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The pH-dependent induction of lipid membrane ionic permeability by N-terminally lysine-substituted analogs of gramicidin A

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Abstract Insertion of charged groups at the N-terminus of the gramicidin A (gA) amino acid sequence is considered to be fatal for peptide channel-forming activity because of hindrance to the head-to-head dimer formation. Here the induction of ionic conductivity in planar bilayer lipid membranes (BLM) was studied with gA analogs having lysine either in the first ([Lys1]gA) or the third ([Lys3]gA) position. If added to the bathing solution at neutral or acidic pH, these analogs, being protonated and thus positively charged, were unable to induce ionic current across BLM. By contrast, at pH 11 the induction of BLM conductivity was observed with both lysine-substituted analogs. Based on the dependence of the macroscopic current on the side of the peptide addition, sensitivity to calcium ions and susceptibility to sensitized photoinactivation, as well as on the single-channel properties of the analogs, we surmise that at alkaline pH [Lys1]gA formed channels with predominantly single-stranded structure of head-to-head helical dimers, whereas [Lys3]gA open channels had the double-stranded helical structure. CD spectra of the lysine-substituted analogs in liposomes were shown to be pH-dependent.

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Introduction

The pentadecapeptide gramicidin A (gA) is known to form ionic channels in bilayer lipid membranes exhibiting high selectivity for monovalent cations (Hladky and Haydon 1984; Andersen and Koeppe 1992; Woolley and Wallace 1992; Busath 1993). It is generally considered that the predominant conducting form of gA represents a transmembrane dimer in which monomers having a structure of a single-stranded $\beta^{6.3}$-helix are linked head-to-head by hydrogen bonds (Urry 1971; Arseniev et al. 1985; Smith et al. 1989; Lomize et al. 1992; Ketchem et al. 1993, 1997; Separovic et al. 1994, 2011). However, in membranes of certain lipid composition, monovalent cation-selective channels formed by gA and its analogs might have the structure of a double-stranded $\beta^{5.6}$ helix (Durkin et al. 1992; Koeppe and Andersen 1996; Sychev et al. 1996; Mobashery et al. 1997; Saparov et al. 2000; Dutseva et al. 2007) found both in crystals (Langs 1988; Wallace and Ravikumar 1988; Wallace 1998) and some lipid membrane environments (Dzikovski et al. 2011). The double-helical structure is also implied for the conducting form of gA-related peptides with a D,L-alternating amino acid sequence (Kusel et al. 2007; Schneggenburger et al. 2010; Schramm and Hofmann 2010). Though gA structure and function have long been studied, the ideas of the nature of gA conducting states and the mechanism of channel opening/closing still remain points of controversy (Jones et al. 2010); in particular, the contribution of the double-helical structure (Veatch et al. 1974) to gA-mediated membrane

conductance is continuously debated (Burkhart et al. 1998; Andersen et al. 1999; Cross et al. 1999; Siu and Bockmann 2009). Much progress in related studies has been achieved by using gramicidin analogs with amino acid substitutions and other chemical modifications. Those altering the N-terminus of gA, e.g., substitution of amino acid residues in positions 1 (Morrow et al. 1979; Mattice et al. 1995) and 3 (Daily et al. 2010) or replacement of *N*-formyl with the *N*-acetyl group (Szabo and Urry 1979; Seoh and Busath 1993), led to considerable destabilization of the channel manifesting in a sharp reduction of single channel lifetime, thereby supporting the head-to-head dimer structure as a prevailing gA channel form. To this end, the gA analog with the negatively charged *N*-pyromellityl group placed instead of *N*-formyl did not induce membrane conductance comparable to that seen with the parent gA at pH 6 (Bamberg et al. 1977). By contrast, attachment of charged groups to the C-terminus of gA did not provoke marked changes in its channel characteristics (Apell et al. 1977; Roeske et al. 1989; Woolley et al. 1995, 1997; Separovic et al. 1999; Antonenko et al. 2002, 2005, 2006; Stoilova et al. 2007). According to early works (Goodall 1971; Urry 1971; Bezrukov et al. 1984), elimination of the *N*-formyl group resulted in loss of typical gA channel activity. Based on later experimental and theoretical studies (Saparov et al. 2000; de Groot et al. 2002), a low but noticeable membrane conductance induced by desformylgramicidin was ascribed to the double-helical form of the peptide.

It is important that the structure of gA head-to-head transmembrane dimer implies that N-termini of both monomers reside in the middle of the hydrophobic core of a bilayer lipid membrane (BLM). Therefore, introduction of an electrical charge to the gA N-terminus should have dramatic consequences for the channel activity. In line with this prediction, the analog with the *N*-formyl substituted by the *N*-succinyl residue demonstrated strong pH dependence: at low pH (<5) the *N*-succinyl derivative showed high channel activity, whereas at high pH (>7) when carboxyl groups became negatively charged, the activity was sharply reduced or disappeared totally (Bamberg et al. 1979). Similar observations were made with a gA analog containing lysine instead of valine in position 1 ([Lys1]gA): it exhibited normal gA conductance at pH 11 and negligibly low conductance at pH 7 when it became positively charged (Borisenko et al. 2003). These results prove the location of charged moieties inside the membrane interior to be extremely unbeneficial. However, studies on channel activity of the 18-residue peptide alamethicin with a negative charge introduced into position 7 (Glu7) revealed a change in the channel selectivity, but no suppression of the activity (Asami et al. 2002). Though the barrel-stave mechanism of alamethicin pore formation is quite different from that of gA (Woolley and Wallace 1992), Glu7 is supposed to reside rather deeply in the membrane. This prompted us to apply a set of different approaches for thorough examination of behavior of gA analogs with a dissociable residue (lysine) introduced to position 1 or 3, which renders the peptides positively charged upon lowering the pH of a membrane-bathing solution. We believe that this study is relevant for uncovering the mechanistic details of immersion of charged residues into the hydrophobic membrane interior, which are of general importance because such a process underlies functioning of voltage-gated ionic channels, translocation of polycationic peptides across cellular membranes, etc. Here, both of the N-terminally lysine-substituted gA analogs were shown to be potent channel formers at alkaline pH; however, their predominant conducting forms appeared to be quite different.

Materials and methods

Analogs of gramicidin A, i.e., [Lys1]gA (HCO-KGALA VVVWLWLWLW-NH$(CH_2)_2$OH) and [Lys3]gA (HCO-VGKLAVVVWLWLWLW-NH$(CH_2)_2$OH), were prepared by standard solid-phase Nα-Fmoc methodology on Rink amide resin [4(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin] using the diisopropylcarbodiimide/1-hydroxybenzotriazole coupling system. N-terminal formylation of peptides was conducted in the presence of *N*-ethyldiisopropylamine using 2-nitrophenyl formate. The peptide resins were treated with trifluoroacetic acid–ethandithiol–water (94:3:3) for 2.5 h. HPLC purification of the samples gave pure peptides (purity >95%). The fidelity of the peptides was confirmed by MALDI-TOF MS. Gramicidin A (gA) was from Fluka.

Planar bilayer lipid membranes (BLM) were formed from a 2% solution of diphytanoylphosphatidylcholine (DPhPC) (Avanti Polar Lipids, Alabaster, AL) in *n*-decane on a hole in a Teflon partition (0.5-mm diameter) separating two compartments of a cell containing aqueous buffer solutions (Mueller et al. 1963). The membrane bathing solution was 1 M KCl, 10 mM Tris, 10 mM MES, and 10 mM β-alanine adjusted to the appropriate pH. Most of the experiments were carried out at room temperature (23–25°C). The electric current (I) was recorded under voltage-clamp conditions. Voltage was applied to BLMs with Ag–AgCl electrodes placed directly into the cell. The current measured by means of a patch-clamp amplifier (Warner Instruments, Hamden, CT, model BC-525C) was digitized using an NI-DAQmx (National Instruments, Austin, TX) and analyzed with a personal computer with the use of WinWCP Strathclyde Electrophysiology Software designed by J. Dempster (University of Strathclyde, UK). Multichannel measurements were carried out using a

home-made current amplifier with a feedback resistor of 10^8 Ohm.

Liposomes for CD measurements were prepared as described by Killian et al. (1988) with small modifications (Sychev et al. 1996). Stock solutions of an appropriate amount of a peptide (0.1–0.5 mg) and lipids were prepared in methanol (0.8 ml) and chloroform (0.8 ml), respectively. The solutions were mixed and solvents were removed in a rotary evaporator. The dry mixture of the peptide and egg yolk phosphatidylcholine (EggPC) was dissolved in TFE or ethanol (1.6 ml) to obtain the peptide concentration of 10^{-4} M. After incubation for 2 h at 20°C the solvent was removed by rotary evaporation (at 45°C), and samples were dried for 2 h under vacuum. The dry lipid-peptide film was hydrated in a buffer to 5×10^{-4} M concentration of the peptide. The samples were allowed to swell for 30 min and then were sonicated at 20°C by a Braunsonic 1510 sonicator two or three times until they became optically transparent. The samples were subjected to "heat treatment" by overnight incubation at 80°C under argon. CD spectra were recorded at 20°C with a Jasco 500 C dichrograph in demountable cells (Hellma) with 10^{-2} cm optical path length. All the spectra reported are the average of four scans. A spectrum of an appropriate suspension of liposomes without peptides was used as the baseline. Contributions of different gramicidin forms to the CD spectra were analyzed as described previously (Sychev et al. 1993, 1996).

Results

Gramicidin A and its analogs are known to effectively induce the current of potassium ions across BLM in a wide range of pHs (Rostovtseva et al. 1998; Borisenko et al. 2002). Based on the head-to-head dimer structure of the gA channel, it is reasonable to expect that the channel activity of gA analogs having a dissociable residue in position 1 or 3 should exhibit strong pH dependence. In particular, with lysine-containing analogs, the channels similar to those of gA should be observed at alkaline pH (pK of lysine in water solution is about 11). Actually, in our experiments, the addition of both [Lys1]gA and [Lys3]gA to the bathing solutions containing 1 M KCl at pH 11 led to induction of substantial macroscopic currents across BLM. As the channel activity of gA associated with formation of single-stranded head-to-head dimers requires the peptide to be added at both sides of BLM, it was important to determine how the channel-forming potency of the analogs depends on the side of their addition. As seen from Fig. 1a, the addition of [Lys3]gA to one side of BLM induced an increase in the current in the time scale of tens of minutes, whereas no increase in the current was detected under similar conditions with gA, i.e., gA displayed no channel activity if added to only one side of the membrane. In accord with well-known properties of gA, a dramatic increase in the transmembrane current is observed after the subsequent addition of gA to the opposite side of the bilayer. By contrast, no change in the slope of the current versus time dependence occurs after the corresponding addition of [Lys3]gA to the opposite side of the bilayer. Interestingly, the behavior of [Lys1]gA appeared to be intermediate between that of gA and [Lys3]gA, namely: the transmembrane current rose gradually with time after the addition of [Lys1]gA to one side of BLM until a sharp change in the slope of the current recording took place after

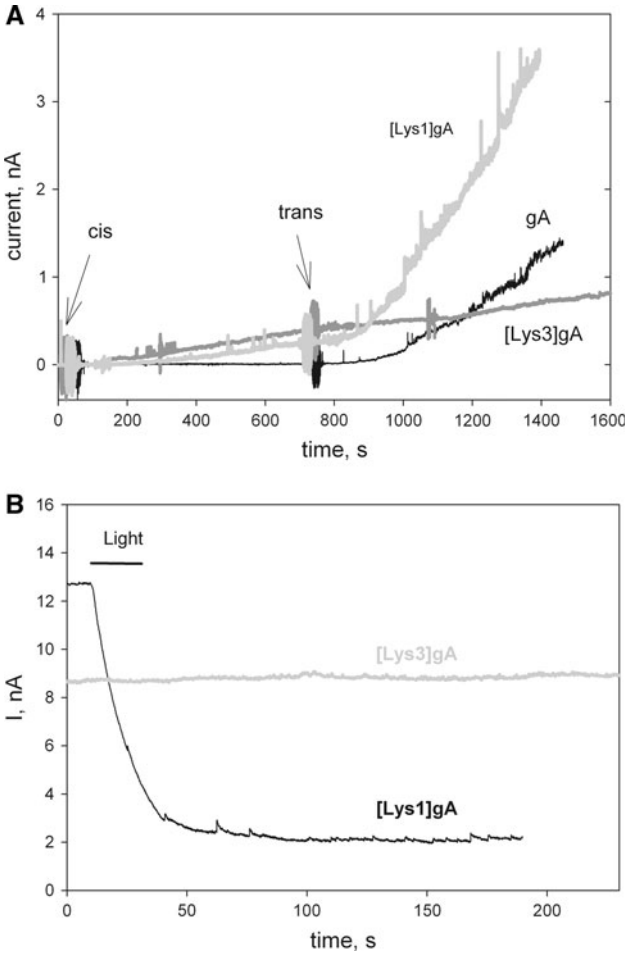

Fig. 1 **a** The current across planar BLM after asymmetric and symmetric additions of gA and its analogs. Concentrations of peptides upon additions were: [Lys1]gA 2.5 nM, [Lys3]gA 50 pM, and gA 70 pM. The solution was 10 mM β-alanine and 1 M KCl, pH 11. BLM voltage, 50 mV. **b** Different susceptibility of lysine-substituted gA analogs to sensitized photoinactivation. Effect of 20-s exposure of BLM to *red light* (marked by a *line bar*) in the presence of a photosensitizer, Rose Bengal (1 μM), on the electrical current mediated by [Lys1]gA (2.5 nM) and [Lys3]gA (70 pM). The solution was 10 mM Tris, 10 mM MES, 10 mM β-alanine, and 1 M KCl, pH 11. BLM voltage, 50 mV

the addition of [Lys1]gA to the opposite side of BLM. Notably, to induce comparable currents, a much higher concentration was required with [Lys1]gA (2.5 nM) than with [Lys3]gA (50 pM) and gA (70 pM).

According to earlier works (Strassle and Stark 1992; Rokitskaya et al. 1993, 1996), classical gA channels are highly susceptible to photoinactivation in the presence of a photosensitizer, e.g., Rose Bengal, whereas covalently linked gramicidin dimers proved to be insensitive to the photodynamic treatment (Rokitskaya et al. 1996). Here, illumination of BLM in the presence of Rose Bengal (Fig. 1b) led to suppression of the current induced by [Lys1]gA, but not of that induced by [Lys3]gA, thereby

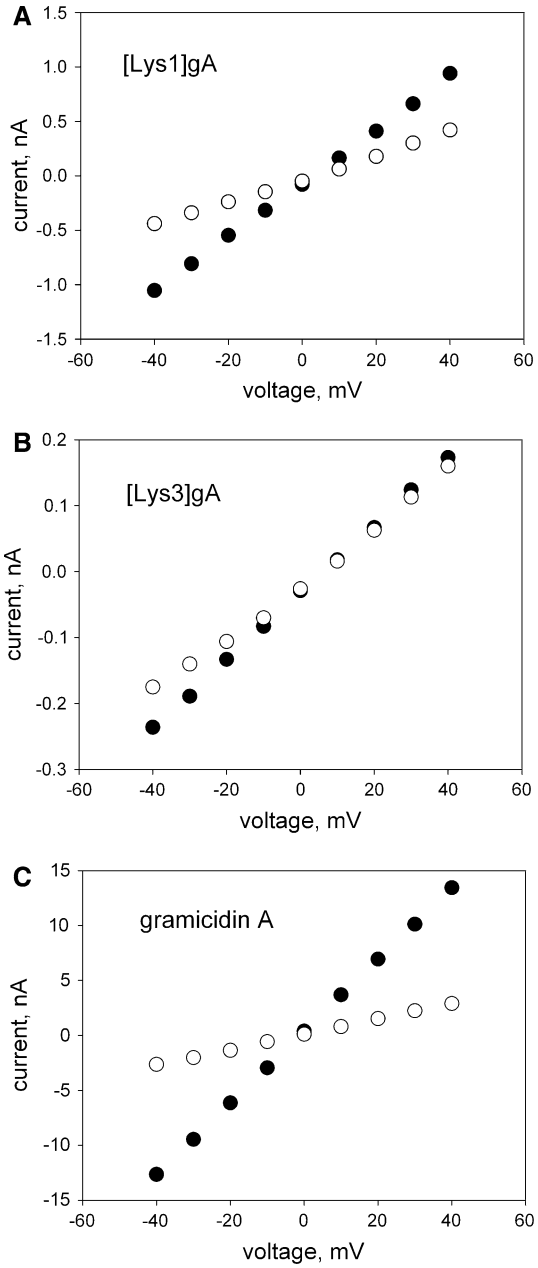

Fig. 2 Current-voltage dependences for [Lys1]gA-, [Lys3]gA-, and gA-mediated electrical current before (*closed circles*) and after (*open circles*) addition of 60 mM $CaCl_2$ at the cis-side of the bilayer (positive potential). The solution was 10 mM β-alanine and 250 mM KCl, pH 11

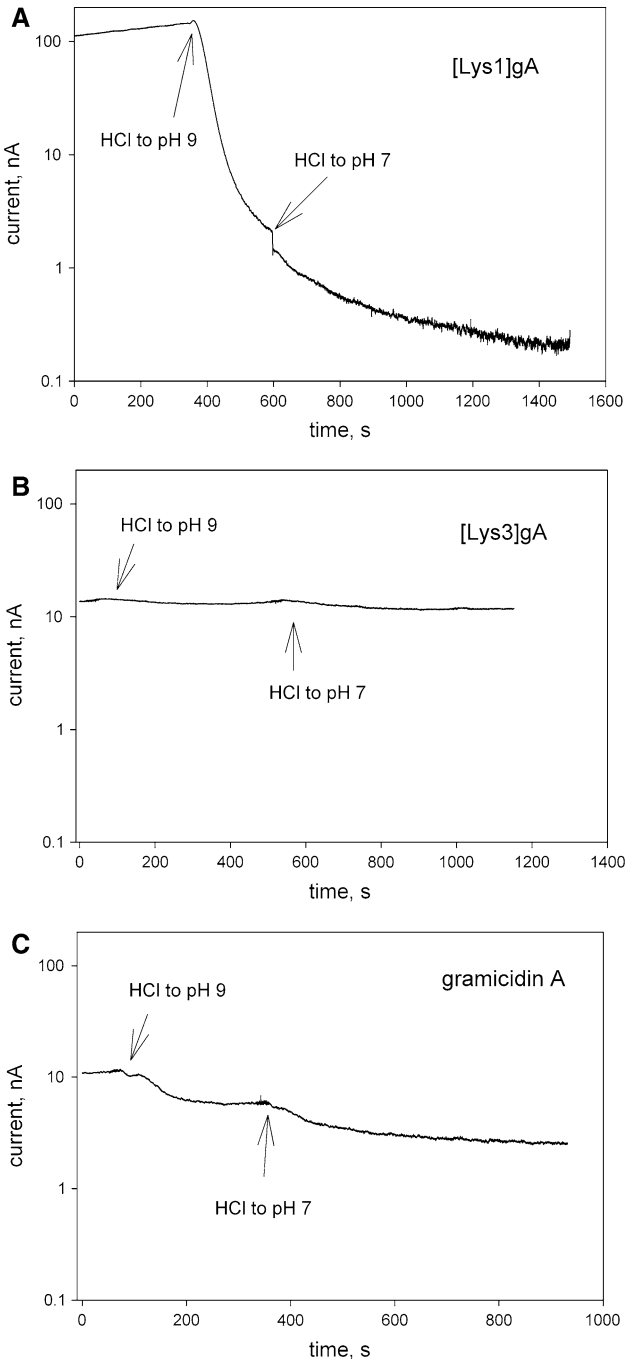

Fig. 3 The pH dependence of the peptide-induced current across planar BLM. *Panels* **a–c** show typical records with [Lys1]gA (2 nM, *panel* **a**), [Lys3]gA (150 pM, *panel* **b**), and gA (100 pM, *panel* **c**). All the peptides were added at both sides of BLM. The initial solution was 10 mM Tris, 10 mM MES, 10 mM β-alanine, and 1 M KCl, pH 11. BLM voltage, 50 mV

showing the difference between conducting states of [Lys1]gA and [Lys3]gA.

Calcium ions are known to block gramicidin-mediated potassium currents in millimolar concentrations (Bamberg and Lauger 1977; Heitz and Gavach 1983; Gambale et al. 1987). To determine the calcium sensitivity of [Lys1]gA, [Lys3]gA, and gA at pH 11, current-voltage dependences were measured before (Fig. 2, closed circles) and after the addition of 60 mM $CaCl_2$ to one side of the membrane (panels a, b, and c, respectively). As expected, calcium ions blocked gA- and [Lys1]gA-mediated currents (panel a and c of Fig. 2). However, in the case of [Lys3]gA, the blockage was very poor (Fig. 2b), thereby supporting a difference in the nature of conducting states.

To study an impact of introducing electric charge to the peptide N-terminus on the channel activity, we examined an effect of acidification of the bathing solution on the transmembrane current induced by the lysine-substituted analogs. Upon lowering the pH of the bathing solution from 11 to 5 by successive additions of HCl, complete abolishment of the current was observed with [Lys1]gA, while an approximately threefold decrease in the current was seen with [Lys3]gA and about tenfold decrease with gA (Fig. 3). Therefore, after insertion into the membrane at pH 11, [Lys3]gA was able to maintain rather high membrane conductance at neutral and acidic pH. However, if [Lys3]gA was added to the bathing solution at pH 5–7, no current was detected, just as with [Lys1]gA, which is in

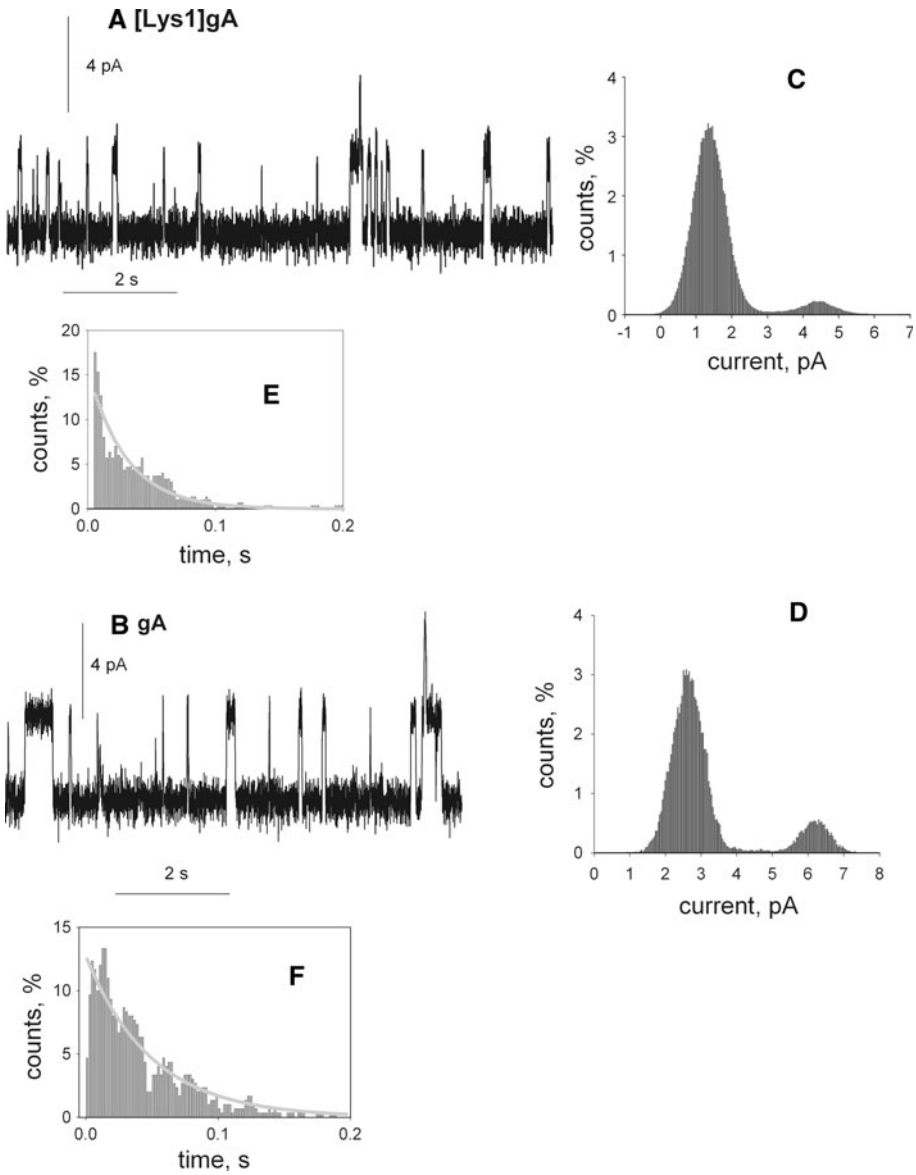

Fig. 4 Single channel recordings of [Lys1]gA (**a**) and gA (**b**) with planar BLM and corresponding current amplitude (*panels* **c**, **d**) and channel lifetime (*panels* **e**, **f**) histograms. The solution was 10 mM Tris, 10 mM MES, 10 mM β-alanine, and 1 M KCl, pH 11. BLM voltage, 100 mV

striking contrast to the well-known fact that gA effectively facilitates transmembrane ionic current at neutral pH.

To ascertain quantitative parameters of channels formed by the lysine-substituted gA analogs, we performed single-channel experiments at pH 11. In line with the earlier data (Borisenko et al. 2003), [Lys1]gA exhibited homogeneous channels (Fig. 4, panel a) with the single-channel conductance of 30 pS and the lifetime of 30 ms. Rather similar channels were formed by gA (Fig. 4, panel b) under these conditions, i.e., at pH 11: they had the single-channel conductance of 35 pS and the lifetime of 49 ms. Single-channel recordings made with [Lys3]gA (Fig. 5) contained two kinds of events: (1) "fast" channels (panel a) with the conductance of 40 pS and the lifetime of 100 ms, and (2) "slow" channels (panel b) with a long-lived (having the lifetime of the order of 100 s) open state characterized by a decreased conductance (22 pS).

In view of the marked differences in the pH-dependent behavior of gA and its lysine-substituted analogs in planar bilayers, it was important to compare their structure in lipid bilayer membranes at different pH values. Therefore, we measured CD spectra of the peptides incorporated into EggPC liposomes after heat incubation (as described in "Materials and Methods") at alkaline and neutral pHs (Fig. 6). Importantly, a CD spectrum of [Lys3]gA measured at pH 11 differed greatly from that recorded at pH 7 (Fig. 5a, curves 1, 2). In particular, the pH 11 spectrum was dominated by a positive peak with its maximum at 220 nm, typical for single-stranded $\beta^{6.3}$ helical dimers, whereas the pH 7 spectrum was dominated by a negative peak with its maximum at 228 nm, corresponding to double-stranded $\beta^{5.6}$ helical dimers. The CD spectra of [Lys1]gA (Fig. 5b, curves 1, 2) showed less variability at neutral and alkaline pHs than those of [Lys3]gA.

Discussion

This study of gA analogs with protonatable residues placed at the N-terminus of the peptide sequence revealed their ability to form ion-conducting channels with the efficacy depending on the pH of the bathing solution, namely: the lysine-substituted analogs formed ionic channels only if added to the bathing solution at alkaline pH, when the peptides are neutral. Taking into account the data obtained upon addition of the peptides to either one or both sides of the bilayer, different structures are suggested to contribute predominantly to the BLM conductivity mediated by the gA analogs, namely: the single-stranded $\beta^{6.3}$-helical dimer (HD)—in the case of [Lys1]gA, and the double-stranded dimeric helix (DH)—in the case of [Lys3]gA. From the two types of events observed in single-channel recordings of [Lys3]gA (Fig. 5), the long-lived channels with a conductance of 22 pS (Fig. 5) are likely to have a double-stranded structure, while the short-lived events apparently correspond to channels of conventional single-stranded structure, which contribute insignificantly to the macroscopic current induced by [Lys3]gA. This assumption is based upon the properties of the gA analog (gLW) described in Koeppe and Andersen (1996), which also exhibited two types of channels with double-helical ones having two orders of magnitude longer duration. Such a stabilization of double-helical channels could be associated with a much larger number (28) of intermolecular

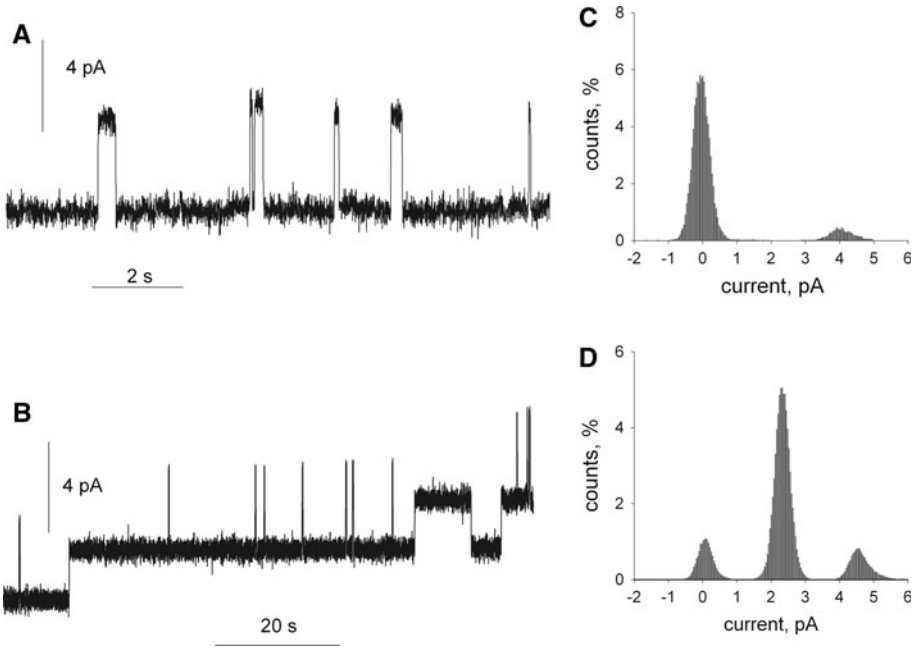

Fig. 5 Single channel recordings of [Lys3]gA (**a**, **b**) with planar BLM and corresponding current amplitude histograms (*panels* **c**, **d**). The solution was 10 mM Tris, 10 mM MES, 10 mM β-alanine, and 1 M KCl, pH 11. BLM voltage, 100 mV

hydrogen bonds maintaining DH structure as compared to only 6 of them in HD structure (Wallace 1998).

Of the different types of DH found in solution (Veatch et al. 1974), an antiparallel one, having a central lumen sufficiently wide to accommodate an alkali metal cation, is the most probable candidate for the conducting double-helical form of [Lys3]gA (Wallace 1998; de Groot et al. 2002), because a central lumen of a parallel DH is too narrow for a cation to enter and traverse it. According to molecular dynamics simulations (de Groot et al. 2002), the diameter of a hydrophilic cylinder inside the antiparallel DH is even larger by 0.2 Å than that of the ion-conducting pathway of HD. Importantly, HD was shown to readily form complexes with calcium ions (Jing and Urry 1995), resulting in partial suppression of channel conductivity (Bamberg and Lauger 1977; Heitz and Gavach 1983; Gambale et al. 1987). By contrast, the antiparallel DH does not bind calcium ions (Chen and Wallace 1997; Wallace 1998). Therefore, our assignment of [Lys3]gA-mediated conductivity to the antiparallel double-helical form of this peptide is supported by a substantial difference in calcium effects on gA- and [Lys3]gA-induced currents (Fig. 2).

The CD spectra showed that at pH 11 both lysine-substituted analogs contained two types of helical structure: single-stranded and double-stranded, with the portion of the latter being even higher than that of the former in the case of [Lys1]gA (Table 1). It can be supposed that these structures have different functional activity depending on the position of a lysine residue. In particular, with [Lys1]gA, single-stranded helical dimers are more potent in forming conducting channels than double-stranded dimers, whereas with [Lys3]gA, it is obviously not the case. To explain why insertion of a lysine residue in position 3 compromises the channel activity of HD structure, while insertion of a lysine residue in position 1 hinders the activity of DH, one should take into account that intermolecular hydrogen bonds in HD are formed between residues in positions 1 and 5 of two monomers and between residues in positions 3 and 3 (Wallace 1998). We may speculate that lysine placed in position 1 appears not to disturb seriously an ion-conducting pathway in HD, whereas with lysine in position 3, we have got two lysines facing each other in HD, which might cause local bending of the molecular structure, thereby completely blocking ion movement through the channel. On the other hand, in the case of DH, insertion of lysine in position 1, which is close to the channel entrance (in contrast to position 1 in HD located near the membrane center), might hamper cation influx or efflux from the channel, while lysine in position 3 resides farther from the channel entrance, thus producing a weaker effect on the conductivity.

Noteworthily, the conductivity measurements were performed with decane-containing membranes known to be thicker than solvent-free bilayers (Chernyshev et al. 2003) used in the CD experiments. Given the dependence of gA conformational preference on membrane thickness (Mobashery et al. 1997; Andersen and Koeppe 2007), CD spectra might not reflect the ratio of single-stranded and double-stranded helices occurring in the electrophysiological experiments. To this end, it should be noted that according to Svensson et al. (2011), linear dichroism of gA in the presumed double-helical state in lipid membranes is incompatible with the double-helical conformation observed in gA crystals and might be ascribed to an extended, partially intertwined structure where the

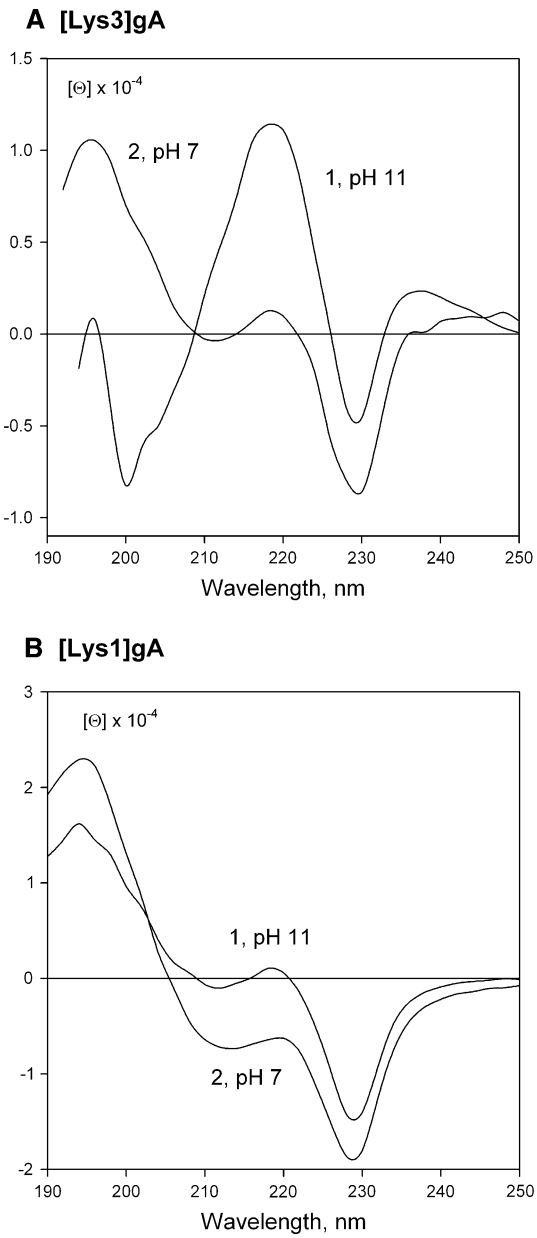

Fig. 6 CD spectra of [Lys3]gA (*panel* **a**) and [Lys1]gA (*panel* **b**) in EggPC liposomes measured at pH 7 and 11

Table 1 Contributions of different equilibrium conformational states of [Lys1]gA and [Lys3]gA in egg PC liposomes as calculated from the CD spectra

Peptide	PH	Parallel $\beta^{5.6}$ double helix	Antiparallel $\beta^{5.6}$ double helix	Single-stranded $\beta^{6.3}$ helical dimer
[Lys3]gA	7	0.10	0.40	0.50
	11	0.10	0.10	0.80
[Lys1]gA	7	0.30	0.50	0.20
	11	0.15	0.45	0.40

backbones of the two monomer units are intertwined in the hydrocarbon core and the C-terminal parts of the backbone are arranged in a single-stranded helical manner near the membrane surface.

At lower pH values, lysine residues of the peptides become protonated and acquire a positive charge, thereby making formation of head-to-head dimers energetically unfavorable because of electrostatic repulsion between N-termini of monomers. Accordingly, the content of these dimers estimated from the CD spectra decreased upon acidification (Table 1). It should be taken into account that with [Lys3]gA channels having most probably the antiparallel double-stranded helical structure where lysine residues are located close to the water-membrane interface, the appearance of positive charges on lysine residues upon their protonation is not expected to provoke such dramatic consequences for the conductance as found with [Lys1]gA channels having the structure of single-stranded head-to-head dimers where lysine residues are supposed to reside in the middle of membrane hydrophobic core. Actually upon the transition from pH 11 to pH 7, only modest decrease in the current was seen with [Lys3]gA, in contrast to the complete suppression of the current with [Lys1]gA. Supposedly, acidification did not result in protonation of the [Lys3]gA peptide because of poor accessibility of Lys3 in DH channels to bulk protons; otherwise, protonated Lys3 in the [Lys3]gA peptide would distort an ion-conducting pathway, leading to non-functional channels. Restricted access of bulk protons to membrane-buried amino or carboxyl groups is a characteristic feature of many proteins operating as proton pumps, e.g., bacteriorhodopsin (Neutze et al. 2002) and cytochrome C oxidase (Yoshikawa et al. 2011).

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