

Amiloride-sensitive Trypsinization of Apical Sodium Channels

Analysis of Hormonal Regulation of Sodium Transport in Toad Bladder

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ABSTRACT Incubation of the mucosal surface of the toad urinary bladder with trypsin (1 mg/ml) irreversibly decreased the short-circuit current to 50% of the initial value. This decrease was accompanied by a proportionate decrease in apical Na permeability, estimated from the change in amiloride-sensitive resistance in depolarized preparations. In contrast, the paracellular resistance was unaffected by trypsinization. Amiloride, a specific blocker of the apical Na channels, prevented inactivation by trypsin. Inhibition of Na transport by substitution of mucosal Na, however, had no effect on the response to trypsin. Trypsinization of the apical membrane was also used to study regulation of Na transport by anti-diuretic hormone (ADH) and aldosterone. Prior exposure of the apical surface to trypsin did not reduce the response to ADH, which indicates that the ADH-induced Na channels were inaccessible to trypsin before addition of the hormone. On the other hand, stimulation of short-circuit current by aldosterone or pyruvate (added to substrate-depleted, aldosterone-repleted bladders) was substantially reduced by prior trypsinization of the apical surface. Thus, the increase in apical Na permeability elicited by aldosterone or substrate involves activation of Na channels that are continuously present in the apical membrane in nonconductive but trypsin-sensitive forms.

INTRODUCTION

Transepithelial Na transport by the toad urinary bladder is considered to be a two-step process: according to this model, Na diffuses passively from the lumen across the apical membrane, via amiloride-inhibitable Na channels, and is actively extruded into the interstitial space by the Na pump (1, 2). Many studies have implicated regulation of apical Na permeability as a key

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step in hormone action on epithelia. For instance, hormones that stimulate transepithelial Na transport, such as aldosterone and anti-diuretic hormone (ADH),¹ increase the estimated rate of Na uptake from the lumen (2-6).

In a recent study on toad bladder, Palmer et al. (7) showed that raising the serosal K activity depolarizes the basolateral membrane, apparently completely, thereby allowing estimation of the apical Na permeability from transepithelial measurements. This preparation was used to study the effects of aldosterone, ADH, and pyruvate on I_{Na} by analysis of current-voltage relations and current fluctuation (6, 8). In all three cases, proportionate increases in I_{Na} and P_{Na} were elicited. The aldosterone- and ADH-dependent increases in P_{Na} were the result of equivalent increases in the population of Na-conducting channels with no significant effect on the single-channel transport rates (6, 8). The fact that aldosterone and ADH have similar effects on P_{Na} , both quantitatively and qualitatively, raises the possibility that the two hormones share the final steps in activating Na channels, i.e., the hormones recruit active channels from the same pool of electrically silent precursors.

In this paper, we describe irreversible decreases in I_{Na} induced by trypsinizing the apical surface. This effect was prevented if amiloride was added to the mucosal solution before exposure to the enzyme. The ability of trypsin to block Na channels irreversibly was used to study hormone effects: 50% inhibition of the baseline current had no effect on the response to ADH. In contrast, pretreatment with trypsin reduced the baseline current and the response to aldosterone or pyruvate proportionately. These results imply the existence of two spatially distinct precursors of the Na channels under differential control by ADH and aldosterone.

MATERIALS AND METHODS

Toads (*Bufo marinus*, Dominican Republic origin) were obtained from National Reagents (Bridgeport, CT) and kept partially submerged in tap water for at least 3 d before use. The animals were double-pithed and the urinary bladders were excised and mounted as flat sheets in a double chamber. With one hemibladder mounted across such a double chamber, one quarter served as the tested tissue and the other as control. The exposed cross-sectional area was 2 cm²/quarter-bladder. The hemibladders were supported by nylon mesh from the serosal side and silicon grease was used to minimize edge damage. The mucosal and serosal compartments had volumes of 13 and 4.5 ml, respectively, and were stirred by aeration. A hydrostatic pressure difference of 5 cm H₂O was used to hold the bladder against the mesh. Electrical connections were made through agar bridges to Ag/AgCl (current) or calomel (voltage) electrodes. Short-circuit current and transepithelial resistance (I_{sc} and R) were measured as described previously (9). Substitutions of the mucosal Ringer's were done by perfusing the mucosal compartment with the new solution for 5 min at a rate of 30 ml/min. This exchange procedure ensures complete replacement

¹ Abbreviations used in this paper: ADH, anti-diuretic hormone (in this study, vasopressin was used); DSA, diazosulfanilic acid; I_{sc} , short-circuit current; I_{sc0} , short-circuit current at time zero; P_{Na} , apical sodium permeability; R , transepithelial electrical resistance; R_{Na} , sodium-specific (amiloride-sensitive) resistance; R_s , paracellular (shunt) resistance; TAME, *p*-toluenesulfonyl-L-arginine methyl ester.

with the substitute solution and was not accompanied by any significant perturbation in I_{sc} or R . At higher flow rates, however, I_{sc} tended to increase and R to decrease, presumably because of mechanical stretch effects.

The serosal solutions consisted either of standard NaCl Ringer's containing (in mM): 110 NaCl, 1 CaCl₂, 0.5 MgCl₂, 3.5 K-phosphate (adjusted to pH 7.5), and 5 pyruvate (unless otherwise indicated), or high-K sucrose Ringer's in which all of the NaCl was replaced by 85 mM KCl plus 50 mM sucrose (7). The mucosal solutions consisted either of NaCl Ringer's (composition as described above) or Na-free Ringer's in which NaCl was replaced by 110 mM KCl, or Na₂SO₄ Ringer's containing (in mM): 18.2 Na₂SO₄, 36.3 K₂SO₄, 1 Ca gluconate, and 5 K-phosphate (pH 7.5). The Na activity in Na₂SO₄ Ringer's is 20 mM.

To ensure that variations in the effects of trypsin on I_{sc} or R were not a consequence of changes in the compositions of the mucosal solutions, proteolytic potency of trypsin was assayed by measuring the hydrolysis of *p*-toluenesulfonyl-L-arginine methyl ester (TAME) as described in reference 10. The reaction mixture consisted either of 40 mM Tris, 10 mM CaCl₂, and 1 mM TAME, or one of the above-described mucosal solutions (i.e., NaCl Ringer's, Na-free Ringer's, or Na₂SO₄ Ringer's) plus 1 mM TAME. In the presence of 0.15 μ g/ml enzyme the absorption changes were linear for at least 3 min.

Statistics

All data are expressed as mean \pm SEM and the probabilities were calculated using the paired *t* test.

Materials

Trypsin (thrice-crystallized, 180–220 TAME U/mg) was obtained from Worthington Biochemical Corp., Freehold, NJ. Soybean trypsin inhibitor (type I-S), TAME HCl, and *d*-aldosterone were obtained from Sigma Chemical Co., St. Louis, MO. Vasopressin (20 pressor units/ml) was obtained from Parke, Davis, & Co., Detroit, MI, and lyophilized penicillin-streptomycin mixture was obtained from Gibco Laboratories, Grand Island, NY. All conventional reagents were of analytical grade.

RESULTS

Trypsin Effects on I_{sc} and R

The action of trypsin was assessed by adding the enzyme (final concentration = 1 mg/ml) to the mucosal media of pairs of quarter-bladders; one of each pair was pretreated with soybean trypsin inhibitor (final concentration = 0.5 mg/ml). The enzyme evoked a monotonic decrease in I_{sc} to 50% of the pretreatment value at 40 min (Fig. 1). The quarter-bladders protected by trypsin inhibitor exhibited no significant change in I_{sc} for up to 75 min. Addition of the trypsin inhibitor (0.5 mg/ml) to the mucosal media of the experimental quarter-bladders at 40 min prevented any further fall in I_{sc} and was attended by a limited reversal in the response (i.e., a rebound to 60% of the pretreatment control at 80 min). The further addition of amiloride (80 μ M) to the mucosal media at 80 min completely inhibited the residual I_{sc} (data not shown). This finding implies that the conducting Na channels retained the amiloride-binding site. In these experiments the mucosal and serosal compartments contained identical solutions. Thus, the decrease in I_{sc}

must indicate a corresponding decrease in Na flux across the epithelial cells, presumably caused by proteolytic digestion of a component of the mucosal Na channels. The slow partial recovery observed after blocking the enzyme with the trypsin inhibitor may reflect an activation process triggered by the decreased Na uptake from the mucosal side (11, 12).

Table I summarizes the effects of exposure to mucosal trypsin for 1 h on I_{sc} and R : the enzyme decreased I_{sc} by $50 \pm 2\%$, accompanied by a small but

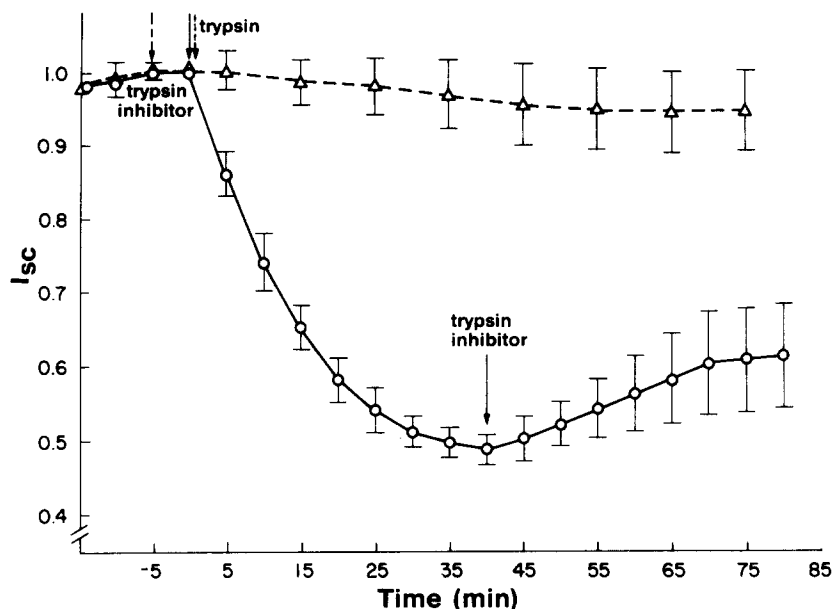


FIGURE 1. The effect of trypsin on I_{sc} . Pairs of quarter-bladders were equilibrated in NaCl Ringer's (both mucosal and serosal) until I_{sc} reached near-steady values (2–4 h). Soybean trypsin inhibitor (0.5 mg/ml) was added to the mucosal side of one quarter-bladder and 5 min later ($t = 0$) trypsin (1 mg/ml) was added to the mucosal media of both quarter-bladders. At 40 min, soybean trypsin inhibitor (0.5 mg/ml) was added to the experimental quarter-hemibladder. Both the enzyme and inhibitor were added from 100-fold-concentrated solutions in NaCl Ringer's. I_{sc} was measured every 5–10 min (and maintained open-circuited between measurements) and the fractional change I_{sc}/I_{sc_0} is plotted vs. time. The vertical bars are the standard errors of the means. $n = 7$ pairs of quarter-bladders. I_{sc_0} was 20.5 ± 3.0 and $21.7 \pm 3.5 \mu\text{A}/\text{cm}^2$ in the trypsinized and control bladders, respectively.

significant increase in mean resistance ($P < 0.001$). The control quarter-bladders did not show any significant changes from the initial values ($P > 0.1$). From time to time, quarter-bladders proved to be almost completely resistant to trypsin-mediated inhibition of I_{sc} (~ 1 in seven toad bladders). These bladders were not used in any of the reported experiments. In some of these experiments reversibility of the changes in I_{sc} was assessed either by blocking trypsin action with soybean trypsin inhibitor ($n = 10$) or by substi-

tuting with enzyme-free mucosal solutions ($n = 12$). The rebound increase in I_{sc} was about the same, regardless of the method used to stop the enzyme reaction. For the combined groups ($n = 22$), the ratio I_{sc}/I_{sc_0} increased from 0.52 ± 0.02 to 0.62 ± 0.06 at the end of 1 h after termination of trypsinization.

In contrast to the marked decrease in I_{sc} , the change in R with trypsinization was limited. Since the total resistance is made up of at least three components, the apical membrane, the basolateral membrane, and the paracellular pathway, the change in R may be small because only one of these components, the apical site, may be subject to proteolytic attack. To distinguish between effects on the apical, basolateral, and paracellular resistances, we measured I_{sc}/I_{sc_0} and R/R_0 in trypsinized, K-depolarized bladders. The high serosal K in this preparation causes large decreases in the basolateral resistance; under these

TABLE I
EFFECTS OF TRYPSIN ON I_{sc} AND R

(A) NaCl Ringer's	Initial values		Fractional change		Number of bladders
	I_{sc_0}	R_0	I_{sc}/I_{sc_0}	R/R_0	
Trypsinized	28.8 ± 3.0	1.25 ± 0.09	0.5 ± 0.02	1.1 ± 0.02	36
Control	28.4 ± 3.5	1.34 ± 0.09	1.0 ± 0.03	0.95 ± 0.03	36

(B) K-sucrose Ringer's	Initial values			Fractional change			Number of bladders
	I_{Na}	R_{Na}	R_s	I_{Na}/I_{Na_0}	R_{Na}/R_{Na_0}	R_s/R_{s_0}	
	29.0 ± 4.8	1.61 ± 0.27	3.12 ± 1.04	0.4 ± 0.04	2.7 ± 0.3	0.9 ± 0.06	8

(A) The experimental protocol is as shown in Fig. 1. The control quarter-bladders received either trypsin (1 mg/ml) plus soybean trypsin inhibitor (0.5 mg/ml) ($n = 15$ pairs) or had no additions to the mucosal media ($n = 21$ pairs). The effects were measured 1 h after incubation with the enzyme.

(B) Quarter-bladders were incubated in Na_2SO_4 Ringer's (mucosal) and K-sucrose Ringer's (serosal). The effects of trypsin of I_{sc} and R were assayed for 1 h. The Na-specific values of these parameters were evaluated by adding amiloride (80 μM) to the mucosal medium before and 1 h after the addition of trypsin (1 mg/ml). The units were: $I_{sc} = \mu\text{A}/\text{cm}^2$; $R = \text{K}\Omega \cdot \text{cm}^2$.

conditions the sodium-specific apical resistance (R_{Na}) can be estimated from the difference between R and R_s (7). Thus, R_s was measured before and after trypsinization by blocking Na channels with 80 μM amiloride. In these depolarized bladders, 1 h of incubation with trypsin caused a 2.7 ± 0.3 -fold increase in R_{Na}/R_{Na_0} and had no significant effect ($P > 0.1$) on R_s/R_{s_0} (Table I, part B). This result indicates that proteolytic digestion affected I_{sc} by decreasing P_{Na} , probably because of inactivation of Na channels, and that the paracellular shunt does not open under these experimental conditions. This inference is based on the quantitative relationship between the reciprocal of R_{Na}/R_{Na_0} and I_{sc}/I_{sc_0} , both of which are ~ 0.4 (Table I, part B).

The use of a serosally depolarized preparation to evaluate effects of reagents such as trypsin on apical conductivity depends on the expectation that serosal depolarization will not in itself alter the properties of the apical boundary. That this is indeed the case is indicated by the findings that the magnitudes

and time courses of the I_{sc} responses to hormones (aldosterone, ADH), metabolic substrates (glucose, pyruvate), and the apical reagent (amiloride) under depolarizing conditions are all indistinguishable from those obtained with standard NaCl Ringer's solution (6, 7, 8).

Effects of Amiloride and Na-free Media

The specificity of the proteolytic attack on the Na channel protein(s) was assessed by evaluating the degree of protection afforded by pretreatment with amiloride. Two protocols were used: in the first, one of each pair of quarter-bladders was exposed to 30 μ M amiloride, and 2 min later both quarter-bladders received 1 mg/ml trypsin (Fig. 2). When I_{sc} reached a new steady

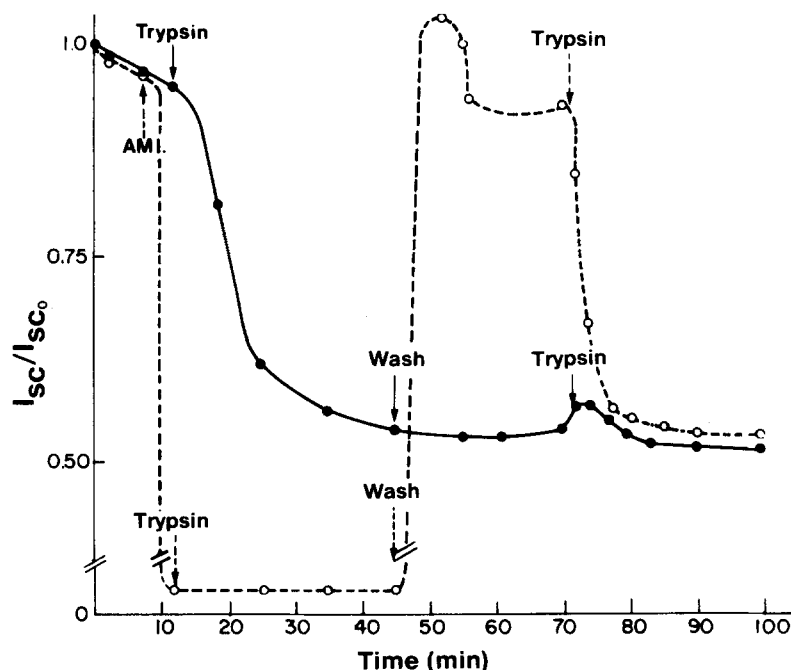


FIGURE 2. Protective effect of amiloride on inhibition of I_{sc} by trypsin. Paired quarter-bladders were mounted and equilibrated in NaCl Ringer's (both mucosal and serosal). Amiloride (AMI) (30 μ M) was added to the mucosal side of one quarter-bladder, and 2 min later both quarter-bladders received trypsin, 1 mg/ml (mucosal). After an additional 45 min, trypsin was removed from the mucosal compartments by perfusion for 5 min with NaCl Ringer's and when the steady-state I_{sc} was re-established, a second addition of trypsin was made. (One representative experiment.)

value, amiloride and trypsin were removed from the mucosal compartment by perfusing it with NaCl Ringer's for 5 min. This wash had little effect on the quarter-bladder that received trypsin only. In contrast, in the amiloride-treated quarter-bladder, removal of amiloride and trypsin restored I_{sc} to the pretreatment value, which indicates that amiloride protected against the

attack by trypsin. A second addition of trypsin was made 30 min after the washout. This maneuver had almost no effect on the I_{sc} of the quarter-bladder that received trypsin alone (control), but decreased the I_{sc} of the quarter-bladder that was previously exposed to trypsin in the presence of amiloride to the same value as that of the control.

In the second set of experiments, the following additions were made in four quarter-bladders harvested from the same toad (in each case): (a) trypsin alone; (b) trypsin plus amiloride; (c) amiloride alone; (d) diluent alone. In the quarter-bladders that received amiloride, with or without trypsin, I_{sc} decreased immediately to 0.13 ± 0.01 of the initial value. 1 h after the additions, the mucosal compartments were perfused with NaCl Ringer's, and I_{sc} was re-measured. Trypsin inhibited I_{sc} to 0.56 ± 0.06 of the initial value (Table II). On the other hand, incubation with amiloride plus trypsin had no effect on the post-washout I_{sc} , as compared with either amiloride alone or diluent.

TABLE II
INHIBITION OF THE ACTION OF TRYPSIN BY AMILORIDE

Treatment	I_{sc_0}	Fractional change	Number of quarter-bladders
(A) Trypsin	24.7±5.8	0.56±0.06	11
(B) Trypsin + amiloride	29.5±10.2	1.0±0.13	11
(C) Amiloride	39.8±13.9	1.14±0.10	8
(D) Diluent	15.8±3.0	1.0±0.10	8

Four quarter-bladders from each toad were incubated simultaneously with (A) trypsin (1 mg/ml), (B) trypsin (1 mg/ml) plus amiloride (30 μ M), (C) amiloride (30 μ M), or (D) diluent on the mucosal side. 1 h later all four mucosal compartments were perfused with NaCl Ringer's solution to remove all of the reagents ($n = 8$). In three other experiments only conditions A and B applied. The fractional change in I_{sc} is the ratio of the I_{sc} measured after perfusion to that measured before the additions. The I_{sc_0} (μ A/cm²) is the I_{sc} value measured immediately before the additions.

To ensure that amiloride does not prevent the decrease in I_{sc} by inactivating trypsin directly, we assayed enzyme activity in the presence and absence of 30 μ M amiloride. In the various mucosal solutions (i.e., NaCl, Na₂SO₄, Na-free Ringer's) the enzymatic activity varied from one-third to two-thirds of that in the reference buffer, Tris-CaCl₂ (Table III). Amiloride, however, had no effect on trypsin activity assayed either in the Tris-CaCl₂ or in NaCl Ringer's solutions.

The ability of amiloride to protect Na channels from trypsin digestion can be explained in one of two ways: (a) amiloride directly either masks the cleavage site or induces a conformational change that in turn masks the cleavage site; or (b) the abolition of Na transport by amiloride in turn protects Na channels from trypsin (e.g., because of changes in local Na concentration or membrane potential).

To distinguish between these possibilities, we examined the effect of trypsin on bladders in which Na transport was abolished by substituting Na-free Ringer's for NaCl Ringer's on the mucosal side. In Na-free mucosal solutions,

TABLE III
 ENZYME ACTIVITY IN VARIOUS RINGER'S SOLUTIONS

Medium	Trypsin activity	Number of assays
Tris-CaCl ₂ solution	220±11	13
Tris-CaCl ₂ solution + amiloride	215±6.5	5
NaCl Ringer's	83.1±6.5	5
NaCl Ringer's + amiloride	92.9±4.3	5
Na-free Ringer's	73.4±6.5	5
Na ₂ SO ₄ Ringer's	166.2±2.5	4

Enzyme activity was assayed as described in Materials and Methods. The reaction mixture was one of the above media plus 1 mM TAME plus 30 μ M amiloride or an equal volume of diluent (water). Trypsin (1 mg/ml) was preincubated for 30 min with amiloride (30 μ M) or the diluent at room temperature. The incubation mixture was diluted at time zero with the reaction mixture, which then contained (final concentration) TAME (1 mM), trypsin (0.1–0.3 μ g/ml), \pm 30 μ M amiloride. Three different batches of trypsin were assayed. Activity was expressed as units of TAME per milligram of trypsin.

trypsin reduced I_{bc} to 0.79 ± 0.07 of the initial current, a value that differs significantly from the fractional change measured in the presence of Na (0.48 ± 0.05 ; $P < 0.01$) (Table IV). Incubation in Na-free mucosal solutions alone, however, increased I_{bc} by 40%. Thus, the fractional changes after exposure to trypsin were normalized to the control ratios that gave the following results: trypsinization in the presence of Na decreased the normalized current to 0.61 ± 0.15 ($n = 8$) and in the absence of Na to 0.64 ± 0.08 ($n = 11$). These results imply that amiloride-dependent inactivation of the effect of trypsin on P_{Na} is not secondary to abolition of Na transport.

Method of Analysis of Hormonal Effects in Trypsinized Bladders

The ability of trypsin to block irreversibly apical Na channels was used to distinguish between various possible mechanisms for activating Na channels by aldosterone, ADH, and metabolic substrates. The experimental protocol used in these experiments is shown in Fig. 3. Trypsin was added to the mucosal

TABLE IV
 EFFECT OF MUCOSAL Na ON THE RESPONSE TO TRYPSIN

Mucosal medium	I_{bc0}	Fractional change	Number of quarter-bladders
NaCl Ringer's	31.9±6.3	1.10±0.17	8
NaCl Ringer's + trypsin	33.8±4.7	0.48±0.05	8
Na free-Ringer's	32.7±4.4	1.39±0.13	11
Na free-Ringer's + trypsin	30.8±4.0	0.79±0.07	11

Four quarter-bladders from the same toad were perfused for 5 min in either NaCl Ringer's or Na-free Ringer's on the mucosal side. Trypsin (1 mg/ml) was added to pairs of quarter-bladders, one of which was perfused in NaCl Ringer's and the other in Na-free Ringer's. 1 h later all of the quarter-bladders were perfused with NaCl Ringer's ($n = 8$). In three other experiments only Na-free Ringer's was used. The fractional change in current is the ratio of I_{bc} measured after the second perfusion to that obtained before the first perfusion. I_{bc0} (μ A/cm²) was measured just before the first perfusion.

side of one of the paired quarter-bladders, and after I_{sc} decreased to $\sim 50\%$ of the initial value, the enzyme was either inhibited with soybean trypsin inhibitor or removed by perfusing the mucosal compartment with NaCl Ringer's (or both). The pairs of quarters-bladders were then challenged with ADH, aldosterone, or pyruvate (added to the serosal compartment), and the response was monitored until maximal stimulation of I_{sc} was established.

To compare the responses of the control and trypsinized quarter-bladders, two parameters were calculated: (a) the fractional change in current, I_{sc2}/I_{sc1} , and (b) the normalized absolute increment in current $(I_{sc2} - I_{sc1})/I_{sc0}$.

In the analysis of the responses of the control and trypsinized bladders to aldosterone, ADH, and pyruvate, we considered two possible models: (I) The hormone-induced channels are present in the apical membrane in inactive

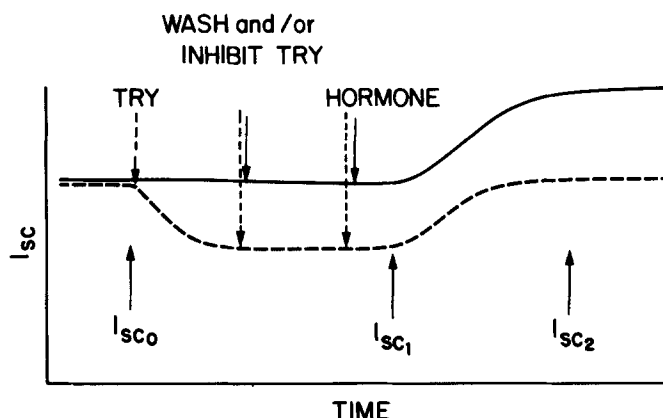


FIGURE 3. Schematic representation of the protocol used to compare the responses of control and trypsinized (TRY) quarter-bladders to ADH, aldosterone, and pyruvate. I_{sc0} denotes the current at the time of addition of trypsin to the mucosal medium, I_{sc1} that just before the beginning of the response to the stimulants, and I_{sc2} at the maximum of the response.

form before the addition of hormone or substrates and are subject to the same extent of trypsin inactivation as the baseline active channels. In this case, $I_{sc2}^{try}/I_{sc1}^{try}$ should be equal to $I_{sc2}^{cont}/I_{sc1}^{cont}$, but $(I_{sc2}^{try} - I_{sc1}^{try})/I_{sc0}^{try}$ should be smaller than $(I_{sc2}^{cont} - I_{sc1}^{cont})/I_{sc0}^{cont}$ in proportion to $I_{sc1}^{try}/I_{sc0}^{try}$ (providing that $I_{sc0}^{cont} = I_{sc1}^{cont}$). (II) The hormone-induced channels are completely protected from trypsinization (before the addition of hormone), i.e., either they are not present in the membrane or they exist in a trypsin-insensitive conformation. In this case, $(I_{sc2}^{try} - I_{sc1}^{try})/I_{sc0}^{try}$ should be equal to $(I_{sc2}^{cont} - I_{sc1}^{cont})/I_{sc0}^{cont}$, but $I_{sc2}^{try}/I_{sc1}^{try}$ would be larger than $I_{sc2}^{cont}/I_{sc1}^{cont}$ by a factor determined by $I_{sc1}^{try}/I_{sc0}^{try}$. It should be emphasized that since trypsin is applied to the mucosal side, whereas ADH, aldosterone, and pyruvate are added from the serosal side only, trypsin should not affect cellular entry of aldosterone and pyruvate or binding of ADH to its receptor.

Thus, by comparing the experimental values of I_{sc_2}/I_{sc_1} or $(I_{sc_2} - I_{sc_1})/I_{sc_0}$ with those predicted by the models, some features of the mechanisms by which Na channels are activated in response to aldosterone, ADH, or substrates should become apparent.

ADH Stimulation of Trypsinized Bladders

In the control quarter-bladders, the ADH-dependent increase in I_{sc} reached a maximal value at ~ 10 min after the addition of ADH (Fig. 4). In the trypsinized quarter-bladders the same time course was observed. The fractional and incremental ADH-induced increase in I_{sc} in control and trypsinized bladders, together with the values predicted by the models, are shown in Fig.

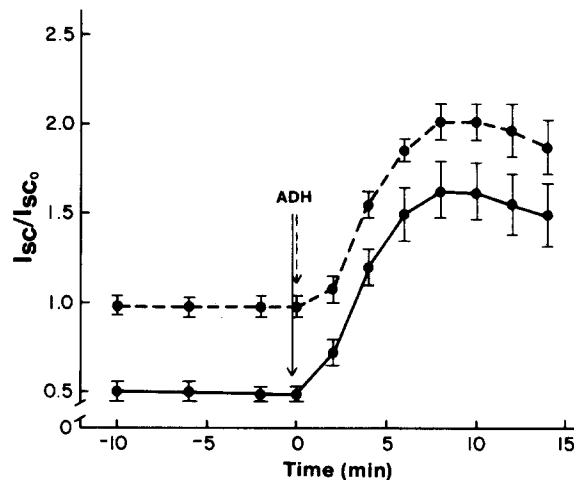


FIGURE 4. The effect of trypsin on the response to ADH. Paired quarter-bladders were treated as shown in Fig. 3. The trypsinized quarter-bladder is denoted by the solid (lower) curve and the control quarter-bladder by the dashed (upper) curve. Trypsin (1 mg/ml) was added to the mucosal side of the experimental quarter-bladders 60 min before challenge with ADH (50 mU/ml), and soybean trypsin inhibitor (0.5 mg/ml) was added 30 min before challenge with ADH at $t = 0$. $n = 6$ pairs of quarter-bladders. The vertical lines are the standard errors of the mean.

5. Trypsinization increased I_{sc_2}/I_{sc_1} by 50% (from 2.2 ± 0.1 to 3.4 ± 0.3), but had no apparent effect on $(I_{sc_2} - I_{sc_1})/I_{sc_0}$ (1.1 ± 0.1 and 1.2 ± 0.2 for the control and trypsinized bladder, respectively). These data are in full agreement with model II, which predicts $I_{sc_2}/I_{sc_1} = 3.5$ and $(I_{sc_2} - I_{sc_1})/I_{sc_0} = 1.1$, and do not fit model I, which predicts $I_{sc_2}/I_{sc_1} = 2.2$ and $(I_{sc_2} - I_{sc_1})/I_{sc_0} = 0.5$. This result indicates that ADH-induced channels are either not present in the apical membrane during trypsinization or are in a trypsin-insensitive conformation before activation.

This conclusion is confirmed by the experiment shown in Fig. 6. In this case, paired quarter-bladders were first exposed to 1 mg/ml trypsin and 1 h later trypsin inhibitor was added to one of each pair. 10 min thereafter, all

quarter-bladders were challenged with ADH. No significant difference in I_{sc} was detected before the addition of the hormone or during the initial stage of hormone stimulation (i.e., for the first 5 min). Activation of I_{sc} in the presence of mucosal trypsin, however, resulted in a truncation of the response and a progressive divergence (greater fall in the presence of uninhibited trypsin) in the I_{sc} 's. This experiment indicates that ADH exposes "Na-conducting substrate" (which was not available before stimulation) to the protease.

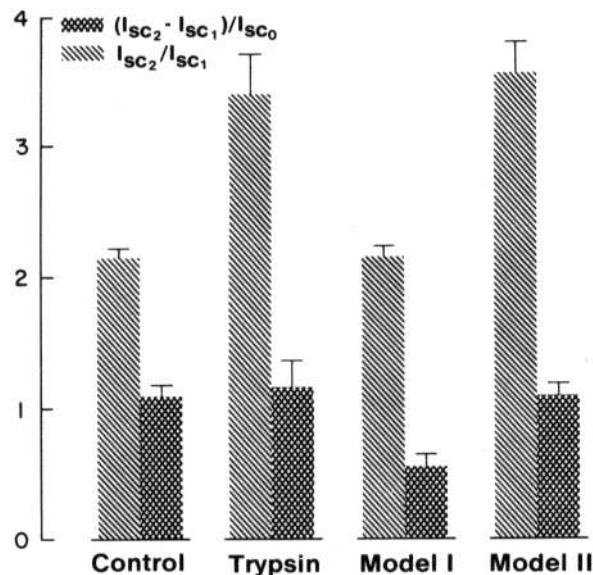


FIGURE 5. Model analysis of the response to ADH after pretreatment with trypsin. The fractional (I_{sc_2}/I_{sc_1}) and the incremental ($(I_{sc_2} - I_{sc_1})/I_{sc_0}$) responses to ADH (50 mU/ml) in control and trypsin-pretreated (1 mg/ml) quarter-bladders are represented in the two pairs of bars on the left. The I_{sc_0} values were 26.7 ± 9.3 and $25.9 \pm 10.6 \mu\text{A}/\text{cm}^2$ for the control and experimental groups, respectively ($n = 12$ pairs of quarter-bladders). The predicted fractional and incremental response to ADH after trypsinization were computed for the models (I, II) as described in the text and are represented by the two pairs of bars on the right.

Aldosterone and Pyruvate Stimulation of Trypsinized Bladders

As was the case with ADH, prior trypsinization did not alter the time course of the I_{sc} response to aldosterone, but the absolute increase in I_{sc} was much smaller in the trypsinized quarter-bladder than in the control (Fig. 7). In response to aldosterone, I_{sc_2}/I_{sc_1} had similar values in the control (2.4 ± 0.4) and trypsinized (1.9 ± 0.3) quarter-bladders in agreement with model I (Fig. 8). According to model II, a value of 3.5 is predicted for $I_{sc_2}^{\text{try}}/I_{sc_1}^{\text{try}}$. The normalized incremental increase in I_{sc} in the trypsinized bladder was 0.8 ± 0.2 , significantly lower than the value measured for the control quarter-bladder (1.45 ± 0.4). This result fits well with model I, which predicts a value

of 0.8. The response of the trypsinized bladders to aldosterone, therefore, fits model I but not model II, i.e., the aldosterone-induced Na channels are subject to the same degree of inactivation by trypsin as the baseline channels. These results imply that the aldosterone-induced channels were present in the apical membrane in nonconductive form before the addition of the hormone. However, an alternative possibility is that trypsinization of other apical proteins impaired the ability of aldosterone to insert new channels in the membrane. To distinguish between these possibilities, we tested the effect of aldosterone in quarter-bladders that were trypsinized in the presence of amiloride. As

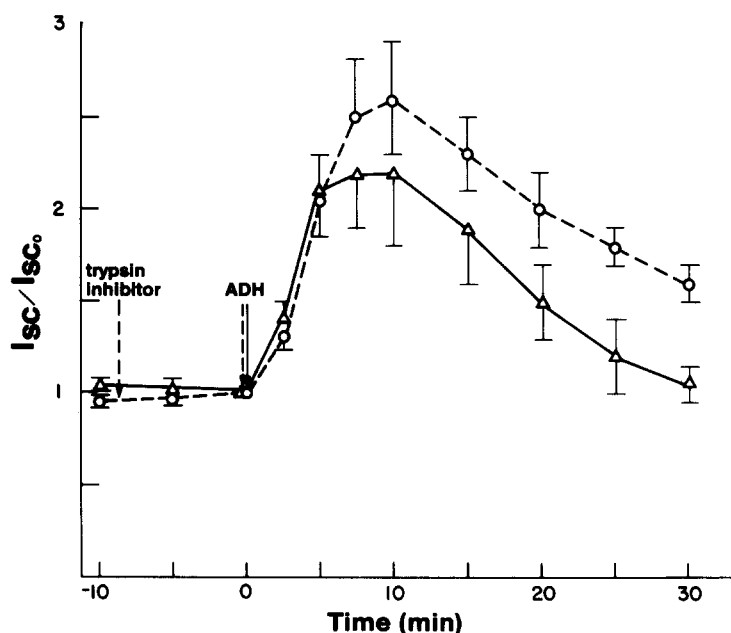


FIGURE 6. The effect of continuous exposure to trypsin on the response to ADH. Paired quarter-bladders were exposed to trypsin (1 mg/ml, mucosal side) for 1 h and then soybean trypsin inhibitor (0.5 mg/ml) was added to one of each pair (solid line). 10 min later ADH (50 mU/ml) was added to the serosal side of both quarter-bladders. ($n = 9$ pairs of quarter-bladders.) Vertical lines represent the standard errors of the means.

shown above, amiloride prevents inactivation of Na channels by trypsin and presumably has no effect on trypsinization of other non-amiloride-binding proteins. Thus, impairment of the response to aldosterone caused by trypsinization of membrane proteins, other than apical Na channels, should not be affected by amiloride. In eight pairs of bladders, no significant difference in the response to aldosterone could be detected between the quarter-bladder trypsinized in the presence of amiloride and the control quarter-bladders, which were not exposed to trypsin or amiloride or received amiloride only ($P > 0.5$ both for the fractional and incremental current changes) (data not shown). The power of the t test to detect 50% changes under these conditions

is estimated to be >0.9 . Thus, amiloride prevented trypsin impairment of the aldosterone-induced channels as well as baseline channels. This result indicates not only that the channels activated by aldosterone were exposed to trypsin before hormone stimulation (i.e., constantly present in the apical membrane), but also that amiloride protects the inactive channels (inducible by aldosterone) from the action of trypsin to the same extent as the active channels.

It is well documented (13, 14) that substrate-depleted bladders respond minimally to aldosterone and that the subsequent addition of substrates to substrate-depleted, aldosterone-treated bladders induces a twofold (or greater)

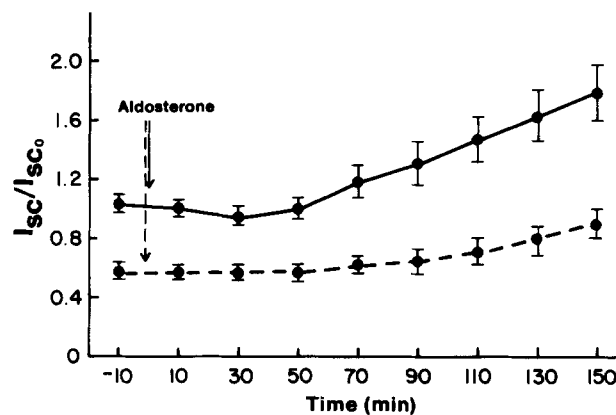


FIGURE 7. The effect of trypsin on the response to aldosterone. Paired quarter-bladders were incubated for 14–18 h in Na-free Ringer's (mucosal) and NaCl Ringer's plus 5 mM pyruvate (serosal). Both solutions contained 100 U/ml penicillin and 100 μ g/ml streptomycin to prevent bacterial growth. At the end of this period, the mucosal and serosal compartments were perfused with fresh NaCl Ringer's, and after I_{sc} reached steady values (30–60 min), trypsin (1 mg/ml) was added to the mucosal medium of one quarter-bladder. 1 h later, the enzyme was blocked with soybean trypsin inhibitor (0.5 mg/ml) and aldosterone (5×10^{-7} M) was applied to the serosal media of both hemibladders ($t = 0$) (current changes were followed for several hours). $n = 6$ pairs of quarter-bladders. The vertical lines are the standard errors of the means. See Fig. 8 for a summary of all the results.

increase in I_{sc} with a very brief latent period. Recently (14), we showed that this increase reflects metabolic-dependent regulation of the apical Na channels.

This mechanism is, in fact, evident even in the absence of aldosterone, but the increase in P_{Na} upon adding substrates is more pronounced in aldosterone-repleted bladders. To evaluate whether the substrate-induced increase in I_{sc} results from activation of Na channels previously accessible to trypsin (as in the case of aldosterone) or channels that are not available to trypsinization before substrate stimulation, a comparison was made of pyruvate effects in control and trypsinized quarter-bladders. The experimental protocol was similar to the one used for aldosterone (see the legend to Fig. 9). Pyruvate

induced an immediate and progressive increase in I_{sc} that reached a maximal value after 2 h (Fig. 9). No difference was noted in the time courses of these responses in the control and trypsinized quarter-bladders. I_{sc_2}/I_{sc_1} was 1.9 ± 0.2 and 1.85 ± 0.15 for the control and trypsinized quarter-bladders, respectively, and $(I_{sc_2} - I_{sc_1})/I_{sc_0}$ was 1.2 ± 0.2 and 0.65 ± 0.15 for the control and trypsinized hemibladders. Thus, no significant difference was detected in the fractional effect, but a nearly twofold difference in the incremental effect was obtained. The substrate-induced increase in I_{sc} , therefore, fits model I but not model II, i.e., it reflects activation of apical Na channels that were accessible

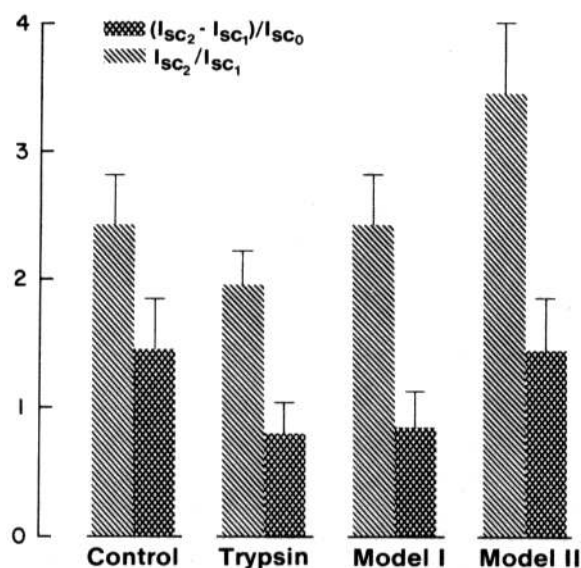


FIGURE 8. Model analysis of the response to aldosterone after pretreatment with trypsin. Average fractional (I_{sc_2}/I_{sc_1}) and incremental [$(I_{sc_2} - I_{sc_1})/I_{sc_0}$] responses to aldosterone (5×10^{-7} M) in control and trypsin-pretreated (1 mg/ml) quarter-bladders are represented by two pairs of bars on the left. I_{sc_0} was 21.7 ± 4.5 and $17.1 \pm 4.8 \mu\text{A}/\text{cm}^2$ for the control and trypsinized quarter-bladders, respectively ($n = 12$ pairs of quarter-bladders). The predicted fractional and incremental responses to aldosterone after trypsinization were computed for the models (I, II) as described in the text and are represented by the two pairs of bars on the right.

to trypsin before the addition of substrate, as was the case with aldosterone.

The time courses of the increases in P_{Na} induced by ADH vs. that induced by aldosterone or substrates differ significantly. I_{sc} peaks 5–10 min after addition of ADH, 2 h after the addition of substrate, and 5–6 h after the addition of aldosterone. In the latter instances, the quarter-bladders were preincubated for 14 h before challenge, and in the former, only 1–2 h before use. Thus, a remote possibility exists that trypsin may have differing effects in fresh and preincubated bladders. To rule out such a possibility, some of the quarter-bladders that were used to assess responses to aldosterone and pyruvate

after overnight incubation were subsequently challenged with ADH (50 mU/ml). In eight pairs of quarter-bladders (pretreated with aldosterone and pyruvate after overnight incubation), ADH induced 2.2 ± 0.2 - and 3.1 ± 0.3 -fold increases in I_{sc} in the control and trypsinized quarter-bladders, respectively. The aldosterone- or pyruvate-induced fractional increases in I_{sc} in these same quarter-bladders were 2.1 ± 0.1 and 1.6 ± 0.2 for the control and trypsinized groups, respectively. Thus, in the same quarter-bladders that

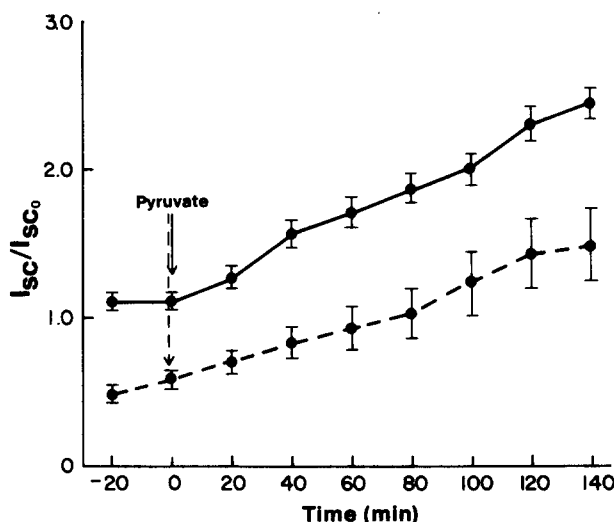


FIGURE 9. The effect of trypsin on the response to pyruvate. Paired quarter-bladders were incubated for 14–18 h in Na-free Ringer's (mucosal) and substrate-free NaCl Ringer's plus 5×10^{-7} M aldosterone (serosal). Both solutions contained 100 U/ml penicillin and 100 μ g/ml streptomycin to prevent bacterial growth. At the end of this period, the mucosal and serosal compartments were perfused with NaCl Ringer's free of substrate or aldosterone. One of the two quarter-bladders received trypsin (1 mg/ml) and 1 h later the enzyme was blocked with soybean trypsin inhibitor (0.5 mg/ml). Pyruvate (5 mM) was added to the serosal compartment of both quarter-bladders ($t = 0$) and changes in I_{sc} were followed for several hours. $n = 9$ pairs of quarter-bladders. I_{sc} was 12.5 ± 2.1 and 18.3 ± 2.7 μ A/cm² for the control and trypsinized quarter-bladders, respectively.

responded to aldosterone or pyruvate according to model I, the response to ADH fits model II, which implies that there are basic differences in the activation mechanisms of these agents.

DISCUSSION

A number of findings suggest that the observed decrease in I_{sc} upon addition of trypsin to the mucosal compartment is the outcome of closure of apical Na channels as a result of proteolytic cleavage. (a) The decrease in I_{sc} is accompanied by a proportionate increase in the apical resistance, R_{Na} , with no

change in the paracellular resistance, R_s (Table I). (b) Amiloride at a concentration that was just sufficient to abolish I_{Na} prevented the trypsin-induced decrease in I_{sc} . In contrast, eliminating I_{Na} by removing all Na from the mucosal medium had no effect on the sensitivity to trypsin. (c) Much of the decrease in I_{sc} was irreversible and was blocked by trypsin inhibitor. These findings imply a relationship between the amiloride-binding site and the site cleaved by trypsin and denies the possibility of nonspecific damage to the apical membranes as the source of the change in I_{sc} .

Consideration should also be given to the possibility of trypsin-induced changes in apical ion selectivity, and particularly to K, which would complicate the interpretation of these results. This possibility, however, was contradicted by two sets of findings. (a) If trypsin rendered the apical surface permeable to K, the K concentration gradient (mucosal to serosal) imposed by the use of Na-free, high-K solutions on the mucosal side (Table IV) should have elicited a positive current in the presence of the enzyme. This was not the case in that perfusion with the Na-free, high-K solution gave current ratios (I_{sc}/I_{sc0}) in control and experimental hemibladders of 0.12 ± 0.02 and 0.15 ± 0.02 , respectively, before trypsinization, and 0.14 ± 0.02 and 0.16 ± 0.02 , respectively, after trypsinization. Indeed, addition of the ionophore nystatin to the mucosal solution in the presence of a positive K gradient elicited the predicted positive current (data not shown). (b) A trypsin-induced increase in apical K permeability should have increased apical conductance. The opposite effect, however, was recorded, in that trypsin increased the amiloride-sensitive resistance 2.7-fold, with no change in the shunt resistance (Table I).

A highly reproducible observation was that trypsin blocked only 50% of the original current. In an earlier study, Cuthbert and Painter (15) noted that application of chymotrypsin to the external (mucosal) side of frog skin reduced I_{sc} ~50% after 2 h. These observations suggest either that 50% of the Na channels are not accessible to the enzyme (e.g., due to steric hindrance) or that all of the channels have been partially blocked and continue to conduct Na at 50% of the original rate. The latter explanation seems unlikely in that all previous studies based on noise analysis indicate that the apical Na channels of both toad bladder and frog skin operate in an "all-or-none" mode (6, 8). Further studies are needed, however, to confirm the all-or-none mechanism in the response to trypsin.

In the toad bladder, changes in I_{sc} induced by aldosterone, ADH, and metabolic substrates all appear to be mediated, at least in part, by changes in the density of open (i.e., electrically detectable) apical Na channels (6, 8). Some of the possible mechanisms that can account for the increase in channel density are: (a) activation of a pre-existing set of channels in the apical membrane by covalent chemical modification (16, 17); (b) activation of a pre-existing set of channels by cytoplasmic factors such as Na activity, Ca activity, or pH (i.e., noncovalent modification) (11, 12, 18, 19); (c) recruitment of channels from subapical vesicles (20); (d) recruitment of channels by *de novo* synthesis of the components (13).

The ability of trypsin to block, irreversibly, apical Na permeability was

used to distinguish between the above mechanisms (*a* or *b* vs. *c* or *d*). In these experiments, changes in I_{sc} were used as indicators of changes in P_{Na} for the following reasons. Stimulation of I_{sc} by ADH, aldosterone, and metabolic substrates, and inhibition by trypsin are proportionate (i.e., 1:1) to changes in P_{Na} or an index of P_{Na} , such as R_{Na} (6-8, 14). It is very likely, therefore, that changes in I_{sc} evoked by combinations of trypsin and one of the activating agents will also be accompanied by corresponding changes in P_{Na} . Since the hormones and substrates were added to the serosal medium only, effects of trypsin on the primary interactions between these reagents and the epithelium are highly unlikely.

Pretreatment with trypsin inhibited the responses to aldosterone and pyruvate to the same extent as the baseline current, which indicates that the aldosterone- and pyruvate-induced channels were accessible to trypsin and presumably present in the apical membrane before stimulation by these agents. Thus, *de novo* synthesis of channel components or acquisition of new channels by fusion of vesicles with the membrane should be excluded as the activation mechanism in these cases. Since the effect was abolished when amiloride was present during exposure to trypsin, susceptibility of the latent channels to trypsin probably involves cleavage of amiloride-binding proteins. In contrast, the ADH-induced increase in I_{sc} was totally insensitive to the proteolytic attack: in this case, the increase in I_{sc} appears to involve either fusion of channel-containing vesicles with the apical membrane or recruitment from some other precursor pool that is inaccessible to trypsin.

In an earlier study by Palmer and Edelman (21), diazosulfanilic acid (DSA), an impermeant protein-modifying agent, was used to inhibit Na conductance of apical channels irreversibly in toad bladders. As in the present study, pretreatment with DSA inhibited the basal and aldosterone-stimulated I_{sc} to the same extent. In contrast to the present results, however, the pool of electrically silent channels recruited by vasopressin was accessible to DSA. A possible explanation for the discrepancy between these results and those obtained with trypsin is that DSA may also modify the apical plasma membrane sites mediating the incorporation of channel-containing vesicles into the surface. A similar explanation may apply to the results of Park and Fanestil (22). They noted that a tyrosine-reactive reagent inhibited basal and ADH-stimulated I_{sc} proportionately.

Inhibition of the basal current may either inhibit or enhance the response to hormone or substrates, irrespective of the means by which the initial current was reduced. In earlier studies (14, 23), however, the aldosterone-induced increase in current was independent of the magnitude of the baseline current. Effects of the initial current on the response to ADH were indeed demonstrated, but such effects were associated with modified kinetics not observed in our experiments (24). Furthermore, the experiment summarized in Fig. 6 clearly shows that ADH exposes previously inaccessible channels to trypsin. Thus, the differences in the responses of the control and trypsinized bladders are not attributable simply to differences in the initial currents.

An important conclusion from our results is that aldosterone and ADH do

not share the same pool of inactive channels. The possibility of "protocol-dependent" results was excluded by measuring the effect of ADH on the same bladders that were used to measure the effect of aldosterone. Thus, at least two distinct mechanisms to increase the density of Na-conducting channels seem to operate in the toad bladder epithelium.

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