Calmodulin Interaction with Mesocaine-Modified Lipid Bilayer

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Abstract. Calmodulin (CaM) interactions with bilayer lipid membranes (BLM) were studied by measuring modulus of elasticity in direction perpendicular to the membrane plane ($E_\perp$) and intramembrane potential $\Delta\psi$. Upon interaction of CaM with egg phosphatidylcholine and asolectin BLM the parameter $E_\perp$ grew slightly (not more than by 10 % as compared to the respective value for nonmodified BLM), suggesting a weak effect on the ordering of the hydrophobic moiety of the lipid bilayer. In the presence of mesocaine (Mes), a calmodulin inhibitor, CaM affected the incorporation of Mes into the membrane. It can be concluded that CaM affects the ordering of the polar (superficial) membrane region.

Key words: Calmodulin — Mesocaine — Lipid bilayers — Modulus of elasticity — Intramembrane potential

Introduction

Calmodulin (CaM) is a cytosolic protein. It activates a number of membrane systems such as adenylate cyclase or Ca^{2+}-ATPase (Cheung 1980). CaM is activated at Ca^{2+} concentrations of 1—10 μmol/l. It undergoes conformation changes which stabilize its tertiary structure, and expose its hydrophobic region (Babu et al. 1985). Via this region calmodulin interacts with several enzymes and agents, including drugs. Several agents (neuroleptics, local anesthetics) inhibit

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calmodulin. They bind to its hydrophobic region and prevent it from activating the respective enzymes (Tanaka and Hidaka 1981).

The binding of calmodulin to membrane fragments and/or lipids may play a crucial role in its regulatory function (Permyakov et al. 1988). Methods employing protein incorporation into artificial bilayer lipid membranes (BLM) are being increasingly used to study protein-lipid interactions as they simulate in their physico-chemical characteristics, genuine biomembranes (Tien 1974). The determination of viscoelastic characteristics of membranes, which are sensitive to structural alterations of lipid bilayer induced by protein incorporation, is a valuable tool for studying protein-BLM interactions (Passechnik et al. 1981; Hianik et al. 1988).

The aim of the present work was to investigate the mechanism of interaction of calmodulin with lipids and with BLM modified by local anesthetic (mesocaine). It was attempted to establish whether CaM can incorporate into the hydrophobic membrane region and alter its ordering, or whether CaM only interacts with the polar surface of the BLM, and how this interaction is modified by the presence of mesocaine (Mes) in BLM. Interactions of CaM with BLM and Mes were studied by measuring modulus of elasticity $E_\perp$ in direction perpendicular to membrane surface, and of intramembrane potential difference $\Delta\psi$. Parameter $E_\perp$ characterizes the elasticity of the inner hydrophobic membrane region upon its deformation induced by transversal pressure p. A strongly ordered bilayer is characterized by slight changes in membrane thickness $\Delta h/h$, and thus by a higher value of $E_\perp$ ($E_\perp = -p/(\Delta h/h)$) as compared with disturbed ordering of the hydrophobic region.

Materials and Methods

BLM were prepared according to the method of Mueller et al. (1962) on a circular hole (with a diameter $d \sim 0.5$ mm) in the wall of a teflon cell. The cell was divided by the wall into two identical compartments, 3 ml each. Both compartments were filled with electrolyte. BLM were prepared of a mixture of egg phosphatidylcholine (egg PC) (Kharkov Plant of Chemical Preparations, USSR) with cholesterol (CH) (Fluka) (4 : 1 w/w) in *n*-heptane (Kodak) in a concentration of 20 mg/ml, and of a mixture of asolectin (AL) (Sigma) with cholesterol (4 : 1 w/w) in *n*-heptane (20 mg/ml). BLM prepared of the above mixtures differ from each other by their charges. At the electrolyte pH used (7.2—7.4), egg PC + CH BLM are electrically neutral, whereas BLM of AL + CH bear a marked negative charge (Sokolov et al. 1980). The electrolyte used was 0.1 mol/l KCl + 5 mmol/l Tris-HCl (pH 7.2—7.4). Calcium concentration in the electrolyte was checked by ion selective electrode, and values of 0.1 mmol/l were characteristic for all series of experiments. At this concentration, all the four binding sites of CaM were occupied by calcium. On the other hand at the concentration of 0.1 mmol/l, calcium does not influence mechanical properties of BLM (see Passechnik et al. 1981). The preparations used were of analytical purity.

CaM was isolated in Centre of Physiological Sciences, Slovak Academy of Sciences (Križanová et al. 1986), with a 91 % purity. Mesocaine (Léčiva, Prague) was used as CaM inhibitor.

Parameters $E_\perp$ and $\Delta\psi$ were measured with the use of the electrostriction method. Membrane elasticity was measured using a special method as described by Passechnik and Hianik (1977). The basic principles of the method are as follows. Alternating voltage $U = U_0 \sin 2\pi ft$ (where U_0 is the voltage amplitude, f is its frequency, and t is time) applied to the membrane produces pressure $p = C_s U^2/2h$ (where C_s is the specific membrane capacity, and h is the membrane thickness), resulting in membrane attenuation due to electrostriction.

Membrane compression by alternating electric field results in modulation of alternating current flowing through the membrane. A current component with frequency $3f$ and amplitude A_3 arises in current with frequency f and amplitude A_1 ($A_1 = 2\pi fCU_0$), whereby $A_3 \ll A_1$. The capacity of membrane to change its thickness in response to the action of an external force is characterized by modulus of elasticity in direction perpendicular to the membrane plane, $E_\perp$. Parameter $E_\perp$ can be described by

$$E_\perp = 3C_s U_0^2 A_1/4hA_3 \tag{1}$$

Voltage $U_0 = 140$ mV was applied to the membrane. The following parameter values were used to calculate $E_\perp$: $C_s = 3.4 \times 10^{-3}$ F/m^2, $h = 5.5$ nm (Hianik et al. 1984). BLM of egg PC + CH and those of AL + CH had similar values of C_s and h.

Intramembrane potential, $\Delta\psi$, is produced as the result of adsorption of charged particles to membrane. In this case, the second current harmonic with amplitude A_2 and frequency $2f$ will be generated in addition to the third current harmonic (Carius 1976). The value of $\Delta\psi$ is then calculated by

$$\Delta\psi = U_0 A_2/4A_3 \tag{2}$$

This means that it is sufficent to measure amplitudes A_1, A_2, and A_3 to determine $E_\perp$ and $\Delta\psi$. This can be done with the use of a standard electronic apparatus including resonance amplifiers (Passechnik and Hianik 1977).

To study BLM interaction, CaM (0.01—1 μmol/l) was added to the electrolyte at one side of formed membrane approximately 10 min after the start of membrane formation, or directly into the lipid solution used to prepare the membrane. CaM concentrations in latter case were 60—120 μg/ml. Either lyophilized CaM or 0.10% ethanolic solution was added to the lipid solution. In one experimental series, the lipid solution containing CaM was sonicated in an ultrasound disperger (Tesla) according to Mirsky et al. (1984), to obtain homogenous volume distribution of the protein.

In experiments with Mes, the local anesthetic was added to the electrolyte at both sides of the membrane in concentrations of 0.6—12 mmol/l.

Results

1. CaM-BLM interactions

Two methodical approaches were employed to determine the degree to which CaM is able to modify mechanical characteristics of BLM: CaM was added to the electrolyte or directly to the lipid solution used to prepare BLM.

1.1. CaM in the electrolyte

CaM was added to the electrolyte in increasing concentrations of 0.01, 0.1, and 1 μmol/l. The addition of 0.01 or 0.1 μmol/l CaM to the electrolyte at one

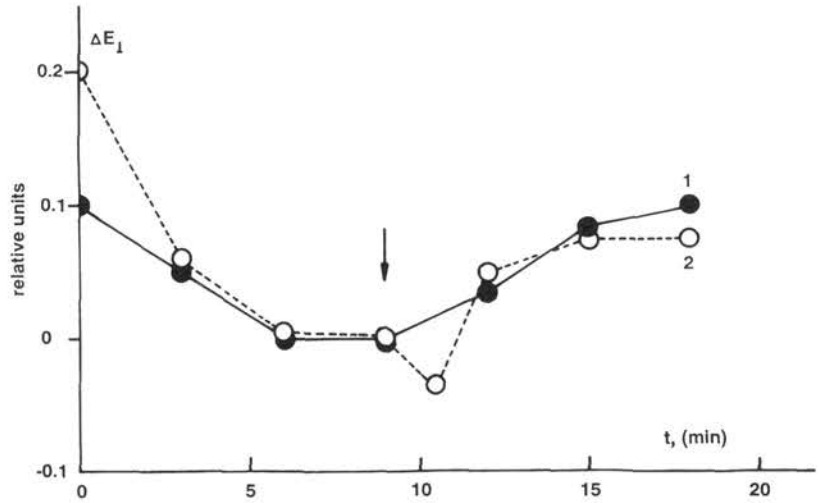

Fig. 1. Kinetics of relative changes of modulus of elasticity $\Delta E_\perp = (E_\perp - E_0)/E_0$, for the two BLM types following the addition of CaM (final concentration 1 μmol/l). 1-egg PC + CH, 2-AL + CH. The arrow shows CaM addition.

side of egg PC + CH and/or AL + CH BLM did not result in any changes in $E_\perp$. At 1 μmol/l CaM, a slight increase in BLM modulus of elasticity was observed. Changes in relative modulus of elasticity $\Delta E_\perp = = (E_\perp - E_0)/E_0$ (where E_0 is the modulus of elasticity prior to CaM addition) for egg PC + CH BLM (Fig. 1, curve 1) and for those of AL + CH (Fig. 1, curve 2) did not exceed 10%. The transmembrane potential remained unchanged, reaching values of approx. 0.05 mV. The initial decrease of $\Delta E_\perp$ is associated with BLM formation (Passechnik and Hianik 1977). Fig. 1 shows that the process of CaM-BLM interaction is associated with a slight increase of modulus of elasticity, i.e. the ordering of the lipid bilayer is altered but weakly.

1.2 CaM in the lipid solution

In this experimental series, CaM was added directly to the lipid solution of egg PC + CH, used to form BLM. The values of $E_\perp$ for 9—16 membranes in each of the 5 series are shown in Table 1. Series II—V differ in CaM concentrations and in conditions of CaM dissolution in the lipid solution. Statistically significant differences (according to Student's t-test) were observed in the value of $E_\perp$ only for series II and III. This could be probably due to the presence of ethanol in the lipid solution. During BLM formation ethanol dispersed out into the

Table 1. The values of $E_\perp$ for BLM of egg PC + CH (4 : 1 w/w) (I) and for the same BLM formed of lipid solution containing different concentrations of CaM under different conditions. II — CaM dissolved in 0.1 % ethanol and added into the lipid solution (60 μg protein per 1 ml lipid solution). III — the same as II, CaM concentration 120 μg/ml. IV — lyophilized CaM added into the lipid solution at 120 μg/ml. V — the same as IV, but the lipid solution containing CaM sonicated in an ultrasound disperger.

Series of experiments	Number of membranes	($\bar{E}_\perp \pm$ S.E.) 10^7 Pa
I	10	4.66 ± 0.54
II	10	7.49 ± 1.35
III	16	7.54 ± 0.77
IV	9	3.15 ± 0.30
V	9	3.99 ± 0.10

electrolyte and the membrane becomes less compressible because of the decreasing concentration of membrane solvent (see Passechnik and Hianik 1977). These experiments showed that BLM interaction with CaM is not markedly reflected in changes of modulus of elasticity $E_\perp$ or of intramembrane potential difference $\Delta\psi$. Therefore it is likely that CaM-membrane interaction requires presence of specific receptors in the lipid bilayer, or at least, the existence of defined sites (polar groups of certain agents) on the membrane surface. This prompted us to try to model a similar specific interaction of CaM with membrane. Local anesthetic mesocaine was employed as the binding site model. Mes can interact with both CaM (Orrego et al. 1983) and membrane (Ondriáš 1984).

2. CaM interaction with Mes-modified BLM

2.1 Mes interaction with BLM

We have shown (Hianik and Palacková 1985) that the local anesthetics carbisocaine and lidocaine affect the mechanical characteristics of membranes. The first experiments were therefore designed to examine the effects of Mes on mechanical characteristics of BLM. Mes is very similar in its structure to lidocaine (Fig. 2*a*), the only difference being an additional CH_3 group in the Mes molecule. This group is attached to the phenyl ring in position C_4. Mes was added to the electrolyte at both sides of AL + CH membrane. The final concentrations of Mes in the solution ranged between 0.6 and 12 mmol/l. The measurements were performed on 13 membranes, and changes in modulus of elasticity $E_\perp$ and of intramembrane potential difference $\Delta\psi$ were determined in dependence on Mes concentration in the electrolyte. Fig 2*b* shows the depen-

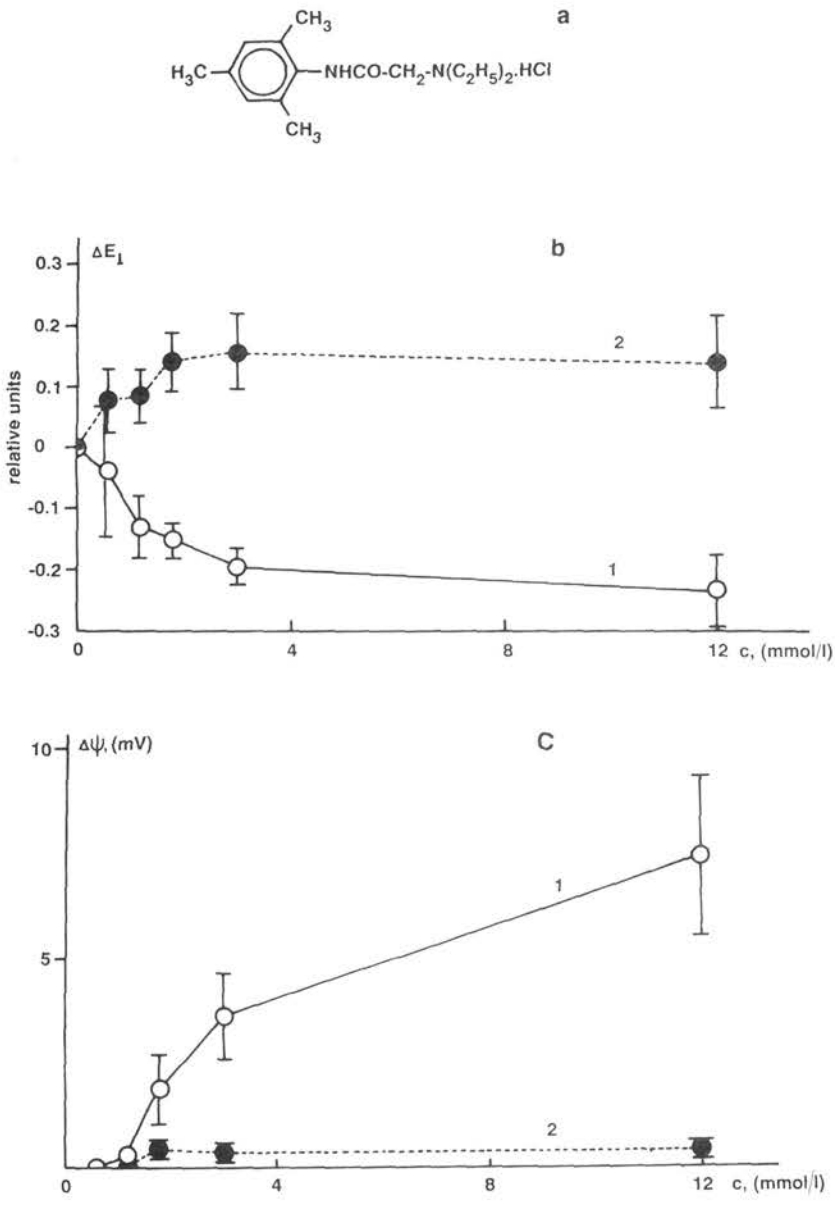

Fig. 2. *a*. Structural formula of mesocaine. *b*. The dependences of relative change of modulus of elasticity $\Delta E_\perp$ and of intramembrane potential difference. *c*. On Mes concentration in the electrolyte for two groups of membranes of identical composition showing different patterns of changes of $\Delta E_\perp$ and $\Delta\psi$. 1-$\Delta E_\perp > 0$, $\Delta\psi \cong 0$, 2-$\Delta E_\perp < 0$, $\Delta\psi > 0$.

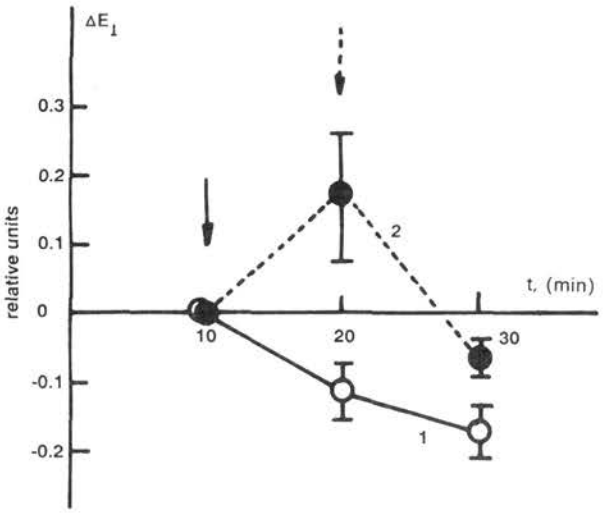

Fig. 3. Kinetics of changes $\Delta E_\perp$ during the addition of Mes and CaM. ↓-addition of Mes in a final concentration in the electrolyte of 12 mmol/l, ↓-addition of CaM in a final concentration in the electrolyte of 1 μmol/l. 1,2- two groups of membranes (see Fig. 2).

dence of relative changes of modulus of elasticity $\Delta E_\perp = (E_\perp - E_0)/E_0$ (where E_0 is the modulus of elasticity of nonmodified BLM, and $E_\perp$ is that following the addition of the respective concentration of Mes) on Mes concentration in the electrolyte for two different groups of membranes. Growth of $E_\perp$ ($\Delta E_\perp > 0$, curve 2) was observed for one group of membranes ($n = 5$), whereas the other group of membranes ($n = 8$) showed a decrease of this parameter ($\Delta E_\perp < 0$, curve 1). The respective curves of $E_\perp$ kinetics showed, in several cases, two-phase pattern (monotonous and nonmonotonous growth or decrease). No changes of parameter $\Delta\psi$ were observed for the first group ($\Delta E_\perp > 0$) (Fig 2*c* curve 2), whereas an increase of $\Delta\psi$ was observed for membranes with the opposite kinetics of changes of modulus of elasticity (Fig. 2*c*, curve 1).

2.2. CaM interaction with BLM in the presence of Mes

Based on the assumption that CaM is able to interact with Mes (Mes is CaM inhibitor, see Tanaka and Hidaka 1981), and on the observation that Mes is also able to bind to the membrane, experiments were designed to study CaM interactions with Mes and BLM in dependence on the sequence of the additions of

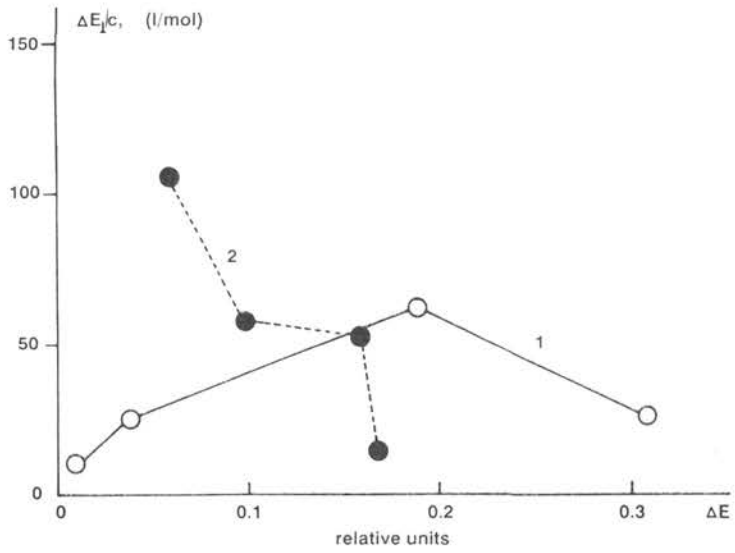

Fig. 4. Typical example of the Mes concentration dependence of relative changes of modulus of elasticity $\Delta E_\perp$. Scatchard plots for membranes with increasing (1) and decreasing (2) value of $E_\perp$.

these agents into the electrolyte. CaM was added up to a final concentration of 1 μmol/l to one membrane side, Mes (up to a final concentration of 12 mmol/l) to both sides of AL + CH membrane. Fig. 3 shows the kinetics of the dependencies of relative change of modulus of elasticity, $\Delta E_\perp$ for two different groups of membranes. Mean values obtained for 8—9 membranes in each experimental series are shown. Fig. 3 shows that when Mes was added, the effects were similar as those described in previous section. Subsequent addition of CaM always resulted in a decrease of modulus of elasticity (Fig. 3) curves 1, 2. Curves drawn through filled and empty circles represent the respective membrane groups (see Fig. 2).

Discussion

Let us now evalute the results obtained in experiments with the CaM incorporation into BLM (1), with the Mes interaction with the membrane (2), and with CaM interactions with both Mes and lipid bilayer (3).

1. CaM is a globular protein of peripherical type, predominantly containing hydrophilic regions and carrying negative charge at neutral pH of the

electrolyte. Hydrophobic interactions are possible but after CaM has been saturated with calcium. Then the helicity of the protein changes from 5—10% to 40—55%, thus exposing the hydrophobic part (Babu et al. 1985). The coefficient of hydrophobicity calculated from bovine brain CaM structure (Permyakov 1985) using the method of Tanford (1978), reaches the value of $H = 1.076$; this value is intermediate between the values of H typical of cytosol and those of peripheral proteins (Cantor and Shimmel 1980). Moreover, both the composition and the geometry of CaM molecule do not allow interaction with the hydrophobic part of the membrane. Hence, the slight growth of $E_\perp$ observed with BLM upon the addition of CaM to the electrolyte may be due to the action of the molecule on the polar part of the lipid bilayer. With another process of CaM membrane incorporation (CaM added directly to the lipid solution used to prepare BLM of), hydrophobic interactions are also difficult to occur. Although the CaM molecule has the form of a dumbbell with two pairs of binding sites for calcium (~ 6.5 nm from each other), the "handle" contains large numbers of polar amino acids. This does not allow CaM to become localized transversally in the membrane. Hence, during BLM formation CaM most likely is expulsed from the lipid bilayer to the membrane surface.

2. Results obtained in experiments with the action of Mes on BLM pointed to the existence of the two patterns of changes of modulus of elasticity. $E_\perp$ grew for one group of membranes, while decreasing for the other group of membranes (Fig. 2*b*). The results obtained make us to conclude that the observed changes of $E_\perp$ might be associated with membrane inhomogeneity as well as with the existence of a certain asymmetry of the arragement of the individual monolayers. This inhomogeneity and asymmetry can result from a certain oscillation of cholesterol concentrations in BLM and from the dynamics of the formation of mixed clusters (of cholesterol and egg PC) (Hianik et al. 1984). Then, the intramembrane potential can also be generated due to different rates of Mes binding to the individual inhomogenous regions at both sides of the membrane.

To analyze the pattern of Mes incorporation into the membrane, Scatchard plots of $\Delta E_\perp/c$ vs. $\Delta E_\perp$ i.e. the concentration dependence of $\Delta E_\perp$ were constructed for individual membrane groups (c is the concentration of Mes in electrolyte). This approach allowed us to analyze in more details the cooperativity pattern. For the above relationship holds

$$\Delta E_\perp/c = c^{m-1}K(N - \Delta E_\perp),$$

where K is the binding constant, N is the number of binding sites at the membrane, and $\Delta E_\perp/c$ represents the distribution coefficient for the concentrations of free and bound agent. Three different cooperativity patterns can be

obtained depending on the Hill coefficient m: for $m > 1$ the processes are positively cooperative, for $m = 1$ they are non-cooperative, and for $m < 1$ they show negative cooperativity. Fig. 4 shows typical dependences $\Delta E_\perp/c$ ($\Delta E_\perp$) for two membrane groups, one with growing (1) and the other with decreasing (2) modulus of elasticity. Positive cooperation was detected in the membranes with decreasing modulus of elasticity, wheras non-cooperative and/or negatively cooperative behavior was observed with membranes showing increasing $E_\perp$.

Positive cooperativity means that Mes incorporated into BLM facilitates the incorporation of further molecules. Since this is associated with a decrease of modulus of elasticity, this process can be considered, from the structural point of view, as being associated with decreased ordering of the membrane. Hence, Mes can more easily incorporate into membrane sites showing less ordered structure. The facilitated incorporation of Mes secondary to the mentioned process of cooperativity can give rise to intramembrane potential, if the process is asymmetrical. In contrast, non-cooperative incorporation of Mes observed with BLM showing increasing $E_\perp$ may correspond to the incorporation of the local anesthetic into non-ordered regions. There, Mes can induce a partial increase of ordering. Moreover, transmembrane diffusion of Mes is facilitated in non-ordered membrane regions, so that no charge asymmetry arises ($\Delta\psi = 0$). The polar membrane region most likely playes the crucial role in the way Mes is incorporated into BLM. This is due to the fact that the two membrane groups (those with increasing and those with decreasing $E_\perp$) cannot be subdivided into groups according to the initial modulus of elasticity E_0 of nonmodified membranes. It should be noted that for membranes of identical lipid composition (AL + CH), the value of E_0 varied between 3×10^7—1.4×10^8 Pa, due to oscillations of solvent (*n*-heptane) concentrations during BLM formation according to Mueller.

Based on the above results it can be concluded that the pattern of Mes incorporation is associated with a defined type of the cooperative mechanism, which in turn is linked to the BLM structure.

3. As follows from Fig. 3, CaM influences parameter $E_\perp$ of BLM modified by Mes. CaM can affect, at least in two ways, parameter $E_\perp$ of local anesthetic-modified BLM: 1. By pulling Mes out of the membrane, due to which the BLM ordering can change. 2.CaM can interact with the membrane surface thus influencing the incorporation of further Mes molecules into BLM. With the molar CaM/Mes polar ratio used (approx. 10^{-4}) CaM in the solution is unlikely to pull Mes out BLM. The major action of CaM obviously is that on the membrane polar region. This may result in enlargement of area per one lipid and a consequent decrease of intramolecular interactions between lipid hydrocarbon chains. Van Dael and Van Cauwelaert (1988) came to a similar conclusion in studying effects of low concentrations of another calcium binding protein,

α-lactalbumin (BLA) with unilamellar vesicles: in their experiments BLA behaved as a peripheral protein. The less ordered membrane structure may then be better accessible for the incorporation of further Mes molecules.

Thus, our results support the idea that calmodulin interacts with the membrane. Responsible for this interaction is mainly the polar membrane region.

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