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Effect of Protamine Sulfate on the Permeability Properties of the Mammalian Urinary Bladder

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Summary. Protamine sulfate (PS, an arginine-rich protein of molecular weight 5,000) has been reported to affect the ionic permeability of gallbladder epithelium, the permeability of cultured epithelial cells to mannitol, and the permeability of endothelial cell layers to albumin. Although the effect of PS has been widely investigated, the mechanism of its action on membrane permeability is presently unknown.

The effect of PS on the rabbit urinary bladder epithelium was studied using both transepithelial and intracellular microelectrode techniques in conjunction with equivalent circuit analysis. The addition of 100 μg/ml of PS to a NaCl-containing mucosal solution caused (over a 40-min period) a large increase in the transepithelial conductance (G_t) and a transient hyperpolarization of the transepithelial voltage (V_t) followed by a depolarization of V_t. This secondary depolarization of V_t was not present if the mucosal solution was a KCl or a K-gluconate Ringer. The PS-induced increase in G_t was due to an increase in the apical membrane permeability to both cations (Na^+ or K^+) and anions (Cl^- or gluconate). Further studies revealed the following features of the PS-induced conductance. (i) Trypsin inhibits the PS effect; however, this was due to PS hydrolysis by trypsin and not a membrane effect. (ii) Mucosal PS partially inhibited the PS-induced apical membrane conductance. (iii) The ability of PS to increase the membrane conductance was enhanced when the apical membrane potential was cell interior negative. (iv) The rate of conductance change (at any given membrane potential) was a saturating function of the PS concentration. This finding suggests that PS must interact with a membrane binding site before it can induce a change in the membrane conductance. (v) Lanthanum inhibited the PS-dependent conductance by two different mechanisms. One was as a reversible blocker of the PS-induced conductance. The other was by inhibiting the interaction between PS and a membrane binding site. A kinetic model is developed to describe the steps involved in the increase in membrane conductance.

Key Words epithelial permeability · protamine · polycations · voltage sensitive conductance · protein binding

Introduction

Heparin (an acid mucopolysaccharide) is an anticoagulant used to treat thrombosis-related diseases (Jorpes, 1962). Due to the narrow therapeutic range, heparin is hazardous to use because of the increased risk of bleeding due to over-heparinization. Protamine sulfate (PS: a basic polycationic protein) is used clinically as an antidote to such over-heparinization. Unfortunately, protamine sulfate has been shown to produce adverse side effects (*see* Horrow, 1985 for review) such as systemic hypotension, bradycardia (reduced heart rate), pulmonary artery hypertension (Lowenstein et al., 1983), neutropenia (a decrease in the number of neutrophilic leukocytes in the blood), and thrombocytopenia (a decrease in the number of platelets).

In addition to the above clinical manifestations, protamine sulfate and other basic polycationic proteins such as polyarginine and polylysine mimic renal pathophysiological conditions such as minimal change nephrotic syndrome (Firth, 1990), and chronic serum sickness glomerulonephritis (Furness, 1990). Other effects of PS include an increase in pulmonary endothelial permeability (Chang et al., 1987; Peterson, Stone & Shasby, 1987; Sunnerguen et al., 1987); stimulation of arachidonic acid and prostaglandin production in glomerular epithelial and mesangial cells (Pugliese et al., 1987; Alavi 1990); inhibition of tubulin carboxypeptidase (Modesti et al., 1984; Lopez et al., 1990); secretion of thromboxane from platelets (Montalescot et al., 1990; Yoshioka et al., 1991); an increase in the release in creatine kinase and lactate dehydrogenase from frog muscle (Suarez-Kurtz, 1985) and increased enzyme secretion by neutrophils (Elferink & Deierkauf, 1986).

Although PS alters the permeability properties of almost all epithelia studied, the direction of the permeability change (increase or decrease) and the proposed site of the permeability change (paracellular pathway, apical membrane or membrane-associated fixed negative charge) depends not only on the type of epithelium studied, but also on the experimental conditions and concentration of PS used.

In the *Necturus* gallbladder (Bentzel et al., 1987; Fromm et al., 1990) and in the ascending thin limb of Henle's loop (Koyama, Yoshitomi & Imai, 1991), low concentrations of PS have been shown to reduce the cation permeability of the paracellular pathway, i.e., the paracellular conductance is decreased without affecting the cellular resistance. This is opposite to the report by Poler and Reuss (1987) and Sato and Ullrich (1975), who demonstrated, respectively, that PS did not alter the paracellular pathway in the *Necturus* gallbladder or renal proximal tubule, but did alter the apical membrane permeability properties of these tissues. Possible explanations for this difference include different experimental conditions such as PS concentration and solution pH (Fromm et al., 1990), or the intrinsic sensitivity of the tissues to PS (Koyama et al., 1991).

In contrast to the reported decrease (or lack of effect) of PS on the paracellular conductance of the gallbladder and renal tubule, PS has been reported to increase the paracellular permeability of confluent layers of MDCK cells to mannitol (Peterson & Gruenhaupt, 1990). This PS-induced increase in the permeability of substances through the paracellular pathway might be a result of cell injury (Quinton & Philpott, 1973). In the rabbit urinary bladder epithelium, Parsons et al. (1990) proposed that the PS-induced increase in the transepithelial flux of urea, water and calcium was due to an interaction of PS with the negatively charged glycosaminoglycan layer which lies on the surface of the apical membrane. In their model, the main permeability barrier across the urinary bladder is not the apical membrane or paracellular pathway but the glycosaminoglycan layer.

This paper investigates the site of action of PS on the rabbit urinary bladder epithelium (a high resistance epithelium) using electrophysiological methods. The data suggest that PS causes a dose-dependent increase in the apical membrane conductance to both cations and anions and that this increase in membrane conductance is due to an interaction of PS with a binding site at the apical membrane. In addition, long-term exposure (greater than 1 hr) of urinary bladder epithelial cells to PS results in an irreversible decrease in the paracellular resistance which might be due to PS-induced cell lysis.

Materials and Methods

Tissue Preparation

Urinary bladders from male New Zealand White rabbits (3–5 kg) were excised and washed 2–3 times in NaCl-$NaHCO_3$ Ringer solution (*see* Solutions). The bladder was then stretched on a rack immersed in this solution at 37°C, and forceps and a razor blade were used under a binocular microscope to dissect the smooth muscle layers. The epithelium was then mounted on a ring of 2 cm^2 exposed area and transferred to a temperature-controlled modified Ussing chamber (Lewis & Diamond, 1976). The initial bathing solution was NaCl Ringer stirred magnetically, aerated with 95% O_2-5% CO_2 and maintained at 37°C. The tissue usually took 30–60 min before the transepithelial voltage and resistance reached steady-state values.

Solutions

The composition of the NaCl Ringer solution (in mM) was 111.2 NaCl, 25 $NaHCO_3$, 10 glucose, 5.8 KCl, 2 $CaCl_2$, 1.2 KH_2PO_4 and 1.2 $MgSO_4$. In KCl Ringer solution, all Na^+ salts were substituted with the appropriate K^+ salts. In the chloride-free Ringer, all chloride-containing salts were substituted with the appropriate gluconate salt. In the experiments using lanthanum (as a blocker), the solution was buffered by HEPES to yield a HCO_3-free and HPO_4-free solution. Protamine as the sulfate salt (PS; Sigma grade X from salmon sperm, histone free, mol wt ~5,000) was made as a stock solution and microliter quantities were then added to the mucosal solution.

Transepithelial Electrical Measurements

Open circuit potential (V_t) measurements were made with Ag-AgCl wires placed close to and on opposite sides of the epithelium (serosa ground), or through 1 M KCl agar bridges when using Cl^--substituted solutions. Transepithelial resistance (R_t) was determined by passing current (ΔI; from coiled Ag-AgCl electrodes placed in the rear of each chamber) and measuring the transepithelial voltage response (ΔV_t). The short circuit current (I_{sc}) was calculated as V_t/R_t. Both voltage and current electrodes were connected to an automatic voltage clamp.

Microelectrode Measurements

Microelectrodes were fabricated from fiber-filled borosilicate glass tubing (World Precision Instruments, 1B120F-4), pulled to a tip diameter of <1 μm (tip resistance was greater than 20 MΩ) on a Narishige vertical air-cooled puller, and then backfilled with 1 M KCl. Microelectrodes were inserted into Ag-AgCl half-cells, mounted on a three-dimensional electric drive micromanipulator and connected to a high impedance differential electrometer (World Precision Instruments model 750).

The microelectrodes were used to determine the basolateral membrane potential (V_{bl}; serosa ground) and the ratio of the apical to the basolateral membrane resistances. Impalements were made through the apical membrane and referenced to the serosal voltage measuring electrode. The ratio of the apical to the basolateral membrane resistance (R_a/R_{bl}: the resistance ratio or α) was calculated by measuring the change in the apical and basolateral membrane voltage to a transepithelial current pulse. This ratio of voltage deflections is equal to the ratio of the apical to basolateral membrane resistances (Lewis, Eaton & Diamond, 1976).

Data Acquisition

The current (I) and voltage (V_t) outputs of the voltage clamp and the voltage output of the microelectrode amplifier (V_{bl}) were connected to an A/D converter (Axon Instruments) interfaced

to a small laboratory computer. All three parameters were then digitized, stored on hard disk and logged to a printer along with the time of data acquisition and the calculated values for R_t, I_{sc} and the ratio of the apical to the basolateral membrane resistance (α). In addition, all three parameters were continuously monitored on an oscilloscope and a paper chart recorder.

Equivalent Circuit Analysis

Transepithelial Method

To determine the site of action of PS on the urinary bladder epithelium (i.e., cell membrane or tight junction), the method developed by Wills, Lewis and Eaton (1979) was used. This analysis is based on the fact that the urinary bladder epithelium can be modeled as a series combination of a cellular battery (E_c) and resistor (R_c) in parallel with a junctional resistor (R_j). The equation which describes the transepithelial voltage (V_t) is:

$$V_t = E_c - (E_c/R_j)R_t. \quad (1)$$

If only the cell resistance is altered by the added agent, then this is a linear equation and a plot of V_t versus R_t will have an intercept on the ordinate of E_c and a slope of $(-E_c/R_j)$. If the added agent causes a change in the E_c and/or R_j, then a plot of V_t *vs.* R_t will be curvilinear.

Microelectrode Methods

To determine the site of PS action, intracellular microelectrodes in conjunction with transepithelial measurements were used. In brief, the transepithelial conductance (G_t) and the resistance ratio (α) were measured before and during the addition of PS to the mucosal solution. If PS alters only the apical membrane resistance then the apical, basolateral and junctional resistance can be calculated according to the equation (*see* Lewis et al., 1977).

$$G_t = G_j + G_{bl}/(1 + \alpha). \quad (2)$$

If the assumption of only an apical membrane effect is correct, then a plot of G_t *vs.* $(1 + \alpha)^{-1}$ will be linear with an intercept on the ordinate equal to G_j and a slope equal to the basolateral membrane conductance (G_{bl}, the inverse of R_{bl}). The apical membrane conductance can then be calculated from the resistance ratio and the basolateral membrane conductance.

Current-Voltage Relationship

Current-voltage (*I-V*) relationship of the PS-induced conductance was determined using the following protocol. First, a control transepithelial *I-V* relationship was generated by measuring the transepithelial current responses to computer-generated voltage pulses (30 msec duration) of increasing magnitude and alternating polarity. Next, PS was added to the mucosal solution and another computer-generated *I-V* was produced. Since for the control and the experimental condition, the apical membrane conductance is much smaller than the basolateral membrane conductance, then the *I-V* relationship of the control condition approximates that of two parallel conductive pathways (apical membrane: G_a and junction: G_j), and the *I-V* relationship after PS addition (experimental *I-V*) approximates that of three parallel conductors (apical membrane, junction and the PS-induced conductance: G_a^{ps}). The voltage dependence of the current flowing through the PS-induced conductance was then calculated by taking the difference of the currents (experimental minus control) at each voltage step. This DIFFERENCE *I-V* relationship was then curvefit by the constant field equation (Goldman, 1943; Hodgkin & Katz, 1949) to estimate the relative ion permeability of the PS-induced conductance.

Data Analysis

All data are expressed as the mean ± SE. Student's *t*-test (paired or unpaired) was used to determine significance, with $P < 0.05$ being significant. Curve-fitting of the current to voltage relationships was performed on an IBM-AT using NFIT (Island Products, Galveston, TX).

Results

In this section we first demonstrate that protamine sulfate (PS) alters the electrical properties of the rabbit urinary bladder epithelium. Next, microelectrodes are used to demonstrate that the apical membrane is the major site of action of PS. Last, some of the kinetic properties of this induced conductance are described.

Transepithelial Effects of Protamine Sulfate (PS)

The addition of 100 μg/ml of PS (~20 μM) to a NaCl mucosal solution caused a time-dependent decrease in the transepithelial resistance (R_t) of the rabbit urinary bladder epithelium (Fig. 1*a* and Table). During the decrease in R_t, the transepithelial voltage (V_t: serosa ground) initially hyperpolarized over the first 20 min, followed by a depolarization over the next 40 min (Fig. 1*a* and Table). If the primary site of action of PS is on the cell membrane resistance (and not the junctional resistance or cell emf) then plots of V_t *vs.* R_t should be linear. As shown in Fig. 1*b*, a plot of V_t *vs.* R_t (over the first 10 min of PS exposure) is near linear. This suggests that for the short-term response, PS is increasing the cell conductance. The best fit E_c was -32 ± 2.3 mV ($n = 5$) and the R_j was 30,700 ± 6,100 Ωcm^2. The secondary decrease in V_t could be due to either (or both) a decrease in E_c and junctional resistance.

To determine whether the secondary decrease in V_t is due to a decrease in E_c as a result of a loss of cell potassium activity and a concomitant increase in cell Na and/or Cl, PS was added to a mucosal solution composed of either a KCl or a K-gluconate Ringer. The addition of PS to either a mucosal KCl or K-gluconate Ringer, results in a hyperpolarization

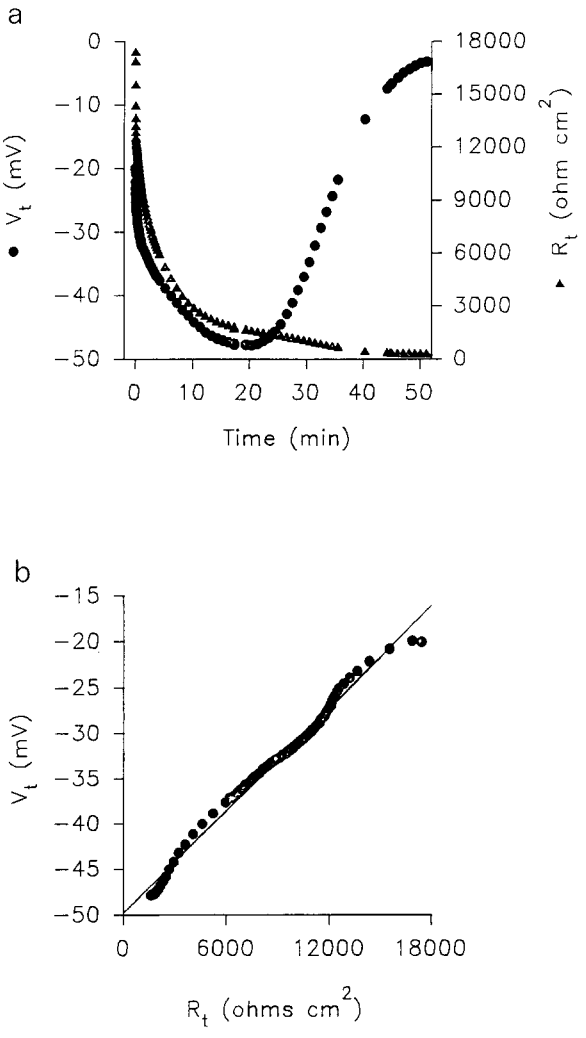


Fig. 1. (*a*) Time course of the effect of protamine sulfate on the transepithelial resistance (R_t; filled triangles) and voltage (V_t; filled circles). Protamine sulfate (100 μg/ml final concentration) was added to the mucosal Na Ringer solution. The transepithelial resistance decreased over the 53 min measurement period. Of interest is that the voltage response is biphasic, i.e., an initial hyperpolarization followed by a secondary depolarization (*see text*). (*b*) Determination of the junctional resistance and cell electromotive force (E_c) using transepithelial measurements of the time course of protamine sulfate effect on the electrical properties of the urinary bladder epithelium when it is bathed in a NaCl Ringer. Using equivalent circuit analysis (*see* Materials and Methods), a plot of V_t *vs.* R_t will be linear only if PS alters only the cell resistance. The predominant effect of PS is at the cell membrane and not the tight junction or (over the first 10 min) the cellular emf. The best fit values are an E_c of −48.4 mV, a R_j of 28,000 Ωcm^2 and a correlation coefficient of 0.9997.

of V_t without the secondary depolarization (Fig. 2*a* and Table, K-gluconate data not shown but similar to that of KCl). Equivalent circuit analysis (Fig. 2*b*, V_t *vs.* R_t plots) demonstrates that the long-term hyperpolarization is due to a decrease in R_c. The best fit value for E_c in KCl Ringer was −46 ± 2.1 mV (n = 9) and R_j was 24,600 ± 3,400 Ωcm^2, and in K-gluconate Ringer was −44.3 ± 1.3 mV (n = 5) and R_j was 36,000 ± 8,500 Ωcm^2. The absence of a secondary depolarization results from a near constant E_c and R_j and suggests that the secondary depolarization in NaCl Ringer resulted from a decrease in E_c due to the loss of cell K^+ in exchange for Na^+.

Figures 1*b* and 2*b*, show that the relationship between V_t and R_t is initially curvilinear. This relationship could be caused by a transient decrease in the junctional resistance in parallel with a decrease in the cell membrane resistance or alternately by a time-dependent change in the emf of the PS-induced membrane conductance with a constant junctional resistance. Since microelectrode data (*see below*) demonstrate that PS does not alter the junctional resistance, this suggests that there is a time-dependent change in the emf (selective permeability) of the PS-induced conductance.

The effect of PS on R_t and V_t was reversed once PS was removed from the mucosal solution by replacement of the mucosal solution with fresh Ringer (Fig. 3), addition of pentosan-polysulfate to the mucosal solution (PPS, a highly negative charged compound known to bind PS), or addition of trypsin (which hydrolyzes PS) to the mucosal solution (*see below*). The extent of the reversal of the PS effect on R_t was dependent on the composition of the mucosal bathing solution, the concentration of PS added to the mucosal solution and the length of time the tissue was exposed to PS. Poor reversibility (of the PS effect on R_t) was noted when the epithelium was exposed for long periods of time to PS, when NaCl was used as a mucosal bathing solution during PS exposure or when high concentrations of PS were used. This decrease in the value of R_t was due to a decrease in the junctional resistance (R_j) i.e., an increase in the paracellular conductance. A possible explanation for the decrease in R_j might be cell death due to a direct cytotoxic effect of PS, the loss of some essential cellular components required for cell survival or an increased influx of bathing solution solutes leading to an increase in intracellular solute content and subsequent cell lysis.

Site of Action of Protamine Sulfate

The above data suggest that PS is causing an increase in the apical membrane conductance. To determine if this is indeed the case, microelectrodes were used to measure the effect of PS on the ratio of the apical to basolateral membrane resistance. If the increase of transepithelial conductance is due to an increase

Table. The time dependent effect of PS on the transepithelial voltage (V_t) and resistance (R_t)

	V_t (mV)					
	0 min $n = 4$	5 min $n = 4$	20 min $n = 4$	40 min $n = 4$	50 min $n = 2$	60 min $n = 2$
NaCl	−17.8 ± 2.7	−34.0 ± 3.5	−41.8 ± 4.5	−32.5 ± 3.2*	−12.0 ± 9.0*	−9.0 ± 7.0*
KCl	−20.5 ± 5.6	−33.5 ± 3.2	−41.3 ± 2.9	−41.8 ± 3.2	−45.0 ± 8.0	−41.5 ± 6.5
	R_t (Ωcm^2)					
NaCl	12,298 ± 1,307	5,303 ± 666	2,832 ± 267	2,077 ± 292	783 ± 443	780 ± 467
KCl	17,158 ± 3,443	6,556 ± 2,152	3,772 ± 983	2,513 ± 566	1,378 ± 43	1,165 ± 51

Protamine sulfate was added at a final concentration 100 μg/ml to the mucosal solution.
* Significantly different from the V_t at 20 min ($P < 0.01$).

of the apical membrane conductance, then the resistance ratio (R_a/R_{bl}) measured using microelectrodes should decrease (Lewis et al., 1977). Addition of PS to the mucosal solution caused a large decrease in R_a/R_{bl} (from 18 ± 3.6 to 0.24 ± 0.15; $n = 4$). This large decrease in R_a/R_{bl} strongly suggests that the decrease in R_t by PS was due to a decrease in the apical membrane resistance (R_a). The linearity of a plot of G_t *vs.* $(1 + \alpha)^{-1}$ (Fig. 4) confirms the above observation that the primary effect of PS is on the apical membrane and not on the tight junction or the basolateral membrane. After reaching a steady-state resistance, microelectrode impalements demonstrated that the resistance ratio of randomly sampled cells were uniformly low. This demonstrates that PS is altering the apical membrane resistance of all cells as opposed to the resistance of a subpopulation of cells.

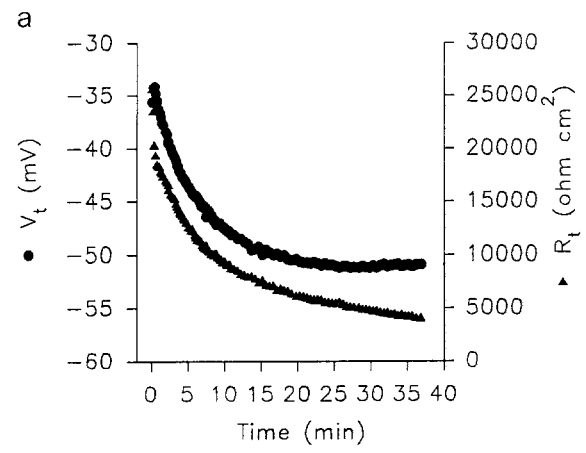
The emf of the PS-induced apical membrane conductance (E_a) was calculated from measurements of E_c (from V_t *vs.* R_t plots, *see above*) and the basolateral membrane emf (E_{bl} of −55 mV, *see* Lewis & Wills, 1981) as $E_a = E_c - E_{bl}$, when the apical bathing solution was KCl, K-gluconate or NaCl. The relative selective permeability was calculated using the constant field equation (Hodgkin & Katz, 1949) and known values for the cell ion activities (Lewis & Wills, 1981). The calculated permeability ratios are for $P_{Cl}/P_K = 1.1 \pm 0.8$ ($n = 9$), $P_{Na}/P_K = 0.58 \pm 0.14$ ($n = 5$) and $P_{gluconate}/P_K = 1 \pm 0.25$ ($n = 5$). Thus, this conductance pathway is relatively nonselective since it does not discriminate between cations and anions. The pathway is also relatively large as it permits the movement of gluconate.

Protamine Effect Is Not Due to a Contaminant

Is the effect of PS on the apical membrane conductance a consequence of a nonprotein contaminant of the PS stock solution, or some other property of PS? To determine whether there is a nonprotein contaminant, PS was pretreated with 0.5 mg/ml trypsin (trypsin will degrade PS into seven fragments; Ando & Watanabe, 1969) and this mixture was then added to the mucosal solution. Figure 5 demonstrates that trypsin-treated PS does not cause a change in the apical membrane conductance. Pretreatment of the apical membrane with trypsin, followed by removal of trypsin and subsequent addition of PS to the mucosal solution did not inhibit the increase in apical membrane conductance (Fig. 5). Since trace quantities of trypsin in the mucosal solution can rapidly decrease the mucosal PS concentration (*data not shown*), we routinely added soybean trypsin inhibitor to the mucosal chamber to totally inhibit all trypsin and subsequently washed this complex from the mucosal bath. The above data demonstrate that PS causes an increase in apical membrane conductance as opposed to a nonprotein contaminant of the stock solution, and that the PS must be intact to alter the membrane conductance, i.e., there seems to be a size requirement for PS action. In addition, since trypsin does not alter the response of the apical membrane conductane to PS, this rules out the possibility of PS interacting with a trypsin-sensitive membrane protein.

Reversibility of Protamine

As mentioned above, the conductance increase induced by protamine sulfate can be reversed by three independent methods: (a) trypsin hydrolysis (b) pentosan-polysulfate precipitation, or (c) mucosal fluid replacement. An unexpected observation was that upon removal of PS from the mucosal bathing solution (using any one of the above methods), there was an initial increase in the apical membrane conductance followed by a decrease in this conductance. This phenomenon is illustrated in Fig. 6, where addition of 0.5 mg/ml of trypsin to the muco-



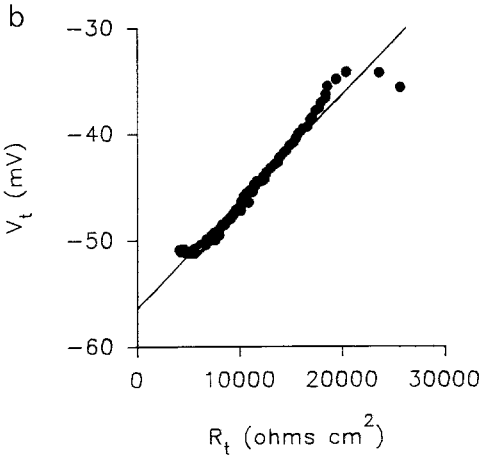


Fig. 2. (*a*) Time course of the effect of protamine sulfate (100 μg/ml) on V_t (filled circles) and R_t (filled triangles) when the mucosal solution is a KCl Ringer. The experimental protocol was the same as in Fig. 1 except that the mucosal bathing solution was KCl Ringer instead of NaCl Ringer. The transepithelial voltage hyperpolarized (more negative) and subsequently plateaued. Note that there was no secondary depolarization. (*b*) Same as in Fig. 1*b* except the mucosal bathing solution is a KCl Riniger. The best fit values are an E_c of -56.4 mV, a R_j of 53,700 Ωcm^2 and a correlation coefficient of 0.9997. Of interest is the transient decrease in V_t, which could be due to a transient decrease in the junctional resistance or to a time-dependent alteration in the cellular emf.

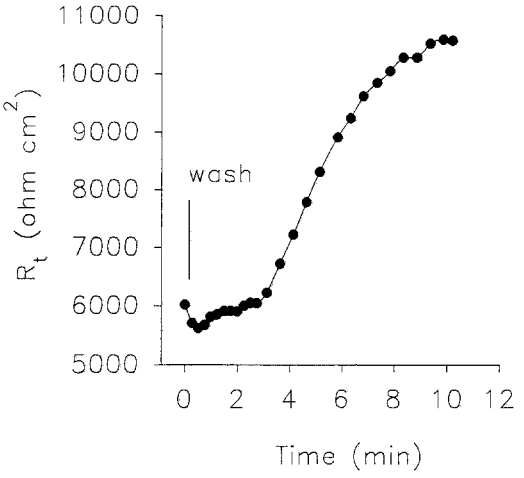


Fig. 3. Time course of the recovery of R_t by replacing the mucosal solution (which contains 100 μg/ml of PS) with a protamine sulfate free NaCl Ringer. The initial lag phase is due in part to an unstirred layer effect.

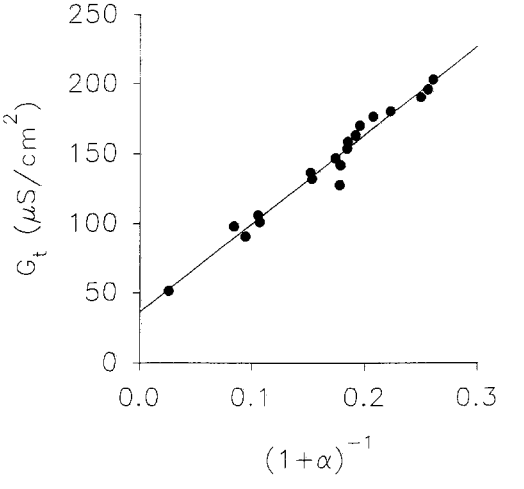


Fig. 4. Mucosal addition of PS (100 μg/ml) results in a linear relationship between the transepithelial conductance (G_t) and $(1 + \alpha)^{-1}$. This suggests that PS is altering only the apical membrane conductance.

sal solution of a tissue preincubated with PS, results in an initial increase in membrane conductance followed by a decrease in conductance to a value close to the membrane conductance before PS addition. A working hypothesis for the above conductance changes is that, not only do the cationic proteins induce an increase in the membrane conductance, but they also act as a partial blocker of this pathway.

A simple model which can describe this response is:

$$G_{ps}^{b} \xrightarrow{k_{ba}} G_{ps}^{a} \xrightarrow{k_{ao}}$$

where, k_{ba} is the rate at which blocked channels change to unblocked channels, k_{ao} is the rate at which PS leaves the membrane, G_{ps}^{b} is the *b*locked PS conductance and G_{ps}^{a} is the *a*ctive PS conductance. The above four parameters can be determined by curvefitting the time course (*see* Fig. 6) using

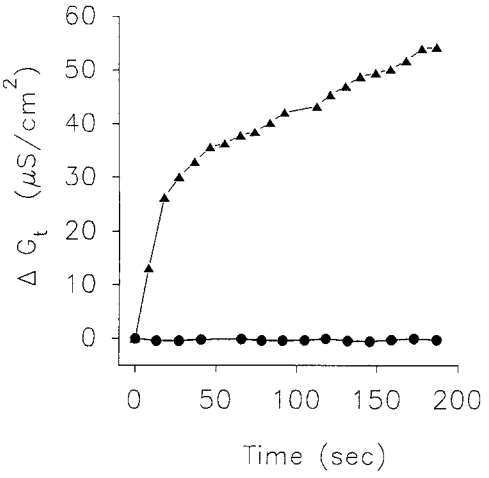


Fig. 5. Preincubation of PS (100 μg/ml) with trypsin (0.5 mg/ml) followed by addition of the PS plus trypsin to the mucosal solution at time 0, eliminates the ability of PS to increase the membrane conductance (filled circles). First, this suggests that it is the polypeptides and not a nonprotein contaminant which is inducing the increase in membrane conductance. Second, it demonstrates that molecular weight is a crucial factor in determining the efficacy of the cationic proteins for increasing the membrane conductance. The filled triangles show the effect of 100 μg/ml of PS on the same tissue after the removal of the mucosal trypsin. This response is not different than that for tissues not pretreated with trypsin, i.e., trypsin is not altering the apical membrane and making it nonreactive to PS.

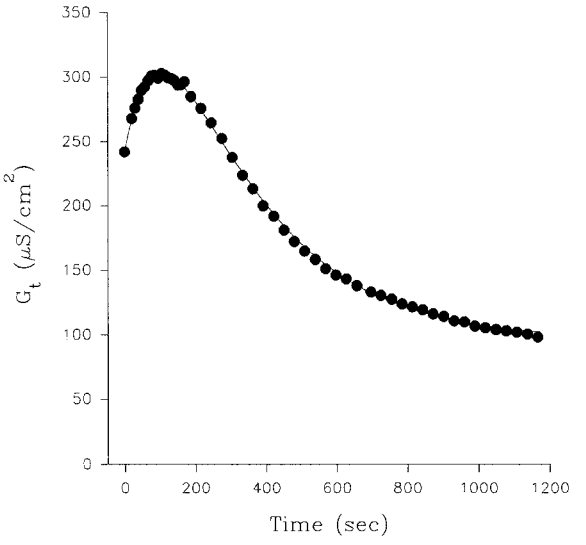


Fig. 6. The time course of the change in G_t when 0.5 mg/ml trypsin was added to the mucosal solution containing 100 μg/ml of PS. The response is biphasic; there is an initial increase in the conductance followed by a return of the conductance to the intrinsic membrane conductance. The above conductance change suggests that not only does PS induce an increase in the membrane conductance, but it also acts as a (partial) blocker of this pathway.

the equation:

$$G_t = \frac{G^b_{ps} k_{ba}}{k_{ao} - k_{ba}} (\exp^{-k_t^{ba}} - \exp^{-k_t^{ao}}) + G^a_{ps} \exp^{-k_t^{ao}} + G_a \quad (3)$$

where, G_t is the measured membrane conductance, t is time (in minutes), and G_a is the normal membrane conductance (membrane conductance when there is no PS in the solution or the membrane). Best fit values for the above parameters are: $k_{ba} = 2.0 \pm 0.7$ min^{-1}; $k_{ao} = 0.145 \pm 0.003$ min^{-1}; $G^b_{ps} = 214 \pm 58$ μS/cm^2; $G^a_{ps} = 296 \pm 70$ μS/cm^2; and $G_a = 94 \pm 20$ μS/cm^2 ($n = 4$). The value of k_{ba} most likely represents the rate of diffusion of trypsin through the unstirred layer to the apical membrane, the rate of hydrolysis of PS by trypsin as well as the rate of dissociation of PS from the conductive unit. It is important to note that this model assumes that PS can only leave the membrane from the conductive state, i.e., a channel which is self-inhibited is stable in the membrane. A more complex model, in which a blocked conductance can also dissociate from the membrane, fits the data as well as the above model; however, the best fit value for G^b_{ps} increases and the best fit value for k_{ba} decreases. Further studies are needed to differentiate between these two models.

Other supportive evidence for self-inhibition is that addition of pentosan-polysulfate (a negatively charged polysaccharide) or simple washout of bulk solution PS show a similar time-dependent change of the membrane conductance. That diffusion might be the major determinant of k_{ba} is supported by the observation that using high levels of PPS (a 50-fold excess will shorten the time for a stoichiometric concentration of PPS to be achieved at the apical membrane) increases the rate constant of the conductance increase to 6.7 ± 1.0 min^{-1} ($n = 3$) without changing k_{ao}.

If PS is self-inhibiting, then an additional dose of PS (to a tissue preincubated with PS), should result in a transient decrease in apical membrane conductance followed by the predicted increase in apical membrane conductance. Such an experiment is shown in Fig. 7 and supports the hypothesis that, not only does PS increase the membrane conductance, but it also acts as a partial inhibitor of this conductance, i.e., demonstrates self-inhibition. In three tissues, the further addition of 400 μg/ml of PS resulted in a small ($9.3 \pm 4.6\%$ $n = 7$) but significant ($P = 0.015$) transient decrease in the PS-induced conductance.

The self-inhibition is also voltage dependent (Fig. 8). The current-voltage relationship of the PS-induced conductance pathway is unique (rectifying

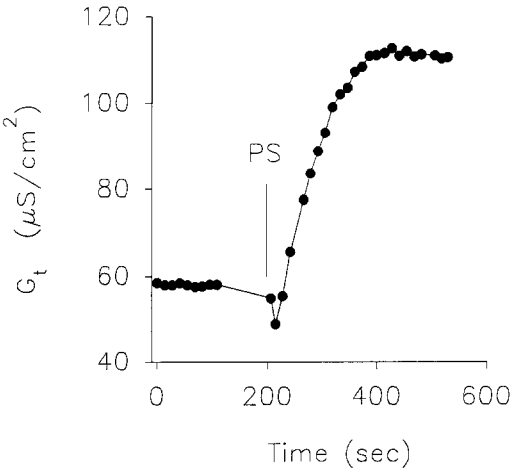


Fig. 7. PS partially inhibits the induced conductance. This is demonstrated by the observation that a further addition of 400 μg/ml of PS to the mucosal solution (which initially contains 100 μg/ml of PS) results in a transient decrease in the membrane conductance (at the arrow) followed by an increase in the membrane conductance. This secondary conductance increase is due to the elevated concentration of mucosal solution PS.

at both high positive and negative voltages). The apical membrane zero current voltage (with a KCl mucosal Ringer) is 3.4 ± 1.9 mV (n = 7; cell interior ground) and suggests that the channel is nonselective. When pentosan-polysulfate was added to the mucosal solution, the *I-V* relationship became linear and could be fitted to the constant field equation. The best fit values for the selective permeabilities are the same as those calculated from the measurement of apical membrane emf (*see above*).

Voltage Sensitivity

Since charge seems to be an absolute requirement for the PS to increase the apical membrane conductance, one might predict the membrane voltage might influence the magnitude of rate of the PS-induced membrane conductance. Figure 9 shows that the ability of PS to alter the apical membrane conductance is very voltage dependent. The addition of 10 μg/ml PS to the mucosal solution of a bladder which was voltage clamped such that the apical membrane potential was approximately −10 mV (cell interior ground; V_t = −60 mV), shows a very slow rate of conductance change when compared to a tissue which is voltage clamped such that the apical membrane potential is 50 mV (cell interior ground; V_t = 0 mV, Fig. 9). This time-dependent change in the membrane conductance is due in part to an unstirred layer effect (i.e., the time taken for the PS to diffuse to the apical membrane). To investigate the voltage dependence of the PS-induced conductance change in the absence of the unstirred layer effect, the bladder was first clamped at a V_t of −60 mV, and then incubated with 10 μg/ml PS for 5 min

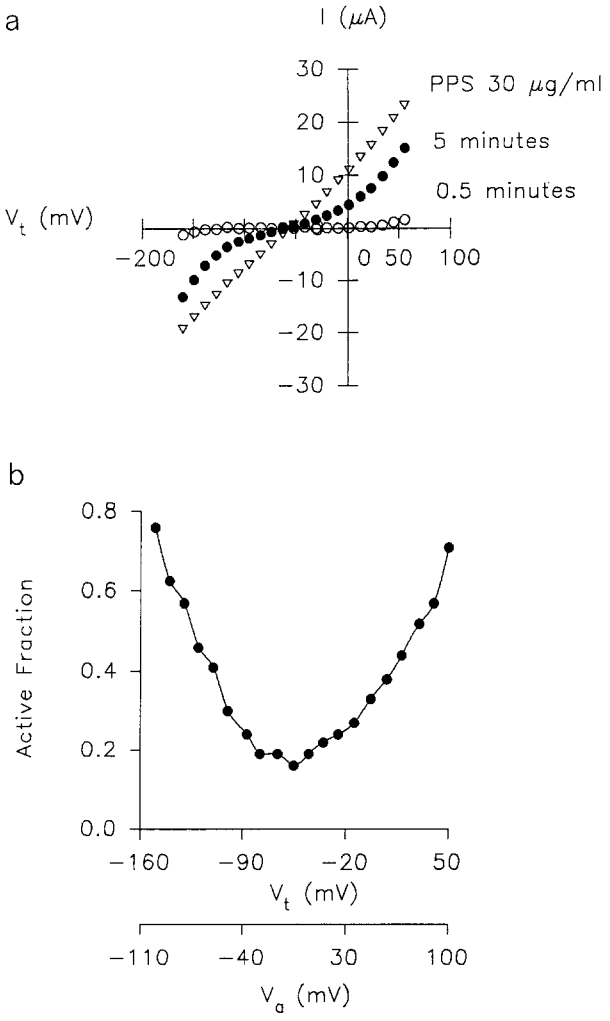


Fig. 8. (*a*) Current-voltage relationship of the PS-induced conductance at time intervals of 30 sec (open circles) and 5 min (filled circles) after adding 10 μg/ml of PS to the mucosal solution. The tissue was normally under open circuit conditions. During the *I-V* relationship, the epithelium was clamped at −50 mV (serosa ground). Of interest is that both of these *I-V* relationships are curvilinear. Thirty seconds after the addition of pentosan-polysulfate to the mucosal solution (to remove the mucosal solution PS), the *I-V* relationship (inverted open triangles) became linear. These data suggest that the PS self-inhibition is voltage sensitive, i.e., large hyperpolarizations or depolarizations relieve the self-inhibition. (*b*) Ratio of the slope conductance of the 5 min *I-V* to the slope conductance of the PPS-treated tissue as a function of voltage. This ratio is equal to the fraction of PS-induced conductance which is not blocked by external PS. The axis labelled V_a is the apical membrane potential with the cell as ground. Note that at an apical membrane voltage of 0 mV (V_t of about −50 mV), 80% of the PS-induced conductance is inhibited by the mucosal solution PS.

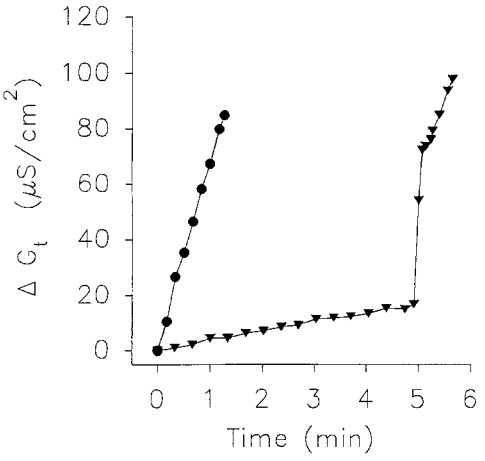


Fig. 9. Voltage dependence of PS-induced membrane conductance. Two experiments are shown in this figure. In the experiment shown by filled inverted triangles, the transepithelial voltage (V_t) was held at −60 mV (giving an apical voltage of −10 mV cell interior ground) and 10 μg/ml of PS was added at time zero. When V_t was clamped to 0 mV at 5 min (giving an apical membrane potential of +50 mV cell interior ground), there was a rapid increase in membrane conductance followed by a slower increase. This time-dependent conductance change can be fit by the sum of an exponential with a rate constant of 4.5/sec and a linear component. In the experiment shown by filled circles, V_t was clamped at 0 mV (apical membrane potential of +50 mV cell interior ground) and 10 μg/ml of PS was added to the mucosal solution. Note that the initial rapid increase of the membrane conductance is absent. This slower time course an be accounted for by an unstirred layer of approximately 80 μm in thickness. These data demonstrate that the apical membrane potential must be cell interior negative before PS can induce a membrane conductance, and also suggest that the PS can sense the membrane voltage field.

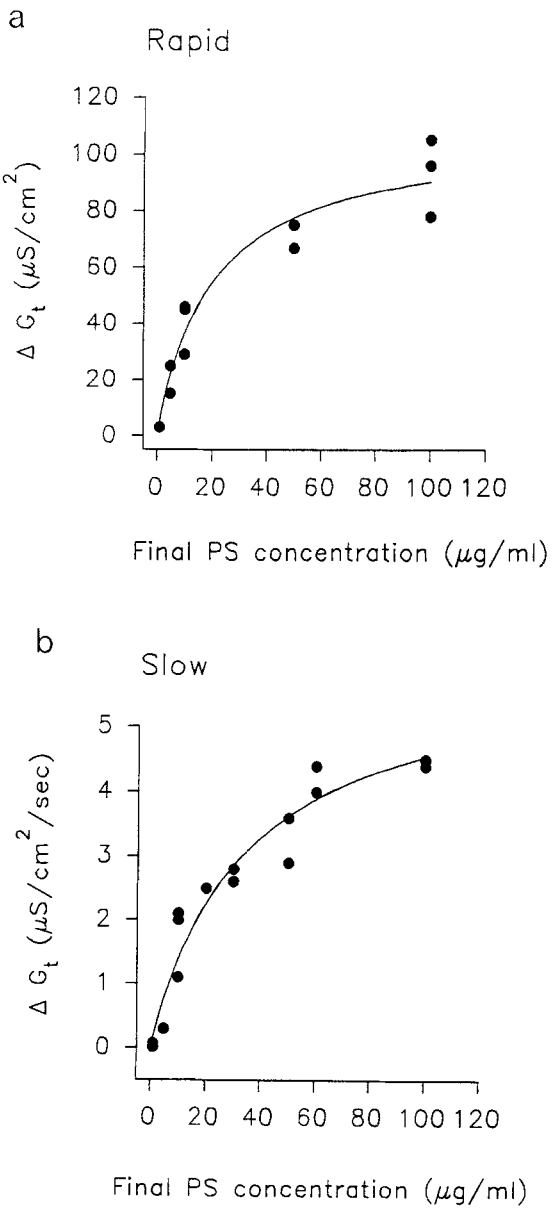


Fig. 10. (*a*) The magnitude of the rapid phase of the conductance change is plotted against the PS concentration. The smooth curve throughout the data points is the best fit to the Michaelis-Menten equation with a K_m of 19.45 ± 5.36 μg/ml and a maximum conductance change of 107.8 ± 9.2 μS/cm² (this change occurs within the first 3 sec after clamping V_t from −60 to 0 mV). (*b*) The rate of change of the slower phase is plotted against the mucosal PS concentration. The smooth curve throughout the data points is the best fit to the Michaelis-Menten equation with a K_m of 34.0 ± 10.8 μg/ml and a maximum rate of conductance change of 6.1 μS/cm²/sec. These data suggest that there is an apical membrane PS-binding component.

before clamping V_t at 0 mV (apical membrane potential of approximately 50 mV with respect to the cell interior). As shown in Fig. 9, there was a rapid exponential-like increase in the membrane conductance of 40 ± 5.5 μS/cm² (n = 3) with a rate constant of about 4.5 ± 0.8 sec^{-1}, followed by a slower rate of increase in the membrane conductance of 1.7 ± 0.3 μS/cm²sec. Incubation of the tissue with 10 μg/ml of PS at V_t = −60 mV, for 10 or 15 min did not alter the magnitude or rate of change in either the fast or slow phase of conductance when the tissue was subsequently clamped to a V_t of 0 mV (*data not shown*).

The concentration dependence of both the rapid and slow phase of conductance is shown in Fig. 10*a,b* along with the best fit of the Michaelis-Menten equation to the data. Of interest is that both the initial rapid phase of the conductance increase, as well as the slow phase of the conductance increase saturate, as a function of PS concentration with similar K_m, the concentration of PS which results in a half-maximal stimulation of conductance (19.5 μg/ml fast phase and 34 μg/ml slow phase). The saturation of the rapid phase of the conductance increase suggests that there is a binding site for PS

at the apical membrane. The following protocol was used to determine whether this site is in rapid (seconds) exchange with the mucosal solution PS. First, V_t was clamped at -60 mV and the tissue incubated with 100 μg/ml of PS for 5 min. Next, a fivefold excess of pentosan-polysulfate was added to the mucosal solution, followed 15 sec later by clamping V_t to 0 mV. If PS falls off the binding site slowly, then when the tissue is clamped at 0 mV the conductance should rapidly increase. If PS falls off the site rapidly, then clamping the tissue to 0 mV should not produce a change in conductance. In four experiments, the above protocol did not produce a significant change in membrane conductance. This suggests that PS bound to the external site is in rapid exchange with the bulk solution.

To determine the effect of different clamp voltages on the rate of conductance change, V_t was clamped at -60 mV in the presence of 10 μg/ml PS. After 5 min, V_t was then clamped at more depolarizing voltages and the rate of conductance change measured. The relationship between the voltage and rate of conductance change is shown in Fig. 11*a*, and can be fit by an exponential function of the form:

$$G_t = G^0 \exp(neV/kT) \quad (4)$$

where G^0 is the rate of conductance change when V_t is zero and has units of μS/cm^2 sec, n is an empirical constant, V is the transepithelial voltage, e is the electron charge, k is the Boltzmann constant and T is temperature. The best fit values for these parameters are given in the figure legend. Since the PS self-inhibition is voltage sensitive (*see* Fig. 8*b*), Fig. 11*a* was corrected for self-inhibition by dividing the rate of conductance change at each voltage (*from* Fig. 11*a*) by the active fraction at the same voltages (Fig. 8*b*). These corrected data are shown in Fig. 11*b* and were also fit by the above equation. The values for the best fit are given in the figure legend. A tentative model to explain the above observations will be presented in the discussion.

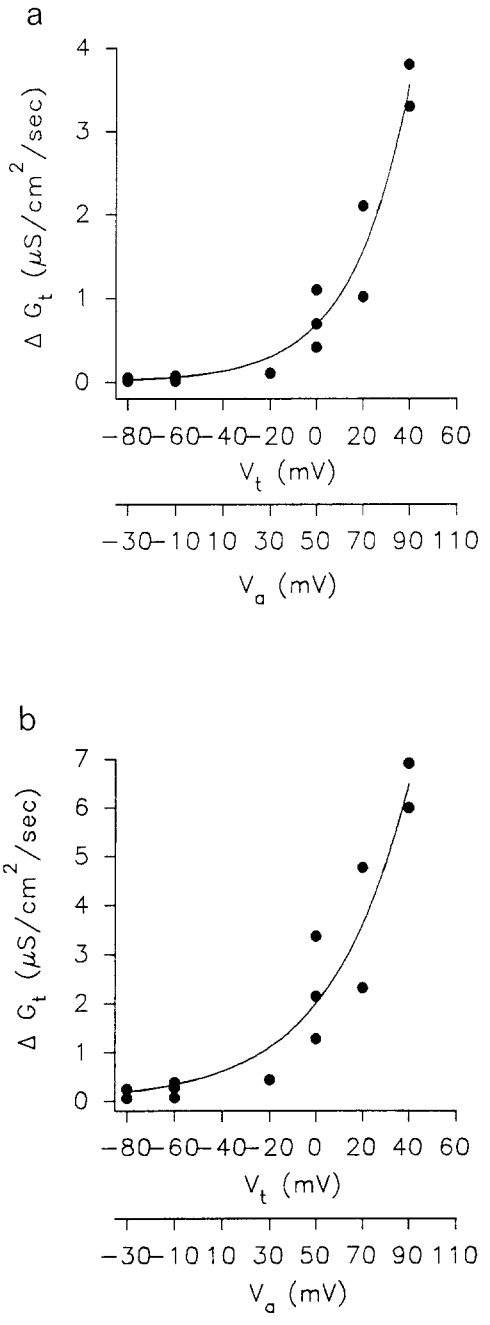


Fig. 11. (*a*) The rate of change of the membrane conductance was measured at a constant concentration of PS (10 μg/ml) but at different transepithelial holding potentials (V_t). The axis labeled V_a is the apical membrane potential with the cell as ground. The smooth curve through the data points is the best fit to an exponential function (*see* Results). The best fit value for n was 1.1 and for G^0 was 0.69 μS/cm^2 sec at a V_t = 0 mV and a G^0 of 0.086 μS/cm^2 sec when V_a = 0 mV. (*b*) Since PS demonstrates a voltage-dependent release from self-inhibition (*see* Fig. 8*b*), the above curve was corrected for this inhibition. The smooth curve through the data points was fit by the above equation and yielded an n = 0.78 and a G^0 of 2.00 μS/cm^2 sec when V_t = 0 mV and a G^0 of 0.46 μS/cm^2 sec when V_a = 0 mV.

Blockers of PS Conductance

The above sections have demonstrated that PS can cause a partial inhibition of the PS-induced apical membrane conductance and that there is a saturable PS binding site at the apical membrane. This section will investigate whether a multivalent cation (in this case La^{3+}) can act as a blocker of the PS-induced conductance and whether such a cation can block the interaction of PS with the membrane binding site. The addition of 10 μg/ml PS to a solution prein-

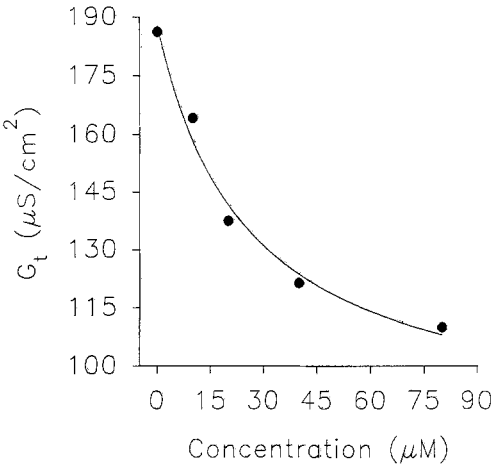


Fig. 12. Dose response of lanthanum blockade of a PS-induced membrane conductance. Mucosal solution PS was removed by replacing the mucosal solution with fresh KCl Ringer. Lanthanum was then added stepwise to the mucosal solution. The resulting dose response curve is adequately described by Michaelis-Menten equation plus a constant which represents the control apical membrane conductance. The best fit value for k_i in this example was 25.5 μM.

cubated with 200 μM lanthanum (La^{3+} itself does not alter the apical membrane conductance) completely blocked the effect of PS on the apical membrane conductance. To determine whether La^{3+} was inhibiting the interaction of PS with the membrane (e.g., interacting with the binding site) or blocking the PS-induced conductance (e.g., open channel blocker), tissues were first incubated in PS. After G_t had reached a steady-state level, PS was rapidly washed from the mucosal solution followed by the stepwise addition of La^{3+} to the mucosal solution. This procedure resulted in a decrease in G_t. Removal of La^{3+} resulted in an increase in G_t. The concentration dependence of the La^{3+}-induced decrease in G_t is shown in Fig. 12. This lanthanum block is adequately described by Michaelis-Menten kinetics (plus the control membrane conductance). The best fit value for k_i (the lanthanum concentration at which half of the PS-induced conductance was blocked) was 26.6 ± 3.6 μM (n = 4). These data suggest that La^{3+} acts as a blocker of the PS-induced membrane conductance.

The above data do not preclude the possibility that La^{3+} can also bind to the PS binding site and thus inhibit the PS-induced increase in membrane conductance. The following experiment was performed to address this question. (i) The tissue (voltage clamped at 0 mV) was first incubated with 200 μM La^{3+}. (ii) Next, 10 μg/ml of PS was added to the mucosal solution (in the presence of La^{3+}). (iii) After five minutes, the PS was removed from the mucosal solution by washing with a Ringer containing 200 μM La^{3+}. (iv) This last wash was then immediately followed by a wash with a control Ringer (no PS or La^{3+}). If La^{3+} does not block the interaction of PS with the membrane binding site, then upon removal of La^{3+} (step iv above), the transepithelial conductance will rapidly increase. However, if La^{3+} inhibits the binding of PS to the membrane binding site, then upon removal of La^{3+} (step iv above) there will be no change in the transepithelial conductance. Using the above protocol, the increase in membrane conductance was only 1.6 ± 1.2% (n = 3) of the PS-induced increase measured in the absence of La^{3+}. This suggests that La^{3+} inhibits the interaction of PS with the membrane binding site.

Role of the Glycosaminoglycan Layer

Parsons et al. (1990) suggested that the PS-induced increase in urea, water and calcium flux across the urinary bladder was due to the binding of PS to the glycosaminoglycan (GAG) layer, and that because of charge neutralization of GAG by PS, this allowed a free flow of solute across the epithelium. To determine whether the GAG layer is indeed the permeability barrier, the apical membrane was treated with a number of enzymes (hyaluronidase II, III, IV-S, VI-S, VIII and chondrotinase ABC, AC from Sigma and hyaluronidase from Worthington) known to hydrolyze GAGs. Each enzyme was added to the mucosal solution at a concentration of 150 μg/ml for at least 30 min, followed by the addition of 100 μg/ml of PS. None of these enzymes alter the rate of increase in G_t by 100 μg/ml of PS (*data not shown*). Of interest is that hyaluronidase caused a small but significant increase in R_t (11.2 ± 1.6 kΩ cm^2 control and 13.3 ± 2.2 kΩ cm^2 n = 14 after enzyme treatment P = 0.046). These data suggest that the GAG layer is not a major permeability barrier for solute or solvent movement across the urinary bladder epithelium and that the external binding site is not the GAG. At the present time it is not clear why hyaluronidase caused an increase in R_t. A possible explanation might be the presence of a contaminant, such as a protease. In this regard, Lewis and Clausen (1991) demonstrated that the apical membrane's ionic conductance was decreased after treating the apical membrane with trypsin, urokinase, plasmin or kallikrein.

Discussion

First, the effects of PS on the urinary bladder epithelium will be summarized. Then, these effects will

be compared to those described in other epithelia. Last, a preliminary model to describe the effects of PS on the membrane permeability will be developed.

Effects on Urinary Bladder Epithelium

The data presented in this paper strongly support the notion that PS increases the apical membrane permeability of the urinary bladder epithelium. First, PS in the micromolar range results in a rapid and reversible decrease in the transepithelial resistance. The extent of the reversibility is dependent upon the composition of the mucosal bathing solution, the concentration of PS applied to the epithelium and the length of time the tissue is exposed to PS. Incomplete reversibility results from a decrease in the resistance of the paracellular pathway. Microelectrodes showed that the site of the initial resistance change was at the apical membrane. The induced conductance seems to be nonselective and allows the movement of sodium, potassium, chloride and gluconate. This selectivity suggests that the size of the conductive unit is large.

An unusual finding was that the PS-induced conductance demonstrated self-inhibition (*see* Figs. 7 and 8). The PS-induced conductance has at least two voltage-sensitive steps. The first is that the apical membrane voltage must be cell interior negative before there is a measurable change in the membrane conductance. This suggests that PS senses the membrane potential field and leads one to speculate that PS might then enter the lipid bilayer in a manner similar to that proposed for excitability-inducing material (Bean et al., 1969). The second is that PS self-inhibition is voltage sensitive, thus an apical membrane voltage more negative than -50 mV or more positive than $+50$ mV results in an increase in the apical membrane conductance only when PS is in the mucosal solution (*see* Fig. 9). Removal of PS from the mucosal solution results in an increase in the membrane conductance with a linear instantaneous current-voltage relationship. The mechanism of this voltage sensitivity might be a result of the ability of membrane voltage to force the blocker through the conductive unit (when the cell interior becomes more negative) or to knock it out of the opening of the conductive unit when the membrane potential becomes positive. Further studies are needed to determine whether this is indeed the mechanism. PS-induced conductance can be reversed by simple washout of mucosal PS, addition of a negatively charged solute (e.g., pentosan-polysulfate) or by addition of the proteolytic enzyme trypsin. In this regard all of the above maneuvers decrease the apical membrane conductance with a near identical time course. This suggests that in the conductive state, PS is inaccessible to trypsin hydrolysis or interaction with PPS and thus PS might be buried in the lipid bilayer of the urinary bladder.

Since trypsin completely eliminates the ability of PS to increase the apical membrane conductance, this demonstrates that it is PS which is increasing the membrane conductance as opposed to a nonprotein contaminant. Is the effect of PS on the membrane conductance due to the high arginine content of PS (80% arginine) or to some other structural feature? Recent studies on the urinary bladder (*unpublished observation*) have demonstrated that synthetic poly-L-arginine (mol wt 11,200) was at least as effective as PS in increasing the apical membrane conductance. This strongly suggests that a major determinant of the PS effect is its cationic nature and not some other structural property.

Comparison with Other Epithelia

Epithelia respond in three different ways to the mucosal addition of PS. First, the tight junctional permeability to cations in the *Necturus* gallbladder, ciliary epithelia and the ascending thin limb of Henle's loop decreases (Bentzel et al.,1987; Koyama et al., 1991; Straub & Wiederholt, 1991). Second, the transepithelial permeability of MDCK monolayers and urinary bladder to large solutes increases (Peterson & Gruenhaupt, 1990; Parsons et al., 1990). Last, there is an alteration of the apical membrane permeability of *Necturus* gallbladder (Poler & Reuss, 1987) and renal proximal tubule (Sato & Ullrich, 1975). The response of endothelia to protamine sulfate is an increase in the transendothelial permeability (Alavi et al., 1982; Olesen & Crone, 1986; Chang et al., 1987).

From studies on perfused rat lung, Chang et al. (1987) concluded that PS (as well as other polycationic molecules) increased the capillary permeability by having a cytotoxic effect on the endothelial cells as demonstrated by electron microscopy. In addition, these authors noted that some of the pulmonary epithelial cells were also damaged. They speculated that the mechanism of this cytotoxic effect (on the endothelial cells) might be a result of an increase in the membrane ionic permeability of the endothelial cells and dissipation of the cell's electrochemical gradient. The present results fully support their speculation. Microelectrode studies (*see* Results) demonstrated that the apical membrane permeability is dramatically increased to both cations and anions, and ion replacement studies (replacing the

mucosal NaCl solution with a KCl solution) reduced the extent of the irreversible loss of transepithelial resistance.

In studies on the high resistance strain of MDCK cells, Peterson and Gruenhaupt (1990) demonstrated that mucosal PS (50 μg/ml) caused a time-dependent decrease in the transepithelial resistance and an increase in the transepithelial flux of mannitol. These effects could be inhibited by heparin, sulfated dextran, bovine serum albumin or fetal bovine serum. The increase in mannitol permeability was a transient phenomenon with an eightfold increase occurring between 2–3 hr after the addition of PS, followed by a decrease in permeability (to threefold higher than control) after 24 hr. There was no explanation given for this reversal, but it could be due to either a time-dependent loss of PS activity (due perhaps to endogenous serine proteases) or an alteration of the tissue sensitivity to PS. Microscopic observation of PS-treated MDCK cells showed areas with intercellular gaps. Again, this is strong evidence for an increase in the conductance of the paracellular pathway. Whether these gaps were due to focal dissociation of the tight junctions was not discussed. In a more recent study Peterson and Gruenhaupt (1992) proposed that there is specific binding of PS to the apical membrane surface of MDCK cells. This binding was not to the glycosaminoglycan layer since heparinase and neuraminidase did not alter the ability of PS to increase the membrane conductance, but rather to some membrane protein since the binding was eliminated by treating the tissue with trypsin. Although our results suggest that the glycosaminoglycan layer is not involved in the PS-induced membrane conductance change, we found that trypsin pretreatment (with subsequent trypsin reversal, *see* Results) did not alter the response of the bladder to PS. Since Peterson and Gruenhaupt (1992) did not state whether the added trypsin was removed (or neutralized) before the mucosal addition of PS to MDCK cells, the possible involvement of a trypsin-sensitive PS binding protein in MDCK cells is still uncertain.

Although the results in this paper, as well as other studies, offer evidence that PS is acting as an ionophore, there are two lines of evidence which suggest that this might not be the case. First, not all epithelia demonstrate a PS-dependent increase in membrane conductance (*see* Bentzel et al., 1987; Koyama et al., 1991; Straub & Wiederholt, 1991). Second, the addition of PS to the serosal bathing solution of MDCK cells (Peterson & Gruenhaupt, 1990) does not change the electrical properties of this epithelium. This difference in sensitivity between the apical and basolateral membranes of the same epithelium suggest that either a specific lipid composition or a protein "receptor" might be a necessity for the PS-induced membrane conductance. The present study offers strong evidence that such a receptor is required for the PS effect on the urinary bladder epithelium. Further studies are needed to determine the membrane components required for the PS effect.

Other Naturally Occurring Cationic Proteins

Protamine sulfate is not the only naturally occurring protein with high levels of basic amino acids. Other examples are histones and proteins present in granules of neutrophils and eosinophils. In a recent review, Spitznagel (1990) described seven proteins associated with the azurophil granules of human neutrophils with demonstrated potent antibiotic activity. Of these seven proteins, four are cyclic peptides with 29–34 amino acids, differ only in the N-terminal amino acid, contain about 13% arginine (no lysine) and are collectively called defensins. These defensins have been demonstrated to form voltage-dependent, ion permeable (weakly anionic) channels in lipid bilayers (Kagan et al., 1990), where making the bilayer potential negative on the side opposite to which the defensin was added, resulted in a rapid (and reversible) increase in bilayer conductance. Other features of the defensins conductive properties are a steep concentration dependence (a slope of 3–4 on a log-log plot) and an exponential dependence of conductance on the membrane voltage. The remaining three proteins, cathespin G (CatG), cationic antimicrobial protein (CAP) with Mr 37,000 "CAP37" and a CAP with Mr of 57,000 "CAP57," contain 16, 11 and 13% cationic amino acids, respectively. It has been proposed that the bactericidal activity of CatG, CAP37 and CAP57 (in the concentration range of 50 μg/ml) is related to the ability of these proteins to cause membrane damage perhaps by an ionophore-like activity. Eosinophils secrete two proteins, termed major basic protein and eosinophil cationic protein, which both have significant levels of positively charged amino acids and have been found to have cytotoxic effects on epithelia (Venge et al., 1988). Young et al. (1986) have demonstrated that eosinophil cationic protein produces single channel events in lipid bilayers and thus can account for its known cytotoxic effect. However, major basic protein does not alter the conductance of lipid bilayer membranes and at the present time its mechanism of action is unknown. It is possible that it might act in a manner similar to that of prot-

amine, i.e., its activity might require a specific membrane component.

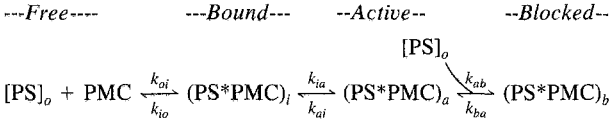
Histones, nuclear binding proteins which are similar in structure to protamine, produce nearly identical changes in the apical membrane conductance of the rabbit urinary bladder (*unpublished observation*). In a recent abstract, Mendizabal and Naftalin (1992) reported that human sperm placed in the colon of rats caused an increase in the paracellular conductance of the colonic epithelium. These authors proposed that a possible mechanism of action was the activation (by acrosin) of collagenase (from procollagenase found in seminal fluid), which then results in the breakdown of the epithelial barrier. These results are of particular interest since such a disruption of the mucosal barrier by sperm might represent an entry pathway for such sexually transmitted diseases as the AIDS virus (Mendizabal & Naftalin, 1992). In light of the effects of histones (a nuclear protein) on urinary bladder permeability, these proteins might represent another component which could aid in the breakdown of the colonic permeability barrier.

A Preliminary Model for PS-Induced Conductance

The data presented in Results, dictate a minimal kinetic model for the PS-induced membrane conductance. This model must have at least three states: the first is a saturable membrane-associated binding domain (*see* Fig. 10*a,b*); the second, an active (conductive) state (*see* Fig. 1), and the third is a self-inhibited state (*see* Figs. 6 and 7). The transition from the binding domain to the active state is voltage dependent (Fig. 11) as is the self-inhibition state (Fig. 8). For simplicity we have not included a diffusional component to account for the unstirred layer.

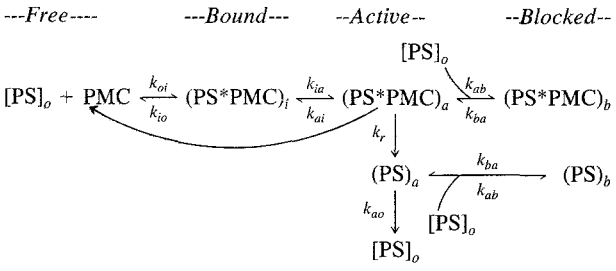
We will consider two models. In the first one the conductive element is a complex formed between PS and the putative membrane component (PMC); the second model assumes that the role of the PMC is catalytic, i.e., once the PS is in the membrane, the PS*PMC complex dissociates thus freeing the PMC to bind additional PS.

Model I



$[PS]_o$ is the concentration of PS immediately adjacent to the membrane; however, there is no interaction between PS and PMC at this stage. $(PS*PMC)_i$ represents a state in which the PS is associated with the PMC in the membrane but is not conductive in this configuration (inactive). k_{oi} and k_{io} are the rate constants for association and dissociation of PS with PMC. $(PS*PMC)_a$ is the conductive unit of the PS-PMC complex. k_{ia} and k_{ai} are the rate constants for activation and inactivation of the conductive unit, respectively, and one or both are voltage dependent. $(PS*PMC)_b$ is the blocked state of the conductance by either the PS (self-inhibition) or lanthanum. k_{ab} and k_{ba} are the on and off rate constants of block, respectively.

Model II



This model differs from Model I in two respects. First, there is a second conductive state labeled $(PS)_a$. This state is a result of the release of PS from the PMC, and occurs with the rate constant k_r (for simplicity this step is assumed to be unidirectional). Thus, the PMC can be continuously utilized to import PS into the apical membrane i.e., it acts as a carrier. Second, the loss of $(PS)_a$ is unidirectional and occurs directly into the bulk solution (or the cell interior) with the rate constant k_{ao}. Some preliminary data suggest that PS can enter the cell; however, at the present time we do not know whether this is due to the partitioning of PS into the cell interior or whether it is due to a finite PS permeability of this conductive pathway.

Both of these models are compatible with most of the presented data: they each have three states, the active state is voltage dependent and both demonstrate self-inhibition. However, Model I cannot account for the experiment shown in Fig. 9. In this experiment, the tissue was preincubated with PS for 5 min at a holding potential of -60 mV, V_t was then clamped to 0 mV and the time-dependent change in membrane conductance measured. Since there is a finite quantity of PMC, Model I predicts that as the bathing solution PS concentration is increased, then the time course of the conductance change will approach that of a single exponential. Model II predicts that the conductance will always change as the sum of two exponentials, the first an initial rapid change in the membrane conductance which will saturate as a function of the bathing solution PS concentration,

followed by a second slower change which will saturate when the rate of entry of the PS into the membrane (which is catalyzed by PMC) is equal to the rate at which PS leaves the membrane. As can be observed in Fig. 10*a,b* the data are most consistent with Model II.

Estimates for some of the rate constants of Model II have been determined:

k_{io}—The protocol used was to first clamp the apical membrane voltage to zero in the presence of PS (this loads the PMC); next to add a sufficient amount of pentosan-polysulfate to the mucosal solution to quickly remove all the PS in bulk solution, then monitor the occupation of PS*PMC by clamping V_t to 0 mV (i.e., apical membrane voltage to +50 mV, cell ground). This decay process is faster than 0.25/sec.

k_{oi}—Since the increase of the slow conductance phase was linear over the range tested, we were unable to determine a rate constant for this process. However, since the rate of change was a saturating function of external PS, this suggests that the value for k_{oi} is faster than for k_{ia}.

k_{ia}—In Fig. 9, the rate constant for the fast phase of conductance increase is 4.5/sec (at a clamp voltage of 0 mV).

k_{ai}—An estimate for this rate constant has not been determined.

k_r—An estimate of this rate constant has not been determined at this time.

k_{ao}—This rate constant was determined from the decrease in the membrane conductance after removing the bulk solution PS. The decrease of membrane conductance was a single inverse exponential which yields a rate constant of 0.145/min at a V_t of 0 mV. This value is probably an overestimate since we have assumed that at this voltage k_{ai} is zero.

k_{ba}—The rate constant of 7/min. was determined from the experiment of adding an excess of PPS to the mucosal solution. However, this value is underestimated as it has a diffusive term.

k_{ab}—An estimate of this rate constant was not possible due to the rate of diffusion through the unstirred layer.

Lanthanum has two sites of action in Model II. The first is that it can inhibit the association step of PS with the PMC, i.e., $k_{oi} = 0$. Whether La^{3+} binds to the same site on the PMC as PS is not known at the present time. The second site of action is by blocking the active conductive states, i.e., $(PS^*PMC)_a$ and $(PS)_a$. At the present time it is not known whether this site of action is the same as the site of PS self-inhibition or another site.

Further studies are needed to determine whether Model II is an adequate representation of the mechanism of PS-induced membrane conductance. One of the interesting aspects of this study is that there is a binding domain for PS in the apical membrane. Such a binding domain has been suggested as a requirement for the antibiotic activity of the neutrophil cationic proteins. Candidate binding sites for the neutrophil cationic proteins include the lipid A phosphoryl groups of lipopolysaccharide (Farley, Shafer & Spitznagel, 1988), penicillin binding protein 2 of gonococci, teichioc acid and lipopolysaccharides (*see* Spitznagel, 1990 for review).

In this paper we have demonstrated that protamine dramatically increases the apical membrane conductance of the rabbit urinary bladder. This conductance increase is voltage dependent and is associated with a membrane binding site. The voltage dependence suggests that the PS binding site is close to or within the membrane electric field. A kinetic model suggests that the binding domain is essential for the PS-induced membrane conductance. The inability of PS to alter the membrane conductance of some epithelia might then be due to the lack of such a binding protein. Our data demonstrate that the reported cytotoxic effect of PS on many other tissues, including epithelia, endothelia, neutrophils and some bacteria (*see* Spitznagel, 1984) might be due to a membrane disruptive effect in which the uncontrolled influx of cations and anions results in an osmotic disequilibrium and cell lysis. Future studies are needed to determine how a relatively hydrophilic molecule such as protamine can increase the cell membrane conductance, whether other polycationic proteins, particularly those from neutrophils and eosinophils, have a similar effect on epithelial permeability and what size molecules can pass through the conductive unit induced by PS.

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