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Tryptophan photolysis is responsible for gramicidin-channel inactivation by ultraviolet light

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The decay of gramicidin fluorescence resulting from ultraviolet exposure was compared to the decay of conductance from gramicidin-containing planar bilayer membranes under the same conditions of illumination. The decay rate was the same for both processes. The fluorescence decay was identical whether gramicidin was dissolved in methanol or incorporated into lipid vesicles, indicating that the peptide conformation does not affect the sensitivity of gramicidin to photolysis. The correlation of fluorescence decay and conductance decay imply that conductance loss from gramicidin-doped membranes illuminated with ultraviolet light is due to photochemical modifications of the channel tryptophans rather than simply to disturbance of the conformation of gramicidin channels.

Introduction

Many protein functions are inactivated by exposure to ultraviolet light. Among these are the sodium channels responsible for inducing excitability in nerve and muscle [1–3]. Busath and Waldbillig [4] have found that gramicidin channels, small peptide channels formed in lipid bilayers [5], are also sensitive to ultraviolet light. Like sodium channels, gramicidin channels are most sensitive to 280 nm ultraviolet light, the peak tryptophan absorption wavelength. Also like sodium channels, gramicidin channels inactivate exponentially with fluence. Gramicidin was found to have a high sensitivity coefficient ($\gamma = 0.028$ cm^2/mW per s [4]) compared to sodium channels ($\gamma =$ 0.0015 cm^2/mW per s [1]). The high sensitivity could occur because channels formed by dimers of gramicidin D contain six to eight tryptophans each. (Gramicidin D is a composite of gramicidins A (72%), B (9%) and C (19%) [6]. Gramicidin A has the structure: HCO-^{1}LVal - Gly - LAla - DLeu - LAla - DVal - LVal - DVal - LTrp - DLeu - LTrp - DLeu - LTrp - DLeu - ^{15}LTrp-NHCH$_2$CH$_2$OH. B and C are pentadecapeptides like A except that Trp-11 in A is replaced with Tyr-11 or Phe-11, respectively.)

It is desirable to correlate the rate of loss of peptide function with that of tryptophan integrity. This allows one to determine whether channel photoinactivation is due to chemical modification of the tryptophans or to some other process such as disruption of the peptide sequence or the peptide conformation, or some other amino acid. Busath and Waldbillig [4] reported a preliminary experiment where they monitored tryptophan in-

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tegrity using its absorbance at 280 nm. However, this method is problematic because the products of gramicidin photooxidation absorb significantly in the same wavelength region, making the determination of tryptophan absorption difficult. We report here that gramicidin fluorescence, like gramicidin-induced membrane conductance, declines monotonically during ultraviolet photolysis. A preliminary report of this work has appeared [6].

Materials and Methods

Vesicles were prepared by co-solubilizing gramicidin D (ICN Nutritional Pharmaceuticals, Cleveland, OH) and dimyristoylphosphatidylcholine (Avanti Polar-Lipids Inc., Birmingham, AL) in methanol (HPLC grade, Fischer) and then gently evaporating the solvent while stirring slowly. Deionized, filtered water (Barnstead Nanopure) was then added to yield a final concentration of 0.1 mg/ml lipid and 0.01 mg/ml peptide. The solutions were vortexed and then sonicated (30–60 min) until the ultraviolet absorption spectrum was similar to those for the comparison samples of gramicidin in methanol (also 0.01 mg/ml). Fluorescence spectra were obtained using a Spex fluorimeter.

For measurements of the gramicidin fluorescence loss, a cuvette containing a cubic sample (1 cm on each side) of dispersed gramicidin was positioned in the unfiltered beam of a 150 W xenon lamp (Photochemical Research Associates, Oak Ridge, TN). Channel photolysis has been shown to result primarily from 280 nm light [4]. We used unfiltered light from the xenon lamp to maximize intensity. Using a small pinhole and a photometer (United Detector) we found that the intensity in the area of the beam exposing the cuvette varied $\pm 25\%$ from the mean. Some heating of the solutions (2–3 Cdeg) results from long illuminations. Heating alone had no effect on the absorbance or fluorescence spectra. In a typical experiment, samples were illuminated for short periods to partially photolyse them and then transferred to the spectrophotometer for the measurement of the fluorescence spectra within about 15 min. This procedure was then repeated for the same sample until no gramicidin fluorescence could be detected. Photolysis from the spectrophotometer lamp during a spectrum scan was undetectable: a second spectrum obtained after a typical scan showed no decrease in fluorescence.

For measurements of membrane conductance loss, planar lipid bilayers were formed on the tip of a polyethylene pipette (40 μm diameter) using monoolein (Nucheck Prep Inc., Elysian, MN) dispersed in *n*-hexadecane (50 mg/ml, Aldrich) as described previously [4]. Gramicidin D (0.01 mg/ml in methanol was added to the chamber in 2-μl aliquots and the membrane current due to a 100 mV applied potential was sampled continuously with the analog-to-digital converter of a Masscomp computer. The membrane was illuminated for photolysis using the same lamp and under the same conditions as the cuvettes described above. The bilayer was placed in an area of the beam which was found using the pinhole mentioned above to have the average light intensity. The membrane conductance decay was measured during both continuous and intermittent exposures with similar results. All measurements were made with solutions equilibrated with air at room temperature.

Results

Figs. 1 and 2 show the progressive changes in the fluorescence spectra after various ultraviolet exposures for methanolic and vesicle-incorporated gramicidin, respectively. At the lamp intensity used, the tryptophan fluorescence peak disappeared irreversibly after 5 min of exposure. Both vesicle and methanol solutions show a steady decline of the 360 nm peak height and a concomitant small increase in nonspecific fluorescence in the 350–450 nm range during photolysis. The small broad-spectrum increase could be due to photoproducts. Scattering artifact peaks at 280 nm, 440 nm and 560 nm are not affected by the photolysis process. Fig. 3 shows a plot of the exponential decay in peak fluorescence versus the total lamp exposure time for methanolic (a) and vesicle-packed (b) gramicidin solutions from an experiment similar to those shown in Figs. 1 and 2. Both solutions show a minor fast component which decays after 3 s of exposure and a slower exponential component which has a time constant of 12.5 s

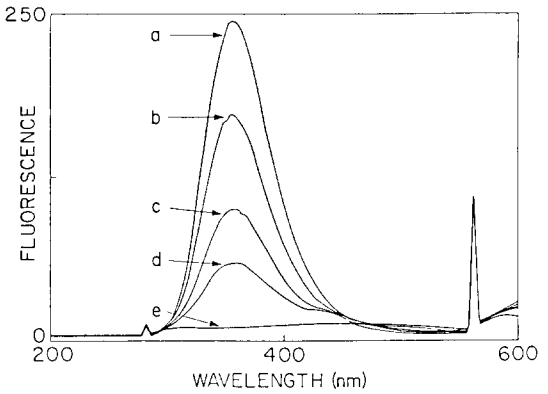

Fig. 1. The decline in fluorescence (arbitrary units) during photolysis. Gramicidin D in methanol was exposed to broad-spectrum ultraviolet light from a xenon lamp. Total exposure times were: curve a, no exposure; b, 5 s; c, 15 s; d, 30 s; and e, 5 min.

in both cases. In five experiments, the main-component time constant averaged 13.5 s (±2.2 s, S.D.) for photolysis of gramicidin dissolved in methanol and 13.7 s (±1.3 s, S.D.) for vesicle-packed gramicidin.

We compared the rate of loss of tryptophan fluorescence to the rate of inactivation of gramicidin channels assayed by the loss of current across a planar bilayer under the same conditions of illumination. Fig. 4 shows data from a photolysis experiment done on a monoolein bilayer. Since the current associated with a single channel is

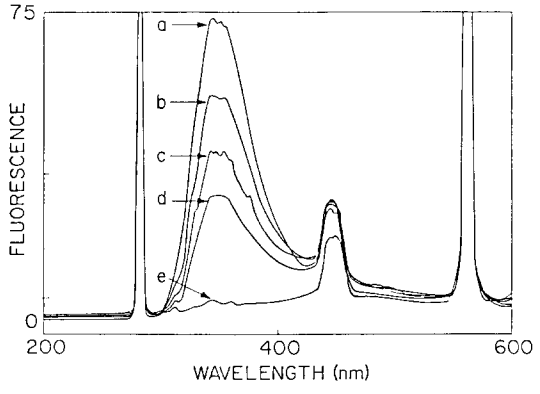

Fig. 2. The decline in fluorescence (arbitrary units) for gramicidin in vesicles. Gramicidin D in DMPC vesicles was exposed to ultraviolet illumination under the same conditions as in Fig. 3. The total exposure times were: curve a, no exposure; b, 5 s; c, 15 s; d, 30 s; e, 5 min.

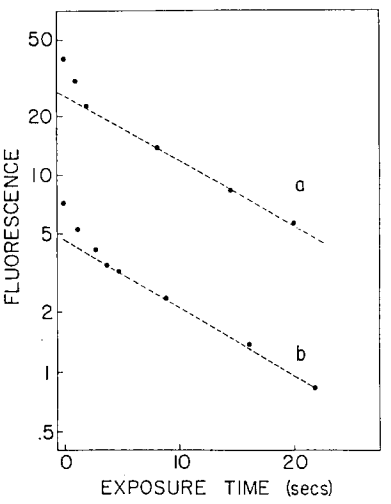

Fig. 3. Peak fluorescence decline (arbitrary units) vs. ultraviolet exposure. Curve a is for a methanol preparation (like that in Fig. 1), curve b for vesicles (like that in Fig. 2). Peak values were obtained by subtracting a baseline value from the highest fluorescence value in the 348–360 nm region. The dashed lines are exponential functions with time constants of 12.5 s.

about 4.7 pA, the prephotolysis current level in Fig. 4 represents approximately 2000 conducting channels. Upon ultraviolet-exposure, the gramicidin-induced membrane current undergoes a rapid initial decline followed by a slower exponential decline with a time constant of 12.5 s. In a group of five conductance decay experiments done under the same conditions of illumination, the main (slower) component had a time constant which

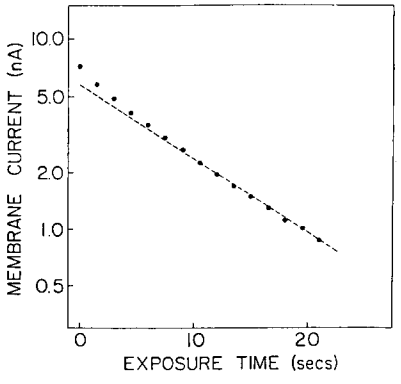

Fig. 4. The decay of conductance in a planar bilayer containing gramicidin D during exposure to ultraviolet illumination. The straight line represents an exponential function having a time constant of 12.5 s.

TABLE I

THE CONDUCTANCE DECAY RATE AS A FUNCTION OF MEMBRANE POTENTIAL

A monolein bilayer containing many gramicidin channels was repeatedly exposed to short flashes of ultraviolet light while being held at any of the four different membrane potentials used in a random sequence. Each flash reduced the membrane current by approx. 5%. The time constant of conductance decay was calculated from the fractional decrese of conductance and the duration of the flash: τ = flash duration/fractional conductance loss.

V_m (mv)	τ (s)	S.D.	n
20	12.3	2.0	3
50	11.3	2.1	3
100	11.4	0.8	3
175	11.6	–	1

averaged 13.1 s ($\pm$2.1 s, S.D.). Thus the ratio of the main-component time constants for fluorescence loss for vesicle-packed gramicidin and conductance loss in the planar bilayer is 1.04.

The experiments with vesicles were performed in conditions that would yield no transmembrane potential, whereas in the conductance decay experiment we utilizied a membrano potential of 100 mV. We examined the effect of membrane potential on the conductance photolysis rate in planar bilayers. The results for the main-component photolysis rate are given in Table I. The main-component time constant is not affected to any degree by membrane potential.

Discussion

The goal of this study was to determine whether photolytic channel inactivation results from chemical modification of the tryptophans or from nondestructive peptide conformation changes. The decay of gramicidin fluorescence during ultraviolet photolysis follows a time course parallel to that of the decay of membrane conductance. The fluorescence decay is most likely related to chemical modification of the tryptophan indole ring rather than changes in peptide conformation alone.

The conformation assumed by the gramicidin peptide depends on its solvent environment. Several lines of evidence indicate that when gramicidin is incorporated into vesicles the predominant structure is the left-handed $\beta^{6.3}$ helix [7–9]. Two monomers associate head-to-head, forming a dimer [10] via hydrogen bonds at their amino termini [11]. The peptide side-chains radiate from the helix into the lipid. The channel through the center of the helix should accomodate water molecules and monovalent cations according to various molecular calculations [12–17] and is the presumed conductance pathway. In organic solvents such as methanol, gramicidin assumes a random-coil conformation when at low concentrations and forms a family of double helices at high concentrations [18–20]. We have compared the absorbance and fluorescence characteristics of gramicidin when in the channel conformation (incorporated into lipid bilayers) to those of the random-coil conformation (dissolved in low concentration in methanol). The fluorescence in vesicles was about 3-fold lower than in methanol (compare traces a in Figs. 1 and 2), suggesting that the channel conformation results in quenching of some of the tryptophans. The fluorescence loss follows the same time course whether the peptide is in the 'channel' or 'random-coil' configuration (Fig. 3).

The small fast component of the decay of fluorescence during illumination (Fig. 3) suggests that the four gramicidin tryptophans might have differing sensitivities to ultraviolet [21]. Busath and Waldbillig [4] found a similar fast component in their studies of membrane conductance photolysis, which we also confirm here (Fig. 4). Boni et al. [22] report that tryptophans in vesicle-packed gramicidin have differing sensitivities to *N*-bromosuccinimide oxidation. From their study it appears that the tryptophans which can fluorescence are more sensitive to *N*-bromosuccinimide because fluorescence decays faster than absorbance with increasing *N*-bromosuccinimide treatment. It is not unreasonable to suppose that some of gramicidin's tryptophans may also be more sensitive to photolysis as well. For instance, it is possible that quenching of Trp-9 by Trp-15 (and vice versa) also protects against the loss of the electron which is thought to lead to tryptophan oxidation [23].

Finally, we note that the exact connection between gramicidin tryptophan photolysis and gramicidin-channel photolysis needs further examination. While we have demonstrated that gramicidin

fluorescence decays as rapidly as channel conductance and thus that tryptophan modification can account for channel inactivation, we cannot deduce which of a channel's six to eight tryptophans is responsible for channel inactivation. If several of the tryptophans could inactivate a channel, we would have expected the conductance decay rate to exceed the fluorescence decay rate. This issue may best be addressed by an examination of gramicidin analogues containing only one chromophore per channel.

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