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Monovalent and Multivalent Binding of Streptavidin to Biotinylated Gramicidin Affects the Kinetic Properties of the Ion Channel

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Abstract—Biotin–avidin (or streptavidin) high affinity binding has been widely applied as a universal tool for basic research as well as diagnostic and therapeutic purposes. Here we studied the interaction of streptavidin with ionic channels formed by biotinylated gramicidin in planar bilayer lipid membranes (BLM) using the method of sensitized photoinactivation. As shown previously, the addition of streptavidin leads to a profound increase in the lifetime (τ) of gA5XB, a biotinylated analog of gramicidin A with a linker arm of five aminocaproyl groups (Rokitskaya et al. (2000) *Biochemistry*, **39**, 13053-13058). The present study has revealed that the increase in τ is related to multivalent interaction of streptavidin with biotinylated gramicidin, i.e., to formation of a complex of streptavidin with several gramicidin channels, whereas binding of streptavidin to a single channel does not change the value of τ. A rather long linker arm attaching biotin to the C-terminus of gramicidin appeared to be required for the multivalent interaction of streptavidin with gramicidin channels, as the increase in τ was not observed with channels formed by gA2XB, the biotinylated gramicidin analog with a linker arm comprising only two aminocaproyl groups. However, the formation of a stoichiometric (1 : 1) complex of streptavidin with gA2XB apparently occurred. The multivalent interaction of streptavidin with gA5XB disappeared if biotinylated lipids were included into the diphytanoylphosphatidylcholine membrane. It is suggested that the slowing of gramicidin channel kinetics provoked by streptavidin binding is due to membrane-mediated elastic interactions between two neighboring channels.

Key words: biotin, sensitized photoinactivation, bilayer lipid membrane, aluminum trisulfophthalocyanine

Functioning of proteinaceous ion channels is known to be crucial for signal transduction across biological membranes. Being regulated via specific membrane receptors, it can be linked to formation and dissociation of ion channel clusters [1-3]. Interaction between clustered channels is modulated by the lipid environment and under certain conditions may result in their concerted opening and closing [4-14] and/or marked stabilization of an open channel state [15-22].

Recently we have shown that avidin and streptavidin can induce significant lengthening of the lifetime of channels formed by the pentadecapeptide gramicidin A modified by attaching a biotin group to its C-terminus through a spacer composed of five aminocaproyl groups (gA5XB) [20, 13]. The ability of tetrameric (strept)avidin for multi-site biotin binding suggests that the channel stabilization is caused by formation of a complex of (strept)avidin with two neighboring channels.

High-affinity binding of biotin to avidin and streptavidin in water solution is well studied and widely employed in both basic and applied research works [23-39]. Currently interaction of (strept)avidin with biotin attached to substances that can insert into membranes has attracted much attention [40-46], as it opens new possibilities for studying translocation of substances across membranes, as well as spatial organization of membrane components. (Strept)avidin binding of biotin groups located near the membrane surface has proved to exhibit certain peculiarities. In particular, it has been shown that

Abbreviations: BLM) bilayer lipid membrane; ROS) reactive oxygen species; τ) characteristic time of photoinactivation; PBS) phosphate buffer solution; gA2XB) biotinylated analog of gramicidin A with a spacer composed of two aminocaproyl groups; gA5XB) biotinylated analog of gramicidin A with a spacer composed of five aminocaproyl groups; biotin-PE) N-(biotinoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt; $AlPcS_3$) aluminum trisulfophthalocyanine.

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(strept)avidin affinity to biotin covalently linked to membrane lipids is substantially lower than that to free biotin [47], which provides reversibility of binding in this system [48]. Examination of the streptavidin/gA5XB model looks promising as an approach to study interaction between (strept)avidin and biotin immobilized at the membrane/water interface.

To prove the validity of explanation of the increase in gA5XB channel lifetime by two-site binding of the channels to streptavidin, we comparatively investigated the effect of streptavidin on channels formed by biotinylated gramicidins with long (gA5XB) and short (gA2XB) spacers, and examined consequences of the presence in membranes of biotinylated lipids competing with gA5XB for binding sites on streptavidin.

MATERIALS AND METHODS

Sensitized photoinactivation technique. Bilayer lipid membranes (BLMs) were formed from a 20 mg/ml solution of diphytanoylphosphatidylcholine (DPhPC, Avanti Polar Lipids, USA) or its mixture (if otherwise stated) with 1 or 10% (w/w) N-(biotinoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (biotin-PE, Molecular Probes, Inc., USA) in *n*-decane (Merck, Germany) by the brush technique [49] on a 0.5-mm diameter hole in a Teflon partition separating two compartments of a cell containing aqueous buffer solutions. The BLM current was measured under voltage-clamped conditions. The BLM voltage was 50 mV in most experiments. In all the experiments the solution was 1 M KCl, 10 mM Tris, 10 mM MES, 10 mM β-alanine, pH 7.0. The biotinylated analogs (generous gift from Prof. F. Separovic, University of Melbourne, Australia) of gramicidin A (Sigma, USA) with a biotin group attached to the C-terminus of gramicidin A through a linker arm comprising five (gA5XB) or two (gA2XB) aminocaproyl groups were added from stock solutions in ethanol to the bathing solutions at both sides of the BLM and routinely incubated for 15 min with constant stirring. Aluminum trisulfophthalocyanine ($AlPcS_3$) from Porphyrin Products (USA) was added to the bathing solution at the *trans*-side (the *cis*-side is the front side with respect to the flash lamp) at a concentration of 1 μM. After transformation to voltage, the BLM current was digitized by using a LabPC 1200 (National Instruments, USA) and analyzed using a personal computer with the help of WinWCP Strathclyde Electrophysiology Software designed by J. Dempster (University of Strathclyde, UK). BLMs were illuminated by single flashes produced by a xenon lamp with flash energy of about 400 mJ/cm^2 and flash duration <2 msec. To avoid electrical artifacts, the electrodes were covered with black plastic tubes.

Typical current traces after a flash of light have two components with characteristic times of <10 msec and about 1 sec [50, 51]. The fast phase, which normally comprises less than 10% of the photoresponse, apparently reflects inactivation of conducting dimers, while the main slow phase corresponds to the monomer–dimer equilibration after damage to gramicidin monomers. We believe that the process of the gramicidin damage includes generation of reactive oxygen species (ROS) due to interaction of excited photosensitizer molecules with oxygen and the attack by ROS of tryptophan residues in gramicidin molecules. The lifetime of ROS is less than 10 msec and the time for gramicidin damage is substantially less than the characteristic time of the major photoinactivation component, which is called below the characteristic time of photoinactivation τ (about 1 sec). Therefore, the current decay represents the relaxation after a concentration jump. The current trace after a flash of light can be described by a single exponential function:

$$J(t) = J_\infty + (J_0 - J_\infty)\exp(-t/\tau),$$

where J_0 and J_∞ correspond to the initial and the final value of the BLM current, respectively, and τ characterizes the photoinactivation kinetics (characteristic time of photoinactivation). According to our experimental data, τ appeared to be close to the gramicidin channel lifetime under a variety of conditions and exhibited similar changes. For example, both the characteristic time of photoinactivation and the single channel lifetime decreased upon increasing the temperature or substituting squalene for decane in the membrane-forming solution [50]. These parameters also displayed similar changes in response to variations of the membrane dipole potential by adding phloretin or 6-ketocholestanol [52-54].

Streptavidin binding by enzyme-linked assay. Streptavidin (S-Avi, batch 30212) and mutant avidin (A-Avi, batch E0328) were kindly provided by Dr. S. P. Domogatsky (IMTEK, Moscow). Shortly, for A-Avi preparation, the avidin gene with a deleted 5′-ended fragment coding 40 amino acids was cloned in *E. coli*. The expressed mutant protein was purified by ion-exchange and size-exclusion chromatography. S-Avi- and A-Avi-biotin binding were characterized by the enzyme-linked assay. These experiments can be divided into two groups.

1. Biotin binding activity assay. A 96-well plate was coated with biotinylated human albumin (20 μg/ml in 0.02 M bicarbonate buffer, pH 9.6, 100 μl per well) at 37°C for 2 h, then washed three times with 200 μl of the phosphate buffer solution (PBS) containing 0.05% Tween-20 per well. The well surface was blocked with 1% BSA/PBS for 30 min at room temperature, then the plate was washed three times. Aliquots of biotin (50 μl) diluted to different concentrations in PBS containing 1% BSA were added into wells and after that 50 μl of the avidin solution (2 μg/ml) in PBS was added into each well. The plate was incubated for 1 h at room temperature. After

three-time washing, biotinylated horseradish peroxidase was added and incubated for 1 h at room temperature. After three-time washing, the solution of *o*-phenylenediamine (OPD, Sigma) in citrate-phosphate buffer (0.4 mg OPD in 50 mM sodium citrate, 25 mM sodium phosphate, 0.012% H_2O_2, pH 5.0) was added and incubated at 37°C until the staining developed. The reaction was quenched by addition of 50 μl of 25% H_2SO_4 per well. Absorption was measured at 492 nm using Multiscan spectrophotometer (LKB, Sweden).

2. Binding of streptavidin to biotinylated gramicidin. A 96-well plate was coated with gA2XB or gA5XB (20 μg/ml in 0.02 M bicarbonate buffer, pH 9.6, 100 μl per well) at 37°C for 2 h, then washed three times with 200 μl PBS containing 0.05% Tween-20 per well. The well surface was blocked with 1% BSA/PBS at room temperature for 30 min, then the plate was washed three times. Streptavidin conjugated with horseradish peroxidase in different dilutions in 1% BSA/PBS was added and incubated for 1 h at room temperature. After three-time washing, the solution of *o*-phenylenediamine in citrate-phosphate buffer (0.4 mg OPD in 50 mM sodium citrate, 25 mM sodium phosphate, 0.012% H_2O_2, pH 5.0) was added and incubated at 37°C until the staining developed. The reaction was quenched by addition of 50 μl of 25% H_2SO_4 per well. Absorption was measured at 492 nm using Multiscan spectrophotometer (LKB).

According to the first approach, we measured the number of the biotin-binding sites on A-Avi and streptavidin. Data of the assay are shown on Fig. 1. Standard streptavidin (S-Avi) has biotin-binding activity 14 U that is equivalent to 4 binding sites per molecule (units equal micrograms of biotin bound to 1 mg of avidin). Activity of A-Avi is 6.8 U that is equivalent to 2 sites of biotin binding per molecule.

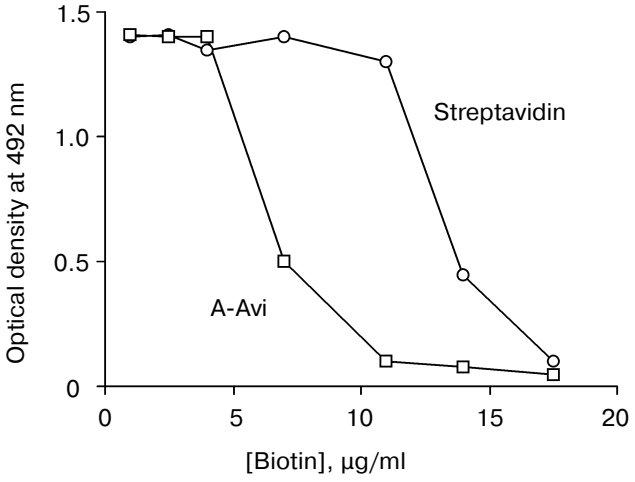

Fig. 1. Inhibition of the interaction of streptavidin and mutant avidin, A-Avi, with biotinylated albumin adsorbed on an immunological plate by biotin. The activity of A-Avi was 6.8 U, while that of streptavidin was 14 U.

RESULTS AND DISCUSSION

As shown in our previous paper, the addition of streptavidin leads to the profound increase in the lifetime (τ) of gA5XB, a biotinylated analog of gramicidin A with a linker arm of five aminocaproyl groups, whereas τ of gA2XB, which has a shorter linker (two aminocaproyl groups), remains unaltered [20]. It was concluded that streptavidin does not bind to gA2XB. Figure 2 shows, however, that the addition of streptavidin to the membrane-bathing solution led to a sharp decrease in the macroscopic current induced by gA2XB across BLM. Subsequent addition of an excess of biotin restored partially the BLM current (Fig. 2). This result supports the idea of direct interaction of gA2XB with streptavidin. However, these data do not necessarily mean that the BLM current in the presence of streptavidin flows via gA2XB–streptavidin complexes because binding of streptavidin to gA2XB can lead to desorption of the complex from the membrane, and the observed BLM current can be determined by free gA2XB channels. Further experiments, however, do not favor that hypothesis.

Figure 3 shows the kinetics of the flash-induced decrease in the BLM current in the absence of streptavidin (curve *1*), and after the addition of 8 nM streptavidin (curve *2*), followed by the addition of an excess of biotin (curve *3*). The addition of streptavidin caused a substantial decrease in the amplitude of the photoinactivation kinetics (α) suggesting the appearance of ROS quenchers near the channels, i.e., the formation of gA2XB–streptavidin complex. This conclusion is based on the estimation showing that the mean diffusion distance of ROS in our system is less than the bilayer width [55, 56]. It is significant that the parameter α represents the properties of the membrane-bound part of the gA2XB population. Remarkably, the effect of streptavidin on α was reversed by the addition of biotin, suggesting the release of streptavidin from the complex under the conditions of excess of biotin.

Figure 4 shows the same type of experiments as in Fig. 3, but with gA5XB instead of gA2XB. These kinetic curves had two major differences compared to those of Fig. 3: 1) the kinetics of gA5XB in the presence of streptavidin was markedly decelerated, and 2) the addition of biotin did not lead to the restoration of the initial amplitude of photoinactivation (6% in the presence of streptavidin and 6% after the addition of 20 μM biotin), although the photoinactivation kinetics was accelerated considerably upon addition of biotin. The latter result showed that the addition of biotin did not lead to desorption of streptavidin from the gA5XB channel. It is important that if biotin had been added before streptavidin, the

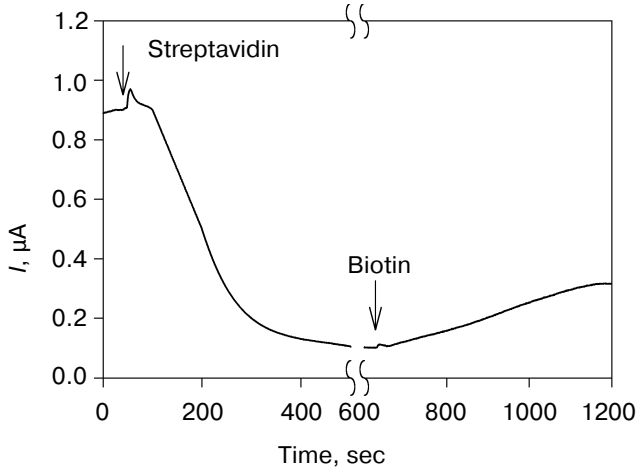

Fig. 2. Effect of the addition of streptavidin (8 nM) at the time indicated by the arrow on the gA2XB-mediated current through a BLM. After the current reached a new steady-state, 20 μM biotin was added on both sides of the BLM at the time marked by the second arrow.

protein affected neither the kinetics, nor the amplitude of the gA5XB photoinactivation, which implied that streptavidin did not bind to gA5XB under these conditions (data not shown).

From the biotin-induced reversal of the streptavidin effect on the photoinactivation kinetics, it can be concluded that biotin released streptavidin from gA5XB channels. On the other hand, biotin did not increase the amplitude of gA5XB photoinactivation suggesting that streptavidin remained bound to the channel. This apparent contradiction can be interpreted by taking into account that streptavidin is a tetramer capable of binding up to four biotin groups [25, 26]. It can be assumed that the decelerating effect resulted from the binding of several gA5XB channels to the same streptavidin molecule, whereas the decrease in the photoinactivation amplitude took place even upon single-site binding of biotinylated channels to the protein. The addition of biotin led to dissociation of a portion of channels from the protein which reduced the binding valence, but gA5XB channels remained still bound to streptavidin (the gA5XB/protein ratio was at least 1 : 1). This model can be applied also for channels formed by gA2XB. In this case, it should be assumed that the short linker prevented multivalent binding of the channels by streptavidin because of steric hindrance. Therefore, it becomes clear why the protein affected only the amplitude of gA2XB photoinactivation. The structural aspects of this model will be discussed in the following section.

This model is in accordance with the experiments performed on membranes containing PE derivative with covalently bound biotin group (biotin-PE). Figure 5a shows typical experiments with gA5XB-mediated current across bilayers containing 1% biotin-PE. The addition of

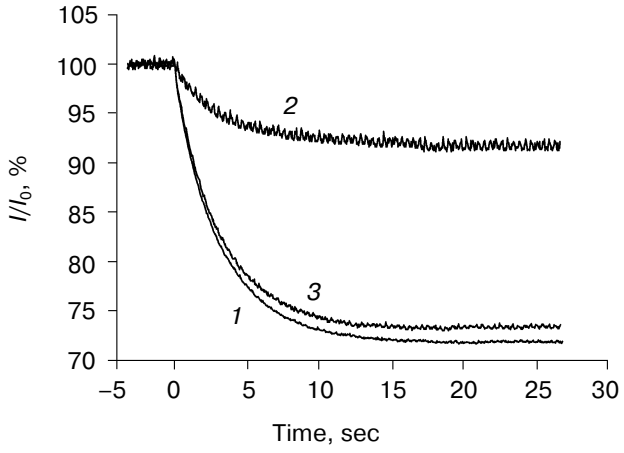

Fig. 3. Effect of streptavidin on the time course of the decrease in the gA2XB-mediated current across the BLM after a flash of visible light in the presence of 1 μM $AlPcS_3$. Curve *1* (control) is the time course of the current decrease in the absence of streptavidin (τ = 3.1 sec, α = 28%). Curve *2* was obtained after the addition of 8 nM streptavidin (on both sides of the BLM) (τ = 3.8 sec, α = 8.5%). Curve *3* was obtained after the addition of 20 μM biotin to the bathing solutions on both sides of the BLM in the presence of 8 nM streptavidin (τ = 3.1 sec, α = 27%). The normalized values of the current (I/I_0) are plotted versus the time (t). The initial value of the current (I_0) was approximately 1 μA. The BLM voltage was 50 mV.

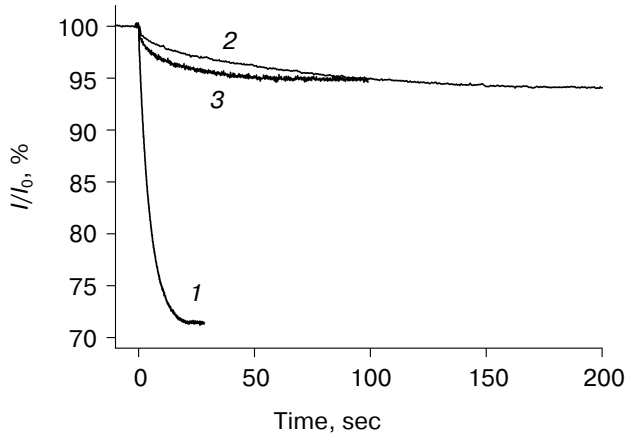

Fig. 4. Effect of streptavidin on the time course of the decrease in the gA5XB-mediated current across a BLM after a flash of visible light in the presence of 1 μM $AlPcS_3$. Curve *1* (control) is the time course of the current decrease in the absence of streptavidin (τ = 3.7 sec, α = 29%). Curve *2* was obtained after the addition of 8 nM streptavidin (on both sides of the BLM) (τ = 110 sec, α = 6%). Curve *3* was obtained after the addition of 20 μM biotin to the bathing solutions on both sides of the BLM in the presence of 8 nM streptavidin (τ = 14 sec, α = 6%). The normalized values of the current (I/I_0) are plotted versus the time (t). The initial value of the current (I_0) was approximately 1 μA. The BLM voltage was 50 mV.

streptavidin did not decelerate the kinetics of photoinactivation under these conditions, but decreased the amplitude of photoinactivation, thereby supporting the binding of gA5XB channels by streptavidin. To distinguish between the binding of biotinylated channels and biotin-PE to streptavidin, the control experiments with gramicidin A (lacking a biotin group) were performed for bilayers containing biotin-PE. As seen from Fig. 5b, the amplitude of photoinactivation remained unaltered after the addition of streptavidin (α was 18 ± 1% in the control and 20 ± 2% in the presence of streptavidin), although 10% of biotin-PE was present in the membrane in this case. The insensitivity of the amplitude of the gramicidin A photoresponse to the binding of streptavidin to the bilayer can be accounted for by long distances between gramicidins and bound streptavidin in the lateral direction (more than several nanometers) that prevent effective quenching of ROS interacting with gramicidin channels [55, 56].

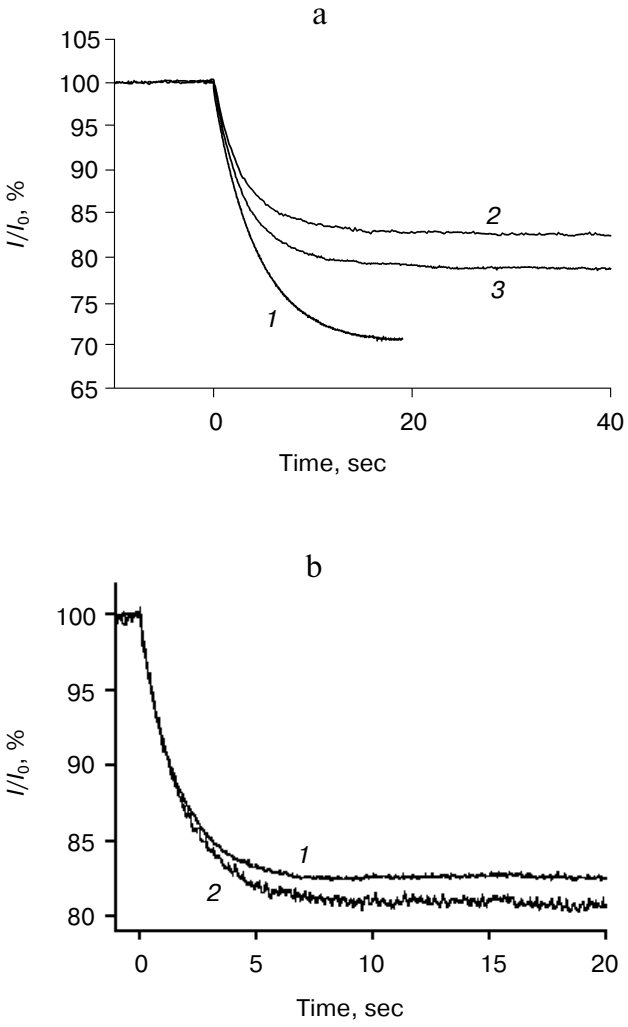

Fig. 5. a) Effect of streptavidin in the presence of 1% biotin-PE in the membrane-forming solution on the time course of the decrease in the gA5XB-mediated current across a BLM after a flash of visible light in the presence of 1 μM $AlPcS_3$. Curve *1* (control) is the time course of the current decrease in the absence of streptavidin (τ = 4.3 sec, α = 30%). Curve *2* was obtained after the addition of 8 nM streptavidin (on both sides of the BLM) (τ = 3.7 sec, α = 18%). Curve *3* was obtained after the addition of 20 μM biotin to the bathing solutions on both sides of the BLM in the presence of 8 nM streptavidin (τ = 3.5 sec, α = 21%). b) Control experiments showing the effect of streptavidin in the presence of 10% biotin-PE in the membrane-forming solution on the time course of the decrease in the gramicidin A-mediated current across a BLM after a flash of visible light in the presence of 1 μM $AlPcS_3$. Curve *1* (control) is the time course of the current decrease in the absence of streptavidin (τ = 1.6 sec, α = 17.5%). Curve *2* was obtained after the addition of 8 nM streptavidin (on both sides of the BLM) (τ = 1.7 sec, α = 19%). The normalized values of the current (I/I_0) are plotted versus the time (t). The initial value of the current (I_0) was approximately 1 μA. The BLM voltage was 50 mV.

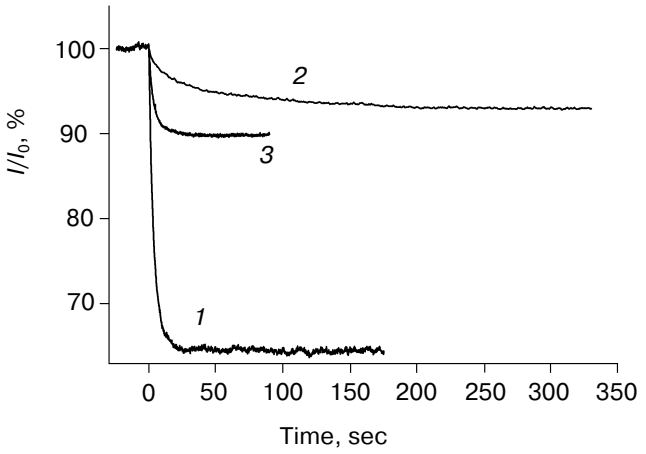

Fig. 6. Effect of mutant avidin (A-Avi) on the time course of the decrease in the gA5XB-mediated current across a BLM after a flash of visible light in the presence of 1 μM $AlPcS_3$. Curve *1* (control) is the time course of the current decrease in the absence of A-Avi (τ = 4.2 sec, α = 35%). Curve *2* was obtained after the edition of 10 nM A-Avi (on both sides of the BLM) (τ = 53 sec, α = 7%). Curve *3* was obtained after the addition of 20 μM biotin to the bathing solutions on both sides of the BLM in the presence of 10 nM A-Avi (τ = 4.4 sec, α = 10%). The normalized values of the current (I/I_0) are plotted versus the time (t). The initial value of the current (I_0) was approximately 1 μA. The BLM voltage was 50 mV.

Therefore, binding of the protein to the lipid part of the membrane did not protect gramicidin from the attack of ROS and consequently did not decrease the α value. Thus, the experiments with gA5XB channels (Fig. 5) suggested that even in the presence of biotin-PE, complexes of streptavidin with gA5XB channels were formed, though their kinetic properties were the same as in the control without the protein. These results are consistent with the model assuming multivalent binding as a cause of deceleration of the photoinactivation kinetics, because multivalent binding is apparently impossible under the conditions of excess of biotin groups on the membrane surface.

Experiments with mutant avidin (A-Avi) showed that the binding of two channels by one streptavidin is enough

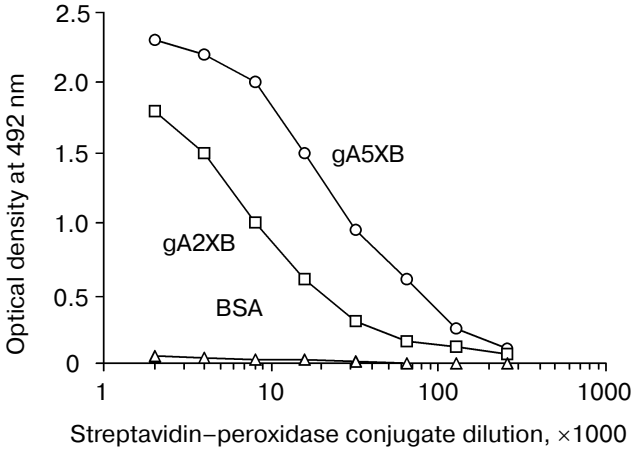

Fig. 7. Interaction of gA2XB and gA5XB adsorbed on an immunological plate with streptavidin–peroxidase conjugate.

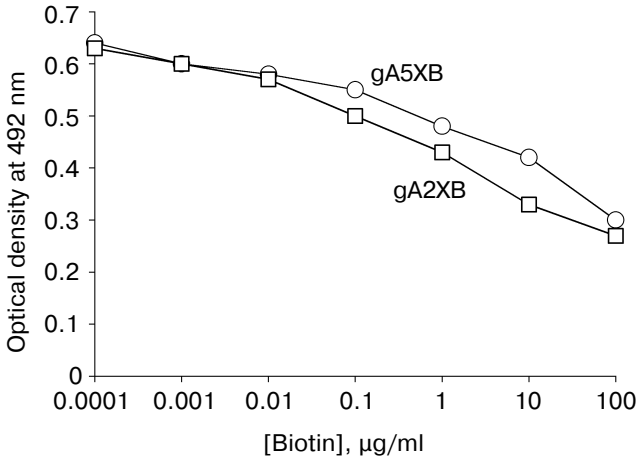

Fig. 8. Effect of biotin on the release of streptavidin–peroxidase conjugate bound to immobilized gA2XB or gA5XB.

to induce the deceleration of the channel kinetics. As shown in the "Materials and Methods" section, A-Avi has only two biotin binding sites. However, the addition of A-Avi caused strong deceleration of the kinetics close to that of usual streptavidin (Fig. 6). It should be pointed out that the photoinactivation kinetics in the presence of A-Avi differed from that of streptavidin. Namely, it contained two components with approximately equal contributions, $\tau_1 = 10$ sec (43%) and $\tau_2 = 83$ sec (57%). Besides, there was a small increase in the amplitude of photoinactivation after the addition of biotin (35% in the control, 7% in the presence of A-Avi, and 10% after the addition of biotin). These experiments suggest that the decelerating effect can be attributed to the binding of two gA5XB channels to streptavidin molecule and that the assumption of higher stoichiometry is not necessary.

To show independently the interaction of streptavidin with biotinylated gramicidin and biotin, we conducted experiments with a conjugate of streptavidin with peroxidase and immobilized gA5XB (gA2XB) by enzyme-linked assay. As seen from Fig. 7, streptavidin conjugated to horseradish peroxidase showed higher binding activity to gA5XB than to gA2XB adsorbed on an immunological plate. Subsequent addition of biotin led to only partial release of the streptavidin–peroxidase conjugate (Fig. 8), which was more pronounced in the case of gA2XB. These results, despite considerable correlation with the bilayer data, displayed some difference. In particular, the biotin-induced reversal of the streptavidin effect on gA2XB was nearly complete in bilayers, while even the excess of biotin in the system of enzyme-linked assay led to only partial reversal of the binding. This difference can be attributed to the ability of gramicidin to diffuse freely in lipid bilayers, in contrast to the case of immunological plates where gramicidin might be effectively immobilized.

Figure 9 shows the scheme of the formation of the complex of streptavidin with two channels of biotinylated gramicidin. The essential feature of this complex is the presence of streptavidin on both sides of the membrane, which leads to the formation of two cross-linked semi-

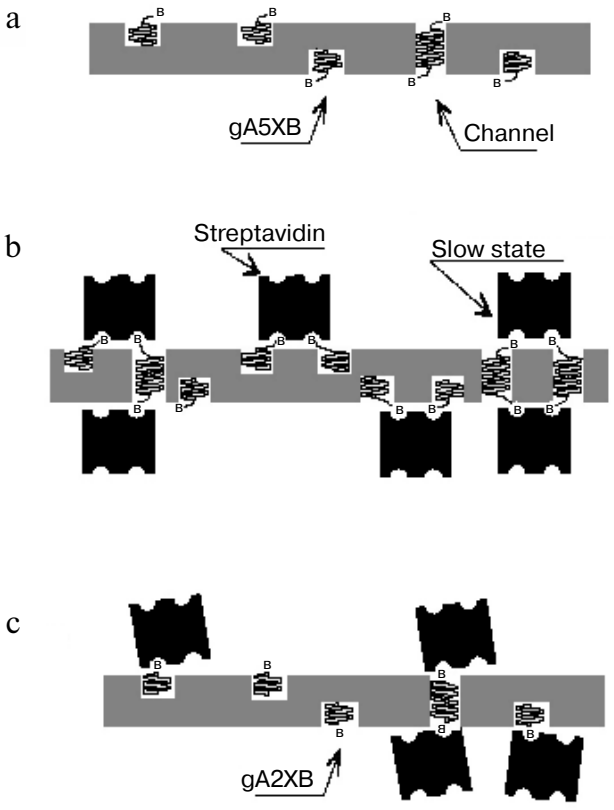

Fig. 9. Scheme of the interaction of streptavidin with biotinylated gramicidin gA5XB (b) or gA2XB (c).

channels in two lipid monolayers. Transmembrane association of these semi-channels leads to formation of cross-linked tandem channels, which have increased lifetime compared to unbound channels. The channel stabilization can result from doubling of the number of hydrogen bonds between N-termini of gramicidin molecules in the case of tandem channels. Besides, the cooperativity in the formation of tandem channels, associated with the disturbance of the lipid bilayer structure, which inevitably accompanies the formation of gramicidin channels [57, 22], should be taken into account. Figure 9c shows the interaction of streptavidin with gA2XB. Due to a shorter linker group between gramicidin and biotin, the interaction of streptavidin with two gA2XB channels is sterically ineffective. This apparently prevents the formation of the structure shown in Fig. 9b.

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