

Angiotensin II-Induced Formation of Ionic Channels in Bilayer Lipid Membranes

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Abstract. The interaction of angiotensin II (ANG II) with membrane was studied by measuring conductance and current-voltage characteristics (IVC) of bilayer lipid membranes (BLM) prepared of a mixture of egg lecithin with cholesterol, and of gramicidin D-modified membranes of the same composition. Addition of physiological concentrations of ANG II (approx. 15 $\mu\text{mol/l}$) into the electrolyte (1 mol/l KCl, pH = 7) in contact with one side of BLM resulted in the appearance of discrete membrane conductance $A = (39.5 \pm 1.07)$ pS with a duration of the conductivity state $\tau = (52.15 \pm 6.44)$ s. Raising ANG II concentration to 75 $\mu\text{mol/l}$ resulted in an additional conductance level of approx. 130 pS with a lifetime of approx. 1 s. The electrolyte pH markedly influenced ANG II modified BLM conductance. A decrease of the electrolyte pH to 2.8 resulted in a reduction of the discrete conductance level to approx. 14 pS, whereas ANG did not induce any conductivity at pH = 11.5. The results obtained suggest that ion channels are formed consisting at least of two ANG II molecules. IVC of ANG II-modified BLM are superlinear within the range of electrolyte concentrations studied (between 0.01 and 3 mol/l KCl), i.e. the limiting stage of ion transport is the internal area of the conducting pore. ANG II affects in a cooperative manner the gramicidin D (GRD)-mediated transport, most likely by forming ANG II aggregates in the area of local inhomogeneities in the BLM structure of GRD channels.

Key words: Angiotensin II — Bilayer lipid membranes — Ionic channels — Current-voltage characteristics — Gramicidin D

Introduction

Angiotensins are biologically active peptides able to affect smooth muscle motorics. They play a significant role in the maintenance of extracellular fluid homeostasis and in the central nervous system (CNS) control of blood pressure

(Peach 1977; Philips 1980). Angiotensin II (ANG II) is one biologically active agent of a number of different angiotensins. It is a linear octapeptide: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe. In studying the mechanism of ANG II action, the site of its initial action is of special interest. Paglin et al. (1987) observed a weak nonspecific interaction of ANG II binding to the same membrane; however, the authors did not further investigate nonspecific binding, considering it as being the result of the radioligand entering the liquid membrane phase during the incubation. It is generally accepted that hydrophobic effects play a significant role in the incorporation of short peptide chains, such as angiotensin, into lipid bilayer membranes (Jain and Zakim 1987). Only amino acids with aliphatic or aromatic side chains can reach the nonpolar part of the bilayer from the water solution. The transfer of the other functional groups is energetically disadvantageous. The side chains of ANG include, a.o. Phe, Tyr, Val, and Ile residues, i.e. aromatic and aliphatic residues; this could effectively allow ANG to penetrate into the lipid bilayer.

Similarly as the site of ANG action, also processes which follow ANG binding to the membrane are subject of intense studies. Most authors are prone to believe that the mechanism underlying the action of vasoactive hormones is the ability of the hormones to regulate ion transport. However, numerous hypotheses have been proposed as for the actual operation of such a regulation. According to Smith et al. (1985), ANG II stimulates the production of inositol trisphosphate which in turn induces, by opening intracellular Ca^{2+} channels, the release of bound calcium into the cytoplasm. On the other hand, intravenous infusion of the octapeptide ANG II was shown to raise (by 1/3) the concentration of K^+ in arterial blood plasma of anesthetized sheep (Osborn et al. 1985), with a simultaneous decrease in plasma Na^+ and Cl^- concentrations at unchanged plasma Ca^{2+} concentration. The increase of the K^+ levels in the circulation was ANG-dose dependent. According to Smith and Smith (1987), ANG II affects the activity of the furosemide-inhibitable $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransport in vascular smooth muscle cells, acting similarly as do the two calcium transporting ionophores A 23187 and ionomycin. Removal of extracellular Ca^{2+} abolished the cotransport stimulation induced by A 23187 and ionomycin, but not by ANG. Prompted by the so far unexplained questions concerning the action of ANG II on biological membranes, in particular the nonspecific effects on membranes and transport systems within them, we tried to investigate the effect of ANG II on bilayer lipid membranes (BLM) which represent an adequate model of biological membranes (Tien 1974). Moreover, ANG II effects on gramicidin D (GRD)-modified BLM were studied. In one series of experiments the kinetics of BLM conductance changes were determined. Another series of experiments were designed to estimate coefficient of nonlinearity of current-voltage characteristics (IVC) of membranes modified with ANG II.

The methods employed for the present experiments give a comprehensive picture of transport processes operative in membranes modified by a drug. Kinetic measurements provide information concerning the nature of conductance changes in dependence on the modifier concentration, lifetime, etc.: based on this, conclusions can be drawn as to the nature of drug induced changes in membrane conductance. Measurements of coefficient of nonlinearity β of IVC allow to study the dynamics of ion transport across membranes. In this way, transport mediated by a channel-forming agent and/or a carrier can be distinguished from each other (Hianik et al. 1987a), conclusions can be drawn as to the limiting stage of ion transport across the membrane under the given experimental conditions (Passechnik et al. 1985), suggestion can be made as to the site and relative heights of potential barriers to ion transport across the membrane.

Materials and Methods

In both types of experiments (determination of conductance kinetics and measurement of coefficient β) BLM prepared by the method developed by Mueller et al. (1962) were used. According to this method, BLM are formed on a circular hole (approx. 0.5 mm in diameter) in the wall of a teflon cup. The wall divides the cup into two compartments of equal volume (approx. 3 ml each), filled with electrolyte, such as water solution of KCl. With the aid of a glass pipette, a small volume of lipid solution (containing lipid and hydrocarbon solvent, such as *n*-heptane) is applied on the hole. Due to hydrophobic interactions, a lipid bilayer starts forming within a short interval of time (approx. 1 min), reaching a thickness of approx. 5 nm. The lipid solution used to form BLM contained egg lecithin (Kharkov Plant of Chemical Preparations, USSR) and cholesterol (Fluka), in a 4:1 weight ratio, dissolved in *n*-heptane (Fluka), 20 mg/ml. At first approximation, BLM of this composition represent an adequate model of the lipid bilayer of the plasma membrane; at the same time, the model is a matrix suitable for spontaneous incorporation of ANG. According to the hypothesis forwarded e.g. by Jain and Zakim (1987), the process of incorporation requires the occurrence of local "defects" in the bilayer; these defects can be induced by the presence of another, not bilayer, phase, by lysophospholipids, fatty acids, detergents, or cholesterol. In studying the conductance kinetics, 1 mol/l KCl in redistilled water was used as the electrolyte. Different KCl concentrations (0.01; 0.03; 0.1; 0.3; 1; and 3 mol/l) were used to measure coefficient of nonlinearity β of IVC. Angiotensin (kindly supplied by T. L. Davydovskaya, Kiev State University, USSR) was added to the electrolyte in contact with one side of the membrane, in final concentrations in the electrolyte between approx. 10^{-4} and 10^{-5} mol/l. This concentration range is considered physiologically effective (Piskorskaya et al. 1983). Ethanol dissolved GRD (GRD contains about 80% of gramicidin A) (P-1 Biochemicals) was added in a similar way (i.e. to the electrolyte in contact with one side of the membrane). The concentrations of ethanol in the electrolyte did not exceed 0.7%. The chemicals used were all of analytical grade. The experiments were performed at $T = 20^\circ\text{C}$. The measurements of BLM conductance kinetics were performed using the known method of Hladky and Haydon (1972). One calomel electrode, immersed into one compartment, was connected to a DC voltage source, and the other one, immersed into the other compartment of the teflon cup, was connected to the high-resistance input of an operational amplifier, type WSH 223 (Tesla) (Dostál 1981). The apparatus allowed recording of currents $i \sim 10^{-13}$ A, using a TZ4100 recorder (Labora-

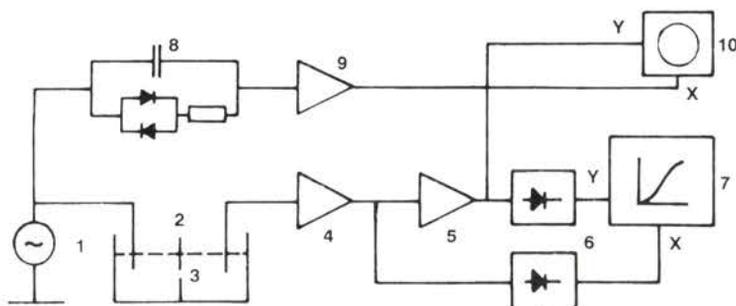


Fig. 1 Block diagram of the apparatus employed to measure coefficient of nonlinearity β of membrane current-voltage characteristics. See text for explanation.

tory Devices, Prague) with a time discrimination of approx. 10 ms. Voltage $V = 100$ mV was applied to the membranes.

Measurements of coefficient of nonlinearity of IVC were performed using the method described in detail by Passechnik et al. (1985). The employed method of direct measurement of IVC of modified membranes is based on the recording of the third current harmonic produced in a BLM with nonlinear current-voltage relationship. Here, we shall show but the principal relationships. At first approximation, membrane IVC can be expressed as $i = gV(1 + \beta V^2)$, where g is the conductance, β is the coefficient of nonlinearity. If alternating voltage $V = V_0 \sin 2\pi ft$ with a sufficiently low frequency (to eliminate the capacitive current component) is applied to a membrane, a current with frequency $3f$ and amplitude A_3 will flow across the membrane in addition to current with frequency f and amplitude A_1 . Coefficient of nonlinearity is then determined by $\beta = -4A_0(1 + rg)^3 E_0^2 A_1$, where r is the resistance of both electrolyte and the electrodes, $g = A_1/(E_0 - rA_1)$, E_0 is the amplitude of the alternating voltage applied to the system electrodes-electrolyte-membrane. For total voltage amplitude E_0 and that of voltage decrement across membrane, V_0 , holds: $V_0 = E_0 - A_1 r$. Thus, a membrane IVC pattern can be determined by measuring A_1 , A_3 , and resistance r . In our experiments, alternating voltage with amplitude $E_0 = 100$ mV was applied to membrane (3) from a generator I (Fig. 1) with a small harmonic distortion, via calomel electrodes (2) and electrolyte. Current flowing through the membrane was amplified using operational amplifiers WSH 220 (Tesla) (4). Third current harmonic was amplified via two-step selective amplifier (5). Signals of the first and third harmonics were then rectified using amplitude detector (6), and were fed to the input of an XY recorder TZ4103 (Laboratory Devices, Prague) which recorded the dependence $A_3(A_1)$. The phase shift of the third current harmonic from the basic signal could be observed with the aid of Lissajous patterns on the oscilloscope screen (10): signal through the electric model of the membrane (8) and a selective amplifier (9) of the same type as (5) was fed to the input of the oscilloscope. Phase shift φ was needed for the determination of the sign of coefficient β : it can be negative ($\varphi = 0$) (IVC is sublinear) or positive ($\varphi = 180^\circ$) (IVC is superlinear).

In a previous work (Hianik et al. 1987a) we could show that the IVC pattern of BLM modified by channel formers differs from that of carriers-modified BLM, and that they depend on the electrolyte concentration. Therefore, the dependence of coefficient β of modified BLM on electrolyte concentration has to be allowed for in any experiment of this kind.

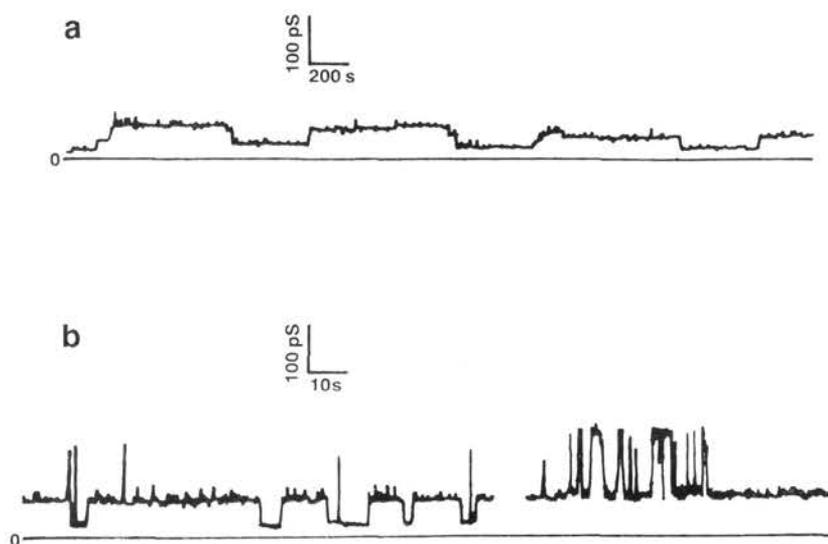


Fig. 2. A conductance record of a membrane of egg lecithin and cholesterol (weight ratio 4:1). Angiotensin concentration in the electrolyte: *a* — 15 $\mu\text{mol/l}$; *b* — 75 $\mu\text{mol/l}$; Electrolyte: 1 mol/l KCl, pH = 7, $T = 20^\circ\text{C}$. $V_0 = 100$ mV.

Results and Discussion

Kinetics of BLM conductance. Its dependences on ANG II concentration and electrolyte pH

Fig. 2 shows the kinetics of BLM conductance recorded several minutes after addition of 15 $\mu\text{mol/l}$ (*a*) and 75 $\mu\text{mol/l}$ (*b*) ANG II into the electrolyte. Obviously, the conductance changes show a discrete pattern typical of BLM conductance induced by the known channel forming agents. At ANG concentration of 15 $\mu\text{mol/l}$ discrete levels were of similar conductances $\Lambda = (39.5 \pm 1.07)$ pS and lifetime of (52.15 ± 6.44) s (number of conductance jumps measured $n = 66$). With the higher ANG concentration tested (75 $\mu\text{mol/l}$), also "rapid" changes of conductance were observed, reaching values of approx. 130 pS and lifetime of approx. 1 s (Fig. 2*b*).

The results obtained suggest that ANG II is able to form conductive pores in the membrane, resembling ion channels. The comparison of parameters of ANG II channels with dose induced by the extensively studied pentadecapep-

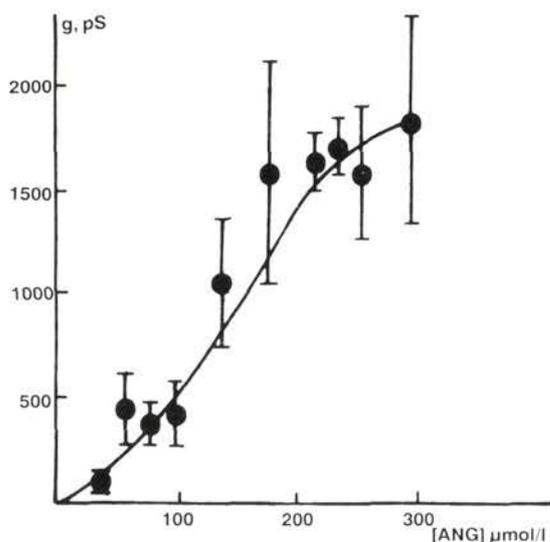


Fig. 3. The dependence of the conductance g of lipid bilayer membrane from egg lecithin and cholesterol (weight ratio 4:1) in electrolyte (1 mol/l KCl) on angiotensin concentration [ANG].

tide gramicidin A shows that, interestingly, the conductance of ANG II channels at 15 $\mu\text{mol/l}$ in 1 mol/l KCl is effectively the same as that of gramicidin A channels. However, the lifetime of ANG II channels is almost 100-fold longer than that of gramicidin A channels. According to the hypothesis proposed by Urry et al. (1971), the gramicidin A channel consists of two monomers, as suggested by the square dependence of membrane conductance on monomer concentration in the electrolyte (Bamberg et al. 1976). What is the possible structure of ANG II channels like? In order to approach this problem, BLM conductance was plotted against angiotensin concentration in the electrolyte, [ANG] (Fig. 3). This dependence can be approximated by an S-shaped curve. Thus, the interaction of ANG II with the membrane is an apparently co-operative process. At relatively low ANG II concentrations (0–100 $\mu\text{mol/l}$), the above dependence can be approximated by: $g = 26.78[\text{ANG}]^{1.315}$. Allowing for the possible adsorption of ANG to the walls of the teflon cup, the exponential coefficient for the remaining lipid solution will exceed 1.315. Thus, a process occurs in the membrane, which differs from monomolecular reaction and involves the formation of ANG II complexes, consisting of at least two molecules. In support of similar conclusions are also structural characteristics of ANG II molecule. The length of the angiotensin peptide chain is insufficient to allow the molecule monomers to form in the membrane spiral structures as does grami-

cidin. Admitting that ANG II forms β -chains, the length of the formation (2.66 nm) is comparable with that of gramicidin dimer. Nevertheless, this value is less than the thickness of the lipid bilayer and the membrane is distorted in the region, where molecules of ANG II are incorporated. As a result of the distortion of membrane homogeneity due to the incorporation of the first ANG II molecules, the following peptides can more easily be incorporated into this region. Hence, we assume that at higher concentrations of ANG II larger aggregates can arise. This may be one of the possible reasons which gives rise to a new additional conductance level at ANG II concentration $\sim 75 \mu\text{mol/l}$.

The angiotensin molecule includes 8 amino acid residues, some of which are hydrophilic at neutral pH and some are hydrophobic. At pH = 7 the first ones, Asp and Arg, have charged side groups. The remainder chain Val-Tyr-Ile-His-Pro-Phe seems to be mainly nonpolar as a whole, its hydrophobic coefficient evaluated according to Tanford (1978) and Cantor and Schimmel (1980) is rather high, $H \sim 8.28 \text{ kJ/mol}$, and is comparable with the H value of the hydrophobic parts of integral proteins. This might facilitate its incorporation into the bilayer. Simultaneously with the incorporation of monomers into the bilayer, also angiotensin dimers could be incorporated, longitudinally linked with each other by intramolecular hydrogen bonds. The question arises, how ions are transported across ANG-modified lipid bilayers. According to some authors (Rostovceva et al. 1987), bilayers get deformed upon the incorporation of polypeptide molecules into bilayer membranes, and structures resembling hydrophilic pores are formed in them. Transmembrane ion transport then proceeds via diffusion and migration along the surface of the lipid which lines the pore interior. It is assumed that ions "jump" from one binding site on the lipid surface to another. It may be that ions are transported in a similar way in angiotensin-modified membranes as well.

The mechanism of ANG incorporation into BLM can be studied in more detail by varying the ANG charge. At neutral pH (around 7), the Asp-Arg side of the molecule is mainly zwitterionic and the remaining part is mainly nonpolar. Therefore, it can be expected that electrolyte pH affects ion transport mediated by ANG-modified membranes, and this is reflected on the conformation of the ANG molecule itself. Based on these considerations, we studied the effect of the environmental pH on the conductance kinetics of angiotensin-treated membranes. At pH = 2.8 (i.e. under conditions when the angiotensin molecule carries an overall positive charge), membrane conductance is reduced to that measured at pH ~ 7 , reaching approx. 14 pS. In basic environment (pH = 11.5), with the overall charge being markedly negative, ANG II did not induce any changes in membrane conductance.

In view of the above assumption concerning the possibility of angiotensin-induced pore formation in lipid bilayers, we can also explain the environmental

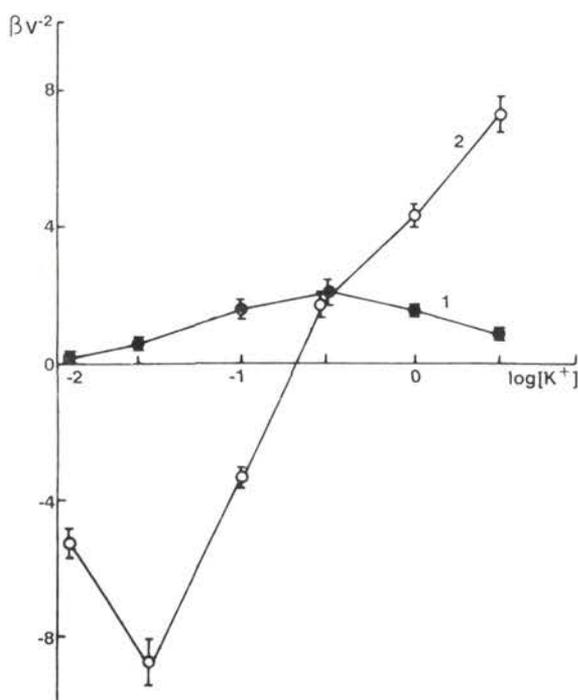


Fig. 4. 1: The dependence of coefficient of nonlinearity of current-voltage characteristics of angiotensin-modified membranes from egg lecithin and cholesterol (weight ratio 4:1) on the electrolyte concentration. The angiotensin concentration in the electrolyte was 380 $\mu\text{mol/l}$. 2: The dependence $\beta(K^+)$ for gramicidin D-modified BLM of identical composition. The concentration of gramicidin D in the electrolyte was 1 nmol/l.

effects on angiotensin-mediated ion transport. Since the conductance of ion channel reflects the overall changes in the transport energetics, transport reduction observed in strongly acid environment can be due to effects in the pore mouth as well as in the central part. Under these conditions, another group of the ANG chain (the imino group of the histidine residue) gets protonized. This can explain the decreased effectivity of K^+ transport across ANG-modified membranes, as reflected in a decrease of membrane conductance in comparison with that measured at neutral pH. At basic pH, when angiotensin cannot develop its effect, no conclusions are possible as to whether changes occur in its structure that abolish its ability to mediate transport following its penetration into membrane, or whether the changed molecule charge hampers the penetra-

tion of the molecule into the membrane. Results obtained by Hianik et al. (1987b), who studied insulin interaction with BLM, support the latter possibility. Insulin is a larger polypeptide than ANG II. The negative charge of insulin prevents its incorporation into BLM. On the contrary, as soon as the negative membrane surface has been neutralized by Ca^{2+} (10 mmol/l), insulin starts incorporating into the lipid bilayer. We studied the BLM conductance at physiological and/or higher than physiological concentrations of ANG II. However, we cannot exclude that ANG II may induce membrane conductance also at lower concentrations, especially in the case of rather inhomogeneous biomembranes.

Current-voltage relationship of ANG-modified BLM

The mechanism of ion transport can also be analyzed by measuring current-voltage characteristics (IVC) of modified membranes. Generally, the IVC modified membranes are nonlinear. The nature of this nonlinearity is one of the aspects that allow assumptions as to the dynamics of ion transport across membranes modified by channel formers (e.g. gramicidin) or carriers (e.g. valinomycin). The measure of nonlinearity depends, among others, on the electrolyte concentration and BLM composition. The second part of our experiments was therefore aimed at investigating the effects of electrolyte on IVC of bilayer lipid membranes modified by angiotensin, and comparing them with the effects of channel forming agents and/or carriers.

The method based on the measurement of higher harmonics of membrane current as described above was employed for the study of membrane IVC nonlinearity. Fig. 4 (curve 1) illustrates the dependence of coefficient of nonlinearity β on the electrolyte (KCl) concentration under symmetrical conditions, for BLM of egg lecithin and cholesterol (weight ratio 4:1) in the presence of 380 $\mu\text{mol/l}$ angiotensin in the electrolyte in contact with one side of the membrane. The angiotensin concentration tested warrants a high membrane conductance (see the problem of concentrational dependence). Each point represents averaged experimental value of β obtained for 10 membranes and each of the concentrations tested. It is obvious from the relationship shown that coefficient β remains positive within the entire range of concentrations tested, i.e. the IVC of angiotensin-modified membranes are superlinear both at high and at low electrolyte concentrations. At low electrolyte concentrations no effects similar to those as observed with coefficient of nonlinearity β of IVC membranes modified by the channel forming agent GRD occur (Fig. 4, curve 2): in the latter case, β is negative, and ion transport is limited by the potential barrier of the channel entrance.

Hence, the transport of ions through ANG-modified membranes is limited

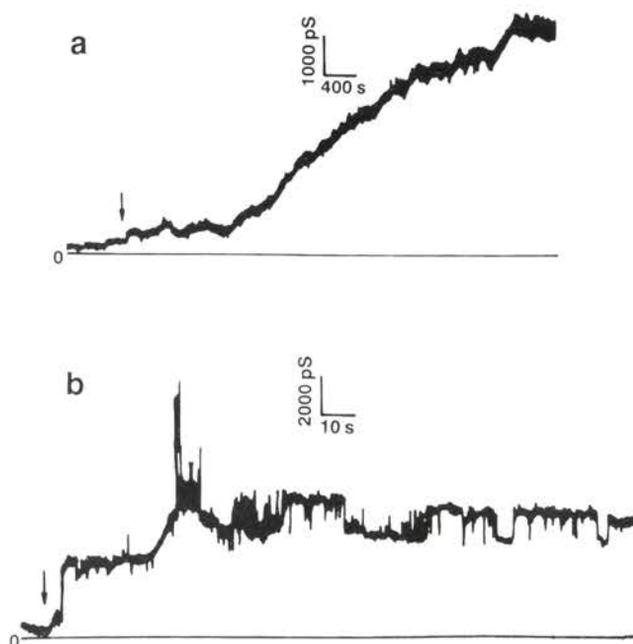


Fig. 5. A record of conductance kinetics of gramicidin D-modified BLM (gramicidin concentration in the electrolyte: 30 nmol/l) from egg lecithin and cholesterol, following angiotensin addition (arrow) into the electrolyte at one membrane side. Concentration: *a* — 75 μ mol/l; *b* — 115 μ mol/l. Electrolyte: 1 mol/l KCl, pH = 7, $T = 20^\circ\text{C}$, $V_0 = 100$ mV.

by the central part of the lipid bilayer. This is suggested also by the above considerations concerning the possible position of the angiotensin molecule in the bilayer and the mechanism of angiotensin-mediated transport. Obviously, the pore mouths formed by deformation of the lipid bilayer are sufficiently wide; then the barrier to the transport is the central part of the bilayer, which thanks to the hydrophobic nature of the central part of ANG molecule, maintains the original ordering of the lipid molecules.

Effects of ANG II on gramicidin-D modified BLM

The effects of ANG II on the operation of ion channels was studied using gramicidin D-modified BLM. ANG was added into the electrolyte as soon as the conductance of membrane containing gramicidin channels stabilized and

reached steady-state level of approx. 900 pS. In the presence of 75 $\mu\text{mol/l}$ ANG in the electrolyte, the conductance of GRD-modified membrane grew, and was sigmoid in shape (Fig. 5a), suggesting possible co-operativity effects in the system studied. The curve had a plateau at approx. 5300 pS from the conductance of GRD-modified membrane. This change exceeds manifold the effects of identical concentrations of ANG observed in the absence of GRD. ANG concentrations above 100 $\mu\text{mol/l}$ induced qualitatively different changes in the conductance of GRD-modified membrane. After addition, membrane conductance changed in a discrete manner, with the lowest level observed showing a value of 1400 pS, and the other levels being 2 and/or 3 times higher (at ANG concentration 115 $\mu\text{mol/l}$) (Fig. 5b).

The observed effects can be explained as follows. In GRD-modified membranes, ANG may become incorporated predominantly in the neighbourhood of ionic channels formed by GRD, where the lipid bilayer has been strongly deformed and is thinner than it would be in the absence of gramicidin (Jordan and Vayl 1985). Upon increasing angiotensin concentration above 100 $\mu\text{mol/l}$, larger aggregates of ANG molecules might be formed; their formation and/or dissociation may cause discrete changes in conductivity on the background of conductivity induced by the presence of gramicidin.

Thus, angiotensin affects the conductance of bilayer lipid membranes. Most conspicuously, the changes in conductance occur at electrolyte pH values close to the physiological levels. Probably, ANG forms pores in the lipid bilayer, composed of ANG aggregates, and thus so called "barrel channels" can arise. On the other hand, it cannot be excluded that ANG II induces a structural transition in lipid bilayers, and conducting pores are formed not only by means of ANG II itself but also as a complex of ANG II and altered lipid bilayer phase. At the same time, it also affects ion transport mediated by the channel forming agent gramicidin. This suggests that ANG II is able to regulate ion transport.

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