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Protamine Alters Structure and Conductance of *Necturus* Gallbladder Tight Junctions Without Major Electrical Effects on the Apical Cell Membrane

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Summary. Protamine is a naturally occurring basic protein (pI; 9.7 to 12.0). We have recently reported that protamine dissolved in the mucosal bath (2 to 20 μM), induces about a twofold increase in transepithelial resistance in *Necturus* gallbladder within 10 min. Conductance decreased concomitantly with cation selectivity.

In this leaky epithelium, where >90% of an applied current passes between cells, an increment in resistance of this magnitude suggests a paracellular action *a priori*. To confirm this, ionic conductance across the apical cell membrane was studied with microelectrodes. Protamine increased transepithelial resistance without changing apical cell membrane voltage or fractional membrane resistance. Variation in extracellular K concentration (6 to 50 mM) caused changes in apical membrane voltage not different from control.

To determine if protamine-induced resistance changes were associated with structural alteration of tight junctions, gallbladders were fixed *in situ* at peak response and analyzed by freeze-fracture electron microscopy. According to a morphometrical analysis, the tight junctional intramembranous domain expands vertically due to incorporation of new strands (fibrils) into the main compact fibrillar meshwork.

Since morphologic changes are complete within 10 min, strands are probably recycled into and out of the tight junctional membrane domain possibly by the cytoskeleton either from cytoplasmic vesicles or from intramembranous precursors. Regulation of tight junctional permeability by protamine and other perturbations may constitute a common mechanism by which leaky epithelia regulate transport, and protamine, in concentrations employed in this study, seems reasonably specific for the tight junction.

Key Words protamine · tight junction · transepithelial resistance · tight junction morphology · tight junction strands · *Necturus* gallbladder

Introduction

Finding a specific blocker of transjunctional ion conductance in a leaky epithelium may add considerable investigative potential. More specifically, the conductive properties of the shunt pathway in leaky epithelia can be more clearly defined if alterations in transcellular ion movement do not occur simultaneously.

We have recently reported (Fromm, Palant, Bentzel & Hegel, 1985) that the naturally occurring polycationic protein, protamine, reversibly decreases paracellular permeability in *Necturus* gallbladder. The resistance response begins within 1 min, peaks in 10 min, averaging 89% above baseline, and is reversed by heparin neutralization. Briefly, the protamine response consists of: (1) a rapid increase in transepithelial resistance with a variable effect on transepithelial potential difference; (2) a decrease in partial ionic conductance for Na and K but not for Cl ions; (3) a reversible increase in net water transport from mucosa to serosa; (4) an "all-or-none type" dose-response relationship, indicating that a near maximal response was observed when mucosal concentrations of protamine equalled or exceeded 10 μg/ml; (5) no effect when protamine is applied to the basolateral cell membrane; (6) no toxic effects even after repeated exposures in the same gallbladder using mucosal concentrations up to 100 μg/ml (~20 μM); (7) transepithelial resistance responses were elicited even when serosal hydrostatic outflow pressure was increased to dilate intercellular spaces, suggesting that the major site of action is the tight junction.

We have now extended the study of protamine responses by measuring apical cell membrane potential difference, fractional membrane resistance, and K permeability. The results obtained suggest that mucosal protamine (at concentrations studied) reversibly decreases shunt conductivity without inducing major effects on the conductance of the apical cell membrane.

Most importantly, a primary effect on the tight junction is confirmed by distinctive modification in the intramembranous meshwork of tight junctional

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strands observed by freeze-fracture electron microscopy. This latter observation suggests that protamine exerts its effect primarily through a secondary action via cellular regulatory mechanisms rather than by direct neutralization of fixed negative charges with loss of cationic selectivity in the junctional channel, a mechanism previously postulated by Moreno (1975) to explain decreased cation conductance induced by 2, 4, 6-triaminopyrimidinium (TAP), an organic cation.

This cytoplasmic mode of action is also compatible with the finding that similar tight junction structure/function changes in this epithelium can be reversibly induced by other intracellular perturbations including microfilament-active agents (Bentzel et al., 1980), cyclic nucleotides (Duffey, Hainau, Ho & Bentzel, 1981) and/or calcium ions (Palant, Duffey, Mookerjee & Bentzel, 1983).

Symbols and Definitions

ψ^{ms}	spontaneous transepithelial voltage of the mucosal side with respect to the serosal bath (mV)
ψ^{mc}	spontaneous voltage of the cell interior with respect to the mucosal bath (mV)
$\Delta\psi^{ms}$	transepithelial voltage deflections (mV) induced by short bipolar constant current pulses
$\Delta\psi^{mc}$	voltage deflections across the apical membrane (mV) induced by current pulses
$\Delta\psi^{sc}$	voltage deflections across the basolateral membrane (mV) induced by current pulses
ψ^{me}	spontaneous voltage measured across a microelectrode placed in the mucosal bath with respect to mucosal bath (mV)
$\Delta\psi^{mfl}$	voltage deflections of a microelectrode positioned close to the apical membrane (mV) induced by current pulses
$\Delta\psi^{sfl}$	voltage deflections of a microelectrode positioned close to the basolateral membrane (mV) induced by current pulses
fR^a	fractional resistance of the apical cell membrane
R^a/R^b	ratio of apical to basolateral membrane resistances; $R^a/R^b = (\Delta\psi^{mc} - \Delta\psi^{mfl})/(\Delta\psi^{sc} - \Delta\psi^{sfl})$
R^t	tissue resistance (Ωcm^2)
R^e	transepithelial resistance (Ωcm^2)
R^s	subepithelial resistance (Ωcm^2)
Number of strands:	number of more or less horizontally oriented strands intersecting with one grid line. Number of strands is given for the total junction and for the compact meshwork
Compact meshwork:	vertical expansion of the main meshwork of interconnecting tight junctional strands forming closed loops
Total junction:	vertical expansion of all strands, including strands connected to or isolated from, the basal extension of the compact meshwork

Materials and Methods

Electrophysiological Techniques

Gallbladders, obtained from anesthetized (tricaine, Sandoz, Nürnberg, FRG) adult mudpuppies, (*Necturus maculosus*, Nasco, Oshkosh, WI), were mounted mucosa side up in a horizontal 4-electrode chamber (exposed area, 0.20 cm^2). The serosal side of the tissue was supported by fine nylon mesh and edge damage was minimized by use of high viscosity silicon grease as a sealant between half chambers. The serosal outflow was kept 0.5 to 1.0 cm below the mucosal outflow throughout the experiment. Continuous perfusion of mucosal and serosal compartments was achieved by gravity with an amphibian NaCl Ringer's solution at a rate of approximately 1.0 ml/min. The composition of the bathing solution was (mM): NaCl 95, $NaHCO_3$ 13, KCl 4.5, $CaCl_2$ 1.8, $MgCl_2$ 1.0, NaH_2PO_4 0.7. In some experiments HEPES buffer 5.0 was also added to the bathing solution. Solutions were gassed with a mixture of 95% O_2 and 5% CO_2, and pH remained stable at 7.3–7.5 throughout all experiments. Protamine was dissolved in the mucosal bathing solution as either Protamine-HCl (La Roche, FRG) or Protamine-SO_4 (Lilly, Indianapolis, IN) at concentrations of 30, 35, 50 or 100 μg/ml (about 6 to 20 μM, respectively). As pointed out in our previous paper (Fromm et al., 1985), both protamines acted in the same way and the results were analyzed together. Heparin, added to the mucosal medium to counteract the protamine response, was used at twofold neutralizing concentration (2 units of heparin to 1 μg or unit of protamine).

Current clamping was performed using an automatic voltage/current clamp (H. Güllentops, Leuven, Belgium or Industrial Associates, Houston, Texas). For intracellular recordings the microelectrode was connected to a F-223A dual electrometer (WPI, New Haven, CN). In some experiments, an additional subepithelially positioned microelectrode (*see below*) was connected to a second electrometer (M-4A, WPI, New Haven, CT). The mucosal (upper) bath was connected as the electrical reference point to all amplifiers. (However, spontaneous transepithelial potential difference, ψ^{ms}, is given as mucosal voltage with respect to serosa.)

Transepithelial resistance was determined by passing bipolar rectangular current pulses of 20 or 40 $\mu A/cm^2$, 0.2 sec duration every 10 sec, across the gallbladder. After correction for the fluid resistance between voltage-sensing electrodes, transepithelial tissue resistance (R^t) could be calculated from resulting voltage deflections. Epithelial and subepithelial resistance (R^e and R^s) were determined using two methods:

(i) A microelectrode was advanced just through the basolateral cell membrane. The voltage deflections at this position minus those at the position immediately adjacent to the apical cell membrane yield R^e. R^s is then given by $R^t - R^e$.

(ii) An additional microelectrode was positioned just below the basolateral cell membrane and was used in the same way as the micropuncture electrode of method *i*. Methods *i* and *ii* gave identical results within a range of ± 2 Ωcm^2. Thus, both methods were used, alternately or together, for measuring R^e and R^s. Since R^s was very consistent (15 or 16 Ωcm^2) in this study, we refer to R^e and R^t interchangeably.

Microelectrodes were drawn from borosilicate glass with an inner filament (1.5 mm OD, 0.85 mm ID; Hilgenberg, Malsfeld, FRG) using a two-stage puller (PD-5, Narishige, Tokyo, Japan). Electrodes were backfilled with 0.5 M KCl to reduce KCl leakage

out of the tip. Microelectrode tip resistance was 50–85 mΩ when tested in 0.5 M KCl.

Cell impalements were done vertically with the aid of a piezoelectric micropositioner (Fromm, Weskamp & Hegel, 1980). The driver was mounted directly on a manual micromanipulator (CP-5, Brinkmann, USA).

Criteria for acceptable cellular impalements were: (i) an abrupt voltage deflection when entering the cell; (ii) less than 10% variation in ψ^{mc} for 1 min, (iii) less than 5% variation in $\Delta\psi^{mc}$ for 1 min; (iv) a sudden return to baseline within ±2 mV after withdrawal from the cell; (v) no significant change in electrode tip resistance after withdrawal from the cell. About 60% of all impalements fulfilled these criteria.

The fractional resistance of the apical cell membrane was calculated from $fR^a = (\Delta\psi^{mc} - \Delta\psi^{mfl})/(\Delta\psi^{ms*} - \Delta\psi^{mfl})$, where $\Delta\psi^{mfl}$ are voltage deflections of the microelectrode positioned close to the apical membrane and $\Delta\psi^{ms*}$ is Δc^{ms} after correction for contribution of the subepithelium and serosal bathing fluid.

Each gallbladder was studied for 1–2 hr. Cell punctures were performed using two technical protocols. Either different cells were independently punctured during each experimental condition and solutions were switched while the microelectrode was withdrawn from the cell ("discrete" punctures) or the microelectrode tip was continuously kept within a single cell during solution changes ("continuous" punctures). The experimental protocol always included a control Ringer's period and sequential mucosal perfusions of protamine, and to reverse the protamine effect, heparin in Ringer's. Heparin alone, dissolved in Ringer's, had no effect on electrical parameters.

Apical Membrane Permeability to K

To test whether membrane conductance to potassium was altered after exposure to protamine, mucosal and serosal surfaces were bathed in solutions containing different concentrations of potassium. Sodium concentration was maintained above 40 mM by substitution with choline. Solution composition was: Control: (mM) NaCl 87; KCl 4.5; $CaCl_2$ 1.8; $MgCl_2$ 1.0; KH_2PO_4 0.2; K_2HPO_4 0.7; $NaHCO_3$ 7.5; NaH_2PO_4 0.8; HEPES 5.0; pH 7.4; 195 mOsm. In addition to the control solution (total K = 6.4 mM), substitutions were made for NaCl by adding KCl and choline Cl to yield three additional test solutions containing 14 mM K, 15 mM choline Cl; 29.6 mM K, 25 mM choline Cl and 50 mM K; 5 mM choline Cl. Protamine (35 μg/ml) or heparin was added to mucosal test bathing solutions where appropriate.

A total of nine gallbladders yielded baseline control intracellular potentials. Of these gallbladders, five were also perfused with mucosal protamine, and in three of these the protamine effect was reversed with heparin. In these experiments, since heparin reversal measurements were not different from control, they were analyzed with the control data.

Morphological Methods

All tissues were fixed in the Ussing chamber 10 to 15 min after the appropriate electrophysiologic response had been recorded, i.e., at the peak resistance response to protamine or after complete reversal by heparin. The experiment was stopped at the end of either control (gallbladder mounted and perfused 10 to 15 min with Ringer's), protamine or heparin reversal by flushing both sides of the chamber with 2% phosphate-buffered glutaraldehyde delivered through the serosal and musocal perfusing tubing at the same rate as in preceding physiological monitoring. Since we have noted seasonal variations in tight junction ultrastructure, gallbladders fixed during protamine exposure were bracketed in time (within a few days) with gallbladders fixed during control and heparin reversal experiments using the same batch of animals.

Tissue was either dehydrated in ethanol, embedded in Epon and cut and stained with uranyl acetate and lead citrate (thin sections) or frozen in Freon and liquid nitrogen (−100°C) and shadowed with platinum and carbon in a Denton DFE-3 apparatus (freeze fracture). Replicas and sections were examined with a Siemens 1A electron microscope. Freeze-fracture replicas were scanned (×20,000) and electron micrographs were taken of all tight junctional regions where both the luminal and abluminal strand in the meshwork could be discerned. To avoid bias, no tight junction meeting this criteria was passed over. Electron micrographs were printed ×3 and morhometric analyses were done on coded prints to exclude prior knowledge of experimental conditions.

Morphometric Analysis

As previously described (Bentzel et al., 1980), grid lines were drawn on electron-micrograph prints perpendicular to the most luminal strand of the junction at 80-nm intervals (equivalent to 5 mm on the print). The number of intersections between grid lines and strands within and below the main compact meshwork were counted, and the means were defined as the number of strands within the "compact meshwork" and in the total junction, respectively. The vertical expansion (depth) of the total tight junction is the distance between the most luminal and contraluminal strand even though that strand might be attached to and extend below or be separate from the "compact meshwork." The terms "strand number" and "depth" are applied to the "compact meshwork" or to the total junction which includes the compact meshwork.

All results were given as arithmetic mean ± SEM. Significances were evaluated using unpaired *t*-test and two-tailed probabilities.

Results

Cell Puncture

The typical effect of protamine to reversibly increase transepithelial resistance by about 90% (without affecting subepithelial resistance), was confirmed in this study (Tables 1 and 2). Spontaneous transepithelial potential difference, ψ^{ms} was near zero under control conditions and did not change significantly after protamine or heparin reversal. As discussed in our previous study (Fromm et al., 1985), ψ^{ms} turned to slightly mucosal negative values in some experimental groups of gallbladders but his response was not consistent.

The voltage across the apical membrane, ψ^{mc}, did not significantly change during protamine or

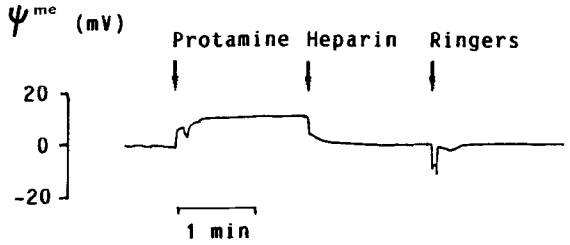

Fig. 1. A microelectrode was placed in the mucosal bath. An +11 mV tip potential (ψ^{me}) develops within 2–3 min when Ringer's containing 50 μg/ml protamine (1st arrow) is perfused into the mucosal bath. This tip potential is readily reversed by heparin Ringer's (2nd arrow). Artifacts are noted in potential recordings

Table 1. Protamine effect on apical membrane using "discrete" punctures

	Control	Protamine 100 μg/ml	Heparin
ψ^{ms} (mV)	+0.5 ± 0.5	+1.0 ± 0.9	+1.3 ± 0.7
ψ^{cm} (mV)	−55.2 ± 4.6	−55.6 ± 2.3	−55.9 ± 1.4
R^e (Ωcm^2)	104 ± 9	176 ± 9	101 ± 5
R^s (Ωcm^2)	15 ± 3	16 ± 4	16 ± 4
fR^a	0.78 ± 0.03	0.74 ± 0.03	0.74 ± 0.02
n	20	37	41

ψ^{ms} given with respect to serosal bath; Only R^e is significant when compared: control *vs.* protamine: $P < 0.0008$; protamine *vs.* heparin: $P < 0.0001$; control *vs.* heparin: NS; n = number of punctures, 5 gallbladders.

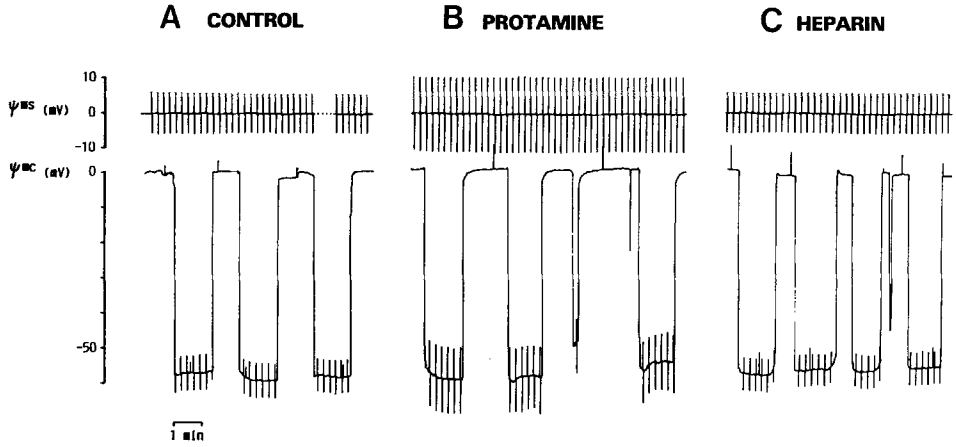

Fig. 2. Typical "discrete" cell punctures: Effect of: control (*A*); protamine (30 μg/ml), mucosal bath (*B*); heparin reversal (*C*); upper trace (ordinate on the left) is transepithelial voltage (ψ^{ms}). Lower tracing (ordinate on left) is voltage at microelectrode tip (ψ^{mc}). Spontaneous transepithelial potential, ψ^{ms}, determined when no current is passed (mid point, upper trace) and ψ^{mc} does not change during protamine exposure (about −57 mV in *A*, *B*, and *C*). In *B* (protamine), transepithelial resistance (calculated from $\Delta\psi^{ms}$, top tracing) increases to the same extent as $\Delta\psi^{mc}$ so that fR^a remains constant during protamine exposure. Voltage deflections return to baseline in *C* (heparin reversal). Tip potential was not zeroed prior to puncture and microelectrodes were changed before each group of punctures. Cells were quickly punctured to minimize tip potentials caused by protamine

heparin reversal using two different experimental protocols, "discrete" and "continuous" punctures (*see* Materials and Methods). The reason for using two protocols was that in many discrete punctures, protamine induced a tip potential of about +11 mV within several minutes after immersion in the mucosal bath (Fig. 1). The tip potential is reversed by heparin. If the tip potential is zeroed off prior to cellular puncture, ψ^{mc} will appear hyperpolarized by 11 mV. Except for experiments testing K permeability, tip potentials were not zeroed off. Figure 2 (discrete puncture) and Figure 3 (continuous puncture) show typical experiments using the two protocols. Statistical data are given in Table 1 (discrete punctures) and Table 2 (continuous punctures). Both methods gave nearly identical results, i.e. apical membrane potential difference, ψ^{mc}, did not change after protamine or after heparin reversal. Mean values of discrete punctures, which were performed at a different season of the year on a different batch of animals, were slightly lower than those measured with continuous punctures, but the difference was not significant (*compare* protamine, 100 μg/ml in Tables 1 and 2).

Fractional resistance of the apical membrane, fR^a, as determined using both cellular puncture protocols, remained fairly constant between 0.72 and 0.78 and was not consistently altered during protamine exposure (Tables 1 and 2). The only observed change in fR^a was found between control

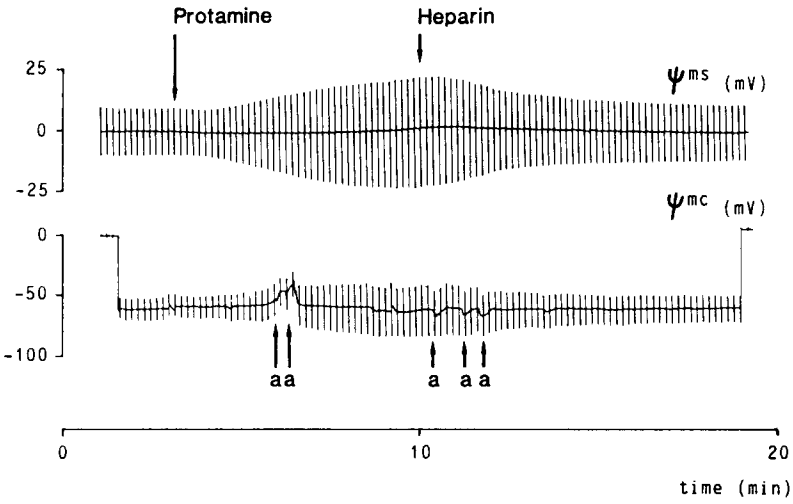

Fig. 3. Typical "continuous" puncture: Upper tracing gives transepithelial voltage, ψ^{ms}. Lower tracing, voltage at microelectrode tip, ψ^{mc}. Transepithelial resistance is calculated from $\Delta\psi^{ms}$. Note that several minutes after addition of protamine (30 μg/ml) to the mucosal bath, $\Delta\psi^{ms}$ begins to increase. $\Delta\psi^{mc}$ also increases so that fR^a is unchanged. ψ^{ms}, spontaneous transepithelial potential differences (midline, upper tracing) does not change during protamine. Heparin (2nd arrow) returns $\Delta\psi^{ms}$ and $\Delta\psi^{mc}$ to baseline. *a* = stirring artifacts to insure protamine dispersion at the apical surface

Table 2. Protamine effect on apical membrane using "continuous" punctures

	Control "C"	Protamine 30 μg/ml "P30"	Protamine 100 μg/ml "P100"	Heparin "H"
ψ^{ms}	−0.1 ± 0.3	+0.9 ± 0.8	+0.6 ± 0.3	+0.5 ± 0.2
ψ^{mc}	−60.8 ± 1.3	−60.3 ± 0.9	−58.3 ± 1.5	−60.1 ± 1.0
R^t	126 ± 5	201 ± 7	212 ± 17	142 ± 4
fR^a	0.72 ± 0.01	0.78 ± 0.02	0.78 ± 0.02	0.74 ± 0.02
n	20	15	14	28

Significances

	P<					
	C/*H*	*C*/P30	*C*/P100	P30/P100	P30/*H*	P100/*H*
ψ^{ms}	NS	0.05	NS	NS	NS	NS
ψ^{mc}	NS	NS	NS	NS	NS	NS
R^t	0.01	0.0001	0.0001	NS	0.0001	0.0001
fR^a	NS	0.005	0.005	NS	NS	NS

NS = not significant; ψ^{ms} with respect to serosal bath; *n* = number of punctures, 5 gallbladders; *C* = control; P30,100 = protamine 30, 100 μg/ml; *H* = heparin reversal.

and protamine (30 and 100 μg/ml) but heparin did not reverse the change (Table 2). In three other gallbladders, R^a/R^b averaged 3.4 ± 0.6 (10 control punctures) and was 4.7 ± 0.7 (15 punctures) during mucosal exposure to protamine, 35 μg/ml. This change was not significant, $P < 0.1$.

The finding that protamine does not induce changes in apical cell membrane permeability to potassium is predictable from its lack of effect on ψ^{mc} and fR^a. K for Na substitution in both baths yielded a steady-state relationship between ψ^{mc} and extracellular K concentration of 29 mV per decade K change (Fig. 4). This is close to the slope reported by Reuss and Finn (1975) and indicates that the apical membrane is highly K permeable. Mucosal protamine (35 μg/ml) yields a similar slope of 31 mV per decade K change (Fig. 4). Therefore, at a concentration which rapidly increases transepithelial resistance, protamine has no major effect on apical cell membrane permeability to K. We should emphasize that protamine may well alter membrane permeability to other ions (e.g. calcium) which we have not explored. We have, however, observed that protamine did not alter intracellular cAMP concentration when incubated with *Necturus* gallbladders *in vitro*, whereas methylisobutylxanthine (MIX) doubled intracellular cAMP within 30 min (*unpublished observation*).

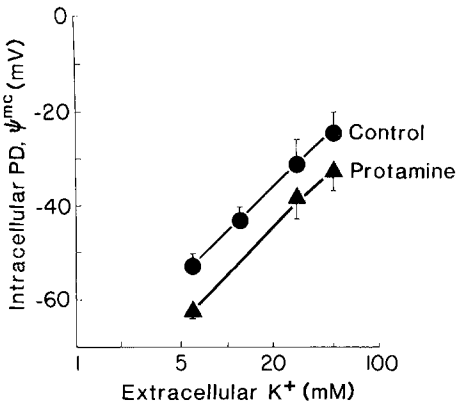

Fig. 4. Effect of extracellular K on apical membrane voltage. Apical membrane voltage, ψ^{mc}, is given on the ordinate and K concentration (mM) of the bathing solution is given on the abscissa. Tip potential during protamine (about +11 mV) was zeroed prior to cell impalement in these experiments, so that the constant voltage difference between the two lines, control (●) and protamine (▲), differ approximately by this tip potential. Slopes during control of 29 mV per decade K and protamine (35 μg/ml, mucosal surface) of 31 mV per decade K did not differ, indicating that apical cell membrane permeability to K was unchanged

Morphology

Thin Sections

Necturus gallbladders exposed to mucosal protamine (50 μg/ml) and fixed while mounted in the chamber, frequently showed striking changes in apical regions when compared to control and heparin reversal. In gallbladders showing a change, there was distortion and rounding out of microvilli, particularly in the vicinity of tight junctions. In these regions, microfilaments appeared to lose definition and appear as darkly stained cytoplasm. However, this abnormal appearance in the microfilaments was not consistent in all gallbladders and, in fact, in most tissues examined, protamine induced little change either in microvilli architecture or in the core of microfilaments.

Freeze Fracture

Control (*Fig. 5*). Clearly, the major morphological change induced by protamine was on the intramembranous structure of the tight junction as revealed by freeze-fracture electron microscopy. Under control conditions, tight junctional structure did not differ substantially from that previously reported. The junction consists of a regular array of interconnecting strands which we call the "compact" meshwork of strands (fibrils), which encircles each cell separating the apical from the basolateral membrane.

Table 3. Effect of protamine on total junction

	n	Number of strands	Depth (μm)
Control	409	7.17 ± 0.13	0.43 ± 0.01
Protamine (50 μg/ml)	332	8.91 ± 0.14	0.60 ± 0.01
Heparin reversal	220	8.15 ± 0.14	0.58 ± 0.02

n = number of grid lines analyzed at 0.08-μm intervals. Strand number and depth: Protamine *vs.* control and heparin *vs.* control: $P < 0.001$. Heparin *vs.* protamine: (depth) *ns*, (strand number); $P < 0.001$.
Total junction includes "compact" meshwork plus strands below the meshwork.

Occasionally a strand extends down from the compact meshwork (Fig. 5), but it is rare to see a strand isolated from the compact meshwork.

Protamine (*Fig. 6*). After 10 to 15 min of protamine exposure (50 μg/ml mucosal surface), the freeze-fracture appearance of the tight junction is altered in the following manner: (i) changes are focal, that is to say progressing laterally along the junction, some junctional regions appear to have undergone more change than other regions, (ii) on the average, the compact meshwork is expanded vertically so that depth of the compact meshwork is increased primarily because more strands appear within the meshwork, (iii) strands seem to originate from below the compact tight junctional meshwork, i.e., from the zonula adherens region of the basolateral membrane. In these regions, not only are more strands seen, but also there is an aggregation of intramembranous particles; (iv) morphologic changes occur within 10 to 15 min following protamine exposure when peak resistance changes were complete. As in electrophysiological measurements, changes in junctional ultrastructure are not dose dependent, i.e., 15 and 100 μg/ml mucosal protamine induces approximately the same change as 50 μg/ml (only 50 μg/ml data are given).

Heparin Reversal (*Fig. 7*). After the resistance response was reversed by heparin, gallbladders have the following features: (i) Retraction of the compact meshwork so that meshwork depth decreases to a level not statistically different from control; (ii) Although junctional strands are somewhat reduced, many strands are observed below the compact meshwork and are frequently disconnected from the compact meshwork. This gives the appearance that strands are being "left behind" as the compact meshwork retracts. For this reason, the depth of the total junction was comparable to that observed after protamine; and (iii). The total

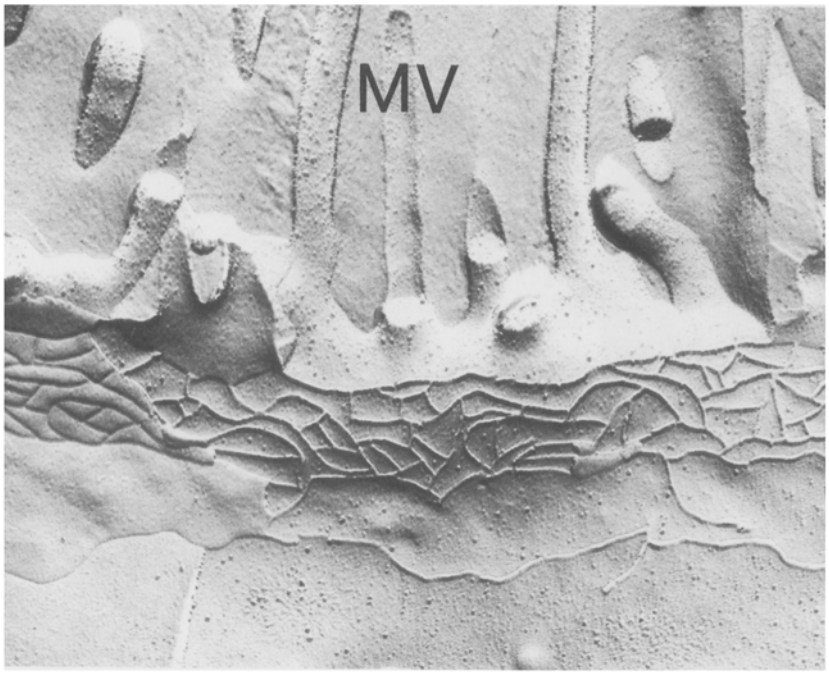

Fig. 5. Control: Freeze fracture replica of *Necturus* gallbladder fixed in chamber during perfusion with NaCl Ringers showing intramembranous appearance of tight junctional membrane domain. Microvilli (*MV*) indicate the mucosal surface. The tight junction is composed of an interdigitating meshwork of closed loop strands or fibrils ("compact" meshwork) forming a continuous belt around the apical region of the epithelial cell. Occasional strands extend down from a reasonably compact main meshwork into the zonula adherens region below the junction. P face ×70,000

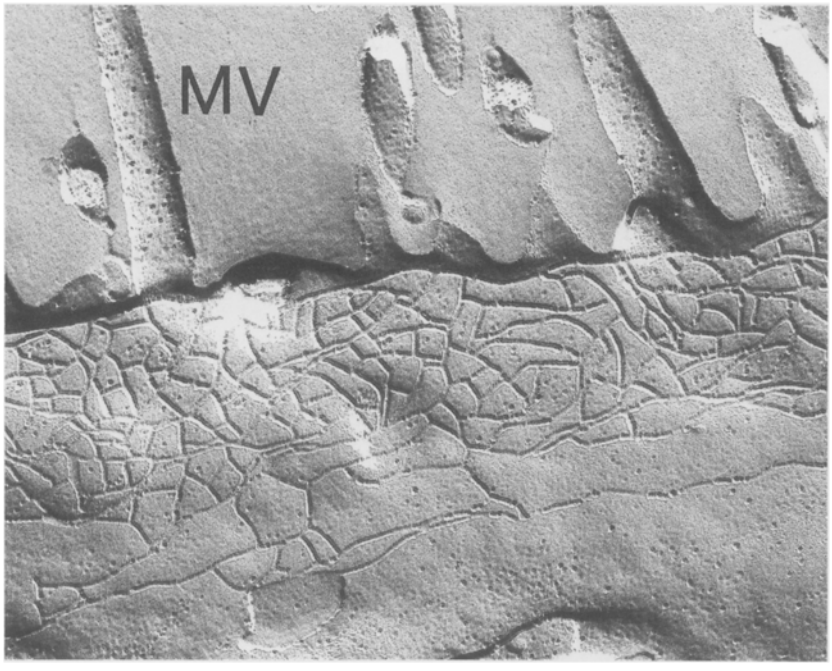

Fig. 6. Protamine response: Gallbladder fixed and freeze fractured (P face) 10 min after mucosal exposure to protamine (50 μg/ml). Note the main compact meshwork is expanded and more strands are seen extending down from the main meshwork. Strands below the main compact meshwork do not form closed loops and may even be isolated from the meshwork. Although not well observed on this electron micrograph, in many subjunctional regions there was increased aggregation of intramembranous particles, particularly where strand activity was prominent. Microvilli (*MV*) are labeled. ×70,000

number of strands in the junction is reduced slightly, but strands in the compact meshwork are approximately equal to that observed in control.

Morphometric Analysis (Tables 3 and 4)

Figure 8 is a schematic representation of the changes in tight junctional structure induced by protamine and reversed by heparin. A sample grid line is drawn to illustrate the procedure of morphometric analysis. Note that strand number and vertical expansion (depth) were measured in the total junction and in the compact meshwork, the latter consisting of only those strands that form interconnecting closed loops.

The total tight junction under control conditions (Table 3) consisted, on the average, of 7.17 more or less horizontally-oriented strands with a vertical expansion of 0.43 μm. These values are in agreement

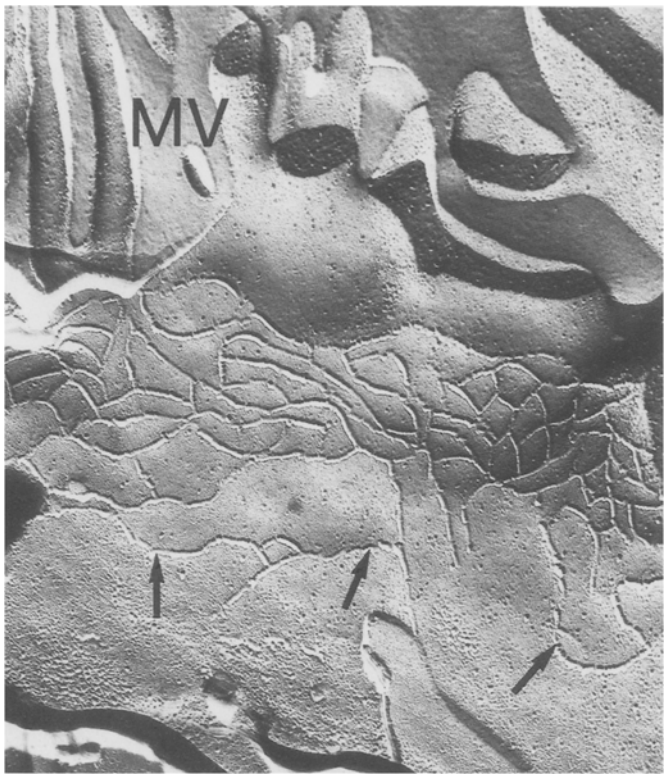

Fig. 7. Heparin reversal: Gallbladder was previously exposed for 10 to 15 min to protamine (50 μg/ml), then transepithelial resistance was returned to baseline by heparinized Ringer's before fixation and freeze fracture (P face). The depth of the compact meshwork is approximately the same as control (Fig. 5) but many strands are located beneath the meshwork, some of which are open ended (arrows) and disconnected from the compact meshwork. In many other replicas, this feature was even more prominent. Microvilli (*MV*) are labeled. ×70,000

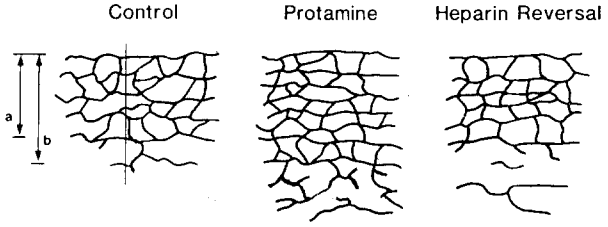

Fig. 8. A pictorial summary of structural changes measured under the three experimental conditions. A single grid line is shown to illustrate parameters measured. *a* is the vertical expansion (depth) of the compact meshwork and *b* is the vertical expansion (depth) of the total junction. In this illustration the grid line intersects seven strands in the total junctional region and six strands in the compact meshwork. More strands appear both below and within the compact junctional meshwork during protamine. After heparin reversal, the meshwork retracts back to control dimensions "leaving" some isolated strands below. Thus, the vertical expansion of the total junction has only partially recovered to control dimensions

Table 4. Effect of protamine on compact meshwork

	n	Number of strands	Depth (μm)
Control	182	5.44 ± 0.15	0.25 ± 0.01
Protamine (50 μg/ml)	152	6.97 ± 0.18	0.34 ± 0.01
Heparin reversal	118	5.81 ± 0.15	0.28 ± 0.01

n = number of grid lines analyzed at 0.16 μm intervals. Control *vs.* protamine and protamine *vs.* heparin reversal: $P < 0.001$. Control *vs.* heparin reversal: NS.

with recent previous data of this laboratory (Alavi, Lianos, Palant & Bentzel, 1983; Palant et al., 1983). Both strand number and depth of the total junction increased during protamine exposure, but did not return to control values after heparin. This means that after complete reversal of the resistance response by heparin, an average of one isolated strand was found beneath the compact meshwork. This point is made clear comparing Tables 3 and 4. When strand number and depth measurements are restricted to the compact meshwork, both the number of strands and the depth increased after protamine. After reversal by heparin, these parameters returned to values not significantly different from control. Thus, the observed resistance changes correlate better with changes in strand number and depth of the compact meshwork than with changes in the total junction.

Discussion

In a previous study (Fromm et al., 1985) we concluded that protamine decreased tight junction cat-

ion selectivity in this epithelium, but additional effects at the cell membrane level were not investigated. Two major conclusions have now emerged from the current study. First, protamine (at mucosal concentrations up to 20 μM) acts, for the most part, on the paracellular path or more explicitly, on the tight junction. Second, a rapid and largely reversible increase in intramembranous fibrillar elements which characterizes tight junctional ultrastructure parallels changes in transepithelial resistance.

Effect of Protamine at the Apical Cell Membrane

Besides diminishing conductance of the paracellular pathway, which was supported in this study by morphological findings, protamine with its strong positive charge might also be expected to alter electrical parameters of the apical membrane. Under control conditions, the voltage across the apical membrane, ψ^{mc}, as well as the fractional resistance of the apical membrane, fR^a, were both in accord with other values published (Suzuki & Frömter, 1977; Graf & Giebisch, 1979; Reuss & Grady, 1979; Palant et al., 1983). ψ^{mc} did not change after exposure to 30 or 100 μg/ml protamine or after heparin reversal.

The fractional resistance of the apical membrane, in one set of experiments, increased after mucosal protamine exposure, from 0.72 to 0.78, but exhibited no further significant changes after switching solutions to one containing heparin or after repeated switching between heparin and protamine in the same experiment. This initial increase in fR^a occurred only using the "continuous" protocol puncture, whereas in another series of experiments using "discrete" punctures fR^a never changed. Also, if measured as R^a/R^b, changes reached no higher degrees of significance. Fractional resistances in a leaky epithelia could theoretically be overestimated by a decrease in resistance of the lateral intercellular space and/or an increase in resistance of the tight junction (Boulpaep & Sackin, 1980; Nagel, Garcia-Diaz & Essig, 1983). Respective resistances of junction and interspace were not measured in this study, but one may argue that protamine increased tight junctional resistance (because of morphological changes) and probably had no effect or even decreased lateral intercellular space resistance (since net fluid transport increases; Fromm et al., 1985). Under this assumption, measured fR^a would slightly increase with protamine, even if true membrane resistances were not altered. Turning this argument around, true apical membrane resistance may even have decreased slightly under protamine, since no change in fR^a was measured. When R^a/R^b showed a tendency to increase during protamine, $\Delta\psi^{mc}$ increased while $\Delta\psi^{sc}$ changed less, but to conclude that R^b decreased is not justified. It is unlikely that protamine even reached the basolateral membrane, considering its molecular size and charge.

In a recent abstract, Poler and Reuss (1985) reported intracellular electrical measurements after protamine exposure in *Necturus* gallbladder using a much higher concentration of 1 mg/ml, which is 10 to 100 times higher than concentrations used in this and our previous study. They confirmed our findings on transepithelial resistance change, but observed a 10-mV decrease in both membrane voltages and a slight decrease in fR^a from 0.78 to 0.75. Testing this high concentration in three gallbladders, we observed a steady decline in ψ^{mc} and fR^a and a lumen positive shift in ψ^{ms} of 5 mV. R^t changes were not different from responses at lower concentrations. This suggests that the effect of protamine on the cell membrane is dose and/or time dependent. Therefore, for the moment, the safest conclusion is that exposure of the apical membrane to protamine at concentrations of 1 to 100 μg/ml does not appreciably alter cell membrane conductance.

The lack of a significant effect of protamine on apical membrane conductance at micromolar concentrations was also verified by the finding that the relation between extracellular K concentration and apical membrane voltage did not change after protamine (Fig. 4).

This means that even before knowing the morphometric results one could predict that protamine acts mainly on tight junction resistance and has only minor effects on electrical parameters of the cell membranes.

Structural Correlates of the Protamine-Induced Resistance Changes

Interpretations of any changes in tight junction structure, at least in this tissue, is hampered by variation not only between gallbladders, but also within the same gallbladder. For example, considering individual gallbladders under control conditions (n = 4 gallbladders), total junctional depth ranged from 0.30 to 0.58 with a mean of 0.40 μm. Strand number varied from 4.9 to 9.3 with a mean of 6.7. (Compare these values with data presented in Table 3.) After protamine exposure (n = 5 gallbladders), total depth ranged from 0.47 to 0.76 with a mean of 0.65 μm and strand number, from 7.6 to 11.3 with a

mean of 9.7. The increase in strand number and total depth in gallbladders exposed to protamine was still different from control if the number of gallbladders was used as "n," but the level of significance was less, $P < 0.05$ and $P < 0.02$, respectively. After heparin reversal ($n = 6$ gallbladders), the conclusions are not different from that listed on Table 3, only the level of significance is reduced. Dimensions of the compact junction varied less from gallbladder to gallbladder and within the same gallbladder. Seasonal differences were clearly discernible, with shallow junctions found particularly in gallbladders obtained from animals stored during the summer, but since up to threefold differences were observed in strand number and total depth in the same gallbladder, it seemed more accurate to analyze the total longitudinal extent of junctions revealed under the three experimental conditions using all replicas. Data analyzed with this procedure are therefore given in Tables 3 and 4. With this word of caution said, there are still several conclusions which appear well justified:

(i) Morphological changes, specifically an increase in strand number and depth of the compact meshwork, occur within the time frame of both protamine-induced resistance responses and their reversal with heparin.

(ii) The rapidity of these structural alterations suggest that protamine causes new strands to appear below and within the compact junction, either through a recycling process from adjacent cytoplasmic stores (vacuoles or tubule-like structures) or from intramembranous precursors, but not through *de novo* strand synthesis. Observations made during heparin reversal, not only emphasize the dynamic nature of the process, but also point to the region below the compact meshwork as the site of action.

What tight junctional structural feature correlates with the protamine induced conductance change? This question cannot be answered by this study since so little is known of the ionic permeation pathway through the junction or the biochemical and charge properties of the junctional elements. We can only state that resistance changes correlate best (but not perfectly) with structural modifications of the main or compact meshwork. Applying the two-cell, two-strand model of Chalcroft and Bullivant (1970) one could imagine that complementary strands form in adjacent cells but mirror only the structure of the compact meshwork. Strands located below the compact meshwork (perhaps because they are "new") behave as if there is no complementary strand in the adjacent cell, since in this study they had less influence on ionic conductance. As strands are added to the compact meshwork, more regions of intimate cell-to-cell apposition are created, increasing steric and, if strands are charged, electrostatic hindrance to ion movement. This process if fully reversible.

Claude (1978) developed an empirical relationship between strand number and resistance. If we apply her formula to the data of this study, we obtain the following result: The strand number of the compact meshwork changed by average of 1.34 strands after protamine compared to control and heparin reversal. With this change, one would expect that mean resistance would increase by a factor of 3.1. The observed value of the gallbladders used for electron microscopy was 2.1 ± 0.1. Unfortunately, the relationship between incorporation of strands into the tight junctional membrane domain and decreased ionic conductance does not clearly favor one tight junction model over the other (including the lipid model of Kachar and Reese (1982)) or define the functional ionic pathway through the junction. It does, however, give some basis for the conclusion that the correlation between protamine-induced resistance changes and concomitant structural changes is not only apparent but also causal.

A Common Mechanism of Tight Junction Alteration?

Perturbations other than protamine are able to affect both resistance and structure of the tight junction, all in virtually the same manner:

(i) Bentzel et al. (1980) showed in *Necturus* gallbladder that the plant cytokinins, kinetin and zeatin alter both resistance and structure. Two other substances known to be microfilament-active agents, cytochalasin B (1 μM) and phallodin, increase resistance. These authors found clear-cut alterations in appearance of microfilaments (after cytokinins) and concluded that structural changes of the tight junction were mediated via the cytoskeleton. Similar electrical and morphological responses in the gallbladder could be elicted by cAMP (Duffey et al., 1981). On the other hand, removal of calcium from the mucosal bath led to a decrease in strand number and resistance, whereas an increase in intracellular calcium induced by the ionophore A23187 had a biphasic effect but eventually increased resistance (Palant et al., 1983).

(ii) Madara (1983) studied the effect of a steep osmotic gradient in a leaky epithelium, guinea pig jejunum. Cation permeability decreased within 20 min, and at the same time, strand number and meshwork depth increased.

(iii) Martinez-Palomo, Meza, Beaty and Cereijido (1980) demonstrated on confluent layers of ca-

nine kidney tubule cells (MDCK), a decrease in strand number and resistance in the absence of extracellular calcium. From the effect of cytochalasin B on tight structure, these authors concluded that microtubules and microfilaments are involved in the observed structural alterations (Meza et al., 1980). Furthermore, they conclude from the fast onset of structural changes, which were completed within 10 or 15 min, that additional strand particles are not synthesized *de novo*, but may originate from intramembranous precursors (Meza, Sabanero, Stefani & Cereijido, 1982). These "rapid" effects had to be discriminated from effects within hours, which may be due to *de novo* synthesis of strands.

It remains an open question whether all of the above-mentioned "rapid" triggers, including the most rapid to date, protamine, alter resistance and structural changes of the tight junction via a common mechanism and common response. If so, this would mean that the gallbladder and other leaky epithelia may regulate transport by changing paracellular permeability. Not very much can be said on the mechanism of tight junctional alteration now, but protamine might interact with intracellular calcium transfer or affect the cytoskeleton in other ways, perhaps even by disrupting microfilament attachment to the tight junctional membrane domain through electrostatic neutralization of a linking protein.

Most importantly, the tight junction of leaky epithelia (if one can generalize) is able to regulate net transport rates by altering the paracellular leak through mechanisms residing in the cytoplasm. Basically, this process involves incorporation of strands into the main compact meshwork either from submembranous cytoplasmic vesicle stores as described for insertion of intramembranous plaques into the luminal membrane of toad bladder epithelial cells under influence of ADH (Muller, Kachadorian & DiScala, 1980) or by recruiting intramembranous precursors. Both processes could potentially be mediated through the cytoskeleton, but the stimulus for this action and details of the mechanism remain to be determined. We have already provided some evidence for this regulatory feature since protamine decreases tight junctional permeability while increasing net water transport in *Necturus* gallbladder (Fromm et al., 1985). Thus, it is tempting to speculate that, while tight epithelia adjust transport primarily by changes in apical membrane permeability, leaky epithelia are able to adjust net transport by changes in tight junction permeability.

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