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ION TRANSFER ACROSS LIPID MEMBRANES IN THE PRESENCE OF GRAMICIDIN A

I. STUDIES OF THE UNIT CONDUCTANCE CHANNEL

S. B. HLADKY AND D. A. HAYDON

University of Cambridge, Physiological Laboratory, Downing Street, Cambridge (United Kingdom)

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SUMMARY

The conductance induced by gramicidin A in lipid bilayer membranes has been shown to be made up of discrete, well-defined units. In 0.1 M NaCl, and for 100 mV applied, the integral conductance of the unit channel at 20 °C is $5.8 \cdot 10^{-12}\ \Omega^{-1}$.

The channels are formed by transitions involving inactive gramicidin molecules already in the membrane. The precise nature of a transition is not certain, but circumstantial evidence suggests that the final conducting structure consists of at least two polypeptide molecules. From the temperature coefficient of the average duration of the channels it has been shown that the activation energy for channel closure must be $\gtrsim$ 19 kcal mole^{-1}. The frequency of occurrence and the average duration of the channels both become larger the thinner the membrane. The equilibrium between non-conducting and conducting species therefore shifts towards the conducting species as the membrane thickness decreases.

With one exception, the same unit channel conductance was observed for a range of membranes having hydrocarbon thicknesses from 26 to 64 Å and, from this and other evidence, it has been concluded that the conducting channel is a pore, rather than a carrier. The length of the pore has been estimated to be less than 35 Å.

The pore passes univalent cations but completely excludes polyvalent cations and anions. The selectivity between the univalent cations is not great, and the sequence of the various ion conductances is similar to that for the corresponding electrolytes in aqueous solution. The activation energies for the conduction of the ions are also similar to those in aqueous solution.

The current–voltage relationships for the single channel tend to be curved towards the current axis at high electrolyte concentrations, linear at intermediate concentrations and curved towards the voltage axis at low concentrations. For each of the electrolytes studied the conductance of the single channel tends towards a limiting value at high concentrations.

It is noted that one of the dimeric helical structures (that which contains the $\pi^{6}_{(L,D)}$ helix) proposed by Urry *et al.*[22] could be consistent with some of the properties of the single channel.

Abbreviation: PTFE, polytetrafluorethylene.

INTRODUCTION

The quantitative study of net ion fluxes across lipid membranes in the presence of peptides and proteins poses serious problems, even when the membranes are artificial and in a well-defined environment. Ion fluxes comparable to those encountered in biological membranes may be given by concentrations of peptide in the membrane far too small to be measured by present methods, and thus the properties cannot be related to a given number of molecules. Even if this difficulty is overcome, and the ion transfer properties of a known number of molecules become accessible, such factors as co-operative effects and equilibria between active and inactive units can make the interpretation of the data either impossible or extraordinarily complicated.

For reasons such as these it is very advantageous to be able to measure the properties of a single conducting unit. For many types of system, small water-soluble molecules in particular, the current carried by an individual molecule or complex is likely to be undetectable owing to its being too small, too transient or to a combination of these factors. The macrotetralide antibiotic nonactin is such a molecule, and it was mentioned in a preliminary communication[1] that no stepwise changes in the current across a bilayer membrane could be detected in its presence. Gramicidin A, on the other hand, which has a molecular weight roughly twice that of nonactin, and which is practically insoluble in water, produced clear stepwise fluctuations in the membrane current[1]. At that stage of this investigation, gramicidin A was the only well-characterised substance known to give single channel fluctuations and, as a consequence, was considered worthy of a detailed investigation. The only other obviously analogous fluctuation phenomenon to be reported is that for EIM by Bean *et al.*[2] and, in greater detail, by Ehrenstein *et al.*[3]. The conductance of the unit channel found by these authors is nearly an order of magnitude larger than that of gramicidin A. Although EIM is perhaps of greater interest biologically, its molecular structure is not known.

Valine gramicidin A is a linear polypeptide antibiotic of molecular weight 1883. Its primary structure, as elucidated by Sarges and Witkop[4,5], is illustrated in Fig. 1. The molecule has no ionizable groups, as both the C and N terminals are blocked. This to some extent explains the undetectably low solubility of gramicidin A in water. The polypeptide is also extremely insoluble in hydrocarbons. Such evidence as is available suggests that the solubility is less than 10^{-10} mole l^{-1} in each medium[6]. Gramicidin may, however, be dispersed in hydrocarbon by oil-soluble lipids, and fine dispersions in water, which are stable for appreciable periods, may also be prepared.

HCO–L-Val–Gly–L-Ala–D-Leu–L-Ala–D-Val–L-Val–D-Val–L-Trp–
D-Leu–L-Trp–D-Leu–L-Trp–D-Leu–L-Trp–NH·CH_2CH_2OH

Fig. 1. The structure of valine gramicidin A (after Sarges and Witkop[4,5]).

Several authors (*e.g.* Pressman[7,8], Chappell and Crofts[9], Harris and Pressman[10], Silman and Karlin[11], Podleski and Changeux[12], and Henderson *et al.*[13]) have described the influence of gramicidin on biological membranes. Among the membranes investigated are those of mitochondria, erythrocytes and electroplax and, in each instance, the gramicidin appeared to produce a passive permeability of the membranes to alkali metal ions.

Gramicidin A also produces permeability to alkali metal ions, as opposed to univalent anions, in lipid bilayers[8,14,15], and in this respect resembles the smaller cyclic antibiotics nonactin and valinomycin. Here, as will be shown, the similarity ends. Among other differences, it is found that in contrast to nonactin and valinomycin, which are generally agreed to be carriers, gramicidin forms a pore through the membrane.

MATERIALS AND METHODS

The apparatus and procedure in the present investigation embodies several novel features which it is necessary to describe in some detail.

Electrical circuitry

The small changes in current which accompany membrane conductance fluctuations require for their detection a device with a rapid response and minimal noise in the frequency range of interest. The three principal sources of noise are microphonics, electrostatic pick-up and the input noise inherent in the amplifier used to measure the current.

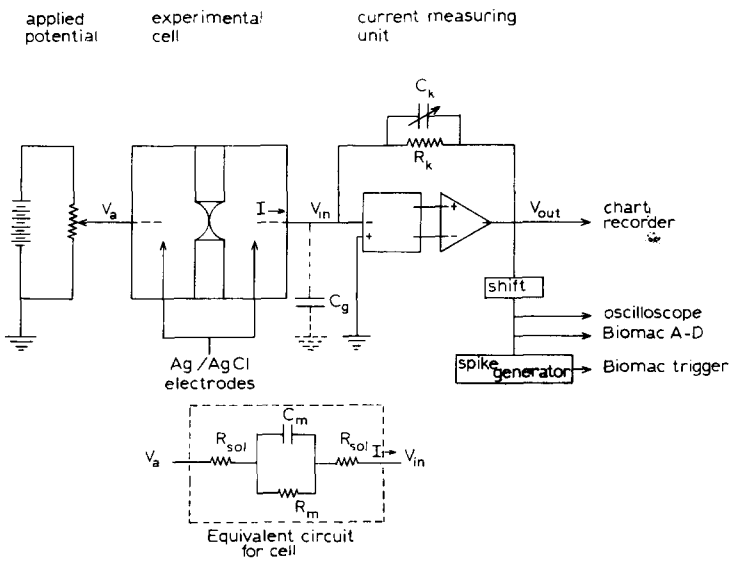


Fig. 2. The schematic circuit diagram. The various features are discussed in the text. The response time, I, of the current measuring unit is dictated by the choice of R_K and C_K. The following values were found suitable:

R_K (Ω)	C_K (pF)	τ (ms)
10^{10}	2	20
10^9	2	2
10^8	10	1
10^7	10	0.1

The different combinations could be selected by means of a PTFE wafer switch.

The circuit is shown schematically in Fig. 2. The potential is applied relative to earth from a 200 ΩHelipot potentiometer (Beckman) in series with a 2 KΩ resistor across a 4.5 V dry battery. The current measuring unit consists of a pair of XE 5886 electrometer pentodes (Plessey) as a preamp for impedance matching, and an ordinary operational amplifier (Analog Devices Ltd. No. 115A) for voltage gain. The preamp circuit is given in Fig. 3. The input current drawn by this device is $\ll 10^{-14}$ A.

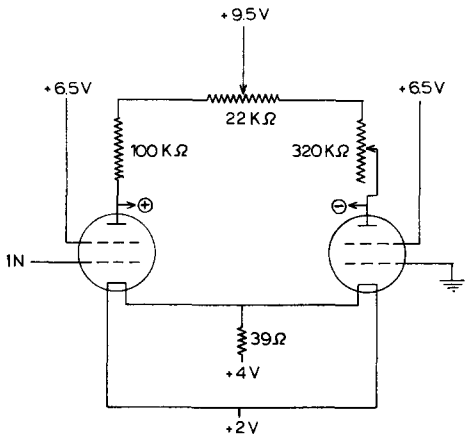


Fig. 3. The high impedance preamp. The XE 5886 electrometer pentodes were mounted between two PTFE blocks, the leads passing through the lower block. A small piece of latex foam was placed on top of the valves and kept in place by pressure of the upper block. The two blocks formed the top and bottom of a light-tight box, the sides of which were of metal in order to screen the input stage from the rest of the amplifier circuitry. Two 60 A/h lead-acid accumulators connected between 0 and +2 V and +2 and +4 V, respectively, provided the bias voltage and heater current. All other current requirements were provided by dry cells. The preamp was run continuously.

In the steady state

$$V_{out} = -\frac{A}{A+1} IR_K \tag{1}$$

where A is the gain of the amplifier. Since $A \gg 1$ (approx. 10^5) the output is effectively IR_K, *i.e.* the unit measures the current. The input voltage is

$$V_{in} = \frac{IR_K}{A+1} \tag{2}$$

By an appropriate choice of R_K, therefore, V_{out} may be made to vary in the range 0–100 mV, while $|V_{in}|$ is always much less than 100 μV. Thus the potential across the experimental cell is just the applied potential from the potentiometer and, provided the resistance of the membrane R_m is much greater than that of the aqueous phases, $2 \cdot R_{sol}$, this is also the potential across the membrane.

The "1/e" response time τ of the circuit may be calculated by standard methods. As long as $AR_m \gg R_K$

$$\tau = R_K C_K + \frac{R_K(C_g + C_m)}{A+1} \tag{3}$$

Thus, to achieve maximum speed of response (*i.e.* small τ) the value of C_K must be kept small. The values of C_g and C_m are not noticeably restricted by Eqn 3.

For frequencies greater than $1/R_K C_K$ the noise at the output V_{on} at a given frequency, due to amplifier noise V_n referred to the input, is approximately

$$|V_{on}| \approx \frac{(C_m + C_g)}{C_K} |V_n| . \tag{4}$$

The value of V_n for the XE 5886 is not known, but for electrometer valves a value much less than 100 μV (0–1 kHz) would be unusual. In order to keep the output noise below 1 mV over the frequency range passed by the amplifier it is therefore necessary that $(C_m + C_g)/C_K$ be small. Although a large C_K could be used, it is obvious from Eqn 3 that the response time of the circuit is then increased. The only acceptable procedure is thus to keep C_g and C_m as small as possible. The input shunt capacity due to the input valve and leads is $\sim$ 25 pF. It is consequently desirable that the membrane capacity should have a comparable value.

The cell and its mounting

The cell, shown in Fig. 4, is designed to allow the formation and control of membranes of small area. It consists of two cups both of which are machined from solid blocks of polytetrafluorethylene (PTFE). The shapes are arbitrary except that

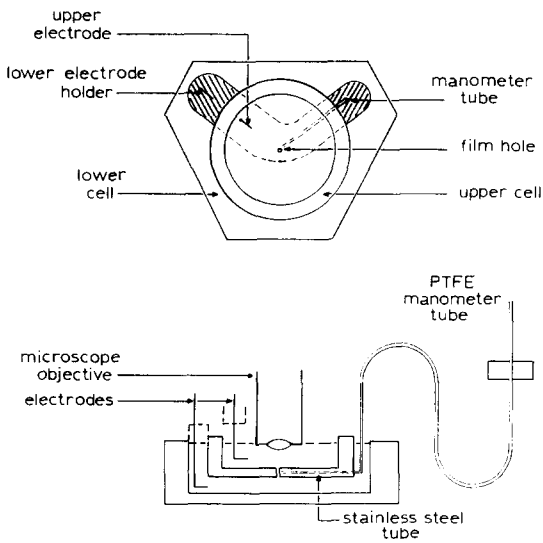


Fig. 4. The experimental cell (see text).

the inner and outer must nest. There are two holes in the inner cup. The larger (diameter 0.7–1 mm) punched vertically through the bottom of the cup, is the site of membrane formation (see Fig. 5). The smaller (diameter approx. 0.3 mm), drilled within the thickness of the base of the cup, connects the membrane and its torus with the hydrocarbon solution of lipid in the manometer. With this apparatus membranes of area down to $3 \cdot 10^{-5}$ cm^2 could be controlled.

The membrane is viewed through a microscope fitted with an incident light illuminator. The objective dips into the upper aqueous phase and has therefore to be insulated (*e.g.* by means of a PTFE collar) from the remainder of the instrument

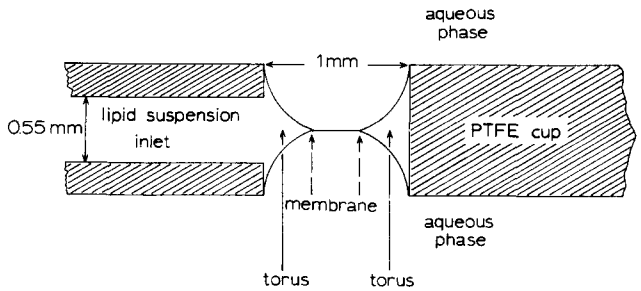


Fig. 5. The lipid membrane shown in relation to the holes in the base of the inner PTFE cup. The horizontal channel on the left is connected to the manometer.

The cell is mounted on an earthed stage which is supported on top of a small platform by stiff polyurethane foam. This platform is supported above a half-inch-thick steel slab by three levelling screws. The microscope and manometer support are rigidly fastened to the slab and the current measuring unit rests on the base-plate next to the cell. The slab forms the bottom of an earthed Faraday cage which completely surrounds the apparatus and to which all protruding components, *e.g.* the microscope eyepiece, are earthed at the point of emergence.

Small changes in the membrane area, and hence also the membrane capacitance, tend to occur as a consequence of mechanical vibration. In this way microphonic currents arise which can completely mask the changes in current due to conductance fluctuations. In order to minimise vibration, therefore, the entire Faraday cage and the power supply are mounted on a concrete slab which is suspended from a steel girder by nylon ropes. The corners of the slab are connected by short rods to oil-filled dashpots mounted on foam pads. With these precautions the major source of vibration is the transmission of sound through the air.

The output from the current measuring unit is fed directly to a Servoscribe (Smith's Industries, London) chart recorder and, *via* a $\times$ 100/$\times$ 1000 preamplifier to an oscilloscope and the various inputs of a Biomac 500 analog-digital computer (Data Laboratories Ltd., London). The Biomac has been used to compile histograms of the amplitudes of pulses and of the intervals between pulses.

For most of the systems examined the electrodes were Ag–AgCl. For the systems which involved $CaCl_2$ or saturated NaCl or KCl, however, a better procedure was to use commercial calomel electrodes connected to the cell by means of wide-bore capillary tubes containing the aqueous phase present in the cell.

Materials

The inorganic salts were of Analytical Reagent grade and, where appropriate, were roasted at 700 °C to remove organic impurities. The water was taken from a commercial still and redistilled from pyrex into a pyrex condenser and collector. Its pH on use was normally approx. 5.7. The *n*-alkanes were from Koch-Light Ltd. and were of puriss grade. They were further purified by passage through columns of

chromatographic alumina. All other solvents were of Analytical Reagent grade. The glyceryl monooleate was obtained from Sigma and was used at a concentration of 7 mM in the hydrocarbon. Glyceryl monopalmitoleate was from Nu-Chek Prep. The valine gramicidin A was a pure specimen kindly provided by Dr. E. Gross, N.I.H., Bethesda. Less pure commercial specimens (*e.g.* from Koch-Light Ltd.) nevertheless gave very similar results.

Cleaning

Glasswear and the syringe needles were cleaned in chromic–sulphuric acid cleaning mixtures. The most satisfactory procedure for cleaning the PTFE cell was found to be to alternate scrubbing in detergent and soaking in the chromic–sulphuric mixture, with intermediate rinsing in water. The use of solvents such as ethanol or propanol followed by a cursory rinse in water and immersion in chromic–sulphuric mixture will often hasten the removal of visible contamination but the products of this procedure appear themselves to be difficult to remove.

Setting up and control behaviour

At the start of an experiment the inner cell is placed loosely into its slot. About 0.1 ml of the lipid solution is introduced into the manometer so as to just fill the tube and the membrane hole of the inner cell. Several drops of the solution are placed on the surface of the lower cell. The aqueous phase is then introduced into the lower compartment. Only then is the inner cell pushed down into position. This procedure helps to avoid trapping air at the points of contact between PTFE surfaces which, in turn, seems to reduce greatly problems over the subsequent release of static charge. When the inner cell is pushed down, the aqueous solution in the lower compartment rises through the membrane hole. Filling of the upper chamber is completed simply by adding aqueous phase.

A membrane is formed by raising the manometer until sufficient lipid solution flows into the hole to form a thick film, then lowering the manometer until the opposite faces of the thick film come into contact. Drainage is spontaneous.

The conductance through the membrane hole may actually fall as the membrane blackens. For glyceryl monooleate in either decane or hexadecane the measurable conductance is across the Plateau border and is constant. The actual membrane conductance is certainly $\lesssim 10^{-9}\ \Omega^{-1}\cdot cm^{-2}$.

RESULTS

In the presence of very small amounts of gramicidin, and for each of the types of membranes examined, the membrane current under fixed applied potential fluctuates in a well-defined step-like manner. A record for a membrane formed from glyceryl monooleate and hexadecane in 0.5 M NaCl is shown in Fig. 6. This particular record was selected because, in addition to the normal fluctuations, it shows two rarely occurring features, the very high peak, α, on the extreme left, and the anomalous plateau heights in the region β. As more gramicidin is added to the system the steps occur with increasing frequency and begin to overlap. When the time-average current reaches a sufficiently high value the fluctuations can no longer be resolved, although there are slower variations in the average current which persist at all levels.

These slow variations, which appear to reflect a variation in the amount of gramicidin in the membrane, make it very difficult to obtain precise statistics of the fluctuation frequency.

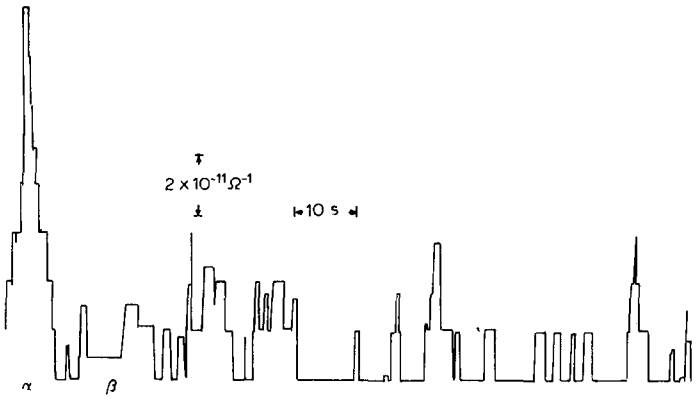


Fig. 6. Conductance transitions for a membrane of glyceryl monooleate and hexadecane in the presence of a very small amount of gramicidin A. The electrolyte was 0.5 M NaCl, the membrane area $8 \cdot 10^{-4}$ cm^2 and the temperature 23 °C. The events marked α and β on the left hand side of the record occurred very infrequently.

At very low levels of the average current, each upward transition is followed by a downward transition equal in magnitude, and these transitions are of sufficient uniformity (see below) for the concept of a basic step height to be introduced. At slightly higher current levels, the most frequently occurring values of the current are multiples of the basic step height. The addition of gramicidin did not give rise to any current other than that produced by the discrete transitions. It is inferred, therefore, that each pair of transitions represents the opening and closing of a single conducting channel. For a given aqueous solution and a given applied potential, the current through a single channel has one value which occurs far more frequently than any other. This is shown for a membrane of glyceryl monooleate and hexadecane in 0.5 M NaCl in Fig. 7. The most probable value for the current is to some extent independent of the type of membrane (see Table II) although there is no reason to suppose this to be generally true.

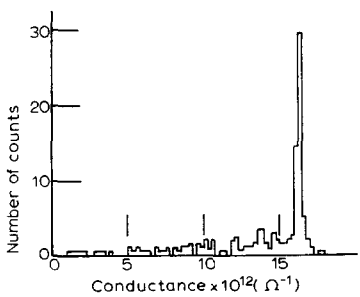


Fig. 7. Histogram showing the distribution of single channel conductances. The data were read from a chart recorder and do not include channels of duration less than 200 ms. The histogram in Fig. 11, however, is not selective for any duration range.

The interval between the opening and closing of a channel, which will be referred to subsequently as the duration, has, in contrast to the step height, a continuous range of values. Fig. 8 shows a typical histogram obtained by plotting the frequency of occurrence against the duration of the channel. The decay in the frequency towards

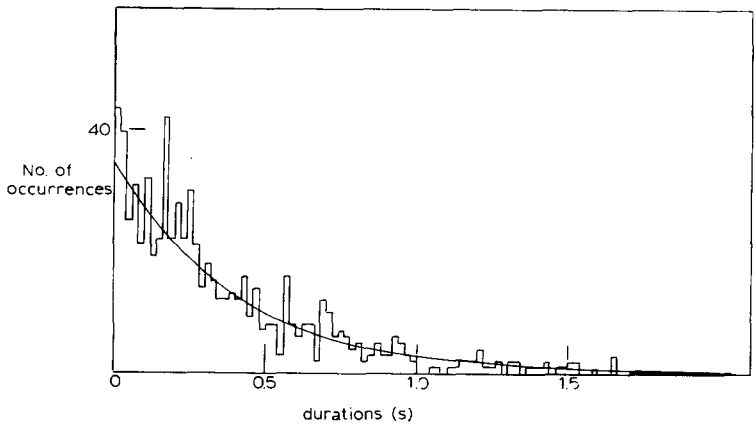
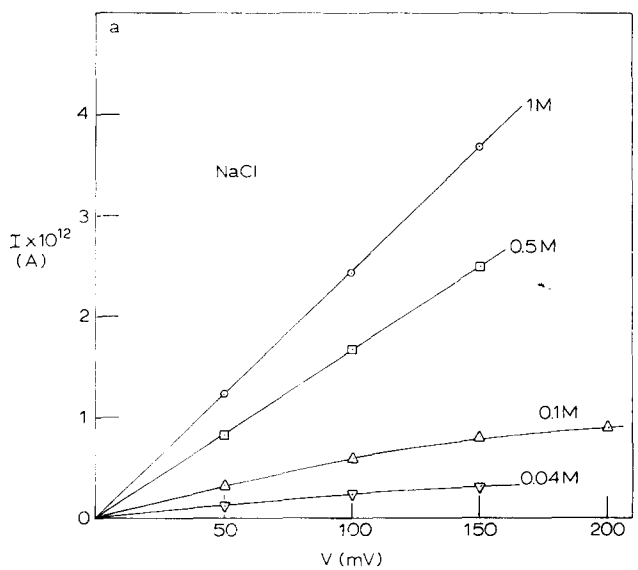


Fig. 8. The frequency distribution for gramicidin channel durations, for membranes of glyceryl monooleate *plus* decane in 0.5 M NaCl. The smooth curve corresponds to the exponential decay expected if the probability of termination of a channel were independent of the lengh of time for which it was open. $T = 23$ C.)

long durations is exponential, as would be expected if the probability of termination of the channel were constant and independent of time.

Both the conductance and the duration of a single channel depend on the temperature, the latter being much more sensitive than the former. The conductance of glyceryl monooleate and decane membranes in 0.5 M electrolyte (where the current–voltage relationships are linear) was measured at 3 and 23 °C. The Q_{20} was found to



be 1.9 and 1.8 for KCl and NaCl, respectively, and the corresponding activation energies 5.4 and 4.9 kcal·mole^{-1}. For the same type of membrane over the same temperature range, the Q_{20} for the rate constant for termination of the channel (*i.e.* the reciprocal of the duration) was $\lesssim$ 10, giving an activation energy of $\lesssim$ 19 kcal·mole^{-1}.

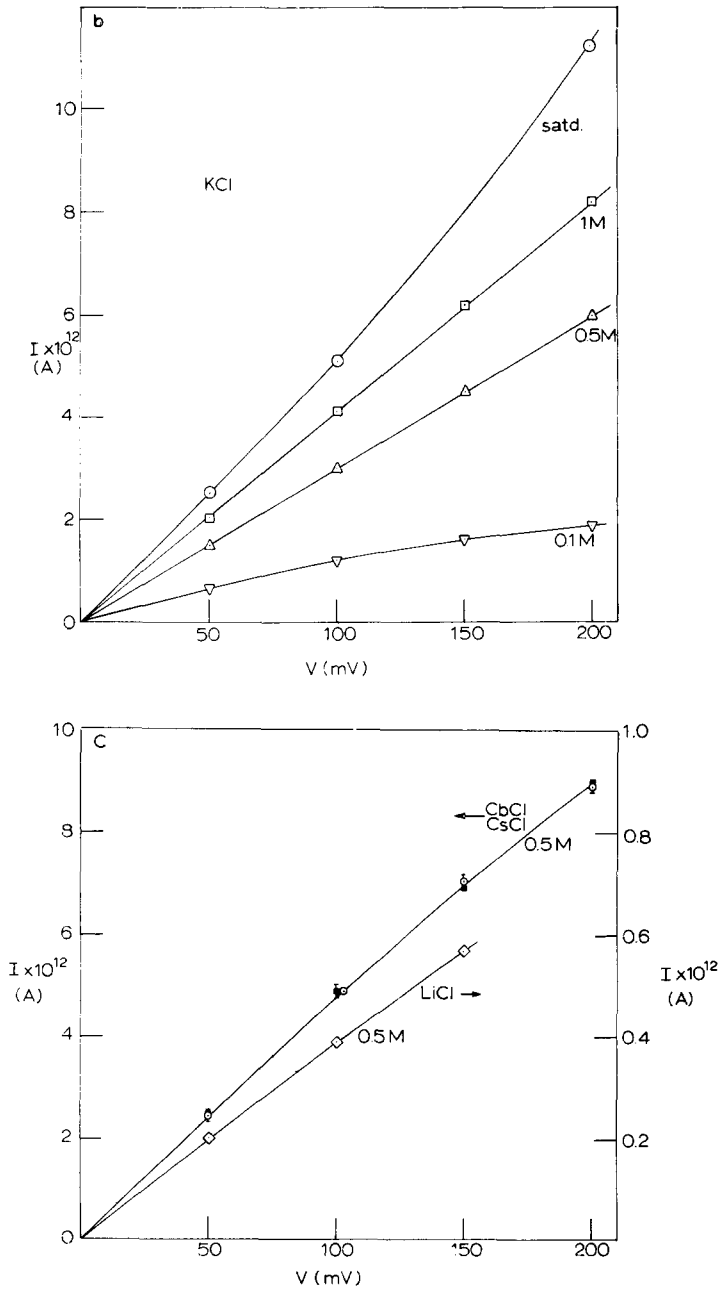


Fig. 9. Current–voltage curves for the single channel of gramicidin. (T = 23 °C.)

The magnitude of this activation energy is dependent, but not strongly so, on the nature of the membrane, as will be seen from results presented below.

Current–voltage relationships for single channels in glyceryl monooleate membranes have been determined for NaCl and KCl for a range of concentrations and for LiCl, RbCl and CsCl at 0.5 M. The results are presented in Fig. 9. A characteristic feature of the plots is that the sign of the curvature is a function of the electrolyte concentration. At low concentrations the curves are concave to the abscissa, while towards saturation they are concave to the ordinate. At intermediate concentrations (approx. 0.25 to 1 M) the plots are approximately linear. The integral conductances (I/V), at 100 mV applied, of the single channel in NaCl, KCl and CsCl are shown as a function of electrolyte concentration in Fig. 10. As can be seen, the currents appear to approach limiting values at high concentrations. In addition to the electrolytes already mentioned the unit channel conductances in HCl and $CaCl_2$, and also in mixtures of KCl and $CaCl_2$, were examined. In HCl and $CaCl_2$ the conductances were very high and very low, respectively (Table I). When added to KCl, $CaCl_2$ produced a slight lowering of the conductance.

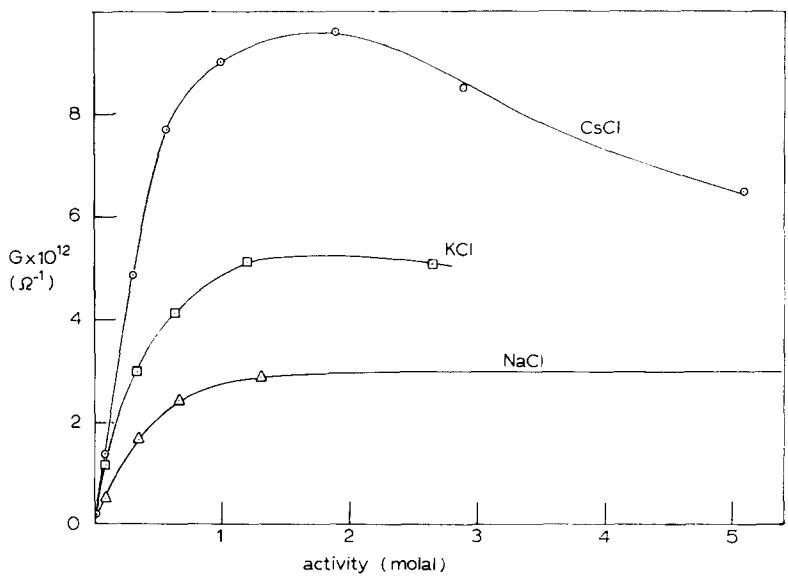


Fig. 10. The integral conductance (at 100 mV applied) of the single channel of gramicidin.

Useful evidence as to the nature of the conducting pathway is obtained by the examination of the unit channel parameters as a function of membrane thickness. A technique for forming membranes of various thickness has been described by Andrews, Manev and Haydon[16] and by Fettiplace, Andrews and Haydon[17]. Using glyceryl monooleate and the *n*-alkanes from decane to hexadecane, membranes are obtained whose hydrocarbon thickness ranges from approx. 47 Å down to approx. 30 Å. Still thinner membranes (26 Å) were obtained by using glyceryl monopalmitoleate and hexadecane and, with certain types of polymer, much thicker membranes (of 64 Å or more) were formed (J. L. Taylor and D. A. Haydon, to be published). The

TABLE I

CONDUCTANCE RATIOS FOR THE SINGLE CHANNEL OF GRAMICIDIN A IN 0.5 M SOLUTIONS, WITH 100 mV APPLIED

	H^+	Li^+	Na^+	K^+	Rb^+	Cs^+	NH_4^+
Single channel conductance ratio G_i/G_{Na^+}	14*	0.23	1.0	1.8	2.9	2.9	2.4
Ratios of single ion conductance at infinite dilution in aqueous solution $(\Lambda_{0,i}/\Lambda_{0,Na^+})_{25\,°C}$	9.1	0.77	1.0	1.5	1.6	1.5	1.5

* at 0.01 M.

TABLE II

THE CONDUCTANCE (AT 100 mV AND 23 °C) AND THE MEAN DURATION OF THE SINGLE CHANNEL OF GRAMICIDIN A AS A FUNCTION OF MEMBRANE THICKNESS AND COMPOSITION

Membrane-forming lipid solution	*Hydrocarbon thickness* (Å)	*Volume fraction of solvent in membrane*[17]	*Single channel conductance in 0.5 M NaCl* ($\Omega^{-1} \cdot 10^{11}$)	*Mean duration of single channel* (s)	*Conductance ratio* G_{K^+}/G_{Na^+} *at 0.5 mole·l*$^{-1}$
Glyceryl monopalmitoleate + *n*-hexadecane	26	(<0.17)	1.7	~60	—
Glyceryl monooleate + *n*-hexadecane	31	0.17	1.7	2.2	1.8
Glyceryl monooleate + *n*-tetradecane	40	0.30	1.7	1.3	—
Glyceryl monooleate + *n*-decane	47	0.47	1.7	0.4	1.8
Polyhydroxystearic acid + glyceryl monooleate + *n*-decane	~64	—	(1.7) (~0.8)	~ 0.03 —	—
Glyceryl monooleate + cholesterol + *n*-decane*	47	—	1.7	~ 0.4	—

* Glyceryl monooleate:cholesterol = 1:1 (mole ratio).

results are summarized in Table II. With one exception, the single channel conductance is accurately constant and independent of membrane thickness for both NaCl and KCl. For the very thick (64 Å) films, although the unit steps of the usual size were observed, there were a significant number of steps of approximately half this size. The frequency of occurrence of the small steps (relative to the large ones) decreased with the applied potential up to 200 mV.

In contrast to the conductances, the duration of the channels is strongly dependent on membrane thickness and increases steadily with decreasing thickness until the membranes are very thin. The activation energy for the termination of a channel therefore increases with decreasing thickness, although this variation does not amount to more than 15% of the 19 kcal·mole^{-1} found for the glyceryl monooleate and decane membranes.

As the membrane thickness varies it can be seen from Table II that the composition also varies, although only in respect to the chain lengths of the hydrocarbon moieties. The composition was also changed by the addition of cholesterol to the glyceryl monooleate and decane systems. As shown in Table II, however, no change occurred in the single channel conductance and average duration.

When small amounts of gramicidin were present in the system, the number of channels open, n, fluctuated about the mean value, $\bar{n}$. The distribution of the values of n about $\bar{n}$ should, in general, depend on the molecularity of the reactions which open and close the channels. Among the more likely mechanisms for the closure of a channel are the rotation of a gramicidin molecule, the spontaneous break-up or desorption of a molecular complex and the fluctuation in thickness of the membrane, all of which would probably be unimolecular processes. If this is so and N, the total number of gramicidin molecules, is much greater than $\bar{n}$, the probability of finding a certain number of channels open at any given time should be given by the Poisson distribution. A test of this point is shown in Fig. 11. The instantaneous value of the

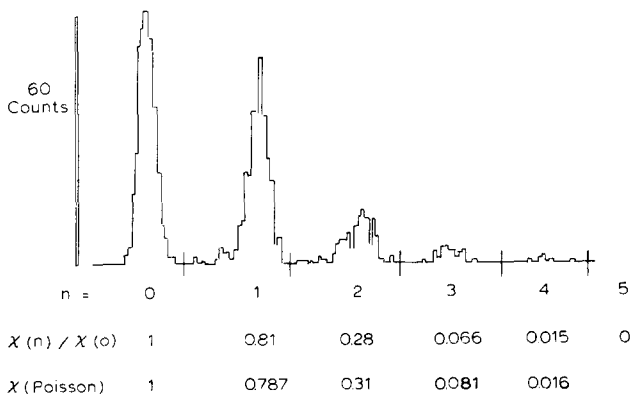


Fig. 11. The frequency of occurrence in a membrane of 0, 1, 2 ... *etc.* simultaneously open channels, obtained by sampling the current at 200 ms intervals. The first row of figures gives the number, n, of channels open, while the second row gives the ratios of the areas, $\chi(n)$, under the peaks for $n = 1, 2, \ldots$ to the area $\chi(0)$ for the peak for $n = 0$. In the third row is given the ratios expected for a Poisson distribution, the mean number, $\bar{n}$, of channels open being 0.787. In order to achieve an adequate time of response the resistance R_K in the current measuring device (see Fig. 2) was kept low ($10^9\ \Omega$). The signal, which is equal to the interval between the peaks, is therefore not much in excess of the noise level, given by the width of the zero peak. The membrane was formed from glyceryl monooleate and *n*-decane.

conductance of the membrane was measured and recorded every 200 ms and the number of occasions that a certain conductance was obtained is represented on the diagram. On the majority of occasions the conductance was close to zero (hence the left hand peak) and the frequency of occurrence of one channel, and of two, three, *etc.* channels simultaneously is given by the subsequent peaks. The areas under the respective peaks relative to that for the peak $n = 0$ are shown in the first set of numbers. The areas expected from the Poisson distribution are given by the second set, the mean $\bar{n}$ having been calculated to be 0.787. The agreement is as close as could be expected.

The frequency of opening of channels was not easy to study in a quantitative manner, especially as between one experiment and another. The main reason for this is that it is impossible to reproduce from one experiment to another the concentrations of gramicidin in the system in a satisfactorily quantitative manner. Nevertheless, although no precise data can be given, it is quite clear that when a given amount of the peptide is added in a given way to a membrane, the frequency of opening of channels is higher the thinner the membrane. Thus, going from membranes formed from glyceryl monooleate in *n*-decane to those formed from *n*-hexadecane (*i.e.* from 47 Å to 31 Å) the increase in frequency is in the region of × 100. Also, again for a given amount of gramicidin added in a given way, it is found that the frequency of opening of channels is greater the higher the applied potential. Experimental difficulties again preclude the presentation of precise data but, for membranes of glyceryl monooleate *plus* decane, an increase of 100 mV produces an increase in frequency of opening of approx. 50–100%. The changes in applied potential which the membranes could stand without breaking did not cause any obvious change in the durations of the channels.

DISCUSSION

The opening and closing of the gramicidin channel

It is assumed that the opening of a channel must occur either by the diffusion into the membrane of an appropriate molecule or complex (all those present being necessarily open) or by a transition involving gramicidin which is already present in the membrane. The evidence strongly favours the latter hypothesis. Thus, diffusion to the membrane from the aqueous phase is certainly not a necessary process as the conducting properties are obtained readily by adding the gramicidin *via* the oil phase. (Passage of the gramicidin from the bulk oil phase into the water and thence back into the membrane may be ruled out from the results of experiments at the oil–water interface in a Langmuir (Brooks) trough[6,18]. These experiments showed that gramicidin persists without detectable loss for many hours as an "insoluble" monolayer and is therefore present to a negligible extent in the adjacent aqueous layers.) Diffusion along the bulk oil–water interface of the torus into the membrane may also be excluded as a necessary process for the following reasons. The Poisson distribution for the occurrence of channels implies that there are many gramicidin molecules available to form channels, although only a small proportion of them do so in any given interval. But it is known from contact angle determinations on black lipid films containing gramicidin (J. Requena, personal communication) that the peptide molecules are fairly evenly distributed between the torus–aqueous solution and black film–aqueous

solution interfaces. Of the large number of inactive gramicidin molecules in the system it is inferred therefore that a proportion, determined largely by the relative areas of the torus and membrane, must be in the latter. These molecules then become conducting by a transition, the nature of which will be discussed later.

The termination of a channel has been assumed in the Results to be unimolecular and has an activation energy of $\gtrsim$ 19 kcal·mole^{-1}. In addition, from the magnitude of τ and the lack of any observable variation of τ with the area of the membrane, it is concluded that the termination does not occur by diffusion along the interface from the Plateau border into the membrane. Thus, for a longitudinal diffusion coefficient $< 10^{-6}$ cm^2·s^{-1}, a value of τ of 400 ms corresponds to a displacement of < 10 μm, whereas the diameter of a membrane is typically 100 to 1000 μm. Furthermore, since the conducting complexes must be distributed fairly evenly over the membrane in order for the conductance to be proportional to membrane area (which it is under comparatively stable conditions), the channels can be terminated far from the edges. It cannot be ruled out, however, that the termination of a channel involves the desorption of a gramicidin molecule or complex from the membrane into the aqueous phase. Thus a solubility of $\lesssim 10^{-15}$ mole·l^{-1} in the aqueous phase would be consistent with an energy of desorption $\gtrsim$ 19 kcal·mole^{-1} and is undetectable by any other known means.

It has been shown that the gramicidin molecules are strongly localised in the membrane (or torus) interfaces and that at any one time a high proportion of them may be inactive. From subsequent considerations it seems likely that the formation of a conducting unit occurs through the association of two or more of these molecules. The equilibrium between the non-conducting and conducting units (perhaps the monomer and dimer, respectively) is very sensitive to the thickness of the membrane, as shown by the frequency and duration data, and is shifted in favour of the conducting species as the membrane thickness decreases. No evidence has been found in the present work for any sort of co-operation between channels, either in the opening or closing process or in the magnitudes of the conductances.

The conductance and selectivity of the channel

The current–voltage curves of Fig. 9 are curious in that curvature both towards and away from the current axis is found, the former at high and the latter at low electrolyte concentrations. The curvature towards the current axis suggests that the number of rate determining energy barriers to the passage of ions through a channel is less than ten. The opposite curvature is consistent with there being energy barriers, across which the electric field strength is small, at each end of the pore. These barriers would tend to become rate limiting at low electrolyte concentrations and high applied potentials. Although the diffusional convergence resistance would also manifest itself in this way, it has to be discounted on the grounds that quantitatively it is too small by about an order of magnitude. The linear, apparently ohmic, plots at intermediate electrolyte concentrations arise presumably from the cancellation of the two effects producing the opposing curvatures.

It will be shown in a later paper that the data of Fig. 9 which, of course, include that of Fig. 10, may be accounted for by a simple pore model.

The effects of the temperature variation on the single channel conductance, and the relative conductances for different electrolytes reveal that the passage through

the membrane channel bears some resemblance to diffusion in water. Thus, the activation energies for the passage of Na^+ and K^+ through the gramicidin channel (4.9 and 5.4 kcal·mole^{-1}, respectively) may be compared with the corresponding values, approx. 4.4 and 4.0 kcal·mole^{-1}, respectively, for diffusion in dilute aqueous solution. Also, the conductance ratios for the uni–univalent ions vary in a more pronounced but nevertheless similar manner to those for dilute aqueous solution.

It is readily calculated that, if a channel is not more than singly occupied, the diffusion coefficient of, say, K^+ in the channel is $\gtrsim 1 \cdot 10^{-6}$ cm^2·s^{-1}. This value may be compared with the value of $\sim 2 \cdot 10^{-5}$ cm^2·s^{-1} for K^+ at infinite dilution in the aqueous phase. If a carrier mechanism were to be responsible for the ion transfer, the diffusion coefficient of the carrier-ion complex would obviously have to be $\gtrsim 10^{-6}$ cm^2·s^{-1}. A molecule of the molecular weight of gramicidin would probably have a diffusion coefficient in water of $1 \cdot 10^{-6}$–$5 \cdot 10^{-6}$ cm^2·s^{-1}, so that even if the membrane interior were no more viscous than water and other types of limitation, *e.g.* image potentials, were absent, the carrier mechanism seems only just feasible.

The dependence of single channel parameters on membrane thickness and composition—the channel as a pore

Some of the evidence already discussed points to the conclusion that the gramicidin forms a pore through the membrane. There is, however, much stronger evidence that this conclusion is correct.

As shown in Table II, both the membrane thickness and composition may be varied substantially without producing a detectable change in either the single channel conductance or the conductance ratio for NaCl and KCl. It is difficult to suggest any explanation for these data other than that, in many systems, the gramicidin forms a pore of fixed structure which is independent of the membrane in which it is situated, It follows that if, for example, the membrane is thicker than the length of this pore. a local thinning or "dimpling" of the membrane must occur when the conducting channel is formed. It might be argued that a carrier mechanism, in which the rate-determining step was at the membrane surface, could also account for the lack of dependence of conductance and selectivity on thickness. It has been pointed out above, however, that diffusion rates alone make the carrier mechanism rather unlikely. For the surface reaction to be rate determining, it would have to be at least an order of magnitude slower than the diffusion across the membrane, and if this were so the observed ion fluxes would be extremely difficult to explain in terms of carriers.

The only single channel conductance which is out of line with the rest is that for the very thick ($\sim$ 64 Å) membranes. This result is of particular interest as the unusually small conductance which is observed is very close to half the normal value. This could reflect a change in the conformation of the gramicidin giving, perhaps, a smaller pore diameter (as yet no selectivity data are available to check this possibility), but it is not clear why a conformational change should be more likely in this rather than in other membranes. A more obvious explanation is that the pore in the thick membranes is twice the normal length. The effect of the applied potential on this system is explicable in terms of the dependence of membrane thickness on electrical fields[16]. The thick membranes are much more compressible than any of those examined previously and at 200 mV their thickness could have decreased to approx. 50 Å (J. L. Taylor, personal communication). At this thickness

the stability of the abnormally long pore is possibly so low that it rarely occurs.

The durations of the channels, in contrast to the conductances, show a steady increase with decreasing thickness of the membrane. It could be argued that this is a consequence of the changing hydrocarbon composition rather than the variation in thickness. On the other hand, it is not obvious how a change merely in the average chain length of the hydrocarbon would produce the effect. Furthermore, addition of cholesterol to the system (so as to produce membranes which, unlike those of pure glyceryl monooleate, interacted with nystatin) changed the composition without altering the thickness, and the duration remained unaffected.

Although no quantitative data could be given it has been stated that the frequency of opening of channels increased with decreasing thickness of the membrane. This observation suggests an explanation for the increase in frequency of opening of the channels in a given membrane with increasing applied potential. It has been demonstrated by Andrews, Manev and Haydon[16] that some of the membranes listed in Table II undergo a significant compression in applied electric fields, those of glyceryl monooleate and decane decreasing in thickness by approx. 3 Å in 100 mV applied. The frequency of opening of channels should therefore increase, if only for this reason. For membranes of glyceryl monooleate and hexadecane, the compressibility is much smaller and, as expected from this hypothesis, so also was the effect of the applied potential on the frequency of channel opening.

It has been argued by several authors[19,20], on the basis of the relationship between the membrane conductance and the gramicidin concentration, that two gramicidin molecules are required to form the conducting channel. While this conclusion may be correct the arguments leading to it are inadequate, for reasons given elsewhere[6].

Urry[21] has proposed from spectroscopic and structural considerations that the gramicidin channel consists of two molecules, each having a lipophilic, left-handed helical conformation, coupled at the formyl ends by means of hydrogen bonds. This $\pi^4_{(L,D)}$ helix has 4.4 residues per turn and a central channel diameter of approx. 1.4 Å, the length of the dimer being 35–39 Å. According to the discussion given above this model is rather too long. The model would also need to deform substantially in order to pass some ions, a requirement which is not obviously consistent with the low activation energies for ion transfer. In a more recent paper Urry *et al.*[22] have preferred the alternative but similar $\pi^6_{(L,D)}$ helical model. This structure has 6.3 residues per turn, a channel diameter of approx. 4 Å and a length for the dimer of 25–30 Å. Both the length and activation energy requirements are better met by this model.

The effects of membrane thickness, whether influenced by applied potential or otherwise, on the frequency and duration of single channels are very important for the understanding of the properties of the highly conducting membranes obtained in the presence of relatively large amounts of gramicidin. A brief discussion of this question has already been given elsewhere[6] and will be treated more fully in a later publication.

The structure of the conducting channel

There is little in the present data to suggest the number of gramicidin molecules required to form a channel, but the dimensions of the pore are indicated to some extent by the results for membranes of different thickness. With the possible exception

of the very thick, polymer-stabilised membranes, the pore is evidently of fixed length, regardless of the thickness of the membrane. Nevertheless, as has been pointed out, over the whole experimental range the stability of the pore increases monotonically with decreasing membrane thickness. As it would seem that the membrane must distort whenever its thickness and the length of the pore differ, and as distortion either inwards or outwards involves an increase in the free energy, it appears that the most stable state of the system should correspond to the membrane thickness and pore being equal. If the total, rather than the hydrocarbon thickness of the membrane is considered (*i.e.* the values of Table II are increased by the thickness, approx. 9 Å, of the polar groups) it follows that according to the above argument the pore length should be less than 35 Å. This distance is ample for a gramicidin molecule coiled in the form of a pore and, in fact, on this view alone, several molecules could be involved.

An attempt has been made to substantiate the dimer hypothesis by the study of *N,N'*-(dideformyl gramicidin A)malonamide, *i.e.* two gramicidin molecules linked chemically head to head[22]. It is claimed that the conductance versus concentration data for this substance in contact with black lipid membranes is of the first order, as might be expected if the dimer were the functional species. As already pointed out for gramicidin, however, the reproducibility of this type of data is very poor and, even if it were reproducible it would still need to be interpreted with considerable caution[6]. (It should also be noted that the single conductance reported by these authors for both the unmodified gramicidin and for the malonyl derivative are apparently much larger than those reported here. It is not clearly stated what electrolyte concentration was used but if this was 100 mM, the conductance given by Urry *et al.*[22] for the unmodified gramicidin is some 15 times the corresponding value in the present work; if it was 1 mM, as seems more likely, the discrepancy increases to about 10^3 times.)

If the conducting channel is formed and terminated by the combination and break-up of two monomers of the type proposed by Urry, it would certainly be reasonable that the activation energy for the termination would, as observed, be $\gtrsim$ 19 kcal·mole^{-1}. As yet, however, there is no direct evidence that even if the conducting unit is a dimer, its formation or disruption is ever rate determining.

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