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Gramicidin-based channel systems for the detection of protein–ligand interaction

Shiroh Futaki,[a,*] Youjun Zhang,[a,†] Tatsuto Kiwada,[a] Ikuhiko Nakase,[a] Takeshi Yagami,[b] Shigetoshi Oiki[c] and Yukio Sugiura[a]

[a]*Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan*
[b]*Division of Medical Devices, National Institute of Health Sciences, Setagaya-ku, Tokyo 158-8501, Japan*
[c]*Department of Molecular Physiology and Biophysics, Fukui Medical University, Yoshida-gun, Fukui 910-1193, Japan*

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Abstract—To detect protein–ligand interaction a gramicidin-based sensor was developed. Biotin was tagged to the C-terminus of gramicidin (Gram-bio **1**). The biotin-moiety, which faces the electrolyte, gave little effect on single-channel conductance. Streptavidin added to the electrolyte was detected by Gram-bio **1** through the monitoring channel current using the planar bilayer system. The suppression of macroscopic currents and the acceleration of their decaying time course were observed in a concentration dependent manner. In the single-channel level, however, no significant effect on the single-channel conductance and the open dwell time was observed upon addition of streptavidin. Therefore, streptavidin neither blocked the open channel nor changed the stability of the conducting dimer. Insertion of a linker between gramicidin and biotin did not change the streptavidin-sensitivity of the current reduction. We conclude that the binding of streptavidin to the Gram-bio **1** shifted the distribution of the complex from the membrane to the electrolyte and, thus, reduced the formation of conducting dimer of Gram-bio **1** in the membrane. Interaction of biotin with an anti-biotin antibody was also observed using this system, indicating that this system is applicable for the detection of protein–ligand interaction having a binding constant of $\sim 10^{8-9}$ M^{-1} or more. Both the adamantane-tagged gramicidin for detection of β-cyclodextrin and the Strep Tag-II-tagged gramicidin for detection of streptavidin (binding constant: $\sim 10^{5}$ M^{-1} or less) failed to respond. Thus, high-affinity ligands upon tagging to gramicidin render the gramicidin-based sensor able to execute as a real-time monitoring system for protein–ligand interaction.

1. Introduction

Artificial ion channels have attracted people to develop molecular devices that function as highly sensitive monitoring systems. Using ion channels is beneficial since an ion channel can detect a single molecule, which can be monitored readily using a single-channel recording electrophysiological system. There are few other analytical methods that can detect with such a high sensitivity. Two strategies have been adopted for developing novel artificial ion channels. One is de novo design of peptides and other synthetic molecules.[1,2] Any molecular architecture having novel characteristics could be designed that can not be attained by natural channels. However, it is not easy to make the designed molecules work as expected. Another approach, which would be more practical than the above approach, is to utilize the structure of natural ion channels. Modifications of alamethicin and gramicidin A are the examples of this approach.[3–7] Attempts to impart novel characteristics to these channels have been reported. The well-defined characteristics of these channel peptides would help the assessment of the channel function thus created.

A channel-forming antibacterial peptide, alamethicin, is a helix-forming peptide. Several alamethicin molecules assemble into a helix bundle, among which an aqueous pore is formed (Fig. 1).[3] On the other hand, gramicidin A takes a characteristic structure called a β-helix. Two molecules of gramicidin A associate in the membrane with their N-terminal sections coming together. The diameter of a $\beta^{6.3}$-helix is large enough to form a pore to

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* Corresponding author. Tel.: +81-774-38-3211; fax: +81-774-32-3038; e-mail: futaki@scl.kyoto-u.ac.jp
† Present address: Chinese Academy of Agricultural Science, Beijing 100081, China.

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allow ions to permeate through the center of the helix. The peptide is composed of 15 amino acid residues and is easy to handle. In addition, the readily channel-forming nature of the peptide is well suitable as a pore module of the molecular device. Based on gramicidin, approaches have been reported to create ion channels in which the ion flux can be controlled by external stimuli, such as the binding of specific ligands and changes in the electrical transmembrane potential.[4]

It has already been reported that, using C-terminal biotin-tagged gramicidin, the interaction of streptavidin with biotin was reflected in the channel current levels.[6,7] In this report, we have further characterized the functions of this channel. We also show that the same concept is applicable to the detection of antigen–antibody interaction using the anti-biotin antibody and discuss the scope and limitation of this gramicidin-based sensing system.

2. Results and discussion

2.1. Dose-dependent suppression of the channel current by the addition of streptavidin

We have reported in a preliminary communication that the channel current of C-terminus biotinylated gramicidin was effectively suppressed by the addition of streptavidin to the electrolyte.[6] The channel current was recorded by the planar-lipid bilayer method using diphytanoylphosphatidylcholine as a lipid.[8] Figure 2 showes a typical channel current record of Gram-bio **1**,

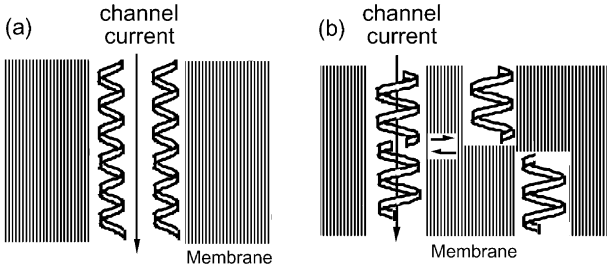

Figure 1. Channel formation of alamethicin (a) and gramicidin A (b).

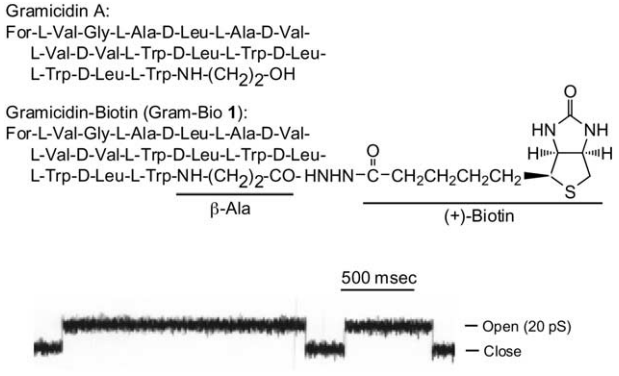

Figure 2. Structure and a single channel current recordings of the biotinylated gramicidin (Gram-Bio **1**) (For = formyl). Peptide concentration, 0.05 nM; applied voltage, +180 mV; electrolyte, 1 M KCl; lipid, diphytanoylphosphatidylcholine.

the conductance of which (20 pS in 1 M KCl at +180 mV) was very similar to that of natural gramicidin A (21 pS under the same condition). C-terminal biotinylation seems to have little effect on the channel characteristics.

Figure 3 shows a channel current recording of **1** where 10–25 channels randomly open in the membrane (i). By the addition of streptavidin (final concentration: 0.10 μM) on both sides of the membrane, the channel current was dramatically reduced to 18% in 1 min compared with the current levels before the addition of streptavidin (ii). After 20 min, almost all the channel current was suppressed (iii). On the other hand, addition of biotin hydrazide (final concentration: 18 μM) recovered the channel current level (iv). Complete suppression of the channel current was not observed when streptavidin was added only to one side of the membrane. Here, a reversible system with a response time of less than min was established.

We next examined the dose-dependency of channel current suppressed by streptavidin (Fig. 4). With the addition of streptavidin (0.13 μM), current level was reduced within 30 s to less than 10% of that observed before the addition of streptavidin. As the streptavidin concentration decreased, the time course of the current suppression was delayed. At a streptavidin concentration of 0.02 μM, almost one h was necessary to gain the similar extent of reduction.

2.2. The mechanisms of channel current suppression

We next focused on the suppression mechanisms of the channel current by the addition of streptavidin. Our initial hypothesis was that streptavidin blocks the open channel upon binding to the biotin moiety of the channel molecule. To obtain reliable kinetic data an experimental condition that only a single channel appears predominantly during recordings was employed. Single-channel current recordings of the Gram-Bio **1** in the absence and presence of streptavidin are shown (Fig. 5). The single-channel current amplitude was obtained from the histogram. In 1 M CsCl, a higher conductance (37 pS at +180 mV) was recorded compared to that in 1 M KCl (20 pS). Addition of streptavidin (1.6 nM) changed neither the single-channel amplitude nor open-channel current fluctuations.

The open dwell-time that represents the lifetime of the gramicidin in conductive dimer was analysed using the QuB program.[9] Current recordings with overlapping multiple channels were eliminated from the analysis. Dwell-time data were collected from a continuous current recordings of ~15 min. The open dwell-time (τ_o) of the Gram-Bio **1** (0.07 nM) in the absence of streptavidin was 437 ms, and in the presence of streptavidin (1.6 nM) was 412 ms (in the first 13 min after the addition of streptavidin) and 490 ms (in the second 13 min after the addition). When 8.3 nM of streptavidin was used, the current disappeared within 22 min after the addition of streptavidin. The τ_o in the absence and in the presence of streptavidin was 459 and 465 ms, respectively. These data suggest that the stability of Gram-Bio **1** is not

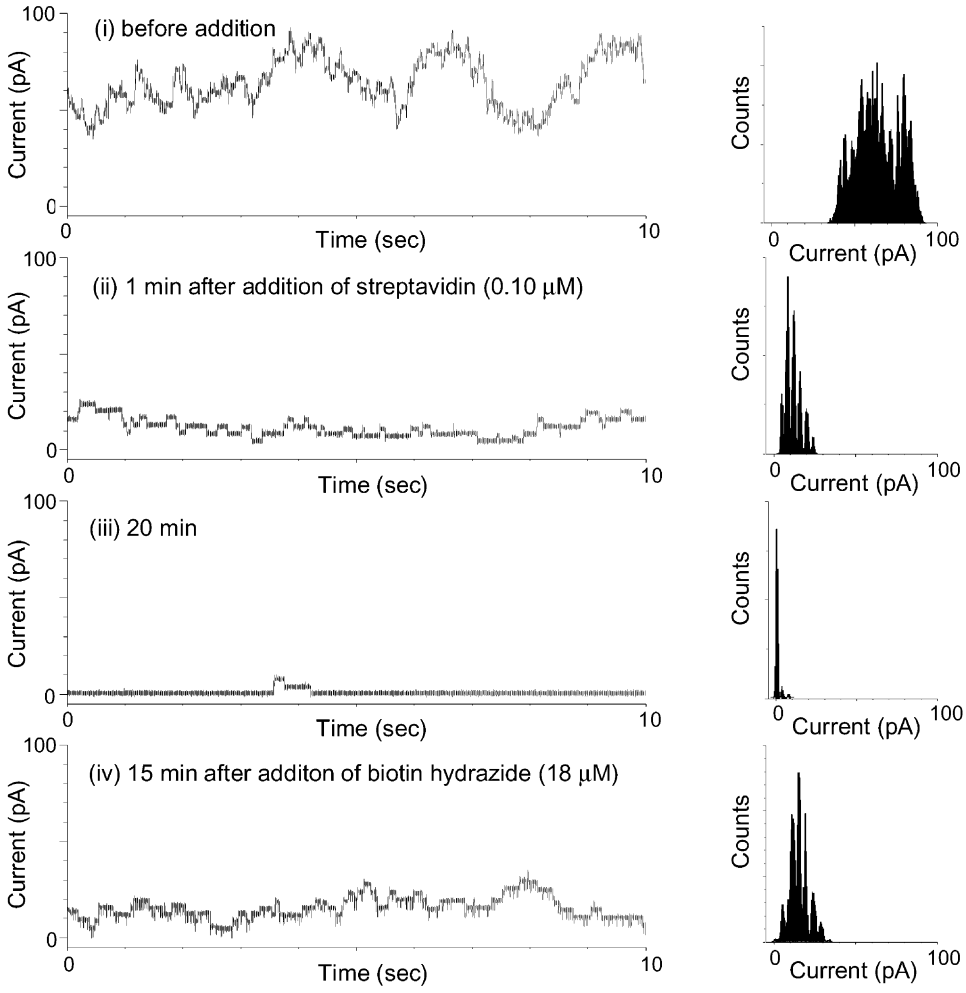

Figure 3. The effect of streptavidin on the channel current of Gram-Bio **1**: (i) before addition of streptavidin; (ii) 1 min and (iii) 20 min after addition of streptavidin (0.10 μM); (iv) recovery of the channel current by biotin hydrazide (18 μM) (15 min after addition of biotin hydrazide). The current histograms are shown at the right of the respective records. Peptide concentration, 0.1 nM; electrolyte, 1 M KCl; applied voltage, +180 mV.

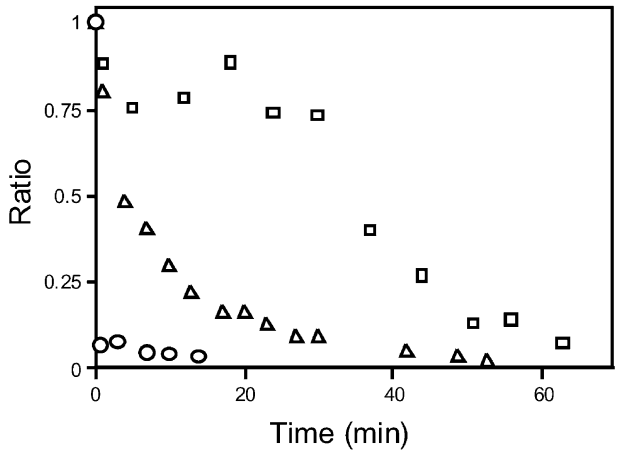

Figure 4. Dose-dependent suppression of the channel current of Gram-Bio **1** by streptavidin. The ordinate: the ratios of the total electric charges carried by the channel during 60-s recordings over those just before addition of streptavidin. Abcissa: time after addition of streptavidin. Streptavidin concentration: 0.13 μM (open circle); 0.07 μM (open triangle); 0.02 μM (open square). Peptide concentration, 0.1 nM; electrolyte, 1 M KCl; applied voltage, +190 mV.

affected by the attachment of streptavidin. Two possibilities remain: Upon streptavidin binding, the lateral diffusion of monomeric Gram-Bio **1** is suppressed; thus, the association rate for dimer formation is reduced; Hydrophilic streptavidin, upon forming a complex with Gram-Bio **1**, shifts the distribution of the complex between the lipid and aqueous phases; thus, the effective concentration of the complex in the membrane phase is reduced.

To obtain more information on the suppression mechanisms, a gramicidin–biotin conjugate with a longer linker was prepared in which an aminohexanoic acid was incorporated into **1** as an extra linker (Gram-ahex-Bio **3**; Fig. 6). The conjugate **3** was similarly prepared as in the case of **1**. Shortly, the gramicidin segment **2** was prepared by the Fmoc-solid-phase peptide synthesis[10] (Fmoc = 9-fluorenylmethyloxycarbonyl) using the 2-chlorotrityl resin[11] as reported.[6] As stated above, the C-terminal ethanolamine of gramicidin A was replaced with β-alanine. Biotinamidohexanoic acid hydrazide was then coupled with this β-alanine. Puri-

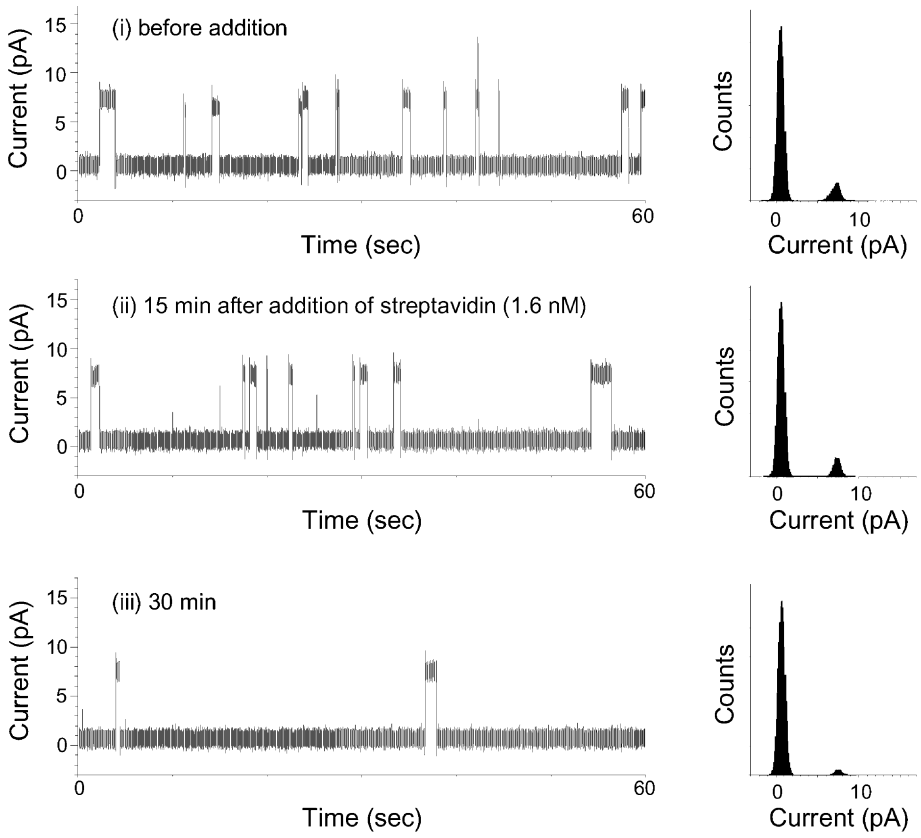

Figure 5. The single-channel current of Gram-Bio **1**: (i) before addition of streptavidin; (ii) 15 min and (iii) 30 min after addition of streptavidin (1.6 nM). The current amplitude histograms are shown at the right of the respective records. Single-channel current amplitudes obtained from the histograms, 6.8 pA (before addition of streptavidin), 6.6 and 6.8 pA (15 and 30 min and after addition of streptavidin); peptide concentration, 0.07 nM; electrolyte, 1 M CsCl; applied voltage, +180 mV.

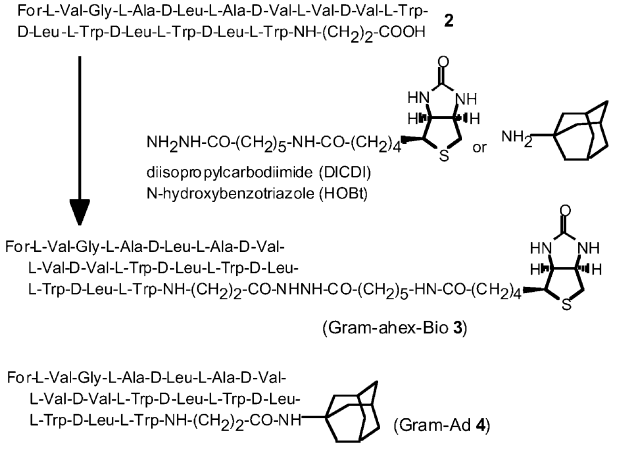

Figure 6. Preparation of Gram-ahex-Bio **3** and Gram-Ad **4**.

fication of the sample by high performance liquid chromatography (HPLC) gave pure **3**. The fidelity of the product was confirmed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS).

The conductance of **3** was identical with that of **1** (22 pS in 1 M KCl, 35 pS in 1 M CsCl). By the addition of streptavidin (0.13 nM), the channel current caused by the peptide (0.08 nM) was significantly suppressed in 1 min (Fig. 7), the sensitivity of which is similar to that of **1**. The aminohexanoic acid linker will give more freedom in lateral diffusion to **3** when attached with streptavidin. Since little difference in the sensitivity to streptavidin was observed for **1** and **3**, we concluded that redistribution of streptavidin-bound channel to the aqueous phase, which leads to the reduction of effective concentration in the membrane phase, causes the current suppression.

Suarez has also reported channel current reduction by streptavidin using another C-terminal biotinylated gramicidin.[7a] Rokitskaya reported that a similar kind of inhibition was observable even when the longer linker (the pentamer of aminohexanoic acid) was employed between gramicidin and biotin.[7b] As Suarez pointed out, addition of streptavidin to both sides of the membrane was necessary for complete suppression of the channel current formed by **1** (data not shown). The issue remains unanswered and further study is necessary.

2.3. Scopes and limitations of the detection of protein–ligand interactions using gramicidin-based channel systems

We next examined the applicability of the above gramicidin-based system for the detection of protein–ligand interactions. As the binding constant between streptavidin and biotin is as high as 10^{13} M^{-1} or more,[12] the binding is almost irreversible. To establish the wider

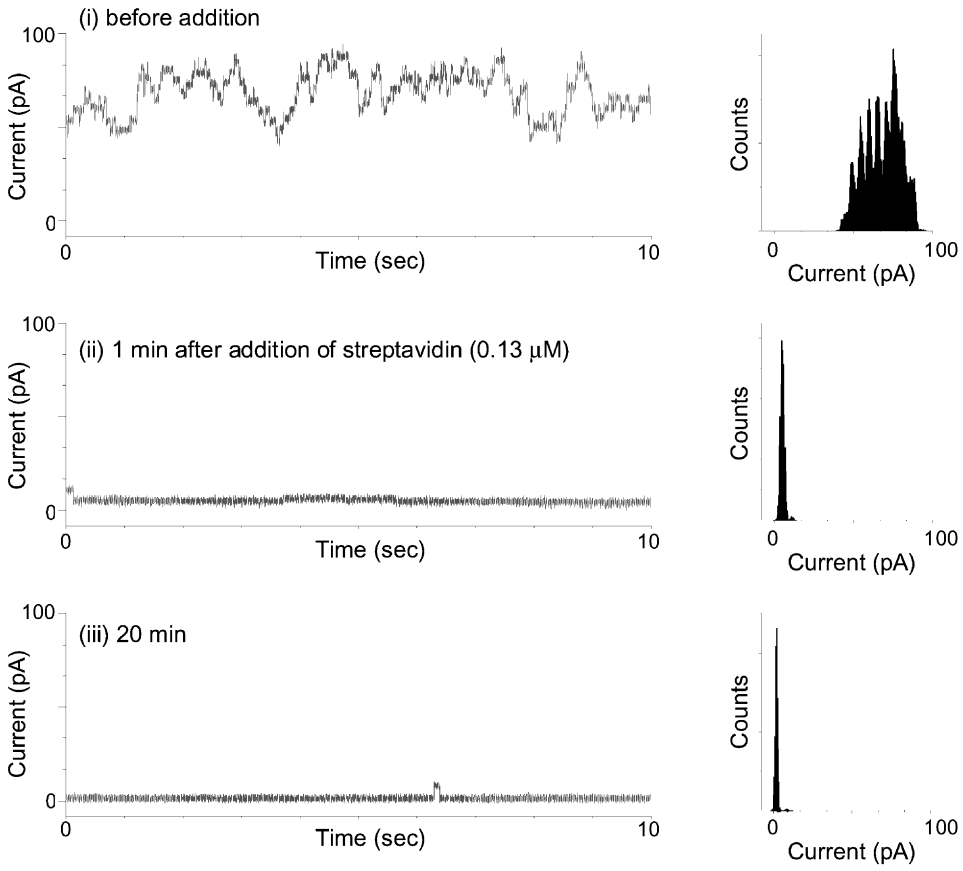

Figure 7. The effect of streptavidin on the channel current of Gram-ahex-Bio **3**: (i) before addition of streptavidin; (ii) 1 min and (iii) 20 min after addition of streptavidin (0.13 μM). The current amplitude histograms are shown at the right of the respective records. Peptide concentration, 0.08 nM; electrolyte, 1 M KCl; applied voltage, +180 mV.

applicability of these systems, we first examined whether the binding of an antibiotin antibody to the biotin was monitorable using the above system. Using an alamethicin-based system, we have already shown that the system works to detect an antigen–antibody interaction.[5i] As was used in the alamethicin–biotin system, goat polyclonal antibody (Vector Laboratories SP-3000, affinity purified) was used as the antibody. As shown in Figure 8, the addition of the antibody (0.13 μM) to both sides of the membrane suppressed the ion channel current of Gram-Bio **1**. The suppression by the antibody seemed to be less effective than that by streptavidin, because a longer time was necessary to obtain the same extent of suppression by streptavidin. The effectiveness of the antibody on the channel current suppression seemed to be less than that of streptavidin. This may be because the binding constant of the antibody to the ligand is, in most cases, not as high as that of streptavidin to biotin. Moreover, the antibody used in this report was a polyclonal antibody, a mixture of the antibody of various binding titers. The difference in the molecular weight between streptavidin and the antibody may affect the rate of diffusion in the electrolytes and cause the difference in the effectiveness of their complex formation with biotin, especially when we consider the pulling-out model as the reason of the current suppression. Even though there is room for improvement, the above result exemplified that the antigen–antibody interaction was detectable in real time through this system. The binding constant of an antigen–antibody interaction is generally speaking around 10^{8-9} M^{-1}. The result suggested that this system is applicable to the detection of a ligand–receptor interaction with a binding constant of this range.

To extend the applicability of this approach for the detection of weaker interactions, we prepared two more analogues of gramicidin. The first one bore the 1-adamantamine moiety on its C-terminus (Gram-Ad **4**). The adamantanamine moiety was reported to form a complex with β-cyclodextrin in water with a binding constant of $\sim 10^5$ M^{-1}.[13] The peptide was prepared by the condensation of the gramicidin segment **2** with 1-adamantamine. The other peptide had the StrepTag-II sequence[14] on its C-terminus (For-D-Val-Gly-D-Ala-D-Leu-D-Ala-D-Val-D-Val-D-Val-D-Trp-D-Leu-D-Trp-D-Leu-D-Trp-D-Leu-L-Trp-NH-$(CH_2)_2$-CONH-Asn-Trp-Ser-His-Pro-Gln-Phe-Glu-Lys-$CONH_2$ Gram-StrepTag **5**). The segment was reported to show affinity for streptavidin even when the segment was placed at the C-terminus of the fusion proteins.[14] The binding constant between streptavidin and the StrepTag-II segment was reported as 2.7×10^4 M^{-1}. The peptide was prepared by the conventional Fmoc-solid-phase synthesis on the Rink amide resin.[10] In the presence of β-cyclodextrin (10 mM) or streptavidin (0.27 μM), no significant suppression of the channel current for these peptides (0.1 and 0.2 nM, respectively) was observed (Fig. 9).

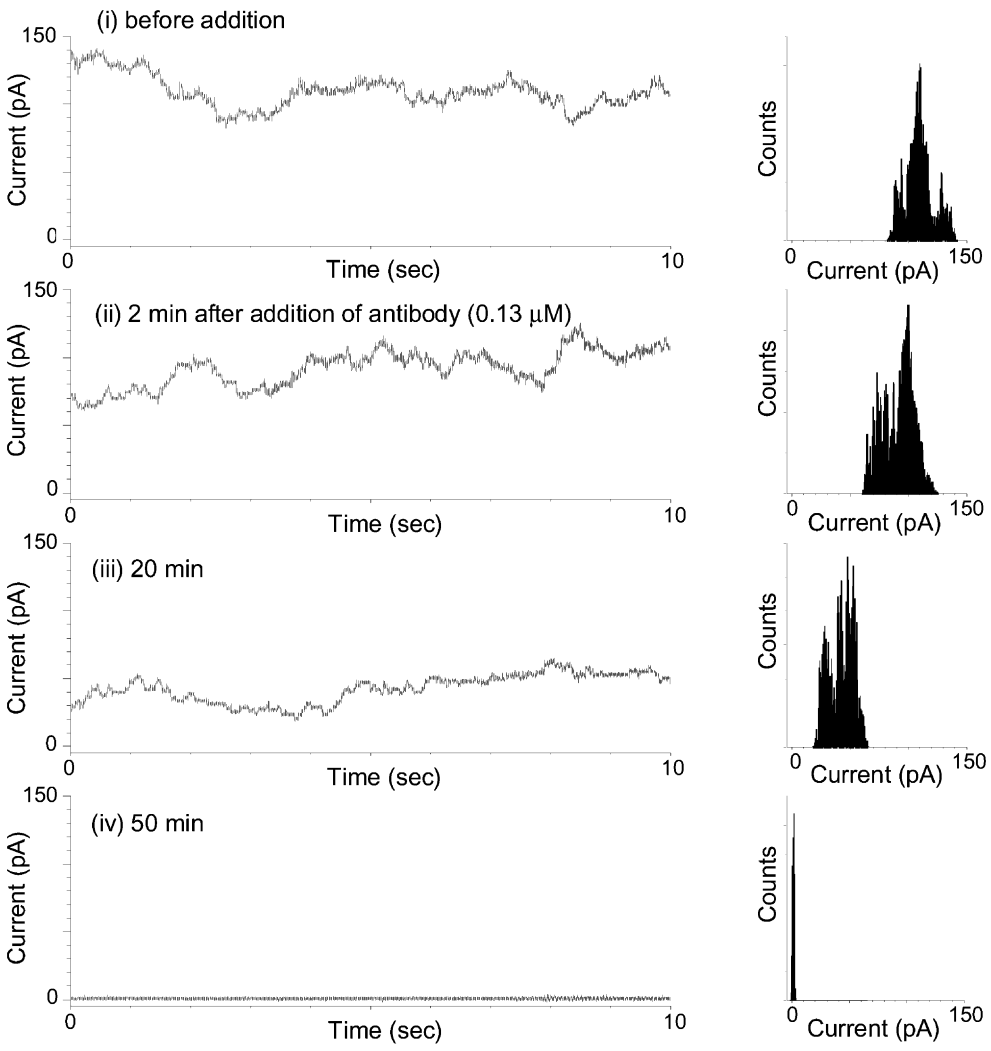

Figure 8. The effect of an anti-biotin antibody on the channel current of Gram-Bio **1**: (i) before addition of the antibody; (ii) 2 min, (iii) 20 min and (iv) 50 min after addition of the antibody (0.13 μM). The current amplitude histograms are shown at the right of the respective records. Peptide concentration, 0.1 nM; electrolyte, 1 M KCl; applied voltage, +150 mV.

3. Conclusion

Using the above gramicidin-based systems, we have shown that the interaction of biotin with streptavidin and an anti-biotin antibody could be detected in real time. The concept can be extended to the real-time detection of various biological interactions having binding constants in the 10^{8-9} M^{-1} range. On the other hand, we were not able to detect interactions having binding constants in the range of 10^5 M^{-1} or less. Further improvement of the apparatus may contribute to an increase in sensitivity. For example, we used a Teflon electrolyte chamber having a volume of 1.5 mL. Reduction of the volume may facilitate the interaction of proteins in electrolytes to the ligands attached to the channel molecules, or may prevent the possible adsorption of the attaching proteins to the apparatus. The results obtained in this study will provide valuable information on the design of artificial ion channels systems.

4. Experimental

4.1. Preparation of Gram-ahex-Bio 3

The peptide segment **2** was prepared using the Fmoc-solid-phase synthesis on 2-chlorotrityl resin as reported. Then the segment 2 (5 mg, 2.6 μmol) was dissolved in dimethylformamide (DMF)-1-methyl-2-pyrrolidone (NMP) (5:1) (120 μL) and treated with biotinamidohexanoic acid hydrazide (Sigma) (2.5 mg, 6.7 μmol), 1-hydroxybenzotriazole (HOBt) (1.0 mg, 7.4 μmol), and diisopropylcarbodiimide (DICDI) (4 μL, 26 μmol) overnight. HPLC purification of the product gave the desired peptide **3** (0.24 mg, 4% from **2**). MALDI-TOFMS, 2264.0 $(M+H)^+$ (theoretical, 2264.3).

4.2. Preparation of Gram-Ad 4

The segment **2** (3.5 mg, 1.8 μmol) was conjugated with 1-adamantanamine (0.31 mg, 2.1 μmol) in the presence of HOBt (0.27 mg, 2.0 μmol), and DICDI (1 μL, 6.4

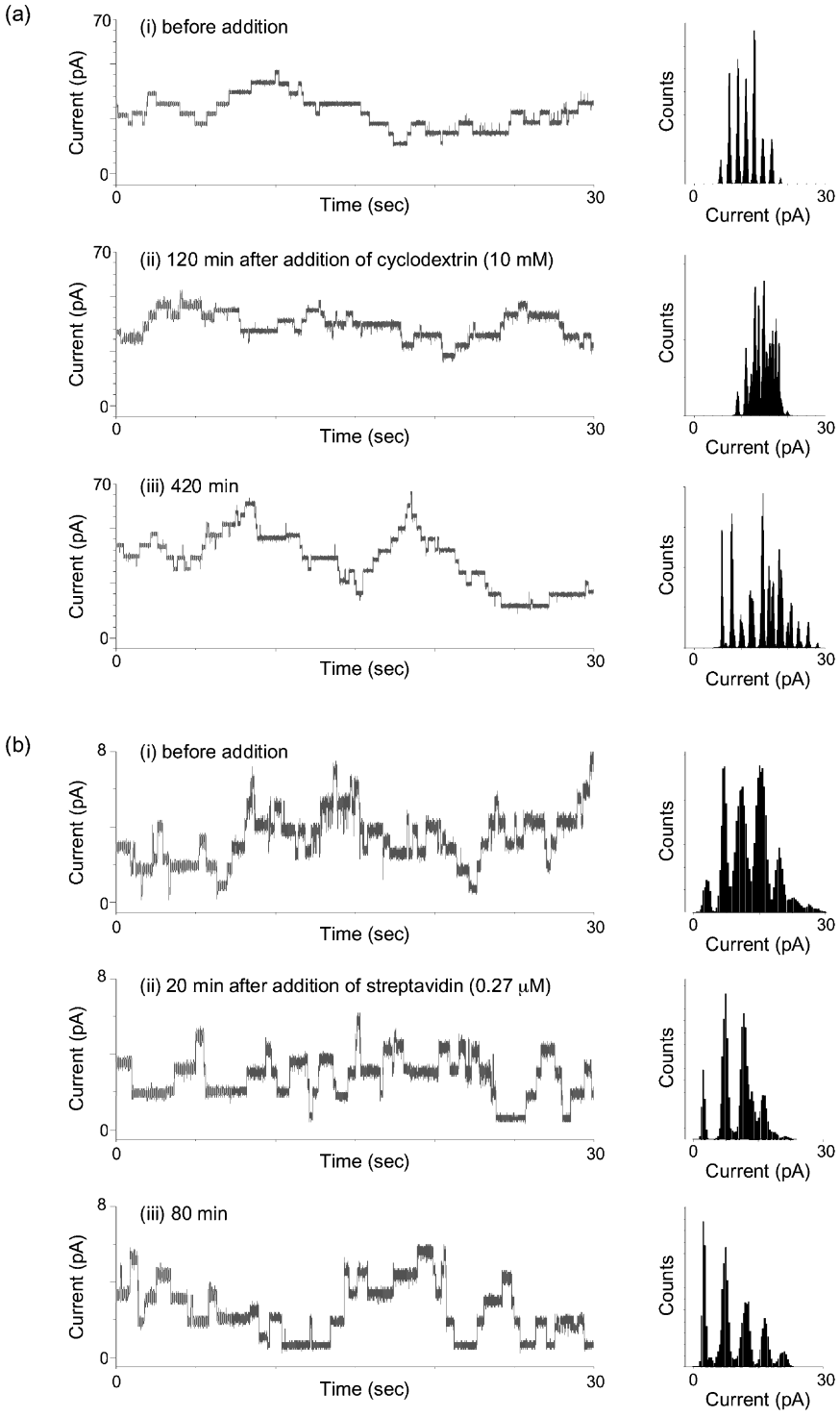

Figure 9. The effect of β-cyclodextrin on the channel current of Gram-Ad **4** (a): (i) before addition of cyclodextrin; (ii) 120 min and (iii) 420 min after addition of β-cyclodextrin (10 mM). Peptide concentration, 0.01 nM; electrolyte, 1 M KCl; applied voltage, +160 mV. The effect of streptavidin on the channel current of Gram-StrepTag **5**: (i) before addition of streptavidin; (ii) 20 min and (iii) 80 min after addition of streptavidin (0.27 μM). Peptide concentration, 0.2 nM; electrolyte, 1 M KCl; applied voltage, +180 mV. The current amplitude histograms are shown at the right of the respective records.

μmol) in DMF (100 μL). HPLC purification of the product gave the desired peptide **4** (0.25 mg, 7% from **2**). MALDI-TOFMS, 2067.9 $(M+Na)^+$ (theoretical, 2067.0).

4.3. Preparation of Gram-StrepTag 5

The peptide chain was constructed using a Shimadzu PSSM-8 synthesizer by its standard protocol of Fmoc-solid-phase synthesis on Rink amide resin. The benzo-

triazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP)[15]–HOBt coupling system was employed. N-Terminal formylation was conducted using 2,4,5-trichlorophenyl formate (Novabiochem) in the presence of NMM. The peptide resin was treated with trifluoroacetic acid (TFA)–ethanedithiol (95:5) for 2.5 h. HPLC-purification of the sample gave a pure peptide **5** (14% from the starting resin). MALDI-TOFMS, 3087.4 $(M+Na)^+$ (theoretical, 3086.6).

4.4. Channel activity measurements

Planar lipid bilayers were formed by the painting method.[8] Diphytanoylphosphatidylcholine (Avanti) dissolved in decane (20 mg/mL) was used as a bilayer-forming lipid. Electrolytes were unbuffered 1 M KCl or CsCl. All the measurements were done at 22 ± 1 °C. A small quantity of the peptides in methanol and proteins (or β-cyclodextrin) in distilled water (usually 1–10 μL) was added to the electrolytes at both sides of the membrane. The applied voltage was defined as the voltage of the *cis* side with respect to the compartment of the *trans* side. The membrane current was measured under voltage clamp conditions using 1 kHz filtering and sampling at 5 kHz using a Nihon Kohden CEZ-2400 patch-clamp amplifier with an Axon Digidata 1322A data acquisition system. The channel kinetics were analyzed using QuB (Research Foundation State University of New York, http://www.qub.buffalo.edu/).[9] Channel conductance is defined as the membrane current divided by the applied voltage.

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