

Tandem Gramicidin Channels Cross-linked by Streptavidin

TATYANA I. ROKITSKAYA, ELENA A. KOTOVA, and YURI N. ANTONENKO

Belozersky Institute, Moscow State University, Moscow 119992, Russia

ABSTRACT The interaction of biotin-binding proteins with biotinylated gramicidin (gA5XB) was studied by monitoring single-channel activity and sensitized photoinactivation kinetics. It was discovered that the addition of streptavidin or avidin to the bathing solutions of a bilayer lipid membrane (BLM) with incorporated gA5XB induced the opening of a channel characterized by approximately doubled single-channel conductance and extremely long open-state duration. We believe that the deceleration of the photoinactivation kinetics observed here with streptavidin and previously (Rokitskaya, T.I., Y.N. Antonenko, E.A. Kotova, A. Anastasiadis, and F. Separovic. 2000. *Biochemistry*. 39:13053–13058) with avidin reflects the formation of long-lived channels of this type. Both opening and closing of the double-conductance channels occurred via a transient sub-state of the conductance coinciding with that of the usual single-channel transition. The appearance of the double-conductance channels after the addition of streptavidin was preceded by bursts of fast fluctuations of the current with the open state duration of the individual events of 60 ms. The streptavidin-induced double-conductance channels appeared to be inherent only to the gramicidin analogue with a biotin group linked to the COOH terminus through a long linker arm. Including biotinylated phosphatidylethanolamine into the BLM prevented the formation of the double-conductance channels even with the excess streptavidin. In view of the results obtained here, it is suggested that the double-conductance channel represents a tandem of two neighboring gA5XB channels with their COOH termini being cross-linked by the bound streptavidin at both sides of the BLM. The finding that streptavidin induces the formation of the tandem gramicidin channel comprising two channels functioning in concert is considered to be relevant to the physiologically important phenomenon of ligand-induced receptor oligomerization.

KEY WORDS: bilayer lipid membrane • sensitized photoinactivation • ligand • clustering

INTRODUCTION

Receptor–ligand interactions play a crucial role in most physiological processes. In particular, chemical signals are converted into electrical ones through ligand gating of ionic channels in cellular membranes (Breitinger, 2001). Clustering of channels is accepted to be the basic mechanism of signal transduction and amplification (Laver and Gage, 1997; Colledge and Froehner, 1998; Swillens et al., 1999; Garner et al., 2000; Neumann, 2000; Breitinger, 2001). Colocalization of PDZ domain-containing or cytoskeletal proteins, on one hand, and receptor channel proteins, on the other hand, leading to formation of their clusters has been visualized for acetylcholine and glutamine receptors as well as for potassium, sodium, calcium, and chloride channels. This coclustering has been reported to activate and immobilize the specific ionic channels (Westenbroek et al., 1990; Joe and Angelides, 1992; Issa and Hudspeth, 1994; Kim et al., 1995; Shieh and Zhu, 1996; Horio et al., 1997; Kurschner et al., 1998; Wood and

Slater, 1998; Zhou et al., 1998; Burke et al., 1999; Caldwell, 2000; Chen et al., 2000; El-Husseini et al., 2000; Nehring et al., 2000; Sheng and Pak, 2000; Tiffany et al., 2000; Wang et al., 2000; Bezprozvanny and Maximov, 2001; Kaplan et al., 2001; Raghuram et al., 2001; Ratcliffe et al., 2001; Anzai et al., 2002; Duggan et al., 2002; Imamura et al., 2002; Tanemoto et al., 2002). Clustering is also one of the main principles of molecular organization of the gap-junction channels (Bukauskas et al., 2000; Falk, 2000).

Synchronous opening and closing of ensembles of ion channels have been observed upon incorporation of channel-forming proteins and peptides into planar bilayer lipid membranes (Volkova et al., 1980; Schindler and Rosenbusch, 1981; Schindler et al., 1984; Hymel et al., 1988; Watras et al., 1991; Mironova et al., 1994; Delcour, 1997; Kaulin et al., 1998; Marx et al., 1998; Ternovsky and Berestovsky, 1998; Dargan et al., 2002; Kullman et al., 2002) and by the patch-clamp method (Kazachenko and Geletyuk, 1984; Geletyuk and Kazachenko, 1989; Meves and Nagy, 1989; Schreibmayer et al., 1989; Honore et al., 1992; Larsen et al., 1996a,b; Neumann et al., 1996; Eghbali et al., 1997; Laver and Gage, 1997). In spite of the fact that studying channel clusters has attracted much attention, the molecular mechanisms of their functioning remain unclear.

Address correspondence to Yuri Antonenko, Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119992, Russia. Fax: (70) 95-939-31-81; E-mail: antonen@genebee.msu.su

*Abbreviations used in this paper: ALPcS₃, aluminum trisulfophthalocyanine; BLM, bilayer lipid membrane; DPhPC, diphytanoylphosphatidylcholine.

Here we demonstrate that one of the simplest and best studied ionic channels formed by a transmembrane dimer of the pentadecapeptide gramicidin A (Koepe and Andersen, 1996; Andersen et al., 1999; Cross et al., 1999) can be rendered ligand gated by attaching a biotin group to the COOH terminus of the channel former. The interaction of biotinylated gramicidin with avidin or streptavidin, having four biotin-binding sites of a remarkably high affinity (Hendrickson et al., 1989; Weber et al., 1989; Green, 1990; Livnah et al., 1993; Pugliese et al., 1993; Sano and Cantor, 1995; Stayton et al., 1999) results in opening of channels with unusual characteristics, namely, extremely long duration and double conductance as compared with the control. Thus, the concerted ligand-gated opening of a couple of gramicidin channels is shown for the first time. This model system can be used to simulate a broad class of the ligand-induced receptor oligomerization phenomena (Metzger, 1992; Lemmon and Schlessinger, 1994; Heldin, 1995; Wells, 1996; Lemmon et al., 1997; Reich et al., 1997; Guo and Levine, 1999; Bouvier, 2001; Cochran et al., 2001), including ion channel regulation via dimerization (Liu et al., 1998; Richards and Gordon, 2000; Yellen, 2001; Schumacher et al., 2001), as well as the formation of coordinated arrays of signaling proteins (Xu et al., 1998; Fanning and Anderson, 1999; Hung and Sheng, 2002). In the accompanying article by Goforth et al. (2003, this issue) the problem is addressed using an independent experimental approach.

Some of these results have appeared in preliminary form (Rokitskaya et al., 2002).

MATERIALS AND METHODS

Bilayer lipid membranes (BLMs)* were formed from a 2% solution of diphytanoylphosphatidylcholine (DPhPC; Avanti Polar Lipids) or its mixture (if otherwise stated) with *N*-(biotinoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (biotin-PE; Molecular Probes, Inc.) in *n*-decane (Merck) by the brush technique on a hole in a Teflon partition separating two compartments of a cell containing aqueous buffer solutions. The cell with the 0.15-mm diameter hole (unless otherwise stated) was used in single-channel experiments, and that with the 0.55-mm diameter hole was used in photoinactivation experiments. The biotinylated analogs of gramicidin A (gifts of F. Separovic, University of Melbourne, Australia) with a biotin group attached to the COOH 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. The structure and synthesis of biotinylated gramicidins were described in (Separovic et al., 1999; Anastasiadis et al., 2001). Streptavidin and avidin were from Fluka. In all the experiments the solution was 1 M KCl, 10 mM Tris, 10 mM MES, 10 mM β -alanine, pH = 7.0. All the experiments were performed at room temperature (22–24°C). In photoinactivation experiments, aluminum trisulphophthalocyanine (AlPcS₃) from Porphyrin Products was added to the bathing solution at the trans-side (the cis-side is the front side with respect to the flash lamp).

The electric currents (*I*) were recorded under voltage-clamp conditions. Voltages were applied to BLMs with Ag-AgCl electrodes placed directly into the cell. The currents, measured by means of a patch-clamp amplifier (OES-2; OPUS) in single-chan-

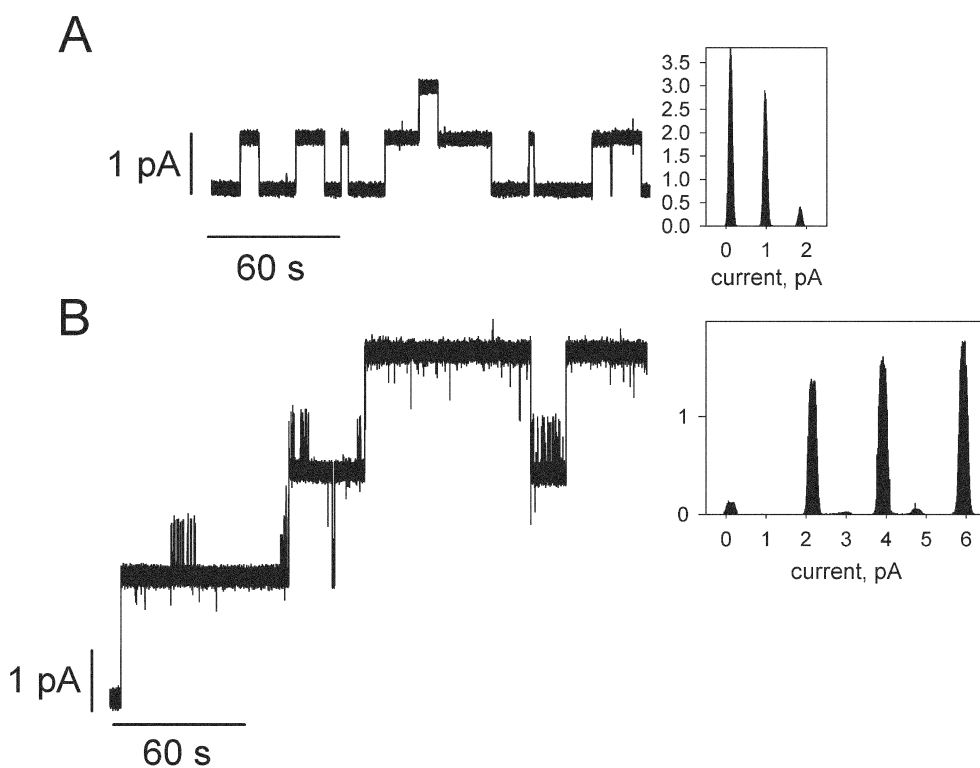


FIGURE 1. (A) Single-channel traces of gA5XB and the corresponding current amplitude histogram. (B) Single-channel traces of gA5XB after incubation with 1 μ g/ml streptavidin added to both sides of a BLM and the corresponding current amplitude histogram. The BLM voltage was 50 mV. The solution was 1 M KCl, 10 mM Tris, 10 mM MES, 10 mM β -alanine, pH = 7.0. Planar bilayers were from DPhPC. The low-pass digital filter with a cutoff frequency of 53 Hz was used in these recordings.

nel experiments and by a U5-11 amplifier in photoinactivation experiments, were digitized by using a LabPC 1200 (National Instruments) and analyzed using a personal computer with the help of WinWCP Strathclyde Electrophysiology Software designed by J. Dempster (University of Strathclyde, UK). Single-channel currents were low-pass filtered with a cutoff frequency of 100 Hz, sampled at 1 kHz and stored directly to the hard disk.

In photoinactivation experiments, BLMs were illuminated by single flashes produced by a xenon lamp with flash energy of ~ 400 mJ/cm² and flash duration < 2 ms.

RESULTS

Fig. 1 presents single-channel recordings and current amplitude histograms of gA5XB (the biotinylated gramicidin with a linker arm comprising five aminocaproyl groups) in the control (A) and after 10-min incubation with 1 μ g/ml streptavidin added to both sides of BLM (B). Remarkably, it is seen that the usual current fluctuations of gA5XB with the open state duration comparable to that of gramicidin A and the single-channel conductance of 17.6 ± 1 pS (recording A) were replaced after the incubation with streptavidin by current fluctuations with a very long-lived open state and the single-channel conductance exceeding that of the control more than twice, 39.5 ± 3 pS (recording B). If presented in the expanded time scale (Fig. 2), the recordings made in the presence of streptavidin revealed that both opening and closing of the double-conductance channels occurred via an intermediate substate of 50 ± 25 -ms duration (mean \pm SD, $n = 17$). The conductance of this transient substate was equal to the single-channel conductance of gA5XB, whereas the conductance of the subsequent step was 25% higher.

It is worth noting that the opening of the long-lived double-conductance channels after the addition of streptavidin was preceded by the appearance of bursts of very short-lived channel events (Fig. 3 A) having the transition amplitude coinciding with that of the control single channels of gA5XB. The open state duration of the individual events in these bursts displayed the exponential distribution with the characteristic time of 60 ± 10 ms. The duration of the closed state varied from 60 to 400 ms in different series of bursts. The duration of the bursts also varied substantially (from 10 to 140 s). After the opening of the double-conductance channels the occurrence of the bursts decreased markedly and their duration on the average became shorter.

In contrast to gA5XB channels, gA2XB (the biotinylated gramicidin having a shorter linker arm) channels never exhibited the double-conductance state in the presence of streptavidin (unpublished data). Nevertheless, the incubation with 1 μ g/ml streptavidin led to a substantial reduction of the number of open gA2XB channels in agreement with the data of (Suarez et al., 1998; Futaki et al., 2001) and the appearance of fast fluctuations of the current with the open state duration of 33 ± 4 ms (Fig. 3 B).

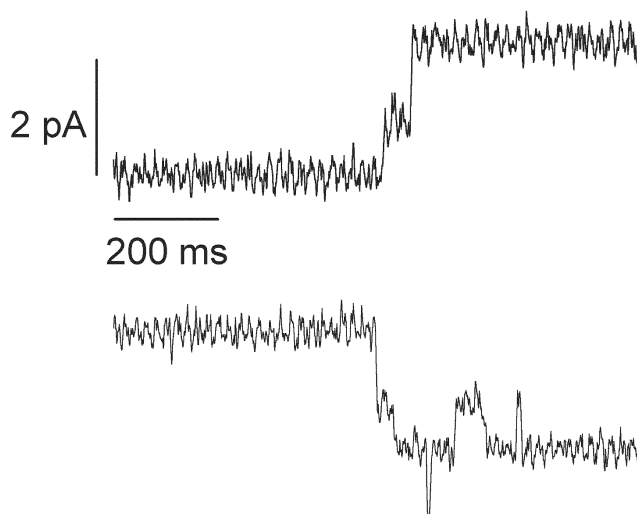


FIGURE 2. Selected parts of the recordings similar to that shown in Fig. 1 B revealing a transient substate in both opening and closing of the double-conductance channel. Here the digital filter was not used.

Fig. 4 displays single-channel recordings and the current-amplitude histogram of gA5XB obtained after incubation with 4 μ g/ml avidin. The experiments using membranes of different areas (A, 0.15-mm diameter hole; B, 0.55-mm diameter hole) permitted us to compare the avidin effect on the parameters of gA5XB single channels at a high (A) and a low (B) surface density of the channel-former. As seen from recording A, the incubation with avidin led to the opening of very long-lived channels with the single-channel conductance of 36.4 ± 2 pS (see the histogram). Thus, avidin induced the formation of double-conductance channels similarly to streptavidin. The transient substate of 50-ms duration was also discernible in this case, with the conductance of the subsequent step exceeding that of the initial step of the channel opening by 7%. Recording B shows, however, that at low surface density of gA5XB the addition of avidin caused the appearance of long-lived channels with the single-channel conductance equal to that of the control channels, as observed previously (Rokitskaya et al., 2000a). The prolonged incubation with avidin resulted ultimately in the appearance of high-conductance channels also with the large-diameter membrane, though the amplitude of these channels was somewhat less than those observed with the small-diameter membrane.

It was reasonable to expect that biotinylated lipid if included in the membrane would compete with biotinylated gramicidin for binding sites on streptavidin and thereby would alter the probability of the formation of the double-conductance channels. In further experiments we studied the effect of including biotinylated phosphatidylethanolamine (biotin-PE) in the

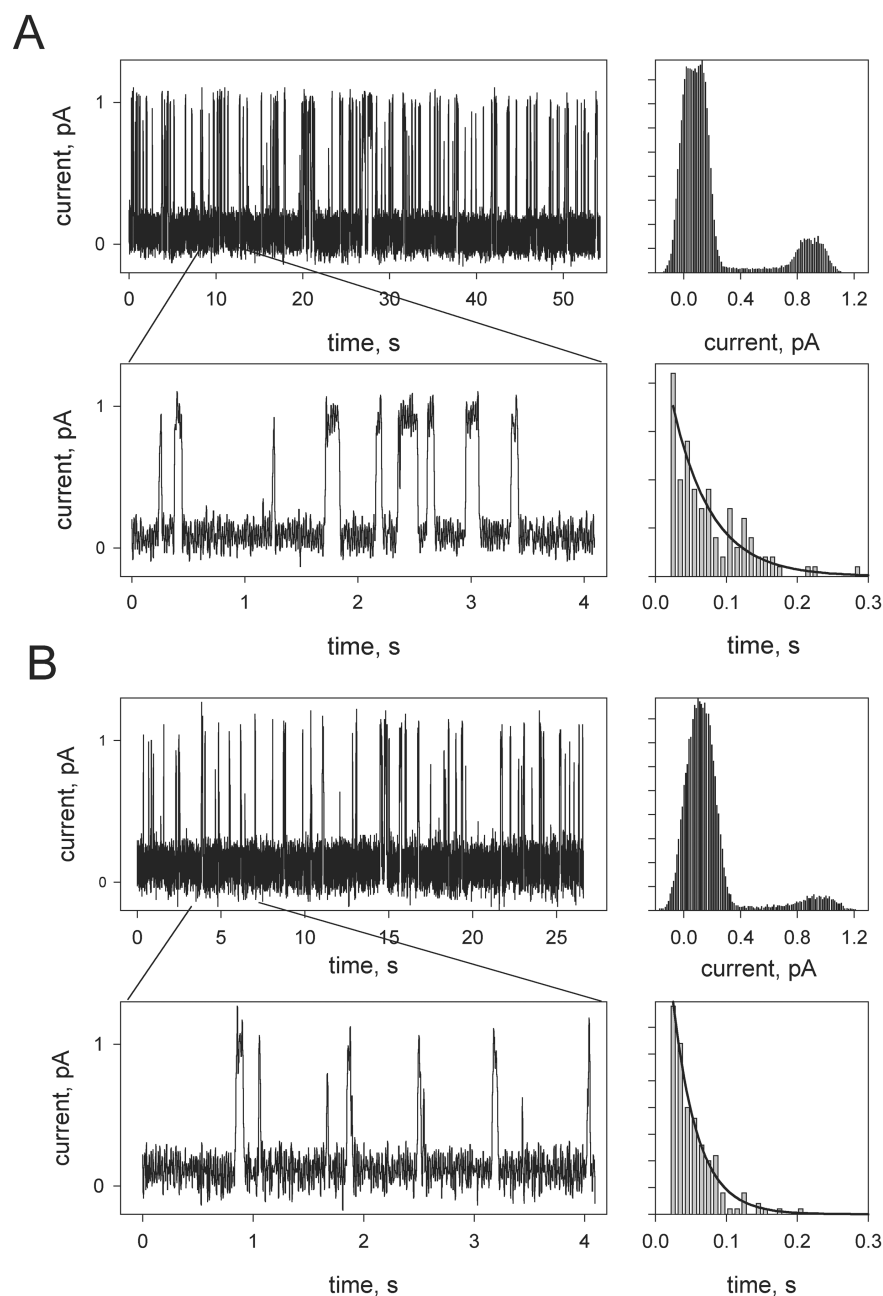


FIGURE 3. (A) Fast fluctuations of the gA5XB single-channel current after the addition of 1 μg/ml streptavidin to both sides of a BLM observed before the appearance of the events shown in Fig. 1 B. (Top right) The corresponding current amplitude histogram. (Bottom right) The open state duration histogram with the exponential fit (the characteristic time is 55 ms). (B) Fast fluctuations of the gA2XB single-channel current after the addition of 1 μg/ml streptavidin to both sides of a BLM. (Top right) The corresponding current amplitude histogram. (Bottom right) The open state duration histogram with the exponential fit (the characteristic time is 35 ms). The BLM voltage was 50 mV. The solution was the same as in Fig. 1. Planar bilayers were from DPhPC. The low-pass digital filter with a cutoff frequency of 53 Hz was used in these recordings.

BLM on the single-channel activity of gA5XB in the presence of streptavidin. It appeared that in the whole range of the biotin-PE content of the membrane studied here the interaction of streptavidin with gA5XB resulted in the reduction of the number of open channels having the standard single-channel conductance of 17 pS (unpublished data), as it was observed in the absence of biotin-PE. Besides, fast fluctuations of the current (bursts of very short-lived channel events having the transition amplitude equal to that of the control single channels) with the same open state duration, as in pure DPhPC membranes, were observed with all membranes containing biotin-PE. On the con-

trary, the occurrence of the double-conductance channels of gA5XB after incubation with streptavidin was extremely sensitive to the presence of biotin-PE (Fig. 5). At the biotin-PE content amounting to $2 \times 10^{-5}\%$ of the membrane lipid (the rest being DPhPC) the double-conductance channels opened with the same frequency as in the absence of biotin-PE. At the biotin-PE content of $2 \times 10^{-4}\%$ the double-conductance channels rarely formed. Beginning from the biotin-PE content of 0.002% the double-conductance channels were not observed at all.

In accord with the streptavidin-induced decrease in the number of open gA5XB channels, the suppression

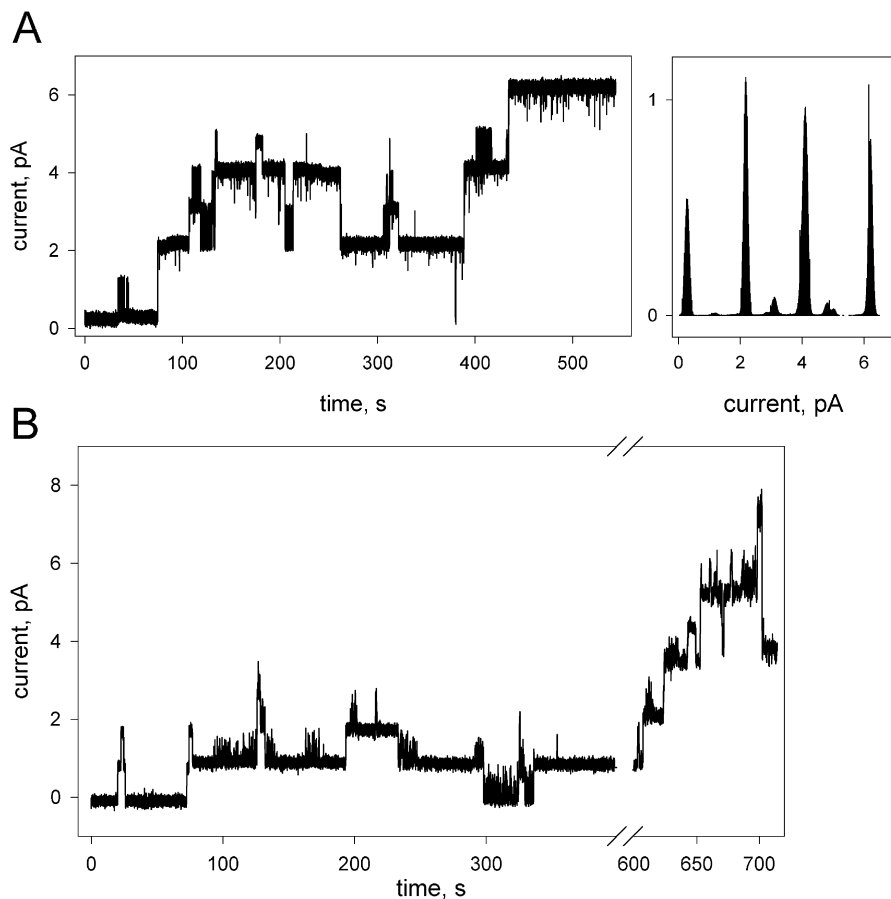


FIGURE 4. Single-channel traces of gA5XB after the addition of 4 $\mu\text{g}/\text{ml}$ avidin to both sides of BLMs formed from DPhPC on the 0.15-mm diameter hole (A) and on the 0.55-mm diameter hole (B), and the current amplitude histogram corresponding to A. The solution was the same as in Fig. 1. The low-pass digital filter with a cutoff frequency of 53 Hz was used in these recordings.

of gA5XB-mediated current across BLM after the addition of streptavidin was also observed at the multichannel level (Fig. 6 A). This effect of streptavidin that had been found earlier (Cornell et al., 1997) appeared to be independent of the presence of the biotinylated lipid; in particular, it was seen at least up to the biotin-PE of 1% (Fig. 6 A). At 0.02% biotin-PE, the time course of the macroscopic current was biphasic (the increase in the current followed its decrease) that can be explained by slow formation of the long-lived double-conductance channels leading to the growth of the macroscopic current. Actually, the magnitude of the gA5XB-mediated current depends not only on the channel lifetime, i.e., on the dissociation rate constant of gA5XB dimers, but also on the frequency of channel openings related to the gA5XB dimerization rate constant, and on the stationary concentration of gA5XB molecules in the membrane.

The interaction of streptavidin with gA5XB channels was studied in the present work also by the method of sensitized photoinactivation (Rokitskaya et al., 1996, 2000b) based on measuring the time course of a flash-induced decrease in the multichannel current across BLM in the presence of a photosensitizer (e.g., aluminum phthalocyanine, AlPcS₃). The sensitized photoin-

activation is thought to result from a damage to tryptophan residues in gramicidin, caused by light-induced generation of reactive oxygen species (Strassle and Stark, 1992; Rokitskaya et al., 1993; Kunz et al., 1995). As shown previously (Rokitskaya et al., 1996, 1997, 2000a), this method makes it possible to get information on channel kinetics from the current measurements at the multichannel level. Fig. 6 B demonstrates the kinetic curves of the flash-induced decrease (below called the kinetics of photoinactivation) in the gA5XB-mediated current in the presence of AlPcS₃ measured before (curve 1) and after (curve 2) the addition of 0.5 $\mu\text{g}/\text{ml}$ streptavidin. It is seen that streptavidin provoked a tremendous deceleration of the gA5XB photoinactivation kinetics that is similar to the effect of avidin described earlier (Rokitskaya et al., 2000a). The control time course of photoinactivation in the absence of streptavidin was well fitted by a monoexponential curve, which allowed us to determine the rate constants of formation (k_R) and dissociation (k_D) of gA5XB channels by plotting the reciprocal of the characteristic time constant, τ , of the kinetic curves versus the square root of the steady-state multichannel conductance (Fig. 7) according to Bamberg and Lauger (1973) and Rokitskaya et al. (1996) (see Appendix I). From the ap-

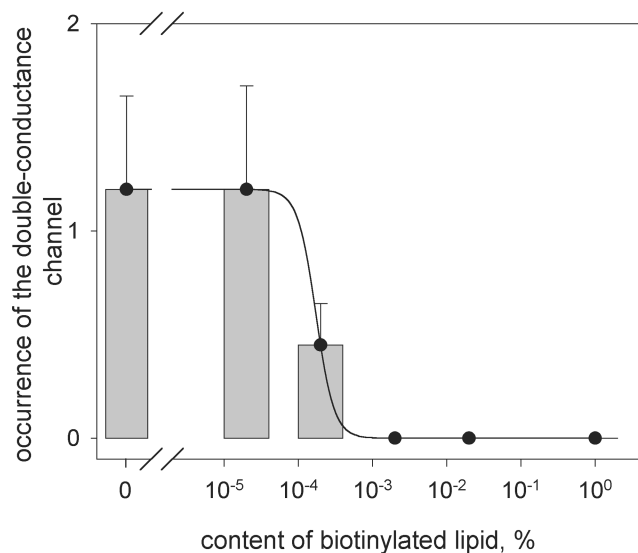


FIGURE 5. The bar chart of the double-conductance channel occurrence plotted versus the content of biotinylated lipid (biotin-PE) in the membrane-forming DPhPC solution. The line was drawn by eye.

proximation of this plot by a linear function we calculated the following values of the rate constants (k_R and k_D) and the equilibrium constant K : $k_D = 0.083 \text{ s}^{-1}$, $k_R = 1.4 \times 10^{14} \text{ s}^{-1} \text{ mole}^{-1} \text{ cm}^2$, $K = k_R/k_D = 1.7 \times 10^{15} \text{ mole}^{-1} \text{ cm}^2$.

The time course of gA5XB photoinactivation recorded after the addition of streptavidin was poorly fitted by a monoexponential curve, but was well described by a sum of two exponentials (Fig. 6 B): $I/I_0 = \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2) + C$, where $\alpha_1 = 1.5\%$, $\tau_1 = 3.2 \text{ s}$, $\alpha_2 = 5.2\%$, $\tau_2 = 90 \text{ s}$, $C = 93.3\%$. As seen from the Fig. 8, A and B, the contribution (α_2) of the slow phase (the component with a longer characteristic time, τ_2) to the overall kinetics decreased upon including biotinylated lipid in the BLM and became negligible at the biotin-PE content of 1% and higher. It should be noted, however, that in contrast to the decrease in the occurrence of the streptavidin-induced double-conductance channels of gA5XB observed at very low biotin-PE content ($2 \times 10^{-4}\%$), the reduction of the slow phase contribution to the photoinactivation kinetics manifested itself noticeably only at the biotin-PE content $>0.02\%$. Thus, the comparison of the effect of biotin-PE on the gA5XB single-channel activity with that on the kinetics of the sensitized photoinactivation of the gA5XB multichannel currents in the presence of streptavidin revealed the pronounced difference between the dependencies of these effects on the biotin-PE content. Namely, the threshold content of biotin-PE, at which its inhibiting effect on the double-conductance channels became noticeable, differed from the

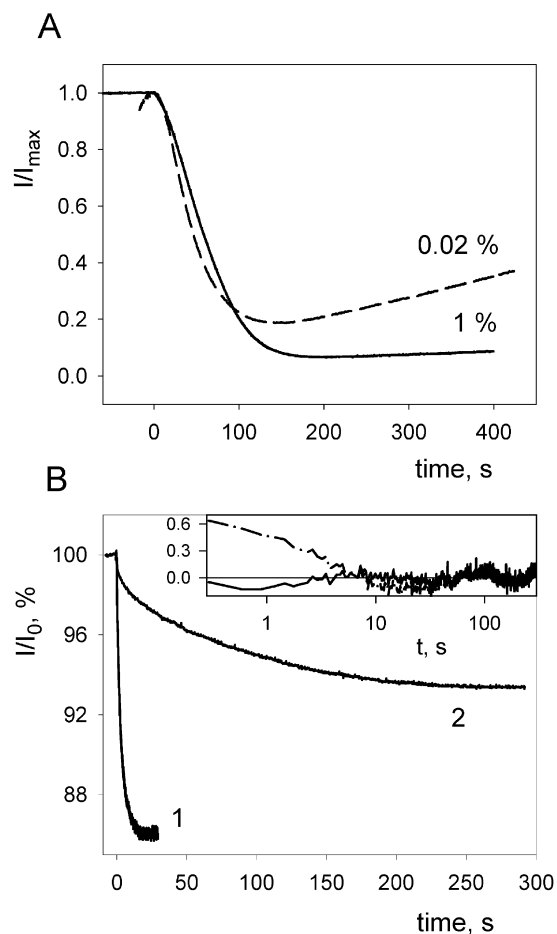


FIGURE 6. (A) Effect of the addition (at $t = 0 \text{ s}$) of $0.5 \mu\text{g/ml}$ streptavidin on the gA5XB-mediated current across BLMs formed from the DPhPC solution with different contents of biotinylated lipid (0.02%, dashed line; 1%, solid line). The normalized values of the current (I/I_{max}) are plotted versus the time (the I_{max} was $\sim 1 \mu\text{A}$). (B) The time courses of the decrease in the gA5XB-mediated current across a BLM after a flash of visible light (at $t = 0 \text{ s}$) in the presence of $1 \mu\text{M AlPcS}_3$ before (curve 1) and after (curve 2) the addition of $0.5 \mu\text{g/ml}$ streptavidin to both sides of the BLM. The normalized values of the current (I/I_0) are plotted versus the time. The initial current (I_0) was $\sim 1 \mu\text{A}$. The BLM voltage was 65 mV . The solution was the same as in Fig. 1. (Inset) Deviations of the curve 2 from monoexponential (dashed-dotted curve) and biexponential (solid curve) fits.

minimum content of biotin-PE giving rise to the inhibition of the streptavidin-induced deceleration of the gA5XB photoinactivation kinetics by two orders of magnitude (compare Figs. 5 and 8). However, this difference became insignificant, if both dependencies were plotted versus the ratio between the surface concentrations of biotin-PE and gA5XB in the membrane (Fig. 8 B, inset). This ratio was calculated as described in the Appendix I. It is seen that the inflection point of both dependencies corresponded to the [biotin-PE]/[gA5XB] ratio of ~ 100 .

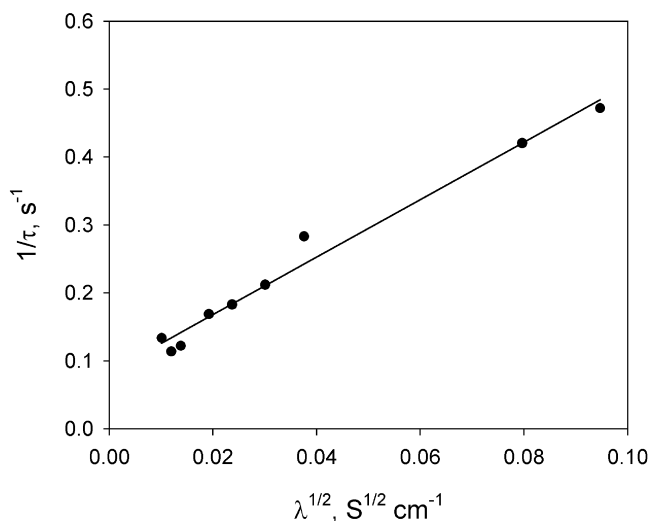


FIGURE 7. The dependence of the reciprocal of the characteristic time constant (τ) of the exponential curve fitting the photoinactivation kinetics (i.e., the time course of the flash-induced decrease in the gA5XB-mediated current in the presence of $1 \mu\text{M}$ AlPcS₃) on the square root of the steady-state multichannel conductance (λ). A solid line represents the linear approximation of the dependence. The example of the photoinactivation kinetics is shown in Fig. 6 B (curve 1). The temperature was 22°C.

It should be pointed out that in all our experiments streptavidin was present in the excess providing infinite stock of biotin-binding sites for gA5XB even at high biotin-PE content. In particular, streptavidin was added at $1 \mu\text{g}/\text{ml}$ concentration corresponding to 70 nM , whereas gA5XB was added at 10 pM and 5 nM in single-channel and multichannel experiments, respectively. The volume concentration of biotin-PE in the experimental cell even at the high content of 1% in the BLM was $<10 \text{ pM}$. It can be easily estimated by dividing the number of biotin-PE molecules in the BLM, calculated as described in the Appendix I, by the cell volume (3 ml).

DISCUSSION

The most striking finding of this work consists in the observation of an unusual state of gramicidin channels that is characterized by the extraordinary long open state duration and the predominant conductance approximately twice as high as the usual single-channel conductance of gA5XB channels (Figs. 1 and 4). This state observed in the presence of the biotin-binding proteins functionally corresponds to the synchronous opening of two gA5XB channels. Similar long-lived open channel state is described in the accompanying article (Goforth et al., 2003) using covalently linked gramicidin analogues.

Taking into account the properties of the double-conductance channels elicited by the interaction of gA5XB

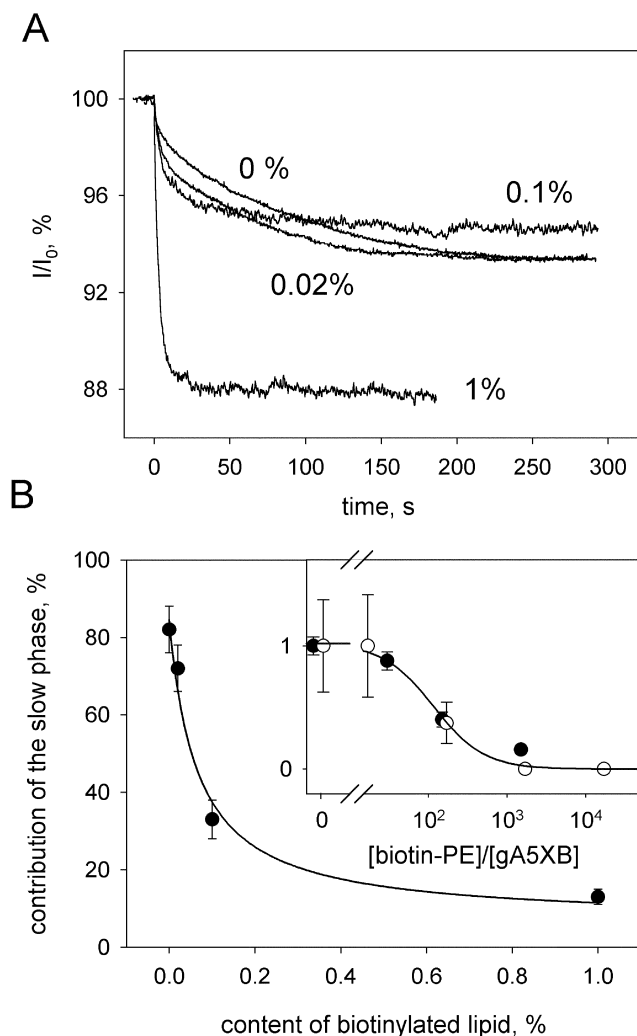


FIGURE 8. Effect of biotinylated lipid (biotin-PE) on the gA5XB photoinactivation kinetics in the presence of streptavidin. (A) The time courses of the decrease in the gA5XB-mediated current across a BLM after a flash of visible light (at $t = 0 \text{ s}$) in the presence of $1 \mu\text{M}$ AlPcS₃ after the addition of $0.5 \mu\text{g}/\text{ml}$ streptavidin to both sides of the BLM formed from the DPhPC solution with different contents of biotinylated lipid (0%, 0.02%, 0.1%, 1%). The normalized values of the current (I/I_0) are plotted versus the time. The initial current (I_0) was $\sim 1 \mu\text{A}$. The BLM voltage was 65 mV . The solution was the same as in Fig. 1. (B) The contribution of the slow phase to the photoinactivation kinetics plotted versus the content of biotinylated lipid in the membrane-forming solution. (Inset) The normalized occurrence of the double-conductance channels (open circles) and the normalized contribution of the slow phase (filled circles) are replotted versus the ratio of the surface concentration of biotin-PE to that of gA5XB. The line was drawn by eye.

with streptavidin and avidin (the value of the predominant conductance level approximately doubled as compared with that of the usual gramicidin single channels and the occurrence of the transient substate [Fig. 2] at the level corresponding to the usual single-channel conductance), it is reasonable to suggest that the double-

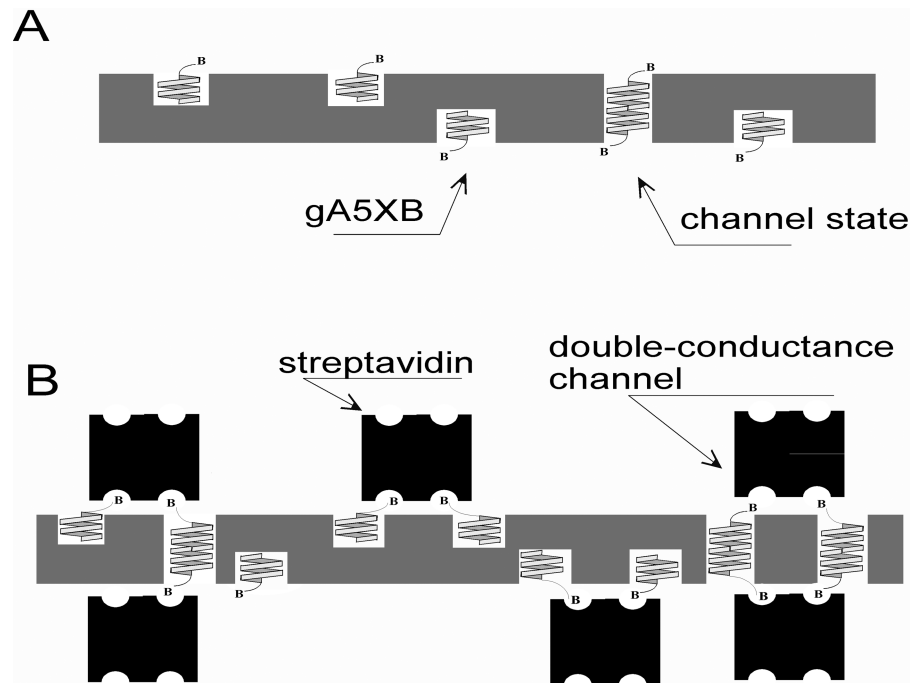


FIGURE 9. Scheme of formation of the tandem channel comprising two gA5XB channels cross-linked by streptavidin.

conductance channels are formed as a result of bivalent binding of two (strept)avidin molecules located at the opposite sides of the BLM to the COOH termini of two transmembrane dimers of gA5XB at both water-membrane interfaces (Fig. 9 B, right). Here the bivalent binding means that two (of the four) biotin-binding sites on (strept)avidin are occupied by biotin groups belonging to two gA5XB molecules. Most likely, the formation of such a complex is preceded by cross-linking of pairs of gA5XB monomers through binding to streptavidin at each side of the BLM. The opening of the double-conductance channel apparently corresponds to the moment when the pairs of gA5XB monomers bound to streptavidin at opposite sides of the BLM associate through hydrogen bonds between their NH₂ termini, thereby forming two neighboring transmembrane dimers. This couple of gA5XB channels appears to be highly stable, obviously due to the increased number of hydrogen bonds stabilizing the complex and/or to a reduction of the elastic force originating from a local thinning of the bilayer that acts on each transmembrane dimer in the couple (see the accompanying paper by Goforth et al. [2003, this issue] and Miloshevsky et al. [2002]). The alternative explanation of the complex formation, assuming that streptavidin binds to two pre-formed transmembrane dimers of gA5XB, seems to be unlikely because in this case it is difficult to explain the suppression of the gA5XB-mediated current observed after the addition of streptavidin both at the single-channel and at the multichannel level (Fig. 6 A).

The transient substate of ~50-ms duration (Fig. 2) may be tentatively ascribed to the process of mutual re-

orientation of the cross-linked pairs of gA5XB monomers, so as the proper conformation for linking into two neighboring head-to-head dimers is achieved. We note that the difference in the two current steps leading to the opening of the double-conductance gA5XB channel is in good agreement with the corresponding property of the double-barreled channels observed by Goforth et al. (2003) with covalently linked gramicidin analogues (the accompanying paper).

We believe that the fast fluctuations of the current (Fig. 3 A) preceding the emergence of the double-conductance channels also reflect certain steps of the mutual reorientation of streptavidin-bound gA5XB pairs that are accompanied by transient formation of transmembrane dimers (but not pairs of dimers) manifesting itself in short-lived openings of channels of the usual conductance. Remarkably, the similar bursting channel activity followed by a two-step transition to the higher current level was observed by Goforth et al. (2003) with covalently linked gramicidin analogues.

If the bivalent binding of streptavidin to a pair of gA5XB channels takes place at only one side of the BLM, whereas at the opposite side of the BLM the monovalent binding of streptavidin to gA5XB (which means that streptavidin has a single biotin-binding site occupied) occurs (Fig. 9 B, left), then another long-lived state of gA5XB channels may be expected to arise. We surmise that due to the reduced gA5XB surface density this state of gA5XB channels was observed with the large-diameter membranes both in this work (Fig. 4) and in the previous study (Rokitskaya et al., 2000a). The prolonged duration of this state having the stan-

dard single-channel conductance may be associated with such factors as lateral interaction of peptide helices in the membrane and an increased molecular weight of the channel former bound to streptavidin.

The fact that gA2XB proved to be unable to form the double-conductance channels in the presence of streptavidin despite the unambiguous evidence in favor of streptavidin binding to gA2XB shows that the long linker arm, obviously providing better access to biotin-binding pockets of streptavidin due to higher flexibility of a biotin group in gA5XB, is needed for the streptavidin interaction with biotinylated gramicidin to result in the formation of the double-conductance channels. Obviously, the cross-linking of two transmembrane dimers of biotinylated gramicidin requires that two binding sites should be located within the reach of biotin groups linked to two gramicidin molecules. The role of linker flexibility and the local monomer concentration is also discussed in the accompanying article by Goforth et al. (2003).

The model of the double-conductance channel representing a tandem of two neighboring gA5XB channels with their COOH termini being cross-linked by the bound streptavidin at both sides of the BLM is supported by the fact that the one-side addition of streptavidin to the bathing solution of the BLM containing gA5XB led neither to the formation of the double-conductance channels, nor to the appearance of the fast current fluctuations (unpublished data). We believe that the formation of the double-conductance channels represents the reason of the deceleration of the gA5XB photoinactivation kinetics observed at the multichannel level here with streptavidin and previously (Rokitskaya et al., 2000a) with avidin. In principle, the double-conductance channels might be formed upon addition of streptavidin at one side of the membrane. However, the probability of the formation of a transmembrane complex from cross-linked gA5XB in one monolayer and a pair of free gA5XB in the opposite monolayer should be substantially lower than that from cross-linked peptides located at both sides of the membrane. In fact, the concentration of gA5XB is very low under the conditions of single-channel measurements and the probability of the simultaneous appearance of two free peptides in close vicinity of the cross-linked gA5XB should be negligible. It is worth noting that at the multichannel level a noticeable deceleration of gA5XB kinetics was observed (Rokitskaya et al., 2000a), even in the case of avidin addition at one side of the BLM.

The data on the prevention of the opening of the double-conductance channels (Fig. 5) and on the inhibition of the deceleration of the photoinactivation kinetics of gA5XB (Fig. 8) by including biotinylated lipids (biotin-PE) into the DPhPC membrane are also in good agreement with the proposed model. Apparently, biotin-PE competing with gA5XB for the binding sites

on streptavidin should prevent the bivalent but not the monovalent binding of the protein to gA5XB, if streptavidin is present in the excess with respect to gA5XB and biotin-PE. This statement was substantiated by a theory (see Appendix II) that considered explicitly the competition between gA5XB and biotin-PE for two binding sites on streptavidin at the membrane surface under the conditions of the excess streptavidin in the bulk phase. The system of equations led to a four-power equation that was solved numerically for a wide range of parameters. This theoretical model allowed us to calculate that the S_{tv} binding constant for gA5XB exceeds that for biotin-PE by a factor of 40.

The conclusions based on the theoretical consideration strongly support the bivalent character of the (strept)avidin interaction with gA5XB. In particular, it was demonstrated that the bivalent binding model readily explained the inhibiting effect of biotin-PE on the formation of the long-lived channel state of gA5XB, whereas the monovalent binding model failed to explain this inhibition.

In summary, it can be concluded that the interaction with streptavidin turns two independent gramicidin channels into a couple of conducting units operating in concert. In other words, streptavidin plays a role of a multivalent ligand, binding of which to membrane receptors (gA5XB) activates the ionic channels by converting them into a new highly stable open state formed by a tandem of the transmembrane dimers. This kind of streptavidin interaction with gA5XB channels models the known phenomenon of ligand-induced receptor dimerization wherein membrane proteins containing single transmembrane α -helices associate to form dimers (or larger oligomers) in the membrane, either as stable complexes or as transient species, in response to the binding of a suitable ligand. Such dimerization or oligomerization have been shown to occur after binding of several polypeptide hormones, cytokines, growth factors, or growth inhibitors to their receptors (Lemmon and Schlessinger, 1994; Heldin, 1995).

APPENDIX I

Assuming that gA5XB monomers (A) and dimers (A_2) floating in the membrane are in equilibrium with each other,



we estimated monomer [N_1] and dimer [N_2] equilibrium concentrations in the membrane from the measured values of gA5XB-mediated current, that is proportional to the number of gA5XB open channels, and the equilibrium constant (K). The equilibrium concentrations of monomers and dimers established as a result

of the reversible dimerization reaction (Eq. A1) obey the following equation:

$$\frac{[N_2]}{[N_1]^2} = \frac{K_R}{K_D} = K. \quad (\text{A2})$$

As shown in (Rokitskaya et al., 1996), the values of K_R , K_D , and K can be calculated from the plot of $1/\tau$ versus $(\lambda_\infty)^{0.5}$, obtained by measuring the flash-induced gramicidin-mediated current transients following exponential kinetics with a characteristic time (τ):

$$1/\tau = K_D + 4 \sqrt{\frac{K_R K_D \lambda_\infty}{N_A \Lambda}}, \quad (\text{A3})$$

where N_A is Avogadro's number, λ_∞ is the steady-state BLM conductance after the light flash, and Λ is the single-channel conductance.

The number of gA5XB open channels, N_2 , is proportional to the concentration of dimers $[N_2]$ according to the equation:

$$N_2 = [N_2] \cdot N_A \cdot S_m, \quad (\text{A4})$$

where S_m is the membrane area.

From Eqs. A2 and A4 it can be derived that,

$$[N_1] = \sqrt{\frac{N_2}{S_m \cdot N_A \cdot K}} \quad (\text{A5})$$

The number of gA5XB monomers in the membrane, N_1 , is proportional to the concentration of monomers $[N_1]$ according to the equation:

$$N_1 = [N_1] \cdot S_m \cdot N_A. \quad (\text{A6})$$

Thus, we obtain:

$$N_1 = \sqrt{\frac{S_m \cdot N_A \cdot K}{K}}. \quad (\text{A7})$$

Bearing in mind that in single-channel experiments the average number of open channels N_2 is equal to 1, we substituted the values of $S_m \approx 10^{-4} \text{ cm}^2$ for the small-diameter membrane and $K = 1.7 \times 10^{15} \text{ mole}^{-1} \text{ cm}^2$ into Eq. A7, and calculated that $N_1 \approx 200$ under single-channel conditions. Based on the known value of lateral area per lipid molecule in a BLM, 0.6 nm^2 (Cornell et al., 1980; Koenig et al., 1997; Nagle and Tristram-Nagle, 2000; Balgavy et al., 2001), we estimated the total number of lipid molecules in the membrane to be 1.7×10^{10} by dividing the value of $S_m \approx 10^{-4} \text{ cm}^2$ by 0.6 nm^2 .

Under multichannel conditions, the number of gA5XB dimers, N_2 , can be estimated from the equation based on Ohm's law:

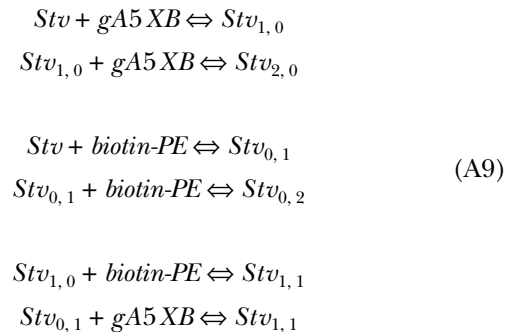
$$N_2 = \frac{1}{U \cdot \Lambda}, \quad (\text{A8})$$

where I is the value of the gA5XB-mediated current, U is the voltage applied to the BLM, and Λ is the single-channel conductance. By substituting the average value of I in multichannel experiments ($1 \mu\text{A}$), $U = 65 \text{ mV}$ and $\Lambda = 17.6 \text{ pS}$ into Eq. A8, we calculated N_2 to be $\sim 10^6$. Taking into account the different value of S_m for the membranes used in multichannel experiments, $2 \times 10^{-3} \text{ cm}^2$, we used Eq. A6 to calculate the number of monomers N_1 under these conditions and obtained $N_1 \approx 10^6$. Thus, the overall number of gA5XB molecules (monomers and dimers) was $\sim 2 \times 10^6$. The total number of lipid molecules in the membrane of this area was estimated to be 3×10^{11} by dividing the value of $S_m \approx 2 \times 10^{-3} \text{ cm}^2$ by 0.6 nm^2 .

Given the total number of lipid molecules and the number of gA5XB molecules in the BLMs in single-channel and in multichannel experiments, we calculated the ratios of biotin-PE and gA5XB surface concentrations corresponding to different values of the biotin-PE content in the BLM for both conditions.

APPENDIX II

Let us consider a system comprising streptavidin (Stv) molecules floating in the bathing solution and those adsorbed on BLM, on one hand, and biotinylated gramicidin (gA5XB) molecules incorporated in BLM, on the other hand. The membrane also contains molecules of biotinylated phosphatidylethanolamine (biotin-PE). We consider here the interaction that takes place at one side of the membrane neglecting the influence of the formation of transmembrane complexes (channels) on the inhibiting effect of biotin-PE. Let's assume that the total concentration of gA5XB and biotin-PE in BLM and the concentration of Stv in the aqueous solution are constant and equal to N , M , and $[\text{Stv}]$, respectively. The latter condition means that the bathing solution represents the streptavidin stock of the infinite capacity which is valid for planar BLM under our experimental conditions (see RESULTS). The interaction of streptavidin with biotinylated gramicidin and biotinylated lipid at one side of the membrane can be described by a system of reversible reactions:



Here it is assumed that two binding sites on streptavidin are effective in the interaction with biotin groups exposed at the membrane surface, because it is known that the biotin-binding pockets are located in pairs at opposite faces of the streptavidin tetramer (Weber et al., 1989). This is consistent with a recent conclusion (Niemeyer et al., 1999) that this tetravalent protein displays a high preference for acting as a bivalent linker molecule. The above equations imply that the interaction of gA5XB and biotin-PE with Stv is weak enough to let these reactions be reversible. The interaction of biotin with streptavidin in solution is hardly reversible and is known as one of the strongest noncovalent bonds (Weber et al., 1989). However, as shown in (Zhao and Reichert, 1992; Perez-Luna et al., 1999), the interaction of Stv with biotinylated lipids at the membrane-water interface is not so strong and is reversible. Obviously, the interaction of (strept)avidin with gA5XB at the interface is also reversible, because the addition of biotin to the bathing solution removed the avidin-induced deceleration of the gA5XB photoinactivation kinetics (Rokitskaya et al., 2000a).

The first and third equations in Eq. A9 are characterized by three-dimensional binding constants K_1 (in M^{-1}) and K_2 (in M^{-1}), whereas the other equations are characterized by two-dimensional binding constants. For the sake of simplicity we assume that all these two-dimensional constants are equal to each other and amount to K_X (in $cm^2/mole$). Because the total concentration of gA5XB is equal to N and that of biotin-PE is equal to M , it can be written:

$$\begin{aligned} [gA5XB] + [Stv_{1,0}] + [Stv_{1,1}] + 2[Stv_{2,0}] &= N \\ [biotin-PE] + [Stv_{0,1}] + [Stv_{1,1}] + 2[Stv_{0,2}] &= M \end{aligned} \quad (A10)$$

The above system of equations can be converted to the following equations by using dimensionless parameters (Perelson, 1981; Stone et al., 2001):

$$\begin{aligned} x + c_1 x + c_1 xy + 2c_1 x^2 &= b_1 \\ y + c_2 x + c_2 xy + 2c_2 y^2 &= b_2 \end{aligned} \quad (A11)$$

where $x = [gA5XB]/K_X$; $c_1 = [Stv]/K_1$; $b_1 = NK_X$; $y = [biotin-PE]/K_X$; $c_2 = [Stv]/K_2$; $b_2 = MK_X$.

This system of two equations was solved numerically. The solution enabled us to calculate the effect of increasing concentration of biotin-PE on the number of $Stv_{2,0}$ complexes that are formed at one side of the membrane and are thought to be the precursors of the double-conductance gA5XB channels. The dependence of $[Stv_{2,0}]$ on c_1 has a bell-like shape with a maximum at $c_1 \approx 1$. So we performed further calculations at $c_1 = 1$, because the Stv-induced deceleration of gA5XB channel kinetics depended weakly on $[Stv]$ under our experimental conditions (unpublished data). Fig. 10 shows the dependence of $[Stv_{2,0}]$ on b_2/b_1 (which is equal to M/N) at $b_1 = 10^3$ and different values of c_2 ,

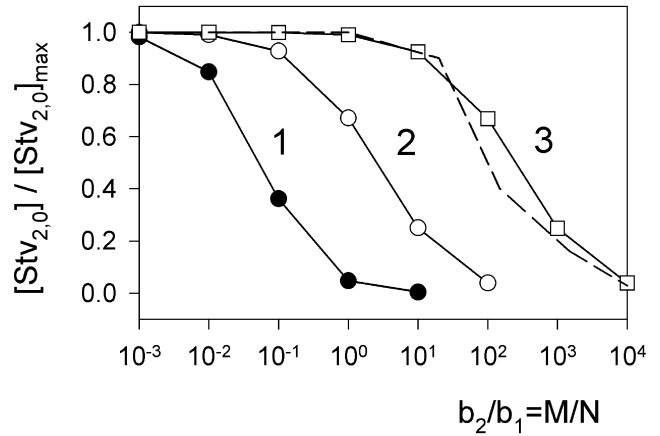


FIGURE 10. Theoretical dependence of the equilibrium concentration of a complex of streptavidin with two gA5XB molecules at the membrane surface ($[Stv_{2,0}]$) on the concentration of biotin-PE in the membrane. The calculations were performed by solving the system of equations (A11) numerically with the following parameters: $c_1 = 1$, $b_1 = 10^3$, $c_2 = 10^2$ (curve 1), $c_2 = 1$ (curve 2), and $c_2 = 10^{-2}$ (curve 3). The dashed line is an averaged experimental curve from the data presented in the inset to Fig. 8 B.

namely $c_2 = 10^2$ (curve 1), $c_2 = 1$ (curve 2), and $c_2 = 10^{-2}$ (curve 3). It means that the ratio of two constants K_1 and K_2 is varied. It is seen from Fig. 10 that the variation of K_1/K_2 is accompanied by a shift of theoretical dependencies of $[Stv_{2,0}]$ on the ratio of b_2/b_1 , so that the value of the b_2/b_1 ratio corresponding to the inflection point of the dependence correlates with the value of the K_1/K_2 ratio. Further calculations have shown that this correlation exists in a wide range of parameters provided that $b_1 \gg 1$, and the b_2/b_1 ratio at the inflection point appears to be independent of b_1 under these conditions. The data presented in Fig. 8 B (inset) proved that the latter statement is valid for our experimental conditions, as the dependence of the occurrence of Stv-induced double-conductance channels recorded at very low gA5XB concentration (low b_1 value) on the ratio of biotin-PE and gA5XB concentrations coincided with the corresponding dependence of the contribution of the slow phase to the photoinactivation kinetics measured at high gA5XB concentration (high b_1 value). Thus, it may be concluded that $b_1 \gg 1$, i.e., $N \gg 1/K_X$, under all our experimental conditions including single-channel recordings, obviously due to extremely high (strept)avidin-biotin binding constant (Weber et al., 1989). The theoretical calculations have shown that the b_2/b_1 ratio at the inflection point essentially depends on b_1 , if $b_1 < 1$.

As seen from Fig. 8, inset, the inflection point of experimental dependencies corresponds to the $[biotin-PE]/[gA5XB]$ ratio, i.e., the b_2/b_1 ratio, of ~ 100 . As follows from Fig. 10, it means that K_1/K_2 is of the order of 100 (curve 3). The precise calculations have given the

value of $K_1/K_2 = 40$. Therefore, we have come to an important conclusion that our theoretical model makes it possible to estimate the ratio of Stv binding constants for gA5XB and biotin-PE. The calculated difference between these binding constants can be explained by taking into account that in both gA5XB and biotin-PE biotin groups are attached to species embedded in the lipid membrane hindering their interaction with water-soluble streptavidin, but a long linker arm present in gA5XB and not in biotin-PE provides higher accessibility of biotin in gA5XB as compared with biotin-PE.

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