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Redox-regulated ion channel activity of a cysteine-containing gramicidin A analogue

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Abstract

According to recent data, gramicidin A analogues having positively charged amino acid sequences at the C-termini exhibit two types of channel activity in lipid membranes: classical cation-selective channels and large unselective pores. The induction of unselective pores was shown here to strongly depend on the redox state of the membrane-bathing solution, if the gramicidin analogue contained a cysteine residue in the sequence GSGPKKKRKVC attached to the C-terminus. In particular, the addition of H_2O_2 led to an increase in the transmembrane current and the loss of cationic selectivity on planar bilayer lipid membranes and an increase in the carboxyfluorescein leakage of liposomes. The effect was observed at high concentration of the peptide while was absent at the single-channel level. It was concluded that oxidation led to possible formation of dimers of the peptide, which promoted the formation of large unselective pores.

Keywords: Ion channel; Transmembrane peptide; Phospholipid membrane

1. Introduction

The activity of membrane ionic channels is known to be modulated reversibly by oxidation of certain amino acid residues, e.g., cysteinyls [1–10]. Oxidation or cross-linking of sulfhydryl groups was reported to promote opening of the permeability transition pore, a cyclosporin A-sensitive channel in the inner mitochondrial membrane [11–13]. It has been suggested that ionic channels may function in vivo as membrane redox sensors [14–16] and in particular as H_2O_2 sensors [17,18]. In view of this, it was of interest to study the effect of thiol oxidation on the activity of simple channel-forming peptides. We addressed this issue using C-terminal derivatives of the pentadecapeptide gramicidin A (gA) with attached cysteinyl.

This system may serve as a model of a device that is sensitive to the redox potential of the medium. Artificial as well as modified natural ion channels have been used successfully as prototypes of sensing devices [19–25]. As in living cells, they can function as amplifiers of signals, since opening of few ion channels leads to a large flux of ions across the membrane. In particular, gramicidin A, a highly potent agent forming ion-conducting channels in lipid membranes, has been used as an element of constructs designed for pH sensing [26] and detecting a series of analytes [19,27–29]. Gramicidin A consists of an alternating sequence of L- and D-amino acids (HCO-VGALAVVVWLWLWLW-NH$(CH_2)_2$OH). In a membrane, two gA monomers associate head-to-head into a transmembrane dimer, thus forming a 4-Å diameter cation-selective pore [30–32]. Here we describe newly synthesized gA analogues that are rendered sensitive to redox potential of the medium. This is achieved by attaching a hydrophylic sequence of 11 amino acid residues with cysteinyl at the end to the water-exposed C-terminus. It is known that elongation of gA at the C-terminus,

Abbreviations: BLM, bilayer lipid membrane; gA, gramicidin A; P4C, peptide gramicidin-βA-GSGPKKKRKVC; P5C, peptide acetyl-gramicidin-βA-GSGPKKKRKVG; P10C, peptide acetyl-gramicidin-βA-GSGPKKKRKVC; CF, carboxyfluorescein; DPhPC, diphytanoylphosphatidylcholine; DPhPG, diphytanoylphosphatidylglycerol

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even by attaching charged groups, does not interfere substantially with the ability of the peptide to conduct cations [33–37].

2. Materials and methods

Peptides 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 formulation and N-terminal acetylation of peptides were conducted in the presence of N-ethyldiisopropylamine using 2-nitrophenyl formate and acetic anhydride, respectively. 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. The expected/measured molecular masses (Da) were: 3077.8/3076.8 (gramicidin-βA-GSGPKKKRKVC, P4C), 3046.7/3046.5 (Ac-gramicidin-βA-GSGPKKKRKVG, P5C), 3091.8/3092.0 (Ac-gramicidin-βA-GSGPKKKRKVC, P10C). The βA linker was added to desethanolamine gA in each case.

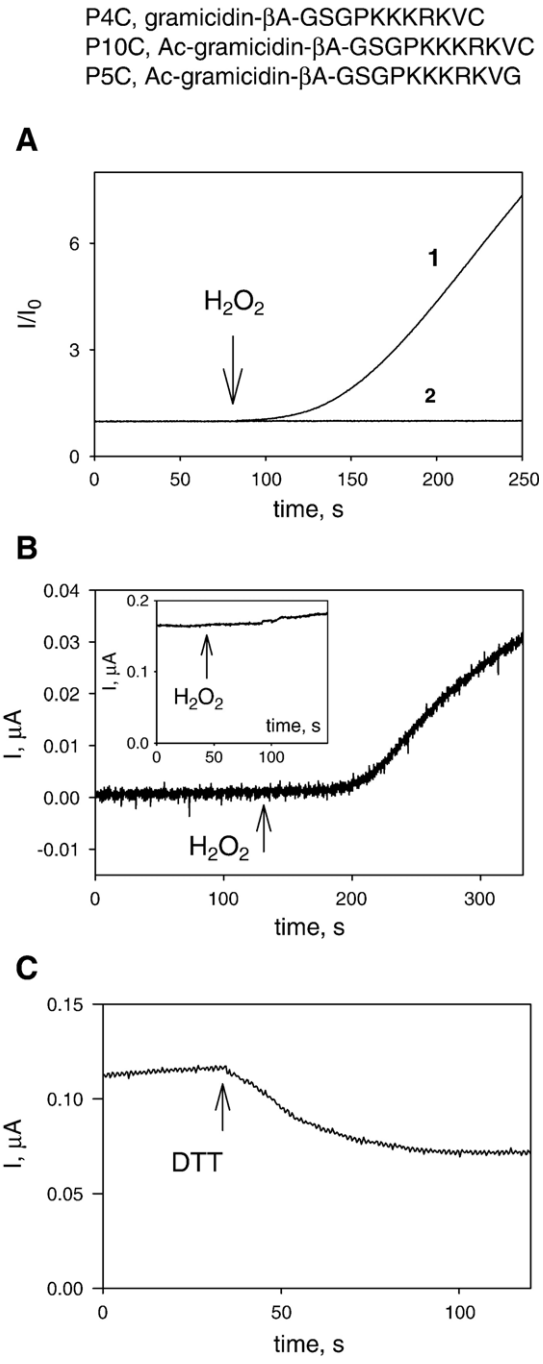

Fig. 1. Effect of H_2O_2 (10 mM, panels A and B) and 1,4-dithio-DL-threitol (3 mM, panel C) on the peptide-induced electrical current across planar bilayer lipid membrane. (A) Curve 1, peptide P4C; curve 2, gA. (B) Peptide P10C, insert: peptide P5C. (C) Peptide P10C. This measurement was recorded in the presence of 1 mM H_2O_2. The solution was 1 M KCl, 30 μM DTT, 10 mM Tris, 10 mM MES, pH 7 except panel C where DTT was absent. I_0 was 0.5 μA.

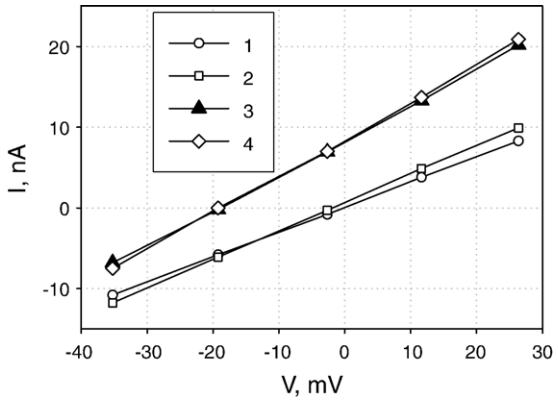

Fig. 2. Measurements of ionic selectivity of P4C under the reducing (30 μM 1,4-dithio-DL-threitol) and oxidizing (10 mM H_2O_2) conditions. The solution was 100 mM KCl, 10 mM Tris, 10 mM MES, pH 7. Curve 1 is a control under symmetrical conditions, while curves 2–4 are measured in the presence of KCl gradient on the membrane (200 mM/100 mM). Curves 1 and 2 are in the presence of 10 mM H_2O_2, curve 3 is in the presence of 30 μM DTT, curve 4 represents measurements for gA.

Planar bilayer lipid membranes (BLMs) were formed from a 2% solution of diphytanoylphosphatidylcholine (DPhPC, Avanti Polar Lipids, Alabaster, AL) in *n*-decane (Merck, Darmstadt, Germany) by the brush technique [38] on a hole in a Teflon partition separating two compartments of a cell containing aqueous buffer solutions. A cell with a 0.15-mm diameter hole was used in single-channel experiments, and one with a 0.55-mm diameter hole was used in multi-channel experiments. BLMs were formed in solutions of 1 M KCl (or 0.1 M KCl), 10 mM Tris, 10 mM MES, 30 μM 1,4-dithio-DL-threitol (DTT), pH = 7.0. DTT was present in all the experiments unless otherwise stated. The electrical current (I) was measured with an amplifier (Keithley 428), digitized by a LabPC 1200 (National Instruments, Austin, TX) and analyzed using a personal computer with the help of WinWCP Strathclyde Electrophysiology Software designed by J. Dempster (University of Strathclyde, UK). A voltage of 30 mV (unless otherwise stated) was applied to BLM with Ag–AgCl electrodes placed directly into the cell. In the case of the measurements of ion-selectivity, agar–agar bridges filled with 100 mM KCl were used with Ag–AgCl electrodes. In single-channel experiments, a patch-clamp amplifier (model BC-525C, Warner Instruments, Hamden, CT) was used for measurements of the electrical current. Aluminum trisulfophthalocyanine ($AlPcS_3$) from Porphyrin Products, Logan, UT, was added to the bathing solution at the *trans*-side (the *cis*-side is the front side with respect to the flash lamp) in the sensitized photoinactivation experiments. BLMs were illuminated by single flashes produced by a xenon lamp with flash energy of about 400 mJ/cm^2 and flash duration <2 ms. A glass filter cutting off light with wavelengths <500 nm was placed in front of the flash lamp. To avoid electrical artefacts, the electrodes were covered by black plastic tubes.

Dye-loaded liposomes were prepared as described previously [39] by using an Avanti Mini-Extruder from a mixture of PDhPC/DPhPG (70/30 mol%). The unbound carboxyfluorescein (CF) was then removed by passage through a Sephadex G-50 coarse column with a buffer solution containing 10 mM β-alanine, 0.12 M KCl, pH 4.0.

The CF leakage of liposomes was measured with a Hitachi F-4000 (Tokyo, Japan) fluorimeter with peak excitation and emission wavelengths of 490 nm and 520 nm (band-pass of both beams, 5 nm). The extent of the CF leakage was

calculated as follows: $(F_f - F_0)/(F_{100} - F_0)$, where F_0 and F_f represent the initial and the final (steady-state) levels of fluorescence before and after the protein addition, and F_{100} is the fluorescence value after complete disruption of liposomes by addition of the detergent, LDAO (lauryldimethylamine-N-oxide, final concentration, 2.4% w/w). The buffer solution 100 mM KCl, 10 mM Tris, 10 mM MES, pH 7, was used for the CF leakage studies.

3. Results

It is known that the addition of several nanomoles of gA to a bathing solution of a planar bilayer lipid membrane (BLM) containing 1 M KCl induces an increase in the macroscopic electric current across BLM from an initial level of less than 10^{-12} A up to 10^{-6} A. The same was observed with P4C, the analogue with a cysteine residue attached through a linker to the C-terminus of gA (gramicidin-βA-GSGPKKKRKVC). Another gA analogue, P10C, being identical to P4C except for N-formyl replaced by N-acetyl, appeared to be much less effective channel former than P4C and gA, as judged by the concentrations of the peptides needed to induce equal transmembrane currents. Hydrogen peroxide stimulated the current mediated by both P4C (Fig. 1A) and P10C (Fig. 1B), but was ineffective with gA (Fig. 1A) and P5C (Fig. 1B, inset). The latter peptide differed from P10C by a single substitution: it had glycine instead of cysteine in the sequence attached to the C-terminus of gA (gramicidin-βA-GSGP-KKKRKVG). The addition of the excess of 1,4-dithio-DL-

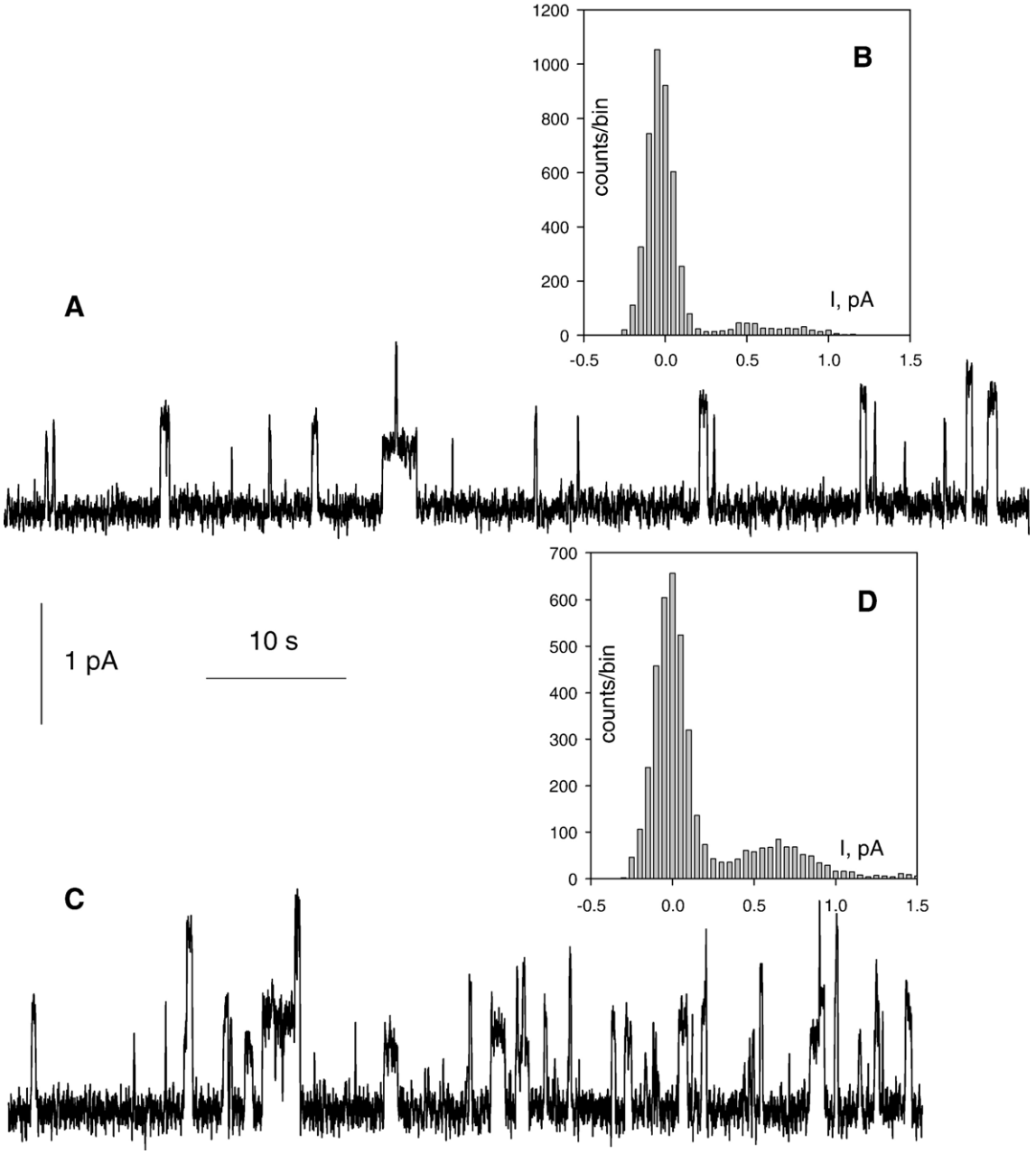

Fig. 3. Single-channel recordings (A, C) and corresponding current histograms (B, D) of the P4C peptide at 80 mV. The solution was as in the caption to Fig. 1. Panel C and D, 10 mM H_2O_2 was added.

threitol (DTT) reversed the effect of H_2O_2 on the current (Fig. 1C).

It is known that the gA-mediated current is cation-selective [40]. This holds true for the P4C-mediated current in the absence of H_2O_2, as evidenced by the value of the zero-current potential (ΔV) measured in the presence of a two-fold gradient of KCl (0.2 M KCl and 0.1 M KCl on the *cis*, *trans* sides) (Fig. 2). The addition of H_2O_2 resulted in the decrease in ΔV for P4C from 17.5 mV to 1.6 mV, which corresponds to a switch from the nearly perfect cationic selectivity to the potassium/chloride permeability ratio of 1.2.

Single channels formed by P4C under the reducing conditions were characterized by an average single-channel lifetime τ_{av} of 0.8±0.2 s and a set of amplitudes in the range of 10 pS (about 0.7 pA at 80 mV, see Fig. 3A, B). As shown earlier [41], amplitude heterogeneity of modified gramicidin channels may correspond to different conformations of C-terminus-attached groups with respect to channel entrances. The addition of H_2O_2 did not alter the characteristics of single channels: τ_{av} was 1.0±0.3 s and similar amplitudes (Fig. 3C, D).

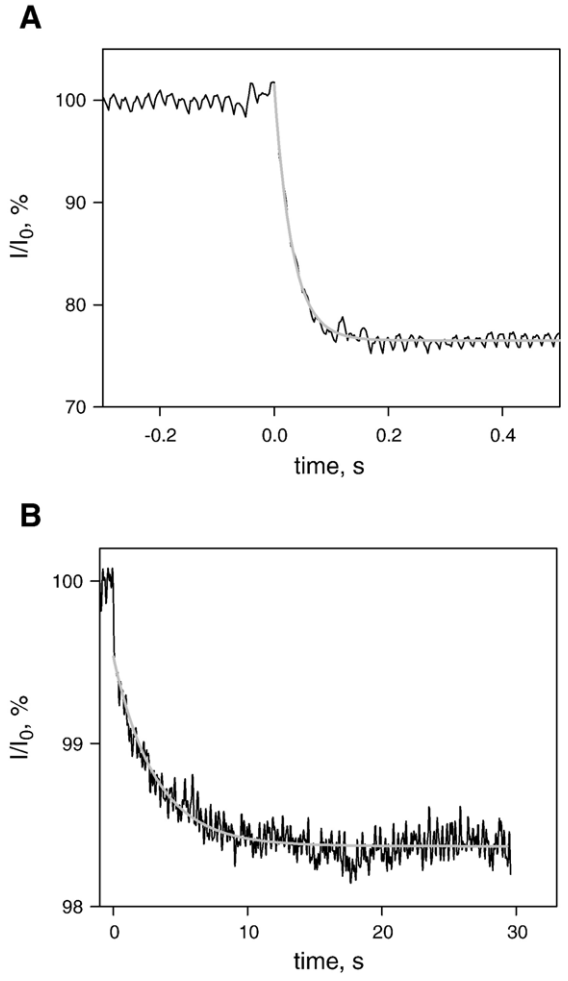

Fig. 4. (A) Sensitized photoinactivation of P10C channels under the reducing (panel A) and oxidizing (panel B) conditions. The time courses of the decrease in the peptide-mediated current across a BLM after a flash of visible light (at $t=0$ s) in the presence of 1 μM $AlPcS_3$. The normalized values of the current (I/I_0) are plotted versus the time. The initial current (I_0) was approximately 0.5 μA. The BLM voltage was 30 mV. The solution was 1 M KCl, 10 mM Tris, 10 mM MES, pH 7. 30 μM DTT was present in panel A, while 10 mM H_2O_2 in panel B. Grey lines show single exponential fits $y=y_0$ + a*exp($-t/\tau$), with $\tau=31$ ms (panel A) and $\tau=3.1$ s (panel B).

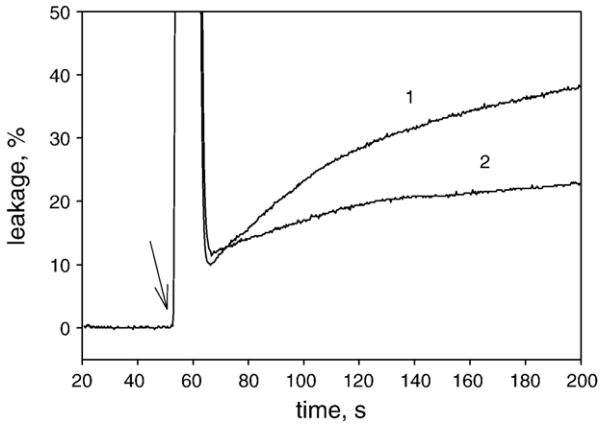

Fig. 5. The CF leakage from liposomes induced by P4C (0.5 μg/ml) in the presence of H_2O_2 (10 mM, curve 1) and DTT (30 μM, curve 2). The peptide was added at the moment marked by an arrow.

We further studied the effect of H_2O_2 on the macroscopic current mediated by gA analogues by using the method of the sensitized photoinactivation. This relaxation technique based on measuring a transient decrease in the gA-mediated current after a flash of visible light in the presence of the photosensitizer provides information on the kinetics of gA channel operation [42,43]. The decrease in the current results from damage to tryptophan residues of gA caused by reactive oxygen species that are generated upon excitation of the photosensitizer [44,45]. The time course of the flash-induced current relaxation is well described by a monoexponential decay curve $I=I_0+(I_0-I_\infty)\exp(-t/\tau)$ with the characteristic time τ correlating with the single-channel lifetime of gA channels under certain conditions. As seen from Fig. 4A and B, the addition of H_2O_2 led to the decrease in the amplitude $\alpha=(I_0-I_\infty)/I_0$ of photoinactivation from 24% to 1.6% and substantially decelerated the photoinactivation kinetics of the current mediated by P10C (τ changed from 31 ms in the presence of DTT to 3.1 s in the presence of H_2O_2). Similar effect of H_2O_2 on the photoinactivation kinetics was observed with P4C. By contrast, the kinetics for gA was independent of the presence of H_2O_2 and DTT in the bathing solution. It should be mentioned that the value of τ for P4C under reducing conditions amounted to 0.6 s; it was much longer than that for P10C and P5C (31 ms and 10 ms, respectively) and close to that of gA (0.7 s).

We have previously shown that at a concentration of 1 μM, P4C and P5C induce the carboxyfluorescein leakage of liposomes [37]. As seen from Fig. 5, the addition of H_2O_2 led to marked stimulation of the P4C-induced CF leakage.

4. Discussion

The results obtained here demonstrate the strong stimulating effect of hydrogene peroxide on the macroscopic electric current mediated by cysteine-containing analogues of gA (Fig. 1A, B). The control experiments showed that the

presence of a cysteine residue at the C-terminus of gA analogues is a prerequisite of the H_2O_2 effect on the current. The reversal of the effect by the addition of DTT favors the oxidation/reduction of SH groups to be the basis of H_2O_2-induced stimulation of the current.

As shown by measurements of the ionic selectivity (Fig. 2), the high conductance of BLM maintained by the cysteine-containing gA analogue, P4C, in the presence of H_2O_2 is practically nonselective. Thus, its nature differs greatly from that of usual cation-selective gA channels. This conclusion is supported by the absence of the H_2O_2 effect on the properties of single channels of P4C recorded at a lower concentration of the peptide (Fig. 3). Oxidation of SH groups of C-terminal cysteine residues may be expected to produce cross-linking of neighboring channels resulting in the formation of coupled tandem channels, as it was observed upon interaction of streptavidin with biotinylated gramicidin [46]. However, no double-conductance channels were observed in the single-channel recordings of P4C (Fig. 3). This may be explained by low probability of oligomerization due to low concentration of the peptide. On the other hand, the formation of tandem channels requires an appropriate length of a spacer between cross-linking (here SH) groups and gA channel-forming moiety [36,47,48]. We surmise that cross-linking of our cysteine-containing gA analogues, resulting from their oxidation by H_2O_2, does not produce coupled double-conductance channels, but leads to an increase in the local concentration of the peptides, which provokes the formation of nonselective pores. According to the model published previously [37], large-conductance nonselective pores are formed by aggregates of certain gA analogues. This model has common features with the barrel-stave model of alamethicin channels [49]. It is of relevance to this point that ionic channels of modified alamethicin [50] are also sensitive to redox regulation.

The H_2O_2 effect on the macroscopic current mediated by cysteine-containing gA analogues also manifested itself in the dramatic deceleration of the sensitized photoinactivation kinetics (Fig. 4). As shown in our earlier works [42,51], in the case of gA, the photoinactivation kinetics reflects equilibration of gA dimers and monomers after a flash-induced concentration jump with τ corresponding to gA single-channel lifetime. In line with this, it is easy to explain very low values of τ measured here for P10C and P5C having N-acetyl instead of N-formyl (31 ms and 10 ms, respectively). Actually, it is known that N-formyl is involved in the formation of hydrogen bonds providing association of monomers into transmembrane dimers, and modification of this group destabilizes substantially gA channels [52–55]. In the case of the large-conductance pores studied here, the photoinactivation kinetics may be tentatively ascribed to equilibration of some conducting and non-conducting states. In view of this assumption, the decelerating effect of H_2O_2 on the photoinactivation kinetics of cysteine-containing gA analogues can be explained by stabilization of some aggregated form of the peptide corresponding to its conducting state.

It is of importance that hydrogen peroxide significantly increased not only the transmembrane electric current but also the dye leakage of liposomes induced by P4C (Fig. 5), thereby confirming the common nature of the two effects. Enhanced membrane permeabilization by a disulfide-dimerized peptide, in particular, the enhanced dye-releasing activity of the dimer at low concentrations, when compared with the corresponding monomeric peptide, was shown earlier for melittin [56] and magainin analogues [57]. It has been suggested that peptide dimerization is rate-limiting for pore formation by the peptide under certain conditions [56].

The phenomenon of the redox regulation of the transmembrane ionic current mediated by cysteine-containing gA analogues can be used as the basis for constructing membrane-located redox sensors. Besides their high activity as channel formers, the advantage of the peptides used in the present work is the high-span response of the membrane electrical current to redox changes, which can be directly transferred to a signal of a potential redox sensor.

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