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Correlations between Changes in Membrane Capacitance Induced by Changes in Ionic Environment and the Conductance of Channels Incorporated into Bilayer Lipid Membranes

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Abstract. The action of metal polycations and pH on ionic channels produced in bilayer lipid membranes (BLM) by three different toxins was studied by measuring membrane capacitance and channel conductance. Here, we show that critical concentrations of Cd^{2+}, La^{3+} or Tb^{3+} induce complex changes in membrane capacitance. The time course of capacitance changes is similar to the time course of channel blocking by these ions at low concentration. No changes in BLM capacitance or conductance were observed in the range of pH 5.8–9.0. A pH shift from 7.4 to 3–4 or 11–12 induced large changes in BLM capacitance and channel conductance. For all studied channel-forming proteins, the initial capacitance increase preceded the conductance decrease caused by addition of polycations or by a change in pH. A close relationship between membrane lipid packing and ion channel protein is suggested.

Key words: BLM — Latrotoxin — Lanthanum — Cadmium — Ionic channels — Membrane capacitance

Introduction

Model systems containing a small number of components simplify the interpretation of experimental results. In some cases, even when using a model as simple as the BLM, it is difficult to determine which component—protein, lipid or a complex of both—plays the major role in an observed effect. In particular, it is known that the properties of ionic channels in membranes depend on the ionic composition of the bathing solution. The interpretation of these data is complicated by ions being able to interact both with the lipid membrane and with protein channels (Cai & Jordan, 1990). Nonetheless, models of ionic transport are usually constructed to analyze only channel-ion interactions, ignoring the influence of ions on the lipid membrane (Laüger, Stephan & Frehland, 1980; Almers & McCleskey, 1984; Teslenko, 1985).

However, the importance of the lipid environment for ion transport through channels has been shown by a number of studies implemented not only on BLM's with such simple channels as gramicidin and monoazomycin (Mueller & Finkelstein, 1972; Gambal, Menini & Rauch, 1987; Osipov, Rostovtseva & Lev, 1987), but also in studies of large proteins in BLM's (Robello et al., 1984), and natural membranes (Misley, Lin & Verkman, 1987). It has been suggested that the physical basis for membrane/channel interaction is either electrostatic (Mueller & Finkelstein, 1972; McLaughlin et al., 1981) or due to conformational changes in proteins that result from changes in lipid ordering (Jacobson & Papahajopoulos, 1975; Evzen, Teising, Svoboda, 1987). Cation binding to the membrane surface is mostly an electrostatic interaction that changes the intramembrane potential gradient. However, it may also alter lipid ordering in the bilayer (Knoll et al., 1986). It is thought that cation adsorption produces a detectable effect only in the case of charged membranes. This is true for monovalent cations and alkaline-earth metal cations (McLaughlin, Szabo & Eisenman, 1971;

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McLaughlin et al., 1981), the ions most commonly used to study the interaction of cations with membranes. Ions that are known blockers of ionic channels, such as lanthanum and cadmium, probably act in similar ways, but also affect uncharged bilayers (Westman & Eriksson, 1979).

Known channel forming proteins α-latrotoxin (α-LTX) (Mironov et al., 1986) and β-latrotoxin (β-LTX) (Salikhov, Tashmukhamedov & Adulbekov, 1982), which were used in this study, have ion transporting properties that are in some aspects similar to the ion transporting properties of natural calcium channels. In our previous paper (Mironov et al., 1986), we explained cadmium blocks of the α-LTX channel in terms of ions binding with high affinity to the selective center. Another protein used, sea anemone (*Radianthus macrodactylus*) toxin (RTX) (Rudnev et al., 1984) is quite different. Ionic channels formed in BLM by any of these proteins can be blocked by cadmium, lanthanum or terbium, which are known blockers of various other ionic channels (Kryshtal, 1976; Menestrina, 1986; Nathan et al., 1988). The influence of these ions on BLM capacitance and channel conductance was investigated to look for correlations between channel conductance and packing of lipids in the membrane.

Materials and Methods

We used routine methods described in the literature in the separate measurement of either BLM conductance or capacitance (Chizmadzev, Abidor & Glazunow, 1985; Mironov et al., 1986). For recording both of these parameters simultaneously, a modified BLM experimental system described earlier (Chanturiya, 1990) was used.

α-Latrotoxin (MW 130 kD) was isolated from the crude venom of the spider, *Latrodectus mactans tredecimguttatus*, by FPLC system (Pharmacia, Sweden) with a MONO-Q column, using a linear NaCl concentration gradient according to published procedures (Chanturiya et al., 1985). α-LTX was eluted at 0.33 M NaCl. At 0.3 M NaCl, a fraction containing equal amounts of 60 and 70 kD (SDS electrophoresis, *data not shown*) proteins was eluted. This fraction corresponded to β-latrotoxin, a channel-former similar to α-LTX (Salikhov et al., 1982). Purified *Radianthus macrodactylus* toxin (MW 20 kD) was the gift of Dr. E.P. Kozlovskaya and Dr. M.M. Monastyrnaya (Pacific Institute of Bioorganic Chemistry, Vladivostok).

BLM's were formed by the Mueller technique (Mueller et al., 1962) across a 0.6 mm diameter hole in a Teflon cup placed in a glass cell, from a mixture of phosphatydylcholine from egg yolk and cholesterol (Serva, Heidelberg), 2:1, w/w. Heptane was used as a solvent; the lipid concentration was 20 mg/ml.

The solution bathing the BLM usually contained 10 mM Tris-HCl (pH 7.4) and 10 mM KCl. In some cases noted later in the text, other compositions were used. Protein (0.5–1 nM LTX, or 5–15 nM RTX) was added to one side of membrane outside the Teflon cup (*cis* side). A perfusion procedure was used to remove the protein not inserted into the membrane. Two Ag/AgCl electrodes were used to measure the current through the bilayer. Zero potential was at the inner (*trans*) side. A 50 mV potential was applied to the BLM. During capacitance measurements, the potential applied was 50 mV with a 30 mV ramp superimposed (rate of change was approximately 2 V/sec).

We investigated the effects of pH on the capacitance and conductance of BLM under conditions that permitted the measurement of single channel conductance. In these experiments, we used very low concentrations of α-LTX to obtain a low frequency of channel insertion (one channel in 3–10 min). Within 20–30 sec of the first channel insertion, the pH of the BLM bathing solution was changed by addition of known volumes of KOH or HCl to the *cis* side of the BLM. The resulting pH value was determined in a separate cell containing a pH electrode by adding proportional volumes of the same chemicals to a larger volume of the same buffer.

BLM capacitance achieved a relatively stable level within 5–15 min after formation. All experiments were conducted when the drift of this parameter was less that 1%/min.

Results

Changes in BLM Capacitance Induced by Channel Blocking Ions

Figure 1 shows the influence of different cadmium concentrations on BLM capacitance. No effect was observed after several additions of $CdCl_2$ below 0.5 mM (final concentration) to the BLM bathing solution. At a concentration between 0.5 and 2.5 mM, a complex process was induced that resulted in a change of capacitance. An initial capacitance increase (1–6%) was usually (in approx. 60% of experiments, $n = 12$) associated with a subsequent decrease (up to 90% of initial membrane capacitance). In some cases, the cadmium induced increase of membrane capacitance was comparable to the spontaneous fluctuation of this parameter. Nonetheless, it was easily recognizable due to the specific shape and consistent delay from the moment of cadmium addition. The decrease correlated with the formation of clearly visible colored areas on the BLM. Ten to fifteen minutes later, the BLM became black again and a further increase in cadmium concentration did not cause any other effects.

The same picture was seen after La^{3+} or Tb^{3+} addition, the only difference being the lower critical concentration of ion. This parameter was also dependent on the ionic composition of the bathing solution (Table). Ca^{2+}, Mg^{2+}, Sr^{2+} and Ba^{2+} had no effect on BLM capacitance.

Correlation between Cation-Induced Capacitance Changes and BLM Conductance

The second part of our study was to compare the observed changes in BLM capacitance with the time course of blocking of channels inserted into the membrane. Simultaneous recordings of capacitance and conductance of a BLM containing either α- or β-LTX are presented in Fig. 2. As can be seen, the capacitance increase preceded the decrease in conductance. A similar picture was obtained using La^{3+}. The data for the

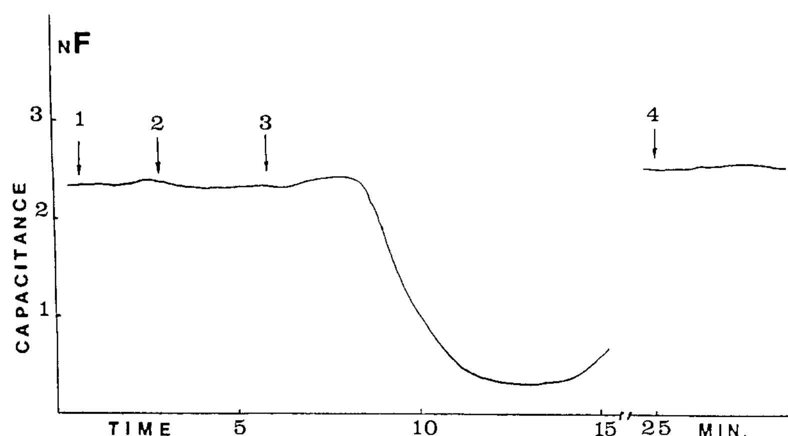

Fig. 1. The time course of cadmium-induced changes in BLM capacitance. The arrows show addition of cadmium to a final concentration of: 1–0.1, 2–0.5, 3–2.5, and 4–5 mM.

Table. Polycation concentration required to trigger capacitance changes in BLM

Solution	Cd^{2+}	mM				Tb^{3+}			μM		La^{3+}		mM		
	0.1	0.5	2.5	5.0	10	0.5	1.0	2.5	5.0	10	0.01	0.05	0.1	0.25	1.0
KCl 10 mM	–	–	+	+	0	–	+	+	0	0	–	–	+	+	0
$CaCl_2$ 10 mM	–	–	–	–	–	–	–	–	–	+	–	–	–	–	+
KCl 100 mM	–	–	–	–	–	0	0	0	0	0	–	–	–	–	+

(+) Effect observed. (−) Effect not observed. (0) No data.

RTX channel did not differ qualitatively from those obtained with α- or β-LTX, but it should be noted that Cd^{2+} was a weaker blocker of RTX channels: 5 mM cadmium added to a 100 mM KCl solution induced only a 50% conductance decrease.

For all proteins, blocking was at least partly reversible. Perfusion of the chamber with 5–7 volumes of blocker-free solution restores a significant part of the initial conductance (up to 100% for Cd^{2+} and 40–70% for La^{3+}-induced block).

Cd^{2+}, La^{3+} and Tb^{3+} Sensitivity of LTX Channels

To determine the minimum concentration of blocking ion needed to produce a detectable decrease of LTX channel conductance, we added different concentrations of blocking ions to channel-containing BLM's. The extent of blocking was calculated by dividing the minimum value of conductance, after addition of the blocker, by its original value. It was found that an addition of 1 μM of $TbCl_3$ to 10 mM KCl solution blocks 90 ± 5% of α-LTX-induced conductance of BLM. Results of a more detailed study of Cd^{2+}- and La^{3+}-induced blocking are presented in Fig. 3. We also observed two interesting effects in the channel blocking induced by low concentrations of La^{3+} or Cd^{2+}. First, the lowest blocker concentrations frequently induced conductance increases instead of decreases. Second, intermediate concentrations (25–100 μM of $CdCl_2$) induced transient conductance changes (Fig. 4) that evolved with time in a very similar way to the capacitance changes induced by high blocking ion concentrations. Only after the addition of blocking ion at concentrations close to those needed to induce capacitance changes did we observe permanent blocking of the LTX channels.

pH-induced Changes in BLM Capacitance and Channel Conductance

In preliminary experiments, we found that a pH change from 7.4 to 5.8–6 or to 8.6–9 did not significantly change either the capacitance of the BLM or the con-

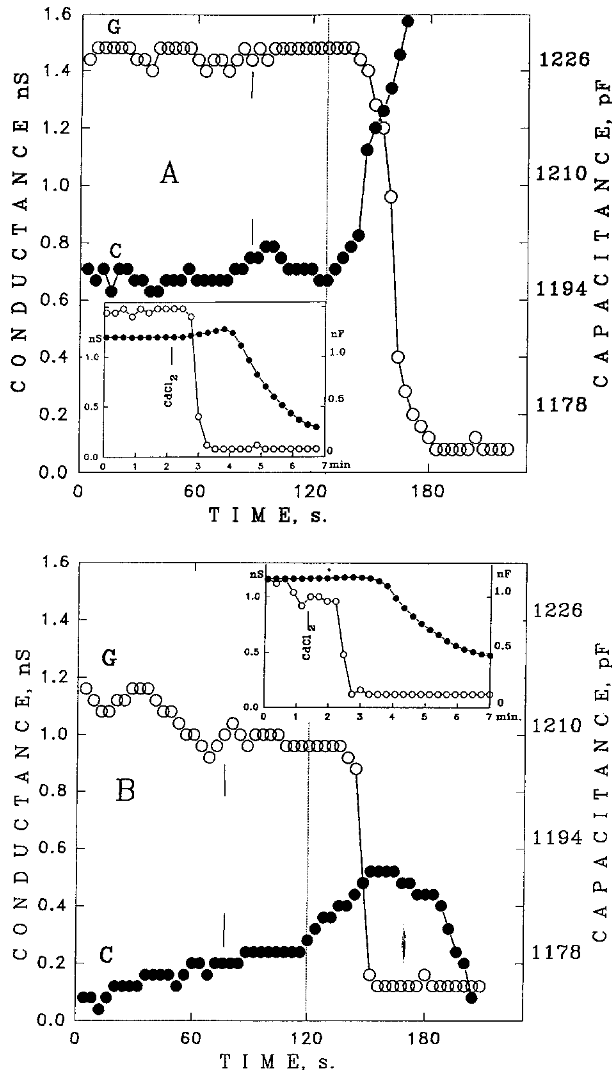

Fig. 2. Cadmium–induced changes in the conductance (open circles) and capacitance (filled circles) of LTX-containing membranes. (*A*) α-LTX. (*B*) β-LTX. Vertical bars indicate the moment of cadmium addition to a final concentration of 3 mM. Insets show same experiments but in full scale for capacitance and over a longer period of time.

ductance of incorporated α-LTX channels (*data not shown*). However, dramatic effects were observed when pH was shifted to either 11–12 or 3–4. A typical record of capacitance/conductance changes induced by KOH addition to the BLM that does not contain channels is shown in the inset of Fig. 5. The period between the beginning of the transient changes of capacitance and a conductance increase followed by membrane disruption is consistently long enough (54 ± 5 sec, $n = 4$) for the registration of the conductance changes of LTX channels. As can be seen (Fig. 5), pH-induced membrane transformation also correlates with a decrease in channel conductance. Two to four additional channels

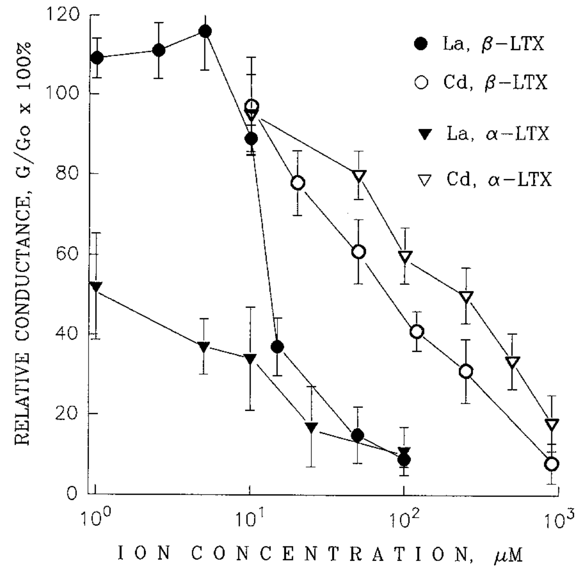

Fig. 3. Dependence of channel blocking on blocker concentration.

always appeared in the membrane after addition of KOH. Nevertheless, it was possible to see a gradual decrease of conductance for a fixed number of channels in the membrane.

We obtained similar records after the addition of HCl to the bathing solution causing a pH shift from pH 7.4 to 3–4 (*data not shown*).

Discussion

It is known that BLM capacitance changes induced by changes in the ionic composition of bathing solution are caused by the adsorption of ions on the phosphate head groups of lipids (Papahajopoulos, Vail & Newton, 1977; McLaughlin, Grathwohl & McLaughlin, 1987). A number of ions, including La^{3+} and H^+, are able to cause dramatic changes in lipid ordering when their concentrations in solution reach some threshold value (Ohki & Duax, 1986). Alteration of lipid packing in the bilayer induces secondary changes in membrane area, in the geometry of microlenses and also in solvent redistribution (Chizmadzev et al., 1985; Omelchenko & Browykin, 1988). The involvement of these last two phenomena is supported by the appearance of visibly colored areas of BLM. These secondary effects indicate that changes in lipid packing in the membrane are quite extensive.

In this connection, the first question we need to answer is about the possibility that the main effect of the conductance decrease is membrane thickening which prevents the protein from spanning the membrane. This possibility can be excluded because parallel capaci-

Fig. 4. The time course of cadmium-induced changes in α-LTX channel conductance. The arrows show addition of cadmium to a final concentration of: 1–25 μM, 2–1 mM.

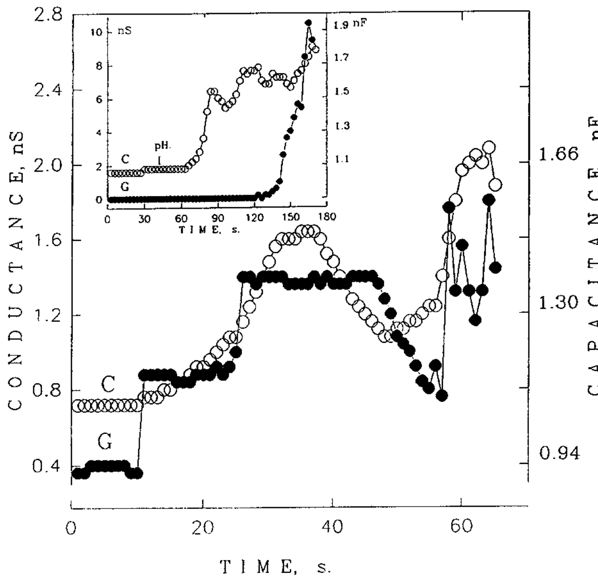

Fig. 5. The time course of pH-induced changes of BLM capacitance and latrotoxin channel conductance. Zero time corresponds to the time of KOH addition. At that time, only one α-LTX channel was present in the membrane. Two other channels were inserted into the BLM after the beginning of the capacitance increase. The inset shows the effect of pH on a BLM that did not contain any ionic channels. A vertical bar and *pH* shows the moment when 50 μl of KOH was added to the *cis* compartment. In all experiments, no changes of membrane conductance were observed for at least 70 sec from this moment. Bathing solution: KCl: 200 mM, Tris: 10 mM, pH: 7.4. Potential across membrane: +50 mV.

tance and conductance measurements show that the membrane conductance decreases to steady-state level at least 30 sec before the beginning of the membrane thickness increase (Fig. 2). Another solvent-related phenomenon, shift of the proteins into the torus, cannot be excluded completely, but it is unlikely to be responsible for a 90% conductance decrease because the perfusion of the chamber with blocker-free solution restores a significant part of the original conductance.

The initial increase of BLM capacitance indicates that the ionic composition of the adjacent buffer layer has changed enough to trigger lipid redistribution. At this moment, the channel conductance is determined by protein/lipid/ion interaction and is not yet affected by the increase of membrane thickness due to solvent entry between monolayers. In the case of blocking ions, the most interesting question is not why and how channels become blocked long after blocker addition, but why they are not blocked when, according to capacitance measurements (Fig. 2), there are high concentrations of blocker near the channel entrance.

It is known that protein-boundary lipids have a highly ordered structure (Smith & Stubbs, 1987; Misley et al., 1987) and might determine the conformation of an ionic channel. Thus, it is possible that these lipids are equally responsible for the channel blocking after replacement of one cation by another. In this case, relatively slow cooperative changes in lipid organization around protein molecules should be observable as changes in channel conductance.

This explanation is consistent with the observed correlation between the propensity of certain cations to induce changes in lipid order (Tb^{3+} > La^{3+} > Cd^{2+}, Table) and the gradation of their blocking potency. It also provides a basis for understanding why the addition of blocking ions induced changes in channel conductance (Fig. 4) that had a similar time course to BLM capacitance changes (Fig. 1). (The "inactivation" of blocking ion shown in Fig. 4, and the increase of channel conductivity at low blocker concentration (Fig. 3), certainly cannot be explained in terms of simple channel-ion interactions.)

Our experiments with pH shifts also provide support for the idea that lipids may be involved in the regulation of ion transport through channels. While the interaction of phospholipid with protons is certainly different from the interaction with polycations, the resulting changes in membranes have much similarity with polycation-induced changes. It is known that for charged lipids, critical pH values can cause a change in molecular packing (Van Dijck et al., 1978; Ohki & Duax, 1986). Just as for Cd^{2+}, La^{3+} or Tb^{3+}-induced effects, there is a threshold concentration of H^+ ion

which induces significant changes in BLM capacitance and channel conductance. It has been shown recently that pH changes may also affect molecular packing of pure PC bilayers (Massari et al., 1991). Taking this into account, we can suppose that for the zwitterionic lipids used in our study, molecular packing changes may be caused by both high or low pH treatment.

Perhaps the most interesting result of our pH experiments is that even when ions cannot directly affect channel conductance by binding to the selective centers, ion-induced changes of the bilayer are sufficient to affect channel conductance. A direct influence of pH on protein conformation is possible, but considering the similarity in action of both high and low pH and an absence of any conductance changes below threshold concentration, it seems more likely that the pH-induced changes in channel conductance are mediated through the bilayer lipids by lipid packing.

In summation, our data suggest that the reorganization of the bilayer lipids, caused by different agents, correlates with the conductance of incorporated protein channels. Two mechanisms may be proposed to explain this effect. First, it may mean that these channels are flexible enough to change the configuration of their ion pathway when lipid-contacting domains are affected by changes in lipid packing—a mechanism similar to one suggested for the regulation of protein kinase C (Epand et al., 1991). Second, it may mean that lipids participate directly in the formation of a pore, as suggested for some short membrane active polypeptides (Grant et al., 1992) or for lipid-protein complexes that form fusion pores (Zimmerberg, Curran & Cohen, 1991). In this context, it is worth mentioning that α-LTX is a protein which can promote membrane fusion (Sokolov, Chanturiya & Lishko, 1987).

Whichever mechanism is true, these channels must be regarded not simply as proteins in a lipid bilayer, but as protein-lipid complexes.

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