was added together with glass beads (1.25 g) into the Perseptive peptide synthesiser vessel.

All 15 peptide residues were successively linked according to the following program: Fmoc-amino acids (4 eq., 0.44 mmol) and HATU (4 eq.) are added in DMF containing 6 eq. DIEA. The coupling step occurs during the loop circulation time of the above solution. Time was controlled by the UV detector system: From 30 min for Trp^{15} until Trp^9 to 60 min for Val^8 and to 90 min for the remaining (from Val^7 to Val^1). After washings with DMF, the deprotection was performed with piperidine solution (10% in DMF) and monitored by the UV detector system.

The Fmoc-Val^1 peptide was cleaved from the resin using the acidic Barlos conditions [8]: the resin was gently stirred for 0.5 h in a mixture of TFA/TFE/DCM in 1/1/8 by volume. The resulting brown mixture was filtered and the filtrate carefully evaporated. The TFA peptide salt was dried, dissolved in 5 ml of methanol and precipitated by adding cold water, filtered again and washed by 1N HCl and water. After drying over P_2O_5 under vacuum, a white powder was obtained and purified by semi-preparative HPLC: the temperature of the oven containing the column was fixed at 30°C and the flow at 5 ml/min. The best eluent composition (MeOH 68%, CH_3CN 20%, water 12%) was determined using the analytical column at a flow reduced by one fifth. This purified peptide (**1b**) obtained in 70% yield was analyzed and its structure confirmed by ^{1}H NMR and by FAB^+ Mass spectrometry, $[M + H]^+ = m/z$ 2076.

Biotinylation

(+)-Biotine *N*-hydroxysuccinimidyl ester (15 mg) which is an easily synthesized or commercially (Sigma) available compound, was added to 4 ml of pyridine containing the peptide (20 mg). The mixture is magnetically stirred for 72 h under [N_2] at 40°C. The pyridine was evaporated and the resulting solid was taken up in MeOH and reprecipitated by adding 1N HCl. After filtration, the white solid was washed with water, saturated $NaHCO_3$ solution and again water and further purified by ion elimination treatment using mixed-bed MB-3 resin in MeOH. After solvent evaporation, a white solid (yield: 70%) was subjected to TLC-silica gel chro-

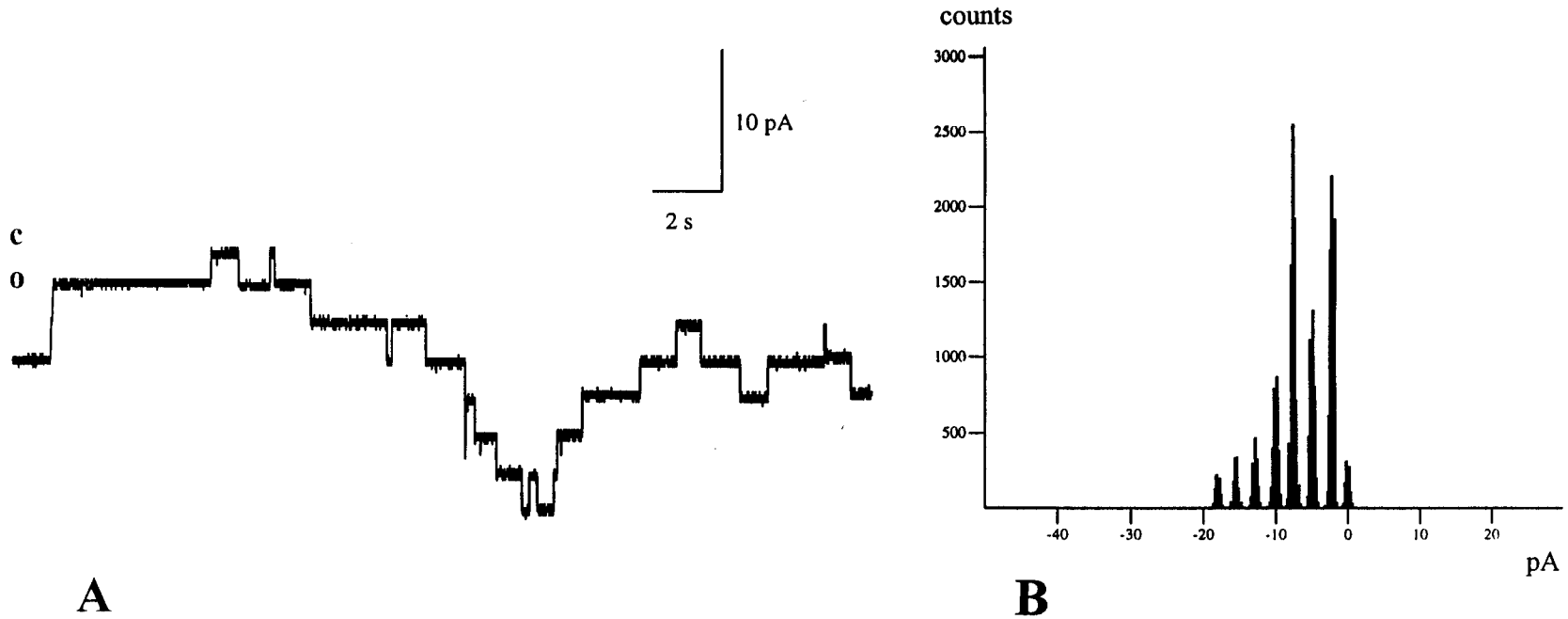

Figure 2 (A) Single-channel recordings induced by the gramicidin A (**1a**, 10^{-10} M) in a DPhPC bilayer. Electrolyte: 1 M KCl; applied voltage: − 100 mV; digitization rate: 1000 Hz; filter: 200 Hz. 'o' for open state; 'c' for closed state. (B) The corresponding amplitude histogram. Current is given as a function of the number of events.

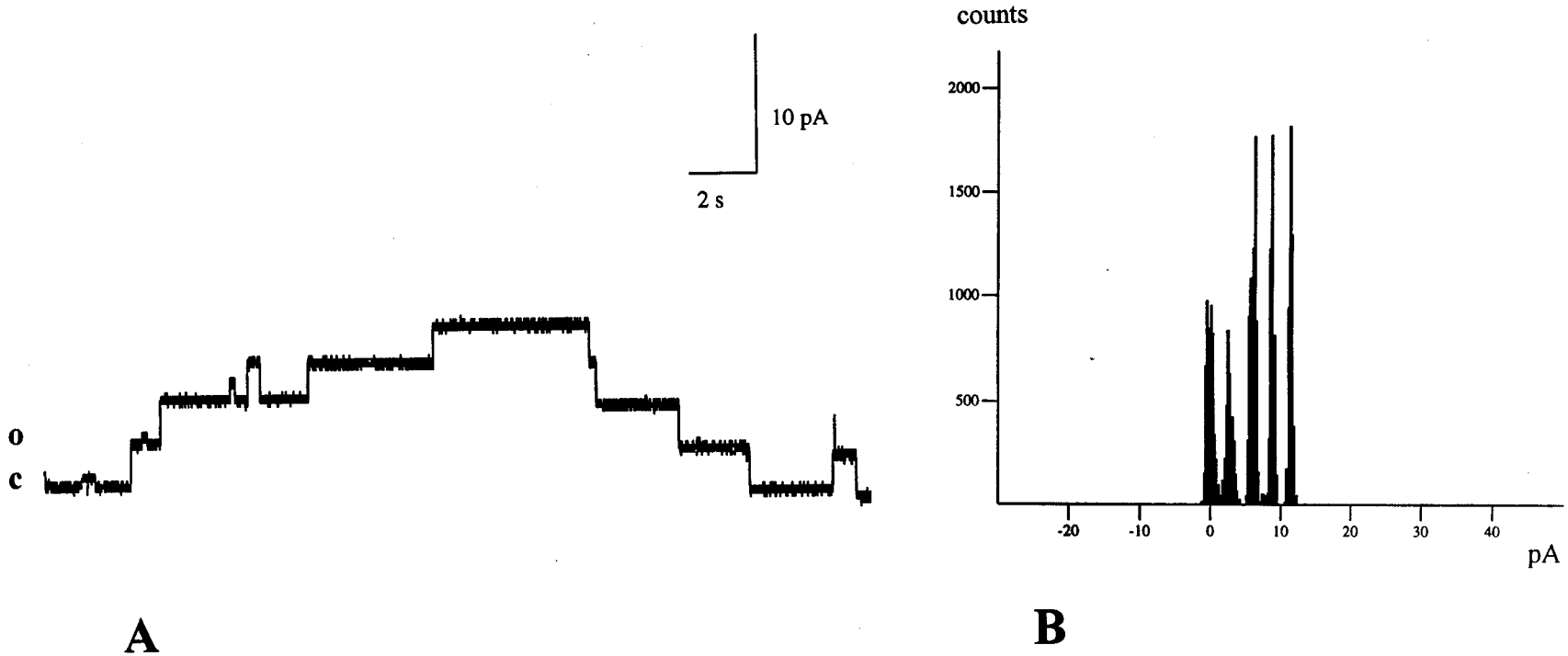

Figure 3 (A) Single-channel traces induced by the New *N*′-biotinyl(EDA)[15]-Gramicidin A analog (**1e**, 10^{-10} M) in a DPhPC bilayer. Electrolyte: 1 M KCl; applied voltage: 100 mV; digitization rate: 1000 Hz; filter: 200 Hz. 'o' for open state; 'c' for closed state. (B) The associated amplitude histogram.

matography: R_f: 0.45 and FAB$^+$ Mass spectrometry, **1c**, $[M + Na]^+ = m/z$ 2324.

Deprotection

The resulting N_α-Fmoc biotinylpeptide (14 mg) was added to a DMF solution (5 ml) containing 20% piperidine and stirred for 30 min. After solvent evaporation, the remaining material was dissolved in MeOH, treated with 1N HCl and the precipitate filtered, washed with water, saturated aqueous $NaHCO_3$ and water then dried under vacuum over P_2O_5. This peptide **1d** (yield 90%) was analyzed by TLC-silica gel chromatography, R_f: 0.3 and FAB$^+$ Mass spectrometry, **1d**, $[M + H]^+ = m/z$ 2080.

N_α-Formylation

The deprotected peptide (10 mg) was solubilized in 0.5 ml HCOOH and kept at 0°C. After 15 min, 0.15 ml Ac_2O was added and the solution stirred for 30 min at 0°C and 4 h at 20°C. The solution was evaporated under vacuum and the residue taken in MeOH and purified from the residual free amine starting peptide through a Dowex 50-X_2 resin column. The final purification step was done by a preparative TLC with the eluent already used (R_f: 0.4). The target peptide purity was checked by analytical HPLC showing one single peak with a retention time (R_t) = 6.67 min under a 1 ml/min flow of the given eluent: MeOH 68%, AcCN 16%, Water 16%. By FAB$^+$ Mass spectrometry, the correct protonated molecular ion of **1e** was detected at $[M + H]^+ = m/z$ 2108.

Reconstitution in Planar Lipid Bilayers

Single-channel experiments were carried out with Montal–Mueller type bilayers [9] formed over a hole (ø = 150 μm) in a Teflon film (thickness: 10 μm) pretreated with hexadecane/hexane (1:40, v/v) separating two half glass cells. Pure diphytanoylphosphatidylcholine (DPhPC) from Avanti Polar Lipids (Alabaster, AL, USA) was used. The electrolyte solutions were 1 M KCl or 1 M CsCl (Merck). The bulk concentration of the re-incorporated peptides (dissolved in methanol) was 10^{-10} M. Bilayer formation was monitored by the capacitance response. The current fluctuations were recorded using a BLM 120 amplifier (Biologic) and stored on a DTR 1202 (Biologic). Recorded signals were transferred to a computer for analysis (amplitude histogram) using a software (Satori) from Intracell (Royston, UK).

RESULTS AND DISCUSSION

After several attempts to synthetize the C-terminal amine from the C-terminal alcohol of the natural peptide (using Mitsunobu reaction, nucleophilic substitution, etc.), a total solid phase synthesis was chosen to obtain this gramicidin analog.

We selected the acid-labile 2-chlorotrityle polystyrene resin, suitable for anchoring by one of the two functions of the diamines or diols [10,11], for the following reasons:

The starting diamine (EDA) can be used *per se* owing to the steric hindrance of the bulky triaryl linker which strongly decreases the cross linking risk if EDA is present in excess.

The resulting supported 2-Cl-trityl-amino group, easily cleaved by a gentle acidolysis allows a Fmoc strategy for the stepwise peptide synthesis without protection of the ^{in}NH-Trp function. The Fmoc-pentadecapeptide was obtained using a Perseptive synthesizer and starting from 0.11 mmol of resin dispersed among glass beads and properly loaded (0.9 meq/g) to avoid aggregation problem due to the hydrophobicity of the peptide.The coupling reagent was the very efficient HATU [12] in DMF containing 0.6 M DIEA. The cleavage of the peptidyl-resin yielded **1b** peptide and was performed before the formylation of the Val^1 because the formyl group is more sensitive to the acidolysis than the N_α-Fmoc group. **1c** was obtained after biotinylation of **1b**, using the active *N*-hydroxysuccinimidyl ester of biotin. As we were already aware of aggregation problems, like dimerization in double helix taking place with the dissolved peptide and impairing the chromatographic purification step of gramicidins [13] the Fmoc-derivatives **1b** and **1c** (after biotinylation of **1b**) were purified by preparative TLC. Then the Fmoc deprotection of **1c** gave **1d** peptide, which conducted to **1e** after formylation and HPLC purification. The final product showing one single peak in HPLC analysis (Tr = 6.67 min) was characterized by ^{1}H NMR analysis at 250 MHz and Mass spectrometry in the positive FAB mode (see Materials and Methods section).

Reconstitution experiments in planar lipid bilayers were carried out with both gramicidin A and the *N′*-biotinyl-(EDA)^{15}-gramicidin A. When the gramicidin A was added to both compartments of the measurement cell *(cis/trans)*, it induced discrete current fluctuations corresponding to conductance values of 27 ± 3 ps and 50 ± 5 ps, in 1 M KCl (Figure 2) and in 1 M CsCl, respectively. In the same experimental conditions, peptide **1e** gave conductance

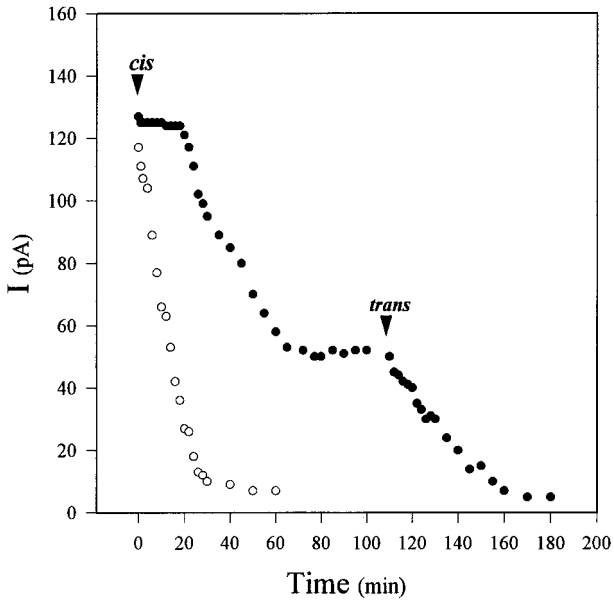

Figure 4 Action of streptavidin on channels induced by the new *N*′-biotinyl-(EDA)^{15}-gramicidin A analog (**1e** at 10^{-11} M) in a DPhPC bilayer: kinetics of channels closure. Both curves show the resulting current (under +80 mV) after addition of streptavidin (1/100, **1e**/streptavidin) as a function of time, (O) for a simultaneous addition of streptavidin (at $t = 0$ min) in *cis* and *trans* sides of the measurement cell. (I) for a first addition of streptavidin in the *cis* side at $t = 0$ min and a second addition in the *trans* side at $t = 110$ min. Arrows show the time of streptavidine addition. Mean error on current values: ±3 pA.

values of 30 ± 3 ps in KCl 1 M (Figure 3) and 55 ± 5 ps in CsCl 1 M. The ohmic response to the applied voltage showed that this ionophore behaviour was not voltage-dependent.

The conductance values obtained for the gramicidin A agree with those found by Koeppe *et al.* [14], (50 ps in 1 M CsCl in same lipid) and Urry *et al.* [15], (26 ps for 1 M KCl). Moreover the anchoring of the biotinyl function does not seem to modify the conductance values and the non-voltage dependence of the channel. Other studies have already shown that a replacement of ethanolamine by a methylamine [4] or by a β-alanine amide [3] at the C-terminal part or its extension [7,16] does not affect channel conductance. However, each modification affecting the N-terminal part has major effects on gramicidin conductance. For instance, the single-channel conductances are reduced by 50% (except in CsCl electrolyte) when the desformylgramicidin is acetylated [17].

In other respects, the effect of the streptavidin–biotin interactions on the gramicidin channel was investigated by conductance measurements. When the peptide **1e** (10^{-11} M) was added to both compartments, a steady-state current was developed after 20 min stirring, and reached a level indicating the presence of 50 active channels in the bilayer ($t = 0$ min, applied voltage +80 mV) (Figure 4).

Interestingly, addition of streptavidin to one side of the bilayer (*cis* side) in a 1/100 (peptide/streptavidin) molar ratio induced a decrease in the membrane current. The latter was stabilized after 60 min, but a complete current shutdown required a new addition of streptavidin (10^{-9} M) to the *trans* side compartment. In these conditions, the current was abolished within 50 min. In contrast, when streptavidin was simultaneously added to both compartments, the whole shutdown occured in less than 50 min. Control experiments with gramicidin A in the bilayer showed that streptavidin did not affect channel activity.

Thus, the interactions between *N*′-biotinyl-(EDA)^{15}-gramicidin A and the streptavidin was shown by the decrease of the membrane conductance when the ligand was added. Our data confirm the impedance spectroscopic measurements observed by Cornell *et al.* [2] with a biotinyl-gramicidin analog which is different from peptide **1e** by a long hydrocarbon chain inserted between gramicidin and biotin via an ester bond. In the same study, the authors proposed an extinction of the channel activity based on the disruption of the channel-forming dimer of biotinylated-gramicidin upon streptavidin addition. Indeed, streptavidin which possess four biotin-binding sites would be able to form cross-linkings with two adjacent biotinalyted-gramicidin dimers inducing a strong constraint between the monomers and consequently disrupting these pore-forming dimers. In our case, the low concentration of **1e** (10^{-11} M) induced a low density of active dimers in the bilayer (about 50 ion channels in a 150 μm-diameter membrane). Thus, the mechanism of current extinction by disruption of conducting dimers consecutive to a cross linking of two adjacent dimers is rather unlikely. The channel closure would more probably be consecutive to the blockage of the pore mouth after binding of the large streptavidin molecule (68 kDa). The absence of the long hydrocarbon chain observed in the biotinylated-gramicidin previously described [2] could cause a lack of accessibility, decreases hence the probability to form the streptavidin–**1e** complex. This hypothesis would be supported by our observations, i.e. slow channel closure mechanism and strong streptavidin/**1e** ratio.

Summary, the steric hindrance of streptavidin–biotin complex, taking place probably at the pore mouth, could prevent the ion conductance.

CONCLUSION

We obtained by solid phase synthesis using the 2-chlorotrityl polystyrene resin a new $(EDA)^{15}$-gramicidin A which was easily grafted through a stable amide linker to the biotinyl moiety. It is worth noting that this procedure would be easily extended to other functionalized ligands such as carboxylic sugars (useful for lectin detection). Moreover, reconstituted in planar lipid bilayers, the biotinyl-gramicidin exhibits the same conductance properties as the native peptide. Finally, preliminary experiments clearly showed a modulation of the *N′*-biotinyl-$(EDA)^{15}$-gramicidin A channel activity by streptavidin, which demonstrates the detection of ligand–receptor interactions at a molecular scale. Therefore, a new functionally active gramicidin-based biosensor can be considered in the future.

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