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Protamine Reversibly Decreases Paracellular Cation Permeability in *Necturus* Gallbladder

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Summary. Protamine, a naturally occurring arginine-rich polycationic protein (pI 9.7 to 12), was tested in *Necturus* gallbladder using a transepithelial AC-impedance technique. Protamine sulfate or hydrochloride (100 μg/ml = 20 μM), dissolved in the mucosal bath, increased transepithelial resistance by 89% without affecting the resistance of subepithelial layers. At the same time, transepithelial voltage (ψ^{ms}) turned from slightly mucosa-positive values to mucosa-negative values of approximately +1 to −5 mV. The effect of protamine on transepithelial resistance was minimal at concentrations below 5 μg/ml but a maximum response was achieved between 10 and 20 μg/ml. Resistance started to increase within 1 min and was maximal after 10 min. These effects were not inhibited by serosal ouabain (5×10^{-4} M) but could be readily reversed by mucosal heparin. The sequence of protamine effect and heparin reversal could be repeated several times in the same gallbladder. Mucosal heparin, a strong negatively charged mucopolysaccharide, or serosal protamine were without effect. Mucosal protamine reversibly decreased the partial ionic conductance of K and Na by a factor of 3, but did not affect Cl conductance. Net water transport from mucosa to serosa was reversibly increased by 60% by protamine. We conclude that protamine reversibly decreases the conductance of the cation-selective pathway through the tight junction. Although this effect is similar to that reported for 2,4,6-triamino-pyrimidinium (TAP), the mechanism of action may differ. We propose that protamine binds to the apical cell membrane and induces a series of intracellular events which leads to a conformational alteration of the tight junction structure resulting in decreased cationic permeability.

Key Words protamine · *Necturus* gallbladder · tight junction · impedance analysis · transepithelial resistance

Introduction

Although most circulating proteins in mammals carry a relatively strong negative electrical charge, natural and synthetic polycationic proteins exert several important effects on biological membranes including an effect on capillary permeability (Turner et al., 1983) and modification of granulocyte and eosinophil action toward bacteria (Zeya & Spitznagel, 1963) and parasites (Butterworth et al., 1979). The action of polycationic proteins such as poly-L-lysine and protamine on epithelial transport and/or structure has been investigated in toad urinary bladder (Mamelak et al., 1969), *Necturus* gallbladder (Fromm et al., 1979; Bentzel et al., 1983; Hegel et al., 1983), trout gill (Greenwald & Kirschner, 1976), rat parotid duct (Mangos & McSherry, 1968), rabbit gallbladder (Quinton & Philpott, 1973), rat kidney proximal tubule (Sato & Ullrich, 1975), rat glomerulus (Seiler et al., 1977) and rat peritoneum (Alavi et al., 1982). Several of the studies above noted an effect, particularly of protamine, on tight junction structure and/or function. However, results are difficult to compare not only because of the differences in species and substrates but also because of a rather wide range of concentrations tested. Of particular note is that infusion of protamine sulfate (20 to 500 μg/ml) into the renal artery of rats, in addition to causing proteinuria (Vehaskari et al., 1982), induces loss of glomerular epithelial cell foot processes associated with migration of slit pore diaphragms away from the basement membrane and induction of tight junctions (Seiler et al., 1977; Kerjaschki, 1978). These latter observations prompted us to undertake an investigation of the action of protamine on both the structure and function of a "leaky" epithelium, the *Necturus* gallbladder.

Protamine is obtained commercially from salmon sperm and is a basic, arginine-rich (70 to 90%) protein of low molecular weight (approximately 5,000 dalton) although chain length can vary depending on species of fish sperm. The isoelectric point of the protamines vary between pH 9.7 and 12.

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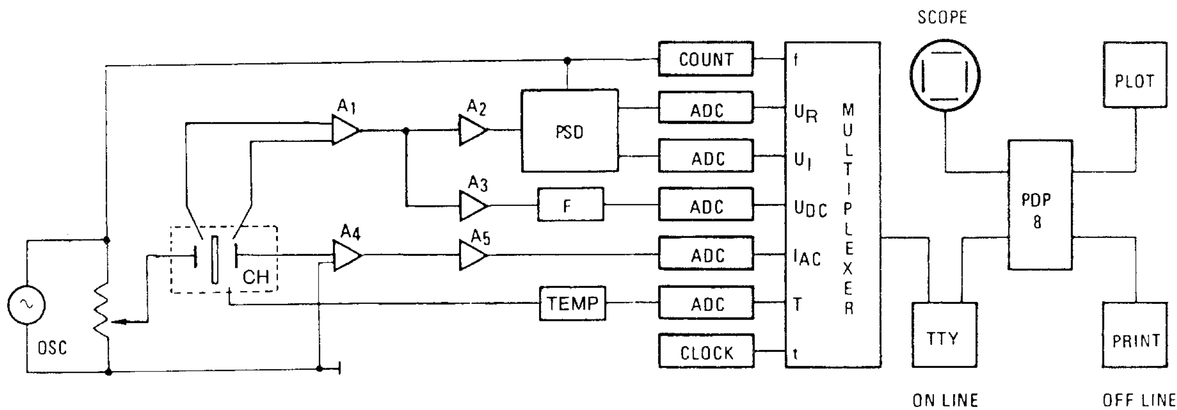

Fig. 1. Schematic electric circuit for measuring transepithelial impedance. OSC = sine wave oscillator; CH = Ussing-style chamber; A_1 = dual electrometer; A_2 = high-pass amplifier; PSD = phase-sensitive detector; A_3 = 1 : 100 amplifier; F = low-pass filter; A_4 = virtual ground amplifier; A_5 = AC amplifier; TEMP = thermocouple thermometer; COUNT = frequency counter; ADC = analog/ digital converter; U_R, U_I = real and imaginary components of AC voltage; U_{DC} = DC voltage = ψ^{ms}; I_{AC} = AC current; TTY = teletype printer; PDP-8 = computer

Protamine is used clinically to reverse the anticoagulant effect of heparin, a naturally occurring, strongly negatively charged sulfated mucopolysaccharide of varying degree of polymerization. Commercially, heparin is isolated from lung and intestine of pig, cow and sheep. The molecular weight of heparin ranges from 3,000 to 57,000 dalton.

The present study indicates that protamine applied to the mucosal surface of *Necturus* gallbladder reversibly decreases paracellular conductance by lowering permeability to Na and K.

Abbreviations

f_O = characteristic frequency of R_PC_P network [H_z]
C_P = apparent capacitance in parallel to R_P [μF/cm^2]
R_P = ohmic resistance in parallel to C_P [Ω cm^2][1]
R_S = ohmic resistance in series to R_P and C_P [Ω cm^2]
R_T = total ohmic resistance of the circuit; thus $R_T = R_P + R_S$ [Ω cm^2]
G_P = $1/R_P$ [mS/cm^2]
G_i = partial ionic conductivity of ion species i [mS/cm^2]
P_i = relative permeability of ion species i [$P_{Cl} \equiv 1$]
ψ^{ms} = spontaneous voltage of mucosal side with reference to serosal side of epithelium (mV)

Materials and Methods

Adult mud puppies (*Necturus maculosus*, Carolina Biological Supply, Burlington, N.C.) were kept unfed at 4°C in aerated water tanks. Animals were anaesthetized in a buffered solution of MS 222 (tricaine, Sandoz, Nürnberg, FRG) for 10 to 20 min. Gallbladders were removed, rinsed in Ringer's solution, glued onto small Plexigals® rings with tissue adhesive (Histoacryl Blau, Braun, Melsungen, FRG) and mounted vertically between conventional Ussing-type half-chambers. Electrical sealing with a minimum of edge damage was provided by soft silicon rubber rings (Silgel 604, Wacker Chemie, München, FRG). The exposed area was 0.28 cm^2.

The bathing solution contained (in mM): NaCl 95, $NaHCO_3$ 13, KCl 4.5, $CaCl_2$ 1.8, $MgCl_2$ 1.0, and NaH_2PO_4 0.7. The solution was gassed with 95% O_2 plus 5% CO_2 and used at room temperature. Under this condition and also after adding protamine and/or heparin, the solution pH was 7.2 to 7.4, the specific conductivity was 91 to 95 Ω cm, and the osmolality was 190 to 195 mOsm.

The experimental protocol consisted of a control period, the subsequent addition of protamine to the mucosal bath and then reversal of protamine action with heparin. This sequence could be repeated up to five times.

Electrical Measurements

All experiments were done under open-circuit conditions. In some experiments the conventional DC pulse-clamp technique was used to measure transepithelial resistance and ψ^{ms} (Bentzel et al., 1980). Most experiments reported here were carried out using a transepithelial AC-impedance technique. This AC electrical setup is shown schematically in Fig. 1.

Alternating currents of 17 μA/cm^2 (peak-peak) were produced by a sine wave generator (Fig. 1, OSC) and fed via Ag/AgCl current-passing electrodes into the Ussing chamber. 15 to 25 discrete frequencies were used in a range of 3 to 20,000 Hz to obtain one complete measurement of the transfer function. The voltage responses were picked up transepithelially by the voltage-sensing electrodes, preamplified by a dual electrometer (Fig. 1, A1) (F-223A, W-P Instruments, New Haven, Conn.), and were detected by a pair of two-phase lock-in amplifiers ("PSD") (Ortec Brookdeal, Bracknell, England). The alternating current signals were determined using a virtual ground amplifier (Fig. 1, A_4) (own design, UH). The complex impedance at each frequency was on-line calculated and stored on a PDP/8 computer (Digital, Maynard, Mass.). In addition, the impedance locus was displayed in the *X*/*Y* mode on a storage oscilloscope. In order to correct for the nonideal ohmic characteristics of the measuring

[1] R_P should not be confused with paracellular resistance.

equipment alone, a complete impedance data set was measured before the epithelium was mounted. All impedance data were then corrected during the measuring procedure for this nonideal behavior of the equipment. Finally, the fluid resistance between the voltage-sensing electrodes was subtracted.

Original impedance plots are shown in Fig. 2. The data were interpreted in terms of a simple resistor-capacitor parallel network (R_P and C_P) in series with an ohmic resistor (R_S) (*cf.* Fromm et al., 1977; Schifferdecker & Frömter, 1978). Total resistance of the circuit is given by $R_T = R_P + R_S$ (*see* inset, Fig. 2). The curvilinear interpretation of the impedance plots was done off-line using a circular least-square fit algorithm. From this, the apparent characteristic frequency f_O was determined by interpolation. Finally, the apparent capacitance C_P was calculated as

$$C_P = 1/(2\pi \cdot f_O \cdot R_P).$$

Complete impedance loci were obtained about every half hour during the experiment. Between these measurements, the impedance at a constant frequency, 3 Hz, was automatically monitored every 100 sec. The ohmic part of this impedance was used as a measure of the resistance of the epithelial layer. For monitoring the spontaneous transepithelial voltage, ψ^{ms}, the DC voltage was filtered out using a simple low-pass filter (Fig. 1 "F") of τ = 5 sec, and stored together with the AC data on the computer.

Ion selectivity was evaluated from dilution and biionic potentials (Barry et al., 1971) when active transport was blocked by serosal ouabain, 5×10^{-4} M. At this ouabain concentration, with Ringer's on both sides, transepithelial voltage was near zero and remained unchanged after 100 μg/ml protamine. Dilution potentials were measured by replacing 50% of the NaCl with osmotically equivalent amounts of sucrose in either the mucosal or serosal bathing solution. Biionic potentials were measured similarly replacing NaCl by KCl.

Transepithelial Net Water Flux

Gallbladders were mounted vertically in a two-piece Plexiglas chamber. Net volume flow into the serosal compartment (1.2 ml) was measured by adjusting the fluid level in a connecting glass capillary tube. This was accomplished by withdrawing a constant bore (1 mm) steel rod attached to a micrometer, thus permitting calculation of displacement volume. Prior to each study, zero flow was established by bathing the gallbladder on both sides in Na_2SO_4 Ringer's. Then, after changing to NaCl Ringer's in both baths, control, protamine and heparin reversal periods were studied sequentially. Each period lasted 30 to 60 min and readings were taken every 5 to 10 min.

Results are given as arithmetic mean ± standard error of the mean. After testing for equality of variances using the *F*-test, significances were evaluated by the appropriate version of the unpaired *t*-test.

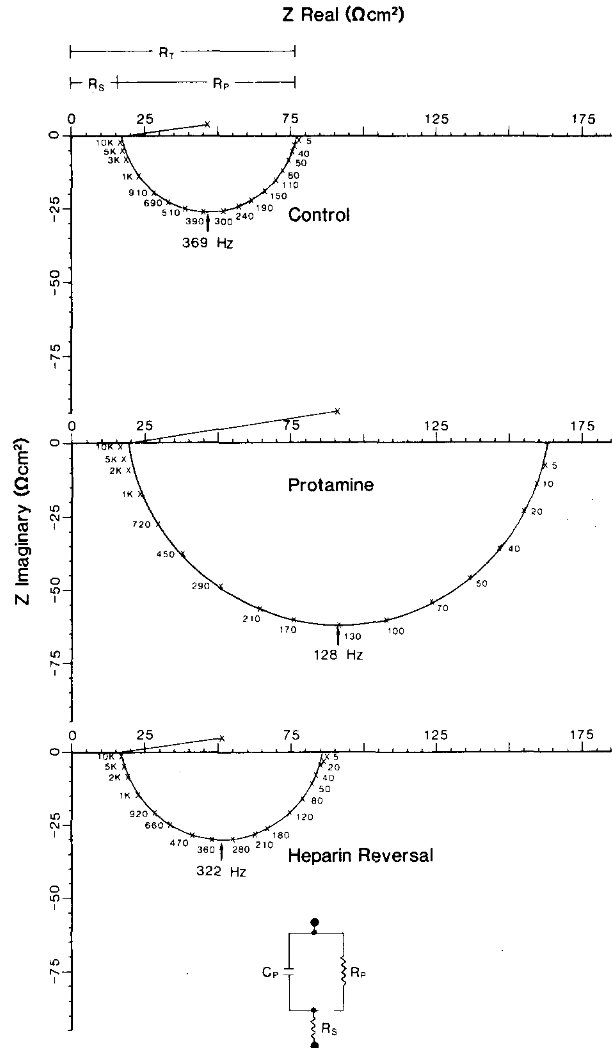

Fig. 2. Impedance plots of a typical experiment. Abscissa gives real (ohmic) and ordinate gives imaginary reactive components of the complex impedance. Symbols are data points measured at given frequencies (H_z). The semicircles shown are best fits to the data points represented by x symbols. Intersections between semicircles and x-axes at high and low frequencies signify series resistance (R_S) and total resistance (R_T), respectively. Note that R_T roughly doubled under protamine, whereas R_S stayed constant. The electrical equivalent circuit used to define R_S, R_P and C_P is given in the inset

Results

Effect on Resistances and Transepithelial Voltage

The typical effect of mucosal protamine on passive electrical properties of the *Necturus* gallbladder is shown in the three impedance plots given in Fig. 2, and the time course plot showing transepithelial resistance and voltage ψ^{ms} in Fig. 3. Statistical data are given in Table 1.

Protamine, at a dose of 100 μg/ml (approximately 20 μM), reversibly increased the total resistance (R_T) by 89%, whereas the series resistance (R_S) and the apparent capacitance (C_P) did not change significantly. Since $R_T = R_P + R_S$, the observed resistance alterations can be fully accounted for by changes of the parallel resistance (R_P). The spontaneous transepithelial voltage ψ^{ms} averaged

Table 1. Effect of protamine (100 μg/ml) and heparin reversal on electrical properties of *Necturus* gallbladder[a]

	Control	Protamine	$P<$	Heparin reversal	$P<$
R_T (Ω cm^2)	100 ± 17	189 ± 24	0.01	101 ± 10	NS
R_P (Ω cm^2)	84 ± 17	175 ± 28	0.02	86 ± 10	NS
R_S (Ω cm^2)	16 ± 1	14 ± 2	NS	15 ± 1	NS
C_P (μF/cm^2)	7.7 ± 1.2	8.2 ± 1.3	NS	7.1 ± 1.1	NS
ψ^{ms} (mV)	+0.7 ± 0.2	−4.6 ± 0.7	0.0001	+0.5 ± 0.2	NS

[a] $n = 8$; protamine P values refer to control and to heparin reversal; heparin reversal P values refer to control.

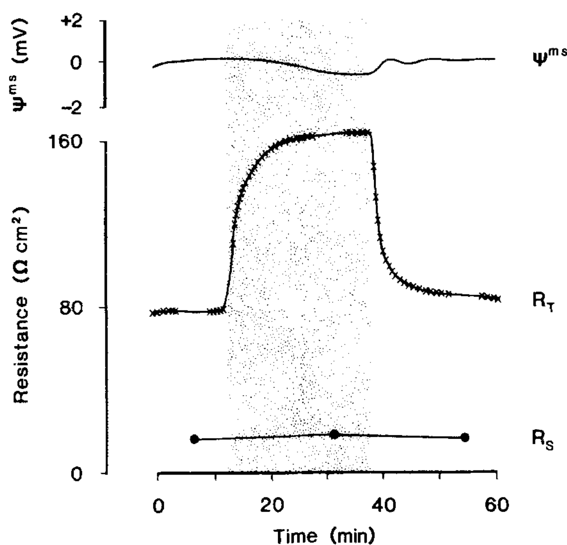

Fig. 3. Time course of the experiment shown in Fig. 2. ψ^{ms} is transepithelial voltage of the mucosal side with reference to serosal side. Total resistance (R_T) was monitored every 100 sec as 3 Hz impedances (*see* Materials and Methods), except where full frequency sweeps were made in order to obtain Cole plots used to extract series resistance (R_S). Protamine exposure (40 μg/ml, mucosal surface) is indicated by the stippled region, 12 to 37 min. R_T and ψ^{ms} return to baseline after heparin neutralization at 37 min

0.7 ± 0.2 mM (mucosa positive) under control conditions. Within 10 min after mucosal exposure to protamine, ψ^{ms} reversed polarity and increased to significantly higher mucosa negative values (Table 1). With a properly mounted intact gallbladder, protamine never failed to promptly increase R_T. However, the protamine effect on ψ^{ms} was more erratic from gallbladder to gallbladder.

The protamine-induced resistance increase could not be reversed by simple washout with fresh Ringer's solution. However, heparin, in a concentration approximately equivalent to the neutralizing potency of protamine (15 μg of protamine-HCl neutralizes the anticoagulant effect of about 1 unit of heparin), readily reverses the protamine response. Heparin alone had no electrophysiological effect when exposed to either the mucosal or serosal surfaces even at up to concentrations of 1,000 units per ml. We normally reversed the protamine effect with approximately a twofold "overdose" of heparin. This sequence of first increasing the resistance by protamine and then reversing it with heparin can be repeated several times in a single experiment (tested up to five sequences with the same gallbladder). Not only heparin, but also other strong anions were able to interfere with the action of protamine. For example, sulfate, at a concentration of 53 mM (Na_2SO_4 Ringer's, both surfaces), reduced the protamine response by 92% when compared to that observed when the same gallbladders were bathed in NaCl Ringer's.

Adding protamine or heparin to the Ringer's solution never changed pH by more than 0.2 units nor did it alter measured osmolality. Also we found no differences in effects of protamine-HCl (Roche, FRG) and protamine-SO_4 (Lilly, Indianapolis, Ind., or Sigma, St. Louis, Mo.), either in magnitude of response or in dose-response relationships. Thus, in this study, we make no distinction between protamine-HCl or protamine sulfate.

Protamine was effective only if dissolved in the mucosal bath. There was no change in any measured parameter if protamine was dissolved in the serosal bath (tested up to concentrations of 10,000 μg/ml). When subepithelial tissue was partially removed, serosal protamine was still without effect.

The increase in resistance after protamine was attenuated by a hydrostatic pressure gradient of 3 cm H_2O, serosa > mucosa: Under this condition, the protamine response in R_T was 43 ± 10% lower ($n = 5$, $P < 0.02$) than values obtained at no pressure gradient. The addition of 5×10^{-4} M ouabain to

Table 2. Time course of protamine and heparin reversal on transepithelial resistance (R_T)

	n	Time after adding protamine				100% effect reversal after:
		1 min	2 min	4 min	10 min	
Protamine	17	41 ± 5%[a]	63 ± 11%	86 ± 6%	99 ± 1%	10.3 ± 2.0 min
Heparin reversal	10	59 ± 10%[a]	69 ± 6%	87 ± 5%	95 ± 5%	11.4 ± 1.8 min

[a] Values are given in % of full (100%) protamine effect or heparin reversal, respectively. There were no significant differences in time course between protamine effect and heparin reversal.

the serosal bath resulted in a zero value for ψ^{ms} within 1 hr. Ouabain had no influence on the resistance response (R_T) to protamine.

Time Course

There was no apparent difference in time course of the protamine resistance response (R_T) at concentrations of 10 μg/ml and above, provided the mucosal bath was well stirred and protamine-containing Ringer's was rapidly exchanged in the mucosal bath.[2] Furthermore, the time course for recovery (heparin neutralization) was not different from the protamine response. A typical time course is given in Fig. 3, whereas Table 2 presents numerical data on the relative action and reversal of protamine on R_T. It can be seen that both the protamine response and the heparin neutralization started within the first minute after mucosal application and that the full effect was recorded after 10 to 11 min.

Dose Response

The dose-response plot (Fig. 4) compiled from all data showed a very sharp increase of protamine effect between 5 and 10 μg/ml (mucosal bath concentration). Taking the scatter of the data into account, it is clear that protamine had only minimal effects at 5 μg/ml and lower, but was almost maximally effective at concentrations of 10 or 20 μg/ml and above. When tested up to 5,000 μg/ml, protamine did not exert any irreversible declines in R_T and ψ^{ms} after brief (10 to 15 min) exposures.

Ion Selectivity

In order to further investigate the mechanism by which protamine induces conductance changes in

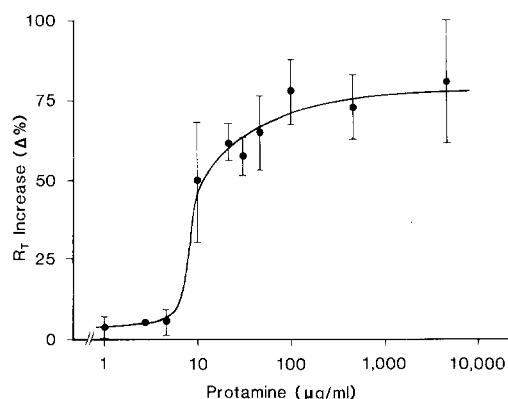

Fig. 4. Dose-response relationship of protamine on R_T. The protamine response on the ordinate is given as % increase in R_T over control. The number of measurements for each point ranged from 2 to 13

the epithelial cell monolayer, ion selectivity of the gallbladder was evaluated according to the procedure introduced by Barry et al. (1971): NaCl dilution potentials and K/Na biionic potentials were measured and the Goldman-Hodgkin-Katz equation was used to calculate relative ionic permeabilities of K, Na and Cl (Cl was defined as 1). We found that $P_K > P_{Na} > P_{Cl}$ under all experimental conditions (Table 3, upper part), but that the relative permeability for both cations decreased by a factor of 3 after maximum response to 100 μg/ml protamine. Adding heparin then led to an almost full recovery to control values. Partial conductivities (G_i) of the three ions (i = K, Na or Cl) were calculated from relative permeabilities (P_i), concentrations of i in bathing media (c_i), and overall epithelial resistance in Ringer's $R_P = 1/G_P$

$$G_i = P_i c_i \cdot G_P / \Sigma (P_i c_i).$$

Steady-state dilution as well as biionic potentials were symmetrical, that is, absolute values of poten-

[2] No attempts were made to numerically determine a time course at doses of 5 μg/ml or below, since the measured effects were small.

Table 3. Relative permeabilities and partial ionic conductivities for K, Na and Cl

		Control		Protamine		Heparin reversal	
Relative permeability; K : Na : Cl		18.3 : 9.1 : 1		6.7 : 3.1 : 1		15.9 : 6.3 : 1	
Partial ionic conductivity of specific or leak pathway (mS/cm²)		Specific	Leak	Specific	Leak	Specific	Leak
	K	0.94	0.05	0.37	0.06	1.09	0.07
	Na	9.17	0.73	3.19	0.87	8.12	0.97
	Cl	—	1.03	—	1.23	—	1.38
	Total	10.11	1.81	3.56	2.16	9.21	2.42

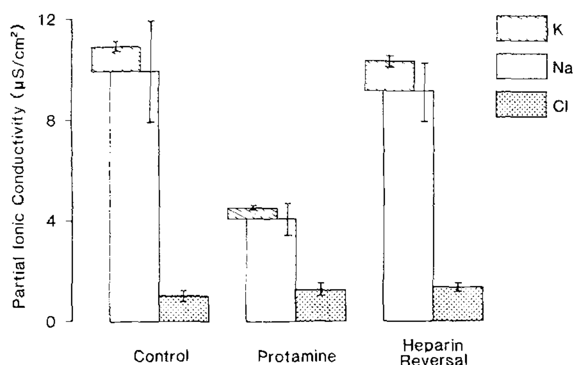

Fig. 5. Partial ionic conductivities of Na, K and Cl. In this plot, no assumptions were made regarding the specificity of the pathways. (For distinction between cation-specific and leak pathways *see* Table 3, lower part.) Error bars refer to variances of R_P measurements only

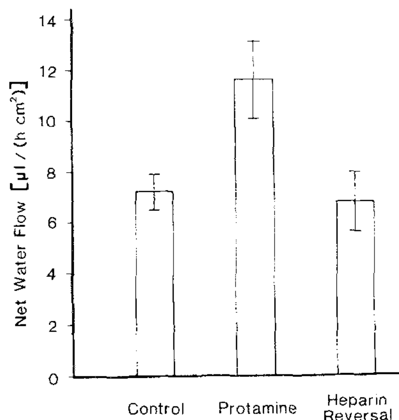

Fig. 6. Effect of protamine on net solute-coupled water transport measured from mucosa to serosa. Protamine values were significantly different from control and from heparin reversal ($P < 0.05$), whereas control and heparin reversal were not significantly different

tials were approximately equal whether dilutions were made on the mucosal or serosal side.

Overall epithelial conductance was largely determined (>90%) by the two cations under control conditions as well as after heparin reversal (Fig. 5). After protamine exposure, where overall epithelial conductance was reduced by approximately one-half, Cl conductance was unaffected and only K and Na conductance were decreased. However, one can further analyze data to distinguish between a "specific" conductance pathway for cations only and a "nonspecific" or "leak" conductance pathway (Moreno, 1975). Besides Cl, this leak conductance path would also carry Na and K proportional to their ionic mobilities and their concentrations.

The lower part of Table 3 gives numerical data on the "specific" and "leak" pathway ion conductivities as defined above. It is assumed that all chloride conductance is "leak" conductance. Under control conditions, the leak conductance contributes 12% of the overall epithelial conductance and its absolute value changes little under either protamine or heparin reversal. In contrast, "specific" conductance decreases after protamine exposure nearly threefold. This effect is also reversed by heparin neutralization.

Net Water Transport

Since protamine reduced cation conductance most probably across the tight junction, we measured net solute-coupled transport of water (Fig. 6). Control transport rates were similar to other values reported in the literature (Persson & Spring, 1982). Under protamine, net water absorption increased by 60% and after heparin reversal, decreased again to values not significantly different from control.

Discussion

AC Method

Transepithelial resistance increases rapidly and reversibly in response to mucosal protamine. This pa-

rameter was measured using a simple transepithelial AC impedance technique. When compared to the conventional DC-clamp method, determination of transepithelial AC impedance yields one piece of additional information. It allows the discrimination between real epithelial resistance (represented by R_P) and resistance of all non- or sub-epithelial structures (represented by R_S). There are several lines of evidence that assignment of these electrically defined parameters to the morphological counterpart structures is correct.

(i) R_P is defined as being in parallel to a capacitance, C_P (*see* inset of Fig. 2). The structural basis of the apparent trans-tissue capacitance is the two epithelial cell membranes in series.[3] By definition, R_P is the resistance across this structure and therefore includes the paracellular as well as the transcellular pathway. R_S is the resistance of all other structures in series to R_P (it does not include solution resistance).

(ii) Removal of part of the subepithelial tissue resulted in decreased R_S values, whereas R_P and C_P remained unchanged.

(iii) Removing part of the epithelial layer resulted in decreased R_P and C_P, but not R_S values (*unpublished results*).

(iv) Epithelial and subepithelial resistance determined from voltage divider ratios after positioning a microelectrode just below the basal-lateral epithelial cell membrane was not significantly different from R_P and R_S measured with the AC technique (Fromm et al., 1984).

Therefore, protamine affected only the epithelial resistance whereas subepithelial tissues were not involved in protamine action.

Is It Paracellular Action?

The increment in transepithelial resistance induced by protamine can be attributed *a priori* to a change in paracellular pathway properties since the shunt path of *Necturus* gallbladder accounts for about 90% of the total epithelial conductivity (Frömter et al., 1981). The shunt path consists of two resistive components connected in series, the junctional complex and the lateral interspace. The junctional complex was estimated to contribute 84% (Spring & Hope, 1979) 98% (Curci & Frömter, 1979) or to shunt path resistivity. Finally, if one considers the decrease in cation conductivity while the anion conductivity remained constant, the lateral interspace cannot be the responsible site since otherwise one would expect a decrease in Cl conductivity too (Wiedner & Wright, 1975). Thus, variations in the shunt path conductivity reflect changes predominantly within the junctional complex, of which the tight junction is the main contributing structure.

Transepithelial Voltage

The effects of protamine on transepithelial resistance and ion selectivity were very uniform and independent of seasonal variability, type of Ussing chamber, or other parameters specific for the two laboratories involved in this study. However, the effect of protamine on ψ^{ms} was quite variable. For instance, in one group of animals studied in late summer (*unreported observations*) ψ^{ms} shifted only by -0.5 to -1 mV in response to protamine. So far, we have no good explanation for this variability except to suggest that animals showing blunted ψ^{ms} responses to protamine may have been in different adaptation states *in vivo*. Thus, if active Na transport is relatively depressed, protamine would not be expected to change ψ^{ms} (*see below*). At least, it would indicate that the observed effects of protamine are not tightly coupled to the intensity (or electrogenicity) of active transport. A more compelling argument supporting this notion is the observation that the resistance response to protamine was not altered in gallbladders previously treated with 5×10^{-4} M ouabain.

However, the ψ^{ms} changes observed under protamine are in agreement with the model proposed by Moreno and Diamond (1975) for gallbladder function: Normally, the gallbladder transports Na and Cl from the lumen to lateral interspace in a slightly electrogenic way which generates a small mucosa negative emf. Na is able to leave the interspace by two pathways, through the basal opening to the serosal side or through the cation-selective tight junction pathway back to the mucosal side. In contrast, Cl can leave only through the serosal side. This asymmetry generates another emf which is mucosa positive across the tight junction. The resulting transepithelial voltage (ψ^{ms}) is determined by the sum of the two opposing emf's and their respective source resistances. Apparently, in control conditions ψ^{ms} is slightly mucosa positive, but during the protamine response, when the source resistance of the tight junction increases, the resulting ψ^{ms} turns toward mucosa negative values. This mechanism also explains our observation that prot-

[3] It should be noted that C_P measured across the epithelium does not *numerically* reflect the sum of the two cell membrane capacitances in moderately tight or leaky epithelia. [For further discussion of this point *see*, e.g., Lewis and Diamond (1976)]. C_P did not change following protamine exposure and this is in accordance with the assumption that lipid membrane area and/or dielectric properties were not altered, but it does not prove this conclusion.

amine does not change ψ^{ms} if active transport is inhibited by ouabain.

Water Transport

The finding that protamine at the same time decreased ion conductance, but increased net water absorption can also be explained by the model stated above. Under control conditions the tight junction is essentially a *back* leak for cations (as argued above). If protamine reduces this leak, it consequently increases net absorptive transport of osmoles. Clearly, osmotically coupled net water transport will increase too.

Just a Big TAP or a Different Mechanism?

Moreno (1975) argues that TAP binds, by virtue of its positive charge, to negative binding sites in or close to openings in the paracellular ion channel, thus neutralizing fixed negative charge resulting in decreased cation selectivity. A similarity between responses to TAP and responses to protamine, both on transepithelial resistance and ion selectivity is apparent. The induced responses to both substances were qualitatively and quantitatively comparable. Nevertheless, several differences in properties of TAP and protamine can be listed:

(i) The doses necessary to produce similar effects are very different. In *Necturus* gallbladder, Reuss and Grady (1979) noted a 65% increment in resistance using 10 mM TAP while in our experiments protamine induced a comparable effect at mucosal concentrations of 4 μM.

(ii) TAP acts on either side of the epithelium and is most effective if given on both sides simultaneously, whereas protamine acts only if added to the mucosal bath.

(iii) The effect of TAP can be reversed by a change of solutions, whereas protamine effects decrease only slightly by this procedure.

(iv) For experimental purposes it may be noted that TAP is active only at a solution pH of below 6, whereas protamine is effective at physiological pH.

The most important finding which limits use of TAP as a potential "tool" to dissect transepithelial conductance paths was reported by Reuss and Grady (1979). These investigators noted that TAP decreased potential and increased fractional resistance of the apical cell membrane of *Necturus* gallbladder epithelial cells. Protamine does not alter apical membrane potential and has only a small effect on membrane fractional resistance (*observations to be published*).

Thus, protamine appears to be a relatively "pure" paracellular conductance probe and does not exhibit some of the experimental disadvantages known for TAP (relatively high concentration and action at unphysiological pH only). It is proposed that TAP neutralizes fixed negative charges in or near the tight junction thereby decreasing cation conductance. Although protamine may act similarly, certain observations are difficult to explain by a direct mode of action: i) a small hydrostatic pressure gradient sufficient to widen the lateral intercellular spaces caused a decreased response to protamine. Similar blunted R_T responses are noted with 8Br-cAMP and are associated with structural changes in the tight junction (Palant & Bentzel, 1984). This demonstrated reponse would not be expected if protamine had a direct effect at the channel site.

(ii) The full effect or full recovery from the protamine-evoked resistance response took about 10 minutes longer than expected for a molecule to directly interact with either a charge or transport site. (Amiloride, for example, inhibits conductive Na entry into the cell within milliseconds and TAP induces a maximal resistance increment within 15 sec.)

However, there are obvious differences in molecular size and probably in membrane binding between TAP and protamine making comparison difficult. To search for a different hypothesis one might consider reported observations on action of protamine in other tissues: Seiler et al. (1977) observed cell swelling and induction of occluding junctions in rat glomerular epithelium perfused through the renal artery with another polycationic substance, poly-L-lysine. These findings were confirmed by Kerjaschki (1978) who infused protamine and presented evidence that microfilaments and microtubules of the cytoskeleton were involved in tight junction formation between glomerular epithelial cell foot processes. Alavi et al. (1982) reported permeability and microfilament alterations in rat peritoneum. Margri et al. (1978) found that protamine induced actin (microfilament) polymerization in muscle spindles. Bentzel et al. (1980) provided evidence for an involvement of microfilaments in regulation of tight junctional permeability in *Necturus* gallbladder and Meza et al. (1980) came to the same conclusion for cultured MDCK cells.

The hypothetical mechanism we assume for protamine action thus includes a "binding" or some type of interaction of the molecule to the apical cell membrane, which in turn, disrupts microfilament connections with the inner side of the membrane.

This loss of microfilament attachment would release a sequence of intracellular events which will finally lead to additional tight junctional strand formation or reallocation of strands within the junctional membrane domain and eventually to the observed decrease in cation permeability.

To add direct evidence in support of this hypothesis, we performed ultrastructural analysis of the zonulae occludentes. The results of this forthcoming study indicate that indeed morphological alterations of tight junctions are observed under the influence of protamine, in accordance with the electrophysiological effects reported in this paper.

At present, we are far from understanding how tight junction strand formation takes place, or even knowing the structural basis of strand allocation within the membrane. However, our own observation that the electrophysiological effect of protamine is attenuated when the lateral interspace is widened by increased serosal hydrostatic pressure could be explained by assuming decreased tight junction strand formation if the adjacent lateral membranes to be drawn together are physically more remote from one another at the outset.

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