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PHOTOLYSIS OF GRAMICIDIN A CHANNELS IN LIPID BILAYERS

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We have tested the hypothesis that peptide tryptophan groups can control the ionic conductance of transmembrane channels. We report here that single gramicidin A channels change conductance state when the peptide tryptophans are flash photolyzed with ultraviolet light. The current flow through planar lipid bilayers containing multiple gramicidin A channels decreases irreversibly when exposed to ultraviolet light. The current-loss action spectrum peaks sharply at the 280 nm absorption maximum of the gramicidin A tryptophans. Gramicidin channel sensitivity to ultraviolet light is found to be about 20-fold higher than that of frog node sodium channels which is even more than expected based on the high tryptophan content of gramicidin. Channels which survive an ultraviolet light exposure exist in a wide variety of different low-conductance forms. The broad distribution of the single channel conductance of these partially photolyzed channels is attributable to the loss of different combinations of the dimer's normal complement of eight tryptophans per channel. Flash photolysis of single channels results in discrete conductance state changes. Partially photolyzed single channels manifest a further conductance cascade when exposed to a second flash of ultraviolet light. Analysis of the photolysis conductance turn-off process indicates that gramicidin A is a multistate electrochemical unit where the peptide tryptophan groups can modulate the flow of ions through the transmembrane channel.

Introduction

Membrane channels are generally thought to be proteinaceous pores which provide a pathway for the flow of ions across the lipid bilayer. The molecular basis for the control of ion movements through transmembrane channels is poorly understood. This report is aimed at evaluating the electrical behavior of individual channels subjected to an instantaneous chemical modification. The experiments described here were designed to test the hypothesis that tryptophan groups modulate the flow of ions through gramicidin A polypeptide channels.

In order to develop an understanding of the molecular aspects of channel electrical function we have taken full advantage of the physicochemical simplicity of planar lipid membranes containing gramicidin A channels. It is well known that the gramicidin A molecule * is a tryptophan-rich peptide [1] which spontaneously and reversibly dimerizes [2] in lipid bilayers to form a β-helical [3] cation conductive [4] channel. While gramicidin A channels typically exhibit only two conductance states (on/off), recent electrical studies by Busath and Szabo [5] have shown that some channels undergo spontaneous transitions between discrete

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* HCO-L-Val[1]-Gly[2]-L-Ala[3]-D-Leu[4]-L-Ala[5]-D-Val[6]-L-Val[7]-D-Val[8]-L-Trp[9]-D-Leu[10]-L-Trp[11]-D-Leu[12]-L-Trp[13]-D-Leu[14]-L-Trp[15]-NH-CH_2-CH_2-OH.

intermediate conductance levels. Intermediate conductance state channels also exhibit current rectification characteristics. It has been suggested [5–8] that they might be conformational variants of fully conducting gramicidin A channels. The results of electrical studies [9] of channels made using chemical analogues of gramicidin A suggest that the peptide aromatic groups influence the channel conductance. In the experiments described here. flash photolysis methods are used to make instantaneous in-situ chemical modifications of electrically patent gramicidin A channels.

Ultraviolet light inactivation of a different type of transmembrane channel, the sodium channel, has been studied by others. Recently, Fox [10] and Oxford and Pooler [11] have demonstrated photolytic decrease in the sodium currents of voltage clamped Ranvier nodes and lobster axons, respectively. Fox et al. [12] reported that gating currents of myelinated nerve are also sensitive to UV irradiation. Stühmer and Almers [13] have monitored the membrane properties of voltage clamped patches of frog sarcolemma which had been photobleached to eliminate sodium currents and found a lack of recovery from photolysis implying that sodium channels are immobile in sarcolemma.

We compare the photosensitivity of gramicidin channels to sodium channels and provide the first examination of single channel behavior during photolysis. A preliminary report of these results has appeared [14].

Methods

Membrane formation and electrical recording techniques used have been described in detail elsewhere [5]. Planar lipid bilayers were made from monoolein dispersed in either *n*-hexadecane or squalene (50 mg/ml). The lipid (99.9%, Nucheck Inc.) and alkane (99%, Aldrich Inc.) were used as supplied whereas the squalene (97%, Eastman Inc.) was passed through activated alumina immediately prior to use. Reagent grade KCl was roasted (500°C, 3 h) and added to high purity deionized water (Millipore) to make the 1 M electrolyte solution. The TFE/LPE membrane chamber was cleaned by sequential washing with solvents of decreasing polarity (water, ethanol, acetone, chloroform, petroleum ether, air) immediately prior to use. The gramicidin A was purified from gramicidin D by isocratic HPLC using a 220 nm detector [15]. Small aliquots (approx. 2 μl) of the peptide/methanol solutions (10^{-2} to 10^{-7} mg/ml) were delivered to the electrolyte near the bilayer.

The bilayers were illuminated with either white or monochromatic (± 3 nm) light from a 150 watt xenon lamp system (Schoeffel Inc.). The focused light beam (3 mm diameter) struck the planar bilayer (< 1 mm diameter) at a near normal angle of incidence. A spectroradiometer (EGG Inc.) was used to measure the light intensity in the plane of the membrane. In certain experiments the light was passed through an ultraviolet light absorbing filter (Jean Glaswerk GG-22). A mechanical photographic shutter was used to control the duration of the light exposure.

Results

This study was designed to test the hypothesis that tryptophan groups are critical components for the control of the ion conductance through polypeptide channels. Since aromatic amino acids are intrinsically photolabile we have used flash photolysis methods to rapidly alter the chemical structure of electrically patent gramicidin A channels.

In our first experiments planar lipid bilayers formed using monoolein/hexadecane mixtures were made highly conductive by injecting about

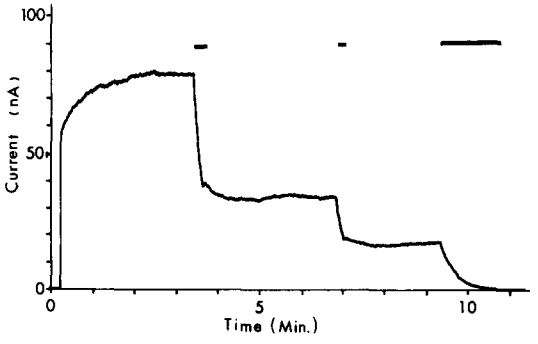

Fig. 1. Photoresponse of the transmembrane current of a bilayer containing many gramicidin A channels. Current flowing through this highly conductive membrane increased sharply upon application of a transbilayer potential (0.1 V) but subsequently decreased abruptly during exposures to xenon light pulses (bars). Notice that the membrane photoresponse is irreversible in character and that prolonged light exposure makes the bilayer virtually non-conductive. Monoolein/hexadecane bilayer; $\cong 80$ mW/cm^2 white xenon light.

10^{-11} mol of purified gramicidin A adjacent to the thin membrane. A 100 mV potential was applied across the unilluminated planar bilayer and the current was observed to rise to a steady-state level. The membrane was then exposed to a series of flashes of white xenon light. The results in Fig. 1 illustrate the bilayer current reponse before, during and after the light exposures. The results show an abrupt current decline during each of three exposure periods. Close examination of the decline shows it to be irreversible and nearly exponential in character. In this regard the bilayer response is similar to that reported for the sodium current of illuminated axons [10,11].

In order to better understand the molecular origin of the bilayer current-loss, experiments were designed to determine the spectral sensitivity of the membrane photoresponse. In these studies freshly made gramicidin A-containing bilayers were exposed to a known dose of monochromatic light and the amount of current loss was measured, providing an estimate of the relative loss-rate. Preliminary studies showed that the current photoresponse is absent in bilayers exposed to visible wavelengths. Consequently our membrane spectral sensitivity measurements were limited to the ultraviolet light region (240–310 nm) of the light spectrum. The results of these and related experiments are shown in Fig. 2.

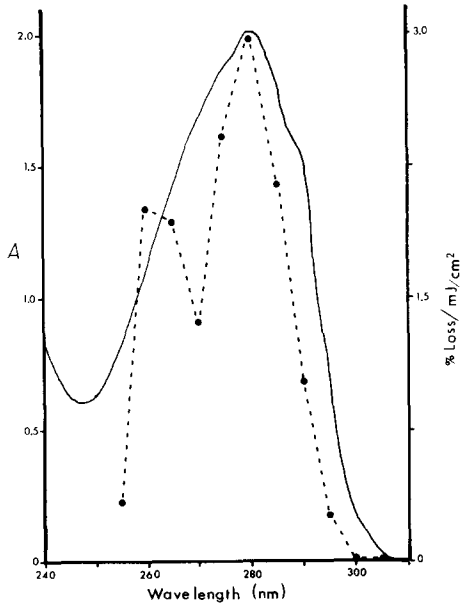

Fig. 2. Photoresponse action spectrum of current loss in gramicidin A-containing bilayer membranes. Current loss occurs only when the polypeptide channels are exposed to ultraviolet light. The membrane photoresponse (dashed line, right hand ordinate) corresponds to the ultraviolet light absorption spectrum (solid line, left hand ordinate) of the purified gramicidin A. The polypeptide tryptophans exhibit a maximal absorption in the 280–290 nm region. Monoolein/hexadecane bilayers; monochromatic intensity at 280 nm: ≅ 0.1 mW/cm^2; 0.1 V; Absorption spectrum from HPLC purified gramicidin A in methanol, 100 μM.

The action spectrum (dashed lines) shows a sharp peak at 280 nm. The membrane photoresponse is minimal for wavelengths above 300 nm or under 260 nm. Moreover the current action spectrum is in excellent agreement with the light absorption spectrum of gramicidin A (solid line, Fig. 2). Tryptophan has an absorption maximum at 280 nm [16] and the gramicidin A channel contains eight tryptophans. Since there are no other ultraviolet light absorbing aromatic amino acids in the gramicidin A molecule it can be concluded the membrane current decline is associated with photo alteration of one or more of the channel tryptophan groups.

In order to quantitatively assess the extent of photoinactivation of the gramicidin channels, we compare the rate constant for the pseudo-exponential decline to the equation:

$$D = D_0 e^{-\gamma I t} \tag{1}$$

where D is the calculated number of channels in the membrane, D_0 is the initial value, I the intensity of incident, 280 nm light (between 0.07 and 0.12 mW/cm^2), t is exposure time, and γ the sensitivity. The sensitivity in four experiments averaged 0.028 ± 0.006 (S.D., $n = 4$) cm$^2 \cdot$ mW$^{-1} \cdot$ s^{-1}. This value is more than one order of magnitude higher than the value reported by Fox et al. [12] for the sensitivity of myelinated nerve sodium channel currents (0.0015 cm$^2 \cdot$ mW$^{-1} \cdot$ s^{-1}) and gating current (0.00065 cm$^2 \cdot$ mW$^{-1} \cdot$ s^{-1}).

We have measured gramicidin's tryptophan photosensitivity by monitoring the rate of loss of ultraviolet light absorbance in a 100 μM sample of gramicidin A dissolved in methanol. Under these conditions, gramicidin does not assume the β-helical conformation, but rather monomeric random coils [17]. The rate of decline of tryptophan ab-

sorbance, corrected by 50% for the increasing photoproduct absorption, yields a tryptophan sensitivity of $\gamma = 0.0005\ cm^2 \cdot mW^{-1} \cdot s^{-1}$. Surprisingly, this value shows much more than the 8-fold decrease over the value for gramicidin-channel inactivation expected based on eight tryptophans per channel.

We have considered the gramicidin dimerization as a factor in the observed current decline. For this purpose the experiment illustrated in Fig. 1 was repeated using covalently linked malonyl gramicidin rather than normal hydrogen bonded gramicidin A channels. The photo results obtained using the linked malonyl analogue were indistinguishable from those obtained using normal gramicidin A channels, the exponential decay indicating a sensitivity of $\gamma = 0.027\ cm^2 \cdot mW^{-1} \cdot s^{-1}$. Thus we can exclude dimer dissociation as the sole source of the bilayer photoinactivation.

We have examined the photoinactivation mechanism at the single-channel level. Mechanisms for the photoinduced current decline include possible changes in the channel occurrence rate, conduction, and mean lifetime. To distinguish between these factors we have measured these characteristics of single channels before and after light exposure. For this study, a small amount of gramicidin A was added to fresh monoolein/hexadecane membranes. Four LD_{50} doses (10 s each, full xenon lamp spectrum) were delivered to the membrane and the discrete transitions of surviving channels were recorded. The results indicate that most of the decline is due to elimination of channels from the conducting population. The total channel occurrence rate dropped from an estimated 20 channels per second (based on the mean membrane conductance and the single channel lifetime) to 1.8 channels per second. This is close to the expected occurrence rate after four halvings, 1.25 channels per second. Following the light exposure the average single-channel conductance is also decreased. This change is particularly evident when the conductance distribution histograms for unexposed and ultraviolet light exposed (Fig. 3) populations are compared. It can be seen that the unexposed control population (inset) has the expected [5] narrow distribution peaked near 46 pS (1 M KCl). However, the channels which survived the four LD_{50} exposures lack this peak and the population has a wide variety of discrete low-conductance forms. The first three doses produced a graded effect. The mean conductance level for the channels in the histogram is 19 pS. No channels had conductance greater than 50 pS. Channels with conductance lower than 1.0 pS were beyond detection resolution. However, we note that we did not see an increase in baseline noise as might be expected to result from large numbers of subthreshold channels. We can conclude that part of the observed ultraviolet light-induced membrane current decline is attributable to a decrease of the average gramicidin single-channel conductance. The channel lifetime following the light exposure was determined for a group of 49 channels. Though the channels had reduced conductances, we found the mean lifetime to be about the same as non-illuminated channels [15], 2.2 seconds. Adding tryptophan, kynurenine, Trp-Leu, photolysed Trp-Leu (approx. 1 mM) or fully photolysed gramicidin (approx. 0.1 μM) to the electrolyte did not alter the typical current transitions. Thus the principle effect of ultraviolet light is to reduce directly the channel occurrence rate.

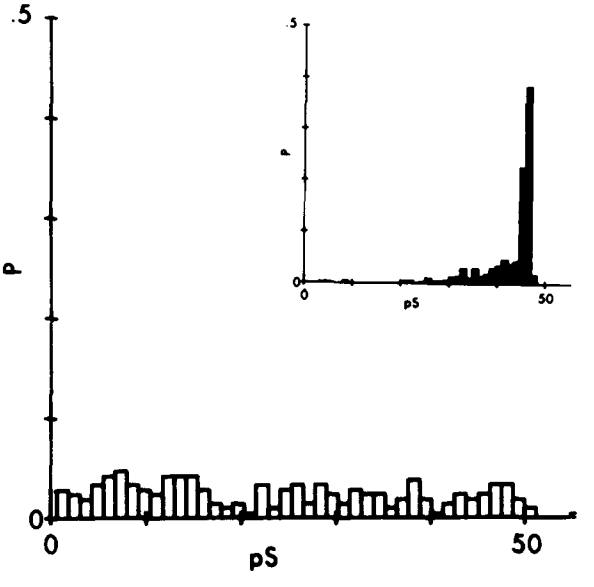

Fig. 3. Single channel conductance histogram from gramicidin-containing bilayers before (inset) and after partial photolysis of the peptide tryptophans using four half lethal doses of white xenon light. Comparison of the histograms indicates that the surviving channels are primarily low conductance units. Monoolein/hexadecane bilayers; 0.1 V.

We have explored the nature of the decrease of

the mean single-channel conductance. First, flash photolysis methods were used to excite the peptide tryptophans of electrically patent intact channels. The probability of coincident spontaneous and flash-induced state changes was decreased ($p < 0.1$) by making long lived ($t = 46$ s) channels in monoolein/squalene bilayers [5]. In these studies the bilayer was exposed to a flash immediately after the spontaneous opening of a normal (46 pS) gramicidin A single channel. The results in Fig. 4 show the response of electrically patent channels to the photolyzing light flashes. Upward deflections are conductance increases.

In the first example (Fig. 4A) a normal, gramicidin A channel that spontaneously opened in the darkened bilayer suffered an abrupt and premature full closure when flashed. In the second example (Fig. 4B) the electrically patent channel switched to a low-conductance state when exposed and then closed prematurely after the flash. The third example (Fig. 4C) likewise shows a normal gramicidin A channel switching to a low fractional conductance state near the end of the ultraviolet light flash. In this experiment and the experiment illustrated in Fig. 5, the number of conductance

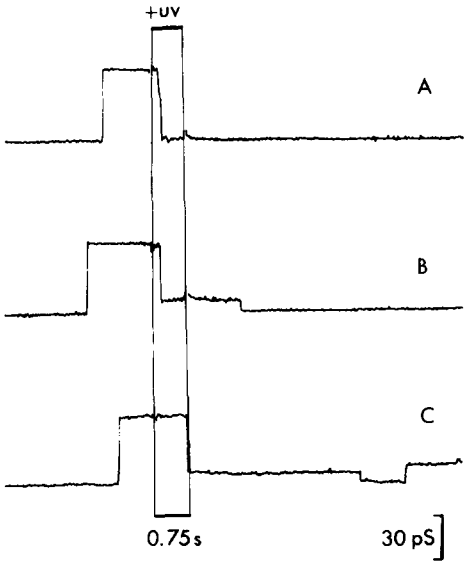

Fig. 4. Flash photolysis of intact gramicidin A channels. Open normal (46 pS) channels were exposed to a flash (0.75 s) of ultraviolet light. The channels prematurely change to lower conductance states when light struck. Monoolein/squalene bilayer; ≅120 mW/cm^2 xenon light.

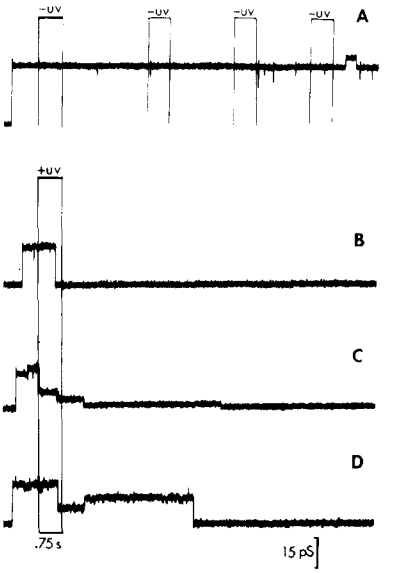

Fig. 5. Flash photolysis of surviving low-conductance gramicidin A channels. After becoming conductive, the channels which had survived previous ultraviolet light exposures, were subjected to a brief (0.75 s) xenon light flash. In example A the xenon light passed through an ultraviolet light absorbing filter (−UV) but in examples B, C and D the filter was absent (+UV). The fractionally conducting channels undergo multistate closure transitions upon flash photolysis of the peptide. Monoolein/squalene bilayer; 0.1 V; ≅120 mW/cm^2 white xenon light.

changes during ultraviolet light exposure far exceeded the expectation in the absence of ultraviolet light ($p < 10^{-23}$). These results confirm the earlier conclusion that ultraviolet light photolysis of the peptide tryptophans yields low conductance forms of the gramicidin A channel.

Next a double exposure experiment was designed to test for a relationship between the conductance level of the channel and the chemical integrity of its eight tryptophan groups. The first exposure converted normal channels into low conductance forms (as in Fig. 3). After a period of dark equilibration the surviving low conductance channels were subjected to a second exposure using the flash photolysis protocol described above. The results of the flash photolysis of the low-conductance survivors are summarized in Fig. 5.

The top record (Fig. 5A) is from a control study where surviving low conductance channels were exposed to ultraviolet-free (> 385 nm) light flashes.

Note that the conductance of the low conductance control channel is unchanged by repeated flashes of visible light. This is interesting because tryptophan's primary photoproducts absorb in the visible region. In the subsequent test records (Fig. 5B–5D) the light flash included both ultraviolet and visible wavelengths. Fig. 5B shows a previously exposed low-conductance survivor (approx. 15 pS) which spontaneously opened in the dark and then switched fully off when struck by the ultraviolet light flash. The record in Fig. 5C shows a surviving low-conductance channel which cascaded through a series of discrete lower conductance levels when flashed. In this record the conductance increase just prior to the light flash is most likely due to a second channel turn on. It could also be a state change in the original channel. However, in the absence of ultraviolet light illumination, careful examination of 40 channels in a membrane previously exposed to four LD_{50} doses of ultraviolet light revealed no evidence of state changes, consistent with the low rate of interconversions found for unexposed channels [7]. Finally, in Fig. 5D another previously exposed low-conductance form of a gramicidin A channel switched to a lower conductance level when flashed and then increased in conductance before turning off. The results of this double exposure experiment suggest that gramicidin channels can exhibit multiple discrete conductance states that are related to the integrity of the peptide's multiple tryptophan groups.

Discussion

We have demonstrated that tryptophan-rich gramicidin channels irreversibly lose activity when irradiated by ultraviolet light. This effect is typical of many enzyme systems (cf. Ref. 18) and is likely to be the result of photooxidation of the peptide tryptophans. The mechanism of tryptophan photooxidation is generally thought to consist of the following steps [19]: (a) photon absorption raises an indole electron to an excited state with high vibrational energy so that it dissociates from the tryptophan (Trp) and is solvated by the aqueous phase; (b) the Trp^+ radical is highly acidic and within microseconds [20] loses H^+ from the nitrogen in the indole ring; (c) the (neutral) Trp radical reacts with O_2 on the sub-millisecond time scale [21] to form *N*-formylkynurenine [22–25] and other stable oxidation products. This photooxidation process occurs in about 1% of photon absorptions [18] for protein tryptophans ($\phi_{ox} = 0.01$). Some photoexcited electrons lose some vibrational energy by dissipation within the molecule [19,26] and then radiate a fluorescent photon having a lower energy than that of the absorbed photons. Tryptophan fluorescence occurs within nanoseconds with a quantum yield $\phi_f = 0.14$ [26]. The remaining fraction of photoexcited electrons resolve to the ground state within microseconds [20] by phosphorescence [19,20] or more rapidly by internal conversion and non-radiative vibrational dissipation [19].

It is most likely that the current loss in Fig. 1 is due to irreversible photooxidation. Photoexcitations followed by fluorescence, phosphorescence, or vibrational dissipation would be expected to result in a reversible current loss, if any. The mechanism underlying the fractional conductance changes when single channels are flashed (Figs. 4 and 5) could be photooxidation of some gramicidin tryptophans. Alternatively dissipation of the vibrational energy might enhance the occurrence of spontaneous state-changes found by Busath and Szabo [5] in the absence of ultraviolet light illumination. These spontaneous events appear to be due to conformational changes near the tryptophan containing mouth of the gramicidin channel [7,8]. It is reasonable to expect that the large amount of energy (174 kT) in a 280 nm photon could induce local conformation changes involving a stable rotational orientation of the tryptophan side chain. Even absorptions leading to fluorescence or phosphorescence are preceded by vibrational dissipation in excess of 35 kT. Busath and Szabo have estimated the energy barrier to normal state changes to be about 37 kT [5].

For many enzyme, viral, and cellular systems, the inactivation spectrum resulting from ultraviolet light photooxidation has been found to be similar to the system's absorption spectrum [27]. For proteins, the absorption spectrum characteristically peaks at 280 nm due to tryptophan absorption [27]. We found the gramicidin photoinactivation spectrum to be peaked at 280 nm like its absorption spectrum. This similarity suggests that

tryptophan absorbance is responsible for photolysis and that the photodestruction rate is proportional to the absorption rate and independent of photon energy [27]. A similar result has been reported by Fox [10] and by Oxford and Pooler [11] for sodium current photolysis in voltage-clamped nerve.

We found the photoinduced decline of membrane conductance to result primarily from a decrease in the occurrence rate of observable channels and secondarily from a decrease in the average single channel conductance. Although channels occurring during a light flash had reduced lifetimes (Fig. 4 and 5), post-illumination channels had normal lifetimes. The lifetime reduction in illuminated channels is probably due to the introduction of the new inactivation pathway, photolysis. Covalently dimerized malonyl gramicidin inactivated much like the normal hydrogen-bonded dimers, indicating that flash inactivation doesn't simply result from enhancement of the usual dimer dissociation. The channel inactivation both for hydrogen bonded and covalently bonded dimers could be due either to production of non-conducting patent channels or to channel exit from the membrane. Either mechanism, channel blockage or membrane exit, would be expected with the formation of kynurenine-like photoproducts. Kynurenine and *N*-formylkynurenine are amino acids which are much more polar than tryptophan. The oxidation of tryptophan's indole ring results in the formation of one or two carbonyls on the side-chain residue. The peptide backbone structure is expected to remain intact. The more polar side chains may inhibit conduction electrostatically or may induce channel exit into the polar aqueous medium.

There are several possible explanations for the variety of intermediate conductance levels assumed by channels exposed to intermediate doses of light (Fig. 3). In a simple model, each of a channel's eight tryptophans could be in either of two states, intact or oxidized. The 256 possible combinations of photoproducts could produce as many differing channel conductances. Further complications that seem likely are: (a) the eight tryptophans probably differ in their influence on channel conductance; (b) tryptophans could have multiple photo-products, some of which may be sensitive to further ultraviolet light photooxidation; (c) some ultraviolet light absorptions may without oxidation cause long-lived conformational changes such as have been suggested [5–8] for the many low-conductance channels observed with purified gramicidin A. The first possibility, variable influence of the different tryptophans, seems plausible because Urry et al. [28] have shown that the carbonyl carbons of the different tryptophans display varying chemical shifts upon ion binding, indicating that ions spend more time associating with ^{11}Trp and ^{13}Trp as they pass through the channel than with ^{9}Trp and ^{15}Trp. The local of ^{11}Trp and ^{13}Trp is thought to be an effective binding site for ions in the channel interior. Photolysis of ^{13}Trp might be expected to perturb the conductance more than that of ^{9}Trp. The second possibility stems from the suggestion of Asquith and Rivett [25] that tryptophan photolysis is a multistep reaction. Initial conversion to *N*-formylkynurenine, which partially hydrolyses to kynurenine is followed by further oxidation to various simpler amino acid products. Kynurenine is susceptible to ultraviolet light photooxidation [25]. The multistep reaction could also explain the multiple stable changes seen in flashed single channels (Figs. 4 and 5). However, the absence of an initial shoulder in the exponential decline in Fig. 1 argues against a multi-hit reaction [17]. The third possibility, ultraviolet light-induced conformation changes, is speculative but not unreasonable considering the large energy delivered to the channel by a single ultraviolet light photon (174 kT).

In order to interpret the sensitivity of gramicidin, γ, in terms of tryptophan quantum yield, ϕ_{ox}, we will examine the relationship between γ and ϕ_{ox} in two stages. As a first approximation we will assume that the gramicidin channel dimers are single molecules and that each channel can be inactivated by destruction of any one of its eight tryptophan residues. Using target theory [27], we can predict the rate of channel photodestruction. For this analysis, we will account for the multiple tryptophan targets by assuming them to be independent and equivalent in effectiveness. The target independence assumption is supported as mentioned above by the absence of an initial shoulder in Fig. 1.

Consider first a membrane containing many channels each having one target which permanently inactivates the channel when photooxidized. It can be shown from Beer's law that a dose of light, F (photons/cm^2) would inactivate a fraction of conducting channels at a rate dependent on the target's absorption cross section, σ (cm^2/target) and on the quantum yield for photooxidation, ϕ_{ox}:

$$D = D_0 \exp(-\sigma\phi_{ox}F) \tag{2}$$

D_0 is the concentration of conducting channels, D, prior to the flash. The molecular cross section σ is related to the molar extinction coefficient, ε(cm^{-1} · M^{-1}), which can be measured independently:

$$\sigma = 1000 \cdot \ln 10 \cdot (\varepsilon/L) \tag{3}$$

L is Avogadro's number. Our observations indicate that the process of gramicidin photolysis depends primarily on elimination of channels and is approximately first order. The channel's eight tryptophans are considered equivalent to one target with a cross section equal to twice that of the gramicidin monomers, σ_g, (roughly eight times that of tryptophan). The quantum yield for photooxidation of the gramicidin tryptophans can then be estimated from our measured sensitivity (Eqn. 1):

$$\phi_{ox} = \gamma/(2\sigma_g h\nu) \tag{4}$$

Here h is Planck's constant and ν the photon frequency. We measured the absorption of gramicidin in methanol at 280 nm over the concentration range of 10 to 100 μM and found the extinction coefficient to be $2.0 \cdot 10^4$ cm^{-1} · M^{-1}. We also confirmed that gramicidin obeys Beer's law in this range [29]. The extinction coefficient of free tryptophan, 5500 cm^{-1} · M^{-1} at 280 nm, varies but little (< 10%) with the polarity of the solvent [30]. We therefore assume the value measured in methanol to approximate well the value expected in the lipid bilayer environment. Using $\varepsilon_g = 2.0 \cdot 10^{-4}$ cm^{-1} · M^{-1} and $\gamma = 0.028$ cm^2 · mW^{-1} · s^{-1} in Eqns. 2 and 3, we estimate that the quantum yield for channel tryptophan photooxidation is $\phi_{ox} = 0.13 \pm 0.03$ (S.D.). This value is considerably higher than the value of around 0.01 usually found for tryptophan photooxidation in proteins [18] even though the increase in sensitivity due to the multiplicity of tryptophans has been taken into account.

This result can be compared to sodium channel photooxidation. Fox et al. [12] measured the sensitivity of gating current in myelinated nerve to be 0.0015 cm^2 · mW^{-1} · s^{-1}. Assuming each gating particle contains one tryptophan, we calculate the quantum yield for sodium channel tryptophan photooxidation to be 0.02. This may suggest that gating particles actually contain more than one tryptophan.

If we now take into account the equilibrium between gramicidin monomers and dimers, we can determine the error in our estimate of ϕ_{ox} resulting from our initial approximation. The dimeric nature of the gramicidin channel can be incorporated into the above analysis by the following means. Consider gramicidin channels having membrane concentration, D, to be in equilibrium with monomers of concentration, M, with an association constant, K:

$$D = KM^2 \tag{5}$$

The total gramicidin membrane concentration, G, can be specified:

$$G = 2D + M \tag{6}$$

Assume the gramicidin molecules to be inactivated if any of their four tryptophans have been photooxidized. Two cases will be distinguished. In the first, inactive molecules leave the membrane and thus do not combine with any other molecules. In the second, the inactive molecules remain in the membrane and may continue to combine with other molecules to form inactive channels. In each case, the rate of loss of active dimers, dD/dF is related to the rate of loss of active molecules, dG/dF:

$$\frac{dD}{dF} = \frac{dD}{dG} \cdot \frac{dG}{dF} \tag{7}$$

Here F is the fluence or light dose. The molecule inactivation rate, dG/dF, is the same as used to derive Eqn. 1 and is again predicted based on the assumptions that the four tryptophans on each monomer comprise a target of cross section σ_g

which inactivates the monomer with probability ϕ_{ox} when hit by a photon:

$$\frac{dG}{dF} = -\sigma_g \phi_{ox} G \tag{8}$$

Under the first assumption, inactivated gramicidin molecules leave the membrane which effectively decreases the gramicidin concentration in the membrane, G. The dimer concentration, D, can be derived from Eqns. 5 and 6:

$$D = \frac{G}{2} + \frac{1-(1+8KG)^{1/2}}{8K} \tag{9}$$

Differentiating both sides of Eqn. 9 with respect to G and combining with Eqns. 7 and 8, and the expression $G = 2D + (D/K)^{1/2}$ from Eqns. 5 and 6 we get:

$$\frac{dD}{dF} = -\sigma_g \phi_{ox} \left(D + (D/K)^{1/2}/2 \right) \times \left(1 - 1/\left(1 + 16KD + 8(KD)^{1/2}\right)^{1/2}\right) \tag{10}$$

A related argument leads to a similar equation for the second case (Appendix):

$$\frac{dD}{dF} = -\sigma_g \phi_{ox} \left(4D + 2(D/K)^{1/2}\right)(KD)^{1/2} \times \frac{\left(1 + 16KD_0 + 8(KD_0)^{1/2}\right)^{1/2} - 1}{2KD_0 + (KD_0)^{1/2}} \tag{11}$$

We have analyzed Eqns. 10 and 11 numerically in order to ascertain the effects of the dimerization process on our estimate of ϕ_{ox}. Upon comparing the decay processes predicted by Eqns. 10 and 11 to that predicted by Eqn. 2 (where $\sigma = 2\sigma_g$), we find that the decay predicted by Eqn. 2 is exponential (linear on a semilog plot), Eqn. 10 is super-exponential (concave downward on semilog) and Eqn. 11 is sub-exponential (concave upward on semilog). The data in Fig. 1 are somewhat subexponential but further assessment of the raw curve shape is not warranted because the channel extinction process is complicated by the shifting mean single-channel conductance demonstrated in Fig. 4. The approximate rate of decline predicted by Eqns. 10 and 11 is always lower than that of Eqn. 2 with the worst case (e.g., Eqn. 10, $KD_0 \gg 1$) being 2-fold lower. If the sublinearity of Eqn. 11 is taken as a basis of preference, than the initial rate of decay under conditions similar to those used in our experiments ($KD_0 \gtrsim 50$) is predicted to be about 10–20% lower than that of Eqn. 2. Therefore, the value of ϕ_{ox} obtained using Eqns. 2 and 4 is probably an underestimate by 10–20% and possibly as much as 100%. In no case does ignoring the dimerization reaction result in an overestimate of ϕ_{ox}.

The high value of ϕ_{ox} found here may be a consequence of the peptide environment or orientation of the gramicidin tryptophans. For instance, Tassin and Borkman [31] found that the photolysis rates of di- and tri-peptides containing tryptophan were up to 12-times the rate for the free amino acid. Since, in their circumstances, there should only be minor changes in absorptivity, it appears that the quantum yield has increased to levels near those we observe. Also, Vladimirov et al. [18] and others (see, for example, Ref. 32) have emphasized apparent differences between ϕ_{ox} for free tryptophan and that observed for tryptophan in proteins. However, Dillon [33] has given evidence that the high quantum yield found by Tassin and Borkman results from an amino-terminus to indole ring-closure mechanism which is not likely to be operative with gramicidin since its amino-terminus is far from the tryptophans and blocked with formyl. Furthermore, our preliminary direct measurement of the rate of gramicidin's tryptophan photooxidation, made by monitoring the rate of loss of methanolic gramicidin ultraviolet light absorption at 280 nm, yielded a low tryptophan sensitivity. Assuming homogeneous tryptophan sensitivities for the random coil gramicidin monomer in this experiment and using Eqn. 4, we calculate the quantum yield for tryptophan photooxidation to be $\phi_{ox} = 0.02$. The similarity of this value to that usually measured for tryptophan photooxidation indicates that the amino acid neighbors of the tryptophans only slightly affect the tryptophan's sensitivity when the molecule is in the random coil conformation. The fact that it is 6-times lower than the estimate based on the channel inactivation rate suggests that the membrane-bound conformation may be more susceptible than normal, perhaps due to the juxtaposition

of tryptophan side chains in the β-helix. An alternative explanation could be that some photon absorptions may irreversibly alter the channel conformation causing channel inactivation without producing photooxidation and thus leading to an erroneous overestimate of ϕ_{ox}.

In summary, we have examined the photo inactivation of gramicidin channel function on the multi-channel and single channel levels. We find inactivation to result largely from a decrease in channel occurrence frequency. Partial modification of some channels fractionally reduces their conductance. Channel lifetimes are decreased in the presence of illumination but partially modified channels having decreased conductance appear to have normal lifetimes in the absence of illumination. The channel photoinactivation process is very sensitive, even more so than expected from the high channel tryptophan content. The wavelength dependence of inactivation is similar to that of tryptophan and gramicidin absorbance. We conclude that gramicidin tryptophan photomodification alters channel conductance.

Appendix

We wish to obtain dD/dG for use in Eqn. 5 for the case where inactivated monomers associate normally with other monomers but form non-conducting dimers. Under these circumstances, it can be proven that during a light exposure, equilibrium between active and inactive monomers and dimers will be retained. This is most easily comprehended by realizing that all active molecules, whether in the dimer or monomer state, are assumed equally sensitive to the light and that association and dissociation constants are unaffected by the conversion. Again, let the active molecule concentration be G, the active monomer concentration M, and the active dimer concentration, D. The chain rule prescribes that

$$\frac{dD}{dG} = \frac{dD}{dM} \cdot \frac{dM}{dG} \tag{A1}$$

The equilibrium condition can be shown to require that

$$\frac{dM}{dG} = \frac{M_0}{G_0} \tag{A2}$$

where M_0 and G_0 are the concentrations of M and G before any photolysis has occurred. Using the equilibrium and conservation of matter equations, Eqns. 5 and 6, it can be shown that

$$\frac{M_0}{G_0} = \left((1+8KG_0)^{1/2}-1\right)/4KG_0$$

$$= \frac{\left(1+16KD_0+8(KD_0)^{1/2}\right)^{1/2}-1}{8KD_0+4(KD_0)^{1/2}} \tag{A3}$$

Also from the equilibrium equation, $D = KM^2$, it is evident that

$$\frac{dD}{dM} = 2(KD)^{1/2} \tag{A4}$$

Substituting Eqns. A3 and A4 into Eqn. A1, we get

$$\frac{dD}{dG} = 2(KD)^{1/2}\frac{\left(1+16KD_0+8(KD_0)^{1/2}\right)^{1/2}-1}{8KD_0+4(KD_0)^{1/2}} \tag{A5}$$

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