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pH dependence of protamine action on apical membrane permeability in *Necturus* gallbladder epithelium

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Protamine reversibly decreases cation permeability and alters the structure of *Necturus* gallbladder tight junctions. Conflicting results, however, have been published whether or not it also affects apical cell membrane permeability. We investigated this issue more systematically by measuring voltage (ψ^{mc}) and fractional resistance (fR^a) of the apical membrane at varying concentrations of protamine, K^+, and H^+ in the bathing solution. At pH 7.6 and [K^+] 2.5 mM, (Poler, M.S. and Reuss, L. (1987) Am. J. Physiol. 253, C662) 6 μM protamine caused ψ^{mc} to depolarize from −58 to −51 mV and fR^a to decrease from 0.74 to 0.67. If we increased pH to 8.1 these effects were even more pronounded. At [K^+] 2.5 mM, but not 4.5 mM, ψ^{mc} transiently hyperpolarized for about 5 min after adding protamine. Most importantly, if [K^+] was 4.5 mM and pH was adjusted to 7.1 (Bentzel et al. (1987) J. Membr. Biol. 95, 9) no significant changes of ψ^{mc} and fR^a occurred. In any case, at a supramaximal concentration of 200 μM, protamine did not further increase the paracellular response but produced decreasing ψ^{mc} and fR^a. We conclude that 6 μM protamine decreases K^+ conductance of the apical membrane, if it is already tuned high by high pH. At low control K^+ conductance as observed at lower pH, protamine action is restricted to the paracellular pathway. Thus, conflicting results were due to different experimental conditions. At a solution pH of 7.1, 6 μM protamine fulfills criteria of a selective tool for reversibly altering structure and function of the tight junction in *Necturus* gallbladder.

Introduction

Protamine, an arginine-rich polycationic protein, reversibly decreases cation permeability [1] and alters the structure of *Necturus* gallbladder tight junctions without major changes of the apical membrane [2]. This would qualify protamine to be an excellent tool for experiments on tight junction regulation.

However, it was also reported, that protamine at very high [3] and also at low concentratins [4] decreases voltage and fractional resistance of the apical cell membrane, which was mainly due to a decrease in K^+ permeability. The paracellular effects of protamine were not questioned by these studies.

Since a simultaneous action on cell membranes and tight junctions would devalue experimental data obtained with protamine we tried to find an explanation for the conflicting results on apical membrane effects of protamine. When the respective experimental protocols were exactly observed, results of both labs proved to be reproducible. Thus, differing experimental conditions had to have caused the incompatible results. The present study demonstrates clearly that at pH 7.1, 6 μM protamine has virtually no side effects on the electrical properties of the apical membrane.

Methods

Gallbladders from *Necturus maculosus* were mounted in Ussing-type chambers and transepithelial voltage (ψ^{ms}), transmembranal voltage (ψ^{mc}), epithelial resistance (R^e), and fractional resistance of the apical membrane (fR^a) were determined exactly as described previously [2].

Briefly, epithelial resistance (R^e) was determined by passing bipolar current pulses (± 50 $\mu A/cm^2$) across the epithelium. For evaluations of R^e and $\Delta\psi^{ms}$ the contributions of the subepithelium and of the fluid located between the voltage sensing electrodes was taken

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TABLE I

Experimental solutions

Solution	K^+ concn.	pH	Gas mixture	Ref.
A	2.5 mmol/l	8.1	100% O_2	
B	2.5 mmol/l	7,6	99% air, 1% CO_2	4
C	2.5 mmol/l	7.1	95% O_2, 5% CO_2	
D	4.5 mmol/l	7.1	95% O_2, 5% CO_2	2

into account. Microelectrodes were filled with 0.5 M KCl [5]. Cell impalements were done with the aid of a piezoelectric micropositioner [6]. Criteria for acceptable impalements were: (i) an abrupt voltage deflection when entering the cell, (ii) less than 10% variation in apical membrane voltage (ψ^{mc}), (iii) less than 5% variation in fractional resistance of the apical membrane (fR^a), (iv) a sudden return to baseline voltage ± 2 mV after withdrawal from the cell, and (v) no significant change in electrode tip resistance after withdrawal from the cell. The fractional resistance of the apical membrane was calculated from $fR^a = \Delta\psi^{mc}/\Delta\psi^{ms}$, where $\Delta\psi^{mc}$ and $\Delta\psi^{ms}$ are the voltage deflections across the apical membrane and the epithelium, respectively, due to the transepithelial current pulses.

The experimental protocol started with a control period, then the mucosal bath was switched to a solution containing 30 μg/ml (≈ 6 μmol/l) protamine, and finally (in order to inactivate protamine) to a solution containing 75 μg/ml heparin. This procedure was carried out using four bathing solutions which differed in K^+ and H^+ concentrations. pH was altered by using different gas mixtures (Table I).

In a separate set of experiments a supramaximal concentration of 1 mg/ml (≈ 200 μmol/l) protamine and 2.5 mg/ml heparin was tested.

Effective protamine concentrations were measured by the method of Bradford [7].

Data are means $\pm$ S.E. Significances were calculated using the paired t-test; $P < 0.05$ was considered significant.

Results

First, what Poler and Reuss [4] suggested was tested, namely that adhesion of protamine to the walls of the chamber and the perfusion system may have caused the differing results. For this, protamine concentrations of a freshly prepared solution were measured in the stock and in the micropuncture chamber. At a nominal concentration of, e.g., 5.8 ± 0.2 μM of the stock solution an effective concentration of 4.6 ± 0.2 μM ($n = 6$) was found in the chamber. As one would expect, this effect was the less pronounced the higher the nominal concentration of protamine was, and *vice versa*.

Table I gives K^+ and H^+ concentrations of the bathing solutions used to test the effect of 6 μM protamine. Solutions B and D were identical to the ones used by Poler and Reuss [4] and Bentzel et al. [2], respectively.

Fig. 1–4 show typical cell punctures using the four different solutions A to D. At [K^+] 2.5 mM and pH 8.1

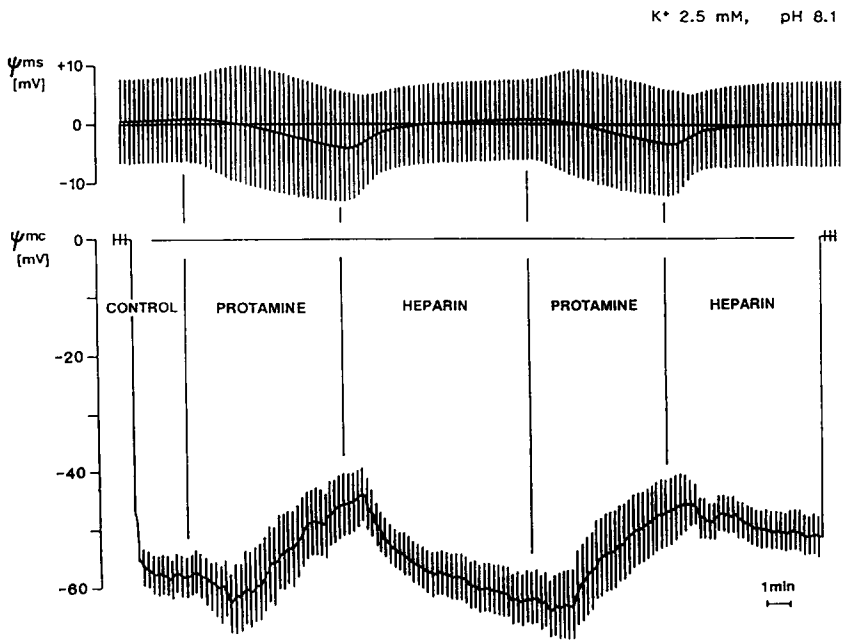

Fig. 1. Typical cell punctures using solution A (pH 8.1, [K^+] = 2.5 mM). Upper trace: transepithelial voltage (ψ^{ms}). The vertical deflections are proportional to transepithelial resistance. Lower trace: Voltage across the apical membrane (ψ^{mc}). The vertical deflections of this trace divided by those of the upper trace represent the fractional resistance of the apical membrane (fR^a).

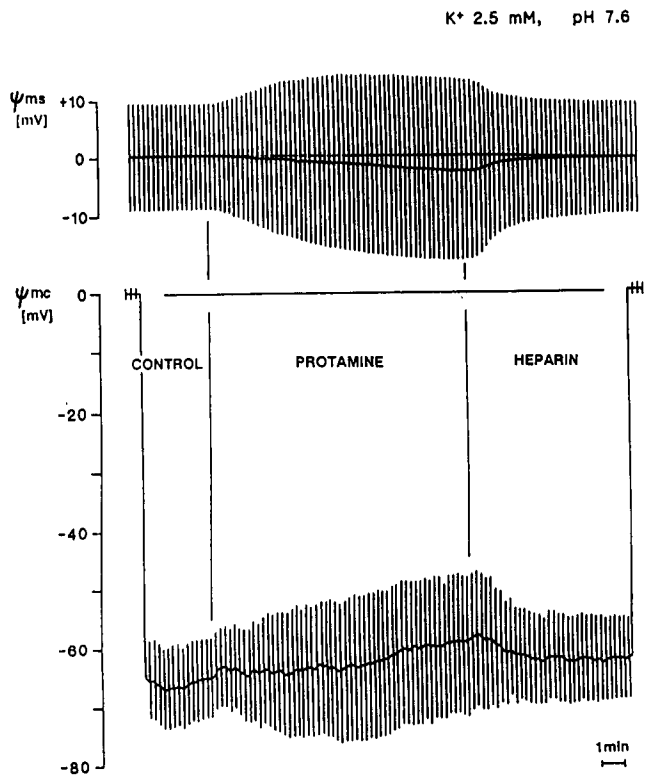

Fig. 2. Typical cell punctures using solution B (pH 7.6, $[K^+] = 2.5$ mM).

(Fig. 1) protamine produces lumen-negative ψ^{ms} and a pronounced decrease of ψ^{mc} and fR^a. At $[K^+]$ 2.5 mM and pH 7.6 (Fig. 2) protamine produces lumen-negative ψ^{ms} and a decrease of ψ^{mc} and fR^a. At $[K^+]$ 2.5 mM and pH 7.1 (Fig. 3) protamine produces slightly lumen-negative ψ^{ms} in most experiments and, after initial hyperpolarization of ψ^{mc}, small changes of ψ^{mc} and fR^a. At $[K^+]$ 4.5 mM and pH 7.1 (Fig. 4) protamine produces slightly lumen-negative ψ^{ms} and no changes of ψ^{mc} and fR^a.

Numerical results using different K^+ and H^+ concentrations are compiled in Table II. Epithelial resistance (R^e) was increased under all experimental conditions by between 51% and 78%. Protamine-induced changes of ψ^{ms} ($\Delta\psi^{ms}$) were correlated with changes in ψ^{mc} ($\Delta\psi^{mc}$, Fig. 5) and $\Delta\psi^{mc}$ was correlated with solution pH (Fig. 6). A similar correlation existed between ΔfR^a and pH. Thus, the lower the bath pH, the smaller were $\Delta\psi^{ms}$, $\Delta\psi^{mc}$ and ΔfR^a. Using solution D ($[K^+]$ 4.5 mM, pH 7.1) both membrane parameters, ψ^{mc} and fR^a, were not significantly altered (Table II).

In a preliminary publication Poler and Reuss [3] reported membrane effects after 200 μM protamine. In order to test this, we first added 6 μM and, after inactivation with heparin, 200 μM protamine. Punctures were performed using solution D. Fig. 7 shows that after 6 μM protamine ψ^{mc} and fR^a did not change, whereas after 200 μM protamine both membrane parameters dramatically decreased. Since this supramaximal dosage did not produce a larger effect on R^e than 6 μM (Table III) its general use cannot be recommended.

Discussion

The contrasting results of both labs cannot be explained by differing effective concentrations of protamine due to adhesion to glass walls, since adhesion

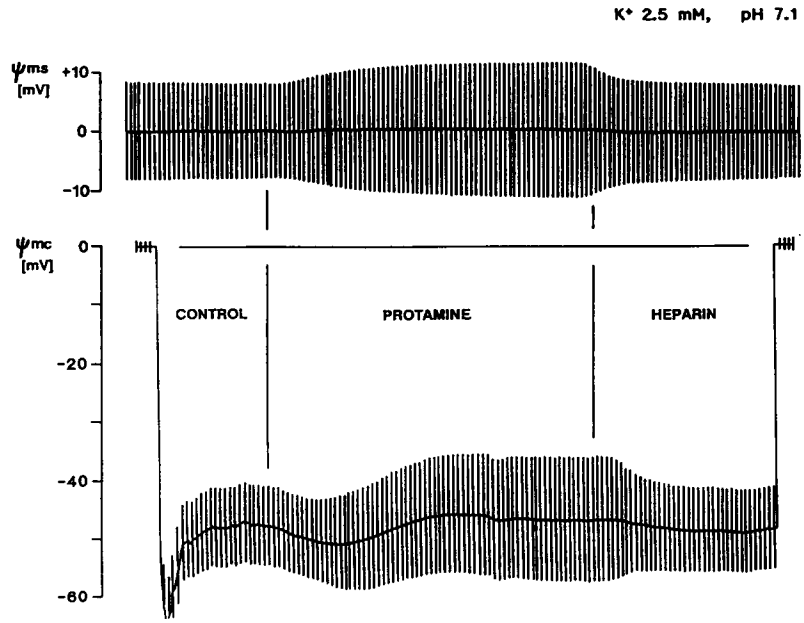

Fig. 3. Typical cell punctures using solution C (pH 7.1, $[K^+] = 2.5$ mM).

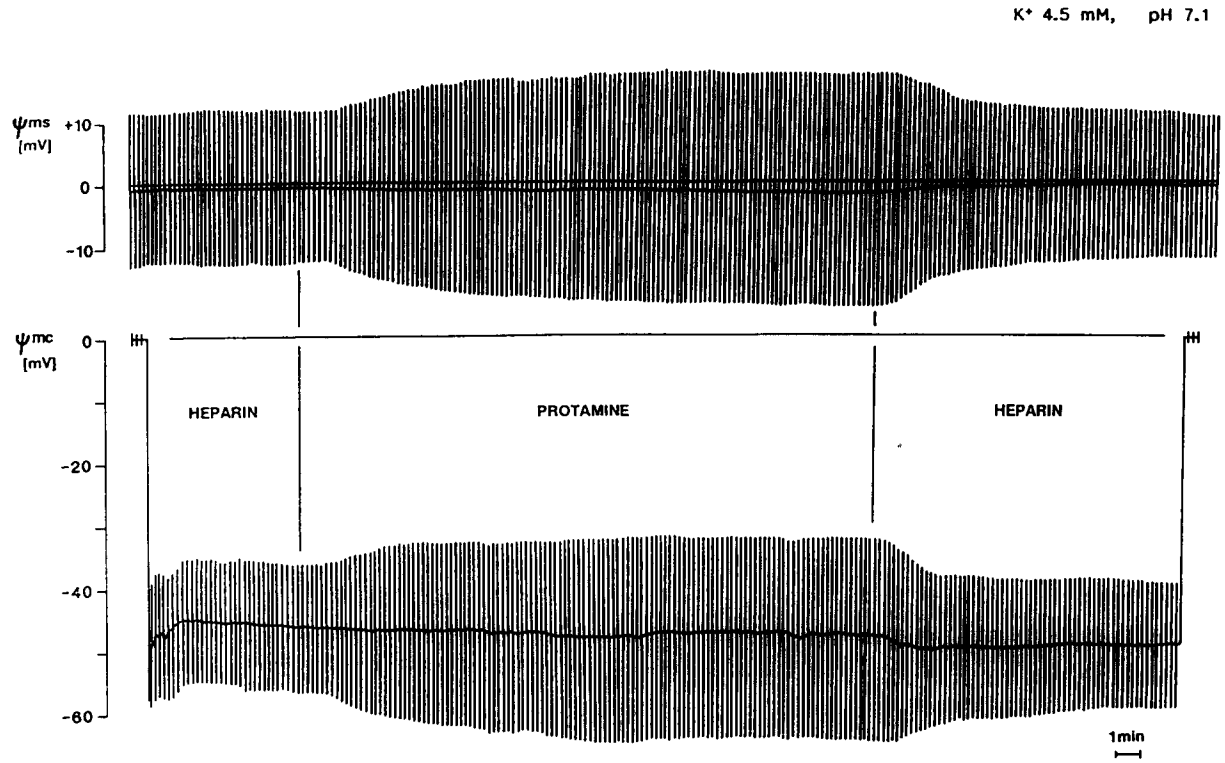

Fig. 4. Typical cell punctures using solution D (pH 7.1, $[K^+] = 4.5$ mM).

may have caused the concentration to drop by 20% at maximum. However, neither increasing nominal protamine concentration by 60% nor a decrease by 100% caused changes in cell puncture parameters (data not shown) or transepithelial resistance [1]. In addition, adhesion may have happened to some extent in experiments of both groups.

To our surprise, all findings of the two labs could

TABLE II

Effects of 6 μM protamine at different K^+ and H^+ bath concentrations

P versus control: ***, ⩽0.0005; **, ⩽0.005; *, ⩽0.05; n.s., not significant.

Solutions			*n*	R^e ($\Omega \cdot cm^2$)		ψ^{ms} (mV)		ψ^{mc} (mV)		fR^a	
	$[K^+]$	pH		control	protamine	control	protamine	control	protamine	control	protamine
A	2.5	8.1	9	113± 6	171±16***	−0.6±0.2	−4.8±0.6***	−62±2	−52±2***	0.60±0.07	0.47±0.07**
B	2.5	7.6	16	118± 5	191±11***	−0.9±0.4	−3.5±0.6**	−58±1	−51±1***	0.74±0.06	0.67±0.07**
C	2.5	7.1	16	135± 9	240±19***	−0.4±0.3	−1.3±0.4*	−52±1	−50±1 n.s.	0.89±0.02	0.85±0.02 n.s.
D	4.5	7.1	7	155±18	244±29***	−0.4±0.2	−1.1±0.1 n.s.	−48±1	−49±1 n.s.	0.95±0.01	0.96±0.01 n.s.

TABLE III

Effect of 6 μM and 200 μM protamine using solution D

P versus control: ***, ⩽0.0005; **, ⩽0.005; n.s., not significant.

n = 13	R^e ($\Omega \cdot cm^2$)	ψ^{ms} (mV)	ψ^{mc} (mV)	fR^a
Control	154±10	+1.0±0.2	−53.0±0.9	0.92±0.02
Protamine 6 μM	238±15***	+0.7±0.2 n.s.	−52.5±1.1 n.s.	0.91±0.02 n.s.
Heparin	155± 8 n.s.	+1.0±0.2 n.s.	−54.2±0.9 n.s.	0.94±0.02 n.s.
Protamine 200 μM	220±19**	−1.6±0.4***	−40.0±2.3***	0.77±0.03***
Heparin	157±10 n.s.	+0.8±0.2 n.s.	−50.2±2.0 n.s.	0.88±0.04 n.s.

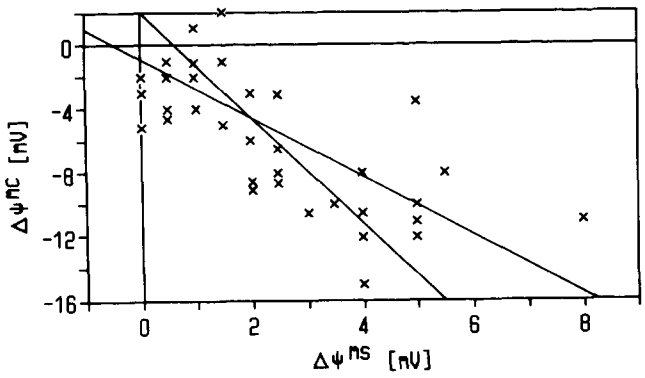

Fig. 5. Protamine-induced changes of ψ^{ms} ($\Delta\psi^{ms}$) plotted against changes in ψ^{mc} ($\Delta\psi^{mc}$). Linear regression using either Y or X as a dependent variable yielded $Y = -0.79 - 1.8 \cdot X$ and $X = 0.65 - 0.31 \cdot Y$ ($r^2 = 0.57$), respectively).

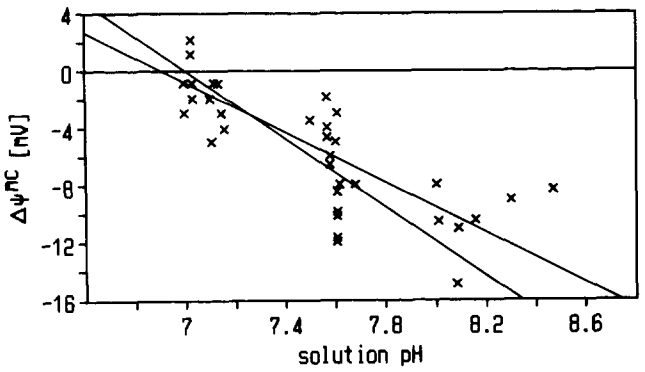

Fig. 6. Protamine-induced ψ^{mc} changes ($\Delta\psi^{mc}$) plotted against solution pH. Linear regression using either Y or X as a dependent variable yielded $Y = 60.6 - 8.8 \cdot X$ and $X = 7.07 - 0.079 \cdot Y$ ($r^2 = 0.70$), respectively.

definitely be confirmed. Conflicting results were demonstrated to be due exclusively to different experimental conditions: Protamine alters apical membrane conductance if the pH of a bath solution with $[K^+]$ 2.5 mM was adjusted to $\geqslant 7.6$ (solution B; [4]) but does not affect the apical membrane at pH 7.1 and $[K^+]$ 4.5 mmol/l (solution D; [2]).

The protamine-induced decrease in ψ^{mc} and fR^a (at pH 7.65) were shown by Poler and Reuss [4] to be due to a (smaller) decrease in K^+ conductance combined with a (larger) increase in Cl^- conductance. They concluded that the decrease of apical K^+ conductance is the initial effect, followed by secondary actions on Cl^- conductance mediated by intracellular events. If Cl^- conductance changes are secondary, above all the changes in K^+ conductance and their dependence on bath pH deserve an explanation.

It has been worked out previously that apical K^+ conductance is positively correlated with luminal bath pH [8,9]. The reduction of apical K^+ permeability produced by luminal acidification was explained (i) by non-specific titration of membrane fixed negative charges, and (ii) by an effect of luminal H^+ activity on the apical K^+ channel [8].

Knowing this, it is suggestive that at high solution pH protamine decreases apical K^+ conductance by virtue of its positive charge. In turn, at low solution pH apical K^+ conductance is tuned low already and protamine does not further decrease that conductance.

On a first view, a bath solution of pH 7.1 may appear unphysiologically low, although pH 7.6 also slightly departs from a blood pH of 7.4. However, for the

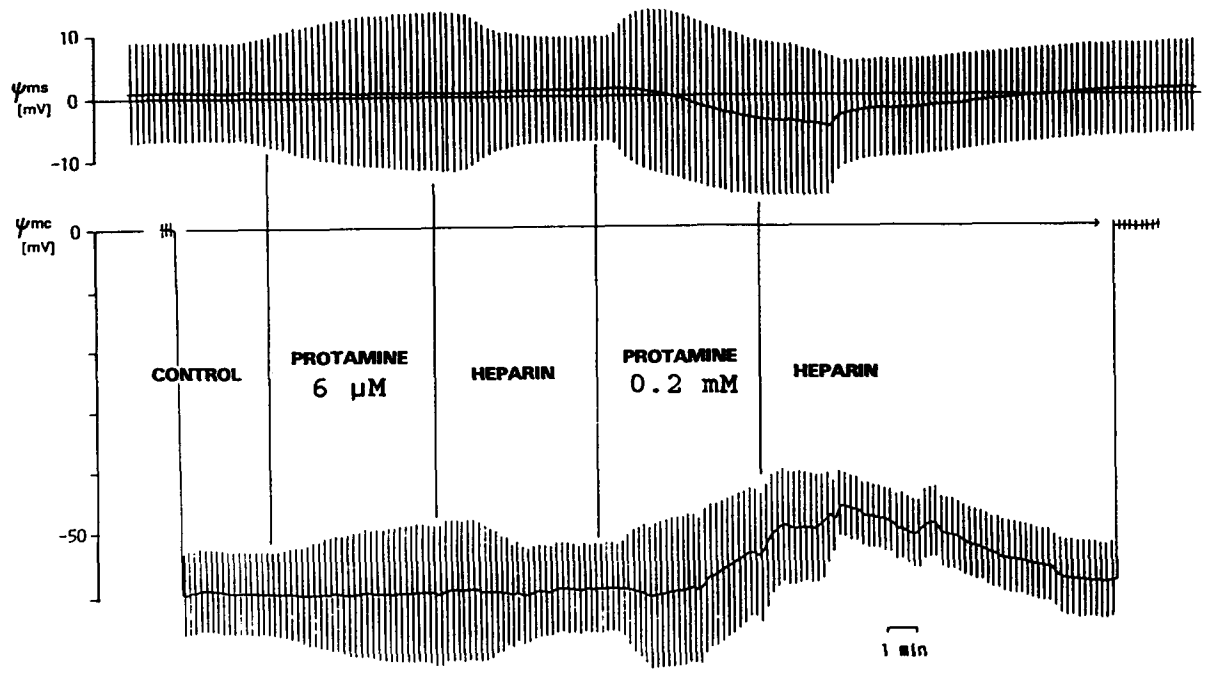

Fig. 7. Typical cell punctures using solution D and 6 μM or 200 μM protamine, respectively.

luminal side of a gallbladder even lower pH of 6.2 (own measurement on pooled fluid of 8 bladders) are physiologic, so that pH 7.1 may be a reasonable compromise. Although these pH variations per se caused changes in tight junction permeability ([10]; see also Table II, R^e), the action of protamine on R^e was not systematically influenced. This was not to be expected since the pI of protamine is much higher (≈ 11).

The transient ψ^{mc} hyperpolarization as seen by Poler and Reuss [4] was due to a second difference in bathing solutions, namely the lower K^+ concentration they used (2.5 mM as compared to 4.5 mM in our studies).

ψ^{ms} changes after protamine as observed at higher pH were attributed by Poler and Reuss [4] to concomitant ψ^{mc} changes. This was confirmed by the present study (Fig. 3).

In conclusion, at pH 7.1 and [K^+] 4.5 mmol/l the action of 6 μM protamine in Necturus gallbladder epithelium is restricted to the paracellular pathway. Under this condition, protamine can serve as a selective tool for intracellularly mediated experimental tight junction regulation in *Necturus* gallbladder.

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