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FEEDBACK INHIBITION OF SODIUM UPTAKE IN K^+-DEPOLARIZED TOAD URINARY BLADDERS

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Ouabain-blocked toad urinary bladders were maintained in Na^+-free mucosal solutions, and a depolarizing solution of high K^+ activity containing only 5 mM Na^+ on the serosal side. Exposure to mucosal sodium (20 mM activity) evoked a transient amiloride-blockable inward current, which decayed to near zero within one hour. The apical sodium conductance increased in the initial phase of the current decay and decreased in the second phase. The conductance decrease required Ca^{2+} to be present on the serosal side and was more rapid when the mucosal Na^+ activity was higher. At 20 mM mucosal Na^+ and 3 mM serosal Ca^{2+} the initial (maximal) rate of inhibition amounted to 20% in 10 min. The conductance decrease could be accelerated by raising the serosal Ca^{2+} activity to 10 mM. The inhibition reversed on lowering the serosal Ca^{2+} to 3 μM and, in addition, the mucosal Na^+ to zero. Exposure of the mucosal surface to the ionophore nystatin abolished the Ca^{2+} sensitivity of the transcellular conductance, showing that the Ca^{2+}-sensitive conductance resides in the apical membrane. The data imply that in the K^+-depolarized epithelia, cellular Ca^{2+}, taken up from the serosal medium by means of a Na^+-Ca^{2+} antiport, cause feedback inhibition by blockage of apical Na^+ channels. However, the rate of inhibition is small, such that this regulatory mechanism will have little effect at 1 mM serosal Ca^{2+} and less than 20 mM cellular Na^+.

Introduction

In tight amphibian epithelia such as toad urinary bladder and frog skin luminal sodium ions enter the cells through the apical membrane by means of amiloride blockable passive channels and are then pumped to the interstitial space by the sodium pump of the basolateral membrane (see, for example, Refs. 1–3). In these tissues and in some other tight epithelia, the apical Na^+ channels are believed to be controlled by negative feedback [4–12]: an increase of the cellular Na^+ activity, resulting from a decreased turnover rate of the pump or an increased apical Na^+ uptake, will induce a drop in the apical Na^+ permeability. The function of this process would be to avoid a large accumulation of Na^+ in the cell. For this inhibition, two different mechanisms have been proposed: (a) direct inhibition of Na^+ channels by cellular Na^+ [5,8], or (b) inhibition of Na^+ channels by the free cellular Ca^{2+}, the activity of which is coupled to the cellular Na^+ activity by means of a basolateral Na^+-

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Abbreviations: I_{sc}, short circuit current. I_{Na}, sodium-specific (amiloride-blockable) component of the short circuit current (I_{sc} minus shunt current). G, transepithelial slope conductance. G_{Na}, sodium-specific apical slope conductance, estimated as G minus G_s. G_s, shunt slope conductance (insensitive to amiloride). I_{sco}, I_{Nao}, G_{Nao}, values of I_{sc}, I_{Na} and G_{Na} measured at the peak of the fast current transient following an upward step of Na_m. X_m, X_c, X_s, mucosal (m), cellular (c), and serosal (s) activities of ion X. P_{Na}, apical Na^+ permeability. $\Delta\tilde{\mu}^m_{Na}$, $\Delta\tilde{\mu}^s_{Na}$, electrochemical potential difference of sodium ions across the mucosal (apical, m) or serosal (basolateral, s) membranes. $\Delta\tilde{\mu}^s_{Ca}$, electrochemical potential difference of calcium ions across the serosal membrane.

Ca^{2+} antiport (see Refs. 4, 6, 7, 11–13). Evidence for the existence of the second mechanism in toad bladder have been recently provided both by studies using the intact epithelium and studies in membrane vesicles isolated from this tissue [4,11,13,14].

In this paper we describe measurements of Na^+ specific currents and conductances in ouabain-blocked K^+-depolarized bladders. This assay system was chosen for the following reasons: (1) Introducing Na^+ to the mucosal solution of ouabain blocked K^+-depolarized bladder causes a progressive increase of the cellular Na^+ activity from zero to a value similar to that of the mucosal Na^+ activity [3]. This 'loading' process resembles the 'in vivo' situation in which Na_c^+ tends to increase as a consequence of increased concentrations of Na^+ in the urine or decreased turnover rate of the pump. It is therefore suitable for studying the proposed feedback inhibition of Na^+ channels, and in particular the time-course at which the apical Na^+ conductance decreases in response to increased Na_c^+. The time-course of the feedback inhibition has not been previously examined. (2) In K^+-depolarized bladders the basolateral conductance is much higher than the apical one. Thus, the apical conductance and the changes induced in it by Na^+ and Ca^{2+} may be estimated from measurements of the transcellular Na^+ specific (amiloride blockable) conductance [3]. (3) K^+-depolarized epithelia were used in numerous studies [3,15–20] and it is of interest to know whether the feedback inhibition remains operative in this state.

By monitoring changes in the Na^+ specific conductance during 'Na^+ loading', we found that a decrease of the apical Na^+ conductance, whose magnitude depends on cellular Na^+ and serosal Ca^{2+} activities can indeed be demonstrated. The rate of this change in conductance, however, is small (approx. 2% per minute) such that the drop in conductance significantly lags behind the increase in cellular Na^+.

In a second set of experiments cells were loaded with 20 mM mucosal Na^+ at minimal serosal concentration of Ca^{2+} (3 μM). After a steady state had been reached, the serosal Ca^{2+} activity was stepped up. The Ca^{2+} addition induced a marked, but slow, decrease of the Na^+ specific conductance, which could be reversed by lowering the cellular Na^+ activity and the serosal Ca^{2+} activity.

The data confirm that Ca^{2+} taken up from the serosal side by means of Ca^{2+}-Na^+ exchange, blocks the apical Na^+ channels. However, in the K^+-depolarized preparation used, the rate of feedback inhibition is relatively slow.

Methods and Materials

Male *Bufo marinus* toads (Mexican origin, obtained from Lemberger, Wisconsin) were maintained in tanks with access to tap water. The animals were double-pithed, and the urinary bladders were removed and mounted in a modified Ussing chamber described before [3,16]. The mucosal compartment was a closed 1 ml volume continuously perfused at a rate of 1–100 ml per min. In this chamber exchanges of the mucosal solution could be made within 1 second without perturbing the mounted bladder mechanically.

The bladders were clamped to zero potential, and I_{sc} was continuously recorded. The transepithelial electrical slope conductance was obtained from the current change observed upon brief (1 s) 10 mV displacements of the clamping potential. The shunt conductance was estimated in either a Na^+-free mucosal solution or in a mucosal solution containing 80 μM amiloride. With 20 mM mucosal Na^+ the amiloride blockable conductance was 0.77 + 0.03 ($n = 20$) of the total transepithelial conductance measured under the same conditions. We expect that the shunt conductance has a paracellular component, and a transcellular component which is partly due to movement of chloride ions.

The mucosal side was perfused with one of the following solutions: (A) Na^+-free sulfate Ringers (denoted NaO) containing (in mM) 55.0 K_2SO_4; 4.0 K_2HPO_4; 1.0 KH_2PO_4; and 1.0 calcium gluconate (pH 7.5). (B) Sodium sulfate Ringers (denoted Na20) containing 18.2 Na_2SO_4; 36.8 K_2SO_4; 4.0 K_2HPO_4; 1.0 KH_2PO_4; and 1.0 calcium gluconate (pH 7.5). (C) 3 : 1 mixture of A and B (denoted Na5). The sodium activities in the above solutions were less than 0.1, 20 and 5 mM, respectively. The serosal Ringers was a depolarizing KCl-sucrose solution (see Ref. 3), containing (in mM) 85.0 KCl; 50.0 sucrose; 5.0 sodium pyruvate; 0.5 $MgCl_2$; 3.5 potassium phosphate (pH 7.5); and 1.0 $CaCl_2$ (unless otherwise indicated). In contrast to Refs. 3 and 21 we used 5 mM rather than 0 mM

Na_s^+ in order to maintain a defined $\Delta\mu^s_{Na}$. In some experiments (Fig. 1B) KCl was substituted by choline chloride. The serosal calcium concentrations indicated are total chemical concentrations. Ca^{2+}-buffering was avoided because all the chelators that can be used to buffer the Ca^{2+} activities to values higher than 1 μM will also bind Mg^{2+} [22].

Materials. Ouabain and nystatin (5400 units/mg) were obtained from Sigma. Amiloride was a gift from Sharp and Dohme GmbH, Munich, F.R.G. All of the conventional chemicals were reagent grade.

Statistics. Data are expressed as means ± (S.E.).

Results

Time-course of Na^+-loading

Hemibladders were mounted and incubated in Na0 (mucosal) and K^+-depolarizing Ringers + 1 mM ouabain (serosal). Since the mucosal solution was practically free of Na^+, I_{sc} assumed near zero values and the transepithelial slope conductance was (by definition) equal to G_s. To assay for time dependent changes in G_{Na} during incubation in Na^+-free solutions, the bladders were occasionally exposed to Na20 (mucosal) for 10–20 s only. As shown before [16] such 'Na^+ pulses' induced an immediate rise in I_{sc} which peaked after 2–4 s (I_{sco}). Upon termination of the Na_m pulse, I_{sc} decreased rapidly, and a small negative (reversal) current was observed. The outward current, which could be blocked with amiloride, returned to zero within 5 min after the Na^+ pulse (cf. Fig. 3 in Ref. 16). The peak current I_{Nao} as well as G_{Nao}, measured with Na_m pulses, reached steady values 2–4 h after mounting. These values were maintained for at least 5 h.

Fig. 1 describes a typical experimental protocol. Bladders preincubated for several hours in Na0, as described above, were exposed to Na20 for about one hour and then reincubated in Na0. On introducing Na^+ to the apical side an immediate rise in I_{sc} and G_{Na} to I_{sco} and G_{Nao} was observed, followed by a decay of the current to near zero values within one hour. Since the pump was blocked by ouabain, this inward current will represent apical Na^+ uptake balanced mainly by cell to serosa K^+ loss. The decrease of I_{sc} with time will at least in

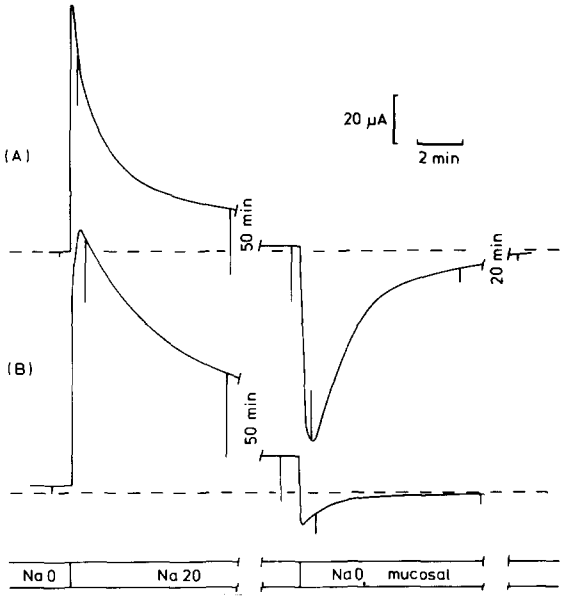

Fig. 1. Passive Na^+ currents in ouabain-blocked bladders. Paired hemibladders were incubated in Na0 (mucosal) and either K^+-depolarizing Ringers + 1 mM ouabain (A) or choline Ringers + 1 mM ouabain (B) (serosal). Ca_s was 1 mM in both cases. At the time indicated in the bottom of the figure, the flowing mucosal Na0 solution was switched to Na20. About one hour later Na^+ was again removed from the mucosal compartment (Na0). I_{sc} was continuously recorded. The vertical spikes are current deflections in response to 10 mV voltage command pulses of 1 s duration.

part reflect abolishment of $\Delta\tilde{\mu}^m_{Na}$. Feedback inhibition may contribute to the decrease in I_{sc}, by reducing G_{Na}, as observed by others under similar conditions (e.g. Refs. 17, 21 and 23).

Subsequent substitution of Na20 by Na0 reverses the direction of the apical Na^+ gradient and induces a negative (reversal) current (Fig. 1A). This current will indicate cell to mucosa Na^+ flow which must be balanced mainly by serosa to cell K^+ flow. Therefore, the outward current may be expected to require a high serosal K^+ concentration. Indeed, if K_s was replaced by choline, the reversal current was much smaller (Fig. 1B). These results, like those of others (see Refs. 3 and 19), confirm that the basolateral K^+-conductance remains large in ouabain-blocked preparations. The outward current decays to zero, apparently because the cytosol is depleted of available sodium ions, and is fully blocked by 80 μM amiloride (not shown).

Palmer et al. [3] presented evidence that the

high KCl-sucrose solution depolarizes the basolateral membrane completely. Under short circuit conditions the apical membrane will then be depolarized too, and since the basolateral conductance is large at high K_c and K_s, $G - G_s$ will estimate G_{Na}. However, during prolonged exposures to Na20, Na_c is expected to rise higher than under the conditions used by Palmer et al. [3], replacing K_c. The resulting decrease in basolateral conductance will cause G_{Na} to be underestimated, as discussed before [15]. Yet, with Na_m no larger than 20 mM, the contribution from the basolateral conductance will be limited. The effect is discussed in conjunction with Fig. 3 and can be studied by changing Na_m in preparations where the apical membrane is shunted, for instance by nystatin (see footnote on p. 95).

In most experiments G_s, measured before applying Na20 as well as after the negative current returned to zero, had practically the same value. In these cases constancy of G_s may be assumed for the whole period shown in Fig. 1. Accordingly G_{Na} was calculated as the difference between G measured at a given time, and G_S measured either before or after the Na20/Na0 substitution. Experiments in which substantial changes in G_s occurred, were not included in the data presented below.

Dependence on Na_m and Ca_s

As discussed above, changes in G_{Na} which develop during the decrease in I_{sc} (first part of Fig. 1) will mainly estimate changes in the apical Na^+ conductance at constant mucosal Na^+ activity and increasing values of Na_c. In this period the feedback inhibition should be established and cause a decrease in P_{Na}. Conductance changes which developed in the period of decreasing current were measured under three conditions: (a) 5 mM mucosal Na^+ and 3 μM serosal Ca^{2+}, (b) 20 mM mucosal Na^+ and 3 μM serosal Ca^{2+}, and (c) 20 mM mucosal Na^+ and 3 mM serosal Ca^{2+}.

Typical changes in G_{Na} and I_{Na}, observed under these conditions, are depicted in Fig. 2. The fractional changes in current showed little dependence on Na_m or Ca_s. In all three cases I_{Na}/I_{Nao} decreased to 0.2 within 5 min. This rapid phase was followed by a slow further decrease. The rapid decrease in I_{Na}/I_{Nao} was associated with an increase in G_{Na}/G_{Nao}. The subsequent phase of slow current decrease was accompanied by a slow decrease of conductance. Its rate was dependent both on Na_m and Ca_s. The initial increase in G_{Na} above G_{Nao} is expected from the increase in Na_c, caused by the surge of inward current. The subsequent decrease in G_{Na} will mainly reflect a decrease in apical Na^+ permeability, which is seen to require Na_c and Ca_s, and may therefore be recognized as feedback inhibition. Under the conditions used, the inhibition appears to have a slow time course. Fig. 2 shows that with 20 mM Na_m and 3 mM Ca_s the maximal, near initial, rate is only 20% in 10 min. The rate can be increased further by adding more Ca^{2+} to the serosal solution (see below). As discussed below, the real rate of inhibition may be somewhat larger or smaller than it appears from the conductance decrease.

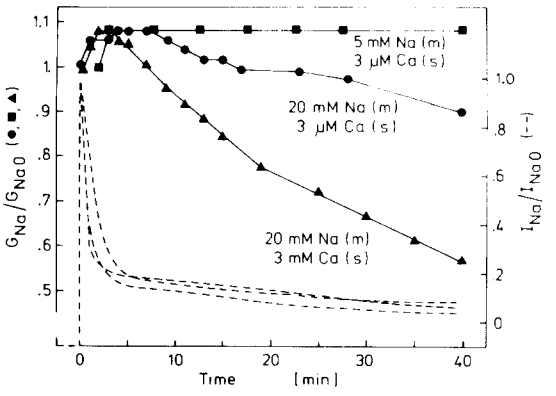

Fig. 2. Time-course of relative I_{Na} and G_{Na} during Na^+ loading. Three hemibladders were incubated for several hours in Na0 (mucosal) and K^+-depolarizing Ringers containing 1 mM ouabain and either 3 μM Ca (■, ●) or 3 mM Ca (▲) (serosal). After I_{Na} and G_{Na} (measured during brief exposures to Na20) had reached steady values, the flowing mucosal solution was at zero-time switched to either by Na20 (●, ▲) or Na5 (■) (see Fig. 1). I_{Na} and G_{Na} were followed for 40 min after the mucosal substitution, and their values relative to those observed at the time of the current peaks were plotted against time.

To further examine the relationship between the changes in I_{Na} and G_{Na} induced by loading the cell with Na^+, the values of I_{Na}/I_{Nao} and G_{Na}/G_{Nao} measured at various times during the Na^+ loading were plotted against each other (Fig. 3). The dashed lines in this figure represent two limiting cases, at which Na_c increases at constant P_{Na}

(upper line), and P_{Na} decreases at zero Na_c (lower line) *. If both Na_c and P_{Na} change, the resulting data points should be found in the area defined by the two lines, as indeed was observed. It can be seen that more than half of the current decay was associated with the initial, linear, increase in G_{Na} above G_{Nao}. This increase did not require Ca_s. It was steeper at $Na_m = 5$ mM than at $Na_m = 20$ mM, but even at 5 mM Na_m it was less than expected for an increase of Na_c at constant P_{Na}. The difference between the two upper curves of Fig. 3 (see Fig. 2 for the time-course) may in part be attributed to the expected larger decrease in the basolateral K^+-conductance caused by displacement of K_c with Na_c in the presence of 20 mM rather than 5 mM Na_m (see footnote on p. 95). In addition, a direct inhibitory effect of Na_c on P_{Na} cannot be excluded as contributing to this difference. This putative effect would also explain the difference between the upper curve and the upper dashed line. The further decrease in I_{Na} was at $Na_m = 5$ mM accompanied by a proportionate further increase in G_{Na}, or at $Na_m = 20$ mM by a net decrease in G_{Na}. The latter effect was much more pronounced in the presence of 3 mM Ca_s, and may for this case be attributed to a decrease of P_{Na} at near constant Na_c which depends on the serosal Ca^{2+} activity i.e. Ca^{2+}-dependent feedback inhibition of the Na^+ channels.

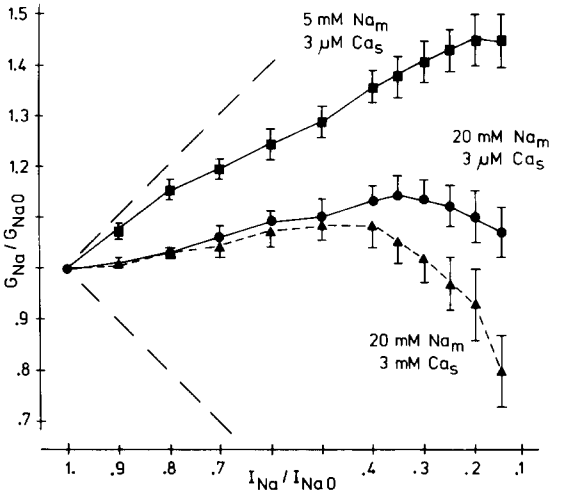

Fig. 3. Relationship of conductance and current responses to a step increase of Na_m from 0 to 5 or 20 mM at Ca_s values of 3 μM or 3 mM. Means ± S.E. from four experiments with the experimental protocol of Fig. 2 (time increases from left to right). The initial values (measured at the time of peak currents following the increase of Na_m) were: 5 mM Na_m, 3 μM Ca_s: $I_{Nao} = 6.9 \pm 3.8$ μA/cm², $G_{Nao} = 0.43 \pm 0.09$ mS/cm²; 20 mM Na_m, 3 μM Ca_s: $I_{Nao} = 58.1 \pm 19.4$ μA/cm², $G_{Nao} = 1.23 \pm 0.43$ mS/cm²; 20 mM Na_m, 3 mM Ca_s: $I_{Nao} = 44.4 \pm 3.6$ μA/cm², $G_{Nao} = 0.92 \pm 0.04$ mS/cm². Periods of observation about 20 min. The dashed lines indicate the expected responses for an increase of Na_c at constant P_{Na} (upper) and a decrease of P_{Na} at constant Na_c (lower dashed line), with the basolateral conductance remaining negligible in both cases.

Delayed addition of Ca_s

In another set of experiments we attempted to demonstrate the Ca_s-dependence of the inhibition by increasing Ca_s after preloading with Na^+ for 40 min in the presence of only 3 μM Ca_s. The advantage of this protocol is that (a) the increase of G_{Na} caused by Na_c, (b) the putative decrease of P_{Na} caused by Na_c, and (c) the decrease of the basolateral conductance caused by replacement of K_c with Na_c should all be near complete at the time when Ca_s is stepped up.

Paired ouabain-blocked hemibladders were exposed to 20 mM Na_m in the presence of 3 μM Ca_s. After near steady state values of G_{Na} were reached, Ca_s was raised to 10 mM in one hemibladder, and was left unchanged in the other (control). Increasing Ca_s induced an immediate acceleration of the decrease in G_{Na} (Fig. 4A). In 9 experiments 10 mM Ca_s decreased G_{Na} to 0.5 ± 0.1 of the value measured immediately before increasing Ca_s. The half time of this Ca induced inhibition was 12.5 ± 1.5 min. In the control hemibladders G_{Na} decreased only to 0.9 ± 0.05 of the original value. Fig. 4B shows an experiment in which Ca_s was raised from 3 μM to 10 mM by three consecutive additions of $CaCl_2$ (1 mM, 4 mM, and 5 mM). The first addition induced an

* The limiting dashed lines in Fig. 3 were calculated as follows: For a short circuited apical membrane $G_{Na}/G_{Nao} = P_{Na}/P_{Nao}\,(1+(Na_c/Na_m))$ and $I_{Na}/I_{Nao} = P_{Na}/P_{NaO}\,(1-(Na_c/Na_m))$. These equations are derived from the expressions for I_{Na} and G_{Na} at zero voltage and are valid for constant field behaviour (cf. Eqns. 20 and 21a in Ref. 24). Thus if P_{Na} stays constant ($= P_{Nao}$) and Na_c rises from zero to Na_m, G_{Na}/G_{Nao} will increase linearly from 1 to 2 as I_{Na}/I_{Nao} decreases from one to zero (upper line). On the other hand, if Na_c remains at zero and P_{Na} decreases, G_{Na}/G_{Nao} will be equal to I_{Na}/I_{Nao} at all times (lower line).

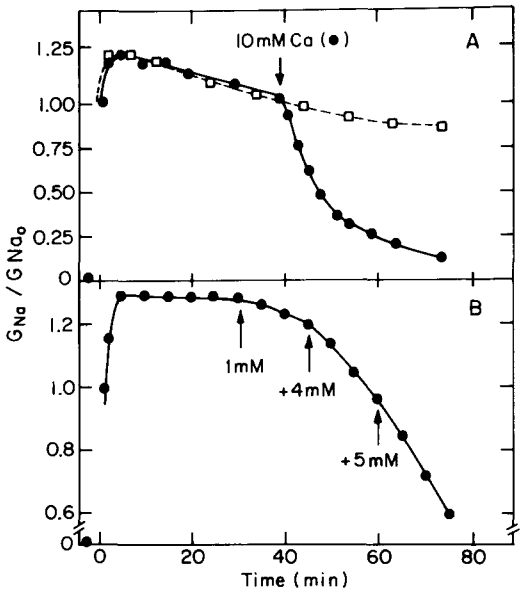

Fig. 4. (A) Ouabain-blocked K^+-depolarized paired hemibladders were exposed to Na20 (mucosal) in the presence of only 3 μM Ca^{2+} in the serosal solution ($t = 0$). 40 min later G_{Na} had reached nearly steady values. At this time the serosal Ca^{2+} activity of one hemibladder (●) was increased to 10 mM. The conductance changes were followed for another 35 min. (B) As A but in this case Ca_s was increased from 3 μM to 10 mM by three consecutive additions of Ca^{2+} (1 mM, 4 mM, and 5 mM) at the times marked by arrows.

only limited decrease of G_{Na}/G_{Nao}. A much larger effect was observed when Ca_s was stepped up to 5 mM by the addition of another 4 mM Ca, and the decrease in conductance became steeper when another 5 mM Ca were added (final 10 mM). These findings confirm the presence of a Ca_s-dependent inhibition of G_{Na} and can be used to estimate the overall rate of this process.

Requirement for Na_c

To establish that the feedback inhibition elicited by a sudden increase in Ca_s requires a high cellular Na^+ activity, we determined G_{Na} in ouabain blocked bladders that were, with only short interruptions, kept in a mucosal solution of zero Na^+ activity (Na0). Since the basolateral Na^+-permeability is usually small, and Na_s was only 5 mM, it may be surmised that Na_c was very low in these preparations. G_{Na} and I_{Na} were measured during brief exposures to Na20, as discussed above and in Ref. 16. After the initial measurement of I_{Na} and G_{Na}, Ca_s in one of each paired hemibladder was raised from 3 μM to 10 mM. I_{Na} and G_{Na} were measured again after 45 min of incubation in Na0. The fractional change in G_{Na} during this period was 0.95 ± 0.05 ($n = 5$) in bladders kept at 10 mM Ca_s, and 1.02 ± 0.04 ($n = 5$) in bladders kept at 3 μM Ca_s. Thus no significant Ca_s dependent decrease in G_{Na} could be seen if Na_c was kept to low values.

Target membrane of the feedback inhibition

The results presented so far are compatible with the possibility that cytosolic Ca, taken up by a basolateral Na^+-Ca^{2+} exchanger, blocks the apical Na^+ channels [11,12]. However, a Ca_c dependent decrease of the basolateral K^+-conductance would also explain the data. To explore this possibility effects of Ca_s in bladders treated with the ionophore nystatin were looked for.

Fig. 5 shows the protocol of these experiments. Paired ouabain-blocked hemibladders were incubated in the presence of 20 mM Na_m and 3 μM Ca_s. After G_{Na} had reached near steady values, the Na^+ channels of one hemibladder were blocked by 80 μM amiloride, and an unspecific permeability for monovalent cations was subse-

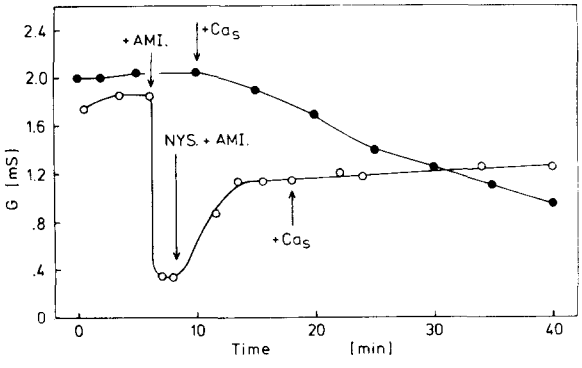

Fig. 5. Ouabain-blocked paired hemibladders were exposed to Na20 (mucosal side) in the presence of 3 μM Ca^{2+} on the serosal side. When G_{Na} had reached steady values, the mucosal solution of one hemibladder (○———○) was replaced by Na20 + 80 μM amiloride (first arrow). Two min later 12.5 μg/ml nystatin were added to the mucosal perfusate of this hemibladder (second arrow). When the nystatin induced conductance had reached a steady value, Ca_s was raised to 10 mM (fourth arrow). In the control hemibladder (●———●) Ca_s was raised to 10 mM (third arrow) without pretreatment with amiloride or nystatin.

quently introduced by incubating the mucosal surface with 12.5 μg/ml nystatin (Fig. 5). The Na^+ channels were thus short-circuited by parallel nystatin channels, which allow Na^+ uptake, but are presumably not inhibited by Ca^{2+}. Therefore, the apical conductance is expected to increase after adding nystatin, while the basolateral conductance should not change much, since Na_c had already been equilibrated with 20 mM Na_m *. In fact, the tissue conductance increased although it did not reach the values observed before addition of amiloride. (With higher nystatin concentrations the conductance increased more, but was less stable.)

Subsequently, Ca_s was increased to 10 mM in both hemibladders. In the nystatin-treated preparation no Ca^{2+}-induced decrease in conductance was detected, while the control hemibladder showed the typical inhibition. Results similar to those of Fig. 5 were obtained in four additional experiments, showing that a decrease in apical P_{Na} and not a decrease in the basolateral K^+ permeability is instrumental in the development of the Ca_s-dependent inhibitory response.

Reversibility of the feedback inhibition

In the experiments described above, Ca_s could not be lowered much below 3 μM. We found that with lower Ca_s-values the shunt conductance increases strongly, presumably because the tight junctions lose their integrity [28]. Also, Na_s may not be raised substantially above 5 mM without abolishment of the depolarization [15]. Therefore, inversion of $\Delta\tilde{\mu}^s_{Na}$ and $\Delta\tilde{\mu}^s_{Ca}$ could not be used to test the reversibility of the inhibition. It proved possible, however, to reverse the Ca^{2+}-induced decrease of G_{Na} by emptying the cells of Na^+ and at the same time lowering Ca_s back to 3 μM.

Fig. 6 describes the protocol of such experiments. Initially (up to 75 min), the sequence of steps was as in Fig. 4A. At 75 min the serosal Ca^{2+} activity of the hemibladder previously exposed to 10 mM Ca, was lowered to 3 μM. This caused a rather limited reversal of the previous conductance decrease. Subsequently, the mucosal solution was changed to Na0, and the negative I_{sc} was allowed to return to zero. The outward current (continuous trace) now observed with the hemibladder previously treated with 10 mM Ca_s was much smaller than the control current.

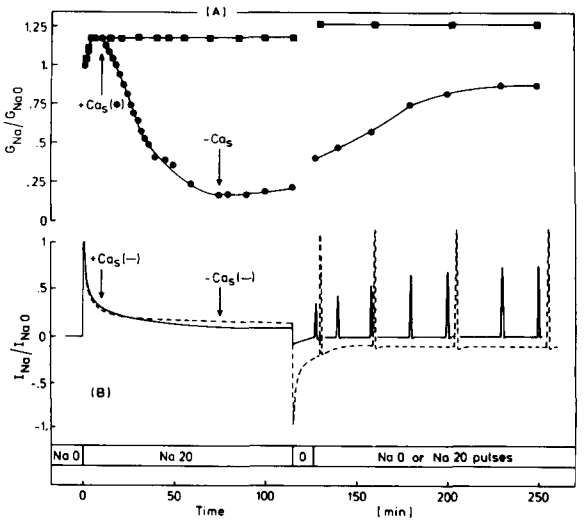

Fig. 6. Reversibility of the feedback inhibition. Paired, ouabain-blocked and K^+-depolarized hemibladders were treated in the initial stage as described for Fig. 4. At t = 75 min Ca_s in the Ca^{2+}-exposed hemibladder was lowered back to 3 μM by four washings of the serosal compartment with a K^+-depolarizing solution containing only 3 μM Ca^{2+} (second arrow). In the control hemibladder the previous serosal medium was renewed at this time. Later both mucosal solutions were substituted by Na0. I_{Na} and G_{Na} were now estimated during brief exposures to Na20. (A) Relative conductance: ●, Ca_s inhibited hemibladder, ■, control hemibladder. (B) Relative current: ———, Ca_s inhibited hemibladder; - - - - -, control hemibladder.

Conductance changes developing in the absence of Na_m were estimated during brief exposures to 20 mM Na_m (causing the current pulses shown in Fig. 6B), as described above and in Ref. 16. A large part of the Na^+ conductance was seen to be restored in the following hours. In six experiments an incubation with 0 mM Na_m and 3 μM Ca_s for 2 h increased G_{Nao} to 0.89 ± 0.05 of the original value. This result demonstrates slow recovery from the Ca_c-dependent inhibition. The speed of this

* It was shown for urinary bladders of *Bufo marinus* and *Bufo bufo* (Garty, H. and Lindemann, B., unpublished data, and Ref. 25), as well as for other epithelial tissues [26,27] that mucosal incubation with nystatin at about this concentration for 30 min does not affect the basolateral conductance. The nystatin-treated preparations studied by us respond with a conductance decrease when Na_m is raised [25]. This decrease is expected from the basolateral membrane when K_c is replaced by Na_c, and would not develop if this membrane was also invaded by the ionophore.

process may be limited by the rate of Ca^{2+} extrusion from the cytosol, by processes other than the basolateral Na^+-Ca^{2+} exchange.

Discussion

In recent years Na^+-Ca^{2+} exchange was demonstrated in vesicles of basolateral membranes obtained from kidney cortex [29] and toad bladder [11]. Its stoichiometry was tentatively estimated to 3 Na^+ per 1 Ca^{2+} [11], as found in other membranes where this antiport stabilises or modulates the cytosolic Ca^{2+} level (see, for example, Refs. 14, 30 and 31). The important studies of the exchange process in vesicle preparations do, however, not yield full information about its physiological significance. This information must be obtained with living epithelial cells where the overall rate of the inhibition process can be assessed while the complex interplay between the various Ca^{2+}-transporting and Ca^{2+}-buffering systems remains functional. First data to this effect came from the mammalian kidney cortex and the proximal tubule [30,32,33].

In frog skin and toad bladder it was shown that replacement of Na_s by choline leads to decreased Na^+ uptake across the apical membrane (see, for example, Refs. 4, 6, 7 and 11), provided Ca_s has not been lowered. Serosal application of calcium ionophores in toad bladders * causes the same kind of inhibition (see, for example, Refs. 6, 10, 12, 35 and 36), which was termed feedback inhibition since it affords a means for transepithelial Na^+ transport to limit itself [12]. The proposed mechanism is, that increased Na^+ uptake at the apical membrane raises Na_c, thus lowering the driving force for Ca^{2+} extrusion by the basolateral exchanger. Leakage of Ca^{2+} into the cell, which may occur at either membrane, would then increase Ca_c, as it does when Na_s is not available [6]. In turn, the increased Ca_c would block the apical Na^+ channels either directly or indirectly. This block was also demonstrated in vesicle preparations [13]. On the whole, therefore, Na^+-feedback inhibition involving a basolateral Na^+/Ca^{2+}-antiport and changes in Ca_c, is reasonably well established, although the changes in Ca_c have not been quantified except in one case [32].

It is less clear, to what extent the feedback inhibition remains operative when the serosal Na^+ is replaced not by choline, which is nonpermeant, but by K^+, which is highly permeant at the basolateral membrane. This replacement was used by several groups (see, for example, Refs. 3, 15, 16, 18, 19 and 37) to decrease voltage and resistance of the basolateral membrane in order to facilitate the study of apical Na^+ transport. Abolishment of the feedback inhibition would be an advantage if the mechanism of apical Na^+ translocation is to be investigated. We analysed for feedback inhibition in K^+-depolarized toad bladders by recording the amiloride-sensitive Na^+ conductance while Na_c was increasing in response to a sudden exposure to mucosal Na^+. The increase in Na_c was maximised by blocking active Na^+ extrusion with ouabain.

On exposing with 20 mM Na_m we observed a step increase in G_{Na} from zero to G_{Nao}, followed by a slow further increase. This response is expected from the apical Na^+ channels * since the mean Na^+ content of conducting Na^+ channels, and therefore G_{Na}, will increase rapidly with Na_m, and will increase further when Na_c rises. The response also shows that the feedback inhibition cannot prevent the initial phase of the increase in Na_c. After about 5 min the net increase in conductance gave way to a decrease, whose rate depended on the serosal Ca^{2+} concentration. At a minimal concentration of 3 μM the decrease was slow (Fig. 4). It may in part be explained by a conductance decrease of the basolateral membrane, caused by replacement of K_c by Na_c [15]. A direct inhibitory

* The use of calcium ionophores, like A23187, to increase Ca_c and study the resulting decrease of P_{Na} (see, for example, Refs. 34 and 36) is problematic since A23187, which exchanges Ca^{2+} against protons, may alter the cellular pH.

* The so-called Na^+ self-inhibition, i.e. the decrease in P_{Na} induced by an increase in Na_m, which is demonstrable at near constant Na_c, has a time constant of seconds [15,38] and would not contribute to changes in G_{Na} observed in these experiments on a timescale of minutes. In view of the conclusions reached by Bevevino and Lacaz-Vieira [23], it is necessary to point out that the demonstration of feedback inhibition, occurring at constant Na_m, should not be used as an argument against the direct P_{Na}-decreasing effect of Na_m (the self-inhibition). As pointed out rather early [38], both regulatory processes may be present. In fact, evidence for Na^+ self-inhibition in yet another tight epithelium, *Necturus* urinary bladder, was recently accured [39].

effect of Na_c on P_{Na} may also contribute, although, at least in the rabbit urinary bladder, this putative mechanism was shown to be absent [40]. When subsequently Ca_s was raised, the decrease in conductance became faster. However, without Na^+ being available to the cells, an increase of Ca_s even to 10 mM did not have this effect. The conductance recovered when Na_c was washed out and Ca_s lowered to 3 μM.

While the rate of conductance change is obvious from our data, the rate of feedback inhibition may differ from it to some extent, since other Na_c-dependent processes contribute to the net change in conductance observed. As mentioned before, these processes are: (1) the increase of G_{Na} with Na_c at constant P_{Na}; (2) a possible decrease of P_{Na} and, therefore, G_{Na} with increasing Na_c; and (3) a decrease of the basolateral conductance in response to replacement of K_c by Na_c, causing G_{Na} to be underestimated.

When starting the feedback inhibition (by increasing Ca_s) about 40 min after increasing Na_m, as was done in the experiment of Fig. 4, processes 1, 2 and 3 should already be completed or have settled down to a small rate. This rate will not be much influenced by the onset of the feedback inhibition since at 40 min Na_c must already be close to its final value. The initial inhibitory rate observed at this time was 8% per min at 10 mM Ca_s and presumably about 20 mM Na_c. The rate of feedback inhibition increased for increasing concentrations of Ca (Fig. 4B). At 1 mM Ca_s, the concentration commonly used in amphibian Ringers, this rate was very small (approx. 0.5%/min). Indeed, with 1 mM Ca_s no evidence for feedback inhibition was found with near-instantaneous current voltage curves of K^+-depolarized toad bladders (Palmer, L.G. and Lindemann, B., in preparation). Similarly, in rabbit urinary bladder exposed to normal Na_s and Ca_s values, no feedback inhibition was found with Na_c rising up to 20 mM [41] or even 30 mM [40]. The low rate of the Ca_s-dependent feedback inhibition also shows that the previous assessment of the much faster Na^+ self-inhibition [15] was not contaminated by the Ca_s-dependent inhibitory process.

In conclusion, our results with K^+-depolarized epithelia are explicable by feedback inhibition involving an increase in Ca_c mediated by basolateral exchange of Na_c against Ca_s. They suggest that at low Na_s (5 mM was used throughout) the Na^+/Ca^{2+} antiport operates not in the Ca^{2+}-extruding but in the Ca^{2+}-uptake direction, if cellular Na^+ is available. This change in the net transport direction was also observed with basolateral vesicles [11].

Since the conditions used in our experiments are somewhat artificial (i.e. low Na_s, high K_s and nearly zero basolateral potential), their possible effect on the rate of feedback inhibition has to be considered. Chase and Al-Awqati [11] estimated values of -28 mV and $+28$ mV for the 'driving force' of an obligatory $3Na^+/1Ca^{2+}$ exchange of non-depolarized preparations at 110 and 2.4 mM Na_m respectively. Substituting $Na_s = 5$ mM, $Na_c = 20$ mM, $Ca_s = 3$ mM and $V_{cs} = 0$ for the values used by them yields a force of -362 mV, i.e. a much larger driving force promoting serosal to cell flow of Ca^{2+} for the K^+-depolarized basolateral membrane *. Therefore if the rate of Na^+-Ca^{2+} exchange was proportional to its driving force the Ca^{2+}-dependent feedback inhibition should be higher in our preparation than under more physiological conditions. Yet, since the K^+-depolarization may have other conceivably inhibitory effects on the rate of Na^+-Ca^{2+} exchange, the estimation of the inhibitory rate under physiological conditions awaits further experimentation.

Thomas, Thompson and Schultz recently suggested (see footnote 5 in Ref. 39), that K^+-depolarization of *Necturus* urinary bladders impairs a mechanism which in the normally polarised epithelia defends Na_c against variations resulting from changes in Na_m. Our results imply that this putative mechanism does not involve feedback inhibition mediated by basolateral Na^+-Ca^{2+} exchange. Therefore, recruitment of basolateral pump-leak units, as suggested by the same authors [39] is

* As in Ref. 11 we assumed $Ca_c = 0.1$ μM. Substituting the serosal Na^+ by K^+ (the depolarization) should in principle increase Ca_c (Na_c-Ca_s exchange). However, in our experiments this substitution was done for zero Na_m (and thus nearly zero Na_c). Moreover the bladders were pre-equilibrated for several hours in the absence of mucosal Na^+, and in most cases this was done for $Ca_s = 3$ μM only. Thus one may expect that the value of Ca_c before 'Na^+ loading' was not higher than under normal physiological conditions.

more likely to keep Na_c constant when the net transport rate increases (data compatible with this 'forward stimulation' were occasionally reported in the pase (see, for example Refs. 9 and 42). In contrast to this mechanism, feedback inhibition appears to be a failsafe device which becomes operative when the deviations from normal ion distribution are more extreme.

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