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The properties of ion channels formed by the coumarin antibiotic, novobiocin, in lipid bilayers

Alexander M. Feigin [a,*], Evgeney V. Aronov [a], John H. Teeter [a,b], Joseph G. Brand [a,b,c]

[a] *Monell Chemical Senses Center, 3500 Market St., Philadelphia, PA 19104-3308, USA*
[b] *University of Pennsylvania, Philadelphia, PA 19104, USA*
[c] *Veterans Affairs Medical Center, Philadelphia, PA 19104, USA*

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Abstract

The coumarin antibiotic novobiocin forms ion channels of varying conductances in lipid bilayers. The conductances (about 20, 22, 14, 7 and 2 pS for 100 mM NH_4Cl, CsCl, KCl, NaCl and LiCl, respectively) and selectivities (cation transference numbers in the range of 0.97–0.98) of one type of novobiocin-induced channel are similar to those found for channels formed by gramicidin A, an antibiotic of very different structure. The conductance of novobiocin channels of this type was independent of the species of the membrane lipid. This observation suggests that novobiocin molecules directly form these channels, and that channels are not formed through defects in lipid structure. The similarity in conductance and ion selectivity between channels induced by novobiocin and those formed by gramicidin A suggests that these structurally different molecules form channels with comparable internal diameter and internal surface charge distribution. Using HPLC purification we argue that the channel-forming activity of novobiocin is related to the activity of the novobiocin molecule itself, and not to a contaminant of the commercially available novobiocin sodium salt preparation.

Keywords: Novobiocin; Ion channel; HPLC purification

1. Introduction

The coumarin antibiotic novobiocin (Fig. 1), known as an inhibitor of topoisomerase [1], also exhibits membrane activity: addition of novobiocin to the mucosal side of frog skin produces a large increase in the short-circuit current [2], transepithelial potential and conductance [3,4]. In preliminary reports [5,6] we showed that commercially available novobiocin itself increased the conductivity of lipid bilayers by forming cation-selective ion channels. This observation was made as a result of our attempts to reconstitute amiloride-blockable sodium channels from rat dorsal lingual epithelium into lipid bilayers to study the mechanism of enhancement of their activity by novobiocin. We found that one type of novobiocin-induced channel displayed relatively low conductance (about 7 pS in 100 mM NaCl), had long dwell times (up to minutes) and expressed significant cation selectivity. Recently, the channel-forming ability of commercially produced novobiocin was independently confirmed by O'Brodovich et al. [7,8].

In this communication we report the basic properties of novobiocin channels and draw tentative conclusions about the mechanism of their formation in lipid bilayers. We present data demonstrating that the conductance and selectivity of one type of novobiocin-induced channel are similar to those found for channels formed by gramicidin A. Similarity in selectivity and conductance is rather surprising given the difference in the molecular structures of these two antibiotics. Unlike gramicidin A, however, novobiocin also forms other types of channels of varying conductance levels.

By using HPLC purified novobiocin, we argue that the channel-forming activity of novobiocin is related to the activity of the novobiocin molecule itself, and not to any contaminant of the commercially available novobiocin sodium salt preparation.

* Corresponding author. Fax: +1 (215) 8982084.

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2. Materials and methods

Synthetic lipids (dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylserine (DOPS), dioleoylphosphatidylethanolamine (DOPE), and diphytanoylphosphatidylcholine (DPhPC) were obtained from Avanti Polar Lipids (Pelham, AL). All electrolytes were reagent grade. All water was doubly distilled and deionized. Salt solutions for bilayer experiments were buffered by Mops (5 mM for 100 mM electrolytes) to pH 6.9. Novobiocin sodium salt was purchased from Sigma (St. Louis, MO) and purified using high-performance liquid chromatography (HPLC) for some studies (see below). All of the solvents (Aldrich, Milwaukee, WI) were HPLC grade and were used without additional purification. Chlorobutane half-saturated with water was prepared according to Tsuji et al. [9].

The chromatographic procedure for separation of novobiocin, its isomers and related compounds has been described [9]. The chromatograph for the purification included a solvent delivery system (model HPXL) and a UV diode array detector (model Dynamax UV-M), both from Rainin Instrument, Woburn, MA. Chromatographic conditions were: column, Zorbax Sil, 4.6 × 250 mm (MAC-MOD Analytical, Chadds Ford, PA); mobile phase, chlorobutane (half-saturated with water): tetrahydrofuran/methanol/acetic acid (88:5:4:3); flow rate, 1.5 ml/min; detection at 390 nm. The separation was monitored using a recording integrator (model 3390A, Hewlett Packard, Avondale, PA). Commercial novobiocin sodium salt (100 mg) was dissolved in 10 ml of the mobile phase under ultrasonication. An aliquot (100 μl) of the resulting solution was injected into the HPLC system and fractions containing novobiocin were collected manually. The eluate was then evaporated and dried in vacuo (oil pump, 0.04 mmHg) for 30 min. The residue was weighed and dissolved in the mobile phase to give a 10 mg/ml solution for the second step of purification. The second step of the purification process involved a repeat of the first step. The eluate was collected, evaporated and dried. The residue was weighed and dissolved in water to give a 1 mg/ml solution. An equivalent amount of 1 M NaOH was added to reconstitute the sodium salt, since in the acid form the drug is not soluble. Care was taken to insure that the pH did not exceed 7.6, since novobiocin is known to isomerize at alkaline pH. The solution obtained was used as a double purified novobiocin in experiments described below.

'Solvent-free' membranes were prepared as described by Montal and Mueller [10]. Two symmetrical halves of a Teflon chamber with solution volumes of 1 cm^3 were divided by a 15 μm Teflon partition containing a round aperture of about 100 μm diameter. Hexadecane in n-pentane (1:10, v/v) was used for aperture pretreatment. 'Virtual ground' was maintained at the *trans* side of the bilayer. A detailed description of methods used for membrane preparation and single channel data analysis may be found elsewhere [11,12]. Novobiocin was added to the aqueous phase from stock solution (10 mM) in water. All experiments were performed at room temperature.

3. Results and discussion

3.1. A variety of channels formed by novobiocin

The singe-channel activities induced by novobiocin, in the form of fluctuations of the novobiocin-induced bilayer current, are presented in Fig. 2. These current fluctuations alternated between discrete levels, consistent with the opening and closing of novobiocin channels. Even taking into account only long-lived channels (open state from several to tens of seconds), a large variability in the amplitude of the current fluctuations was observed, corresponding to single channel conductances of from 3 to 200 pS (for 100 mM KCl or 100 mM NaCl in the bath solutions). An analogous variety of channel conductances was observed for the magainins, peptide antibiotics of animal origin [13,14].

Channels with different levels of conductivity were readily observed, both simultaneously and separately over a novobiocin concentration range of 0.1–0.5 mM. There was no apparent relationship among the level of channel conductance and parameters such as antibiotic concentration, temperature, type of membrane lipids, sign or amplitude of transmembrane voltage, or purity of novobiocin (see below). Also it was not possible to relate the occurrence of a particular type of novobiocin channel to the time after antibiotic addition (such as the occurrence of small channels formed by magainin 2 during the initial incorporation of this antibiotic in the bilayer, followed by

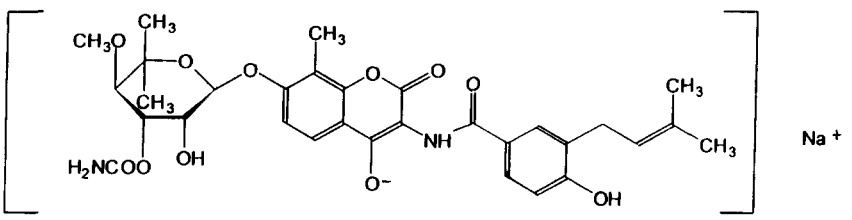

Fig. 1. Structure of novobiocin (from Hinman et al. [37]), a dibasic acid with pK_a values of 4.3 and 9.1 in aqueous solutions and a molecular mass of 635 Da.

formation of much larger channels [14]). The novobiocin channels with smaller amplitudes did not appear to be sub-conducting states of the channels with larger current amplitudes, because the larger channels opened and closed as discrete units.

One type of novobiocin-induced channel was of special interest for several reasons. First, this type of channel (6.8 pS in 100 mM NaCl or 13.5 pS in 100 mM KCl with long open time – tens of seconds) was a predominant one (Figs. 2F and 5). In some experiments these predominant channels were not observed even though channels with other amplitudes and kinetics were found (see examples in Fig. 2A–E). In other experiments we observed the conversion of the channels of this type into channels with different amplitudes and kinetic (see example in Fig. 2F). Yet in many experiments ($n = 72$), these were the only type of channel found. Consequently the electrochemical characteristics of these single channels, as well as their macroscopic conductance parameters, were studied. A preliminary description of these channels appeared in our previous publications [5,6,12]. The same type of novobiocin induced channels was described by O'Brodovich et al. [7,8]. All results described below were obtained in experiments in which only this type of channels was observed.

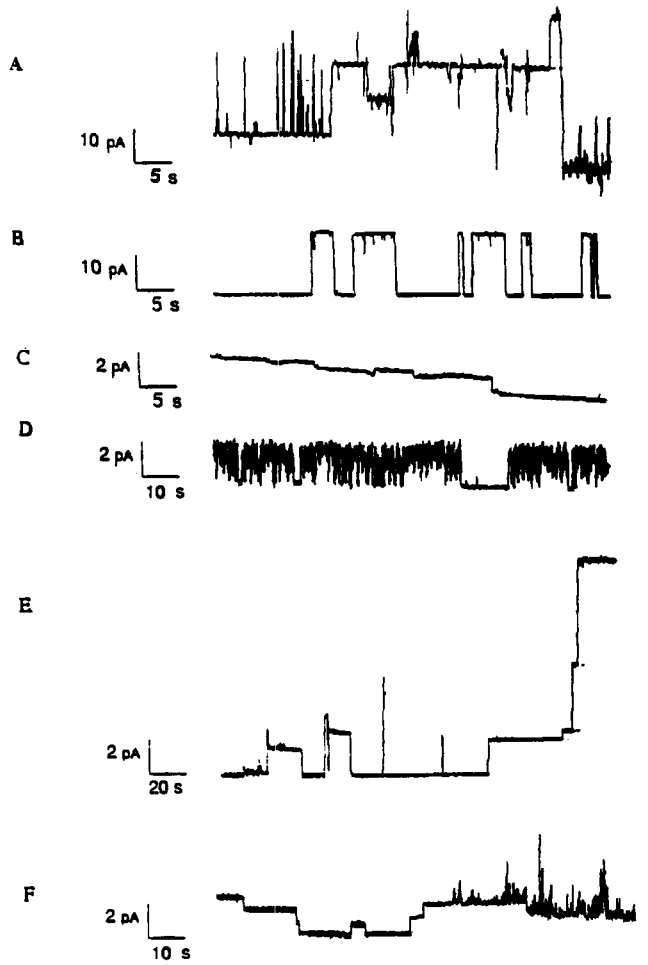

Fig. 2. Single channel activity in lipid bilayers formed from DPhPC induced by addition of 0.2 mM novobiocin to the *cis* side of the membrane. Bath solution: 100 mM KCl (A–D) or NaCl (E,F), 5 mM Mops, pH 6.9. The bilayers were clamped at +100 mV. (A) Record showing discrete current transitions with different amplitudes induced by addition of an HPLC purified-sample of novobiocin. (B) Record showing a 170 pS channel induced by addition of a commercial preparation of novobiocin. (C) Record showing a 3 pS channel induced by HPLC-purified novobiocin. (D) Record showing a 22 pS channel induced by commercial preparation of novobiocin. (E) Record showing channels with different amplitudes induced by a commercial preparation of novobiocin. (F) Record showing the conversion of the dominant 'gramicidin A-like' channels into channels with different amplitudes. These channels were formed by the addition of the HPLC-purified novobiocin.

3.2. Conductance, selectivity and current-voltage characteristics of novobiocin channels

A histogram of novobiocin single-channel conductances together with a 10 min sample of the current record (inset) are shown in Fig. 3. The membrane bathing solution was 0.1 M KCl. Novobiocin was added only to the *cis* side of the bilayer at a concentration of 0.1 mM. Measurements were performed at a transmembrane potential difference of 100 mV, *cis* side positive. At potentials up to +100 mV the current-voltage character of the channel is linear (Fig. 4), so that the values in the histogram represent small-signal conductance of novobiocin channels around zero voltage. The histogram shows a well-defined peak close to 14 pS with a moderately lower conductance tail. Histograms of this shape are typical for channel-forming agents [15–17].

The voltage dependence of the conductance of novobiocin single channels for 100 mM LiCl, NaCl, KCl, CsCl and NH_4Cl was determined. The single-channel current-voltage characteristics plotted in Fig. 4 were obtained by averaging over 20–30 single channel steps at a particular voltage for the indicated salt. Novobiocin was added asymmetrically, to the *cis* solution only. A plus sign corresponds to the *cis* side being positive. Linearity is observed across the range ±100 mV, and the functions saturate at higher voltages. Similar current-voltage characteristics were reported for channels formed by gramicidin A [18].

Conformational equilibrium of an ion channel may strongly depend on the strength and direction of the transmembrane electric field. Voltage-dependent anionic channels from the outer membrane of mitochondria (VDAC), reconstituted in lipid bilayers, and alamethicin channels are well-characterized examples. At the multichannel level, the steady-state VDAC-induced conductance was decreased nearly by half upon increasing transmembrane voltage from 10 to 50 mV with a relaxation time of about 10 s [19]. Much higher voltage sensitivity was displayed by alamethicin, which responded to every 10 mV increment with an approx. 10-fold change in its multichannel conductance [20].

We studied voltage sensitivity of the novobiocin-induced conductivity using multichannel bilayers with asym-

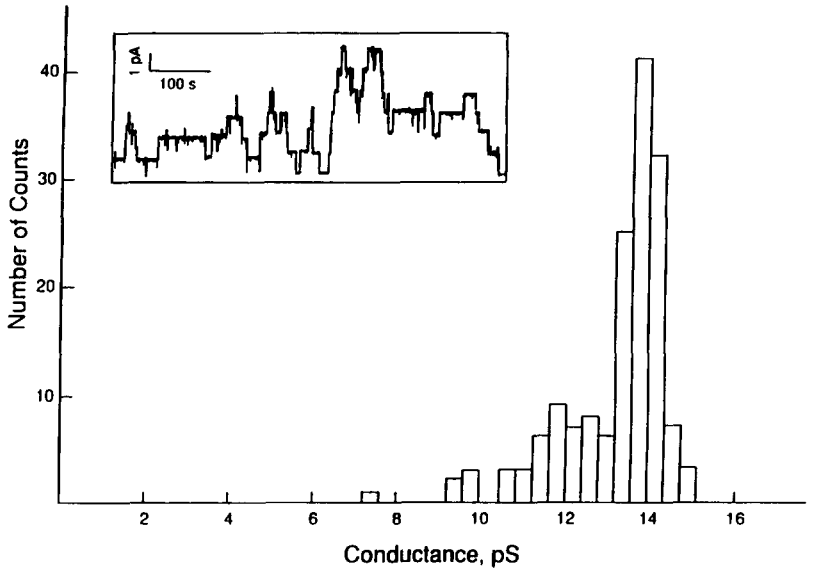

Fig. 3. Histogram presenting the distribution of single channel conductances. Channels were observed in a DPhPC bilayer in 100 mM KCl bathing solution. HPLC-purified novobiocin was added only to *cis* side of the bilayer at concentration of 0.1 mM. Transmembrane voltage was 100 mV, *cis* side positive. Inset shows a 10 min recording of single-channel activity; vertical bar, 1 pA; horizontal bar, 100 s.

metric, *cis* side application of antibiotic. Data of Fig. 5 show that subjecting the bilayer to positive and negative voltages did not produce measurable effects on its conductivity. On the time scale of several minutes currents in different segments of the recording were approximately proportional to the applied voltages. This result is consistent with those from the single-channel current-voltage dependencies shown in Fig. 4. The current fluctuated around their average values within expected limits.

A similarity between novobiocin and gramicidin A channels was found when we analyzed the conductance of novobiocin channels in electrolytes with different monovalent cations: Cs^+, K^+, Na^+, Li^+, and NH_4^+. The conductance values are presented in Table 1 together with data reported by Andersen [18] on the gramicidin A channel reconstituted in the same lipid (DPhPC). Note that novobiocin not only reproduces the gramicidin A channel selectivity sequence: NH_4^+, $Cs^+ > K^+ > Na^+ \gg Li^+$, but also displays very similar conductances in these salt solutions.

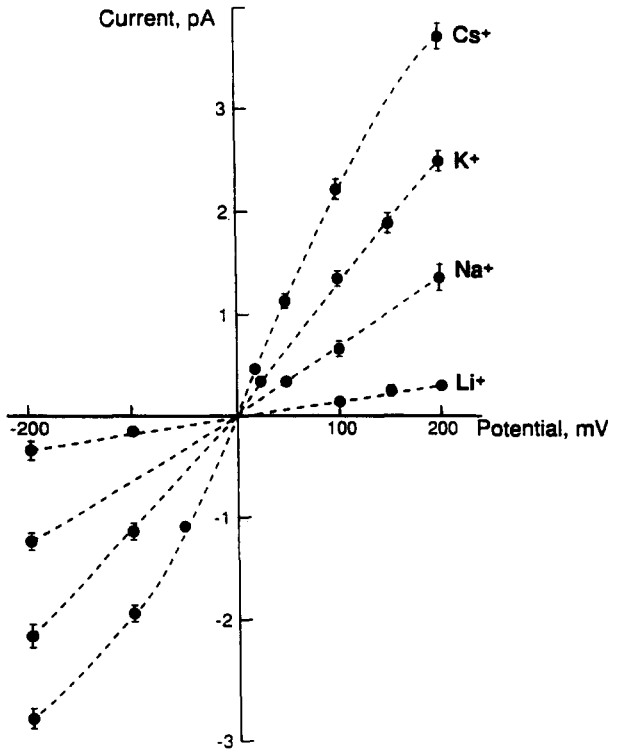

Fig. 4. Single-channel current-voltage characters for different 100 mM salt solutions. HPLC-purified novobiocin in concentrations 0.1–0.2 mM was added only to *cis* side of a DPhPC bilayer. Plus sign in voltage corresponds to *cis* side being positive. Each point represents an average over 20–30 single-channel current 'steps'.

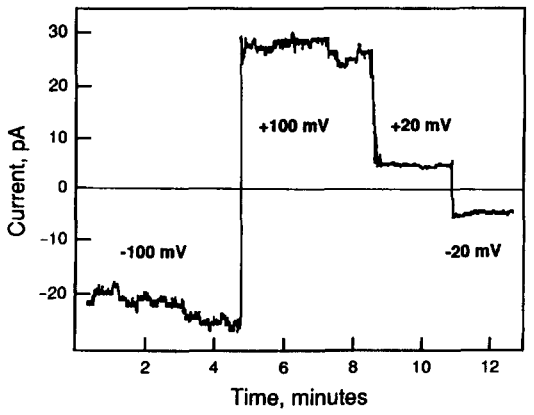

Fig. 5. Multichannel novobiocin-induced conductance at different voltages. DPhPC bilayer in 100 mM KCl bathing solution was exposed to one-sided novobiocin action (3 mM) during 20 min before the recording was started. Voltage plus sign corresponds to *cis* side being positive. Signal was filtered at 100 Hz by a low-pass Bessel filter.

Table 1
Conductance of novobiocin channels in different electrolytes compared with data from gramicidin A channels [18]

	Mean conductance ± S.D. (pS)	
	novobicin	gramicidin A
LiCl	1.7 ± 0.2	1.4
NaCl	6.8 ± 0.5	5.2
KCl	13.5 ± 0.8	12.7
CsCl	22.2 ± 1.2	18.0
NH_4Cl	20.1 ± 2.3	17.8

Novobiocin was added to the *cis* side of a DPhPC bilayer at a concentration of 0.1 mM. Bathing solutions contained 100 mM electrolyte, 5 mM Mops, pH 7.0. Transmembrane voltage was 100 mV, *cis* side positive. The mean conductances were obtained by averaging over 20–30 single channel steps for the indicated salt.

We were not able to resolve single ion channels in 1 M $CaCl_2$ solution. Experiments with multichannel membranes showed novobiocin-induced conductivities of at least two orders of magnitude lower compared with those for KCl or NaCl at the same salt and novobiocin concentrations. Taken together with the data on single-channel conductances in different monovalent salts presented above, this observation indicates that novobiocin forms highly cation selective ion channels.

Cation/anion selectivity measurements also gave results that were very similar to those found for gramicidin

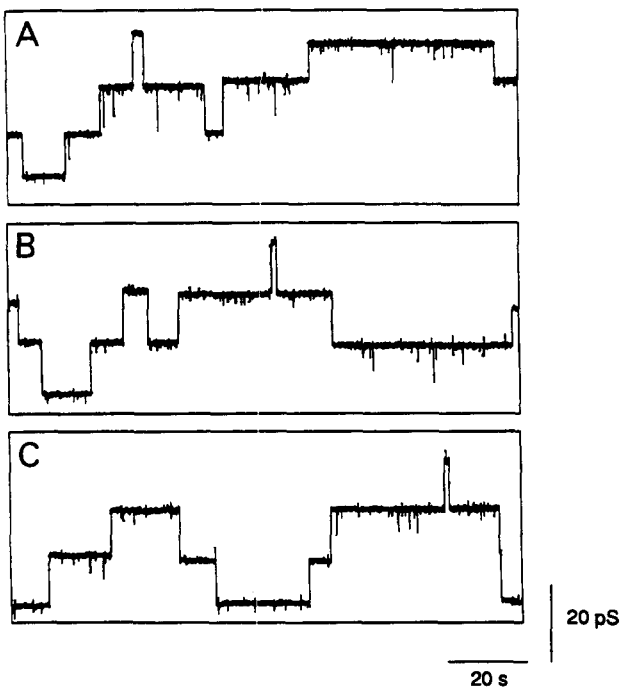

Fig. 6. The single-channel activity of a DPhPC bilayer in the presence of novobiocin. (A) Commercial preparation of novobiocin sodium salt was added to *cis* side in concentration of 0.15 mM. (B) Commercial novobiocin preparation was added to both sides at a concentration of 0.1 mM. (C) HPLC purified novobiocin was added to the *cis* side at a concentration of 0.1 mM. Membrane bathing solution was 100 mM KCl. Transmembrane voltage was 100 mV, *cis* side positive. Signal was filtered at 30 Hz by a low-pass Bessel filter.

Table 2
Zero-current potentials, E, generated by electrolyte concentration difference on a multichannel novobiocin-doped DPhPC membrane and corresponding cation transference numbers, t_+

	LiCl	NaCl	NaCl *	KCl	CsCl	NH_4Cl
E_{theor}, mV	58.6	54.8	54.8	52.9	50.8	52.9
E_{exper}, mV	54.5	52.5	52.2 *	49.4	47.7	49.1
t_+	0.97	0.98	0.98 *	0.97	0.97	0.96
G, nS	0.5	2.6	0.4 *	2.5	4.6	2.2

The concentration gradient was maintained by 100 mM salt at *cis* side and 1.0 M salt at *trans* side of the bilayer. All potential differences applied to nullify membrane current were *cis* side positive. Cation transference numbers were calculated in the usual manner [21]. Theoretical values of the zero-current potential are given for ideal cation selectivity, $t_+ = 1.00$. The steady-state membrane conductance, G, was typically achieved in 20–30 min after symmetrical novobiocin addition.
* Novobiocin was added asymmetrically, to *cis* side only.

data. Table 2 presents zero current (reversal) potentials of novobiocin channels at a 10-fold electrolyte concentration difference together with potentials expected for ideally cationic selective channels. Novobiocin cation transference numbers for alkali metals, calculated from reversal potentials, are in the range of 0.97–0.98, whereas corresponding transport numbers for the gramicidin A channel are reported to be 1.0 [21]. Note also that cation specificity of novobiocin-induced permeability does not depend on the mode of novobiocin addition, because one-sided and two-sided applications of novobiocin generate practically the same reversal potentials.

3.3. Single channels with addition of novobiocin to one or both sides of the bilayer

Properties of ion channels formed by many well-characterized channel formers often depend on the mode of their addition to the bilayer. For example, gramicidin A is about 100–300-times more efficient when added to both sides of the bilayer as opposed to its addition to only one side [22]. One-sided application of polyene antibiotics requires about 30-times higher solution concentration to induce a bilayer conductivity comparable to that of two-sided application [23]. In addition, ion channels produced by polyene antibiotics using these two different procedures are different in their conductance and voltage sensitivity [24,25], in kinetics [26], and in selectivity [23,27].

To check for a possible difference in novobiocin-induced channels using the two modes of antibiotic application, we studied novobiocin action by adding it either to one or to both sides of the bilayer. The channels in DPhPC bilayers formed by novobiocin added from only one or both sides (Fig. 6a and b) were virtually indistinguishable. They had the same conductance (about 14 pS in 0.1 M KCl) and the same long dwell times. The threshold concentrations that induced single channels did not differ significantly: in both cases they were in the 0.05–0.2 mM range. Note that the properties of single channels formed

by gramicidin A are also the same for both modes of application of gramicidin A to the bilayer [22], even though the threshold concentration for one-sided addition is higher than that in two-sided addition.

3.4. HPLC purification of novobiocin. The channel forming activity of the purified antibiotic

The active concentration range of novobiocin in the bath electrolyte begins at a rather high value of 10^{-4} M. This value is many orders of magnitude higher than characteristic concentrations for a number of well-known channel-formers: about 10^{-12} M for gramicidin A [18], 10^{-8} M for amphotericin B [28], and 10^{-8} M for alamethicin [29] [1].

It is clear that even a small contamination of the commercial product with other channel-forming compounds can be misleading. Since novobiocin is a product of microbial synthesis, the commercial product can be expected to be contaminated with other membrane-active compounds. To demonstrate that novobiocin itself, and not a contaminant, is responsible for channel formation, we purified commercially available novobiocin by HPLC [2].

Fig. 7a shows a preparative chromatogram of commercially available novobiocin sodium salt. The major component has a retention time of 12.95 min, corresponding to novobiocin [9]. Because the UV spectrum of this component was identical to the spectrum of the authentic product in acidic medium, no further methods of identification were used. The chromatogram of the purified novobiocin (Fig. 7b) shows that after a single pass through HPLC there were no detectable impurities remaining in the sample.

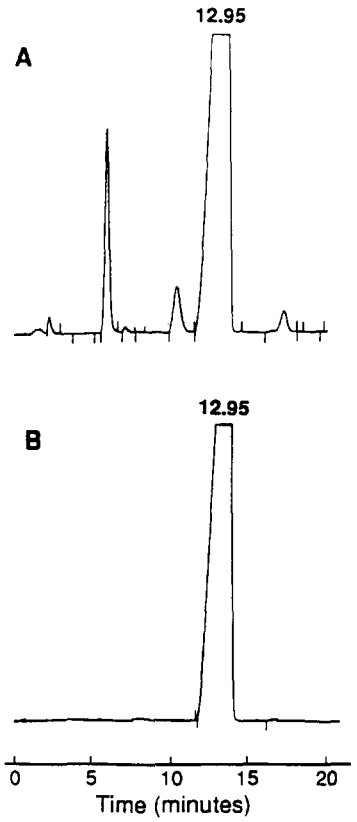

Fig. 7. HPLC separation of novobiocin from its isomers and degradation compounds. The major out of scale component with the retention time 12.95 min corresponds to novobiocin. (A) A preparative chromatogram of commercially available novobiocin sodium salt. (B) A chromatogram of purified novobiocin.

HPLC purification of the novobiocin sodium salt conducted twice proved to have no measurable effect on channel characteristics or the range of active antibiotic concentrations. HPLC purified novobiocin formed gramicidin-like channels identical to those induced by the commercial preparation of novobiocin (Fig. 6C), as well as a wide spectrum of channels with different levels of conductance (Fig. 2D). This observation allows us to conclude that the channel-forming ability is a property of novobiocin molecules themselves, and not of contaminants in the commercial preparation.

3.5. Lipid composition of bilayer and properties of novobiocin channels

It seems unlikely that a single novobiocin molecule (Fig. 1) can form an ion channel. The presence of polar groups should prevent a single novobiocin molecule from being localized in the internal hydrophobic part of a lipid bilayer. Because of this, two models for the formation of the novobiocin channel may be hypothesized. First, the conductive unit may be formed by the assembly of at least several novobiocin molecules arranged to screen the polar groups inside the unit, analogous to an inverted micelle.

[1] It should be noted that channel-former solution concentrations corresponding to single-channel bilayer activity depend on parameters of a particular experiment. Amphotericin B concentration, for example, strongly depends on the cholesterol content of the bilayer, channel-inducing alamethicin concentration is significantly different for different transmembrane voltages, etc. The concentrations presented above give rough order of magnitude estimates only.

[2] In order to demonstrate that novobiocin could be separated from a gramicidin A-like polypeptide using the conditions employed for HPLC purification of novobiocin, we performed analytical thin-layer chromatography (TLC) of the purified novobiocin and gramicidin A (Fluka, St. Louis, MO) using the same stationary and mobile phases as in the HPLC purification of novobiocin. Detection was performed by UV and charing after H_2SO_4 spray. A zone of gramicidin appeared at the origin, while a zone of novobiocin was found at $R_F = 0.12$, which is consistent with its retention under HPLC conditions. As long as the efficiency of an HPLC column is at least one order of magnitude higher than that of a TLC plate, the 'complete' separation of the compounds can be reached by HPLC. It is known from the theory of chromatography [30] that in cases of complete separation (separation of two peaks with a portion of base line between them) each step of HPLC purification decreases an amount of an impurity by at least 10000-fold. This is a strong argument against any peptide contamination of the commercially available preparation being the source of channel-forming activity in our experiments.

Table 3
Conductance of single channels induced by novobiocin in bilayers of different lipid composition

Lipids	Mean conductance ± S.D. (pS)
DOPE	7.6 ± 0.8
DPhPC	7.2 ± 0.9
DOPE/DOPC = 1:1 (mol/mol)	7.1 ± 0.8
DOPS	16.6 ± 1.2

Single channels were obtained by one-sided application of 0.1–0.2 mM of novobiocin. Bathing solutions: 110 mM NaCl, 10 mM Mops, pH 6.9; transmembrane voltage: 100 mV, *cis* side positive. The mean conductances were obtained by averaging over 20–30 single channel steps for the indicated lipid.

Second, novobiocin may induce defects in the lipid bilayer so that the conductive units are some hypothetical phospholipid structures. The latter hypothesis was recently discussed by Pasternak et al. [31] in relation to channel-forming properties of several proteins, and also by Cruciani et al. [14] for channels formed by magainin 2. Cruciani et al. [14] suggested a model for channels induced by magainin 2 in which negatively charged lipids line the aggregate ion pore. Their hypothesis is consistent with their finding that the experimental appearance of magainin 2 conductance was dependent on the presence of the negatively charged lipid, phosphatidylserine, in the lipid solution used to form the bilayers.

If this second model were true, we would anticipate some relationship between the transport properties of the novobiocin-induced channels and the composition of the lipid bilayer (the structure of the lipid polar group). Conversely, if the lipids were not directly involved in channel structure, then we would expect that the lipid composition of the bilayer would not affect channel transport properties, but may influence only conformational equilibrium of the channel between different states [32].

To discriminate between these two models we studied the conductance of novobiocin-induced channels in bilayers of varying phospholipid types. The bilayers were formed from DPhPC, DOPC, DOPE, or DOPS. Table 3 presents mean conductances of single novobiocin channels for these lipids in 110 mM NaCl showing a uniform (about 7 pS) value for all neutral phospholipids. Variation in the structure of the polar head group and the fatty acid tails of a neutral phospholipid does not, therefore, influence single-channel conductance of the novobiocin channel.

However, the presence of negative charge in the polar group region of lipid (DOPS) leads to an increase in channel conductance to about 17 pS. This difference suggests that the presence of negative surface charge on the DOPS membrane increases the local concentration of Na^+ in the area of the channel mouth. To verify this, we studied single-channel conductance as a function of NaCl activity, (across a range of concentrations from 25 mM to 2 M) in the bath solution for membranes formed from neutral and negatively charged lipids.

Fig. 8a shows the conductance vs. sodium activity curves in symmetrical NaCl solutions for novobiocin channels formed in pure DOPE and pure DOPS bilayers. The same data are replotted in Fig. 8b in Eadie-Hofstee coordinates to emphasize the difference in channel conductance in these lipids.

In neutral DOPE our data are described well by a rectangular hyperbola of the form

$$g = g_{max}/\left[1 + K_d/Na^+_{(b)}\right]$$

where g is the conductance of a single channel and $Na^+_{(b)}$ is the activity of sodium ions in the bulk solution, with a maximum conductance $g_{max} = 16.2$ pS, and a half-saturation activity $K_d = 105$ mM. The Eadie-Hofstee plot of the

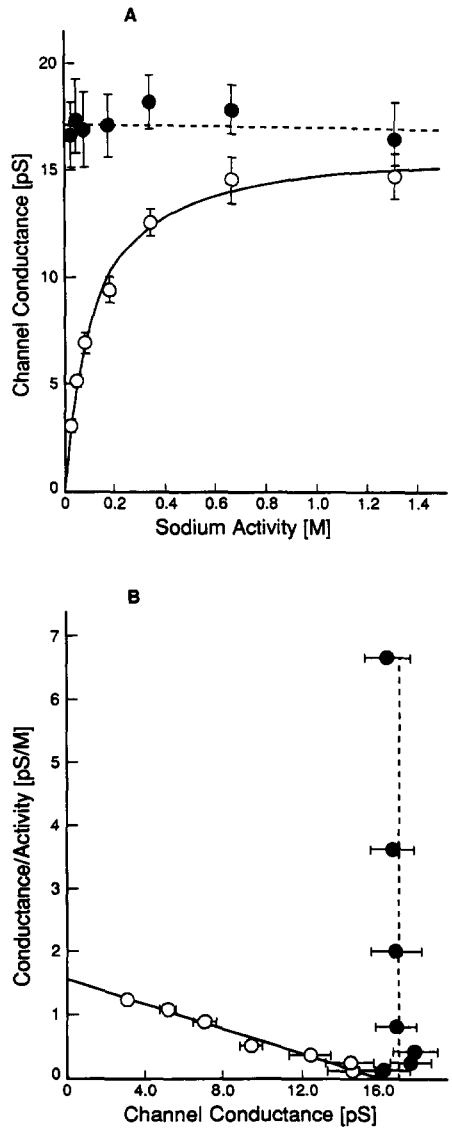

Fig. 8. (A) Conductance of a single novobiocin channel in DOPE and DOPS membranes as a function of NaCl activity in the bath solutions. (B) Eadie-Hofstee representation of the same data. Conductance was determined as an average over 20–30 single-channel 'steps'. Transmembrane voltage was 100 mV, *cis* side positive.

data (Fig. 8b) shows that this behavior is followed well over the entire concentration range.

For negatively charged phospholipid, the conductance of the single channels always displayed a maximum value of about 17 pS across bath NaCl concentrations from 25 mM to 2.0 M (Fig. 8a,b). This value is much higher than the conductance for neutral lipids at low electrolyte concentrations and close to that for the high concentration of 2 M NaCl.

The same type of behavior has been observed for the gramicidin A channel by Apell et al. [33]. In negatively charged lipid, the conductance was practically independent of salt concentration in the 1 mM–1 M range. These authors concluded that in the case of a negatively charged lipid the concentration of cations at the membrane surface is close to 20 M across the entire range of bath concentrations used. This value of the surface concentration is far above the saturation concentration of the channel. Later, Bell and Miller [34] demonstrated that an independence of the channel conductance on bath solution salt concentration in this range can be interpreted as suggesting that the channel mouth opens out to the aqueous phase directly at the lipid surface. If the novobiocin channel extended into the aqueous phase some distance from the membrane surface, we would expect some decrease of conductance with a decrease of salt concentration even for charged lipids. Bell and Miller [34] reported this type of behavior for the K^+ channel of sarcoplasmic reticulum reconstituted in planar bilayers formed from different lipids and concluded that the channel's entryway was located 1–2 nm away from the lipid surface (see also Moczydlowski et al. [35] and Coronado and Affolter [36]).

To summarize, none of our results on the influence of lipid structure on novobiocin channel conductance indicate that lipid molecules directly participate in the formation of novobiocin channels. Neither variations of the polar head groups (DOPC compared with DOPE), nor variations of the fatty acyl chains of phospholipids (DOPC, POPC, DPhPC) influenced the properties of these channels. These observations suggest that novobiocin-induced channels are formed by an assembly of novobiocin molecules isolating an ion pathway from the surrounding phospholipid, rather than by inducing an assembly of lipid molecules. Taking into account two facts – that (1) the structure of novobiocin (Fig. 1) makes it unlikely that a single molecule can form an ion channel in the membrane, and that (2) novobiocin can form channels of varying conductance levels – we hypothesize that novobiocin molecules gathered in assemblies of different sizes are responsible for the formation of channels of different conductances. One way of inferring the existence of these types of assemblies is to measure the molecularity of channel formation. To determine molecularity, the construction of a steady-state conductance/concentration curve for novobiocin would be required. However, we have not succeeded in obtaining this data because of the wide variety of conductivities that are induced by each particular concentration of novobiocin. Additional studies will be necessary to test this hypothesis.

4. Conclusion

We have demonstrated that novobiocin forms ion channels of varying conductance levels in lipid bilayers. HPLC purification of commercially available novobiocin sodium salt has shown that novobiocin itself, not a contaminant, is responsible for channel formation.

One type of novobiocin channel possesses properties that are similar, though not identical, to those of channels formed by gramicidin A:

(1) The single-channel conductances measured by us with chloride salts of alkali metal cations and NH_4^+ are very similar for these two antibiotics. We obtained not only the same sequence: NH_4^+, $Cs^+ > K^+ > Na^+ \gg Li^+$, but surprisingly close values of the conductances themselves.

(2) Novobiocin cation transference numbers for alkali metals calculated from reversal potentials are in the range of 0.97–0.98, whereas corresponding transport numbers for the gramicidin A channel are reported to be 1.0.

(3) Current-voltage characteristics of both types of channels are similar: linear in the range from 0 to +100 mV with saturation at higher voltages.

(4) Similarity is seen in the dependence of single channel conductivity on salt concentration with the neutral and charged lipids.

(5) The properties of both types of channels do not depend on the mode of addition to the bilayer (one or both sides).

The structure of novobiocin is completely different from that of gramicidin A, the oligopeptide formed from hydrophobic amino acid residues. The observation that among the variety of novobiocin channels there is one type with properties similar to those of channels formed by gramicidin A suggests that these structurally different molecules can form channels with comparable internal diameter and internal surface charge distribution.

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