Effect of Avidin on Channel Kinetics of Biotinylated Gramicidin

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Received April 7, 2000; Revised Manuscript Received July 21, 2000

ABSTRACT: Membrane protein functioning basically depends on the supramolecular structure of the proteins which can be modulated by specific interactions with external ligands. The effect of a water-soluble protein bearing specific binding sites on the kinetics of ionic channels formed by gramicidin A (gA) in planar bilayer lipid membranes (BLM) has been studied using three independent approaches: (1) sensitized photoinactivation, (2) single-channel, and (3) autocorrelation measurements of current fluctuations. As shown previously [Rokitskaya, T. I., et al. (1996) *Biochim. Biophys. Acta 1275*, 221], the time course of the flash-induced current decrease in most cases follows a single-exponential decay with an exponential factor (τ) that corresponds to the gA single-channel lifetime. Addition of avidin does not affect τ for gA channels, but causes a dramatic increase in τ for channels formed by gA5XB, a biotinylated analogue of gA. This effect is reversed by addition of an excess of biotin to the bathing solution. The average single-channel duration of gA5XB was about 3.6 s as revealed by single-channel recording of the BLM current. After prolonged incubation with avidin, a long-lasting open state of the gA5XB channel appeared which did not close for more than 10 min. The data on gA5XB photoinactivation kinetics and single-channel measurements were confirmed by analysis of the corresponding power spectra of the current fluctuations obtained in the control, in the presence of avidin, and after the addition of biotin. We infer that avidin produces a deceleration of gA5XB channel kinetics by motional restriction of gA5XB monomers and dimers upon the formation of avidin and gA5XB complexes, which would stabilize the channel state and thus increase the single-channel lifetime.

One of the major routes of signal transduction in biological systems is associated with modulation of the ionic conductivity of cellular membranes. On the molecular level, regulation of ion channel activity in membranes operates through ligand−receptor interactions that form the basis of biological recognition mechanisms. Ionic channels formed by the well-known peptide ionophore gramicidin A (gA)[1] in planar bilayer lipid membranes (BLM) (*1*−*5*) can be used as a simple model to gain insight into these mechanisms. Recently, it has been shown that attachment of certain hydrophilic groups to the C-terminus of gA, which forms the channel mouth on both sides of a BLM, makes the gA channels reactive to specific external ligands (*6*−*10*). As gA channels are generally understood to be transmembrane dimers stabilized by hydrogen bonds between monomers (*11*, *12*), the kinetics of gA channel formation and dissociation is expected to be sensitive to the peptide mobility that can be altered, in particular, by the binding of water-soluble proteins. Here we report studies of a biotinylated analogue of gA with a biotin group covalently attached to the C-terminus of gA through a linker arm comprising five aminocaproyl groups (gA5XB). To study the channel kinetics, we used three independent approaches: (1) the sensitized photoinactivation method, (2) single-channel measurements, and (3) autocorrelation measurements of the current fluctuations.

MATERIALS AND METHODS

BLM were formed from a solution of 2% diphytanoylphosphatidylcholine (DPhPC, Avanti Polar Lipids, Alabaster, AL) in *n*-decane (Merck, Darmstadt, Germany) by the brush technique (*13*) on a 0.55 mm diameter hole in a Teflon partition separating two aqueous compartments. The aqueous bathing solutions contained 1 M KCl, 10 mM MES, 10 mM Tris, and 10 mM β-alanine (pH 7.0). At both sides of the BLM, gramicidin A (Sigma, St. Louis, MO) or its analogue carrying a biotin group at its C-terminus (gA5XB) was added from a stock solution in ethanol to the bathing solutions that were stirred continuously. gA5XB was synthesized according to the method described in ref *6*. The peptide channel former was incorporated spontaneously into the membrane. The membrane conductivity reached steady state usually within 15 min following the addition of gramicidin.

The electric current, *I*, was recorded under voltage-clamp conditions. The currents were measured by means of a picoamperemeter, digitized by DT2814 (Data Translation, Marlborough, MA) and analyzed using a personal computer. Ag−AgCl electrodes were placed directly into the cell. The

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[1] Abbreviations: DPhPC, diphytanoylphosphatidylcholine; gA, gramicidin A; gA5XB, gA with a biotin group covalently attached to the C-terminus of gA through a linker arm comprising five aminocaproyl groups; gA2XB, gA with a biotin group covalently attached to the C-terminus of gA through a linker arm comprising two aminocaproyl groups; $AlPcS_3$, aluminum trisulfophthalocyanine; BLM, bilayer lipid membrane.

10.1021/bi0007876 CCC: $19.00
Published on Web 09/27/2000

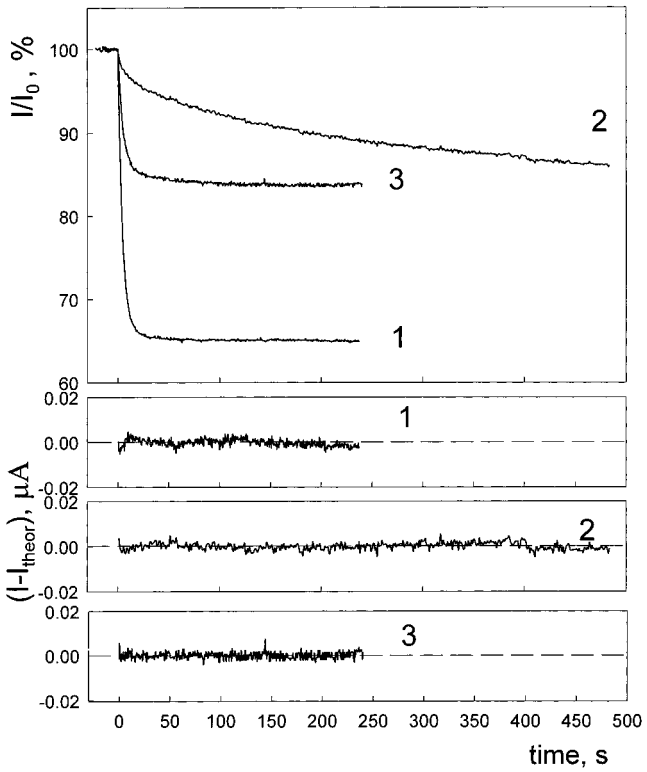

FIGURE 1: Effect of avidin (32 nM on both sides of the BLM) on the time course of the decrease in the gA5XB-mediated current (I) across a BLM after a flash of visible light (at time zero) in the presence of 1 μM $AlPcS_3$ (curve 2) and deviation of the data from the biexponential curve: $I/I_0 = \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2)$, where $\alpha_1 = 21\%$, $\tau_1 = 14.4$ s, $\alpha_2 = 79\%$, and $\tau_2 = 275$ s. Curve 1 is the time course of the current decrease in the absence of avidin ($\tau = 4.7$ s). Curve 3 was obtained after the addition of 22 M biotin to the bathing solution on both sides of the BLM in the presence of 32 nM avidin ($\alpha_1 = 87\%$, $\tau_1 = 4.7$ s, $\alpha_2 = 13\%$, and $\tau_2 = 48$ s). The normalized values of the current (I/I_0) are plotted vs time (t). The initial value of the current (I_0) was approximately 1.0 μA. The BLM voltage was 75 mV, and the temperature was 19 °C. The solution was 1 M KCl, 10 mM MES, 10 mM Tris, and 10 mM β-alanine (pH 7.0).

photosensitizer, aluminum phthalocyanine bearing three sulfonate groups ($AlPcS_3$, Porphyrine Products, Logan, UT), was added to the bathing solution at the trans side of the BLM. The xenon lamp was placed at the cis side of the membrane. Illumination of the bilayer was performed by single flashes (flash energy of about 400 mJ/cm^2 and flash duration of <2 ms). The light passed through a cutoff filter (for wavelengths of <500 nm).

RESULTS AND DISCUSSION

In the photoinactivation method, transients of gA-mediated current across BLM are induced by a flash of visible light in the presence of a photosensitizer. The decrease in the current is believed to result from damage to the tryptophan residues of gramicidin by reactive oxygen species generated upon excitation of a photosensitizer (*14*−*16*). According to our previous work (*17*), the time course of the flash-induced decrease in the gA-mediated current in most cases follows a single-exponential decay with an exponential factor (τ) that corresponds to the gA single-channel lifetime. It is seen from Figure 1 (curve 1) that gA5XB undergoes sensitized photoinactivation in the presence of aluminum phthalocyanine with the kinetics of the flash-induced current decrease similar to that of gA (*17*). In this case, the concentration of the channel former was ~1 nM which induced a current across the BLM on the order of 1 μA. The exponential factor of the decay curve describing the time course of the flash-induced decrease in the gA5XB-mediated current was 4.7 s. The exponential factor (τ) for gA was 2.0 s under these conditions. The addition of avidin (curve 2) caused a dramatic deceleration of the photoinactivation kinetics of gA5XB. Under these conditions, the time course of the current decrease was poorly described by a single-exponential curve, but could be well fitted by a sum of two exponentials: $I/I_0 = \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2)$, where $\alpha_1 = 21\%$, $\tau_1 = 14.4$ s, $\alpha_2 = 79\%$, and $\tau_2 = 275$ s. Similar experiments performed with native gA demonstrated that avidin did not affect τ for gA channels. A remarkable result was that the decelerating effect of avidin on the gA5XB photoinactivation kinetics was reversed by the addition of an excess of biotin (22 μM) to the bathing solution (Figure 1, curve 3, $\alpha_1 = 87\%$, $\tau_1 = 4.7$ s, $\alpha_2 = 13\%$, and $\tau_2 = 48$ s). Experiments with streptavidin have revealed that this protein also causes a tremendous deceleration of the gA5XB photoinactivation kinetics that is reversed by addition of free biotin (data not shown).

It has been found previously that polylysines produce a marked deceleration of the photoinactivation kinetics of channels formed by a negatively charged gramicidin analogue in BLM (*9*). It may be concluded that the effect of avidin studied in the work presented here is similar to the effect of polylysine. However, there are some data that would call into question this conclusion. Namely, to induce deceleration, it is necessary to add polylysine at both sides; in other words, polylysine is ineffective when added to only one side of the BLM. However, in the case of channels formed by gA5XB under conditions identical to those described in the legend of Figure 1, the addition of avidin to one side of the BLM led to considerable deceleration of the photoinactivation kinetics with the following parameters: $\alpha_1 = 55\%$, $\tau_1 = 5.0$ s, $\alpha_2 = 45\%$, and $\tau_2 = 22.5$ s.

It is seen from Figure 1A that avidin not only slowed the kinetics but also decreased the relative amplitude of photoinactivation (from 35 to 14%) which corresponds to the proportion of photoinactivated channels. The reduction of the amplitude could be accounted for by quenching of reactive oxygen species by the protein. As has been shown in refs *17* and *18*, the characteristic time and the amplitude of photoinactivation represent two independent parameters; for instance, variation of the photosensitizer concentration alters markedly the amplitude of photoinactivation, but does not change the characteristic time constant. The study presented here focuses mainly on the effect of avidin on the characteristic time of photoinactivation.

The addition of avidin normally decreased the level of the gA5XB-mediated current (Figure 2), in agreement with refs *6* and *7*. However, it was noticed that the avidin-induced change in the current essentially depended on experimental conditions, namely, on the temperature and stirring conditions. In any case, the effect of avidin on the BLM current was much less pronounced than the effect on the characteristic time constant of photoinactivation (Figure 1).

The avidin-induced reduction of the BLM current was observed also with the biotinylated gramicidin having only two aminocaproyl groups as a linker arm, gA2XB (the data

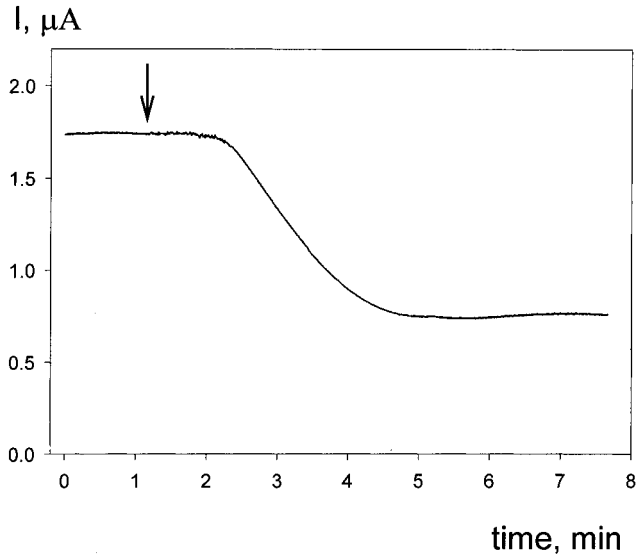

FIGURE 2: Effect of the addition of avidin (32 nM on both sides of the BLM) at the time indicated by the arrow on the gA5XB-mediated current through BLM. The conditions were as described in the legend of Figure 1.

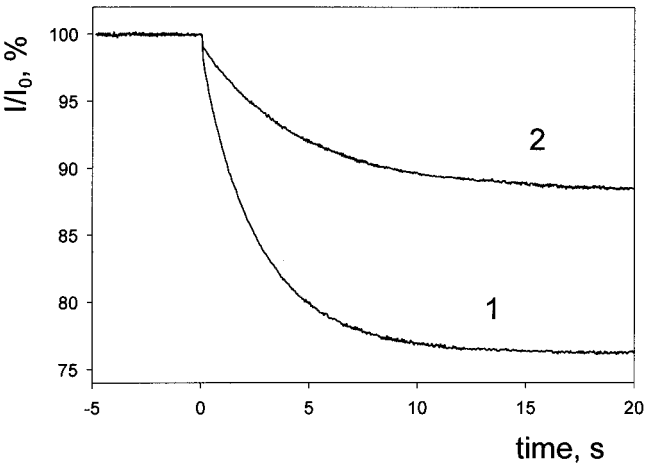

FIGURE 3: Effect of avidin (32 nM on both sides of the BLM) on the time course of the decrease in the gA2XB-mediated current (I) across a BLM after a flash of visible light (at time zero) in the presence of 1 μM $AlPcS_3$ (curve 2). Curve 1 is the time course of the current decrease in the absence of avidin. The normalized values of the current (I/I_0) are plotted vs time (t). Both curves are well fitted by monoexponentials with characteristic time constants of 2.7 (curve 1) and 4.3 s (curve 2). The conditions were as described in the legend of Figure 1.

not shown). However, in contrast to the effect described above for gA5XB, the addition of avidin did not cause a substantial deceleration of the photoinactivation kinetics of gA2XB (Figure 3). The characteristic time of photoinactivation increased in this case from 2.7 to 4.3 s only.

Figure 4 presents the results of single-channel measurements on gA5XB. In these experiments, the concentration of gA5XB was about 1 pM. The value of the average single-channel duration obtained from a histogram of single-channel lifetimes was 3.6 s. After prolonged incubation with avidin, a long-lasting open state of the gA5XB channel appeared which did not close for more than 10 min. It can be seen from the record of single-channel events under these conditions (Figure 4B) that, as well as the new open state of very long duration and usual open states similar to those in the control (Figure 4A), one can observe brief closures. Such flickering behavior has been described in the literature for long-lasting open states of chemically dimerized gA (*19*, *20*) and normal gA at low pH (*21*).

Figure 5 shows the data on current fluctuations across BLM induced by intermediate (0.02 nM) concentrations of gA5XB. Panel A displays successive records of the current fluctuations for the control (trace 1), in the presence of avidin (trace 2), and after the addition of biotin (trace 3). Panel B illustrates the corresponding power spectrum density of the current fluctuations, $S(f)$. $S(f)$ was approximated by the following equation (*22*):

$$S(f) = \frac{4\tau_c\overline{(\delta I)^2}}{1 + (2\pi\tau_c f)^2}$$

where τ_c is the correlation time and $(\delta I)^2$ is the mean square of the current fluctuations. According to ref *22*, $(\delta I)^2$ corresponds to the single-channel amplitude [$\Lambda = (\delta I)^2/I_a$, where I_a is the average current]. Noise analysis revealed that gA5XB generates a Lorentzian type power spectrum for current fluctuations (Figure 5B, curve 1). The following values for $(\delta I)^2$ and τ_c were derived: 5033 pA^2 and 6.1 s, respectively. However, the spectrum obtained in the presence of avidin did not allow us to calculate both parameters. Based on the fact that avidin did not alter the single-channel amplitude of gA5XB (Figure 4), we assumed that the ratio $(\delta I)^2/I_a$ remained unchanged in the presence of avidin. Thus, we could estimate a value for τ_c under these conditions. The calculation revealed an enormous increase in τ_c after the addition of avidin, amounting to 415 s. The effect was reversed by the addition of 2 μM biotin. Under these conditions, the current fluctuations also exhibited a Lorentzian type power spectrum with the following parameters: $(\delta I)^2 = 6059$ pA^2 and $\tau_c = 8.4$ s.

Figure 6 illustrates a possible scheme of interaction between avidin and gA5XB in BLM. The deceleration of the photoinactivation kinetics was attributed to formation of complexes comprising a gA5XB channel and one or two avidin molecules (bound on the opposite sides of the BLM in the latter case). This model is compatible with the results of analysis of the gA5XB photoinactivation kinetics at different concentrations of avidin in the bathing solution (Figure 7). The current decay was fitted by a sum of three exponentials with different values of the characteristic time constant, namely, 4, 30, and 150 s, that correspond to three forms of the conducting channels in the BLM in the presence of avidin (Figure 6). Figure 7B presents the concentration dependencies of the contributions from the three exponential components to the overall photoinactivation kinetics in terms of this model. As the avidin concentration in the solution increased from 2 to 300 nM, the contribution of the slowest component gradually grew, while that of the fastest component decreased monotonically. The contribution of the intermediary component first increased and then decreased in this concentration range. As seen from the solid lines in Figure 7B, this pattern of concentration dependence is consistent with a model involving the interaction of avidin and a gA5XB channel having two protein binding sites with identical affinity constants of 7 nM.

We surmise that avidin produces a deceleration of gA5XB channel kinetics by restricting the mobility of gA5XB

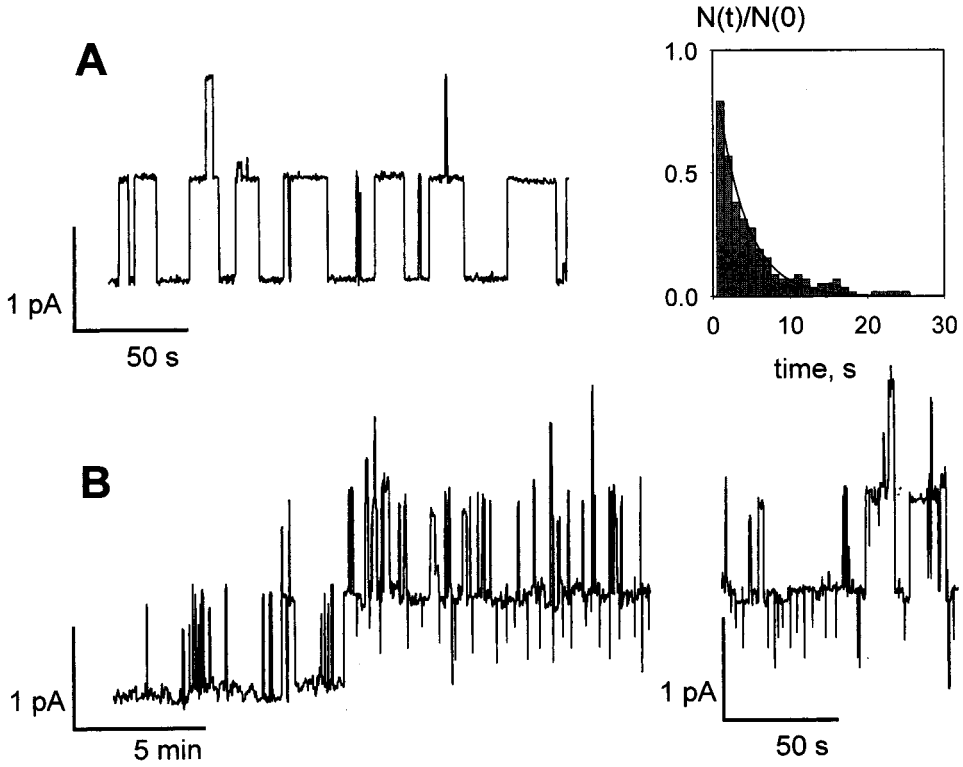

FIGURE 4: (A) Single-channel traces induced by gA5XB and the corresponding histogram of single-channel lifetimes. (B) Single-channel traces of gA5XB after the addition of 32 nM avidin to both sides of the BLM. The BLM voltage was 50 mV. The histogram of single-channel lifetimes was well-fitted by a single exponential with a τ of 3.6 s. The conditions were as described in the legend of Figure 1 except for a lower gA5XB concentration.

monomers and dimers upon formation of avidin and gA5XB complexes (Figure 6) that stabilizes the channel state and, thus, increases the single-channel lifetime. As has been shown by Swamy and Marsh (*23*), binding of avidin causes a strong and selective restriction of the mobility of biotinylated lipids. It also seems relevant to this point that binding of water-soluble proteins to different natural channels constrains their rotational and lateral mobility (*24*−*27*).

It should be noted that, in view of the renowned high affinity of the noncovalent interaction between biotin and avidin (*28*), the biotin-induced reversal of the effect of avidin on the photoinactivation kinetics and the noise spectrum of gA5XB was unexpected. However, this reversal could be explained by taking into account the facts that molecules of gA5XB carrying biotin groups are incorporated into a lipid bilayer and that the avidin association constant for other biotinylated species at the membrane surface (biotinylated lipids) has been found to be 4−6 orders of magnitude lower than the avidin binding constant for free biotin in solution (*29*). The desorption of bound streptavidin from biotin-terminated self-assembled monolayers (SAM) upon exposure to free biotin in solution has been reported recently by Pérez-Luna et al. (*30*).

The effect of the one-side addition of avidin on the channel kinetics, which manifested itself in an about 5-fold (≈22.5/4.7) increase in τ, could be explained by one-side binding of avidin to gA5XB channels. The subsequent addition of avidin to the other side of the BLM led to a 60-fold (≈275/4.7) increase in τ which could be attributed to the formation of two-side complexes of conducting dimers of gA5XB with two avidin molecules (Figure 1). The possibility that a single avidin can bind more than one gA5XB molecule cannot be excluded. However, it is very unlikely that channel cross-linking by avidin is involved in the deceleration of the channel kinetics because the effect of avidin is observed at a very low gA5XB surface density (even at the single-channel level).

The proposed model of the effect of avidin on gA5XB channels differs substantially from the model of the interaction of polylysine with negatively charged gramicidin (*O*-pyromellitylgramicidin, OPg) (*9*) since the latter assumes the segregation of OPg into domains induced by polylysine binding. Because the segregation requires the presence of many gramicidin molecules in the membrane, it has become clear why polylysine does not produce any effect on the single channels of OPg (*31*), while avidin dramatically increases the gA5XB single-channel duration (Figure 2).

According to ref *7*, the interaction between biotinylated gA and streptavidin manifests itself in the ligand-induced decrease in membrane conductance which confirms the results of Cornell et al. (*6*). In agreement with these data, our experiments have demonstrated the avidin-induced suppression of the gA5XB-mediated current across BLM both under single-channel and multichannel conditions. However, it has appeared that this suppression is strongly dependent on the experimental conditions. We propose that the level of the gA5XB-mediated current is a function of three different processes: (1) a decrease in the current due to partial removal of the channels from the BLM induced by avidin binding [a similar phenomenon was observed by Swamy and Marsh (*23*) with biotinylated lipids], (2) an increase in the current due to stabilization of the open channel state of gA5XB caused by interaction with avidin (i.e., lengthening of the channel duration), and (3) a decrease in the current

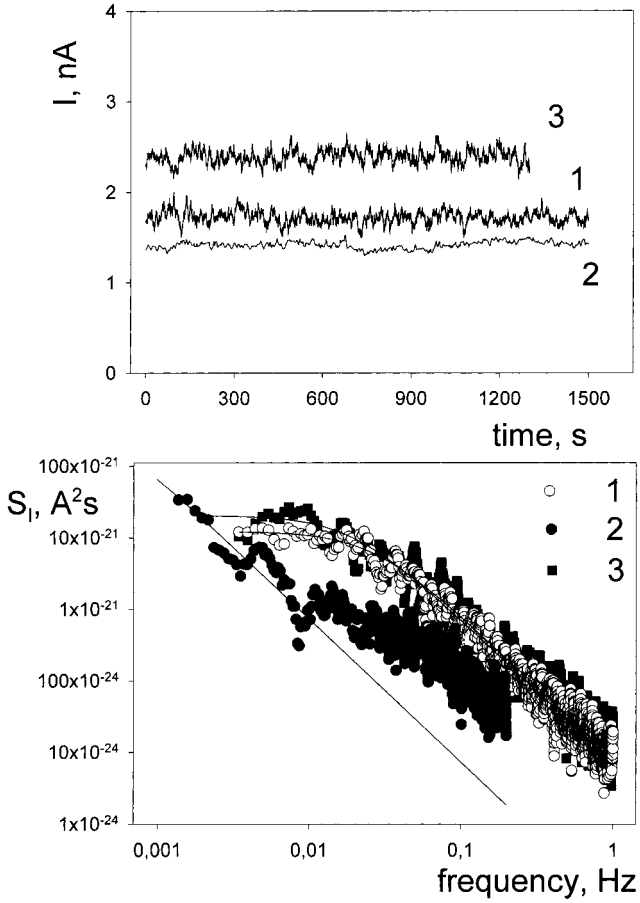

FIGURE 5: Traces of the gA5XB-mediated current across a BLM under multichannel conditions (upper panel) and the corresponding Fourier transforms (lower panel) for the control (curve 1), in the presence of 32 nM avidin (curve 2), and in the presence of 32 nM avidin and 2 μM biotin (curve 3) at 75 mV. The power spectrum density is fitted by eq 1 (solid lines) with the following parameters: $\tau_c = 6.1$ s and $(\delta I)^2 = 5030$ pA^2 (curve 1); $\tau_c = 415$ s and $(\delta I)^2 = 3025$ pA^2; $\tau_c = 8.4$ s and $(\delta I)^2 = 6060$ pA^2. Other conditions were as described in the legend of Figure 1.

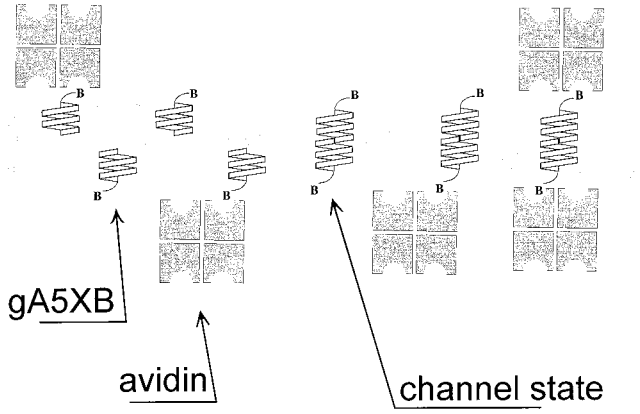

FIGURE 6: Scheme for the interaction of avidin and gA5XB in a lipid membrane at the intermediate concentrations of avidin when bound and unbound species are present in the membrane. Avidin is a tetramer with four biotin binding sites. The concentrations of gA5XB are for purposes of representation only.

due to possible slowing of the channel formation induced by avidin binding to gA5XB monomers. This hypothesis is supported by the data on the gA2XB photoinactivation where avidin induced only the suppression of the BLM current,

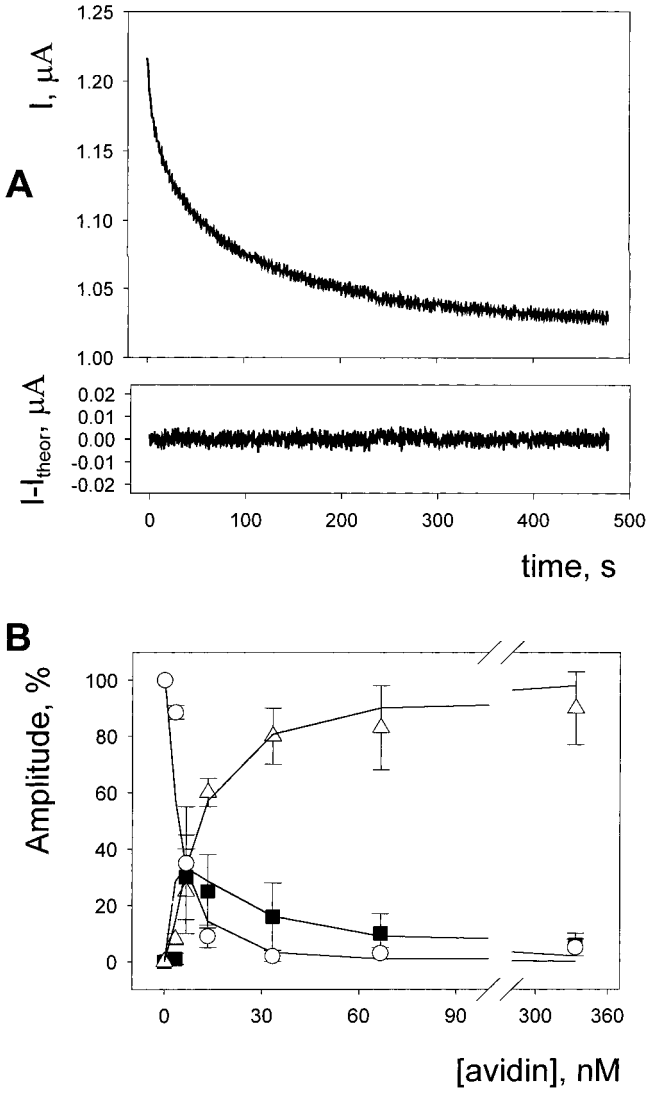

FIGURE 7: (A) Effect of avidin (13 nM on both sides of the BLM) on the time course of the decrease in the gA5XB-mediated current (I) across a BLM after a flash of visible light (at time zero) in the presence of 1 μM $AlPcS_3$ (curve 2) and deviation of the data from the triple-exponential curve: $I = \alpha_1 \exp(-t/4) + \alpha_2 \exp(-t/30) + \alpha_3 \exp(-t/150)$ with normalized amplitudes $\alpha_1 = 23\%$, $\alpha_2 = 30\%$, and $\alpha_3 = 47\%$. The conditions were as described in the legend of Figure 1. (B) The dependences of α_1 (○), α_2 (■), and α_3 (△) on the concentration of avidin. Solid lines represent the best fit curves derived from the theoretical model of gA5XB binding to avidin having two identical binding sites with affinity constants of 7 nM. The experimental points were corrected in this plot by assuming that the portion of the fast component (4 s) tended to zero at high avidin concentrations instead of approximately 20% as was observed. We propose that the avidin-independent fast component originated from a small proportion of nonbiotinylated gramicidin in the preparation of gA5XB.

whereas the characteristic time of photoinactivation was not considerably altered upon the protein addition. The inability of avidin to stabilize gA2XB channels in contrast to gA5XB channels can be explained by steric hindrance to the formation of complexes between water-soluble avidin and biotinylated gramicidin with a short linker arm incorporated in BLM. gA2XB molecules may be prevented from forming dimers or withdrawn from the BLM when complexes with avidin are formed.

In summary, it can be concluded that avidin greatly increased the single-channel duration of gA5XB channels

as revealed by photoinactivation kinetics, noise analysis, and single-channel measurements.

ACKNOWLEDGMENT

We are grateful to Dr. K. Raval (AMBRI-CRC for Molecular Engineering Technology, Sydney, Australia) for help with the synthesis of biotinylated gramicidin. The work was partially supported by the Russian Fund for Basic Research (00-04-48299).

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BI0007876