Characteristics of Single Ionic Channels Induced by Sulfonic Acid Derivatives of Dibenzo-18-Crown-6 in Bilayers

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Abstract

Sulfonic acid derivatives of dibenzo-18-crown-6 (DB18C6) have been shown to induce formation of ion channels permeable to monovalent cations in bilayer lipid membranes (BLM). Some electric characteristics of channels have been studied by means of the voltage clamp method. Channels displayed little interionic discrimination. The voltage dependence as well as the multiple state behaviors of the channels functioning was observed. The channels formation has been shown to be dependent on pH of bath solutions and on the presence of bivalent cations in them. The channels supposed to be formed from aggregates of complexes associates assembled in aqueous salt solutions.

Introduction

Complex-forming and membrane active properties of crown ethers are well established to depend on the sizes of macrocycles and on the nature of substituents. Manipulations with these parameters make it possible to design molecules with predetermined properties. In virtue of their unique complexing abilities macrocyclic polyethers as model of ionophorous antibiotics have contributed significantly to the study of trans-membrane ion transport. Macrocyclic compounds capable of formation of ionic channels in bilayer membranes have been studied intensively in recent years. Successful results in this field were obtained by various groups of researchers [1–4]. On the other hand, lately much attention has been attracted by proton-ionizable crown ethers [5–8], and particularly in connection with ion transport through ultrathin membranes [9–12]. This kind of compounds has an advantage as membrane transporters thanks to their capability of forming electroneutral, lipophilic complexes with cations transferred.

Experimental

Sulfonic acid derivatives of dibenzo-18-crown-6 (DB18C6) were synthesized as in Ref. [13]. Planar bilayer lipid membranes (BLM) were formed as in [14] from 1 to 2% solution of soybean L-α-lecithin (Sigma,

USA) in *n*-octane on the hole ($d \approx 200$ mkm) in the partition of a two-chamber Teflon cell. The conductivities of single ion channels were measured as described elsewhere [15]. The cation–anion selectivity of a channel was estimated by the cation-transfer numbers (t^+), which were calculated by the following Equation [16]:

$$t^+ = (E_0 - E_a)/(E_c - E_a),$$

where E_0 is the zero current potential at the saline concentration gradient, E_c and E_a are theoretical Nernst potentials for cations and anions, respectively.

Zero current potentials were determined from current–voltage curves of single channels as the points in which the curves crossed the voltage axis.

Conductivities of single channels were calculated from the amplitudes of step-current changes of voltage-clamped membranes.

All measurements were carried out at 25 ± 1 °C.

The dissociation constant of 4′-*tert*-butyl-4″(5″)-dibenzo-18-crown-6-sulfonic acid was determined from measurements of its aqueous solution conductivities using an OK 102/1 conductivity meter (Radelkis, Hungary).

Results and discussion

Four water-soluble sulfonic acid derivatives of DB18C6: 4′-*tert*-butyl-4″(5″)-dibenzo-18-crown-6-sulfonic acid (**1**), 4′-acetyl-4″(5″)-dibenzo-18-crown-6-sulfonic acid (**2**), 4′-dibenzo-18-crown-6-sulfonic acid (**3**), 4′-4″(5″)-

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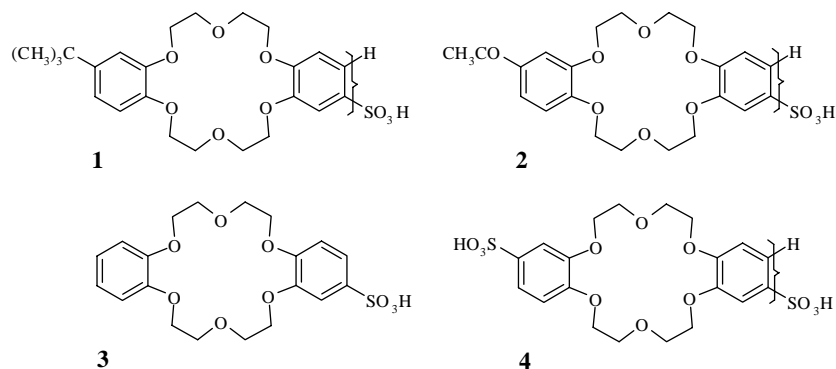Figure 1. (1) 4′-tert-butyl-4″(5″)-dibenzo-18-crown-6-sulfonic acid, (2) 4′-acetyl-4″(5″)-dibenzo-18-crown-6-sulfonic acid, (3) 4′-dibenzo-18-crown-6-sulfonic acid), (4) 4′,4″(5″)-dibenzo-18-crown-6-disulfonic acid.

dibenzo-18-crown-6-disulfonic acid (4) (Figure 1) have been found to induce formation of single ionic channels in BLM in the presence of monovalent cations [17]. They had effect regardless of one-sided or two-sided addition of the compounds to bath solutions was applied. Possessing hydrophobic and hydrophilic groups these compounds have affinity to both the lipid phase of membrane and the aqueous phase.

The minimum channel-formers concentrations required for channels to be formed are as follows: 1, 50, 100 μM, 1 mM for **1**, **2**, **3**, **4**, respectively (in 100 mM KCl).

Single channels induced by each the compounds varied significantly in their conductivities (Figure 2), but the majority of channels were formed in the peak at 6.5 ± 0.5 pS whatever monovalent cation was applied (these channels were the main subject of our analyses).

The cation–anion selectivity of single channels induced by **1–4** was close to ideal cation one (in 10/100 mM KCl, 25 mM Tris–HCl, pH 7.4). Cation–transfer numbers were 0.98 ± 0.02 for all compounds.

The current–voltage characteristics of single channels were superlinear and symmetrical in symmetrical KCl systems and one-sided applications of a channel-former.

Cation specificity sequence investigated for single channels induced by **1** was as follows: Cs > Rb > K > Na > Li. The ratios of permeabilities P_X/P_{Na}
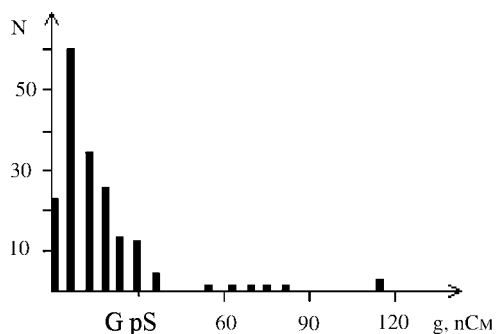
calculated from the measured bi-ionic potentials were 2:1.8:1.4:1:0.5 respectively.

Analysis of channels functioning showed their multiple state behaviors. As can be seen in Figure 3a, channels display two closed states with an average lifetime of one being shorter than 0.2 s whereas the other's is a few seconds. These states are similar to those of amphotericin [18]. Functioning of channels was shown to be voltage dependent. It is seen in Figure 3b that an increase in electric potential clamped results in attempts of a channel to switch off become more frequent. Opening and closing of channels possessing different conductivities occurred in one action (with no sublevel open state) (Figure 3c).

Comparison of characteristics of single ion channels shows that the main properties of the channels formed under identical experimental conditions are much the same. The similarity of the channels properties suggests the similarity of molecular mechanism of channels formation. When planning experiments on investigation of the mechanism we based on the following considerations: taking into account that the sizes of the compounds are small as compared with the thickness of BLM, the channels induced by them can be either inverted phospholipid pores caused by inculcation of the polyethers into a BLM or polyether-based multimolecular structures being large enough to pierce through a BLM. To investigate the molecular mechanism of channels formation we studied dependence of such parameters of single channels as cation–anion selectivity, current–voltage characteristics, channels formation frequency and conductivity distribution on pH and ionic strength of bath solutions. In addition, investigation of the role that permeable cation plays in the process of channel formation was proposed to be of importance as well. In these series of experiments **1** was used as the most effective channel-former (the effective concentration for single channels to be formed by this compound is the lowest one as compared with the others).

Experiments with pH dependence of single channels formation showed the formation frequency to reduce from 120 to 150 (channels of the whole conductivity spectrum per 1 h registration) at pH 7.4 to 10–15
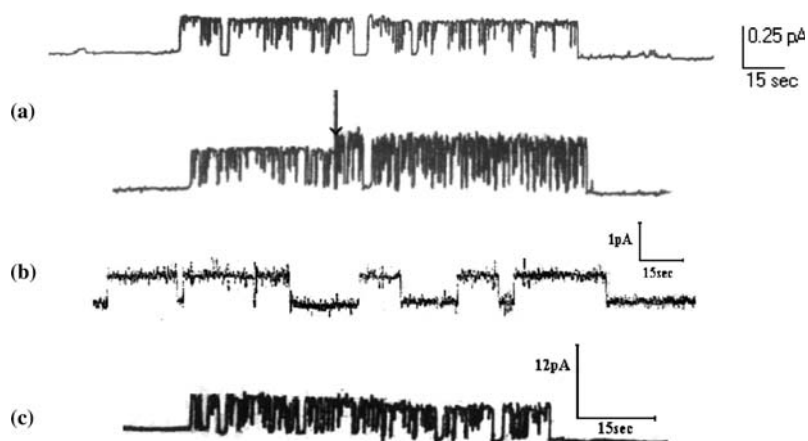

Figure 2. Conductivity histogram of single channels in BLM modified with **1** (experimental conditions: 1 μM **1** (sym), 100 mM KCl (sym), 25 mM Tris–HCl, U_{clamp} = +40 mV).

Figure 3. Multiple state behavior of channels: (a) multiple closed states of a typical channel, (b) voltage-dependence of a channel's functioning. The momentum of voltage change (+60/+80 mV) is pointed out, (c) opening and closing of channels with different conductivities (experimental conditions are as in Figure 2).

channels at pH 5 and to 2–3 channels at pH 3 (1 μM **1** symmetrically, 100 mM KCl, 25 mM Tris–HCl, $U_{\text{clamp}} = +40$ mV). The conductivity distribution of single channels was shown to depend on pH of bath solutions and their widths narrowed with decreasing pH. pH dependence of channels formation indicates that deprotonated sulfonic acid groups may participate directly in formation and stabilization of the channels structure.

To determine the role that permeable cation plays in the process of channel formation we investigated the ability of **1** to form ion channels under the conditions when the channel-former and KCl were added separately to different compartments of the experimental cell (as was shown above the compounds investigated had channel-forming effects regardless of one-sided or two-sided addition of them to bath solutions were applied). Under these experimental conditions (50 mM KCl/ 2 μM **1**, 25 mM Tris–HCl, pH 7.5, U_{clamp} + 40 mV) the compound **1** did not show channel-forming properties. The results obtained suggest that cations not only participate in the transition act but they are also integral elements of ion-conducting structure.

Current–voltage curves of single channels remained superlinear and symmetrical in the pH range 3–7.5 (0.1 M KCl). Their forms did not depend qualitatively on ionic strength of bath solutions over 0.05–0.5 M KCl either. Thus, the current–voltage characteristics proved to be little informative in this case.

Experiments on the cation–anion selectivity of single channels in BLM formed from chromatographically pure lecithin at pH 7.4 showed that channels possessed cation selectivity. High cation selectivity suggests the existence of negatively charged sites in a channel. As a molecule of lecithin is neutral at this pH the cation selectivity can testify either crown ether nature of channels or inverted phospholipid pores with negative charges to be generated by polyether molecules on their entrance or inside a channel. Negative charges in the

molecule of 4′-*tert*-butyl-4″ (5″)-dibenzo-18-crown-6-sulfonic acid can be caused by the sulfonic acid group (pK 4.07) or by oxygen dipoles of the macrocycle. It is known [18] that negative charged groups fixed at an entrance of a channel provide an increase in cation permeability at low concentrations of electrolyte, but they do not work at high concentrations. A charge group inside a channel might provide high selectivity for counter-ion but this group would reduce the efficiency of ion transport at high electrolyte concentrations by making a potential trap for the ion transferred. In our experiments the high value of cation selectivity of single channels measured from zero current potentials in 10/ 100 mM KCl system did not decrease with increasing ionic strength of bath solutions. It remained the same over pH range 3–7.5. Independence of cation–anion selectivity on ionic strength testifies that the first suggestion is not realized. The fact that cation selectivity does not reduce with decreasing pH can provide an evidence against the second suggestion. So, it can be supposed, that this property of channels is determined mainly by dipole sites allocated along a channel.

Cation specificity of single channels corresponds to the 1st sequence of Eisenman. It is known that the first sequences of Eisenman characterize the cation specificity of membranes containing ionophores or channel-formers with weak cation-binding abilities (which crown ethers also belong to) [19, 20].

Summarizing results of experiments on cation–anion and cation–cation specificity it can be assumed that the cation–binding sites and the selectivity centers of channels are macrocycles of the compounds.

Thus, an answer to the question about the channel structures can consist in solution of a task how the investigated molecules could assemble in chains with links of which being bound to each other by means of deprotonated sulfonic acid groups and cations transferred. The structures supposed to be optimal represent chains in which sulfonic acid group of one molecule
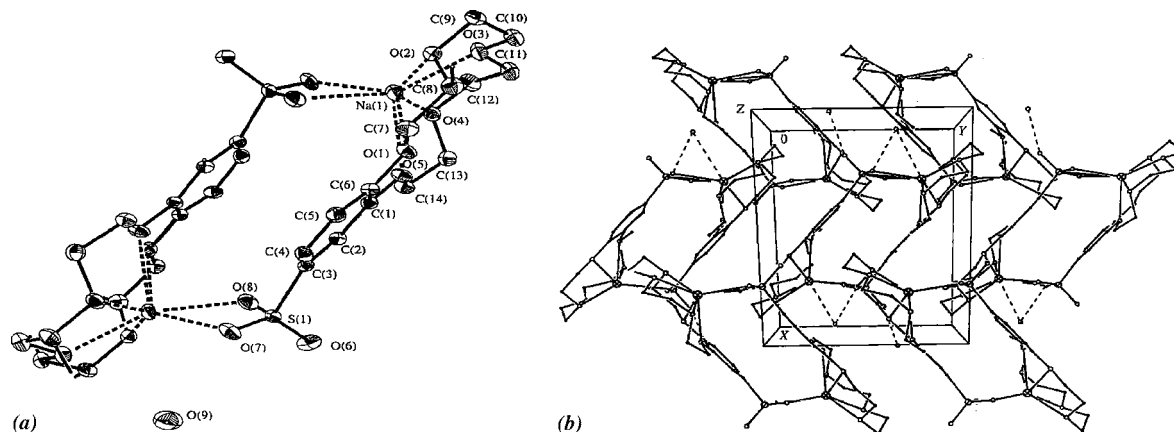
Figure 4. Complex bimolecular associate containing two molecules of 4′-B15C5-sulfonic acid and two sodium ions (a). Crystal structure of Na complex of 4′-B15C5-sulfonic acid (b).

form ionic pair with cation, coordinated by the macrocycle of another molecule. A similar assembly made up of complexes associates was shown [21] for 4′-benzo-15-crown-5-sulfonic acid (Figure 4). This compound formed Na^+-complexes where cation is coordinated by all oxygen atoms of one crown ether molecule and by two oxygen atoms of sulfonic acid group of the other molecule. The complexes formation is followed by deprotonation of sulfonic acid groups.

Thus, an ion-conducting track is supposed to be a chain formed from identical links, representing complexes associates. Such a channel structure suggests linear forms of current–voltage characteristics of channels functioning in symmetrical saline solutions. Observed non-linearity of current–voltage curves can be caused by the influence of electrostatic forces that real themselves near the interface of two phases with different dielectric constants by attracting the cation allocated in the lower dielectric phase to the phase interface, i.e. they make two potential holes near the membrane interfaces for ion transferred [22, 23].

If our suggestion, that the investigated ion-conducting structures represent associates of complexes of crown ethers with cation transferred is correct, then the factors influencing on the complexation process should affect both the channels formation frequency and probability of formation of channels with different conductivity amplitudes. Investigation of influence of

pH on the frequency of the channels formation and on spectra of their conductivity amplitudes showed that a decrease in pH was followed by not only a decrease in channels formation frequency but also a decrease in events of high amplitude channels (Figure 5).

These data can evidence that ion channels are aggregates of complexes associates being of different sizes and therefore possessing different numbers of ion-conducting tracks. Further, if associate aggregates formation occurs in aqueous salt solutions, it is likely that such a factor as "the age of solutions" can have influence on formation of the channels. To verify this suggestion we analyzed conductivity distribution of channels formed in aqueous salt solutions of different "ages". Traces of single channels in BLM, formed in 12 and 24 h-aged solutions are shown in Figure 6. As seen in Figure 6 'aging of solutions' causes an increase in conductivity of typical channels. The "aging" is probably followed by an increase of associate aggregates in sizes and as result an increase in events of high amplitude channels.

Incorporation of supposed associate aggregates into BLM is not very likely to result in structures being strictly symmetrical with respect to the center of a membrane. And it is logically to suggest a decrease in probability for asymmetrical incorporation with increasing sizes of aggregates. Such asymmetry in channels structure would bring about asymmetry in potential
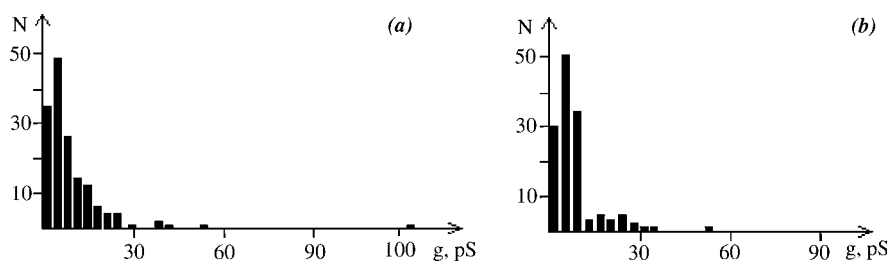

Figure 5. Conductivity histograms of single channels in BLM formed at (a) pH 7.4, (b) pH 5 (experimental conditions: 1 μM **1** (sym), 100 mM KCl (sym), 25 mM Tris–HCl, U_{clamp} + 40 mV).
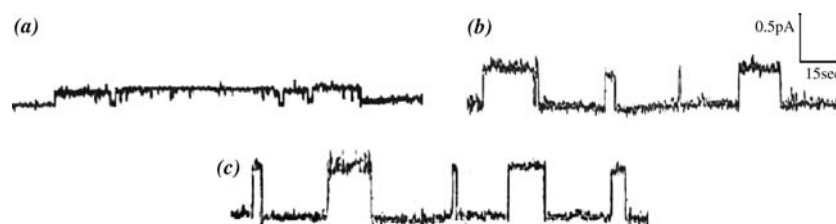
Figure 6. Traces of single channels in BLM, formed in (a) fresh, (b) 12, (c) 24 h-aged solutions (experimental conditions are as in Figure 2).

profile in a channel by changing asymmetrically potential holes at its opposite enters. That in turn would result in the asymmetrical forms of current–voltage curves. Investigation of dependence of current–voltage curves symmetry on the conductivity of single ion channels showed (Figure 7) that an increase in the conductivity was accompanied by appearance and following increase in asymmetry in current–voltage curves.

No increase in conductivity of BLM modified by the compounds was observed in the presence of bivalent cations Ca^{2+} and Mg^{2+} at concentrations of as high as 100 mM. It was found, however, that these cations had
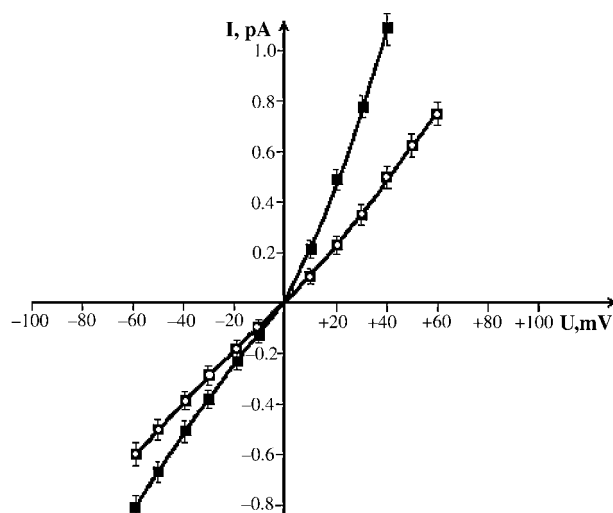

Figure 7. Current–voltage curves of single channels recorded after asymmetrical addition of **1** was applied. Conditions: 100 mM KCl, 25 mM Tris–HCl, pH 7.4.

effect on channel-forming properties of the compounds. The ability of these compounds to form channels was found to diminish, if transferred cation was added to bath solutions, which had contained originally bivalent cations Ca^{2+} or Mg^{2+}. In this case decrease in both frequency of channels formation and events of high amplitude channels was observed. The frequency of the channels formation estimated after addition of 50 μM KCl to a medium having contained originally the channel former **1** and 50, 100, 1000 mM $CaCl_2$ was observed to reduce as much as 2, 10 and 100 orders of magnitude respectively. Conductivity histograms of single channels recorded under described conditions are shown in Figure 8.

As seen from the histograms, an increase in concentration of Ca^{2+} causes a decrease in events of high amplitude channels. The influence of bivalent cations was much less significant if they were added to solutions having contained originally the channel-former and KCl. In this case much higher concentrations of Ca^{2+} or Mg^{2+} were inquired to have the same effect. So, approximately twofold reduction in frequency of the channels formation was observed after addition of as much as 50 mM $CaCl_2$ or $MgCl_2$ was applied. In this concentration bivalent cations had not measurable effect on conductivity distribution but they reduced the conductivity of single channels (Figure 9).

The results of the experiments with bivalent cations allow us to suppose that these cations influence on process of channels formation, taking place in aqueous solutions. The mechanism of the influence can consist in the interaction between bivalent cations and sulfonic acid groups and as result the supposed associates are not formed. The absence of the effects of Ca^{2+} and Mg^{2+} on formed associates and their aggregates can be explained
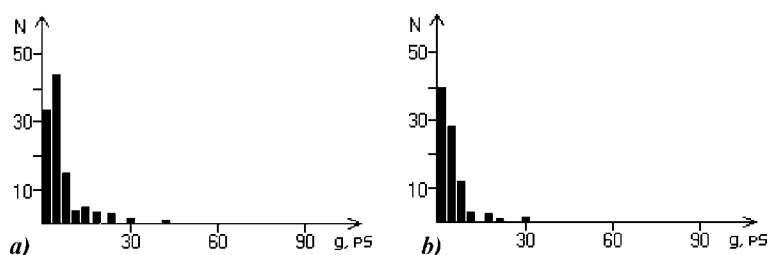

Figure 8. Conductivity histograms of single channels recorded after the addition of 50 mM KCl to bath solutions having contained originally **1** and (a) 50 μM $CaCl_2$, (b) 100 μM $CaCl_2$ (experimental conditions are as in Figure 2).
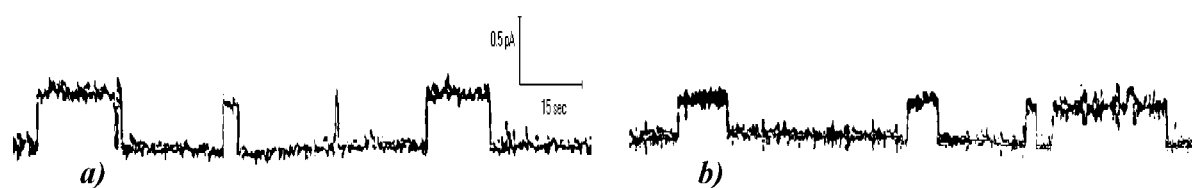
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Figure 9. Traces of single channels made (a) in the absence of Ca^{2+}, (b) after addition of 50 mM $CaCl_2$ (experimental conditions are as in Figure 2).

by their high stability and inaccessibility of sulfonic acid groups for bivalent cations.

Conclusion

The data obtained suggest that the ability for formation of ionic channels is a property of associate aggregates made up complexes of the compounds with cation transferred. Assembled in aqua-saline solutions they inculcate into BLM, forming in them relay chains through which cations jump from one binding site to another. A binding site is supposed to be the macrocycle of one crown ether and the sulfonic acid group of another. The conductance magnitude of a single channel depends on the size of an aggregate and it is determined by the amount of tracks for ion transferred. The open state of a channel (whatever large) corresponds to the aggregate piercing through BLM, hence the sameness of the processes of the opening and the closing of channels, regardless of their conductance.

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