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Ion channel formation by duramycin

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The formation of ion channels by the nonadecapeptide antibiotic duramycin was examined using black lipid membranes and using the patch-clamp technique. In black lipid membranes made from glyceryl monooleate or a phosphatidylcholine/phosphatidylethanolamine mixture, duramycin induced complex fluctuations in membrane conductance, some step-like and some which were incapable of being resolved into discrete conductance states. Both conductance and largest step size increased with time. A similar time-dependent increase in conductance was seen in patch-clamp experiments with HCA-7 Colony 29 human colonic epithelial cells. The channels displayed weak anion selectivity and the smaller channels formed in patches from epithelial cells showed weak inward-rectification. Channel formation by duramycin was achieved at lower concentrations when the black lipid membrane was made with phospholipid rather than with glyceryl monooleate. Lower concentrations were effective in generating conductances in epithelial cells than in bilayers. It is concluded that duramycin forms ion channels in both artificial and biological membranes. Accumulation of duramycin and coalescence of initially small channels into larger ones is considered to be responsible for the recorded behaviour and to final disruption of membranes.

Introduction

Duramycin is a nonadecapeptide antibiotic obtained from *Streptoverticillium cinnamomicus* [1] which has been shown to affect a number of membrane transport properties in various systems [2–7]. Recently it has been reported that duramycin increases chloride secretion and activates Cl^- channel activity in airway epithelium [8–11] and that duramycin increases ion transport (as determined by short-circuit current, I_{sc}, measurements) and stimulates a rise in intracellular Ca^{2+} when applied to the apical membrane of colonic epithelial cells [12]. In the same study it was shown that addition of duramycin to black lipid bilayer membranes caused complex current fluctuations. In some reports [8–12] the potential therapeutic value of duramycin in cystic fibrosis, where there is a disorder of epithelial chloride transport, was discussed. In this study we have employed techniques to study the interaction of duramycin with both artificial and biological membranes to discover the properties of the ion channels formed.

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Materials and Methods

1. Bilayer experiments

Experimental apparatus and formation of black lipid bilayer membranes. A schematic diagram of the apparatus, a modification of earlier designs (Tripathi, S. and Hladky, S.B., unpublished data) is shown in Fig. 1. The upper (open) compartment comprised a 2 ml cup machined from a solid cylinder of Teflon (polytetrafluoroethylene) with a small hole through the bottom [13]. The lower (closed) compartment consisted of Teflon tubes connecting the Teflon cup, a hollow Ag/AgCl cylinder and a Hamilton syringe with a Teflon-tipped plunger. A Ag/AgCl wire formed one electrode and the Ag/AgCl cylinder formed the other [14].

Black lipid films were formed across the end of the Teflon tube (i.d. 0.8 mm, o.d. 1.5 mm) using standard techniques [15,16] from either 3.6 mg/ml glyceryl monooleate (GMO) in *n*-hexadecane or a mixture of 5 mg/ml L-α-dioleoylphosphatidylethanolamine (PE) and 5 mg/l L-α-dioleoylphosphatidylcholine (PC) in *n*-decane. The size and position of the membranes were adjusted by altering the volume of the lower compartment using a micrometer syringe.

Duramycin was added to the electrolyte solution in the upper compartment in concentrations of 2–50 μM,

either before or after formation of the film. The duramycin was thus present on either one or both sides of the membrane. All apparatus which came into contact with aqueous or lipid solutions was cleaned in a sulphuric acid-dichromate solution. Experiments were performed at 23 ± 1°C.

A virtual earth voltage clamp circuit and a potentiometer were used to apply a constant potential difference across the membrane and to measure the current through the membrane [17]. Concentration gradients across the membrane were established by lowering the membrane a small distance into the Teflon tube, replacing the top solution, and raising the membrane back to the end of the tube.

Chemicals. Duramycin (F.W. 2012) was generously supplied by Dr. O.L. Shotwell (U.S. Department of Agriculture, Peoria, IL, USA) and was dissolved in distilled water. The *n*-alkanes were of puriss grade and were obtained from Koch-Light Ltd. They had been passed through an alumina column to remove polar impurities. The GMO was obtained from Sigma Chemical Co. and the phospholipids from Avanti Polar Lipids Inc., Pelham, AL, USA. The inorganic salts were of analytical reagent grade and where appropriate had been roasted at 600°C to remove organic impurities. Aqueous solutions were prepared using distilled water from a commercial still in which all tubing and a seal containing plasticizers had been replaced with Teflon and rubber, respectively.

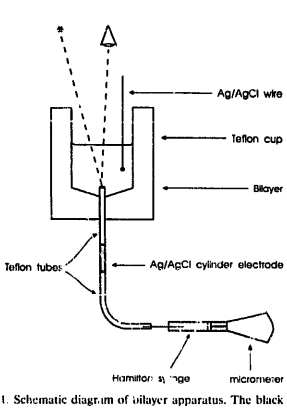


Fig. 1. Schematic diagram of bilayer apparatus. The black lipid film is shown lowered slightly down the Teflon tube that connects the Teflon cell and the Ag/AgCl cylinder electrode. * represents a light source.

TABLE I

Composition of solutions used in patch-clamp experiments

All solutions contained 10 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) titrated to pH 7.4 with NaOH together with 1 mM EGTA (1,2-(di(2-aminoethoxy)ethane-*N,N,N',N'*-tetra-acetic acid).

	NaCl (mM)	KCl (mM)	Sucrose (mM)	Ca^{2+} [a] (M)	Duramycin (M)
Solution P	–	145	–	10^{-6}	–
Solution D	–	145	–	10^{-6}	$4 \cdot 10^{-7}$
Solution B	140	5	–	10^{-3}	–
Solution R	–	20	250	10^{-3}	–

[a] $CaCl_2$ was added to give the free Ca^{2+} activities above.

2. Membrane patch recordings

Cell culture. HCA-7 Colony 29 cells, derived from a human colonic adenocarcinoma [18] were prepared and grown to confluence on Petri dishes as described in detail previously [19,20].

Patch-clamp experiments. Patch-clamp experiments were conducted using the inside-out conformation of current recording [21] as described previously [22]. The composition of solutions used is given in Table I. Free Ca^{2+} was calculated using the equations given in Ref. 23. Solutions were filtered immediately before use with 0.22 μm Acrodisc filters (Gelman Sciences, Ann Arbor, MI, USA). In contrast to Ref. 22 recordings were made at a bath temperature of 33–37°C. The temperature was maintained using a Narishige (USA) MS-C temperature controller and micro-incubator system. When making up the solutions the pH was adjusted to take account of the shift of pH displayed by Hepes buffer when the temperature is raised from 21 to 35°C.

Recordings were made and data analysed as described previously [22].

Results

1. Bilayer experiments

Duramycin, on one or both sides of the membrane, produced fluctuations in membrane conductance after a contact time of between 30 s and 30 min. Some fluctuations were simple and step-like whilst others were complex and could not be resolved into step-like changes. The membrane conductances and the sizes of the resolved steps tended to increase with time. Steps smaller than 100 pS as shown in B were difficult to resolve, partly as a result of the limited recording bandwidth (approx. 40 Hz) at the required sensitivities, and partly because conductances usually increased rapidly beyond this stage. Discrete steps, from 50 pS to 20 nS were observed over 2.5 h in an experiment in which duramycin was present at approx. 10 μM in 1.0 M NaCl on both sides of the phospholipid membrane.

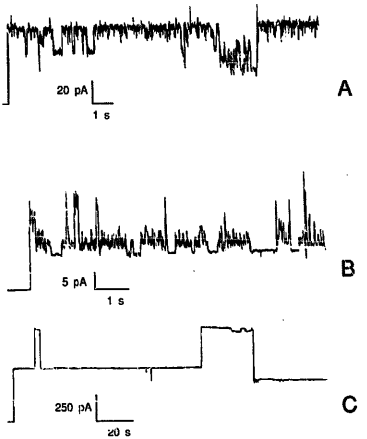


Fig. 2. Fluctuations in membrane current of GMO and phospholipid membranes in the presence of duramycin. In A and B duramycin was present at concentrations of approx. 25 μM in 1.0 M NaCl on both sides of a GMO membrane. The applied potential was −50 mV. In C duramycin was present at concentrations of approximately 10 μM in 1.0 M NaCl on both sides of a phospholipid membrane. The applied potentials was −50 mV. The time-scale lines are placed at the level of the membrane currents in the absence of duramycin, i.e. at the 'closed' level.

Duramycin induced current fluctuations in 'black' films only, and not in thick or 'coloured' films. Duramycin therefore appears to form channels only in bilayers.

Fig. 2 illustrates sample recordings. Ten experiments were performed using GMO membranes and twelve using phospholipid membranes. In Figs. 2A and 2B both simple and complex fluctuations are observed. The lifetime of the channels is extremely variable and transient to very long openings are observed. Fig. 2B

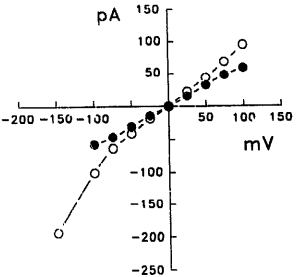


Fig. 3. Current–voltage relationships for duramycin channels. Duramycin was present at concentrations of approx. 4.5 μM (open circles) and 10 μM (filled circles) in 0.1 M NaCl on both sides of a phospholipid membrane.

features a channel of approx. 200 pS conductance that was open for many seconds. In Fig. 2C the membrane appears to contain two very large channels (approx. 8 and 10 nS) which have very long openings, together with several smaller channels.

In both GMO and phospholipid membranes the channel activity elicited by duramycin occurred on a similar time scale and with a similar range of step sizes. The lowest concentrations of duramycin which produced membrane conductance changes were approximately 10 μM for GMO membranes and 2 μM for phospholipid membranes. The highest specific membrane conductances observed under identical conditions of approx. 10 μM duramycin in 1.0 M NaCl were approx. $4 \cdot 10^{-6}$ S/cm^2 for GMO membranes and approx. $8 \cdot 10^{-5}$ S/cm^2 for phospholipid membranes.

Fig. 3 illustrates the results of two experiments in which the membrane conductance remained relatively constant for a sufficient time to allow the determination of a current-voltage relationship. In both curves the conductance is relatively independent of voltage at

TABLE II

Membrane equilibrium potentials (E_m) and permeability ratios (P_{Cl}/P_{Na}) for duramycin channels in artificial bilayers

$E_m = V_{top} - V_{bottom}$. Membrane equilibrium potentials were generally reproducible to within 1 mV. P_{Cl}/P_{Na} was calculated from membrane equilibrium potentials using the Goldman-Hodgkin-Katz equation. The quantitative analysis of equilibrium potentials in systems involving electrolytes other than the 1:1 electrolytes (e.g. $MgCl_2$ and $CaCl_2$) is extremely complex and was not attempted. Specific membrane conductances in these experiments ranged from 10^{-7} to 10^{-5} S/cm^2.

Electrolyte solutions (top solution: bottom solution)	Membrane	E_m (mV)	n	P_{Cl}/P_{Na}
1.0 M NaCl:0.1 M NaCl	GMO + *n*-hexadecane	+7.6	2	1.8
	PE + PC + *n*-decane	+10.4	6	1.7
0.05 M $MgCl_2$:0.1 M NaCl	PE + PC + *n*-decane	−8.2	5	–
0.05 M $CaCl_2$:0.1 M NaCl	PE + PC + *n*-decane	−7.1	2	–

low applied potentials. Under these conditions duramycin channels do not appear to possess substantial rectification properties.

The results of experiments on ion selectivity are shown in Table II. The specific membrane conductances in these experiments were relatively low in order to minimise the influence of the boundary layers in the adjacent aqueous phases on the measured potentials. Exchanges of the top solution were sometimes accompanied by significant decreases in membrane conductance, necessitating the addition of further duramycin.

2. Membrane patch recordings

Duramycin applied to the apical membranes of cultured epithelial cells in a monolayer increases the I_{sc} [12]. To examine this effect at the single-channel level, patch clamp recordings were made from HCA-7 Colony 29 cells at physiological temperatures. Duramycin was present in the pipette solution (Solution D) at 0.4 μM. The strategy for recording was to make a seal on the cell. If no native channels were evident * the patch was excised, briefly air-exposed [21] and held at $\approx +40$ mV. If any channels appeared immediately after replacing the pipette in the bath the patch was discarded. In the absence of duramycin, channels either appear immediately or are absent over recording periods of over 20 min. Typically, channel activity began to appear 4–5 min after seal formation. Single-channel current/voltage relationships, together with a representative recording are shown in Fig. 4. The reversal potential (E_{rev}) for conditions where there were substantial gradients of Na^+ and K^+ but no anion gradient across the patch, $[Na^+]_{pipette} + [K^+]_{pipette} = [Na^+]_{bath} + [K^+]_{bath}$, is approximately 0 mV. As shown in Fig. 4A (filled circles), if the bath solution's ion activity was substantially reduced by isoosmotic replacement of KCl with sucrose, the E_{rev} shifted in the negative direction, implying inward Cl^- current. The E_{rev} for this condition in Fig. 4 is -19 mV whilst the theoretical Nernst potential for the prevailing ionic gradient is -78 mV (for a channel selective for anions). This result suggests that the channel shows relatively poor selectivity for anions ($P_{Cl}/P_K \approx 2.65$) under these conditions.

Four channels which were stable long enough for a comparison of conductances at two potentials to be made showed inward conductances at -40 mV of 45, 61, 33 and 36 pS (mean $\pm$ S.D. = 43.75 $\pm$ 12.5 pS) and outward conductances at $+40$ mV of 27, 45, 20 and 32 pS, respectively (mean $\pm$ S.D. = 31 $\pm$ 10.53 pS). Usually, once channel activity had appeared in an excised patch, channels rapidly proliferated leading to excessively noisy current recordings and ultimately to breakage of the patch. The very large steps recorded in the bilayers were not seen. The conductances increased to levels above the large step size and the patches ruptured in less time than was taken for the large steps to appear in the black lipid membrane experiments. A recording demonstrating the effect of channel proliferation is shown in Fig. 5. The three single point amplitude histograms in Fig. 5(A) represent three consecutive 5.12 s recordings. The three lowest conductance states are evident in the first period; up to five open channels are evident in the second period, and up to six in the third. The number of events at each conductance level increases dramatically during 15 s. Fig. 5(B) shows part of the current recordings from which the data in the first and third curves of Fig. 5(A) were derived. Initially two open states were discernible, whilst later, six states can be identified. It was not feasible to determine a single-channel open probability or the number of conductance states per channel as it

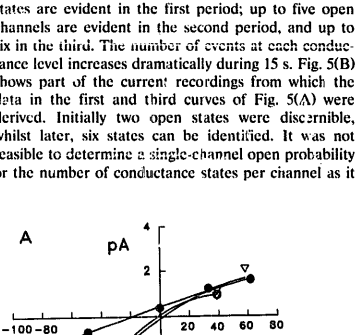
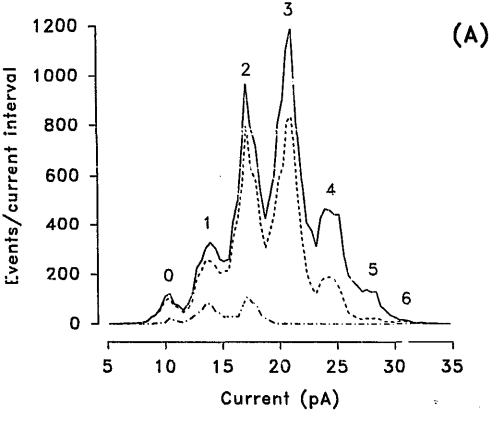


Fig. 4. (A) Current–voltage relationship for single channel appearing in inside-out patch of HCA-7 Colony 29 cell. Open circles: Solution D in pipette and Solution B in bath. Triangle: Solution D in pipette, Solution P in bath. Filled circles: Solution D in pipette, Solution R in bath. (B) Single-channel recording made at a V_h of -53 mV of spontaneously by appearing channel activity. Solution D was in the pipette and Solution B in the bath. The bath temperature was 33°C and the closed level is shown by the arrow.

* Colony 29 cells contain a number of native channel types, the commonest of which are a 20 pS non-selective cation channel with linear conductance which is active in cell attached and inside-out mode, an inward-rectifying potassium channel which runs down on excision and an outward-rectifying chloride channel which is activated by depolarisation or intracellular cAMP [24].

was never possible to be certain how many channels were active in the patch at any one time.

Some caution needs to be exercised in the interpretation of the patch-clamp data inasmuch as it is not possible to be absolute in asserting that the channels seen in this study are the result of duramycin interacting with the membrane. It is possible that the channels seen represent channels native to the Colony 29 cell membrane, and that they are activated by duramycin. However, this explanation would require at least one



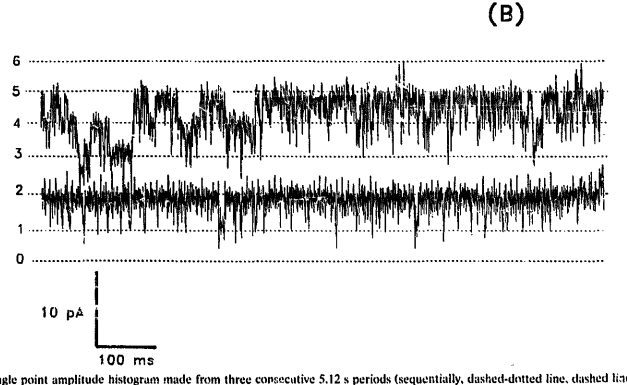


Fig. 5. (A) Single point amplitude histogram made from three consecutive 5.12 s periods (sequentially, dashed-dotted line, dashed line and solid line) from a patch held at −49 mV. Solution P in bath, Solution D in pipette, temperature 36°C. Data points were sampled at 250 μs intervals. (B) Samples from current recordings from which the first (lower trace) and third (upper trace) curves in (A) were made The dotted lines show the open states. The majority of the earlier recording shows up to two open states, while in the later recording up to six open states can be discerned.

thousand 50 pS channels in the approx. 0.4 μM diameter patch to account for the observed currents.

Discussion

In this study duramycin was examined under two somewhat different conditions. In lipid membranes duramycin induced conductance fluctuations only in bilayer membranes and not in thick films. This together with the extremely large channel conductances observed strongly suggests that duramycin channels consist of pores rather that carriers.

The observation of a range of channel conductances in bilayers (50 pS to 20 nS) suggests that channel formation is extremely complex and may be the result of aggregation of many molecules in the plane of the membrane. In Ref. 12 the change in I_{sc} produced by duramycin was highly dependent upon the concentration of duramycin applied and at levels between 2 and 5 μM currents became unstable.

The proposed structure of the duramycin molecule suggests that it possesses two free charges, a positive charge at Lys-2 and a negative charge at Glu-3. This raises the possibility that duramycin molecules may insert into membranes in a preferred orientation in a voltage-dependent manner and that this may lead to duramycin channels possessing rectification properties. There was no evidence for rectification in the bilayer experiments but weak rectification was evident in the cell membranes.

Although duramycin-induced fluctuations in membrane conductance were very similar, the concentrations of duramycin required for channel formation were approx. 5-times higher in GMO than in phospholipid membranes (which in turn required higher concentrations than in the membrane patches), and the largest conductances observed under identical conditions in phospholipid membranes were approx. 20-times higher than those in GMO membranes. This is consistent with the notion that duramycin interacts specifically with PE [3,5,6]. It should also be noted that this specificity does not appear to be absolute. The extremely large channel conductances observed in black lipid bilayers suggest that the selectivity of duramycin channels under these conditions will be the same as for aqueous diffusion. The values of P_{Cl}/P_{Na} obtained (1.5 for GMO membranes and 1.7 for phospholipid membranes) are in good agreement to the ratio of the conductivities for aqueous solutions ($\lambda^{\circ}_{Cl}/\lambda^{\circ}_{Na} = 1.52$ at 25°C). Qualitatively, the low values of the membrane equilibrium potentials obtained with Mg^{2+} and Ca^{2+} (−8.2 and −7.1 mV, respectively) also suggest that duramycin channels are permeable to both monovalent and divalent cations.

Transepithelial ion transport in Colony 29 monolayers [12] stimulated by duramycin was either sodium absorption, with symmetrical bathing solutions or chloride secretion when a suitable gradient was imposed. Again, this suggests that there was little selectivity for anions over cations when duramycin was applied to the apical surface. Since epithelial cells grow in a polarized fashion, apical membrane uppermost and thus accessible to the patch pipette, it is likely that the duramycin-induced channels in the patch-clamp experiments correspond to those responsible for the increase in I_{sc}. It is worth noting that the patch-clamp experiments were performed at physiological temperatures, thus reflecting the situation in Ref. 12 whilst those in the planar lipid bilayers were performed at room temperature. The closely related nonadecapeptide antibiotic Ro09-0198 [25] has been shown to have similar effects to duramycin upon I_{sc} in HCA-7 Colony 29 cells [12] and is thought to form aqueous pores with a radius similar to the hydrodynamic radius of sucrose in liposomal membranes through a specific interaction with PE, resulting in 'clustering' of PE molecules in the membrane [26]. It is likely that duramycin forms analogous pores.

Acknowledgements

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