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Ultraviolet flash photolysis of gramicidin-doped lipid bilayers

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We have examined the rate of gramicidin channel conductance inactivation by ultraviolet photolysis using 0.1 millisecond light flashes. The lower limit on the channel photolysis reaction rate has been reduced by four orders of magnitude over previous observations. Monoolein/hexadecane bilayers formed in 1.0 M KCl were doped with (1–3) · 10^6 gramicidin A′ channels and exposed to a broad-spectrum light flash. The flash reduced membrane conductance abruptly by approx. 16%. Following the flash, a further slow reduction of approx. 3% was observed followed by a slow recovery of approx. 4%. The post-flash decay and recovery may be due to slow chemical reactions, conformational relaxations, or changes in the equilibrium between aqueous, lipid-bound, and channel-forming dimerized gramicidin. Under our experimental conditions, gramicidin M was insensitive to light flashes compared to gramicidin A′, demonstrating that for gramicidin A′ the photolysis mechanism depends specifically on the tryptophan side-chain. Flash photolysis of a membrane containing a small population of channels (approx. 30) indicated that the decay is due to the sudden inactivation of several channels. The recovery appears to result from insertion of normal channels into the membrane. Flash photolysis of single-channel membranes showed that the flash causes abrupt, complete channel inactivation.

Introduction

Many biologically active proteins are inactivated by ultraviolet light [1]. Gramicidin A, a pentadecapeptide containing tryptophans at positions 9, 11, 13, and 15, forms cation-selective channels in cell membranes and lipid bilayers. Busath and Waldbillig [2], who demonstrated gramicidin's photosensitivity, proposed that it could serve as a model for the UV-inactivation of excitable sodium channels in nerve. They compared the sensitivity of the gramicidin channel to the reported sensitivity of sodium channels [3] and found that gramicidin is approx. 20-fold more sensitive to 280 nm light. The difference was ascribed, at least in part, to the high tryptophan content of gramicidin: each gramicidin channel, comprised of two gramicidin molecules, contains a total of eight tryptophans. They identified the 280 nm wavelength as the most potent, consistent with the absorption maximum for tryptophan. Single channels, detected by their potassium-mediated currents, were shown to inactivate when exposed to long flashes (0.75 s) of UV light.

Direct evidence that gramicidin channel inactivation is related to tryptophan photolysis was presented by Joncs et al. [4]. They compared the time-course of the tryptophan fluorescence decay

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from gramicidin dissolved in methanol or incorporated as channels into vesicles to the decay rate of gramicidin-induced bilayer conductance under identical conditions of UV illumination. They found them to be similar, both showing an initial rapid phase followed by a slower phase. The time-constants for the two processes were about the same for all three conditions. They pointed out that gramicidin fluorescence was lower in the vesicle preparation than in methanol and speculated that this may be due in part to mutual quenching between Trp-9 and Trp-15 in the helical channel form which is expected to be absent in the random coil conformation assumed in methanol. They concluded that tryptophan photolysis must be the mechanism for both the fluorescence decay and the channel inactivation.

In this paper we examine the time-course of the gramicidin A′ channel conductance decay during and after brief flashes of UV light. Our objective was to seek evidence for a lower limit in the time scale of membrane conductance changes resulting from the light-induced chemical reaction. Gramicidin A′ is a convenient mixture of gramicidins A, B, and C (in proportions of about 72:9:19, Ref. 5). Gramicidin A has tryptophans at positions 9, 11, 13, and 15. Gramicidins B and C have phenylalanine and tyrosine substitutions at position 11. A fraction of each compound has isoleucine substituted for valine at position 1. The responses examined here should largely reflect the properties of gramicidin A. We report the effects of a brief UV flash on membranes containing large channel populations, small channel populations, and single channels.

It could be asserted that the effects of the broad band light flash we use in the experiments reported here are non-specific, ie are due to some disturbance of the bilayer lipid or to absorption by some component of the peptide rather than its tryptophan side chains. Therefore, as a negative control we measured the responses to identical flashes of membranes doped with gramicidin M, a version of gramicidin with four phenylalanine groups substituted for the four tryptophans at positions 9, 11, 13, and 15 (and whose alternating stereoisomer sequence of amino acids begins with a D stereoisomer at the termini instead of with L). These channels are expected to behave like those formed from gramicidin A except that phenyl groups are much less photosensitive to the flash spectrum used than are the tryptophan indoles.

Methods

Lipid bilayers were formed across the aperture in a teflon partition or in the tip of a polyethylene pipette using the painting technique [6]. The lipid solution consisted of monoolein (50 mg, NuCheck Prep Inc., Elysian, MN) in *n*-hexadecane (1.0 ml, Aldrich Gold Label, Milwaukee, WI). Gramicidin D (A′, ICN Nutritional Pharmaceuticals, Cleveland, OH) or gramicidin M (kindly provided by Drs. Frederic Heitz and Gerard Spach, Centre de Biophysique Moleculaire, Orleans, France) dissolved in MeOH (HPLC grade, Fisher) was diluted approx. 3000 × in the saline bath (filtered 1.0 M KCl prepared using Barnstead Nanopure deionized water). The membrane current was measured via Ag/AgCl electrodes which were shadowed to varying degrees in small beakers wrapped with electrical tape. The beakers contained 1 M KCl and were electrically connected to the bilayer bathing media with 1 M KCl, 3% agar bridges.

The beakers and membrane chamber were placed in a grounded aluminum box with a small window in one end. The box and bilayer chamber were oriented so that the membrane was positioned about 2.5 cm from the lamp in a flash kinetic spectrometer (Applied Photophysics Ltd., London, U.K.). A quartz envelope flash lamp produced a substantial UV spectrum which was used to illuminate the lipid bilayer through a quartz window in front of the bilayer chamber. The flash energy was $\leqslant 40$ J with a flash duration of approx. 100 μs (10% to 90%).

After a bilayer was formed and verified to be of negligible conductance, gramicidin was added to the bath and the bilayer was repeatedly broken and reformed to check for the characteristic time-course of gramicidin channel formation. At a time of stable membrane current, the bilayer was exposed to a flash of light while the membrane current was recorded digitally.

Results and Discussion

During the light flash, the membrane conductance decreased abruptly. This is shown in Fig. 1 where membrane current (sampled at 20 μs intervals) is plotted against time on a fast time scale. An 0.1 ms period starting at the time of the flash artefact is denoted by the horizontal bar. In this figure, the flash artefact obscures the initial decline so that the shape of the decay cannot be related to the time-course of the flash. However, it is evident that the decline in current occurs largely during the flash, with perhaps a slight residual decline following the flash. The stability of the membrane conductance on this time-scale indicates that the light flash induces a stable inactivation of a portion of the gramicidin channels.

On a longer (1 s) time scale, it can be seen that the initial decay is followed by a further decline in conductance and then by a partial recovery. Fig. 2 shows the result of two flashes produced about 2 s apart at arrows a and b. The flash artefact is missing due to the sparse sampling rate used. The fast decay is estimated as the current observed within 10 ms of the flash (see Fig. 1) and the slow

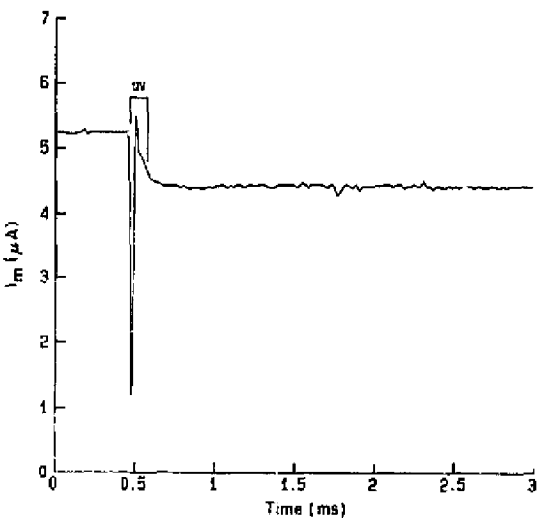

Fig. 1. Membrane current decay during UV flash. Gramicidin A', approx. 1.7 nM, in 1.0 M KCl; 0.83 mm diameter bilayer of monoolein (50 mg) in hexadecane (1 ml); 100 mV applied potential; approx. 23° C; filter cutoff, 31.8 kHz; sample frequency, 50000/s. The Ag/AgCl electrodes were connected to the membrane baths via agar bridges but were not shaded from the lamp flash.

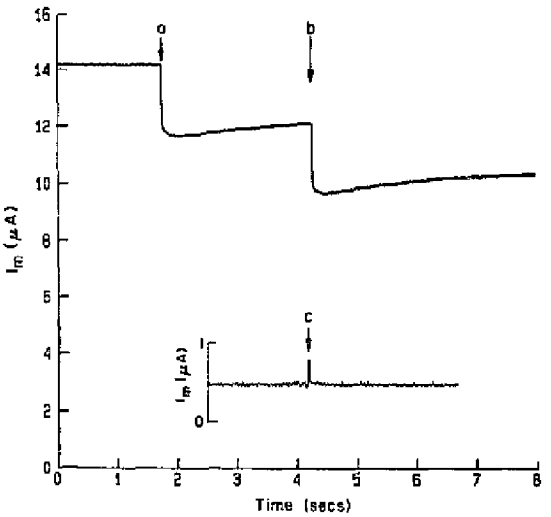

Fig. 2. Membrane current decay on a slow time scale during two flashes (arrows a and b). Gramicidin A', approx. 1.7 nM, in 1.0 M KCl; 0.83 mm diameter bilayer of monoolein (50 mg) in hexadecane (1 ml); 100 mV applied potential; approx. 23° C; filter cutoff, approx. 160 Hz; sample frequency, 100/s; partially shaded electrodes. Inset: The effect of a UV flash at arrow c on membrane current mediated by gramicidin M. Note that the time scale is unchanged but the vertical scale is expanded. The spike is the flash artefact from the electrodes. Gramicidin M, approx. 17 nM. All other conditions unchanged.

decay as the subsequent decay to the nadir which occurs between 0.1 and 0.3 s after the flash. The fast decay averaged $16.3 \pm 2.8\%$ ($n = 4$) of the initial membrane conductance. The slow decay averaged $2.9 \pm 1.1\%$ ($n = 4$) and the slow recovery averaged $4.4 \pm 0.7\%$ ($n = 4$) of the initial membrane conductance.

Light flashes of the same intensity and duration had no effect on the gramicidin M-induced conductance as shown in the inset. Arrow c indicates the flash artefact (note that the time scale is the same as in the main figure, but that the vertical scale is expanded). In experiments using gramicidin M-doped bilayers, the ratio of membrane current after a flash to that before the flash averaged 1.01 ± 0.01 ($n = 6$). In one control experiment, a gramicidin M-doped membrane which was unaffected by a light flash was subsequently exposed to gramicidin D and then flashed. The addition of gramicidin D produced a large increase in membrane conductance which was sensitive to UV light (total flash decay from two flashes: $22.8 \pm$

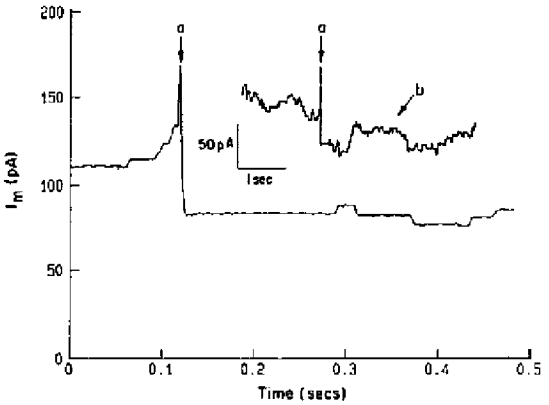

Fig. 3. Flash photolysis of a membrane containing a small population of channels (approx. 28). Gramicidin A', approx. 0.34 pM, in 1.0 M KCl; 0.088 mm diameter bilayer of monoolein (50 mg) in hexadecane (1 ml); 100 mV applied potential; approx. 26°C; filter cutoff, 200 Hz; sample frequency, 400/s; partially shaded electrodes. Inset: The data from which the main figure was extracted shown with reduced scales. Arrow a: flash artefact. Arrow b: the recovery period following the flash.

8.2%) demonstrating that the lipid membrane was normal.

Theoretically, the flash could completely inactivate an integral number of channels or could partially inactivate many channels, i.e., reduce their conductance. To distinguish between these two possibilities, we measured the current for a membrane containing a small population of channels so that the individual channel openings and closings could be distinguished. This small-population approach has proven to be very useful for demonstrating modifications of gramicidin channel occurrence frequency and conductance, even though the individual channel lifetimes cannot be determined. Fig. 3 shows the result of such an experiment. At time zero, the membrane current is about 115 pA, representing approx. 24 channels of 4.7 pA (the average size of single channel current steps in the experiment; baseline current is typically < 2 pA). Four channels turn on prior to arrow a, bringing the total to approx. 28 (133 pA). At arrow a, the UV flash, there is a large ($+34$ pA) electrode artefact. After the artefact terminates, the membrane current is 83 pA (representing approx. 17 channels). The channel occurrence frequency was about the same before the flash as after (9.4 ± 2.4 (S.E.) channels per second before vs. 12.8 ± 1.3 (S.E.) channels per second after). It is not possible to be sure from these data whether the decay represented an integral number of channels because of heterogeneity in the single channel conductances. However, it appears that the main effect of the flash is to abruptly reduce the number of conducting units.

The inset to Fig. 3 shows an overview of the trace from which the main figure was extracted. During the period from one to three seconds after the flash (indicated by arrow b) a recovery in membrane conductance can be seen which is consistant with the recovery demonstrated with the much larger population of channels in Fig. 2. From the data in the inset to Fig. 3, we conclude that the recovery is probably due to a stepwise increase in the average number of channels in the membrane.

These conclusions were also substantiated at lower gramicidin concentrations where the membrane contained only one conducting channel at the time of the flash. Fig. 4 shows a trace obtained with a sub-picomolar concentration of pure gramicidin A in the bath. At time zero, the membrane current is a few picoamps (bilayer resistance

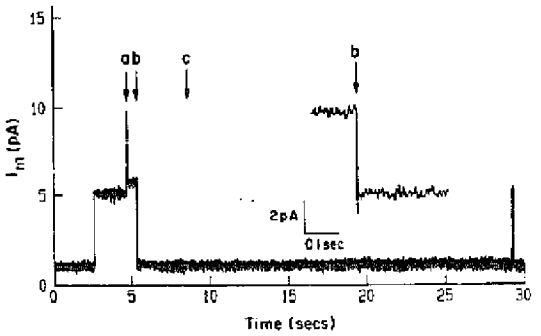

Fig. 4. Flash photolysis of a single standard gramicidin A channel. Gramicidin A, approx. 0.17 pM, in 1.0 M KCl; 0.088 mm diameter bilayer of monoolein (50 mg) in hexadecane (1 ml); 100 mV applied potential; approx. 24°C; filter cutoff, 200 Hz; sample frequency, 400/s, decimated 4-fold for plotting (which removed small flash artefacts at arrows b and c); well shaded electrodes. Arrow a: A second channel (standard conductance) turns on followed immediately by the turn off of the first channel (low conductance). Arrow b: 1st lamp flash. Arrow c: 2nd lamp flash. Inset: Expanded, undecimated view of data at the lamp flash (arrow b). The flash artefact is the small spike extending briefly below the baseline.

approx. 50 GΩ) and no channels are conducting. A low conductance channel turns on at approx. 3 s. It then turns off at arrow a, just after a standard conductance channel (47 pS) turns on. Only the standard channel is on at the time of the flash indicated by arrow b. (A second flash at arrow c had no effect on the zero-channel bilayer current). The inset shows an expansion of the time scale about point b demonstrating that the channel closure is simultaneous with the flash artefact which briefly extends below the baseline. This effect, termination of the channel conductance during the interval containing the flash, was observed in 4 of 31 channels exposed to flashes during this experiment. The mean channel lifetime under these conditions is about 2.5 s, so the probability of a channel turning off spontaneously during the sample interval containing the flash (2.5 ms) is only 0.001, indicating that the appearance of flashed-induced termination is probably not a coincidence. The fraction of single channels terminated with a flash was roughly consistent with the results for a bilayer containing many channels.

The main effect of a brief flash of UV light is an immediate reduction in gramicidin-mediated membrane current due to inactivation of individual channel units. Following the light flash there is a secondary slow decay followed by a slower recovery. The rapid, irreversible decay is postulated to result from photooxidation of channel tryptophans. It is not likely to result from photo-induced effects on the lipid or channel backbone because any such process should have affected the gramicidin M channels as well. The phenylalanine in gramicidin M should also be photosensitive, but its absorption peak is blue-shifted to a region of the spectrum where the lamp is less efficient. Furthermore, the maximum absorption coefficient of phenylalanine is approx. 10-fold lower than that of tryptophan.

Expected tryptophan oxidation products include formylkynurenine and kynurenine which are amino acids like tryptophan but containing polar formyl and ketone groups Other simpler amino acid products would also be expected (e.g. alanine and glycine). Also, more complex products could occur, including polymerization of gramicidin molecules, cross-linking of tryptophan side-chains, oxidation of other parts of the gramicidin molecule, or partial hydrolysis of the peptide.

The bulk of the primary inactivation reaction occurs during the flash as shown in Fig. 1. It is therefore only possible to place a lower limit on its rate. The experiments demonstrate that the time scale for the reaction is $< 100\ \mu s$, four orders of magnitude less than that demonstrated by Busath and Waldbillig [2]. Experiments with shorter (ns) UV flashes should be able to test whether the rate of inactivation exceeds the limits for a diffusion controlled reaction.

The slow decay and recovery that we observe in the multi-channel bilayer after the flash could result from a series of slower chemical reactions. From Figs. 3 and 4, we conclude that the slow decay and recovery are not due to graded changes in single-channel conductance but are statistical sums of underlying all-or-nothing processes. The slow decay may reflect slower light-induced chemical changes, perhaps a long-lived conducting intermediate photoproduct that eventually undergoes a final inactivation step. For the recovery, one possible hypothesis is that the absorbed photons activate a reversible conformation change in some molecules. This hypothesis seems a bit unlikely when one considers that the energy in one photon absorbed by a tryptophan, if converted to kinetic energy by internal conversion, would probably be distributed throughout the peptide, lipid bilayer, and bulk water within picoseconds [7] and thus effectively dissipated before inducing conformational changes in the channel. Another possible mechanism for the recovery is that the dimer-monomer equilibrium, perturbed by elimination from the bilayer of a fraction of the gramicidin molecules, undergoes a slow relaxation. It is possible that experiments on the time-course of recovery using different bath gramicidin concentrations or with covalently dimerized channels will allow these possibilities to be tested.

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