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Heparin influence on α-staphylotoxin formed channel

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Abstract

The effects of heparin on ion channels formed by *Staphylococcus aureus* α-toxin (ST channel) in lipid bilayers were studied under voltage clamp conditions. Heparin concentrations as small as 100 pM induced a sharp dose-dependent increase in channel voltage sensitivity. This was only observed when heparin was added to the negative-potential side of lipid bilayers in the presence of divalent cations. Divalent cations differ in their efficiency: $Zn^{2+} > Ca^{2+} > Mg^{2+}$. The apparent positive gating charge increased 2–3-fold with heparin addition as well as with acidification of the bathing solution. 'Free' carboxyl groups and carboxyl groups in ion pairs of the protein moiety are hypothesized to interact with sulfated groups of heparin through divalent cation bridges. The *cis* mouth of the channel (that protrudes beyond the membrane plane on the side of ST addition and to which voltage was applied) is less sensitive to heparin than the *trans*-mouth. It is suggested that charged residues which interact with heparin at the *cis* mouth of ST channels and which contribute to the effective gating charge at negative voltage may be physically different from those at the *trans* mouth and at positive voltage.

Keywords: Ion channel; Gating; Staphylotoxin; Heparin; Lipid bilayer; Calcium

1. Introduction

Excitability is an essential element in many physiological processes. Planar bilayer and patch-clamp techniques have revealed that this phenomenon results from channels that are opened or closed by a change in membrane potential (voltage-gated channels). This sensitivity to transmembrane potential is not limited to ion channels in biomembranes. Many other types of channels, such as those created by some toxins, peptides and antibiotics, also demonstrate considerable voltage gating. In most cases, channel sensitivity to transmembrane potential (the steepness of the voltage dependence as well as the threshold) is subject to modulation. Channel sensitivity can be modulated by protein kinases and by intra- and extracellular components, such as proteins, glycosaminoglycans and some low molecular weight regulators, including Ca and Mg ions. Despite many studies, the modulation mechanisms are still poorly understood. Since these mechanisms are relevant to our understanding of channel function, we decided to examine some of them using the simplest system, the planar lipid bilayer.

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α-Toxin (ST) is one of the important toxins produced by *Staphylococcus aureus*. It is a single-chain protein with mass of 33.2 kDa [1]. The toxin's ability to form heptameric [2] ion channels in planar lipid bilayers was discovered almost 20 years ago [3,4]. At neutral pH ST channels are usually in a high conductance state and rarely transit to low conductance states. Acidification drastically increases channel sensitivity to transmembrane voltage. At acid pH, the stepwise increase in transmembrane potential facilitates a transition of ST channels from high to low conductance states [5–10]. This transition is as rapid as those observed in excitable biomembranes. Time constants of the transition, τ, are in the range of 0.1–10 ms. Ion composition is also important to kinetic properties of ST channels [5,6,11]. Because of these characteristics, channels formed by *S. aureus* α-toxin were chosen to model the voltage-gated channels of biological membranes.

Heparin is a sulfated polysaccharide with a unique spectrum of pharmacological activity [12]. It contains an average of 3.5 negative charges per disaccharide unit. The high density of negative charges is probably responsible for some of its pharmacological activities. Besides its well-known anticoagulant, antithrombotic and antihemostatic activities, heparin inhibits several enzymes and displays antiviral and antibacterial activities. Moreover, it modifies the activity of some ion channels [13–16]. In view of these data and the fact that related glycosaminoglycans are present on surfaces of cells attacked by α-toxin, we decided to examine heparin's ability to modify properties of the ion channel formed by *S. aureus* α-toxin.

2. Materials and methods

2.1. Chemicals

Wild type *S. aureus* α-toxin was kindly supplied by Dr. K.D. Hungerer of the Behringwerke Laboratories (Marburg, Germany) and Dr. H. Bayley (Texas A and M University, USA). Pure phosphatidylcholine was prepared according to Bergelson et al. [17] or purchased from Sigma (type V-E). Cholesterol was purchased from Sigma. Heparin (as Na-salt) was obtained from Roche. Sulfated dextran came from Merck. Difco supplied Bacto-Agar. Agarose was purchased from Sigma. Agar and agarose, used to prepare agar bridges, were dialyzed against 100 vols. of double distilled water for at least 2 days at 4°C. Water was changed every 12 h. Other chemicals were of analytical grade. Water, double distilled in glass, was used to prepare all buffer solutions. For bilayer experiments, principally two basic solutions were used. The first contained 100 or 200 mM KCl, 10 mM Tris-HCl, pH 7.5 (buffer 1). The second contained 100 mM $CaCl_2$, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5 (buffer 2).

Planar lipid bilayer membranes (PLM) were formed at room temperature (25 ± 2°C) by the technique of Montal and Mueller [18] from a phosphatidylcholine-cholesterol mixture (1:1, by mass). Monolayers from a 10 mg/ml solution of the aforementioned lipids in *n*-hexane were spread on the surfaces of buffered salt solutions placed in two 2 ml compartments of the experimental cell. These were separated by a 25 μm thick Teflon partition in a Teflon experimental cell. A small hole (approx. 0.3 mm diameter) connected the two cells. After evaporation of the solvent, the membrane was formed by raising the monolayers above the level of the hole that had been pretreated with a 2% solution of hexadecane in *n*-hexane. During bilayer formation, a triangle or square wave (amplitude, ± 5–10 mV; frequency, approx. 500 Hz) was utilized and the resulting current was monitored continuously. Electrical capacity of bilayers was about 0.8 μF/cm^2.

Experiments were done under voltage-clamp conditions. Current passing through the bilayers was measured with Ag/AgCl electrodes connected through salt bridges (3% agar or 3% agarose with 3 M KCl) in series with a voltage source and a current amplifier (K284UD1A, USSR). The *trans* compartment of the experimental chamber was connected to the virtual ground. Voltage pulses were applied to the *cis* compartment of the chamber. The amplifier signal was monitored with a Nicolet-2090-III storage oscilloscope (Nicolet Technologies, Madison, WI, USA), and recorded on a strip chart or tape recorder. Conductance of bilayer membranes (G) in symmetrical solutions was defined as $G = I/V$, where I is the transmembrane current flowing through the channels and V corresponds to the fixed potential. Basal conductance of PLM was usually less than 4 pS.

Channel formation in planar lipid bilayers was accomplished as follows. After the membrane was completely formed and stabilized, a few microliters of the stock solution containing *S. aureus* α-toxin (0.5 mg/ml) were always added to the *cis* compartment of the experimental cell, resulting in a final concentration of up to 10 μg/ml. Solutions in both compartments were magnetically stirred. When conductance of the bilayers reached approx. 10 nS, the toxin-containing solution was replaced with fresh buffer. The increase in bilayer conductance stopped within a few minutes. These bilayers, containing α-toxin channels, were then used to measure current voltage characteristics and selectivity. The channels all appear to insert in the same orientation.

Selectivity of the ion channels was measured in the presence of a 3-fold salt concentration gradient, either 300 mM/100 mM KCl or 150 mM/50 mM $CaCl_2$, *cis*/*trans*, respectively. Zero current potential was defined as the potential (V^*) that must be applied to the experimental cell in order to reach a virtual zero transmembrane current equal to that of a symmetrical system with 0 mV applied potential.

To record the instant current-voltage relationship (CVR), the transmembrane potential was initially decreased stepwise from 0 to −110 mV and then gradually increased to +110 mV (during a voltage ramp with duration of 50–100 ms). Finally, the transmembrane potential was returned stepwise to 0 mV. The reversal of the voltage protocol gave the same type of CVR. The relationship between the current passing through the incorporated channels at −100 mV and its value at +100 mV (I_{-100}/I_{+100}) was taken as a quantitative measure of CVR asymmetry (A) (the sign referred to the side of protein addition). To evaluate non-linearity (L) we used a ratio between values of the membrane conductance at ±100 mV and ±20 mV (G_{100}/G_{20}).

Steady-state CVR (sCVR) was measured in multichannel PLM. The method was based upon the finding [8] that time constants (τ) for ion channel transitions from open to closed states are >1000 times larger than time constants for ion channel transitions in the opposite direction. When a large enough voltage (−100 mV or +100 mV) was applied to the membrane, at least five half-times (until 60 min) were allowed to pass before steady-state conditions were achieved. After that, the fixed potential was slowly (<1 mV/s) changed in a linear fashion until it reached 0 mV, allowing steady-state conditions to be reached at all transmembrane potentials. Conductance/voltage dependence was bell-shaped, with low conductance at large voltages (>|70| mV) and maximum conductance at small voltages (approx. 0 mV). Each half of the dependence (at positive and at negative potentials) could be described by equations analogous to that used to fit CVR of other channels [19,20]:

$$g(V) = g_{low} + (g_{max} - g_{low})/[1 + \exp(\Delta G_i/RT + qFV/RT)] \quad (1)$$

where $g(V)$ is the membrane conductance at potential V; g_{low} and g_{max} are minimum and maximum conductance values; ΔG_i and qFV are a 'chemical' voltage-independent and an 'electrical' voltage-dependent component of free energy of a channel opening, respectively; q is the gating charge; R is the gas constant; F is Faraday's constant and T is the absolute temperature.

Although g_{low} and the potential at which the macroscopic conductance is half-saturated (V_o) could be measured directly from experimental data, they were obtained by fitting the data to Eq. 1. The least square method with a minimum random search algorithm [21] was performed on an IBM PC. The values of ΔG_i, g_{low} and g_{max}, q and V_o yielding the minimum sum of the square of deviations of experimental values from theoretical values [$\Sigma(\text{exp}-\text{theor})^2$] were taken as 'true'. In all cases the differences between experimental and theoretical values did not exceed 5%.

On a step transition of the clamp voltage from 0 to ±100 mV the current shows a large initial value which then decreases in an essentially exponential manner to a lower steady-state value. In accordance with earlier observations [5,7], the relaxation does not have a single time constant, but can be fitted adequately with the sum of two purely exponential components, where the faster one makes the principal contribution to the current decrease. Therefore, for simplicity, an effective time constant, that usually nearly equal to the first exponential component, was used in this study to compare the relaxations observed in the presence of non-electrolytes.

Student's t-test was used to determine the signifi-

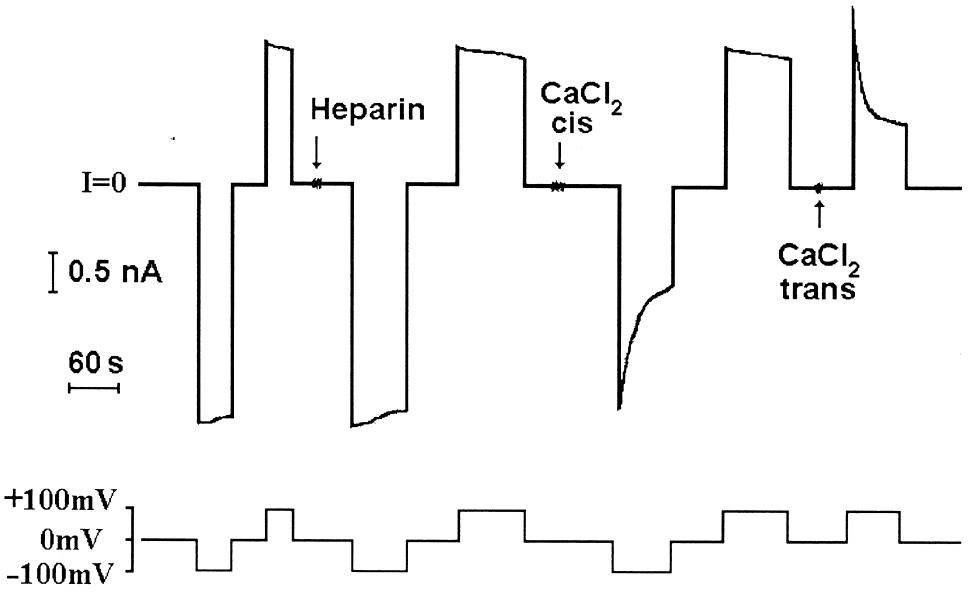

Fig. 1. Effect of heparin and Ca^{2+} on the conductance induced by ST in lipid bilayers. Solutions in both compartments of the experimental cell contained 100 mM KCl, 10 mM Tris, pH 7.5 and were magnetically stirred during experiments. 8–10 μg/ml of ST were added to the *cis* compartment and the membrane was pulsed several times between −20 and +20 mV to accompany channel formation. After bilayer conductance reached approx. 10 nS the solution in the *cis* compartment was replaced with fresh buffer containing no toxin. In a few minutes the increase in bilayer conductance stopped. The record was started with a pulse (±100 mV). Then heparin was added to both compartments of the experimental cell (final concentration 6 μg/ml) and test-voltage pulses (±100 mV) were repeatedly applied. Addition of $CaCl_2$ (final concentration 5 mM) was carried out in two steps. First, it was added to the *cis* compartment and the effect was examined. Then $CaCl_2$ was added to the *trans* compartment. The pulse protocol is shown in the lower part of the figure. Current and time scales are given in the figure.

cance of the difference between mean values obtained under different conditions.

3. Results

3.1. Heparin induces closing of ST channels if applied to the negative-potential side of the membrane in the presence of Ca ions

Planar bilayer membranes modified by *S. aureus* α-toxin were prepared as described in Section 2. After channels had spontaneously formed in the membrane, their voltage dependence was examined by applying an elevated potential. Current instantaneously increased as the increased voltage drove ions through the channels and then diminished as the channels transited to lower conductance states.

Current traces show that if buffer 1 does not contain both heparin and Ca^{2+}, application of 100 mV voltage pulses of either sign to the *cis* compartment failed to induce significant channel closure (Fig. 1). Heparin alone added to solutions on both sides of the bilayer had no detectable effects. Only when $CaCl_2$ was also added, were noteworthy current relaxations observed in response to voltage pulses. The effect of heparin and Ca^{2+} action was side- and voltage sign-dependent.

When $CaCl_2$ was added to the *cis* compartment, the effect could be observed in response to negative voltage pulse. No change was observed in the time course of current when positive voltage pulses were applied. A consecutive addition of $CaCl_2$ to the *trans* compartment caused a sharp reduction of current in response to the application of positive voltage pulses. Sulfated dextran demonstrated effects analogous to those of heparin. However, in this and all other experiments, sulfated dextran decreased the stability (life time) of the lipid bilayers. For this reason, only data obtained with heparin are presented in the present study.

At pH 7.5, Ca ions alone exert little influence on the voltage gating of ST channels. When mixed cation solutions (100 mM KCl+25 mM $CaCl_2$ or 100

mM KCl+50 mM $CaCl_2$) or pure $CaCl_2$ buffer (buffer 2) were used, current relaxation appeared slightly more pronounced to positive voltage than was observed using KCl buffer (buffer 1) (Fig. 1). The relaxation was almost undetectable when negative voltage was applied (Fig. 2). No evidence of anomalous mole fraction effects was seen. When buffer 2 was used, heparin addition to the *trans* compartment (final concentration: 1 ng–10 μg/ml) caused a dose-dependent increase in voltage-induced transition of ST channels to a low conductance state, but only if positive voltage was applied (Fig. 2A). When negative voltage pulses were applied, the time course of current was not affected. However, if heparin was then also added to the *cis* compartment, a sharp relaxation of current in response to negative voltage was observed (Fig. 2A). On the other hand, if heparin was added first to the *cis* compartment, the sensitivity of ST channels to transmembrane voltage was increased only in response to negative voltage pulses, where a fast relaxation of the current was established (Fig. 2B). When heparin was additionally added to the *trans* compartment, a sharp relaxation of current in response to the application of positive voltage pulse was always seen. These data indicate that heparin facilitates the voltage-driven closing of ST channels, but only if present on the negative-potential side of the lipid bilayer, and only in the presence of Ca ions (Figs. 1 and 2).

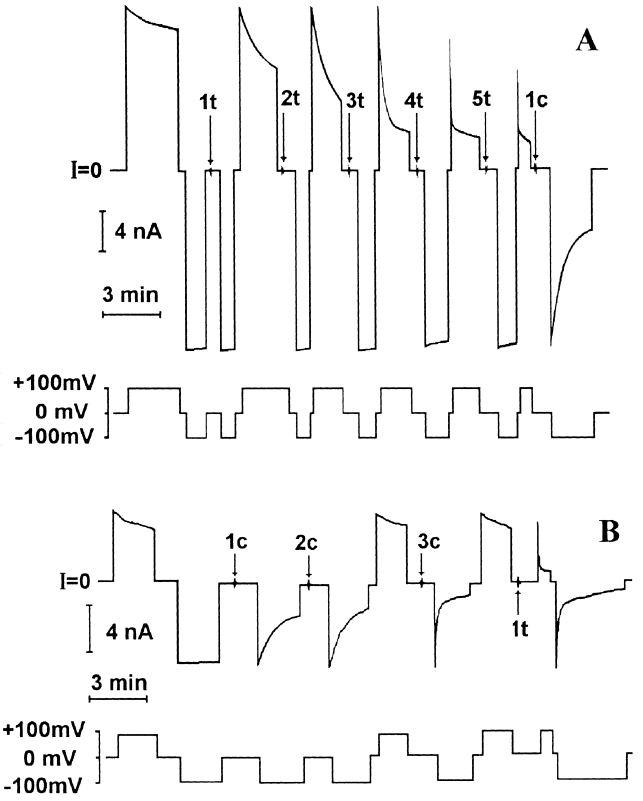

Fig. 2. Dose-dependent effect of heparin on voltage-dependent closure of ST channels. Solutions in both compartments of the experimental cell contained 100 mM $CaCl_2$, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5. (A) ST-modified lipid bilayers were prepared as described for Fig. 1. Heparin was added five times (marked 1t–5t with arrows) to the *trans* compartment with final concentrations of 1 ng/ml, 10 ng/ml, 0.1 μg/ml, 2 μg/ml and 10 μg/ml. The time course of current after application of voltage pulses of ±100 mV is presented. Current courses in response to negative pulses were indistinguishable from those of controls, but when positive voltage pulses were applied, sharp changes were observed. After reaching a large instantaneous value of transmembrane current at the time of the jump to the high voltage, the current relaxed to a lower steady-state value proportional to heparin concentration. Addition of heparin to the *cis* compartment (1c, 1 μg/ml) led to the appearance of current relaxation in response to negative voltage. The pulse protocol is shown in the lower part of the figure. Current and time scales are given in the figure. (B) Three additions of heparin (marked 1c–3c with arrows) were made to the *cis* compartment with final concentrations of 0.2 μg/ml, 1 μg/ml and 5 μg/ml. 1t marks an addition of heparin (1 μg/ml) to the *trans* compartment. Current and time scales are given in the figure.

When concentrations of heparin in the *trans* compartment were greater than 2 μg/ml, amplitudes of instant current in response to the application of positive voltage pulses (see Fig. 2A) failed to reach the value obtained prior to heparin addition. We do not think it is due to insufficient time at 0 mV, because usually a few seconds are sufficient [5,8]. Rather, we suspect that channels opened in response to voltage pulse, but their closing is so fast that the original amplitude of instant current response could not be observed due to the relatively large RC value of bilayers. However, the possibility that a small percentage of channels failed to open cannot be ruled out.

3.2. Asymmetry in heparin-Ca^{2+} effectiveness

Heparin-dependent amplification of ST channel sensitivity to transmembrane voltage depended on the side to which sulfated polysaccharides were added. The experiments were performed at a constant concentration of $CaCl_2$. Under these conditions, when heparin was in the *trans* compartment and a positive pulse was applied to the bilayer, the

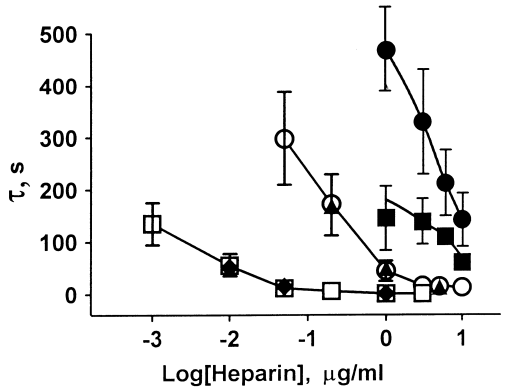

Fig. 3. Time constants (τ, s) of the voltage-dependent relaxation of ST-induced conductance as a function of heparin concentration. When solutions in both compartments of the experimental cell contained 100 mM $CaCl_2$, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5 the following symbols are used: open squares and closed diamonds, results obtained at positive 100 mV pulses; open circles and closed triangles, results obtained at negative 100 mV pulses. Open symbols show data obtained with heparin in both compartments simultaneously; closed symbols demonstrate data obtained in the presence of heparin in only one of the solutions: in the *trans* compartment at positive voltage, or in the *cis* compartment at negative voltage. For the positive voltage control the value of time constant is approx. 200 s, while for negative voltage the relaxation was negligible and the time constant could not be measured. Each value represents the mean of 3–4 separate experiments. When solutions in both compartments of the experimental cell contained 4.0 M NaCl, 50 mM $CaCl_2$, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5 the following symbols are used: closed squares and closed circles, results obtained at positive and at negative 100 mV pulses in the presence of heparin in only one of the solutions: in the *trans* compartment at positive voltage, or in the *cis* compartment at negative voltage. Each value represents the mean of 3–4 separate experiments. If no deviation bars are shown, they were equal to or smaller than the symbols used.

effects were always larger than with *cis* application of heparin and negative voltage pulses (Fig. 2A,B). Such asymmetry in heparin action is illustrated by the relationship between time constant and heparin concentration (Fig. 3), and is in agreement with the asymmetrical structure of the ST channel obtained from a crystallographic study [22]. In the control recording (in the absence of heparin) ST channels remained in a high conductance state when negative voltage pulses (up to 100 mV) were applied; however, if the applied voltage was positive, a very slow current decrease was observed. Heparin sharply decreased the time constant for channel closing. This effect could be seen clearly with concentrations as small as 1.0 ng/ml (less than 10^{-10} M) at positive voltage. At negative voltage the heparin concentration had to be increased more than 100-fold in order to achieve an equivalent increase in voltage-gating behavior.

It should be noted that the effect of heparin-Ca^{2+} was stronger in pure $CaCl_2$ solutions. The addition of electrolytes consisting of monovalent cations and monovalent anions (1:1 electrolyte) always decreased their effectiveness in a concentration-dependent fashion. The result obtained in the presence of one of the mixed cation solutions (4 M NaCl, 50 mM $CaCl_2$) clearly demonstrates this effect (Fig. 3). The influence of 1:1 electrolyte on heparin-Ca^{2+} effect could be caused by the loss of heparin binding sites because of competition between univalent and divalent cations for negatively charged groups. However, the asymmetry in the heparin-Ca^{2+} action continues to be seen.

The other difference between *cis* and *trans* addition of the mixture is the effective concentrations of Ca^{2+}, if heparin concentration was fixed (Fig. 4). At negative voltage, the concentration of Ca^{2+} in the *cis* compartment had to be dozens of times larger than that used in the *trans* compartment with positive voltage (on the *cis* side), in order to induce a similar change in voltage-gating behavior. Hence, the influ-

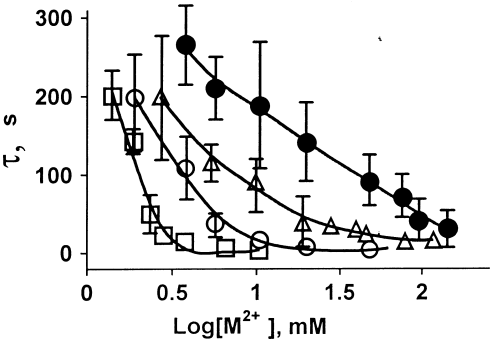

Fig. 4. Dose dependence of effects of divalent cations on the time constant of current relaxation at ST-modified planar lipid membranes. Chloride salts of several cations were added (at the concentration mentioned at the abscissa) to both solutions (100 mM KCl, 6 μg/ml heparin and 10 mM Tris-HCl, pH 7.5) bathing the membrane. Open squares, open circles and open triangles represent Zn^{2+}, Ca^{2+} and Mg^{2+}, respectively, at positive 100 mV pulses. Closed circles represent results obtained at negative 100 mV pulses for Ca^{2+}. Each point represents the average of four or five determinations. If no error bars are shown, they were equal to or smaller than the symbols used.

Table 1
Features of ST channels in KCl and in $CaCl_2$ solutions in the presence or absence of heparin

Parameters	χ	g	V^*	A	L_+	L_-
200 mM KCl	25.8	210 ± 30	7.6 ± 0.4	1.57 ± 0.1	0.84 ± 0.04	1.21 ± 0.04
200 mM KCl+6 μg/ml heparin	25.8	207 ± 28	7.7 ± 0.2	1.53 ± 0.07	0.84 ± 0.06	1.2 ± 0.07
100 mM $CaCl_2$	19.3	214 ± 35	12.7 ± 1.0	1.20 ± 0.06	0.91 ± 0.02	1.08 ± 0.07
100 mM $CaCl_2$+6 μg/ml heparin	19.3	213 ± 30	12.7 ± 0.9	1.15 ± 0.09	0.92 ± 0.05	1.1 ± 0.09

Solutions were maintained at pH 7.5 with 10 mM Tris-HCl buffer. $A = I_{-100}/I_{+100}$ is the asymmetry of the instant current voltage relationships. $L = G_{100}/G_{20}$ is value of the non-linearity of the current voltage relationships. V^* is the zero current potential measured in the presence of a 3-fold salt concentration gradient (300 mM/100 mM KCl or 150 mM/50 mM $CaCl_2$, *cis*/*trans*). g (pS) is the single ST channel conductance in symmetrical solutions when −50 mV was applied on the bilayer. χ (mS/cm) is the conductivity of the solutions. Date are presented as mean ± S.D.

ence of the heparin-Ca^{2+} mixture on the ST channel gating is always weaker when the mixture is added to the *cis* compartment of the experimental cell.

It is important to note that voltage gating of ST channels only requires heparin on the negative side of the bilayer. The simultaneous presence of heparin on the other side apparently did not change τ-heparin concentration dependence (Fig. 3).

3.3. Conductance and selectivity of ST channels in the presence of heparin and Ca ions

ST channels are relatively large in diameter [10,22–24]. Nevertheless, they exhibit certain specificity for charged solutes, i.e., they have a limited selectivity for ions. As was shown recently [23,25], conductance and selectivity of ST channels may be determined by the effective charges at both channel openings and are sensitive to the surface charge of the lipid bilayer. Such sensitivity encouraged us to examine the effects of highly negative charged heparin (approx. 3.5 negative charges per disaccharide unit) on these two channel features.

The lipid bilayer assay allows the evaluation of ion selectivity by measuring the membrane potential under zero-current conditions. It was shown in both KCl and $CaCl_2$ solutions that ST channels possess weak anion selectivity. ST channels are normally more permeable to chloride than to cations and we found that heparin did not alter this selectivity (Table 1). Experiments also demonstrated that heparin failed to alter channel conductance in either buffer system (Table 1). The unmodified conductance indicates that in neither buffer system did heparin alter the resistance of the channel lumen or of the two access resistors, i.e. the hemispheres of buffer at either end of the channel. Possible explanations for this phenomenon include the following. Heparin (with or without Ca^{2+}) (1) does not bind or interact with the channel at high conductance state or this interaction is weak; (2) it localizes so far from the channel openings that it does not detectably alter charges of the channel openings and adjacent lipid surfaces; (3) the change in effective charge is occasionally compensated by some change in channel structure – a change in its functional diameter, for example.

3.4. Evaluation of voltage dependence

The instant CVRs of ST channels obtained in symmetrical solutions are almost linear and slightly asymmetrical (Table 1). Asymmetry is more pronounced in KCl than in $CaCl_2$ solution. Because of voltage gating, the steady-state and instant CVRs of ST-modified lipid bilayers are quite different, especially in the presence of different concentrations of heparin. From these data the steady-state conductance-voltage (G-V) relationships are determined from the relation $G = I/V$ (Fig. 5). As expected, in controls we observed that at negative voltage, ST channels are in a high conductance state, while at positive voltage, a small but definitive decrease in the bilayer conductance could be seen. Heparin modified G-V dependences (Fig. 5), decreasing the level of the low conductance region (g_{low}) in a dose-dependent manner by as much as 92% (Fig. 6). Again, heparin was much more effective from

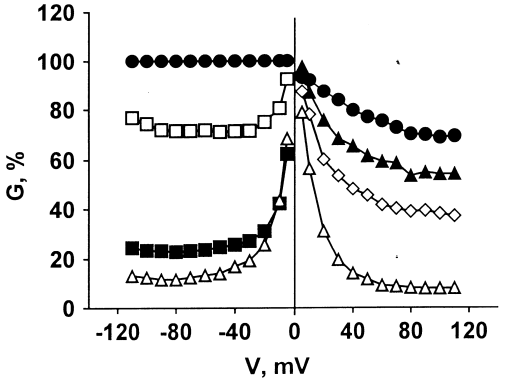

Fig. 5. Dependence of voltage sensitivity of ST channels on heparin concentration. All heparin additions were symmetrical. Each point represents the average of three or four determinations. The results were then normalized to the maximum conductance which was taken as 100%. Data shown were collected in the absence of heparin (closed circles) and in the presence of heparin at 1.0 ng/ml (closed triangles), 10 ng/ml (open diamonds), 50 ng/ml (open squares), 0.5 μg/ml (closed squares) and 3.0 μg/ml (open triangles). The full range of data was collected; however, for clarity only part of the data is presented. Solutions in both compartments of the experimental cell contained 100 mM $CaCl_2$, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5. Other experimental conditions are as in the legend to Fig. 2.

the *trans* than from the *cis* side. The presence of heparin on the *cis* side of the bilayer does not alter the effects of *trans*-heparin and vice versa.

Data analogous to those presented in Fig. 5 were analyzed based on an earlier study of Ehrenstein et al. [26] (for details, see Section 2). This formalism assumes a two-state process. However, ST channels have more than one low conductance state [7,8,10]. Despite this fact, the suggested formalism fits the experimental data well. This fact justifies treating the different low conductance states observed as one state. Hence, this analysis gives averaged values of thermodynamic parameters of the transition of ST channels from high to multiple low conductance states. We used the formalism to establish the voltage-independent energy (ΔG_i) of the channel transition, the apparent gating charge (q) and the potential at which the macroscopic conductance is half-saturated (V_o), reflecting the steepness of the voltage dependence. The dependence of these parameters on heparin concentration is presented in Fig. 7. An increase in heparin concentration (up to 6 μg/ml) increased q about 3-fold and decreased V_o by a comparable amount. Clearly, when V_o becomes smaller and q becomes larger, the voltage dependence is steeper. Hence, these results also indicate that heparin increases the voltage sensitivity of ST channels. The nearly constant value for voltage-independent energy (ΔG_i) at various heparin concentrations indicates that heparin probably targets the ST channel voltage sensor, implying an interaction of heparin with some amino acid residues of the channel.

3.5. Heparin and other divalent cations

Other divalent cations were employed to examine the ionic specificity of heparin on voltage gating of ST channels. When Mg^{2+} and Zn^{2+} were used (Fig. 4), heparin exhibited effects that were qualitatively the same as those seen in the presence of Ca^{2+} (Figs. 1–3 and 5). The only difference observed was the efficiency with which different ions facilitated the transition. The efficiency follows the sequence $Zn^{2+} > Ca^{2+} > Mg^{2+}$, identical to that observed for inhibition of the effects of ST on cells [27–29].

Increasing concentrations of all three cations diminished the time constants of current relaxation in ST-modified bilayers in the presence of a fixed heparin concentration. Intrinsic binding constants for these cations were determined from the dependence of g_{low} upon cation concentration. Experiments were done at positive voltage in the *cis* compartment. At these conditions binding constants appeared to be

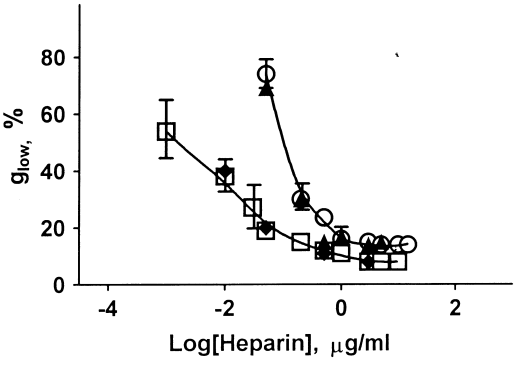

Fig. 6. Dose dependence of heparin effects on minimum values of ST-induced conductance in the lipid bilayers (g_{low}). Results are presented as percentages, where g_{low}% was defined as (g_{low}/g_{max})×100%. Symbols and experimental conditions are as in the legend to Fig. 3. Control values are 68% and 100% for positive and negative voltage, respectively. Each value presents the mean of 3–4 separate experiments. If no error bars are shown, they were equal to or smaller than the symbols used.

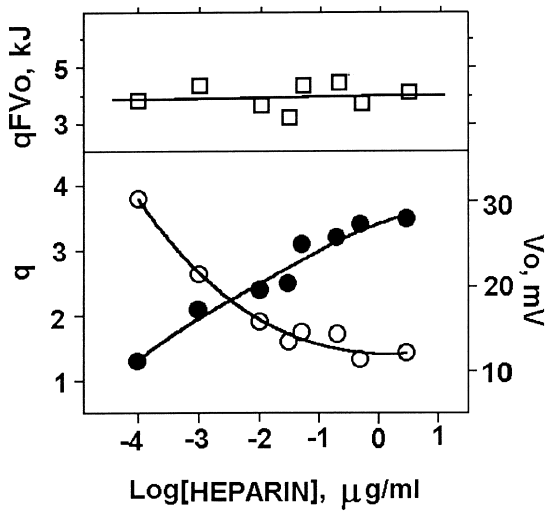

Fig. 7. Quantification of the increase in voltage dependence of ST channels by heparin. Voltage-dependent parameters, q (filled circles) and V_o (open circles), were obtained from results such as those shown in Fig. 5. Values of ΔG_i (open squares) were determined at $V = V_o$ and are given in kilojoules. Under these conditions $\Delta G_i = -qFV_o$. Each point represents the average of three or four determinations.

600, 170 and 90 M^{-1} for Zn^{2+}, Ca^{2+} and Mg^{2+}, respectively. These constants are very different from those obtained for the binding of these ions to the lipids in the bilayer membrane [30]. On the other hand, they appear very close to the intrinsic binding constants of the respective ions to tartaric (480, 63 and 23 M^{-1} [31]) or other organic acids [29,32] where two carboxylic groups are involved in divalent cation binding.

This suggests that there are at least two plausible explanations for the data. The first assumes that heparin interacts principally with phosphatidylcholine via the mechanism proposed by Kim and Nishida [33], where there are direct electrostatic interactions between the phospholipid choline nitrogen and the sulfate groups of heparin while calcium ions cross-link the phosphate groups to the heparin's sulfate groups or to the phosphate groups of neighboring phospholipids. These interactions may not be specific, although multipoint interactions may appear as a relatively high value of the apparent binding constant.

The second, and perhaps more plausible explanation is that heparin-Ca^{2+} interacts with carboxyl groups and with ion pairs of the protein moiety of ST channels. We expect that calcium cross-linking of the carboxyl groups of the protein moiety to the sulfate groups of heparin and/or to iduronate carboxyl groups in heparin which participate in chelation of Ca^{2+} was also demonstrated [34]. The interaction of heparin with ion pairs of the protein moiety may be analogous to the interaction of heparin-Ca^{2+} with the zwitterionic polar head of phosphatidylcholine. Specific interactions could account for the high apparent intrinsic binding constants for heparin.

This constant was evaluated from the dependence of g_{low} upon heparin concentration in the presence of constant Ca^{2+} (buffer 2). Applied voltage had the same sign as in the previous experiments with divalent cations. Under these conditions the apparent intrinsic binding constants for heparin were in the range of $2–15 \times 10^8$ M^{-1}. The established affinity is as high as the analogous value for the specific interaction of heparin with thrombin [35] and myosin ATPase [36], for example. Hence, it is quite possible that there is a specific interaction between heparin and ST channel+lipid bilayer system in the presence of Ca^{2+}. The uncertainty in the established value of this apparent intrinsic binding constant results from the indeterminate molecular mass of heparin, for which detailed product information was not available. In the literature the molecular mass for heparin is given as 10–80 kDa [37] and 12–42 kDa [38].

3.6. Voltage sensor

As previously discussed, it is likely that heparin-Ca^{2+} targets the voltage sensor of ST channels. What is the nature of the sensor? Is it a dipole or a titratable charge? To answer these questions we analyzed the pH dependence of the apparent gating charge q for channel transition. It was found (Fig. 8) that q increased with decreasing pH. Hence, q reflects the involvement of titratable groups and has to be positive. Therefore, when positive voltage is applied on the *cis* side (side of ST addition) those groups have to move toward the *trans* side. Likewise, if negative voltage is applied to the *cis* side, these residues must move toward the *cis* side. As the apparent value of q depends on pH, the voltage sensor of ST channels (as the voltage sensor of voltage-dependent anion-selective channels (VDAC) [39]) may be composed of both positive and negative charges. Because the value of q increases with heparin-Ca^{2+} concentration, it is

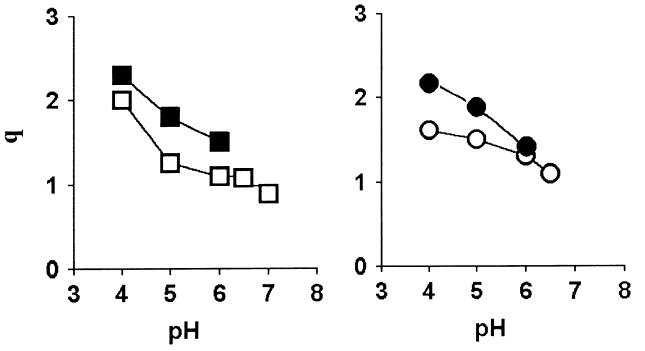

Fig. 8. Influence of pH on the apparent gating charge (q) of ST channels. Solutions in both compartments of the experimental cell contained 100 mM KCl, 10 mM Tris-citric acid with appropriate pH. q was obtained from the analysis of steady-state CVR of multichannel PLM as described in Section 2. Open squares and closed squares represent results obtained at positive voltages; open circles and closed circles represent results obtained at negative voltages. Open symbols show data obtained with symmetrical pH in both compartments of the experimental chamber. Closed symbols demonstrate data obtained in the presence of a pH gradient through the lipid bilayer: pH was shifted by addition of few microliters of citric acid solution (3 M) in the *trans* compartment at positive voltage, or in the *cis* compartment at negative voltage, while the pH value in the opposite compartment was kept at pH 7.0. Each value presents the mean of 3–4 separate experiments.

possible to hypothesize that the negatively charged residues of the protein moiety may be targets for heparin-Ca^{2+} action.

4. Discussion

4.1. Related studies: VDACs

The influence of dextran sulfate and other polyanions on VDACs incorporated in lipid bilayers has been examined recently [40–43]. It was demonstrated that the voltage dependence of VDACs could be dramatically increased by a variety of polyvalent anions such as dextran sulfate. For VDACs, as with ST channels, it appears that polyvalent anions somehow increase the charge of the sensor.

In experiments with VDACs, other researchers have traditionally used buffer solutions containing Ca^{2+} (5 mM) as one of the salt components, but apparently without considering its influence. What was difficult to understand was how the highly charged dextran sulfate anion could increase the charge on a positively charged sensor. The foregoing authors have proposed various hypotheses, including the possibility of voltage-driven accumulation of polyanions on the side of the bilayer that was made negative. We suspect that the observed effects of dextran sulfate on VDAC function are a result of a joint action of sulfated polysaccharides and divalent cations.

4.2. Related studies on ST channels

Menestrina [5,6] observed strong divalent cation effects similar to ours although KCl solution was used in the absence of sulfated polysaccharides. Analogous effects of divalent cations were then described in more detail by Bashford et al. [28,29] and noted by Korchev et al. [44]. The apparent discrepancies between their results and ours probably result from differences in experimental protocols.

The key seems to be that the sensitivity of α-toxin channels to modulation with cations is pH-determined. All experiments presented in the present work were carried out with the pH carefully maintained at 7.5. On the other hand, experiments of other investigators were made at $pH \leq 7.0$. Moreover, divalent cations were added to solutions that usually contained 1 mM EDTA. Such procedures can decrease the pH to approx. 6.0–6.2. At this pH ST channels already possess a considerable ability to spontaneous transition from high to low conductance states and divalent cations facilitate the process.

Another possible explanation for the apparent discrepancy is the agarose bridges used by the authors. Duplicating the conditions described by Menestrina (but under pH control), we also observed a strong effect of divalent cations within minutes after the experiment was started, but only if the agar or agarose used in the salt bridges was not dialyzed before use. It appears that some unidentified components diffuse from the bridges that are effective at very small concentrations, as are heparin and dextran sulfate. In the presence of these unidentified components, divalent cations increase voltage gating of ST channels in a dose-dependent manner. Hence, commercial preparations of agar and agarose are apparently contaminated with some sulfated components that increase ST channel sensitivity to voltage in the

presence of divalent cations. This possibility is emphasized by the difficulty that the authors experienced in explaining the sign dependence in divalent cation effects. They found that divalent cations act only at the negative potentials on the side of addition, i.e. potentials which drive positively charged ions out of the channel into the solution, whereas one should expect the opposite to occur. The presence of a sulfated contaminant explains their results.

It should be noted that neither divalent cations nor heparin (or glycosaminoglycans like dextran sulfate) alone were able to increase dramatically the sensitivity of ST channels to transmembrane voltage at pH 7.5 (Figs. 1–3, 5–7). It appears that only a joint action of sulfated polysaccharides and divalent cations is able to facilitate voltage-induced transition of ST channels to low conductance states. This 'closing' can be seen clearly on a bilayer containing about 15 ST channels (Fig. 9). In all cases monovalent cations stabilize the channel in the open state, while H^+ and divalent cations, especially in the presence of heparin, destabilize it.

4.3. Possible targets for the action of heparin-Ca^{2+} mixture

One can assume that the heparin-Ca^{2+} mixture (or complex) has multi-focal interactions with lipid bilayer-ST channel systems, where one or several of those interactions lead to voltage-dependent channel transition to low conductance states. Direct electrostatic interaction between positively charged residues in proteins and sulfated groups of heparin has been documented for several proteins [45–48]. However, this interaction does not demand the presence of divalent cations. Therefore this type of interaction does not explain the behavior of toxin channels.

Sulfated polysaccharides can interact with zwitterionic polar heads of lipids with the obligatory participation of divalent cations [33,49,50]. This suggests that zwitterionic polar heads of lipid and negative charges and ion pairs of the protein moiety probably participate in the interaction with heparin-Ca^{2+} mixture, where Ca bridges connect the negatively charged groups of targets with sulfated and carboxyl groups of heparin. This interaction can increase the apparent positive gating charge that accompanies increased sensitivity of ST channels to transmembrane voltage. Because the Ca bridge is suggested to be an important element in heparin action on ST channel behavior, all influences that destroy such bridges should reduce the effects of heparin. 1:1 electrolytes may be used for this purpose. We exploited NaCl and found that the addition of this electrolyte (4 M) considerably decreased the action of heparin-Ca^{2+} (Fig. 3). This could be caused by the loss of heparin binding sites due to competition between monovalent and divalent cations for negatively charged groups. Though effects of ion competition are generally more complex [29], under our conditions this explains the data. Moreover, an analogous conclusion about competition between mono- and divalent cations

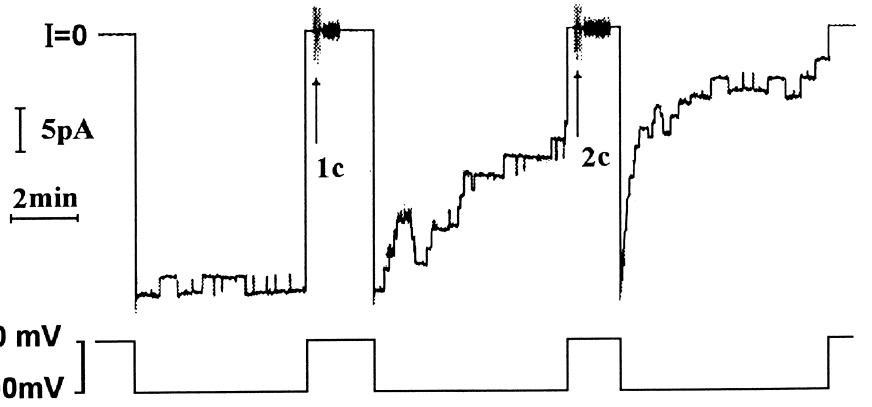

Fig. 9. Current relaxation after application of a 100 mV pulse to bilayers containing about 15 ST channels. Bathing solution was 100 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5. ST-modified lipid bilayers were prepared as described in the legend to Fig. 1. Two additions of heparin (marked 1c and 2c with arrows) were made to the *cis* compartment with final concentrations of 1 μg/ml and 6 μg/ml. The protocol of a voltage change is shown below. Current and time scales are given in the figure.

was made recently [45] regarding the inhibition of Ca-mediated binding of sulfated polysaccharides at the liposomal surface.

As mentioned above, the effects of heparin-Ca^{2+} on ST channel behavior are closely reminiscent of the results reported for the addition of polyanions on VDAC channels. As in the case of VDAC [40], we observed an increase in the steepness of the voltage dependence with virtually no alteration in the membrane surface potential because neither the open ST channel conductance nor cation-anion selectivity was changed by the heparin-Ca^{2+} mixture (Table 1). Moreover, the effects of heparin-Ca^{2+} can be formally described by a model suggested for the VDAC-dextran sulfate system by Mangan and Colombini [40]. The model assumes the voltage dependent build-up of sulfated polysaccharide near the membrane, where they can interact electrostatically with unidentified charges in the channel.

Accumulation of sulfated polysaccharide near the channel mouth requires time for the polysaccharide molecules to migrate from bulk solution. According to the Stokes-Einstein law, solute movement is inversely related to the viscosity of media. The time constant of current relaxation in response to applied voltage pulses should be larger in viscous solutions.

To examine this problem we performed several experiments where the viscosity of the *cis* and *trans* solutions was increased by the addition of one of two very different types of non-electrolyte (20%, w/v): glycerol or polyethylene glycol 200 (PEG200). As shown earlier [51], such concentrations increase the viscosity of the solutions about 1.5-fold, from approx. 0.9 to approx. 1.4 cP. According to the model and the Stokes-Einstein law we expected an increase in the lag period (from heparin addition until full activity) and the time constant of the current relaxation. Although the expected increase in the lag period was observed in the presence of glycerol or PEG200 (100 mM KCl, 50 mM $CaCl_2$, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5), the heparin-affected time constant of the current relaxation diminished even further (6 ± 5 s, $n = 10$; and 0.7 ± 0.4 s, $n = 4$; for glycerol and PEG200, respectively) than in the absence of non-electrolytes (15 ± 5 s, $n = 10$). Hence the hypothesis of a build-up of sulfated polysaccharide near the channel mouth cannot explain the experimental observations. Specific binding of heparin-Ca^{2+} is more probable.

4.4. Partition and plug in heparin-Ca^{2+} action

One might also hypothesize that accumulation of sulfated polysaccharides in the ion channel might by some means induce ST channels to transit to low conductance states. However, heparin partition into the channels has not been observed to decrease channel conductance in the high conductance state, because the very small concentrations of heparin used in these experiments are not able to measurably decrease the conductivity of the standard solutions. In solution heparin assumes two helical forms, bearing linear arrays of sulfate clusters approx. 1.1 nm in diameter and up to 20 nm in length [52,53]. The narrowest diameter of ST channels determined from a crystallographic study is larger (approx. 1.5 nm [22]) than the diameter of heparin. Partitions may appear as short- or long-lived current blockades (shown for polynucleotide molecules [54]) and as a decrease in the time constant of the current relaxation, in response to an applied voltage pulse. Some of these parameters should depend on the viscosity of the solution: the greater the viscosity, the larger the interval between short-lived blockades, the larger the duration of these blockades, the larger the time until the first long-lived current blockade and the larger the time constant of current relaxation. However, as we noted above, increased viscosity decreased the time constant. Hence it is probable that neither partition nor plug participates in the process.

Other facts also support this assumption. As mentioned above, the action of heparin-divalent cation mixtures on voltage gating of ST channels is asymmetric with respect to both the side of mixture addition and the sign of applied voltage pulses. Application of the mixture on the *trans* side induces ST channel 'closing' when positive voltage is applied (on the *cis* side). No effect is observed when a negative potential is applied. Conversely, application of the mixture to the *cis* side induces channel 'closing' if negative transmembrane voltages are used. In this case, no effect is seen when positive potentials are applied. Such side-dependent effects indicate that heparin is essentially unable to traverse the ST channel. Therefore, the absence of a slowing-down of the

heparin-stimulated current relaxation with increased viscosity and the side-dependent effect allow us to reject the partition and the plug hypothesis in the heparin-Ca^{2+} action on ST channel function.

The next consideration is that voltage gating of ST channels is dependent on the presence of heparin+ divalent cations on the side of the bilayer where negative voltage is applied. Addition of the mixture to both sides of the lipid bilayer did not alter the behavior with respect to that observed with unilateral addition of the mixture (Figs. 3 and 6). Such effects provide evidence that the voltage-gating charges (which are plausible targets for heparin-Ca^{2+} action and involved in ST channel transition to low conductance states) are physically different for positive and negative voltage pulses and may be localized on the *trans* and on the *cis* side of the channel-membrane complex, respectively.

Side and voltage sign asymmetries in the action of heparin-Ca^{2+} on voltage gating of ST channels suggest that negative and positive voltages may induce entirely different cascades of structural changes, starting at opposite ends of the channel. For instance, it appears that when positive voltage is applied to the *cis* side of the channel, channel closure commences at the *trans* opening, because the time constant of channel transition diminishes only when heparin-Ca^{2+} is added to (or pH is lowered in) the *trans* compartment (pH data not shown). In both cases we have observed an increase in the apparent gating charge (see Figs. 7 and 8). On the other hand, negative voltage on the *cis* side induces structural reorganization at the *cis* opening after heparin-Ca^{2+} is added to (or pH is lowered in) the *cis* compartment. The analogous conclusion about the voltage sign-dependent structural reorganization was made recently from analysis of pH influence on the voltage-independent component of free energy of ST channel transition (ΔG_i) from high to low conductance state [7]. The present findings strongly support the pH observations.

4.5. Heparin effects and local pH decrease at the vicinity of the ST channels

It is very attractive to explain the effects of heparin through decreased local pH in the vicinity of ST channels. However, this hypothesis predicts a biphasic effect of divalent cations: the activation of ST channel transition to low-conductance states at small concentrations, which is followed by a reversal of the divalent cation's effect with increasing concentrations of divalent ions. The first phase is based on increased binding of heparin to targets (that will increase the local H^+ concentration) and the second phase is based on decreasing the total negative charge of heparin. However, we have never observed this second phase, although we have used relatively high concentrations of divalent cations (see Fig. 4).

Another argument against this hypothesis is that heparin is not able to change such properties of ST channels as selectivity, conductance and asymmetry in current-voltage relationship, properties that are highly sensitive to pH and surface charges located at the vicinity [23,25].

4.6. Facts established

(1) In the presence of Ca^{2+} heparin significantly increases the voltage-dependent closing of ST channels. Heparin is active at a concentration as low as approx. 10^{-10} M. It is the most active polymer known.

(2) The action of heparin-divalent cation mixtures on voltage gating of ST channels is asymmetrical with respect to the side of mixture addition and the sign of applied voltage pulses.

(3) As co-factor Zn^{2+} is more effective and Mg^{2+} is less effective than Ca^{2+}.

(4) Sodium chloride (4 M) considerably diminished the effect of heparin-Ca^{2+} on ST channels.

(5) Increased viscosity of bathing solutions does not decrease the effect of heparin on the time constant of voltage-dependent transmembrane current relaxation.

(6) Both heparin-Ca^{2+} and decreased bathing solution pH increase the effective gating charge of ST channels in a dose-dependent manner.

(7) The steepnesses of q-pH dependences and effects of heparin-Ca^{2+} obtained at opposite voltage signs are different.

4.7. Conclusions

(1) The structural reorganizations of ST channels, which occur at positive and negative voltages, are

suggested to be different. Negative and positive voltages may induce entirely different cascades of structural changes, starting at opposite ends of the channel. Charged residues that contribute to the effective gating charge at negative voltage may differ from those at positive voltage.

(2) The phenomenon of voltage-dependent ST channel closure is an intrinsic property of the channels. H^+, divalent cations alone and, especially, in combination with heparin simply facilitate the process. 'Free' carboxyl groups and carboxyl groups in ion pairs of the protein moiety, together with phosphate groups of phospholipid heads are hypothesized to be plausible targets for heparin action via divalent cation bridges. Such interaction would decrease the appearance of negatively charged groups of the ST channel+lipid bilayer complex. As a result, an increase in the appearance of positively charged groups (some of which contribute to the gating charge) could be seen. Heparin-Ca^{2+} interacts with targets localized at the ST channel+lipid bilayer complex at the side of addition. The unique conformational flexibility of heparin [55] may explain its wide range of biological activity, including its effect on ST channels.

Much remains to be done to establish which residues of the ST molecule interact with heparin. Our data suggest that carboxyl groups may be the targets for heparin-Ca^{2+} action and that the gating charge is positive. Although the structure of the ST channel was recently established at high resolution [22], there is not enough information to determine exactly which groups interact with heparin-Ca^{2+} and which groups are responsible for the gating charge of the channels. Future experiments with point-mutated ST may clarify the problem.

The proposed mechanism of heparin action on ST channels may be applicable to other types of channels as well. A similar mechanism could be responsible for some of the heparin effects demonstrated recently on L-type Ca channels in cardiac myocytes under whole-cell patch clamp conditions [56] and on Ca-gated Ca channel incorporated in planar lipid bilayers [14]. The effects of sulfated dextrans on VDAC [40–43] could also result from an analogous mechanism.

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