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Regulation of Na^+ channel density at the apical surface of rabbit urinary bladder epithelium

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Abstract

We have investigated the effects of various manipulations on Na^+ transport across the rabbit urinary bladder epithelium. After bladders were mounted in Ussing chambers there was a spontaneous and significant (>4-fold) increase in amiloride-sensitive short-circuit current (equivalent to net Na^+ transport) over a 6-h period. The increase in current was almost abolished by brefeldin A, an inhibitor of anterograde vesicular transport, and reduced after a 3-h delay by cycloheximide, an inhibitor of protein synthesis. The spontaneous increase in short-circuit current was potentiated by treatment of bladders with either forskolin, which causes an elevation in cAMP levels, or aldosterone. Acting together, these two agents produced a significant synergistic effect on short-circuit current. The short-circuit current recovered rapidly after reduction in intracellular Na^+ levels, achieved either by lowering the extracellular Na^+ concentration or blockade of epithelial Na^+ channels with the sulphydryl modifying reagent *p*-chloromercuribenzenesulphonic acid (PCMBS). Recovery after PCMBS treatment was partially sensitive to brefeldin A. Short-circuit current saturated as the extracellular Na^+ concentration was increased (EC_{50} = 51 mM). Saturation occurred over a range of Na^+ concentrations in which single channel permeability is known to remain constant, indicating that it depends on a reduction in epithelial Na^+ channel density at the apical plasma membrane. Exposure of bladders to a high Na^+ concentration caused an increase in endocytotic activity, detected through an increase in the uptake of the fluid-phase marker fluorescein isothiocyanate (FITC)–dextran into vesicles located beneath the apical plasma membrane. We conclude that the urinary bladder epithelium is able to respond rapidly and efficiently to changes in its environment by regulating the density of epithelial Na^+ channels in its apical surface.

Keywords: Urinary bladder; Epithelial Na^+ channel; Vesicular transport; Endocytosis

1. Introduction

The rate-limiting step in ion movement across the rabbit urinary bladder, like other Na^+-transporting epithelia, has been identified as the entry of Na^+ ions through channels in the apical membrane (Lewis and Wills, 1983). Net Na^+ transport is determined by three main variables—the open state probability of epithelial Na^+ channels (usually of the order of 0.5; Garty and Palmer, 1997), the single channel conductance and the total number of channels in the membrane. In the rabbit urinary bladder, a number of factors are known to influence the number of functional epithelial Na^+ channels in the apical membrane of the urothelial cells. For example, Lewis and Clausen (1991) showed that enzymes in urine cause a progressive degradation of epithelial Na^+ channels. In addition, the density of epithelial Na^+ channels in the apical membrane is apparently modulated by the insertion or removal of membrane vesicles in response to stretching or relaxing of the epithelium, which occurs naturally as a consequence of bladder filling and emptying (Minsky and Chlapowski, 1978; Lewis and De Moura, 1982, 1984; Loo et al., 1983). It has been suggested that the consequent changes in transepithelial Na^+ transport might control the release of ATP from the basolateral surface, which in turn would have a signalling role via sensory neurones, relaying information about the degree of bladder distension to the spinal cord (Ferguson et al., 1997). Hormonal influences, including aldosterone (Lewis and Diamond, 1976) and factors which raise intracellular cyclic

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adenosine monophosphate levels (Burton et al., 2000), have also been shown to increase the number of functional epithelial Na^+ channels in the apical membrane. Finally, there is evidence that apical epithelial Na^+ channel density in the rabbit bladder is under the control of the Na^+ concentration in the bathing medium. For instance, the Na^+ permeability of epithelial Na^+ channels remains constant over a wide range of Na^+ concentrations (Lewis et al., 1984), whereas short-circuit current saturates (Lewis and Diamond, 1976), indicating that epithelial Na^+ channel density is changing. As reported by Lewis and Diamond (1976), the Na^+ concentration of rabbit urine can vary over a 20-fold range, from 16 to 310 mM, depending on dietary Na^+ intake. It is therefore to be expected that the urinary bladder epithelium should be able to adapt to this changing environment.

In the present study, we have investigated the responses of Na^+ transport across the urinary bladder epithelium to a number of manipulations, including incubation in an environment free of proteolytic enzymes, hormone stimulation, blockade of epithelial Na^+ channels and changes in extracellular Na^+ concentration. We interpret our results in the context of the vesicular delivery and removal of epithelial Na^+ channels at the apical domain of the plasma membrane.

2. Methods

2.1. Animals and materials

The rabbits were cared for according to Home Office guide lines and were killed in accordance with the UK Animals (Scientific Procedures) Act, 1986, and associated guidelines.

Urinary bladders of Dutch rabbits (obtained from Harlan UK) of either gender were used. The animals were killed either by cervical dislocation or by intravenous administration of sodium pentobarbital. Unless otherwise stated, reagents and drugs were obtained from Sigma (Poole, UK).

2.2. Ussing chamber experiments

Bladders were removed, washed in Krebs' solution (124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.1 mM $CaCl_2$, 1.4 mM KH_2PO_4 and 10 mM glucose), and halved vertically. No attempt was made to remove the underlying smooth muscle. The urothelium from each half of the bladder was mounted separately in Ussing chambers of 0.5 cm diameter, giving an exposed tissue area of 0.2 cm^2. One half of each bladder was the experimental preparation to which drugs were applied; the other half acted as a control. A DVC-1000 Dual Voltage Clamp (World Precision Instruments) was used to measure the transepithelial electrical potential difference via KCl-agar bridges. The current necessary to clamp the transepithelial potential difference to zero through Ag–AgCl electrodes is defined as the short-circuit current, which in this tissue has been demonstrated to represent the net transmembrane Na^+ transport (Lewis and Diamond, 1976). The tissue was gassed with 95% O_2 and 5% CO_2 and maintained at 37 °C.

Changes in bathing fluid Na^+ concentration were achieved through the use of three compositions of Krebs' solution. Standard Krebs' solution, containing both NaCl (124 mM) and $NaHCO_3$ (26 mM), had a Na^+ concentration of 150 mM. Low Na^+ Krebs' solution, with a Na^+ concentration of 26 mM, was produced by replacement of NaCl with *N*-methyl-D-glucamine chloride. A nominally Na^+-free Krebs' solution contained *N*-methyl-D-glucamine chloride and 10 mM HEPES as replacements for NaCl and $NaHCO_3$. The calculated osmolarity of all solutions was 330 mosM. Bathing fluid replacement to achieve different Na^+ concentrations was performed in the absence of any hydrostatic pressure gradient. The fluid resistances of the three solutions, as measured by the DVC-1000 voltage clamp, were identical within the limits of measurement.

Results are given as means ± S.E.M. The differences between means were assessed using a Student's *t*-test or an analysis of variance (ANOVA). In most cases, $P < 0.05$ was considered to be statistically significant.

2.3. Morphological studies

Muscle-intact rabbit urinary bladders were divided in half and established in separate Ussing chambers. In both preparations, the Krebs' solution bathing the apical surface was replaced with Krebs' solution containing 26 mM Na^+. A low apical Na^+ concentration was maintained for a 1-h period. In one preparation, the apical Na^+ concentration was then returned to 140 mM by the addition of standard Krebs' solution supplemented with fluorescein isothiocyanate (FITC)–dextran (molecular mass, 70 kDa; 5 mol of FITC per mol of dextran; Sigma), at a final FITC concentration of 1 mM. In the control hemibladder an equal concentration of FITC–dextran was added to the apical surface but the Na^+ concentration was maintained at 26 mM. Bladder preparations were exposed to FITC–dextran for 30 min, washed in Krebs' solution and then fixed in formalin. The tissue was washed, frozen in Tissuetec (Miles, Elkhat, IN, USA) and 10-μm transverse sections were cut. Sections were mounted on slides and examined using a Leica NT-TCS confocal laser scanning microscope, using a 100× objective lens with 1.0 numeric aperture. Images were collected using the 488-nm line of a krypton/argon laser with a 510- to 540-nm band-pass filter.

3. Results

3.1. Spontaneous changes in Na^+ transport with time

The amiloride-sensitive short-circuit current (equivalent to net Na^+ transport) generated by five untreated rabbit

bladders increased spontaneously with time over a 6.5-h period in vitro. The mean increase in short-circuit current was greater than 4-fold as shown in Fig. 1A. The rate of increase fell after about 4 h from the time at which the hemibladder was mounted in the Ussing chamber. Unlike the amiloride-sensitive current, the amiloride-insensitive current remained constant over the same period (Fig. 1A). Fig. 1B shows the amiloride-sensitive current at three time-points as a proportion of the total current. The proportion of the total bladder short-circuit current which was blocked by amiloride increased from 25.8 ± 4.2%, at 0.5 h after mounting the bladder to 67.3 ± 6.9% at 6.5 h (Student's *t*-test: $P < 0.01$, $n = 5$). Since the electrical resistance of the bladder urothelium remained constant (Fig. 1C), the transepithelial potential difference changed in proportion to the short-circuit current (Fig. 1D).

The spontaneous increase in short-circuit current was almost abolished by brefeldin A (5 μM; Fig. 2A). This fungal metabolite causes a rapid (< 15 min) blockade of anterograde vesicular transport, with a collapse of the Golgi complex into the endoplasmic reticulum (Lippincott-Schwartz et al., 1989). Brefeldin A has little effect on protein synthesis, and in contrast to its dramatic effects on the secretory pathway, it does not significantly affect either endocytosis or recycling at the plasma membrane (Lippincott-Schwartz et al., 1991). The protein synthesis inhibitor cycloheximide (100 μM; Fig. 2B) also reduced the spontaneous increase in current; however the effect of cycloheximide was delayed relative to that seen with brefeldin A.

3.2. Changes in Na^+ transport in response to hormones

Elevation of cAMP levels by forskolin or addition of aldosterone resulted in a time-dependent increase in the short-circuit current above control values (Fig. 3). The increase in short-circuit current took a considerable time to develop, and did not reach a maximum over the 6-h incubation period. Interestingly, a combination of the two treatments resulted in a large synergistic increase in Na^+ transport (Fig. 3). The increase in short-circuit current in response to both agents in combination became significant sooner and was of greater magnitude than with either agent alone. A peak increase in the amiloride-sensitive short-circuit current of 59.3 ± 16.6 μA cm^{-2} ($P < 0.05$, $n = 5$) above control values was seen after 5.25 h of aldosterone and forskolin treatment. This denoted a 10.0 ± 1.3-fold increase relative to controls. The synergistic interaction between aldosterone and forskolin was most marked after 3.75 h of drug treatment. At this time point aldosterone and forskolin alone increased the amiloride-sensitive short-circuit current above control values by 5.9 ± 3.3 (not significant, $n = 6$) and 10.9 ± 3.2 μA cm^{-2} ($P < 0.05$, $n = 4$), respectively, whilst in combination the increase in current was 47.3 ± 11.0 μA cm^{-2} ($P < 0.05$, $n = 5$).

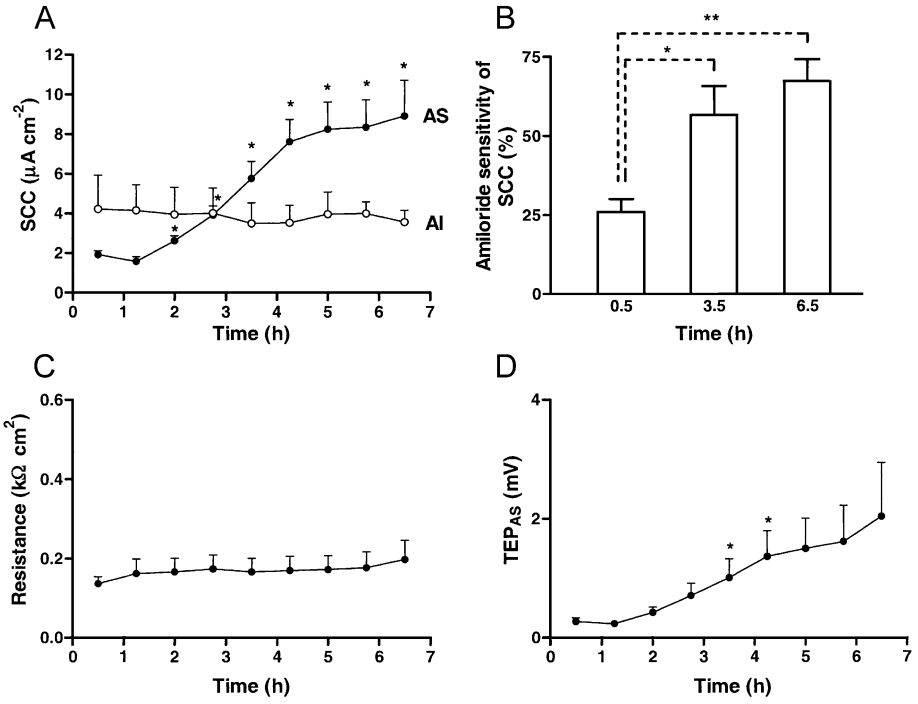

Fig. 1. Spontaneous time-dependent changes in Na^+ transport in rabbit bladders. Hemibladders were mounted in Ussing chambers (bathing fluid $[Na^+] = 150$ mM), and values of short-circuit current (SCC), amiloride-sensitive transepithelial potential difference (TEP_{AS}) and transepithelial resistance were determined at 45-min intervals over a 6.5-h period. (A) Amiloride-sensitive (AS) and amiloride-insensitive (AI) SCC determined by addition of 10 μM amiloride to the apical bathing solution. (B) Amiloride-sensitive SCC at three time-points, expressed as a percentage of total SCC. (C) Transepithelial resistance. (D) Amiloride-sensitive TEP. All values are means ± S.E.M. ($n = 5$). * $P < 0.05$; ** $P < 0.01$, comparison to value at 0.5 h by paired two-tailed Student's *t*-test.

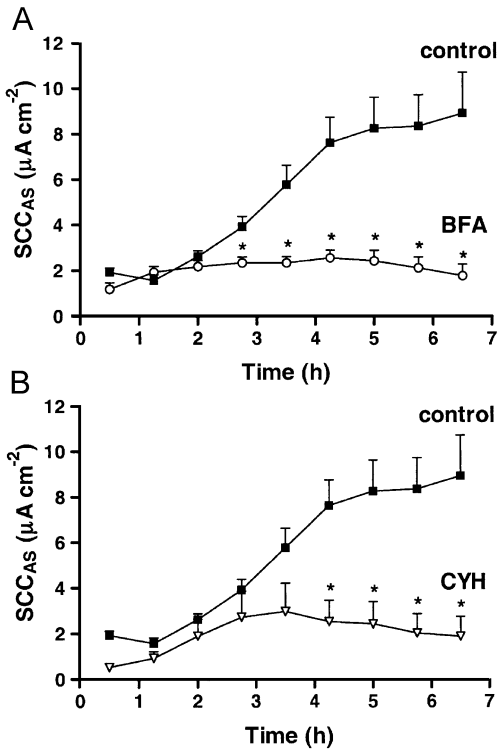

Fig. 2. Effects of brefeldin A and cycloheximide on spontaneous time-dependent changes in amiloride-sensitive SCC. Hemibladders were treated with either brefeldin A (BFA, 5 μM; A) or cycloheximide (CYH, 100 μM, B); the corresponding hemibladders acted as controls (bathing fluid [Na^+] = 150 mM). Reagents were added to both bathing solutions after the first amiloride response, and again after each wash. Amiloride-sensitive short-circuit current (SCC_{AS}) was determined at 45-min intervals over a 6.5-h period. Values are means ± S.E.M. ($n = 5$). * $P < 0.05$, comparison by ANOVA and Dunnett's test.

3.3. Changes in Na^+ transport in response to reduced apical Na^+ entry

The simplest way to reduce the entry of Na^+ across the apical surface of the urothelium is to reduce the apical Na^+ concentration. The short-circuit current response to a reduction in apical Na^+ concentration from 150 to 26 mM is shown in Fig. 4 (representative of three experiments). In the first 5-min period following the reduction in Na^+ concentration the short-circuit current fell to zero. In the subsequent 15-min period, the short-circuit current increased to a new steady state only marginally below the value prior to the reduction in Na^+ concentration. When the apical Na^+ concentration was restored, the new short-circuit current plateau that was attained greatly exceeded that prior to the reduction. The observed pattern of recovery and overshoot of short-circuit current strongly suggests that epithelial Na^+ channel density at the plasma membrane increases in response to the reduction of apical Na^+ concentration. The speed of recovery of the short-circuit current further suggests that no new protein synthesis is involved, but rather that epithelial Na^+ channels are delivered to the plasma membrane from a pre-formed and readily accessible pool.

An alternative means of reducing apical Na^+ entry into the urothelial cells is to treat them with *p*-chloromercuribenzenesulphonic acid (PCMBS). PCMBS is a sulphydryl-reactive agent that is believed to inhibit Na^+ transport irreversibly through a direct interaction with the epithelial Na^+ channel. Its actions have been demonstrated previously on the rabbit descending colon (Gottlieb et al., 1978) and the frog skin (Benos et al., 1980). After 14 min of

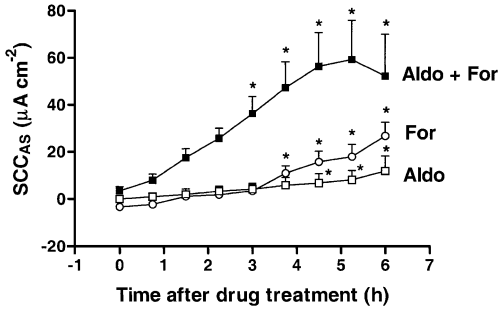

Fig. 3. Stimulation of Na^+ transport by aldosterone and forskolin. Hemibladders were treated with either aldosterone (Aldo, 5 μM in the basolateral bathing solution, $n = 6$), forskolin (For, 3 μM in both bathing solutions, $n = 4$), or both aldosterone and forskolin ($n = 5$); the corresponding hemibladders acted as controls (bathing fluid [Na^+] = 150 mM). Reagents were added to the bathing solutions after the first amiloride response, and again after each wash. Amiloride-sensitive short-circuit current (SCC_{AS}) was determined at 45-min intervals over a 6-h period, and control SCC_{AS} values were subtracted. Values are means ± S.E.M. All three drug treatments significantly increased the amiloride-sensitive SCC over the 6-h period (* $P < 0.05$, comparison by ANOVA and Dunnett's test).

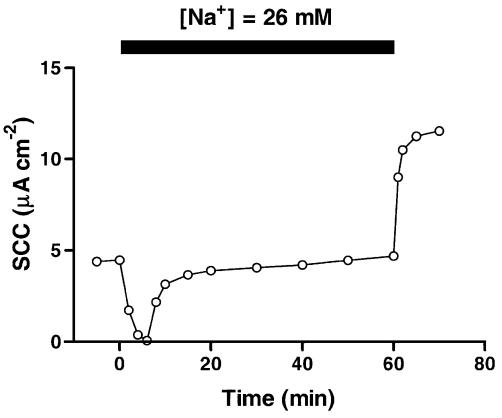

Fig. 4. Short-circuit current (SCC) response to a reduction in apical Na^+ concentration. Hemibladders were mounted in Ussing chambers and left for at least 4 h to allow a maximal amiloride-sensitive SCC to develop. At time zero, the Na^+ concentration of the apical bathing solution was reduced from 150 to 26 mM. After 60 min equilibration in low-Na^+ Krebs' solution (with Na^+ replaced by *N*-methyl-D-glucamine) the Na^+ concentration was restored to 150 mM. The Na^+ concentration of the basolateral reservoir remained unaltered at 150 mM throughout the experiment. The trace shown is representative of three experiments.

PCMBS treatment (2.5 mM) the amiloride-sensitive short-circuit current fell to 21 ± 2% ($P < 0.01$, $n = 7$) of its previous level (Fig. 5), and after removal of PCMBS, the current was restored within 90 min. The early phase of this restoration (i.e., up until 30 min) was completely resistant to brefeldin A, whereas this reagent blocked the rise of short-circuit current at later times (ANOVA: $P < 0.05$, $n = 6$). Our interpretation of this result is that the recovery of short-circuit current depends on the delivery of the epithelial Na^+ channel to the plasma membrane along two pathways—one from an endocytotic compartment, which is brefeldin A-resistant (Lippincott-Schwartz et al., 1991) and quickly exhausted, and another from the secretory pathway, which is brefeldin A-sensitive (Lippincott-Schwartz et al., 1989) and slower, as seen in the experiment illustrated in Fig. 2.

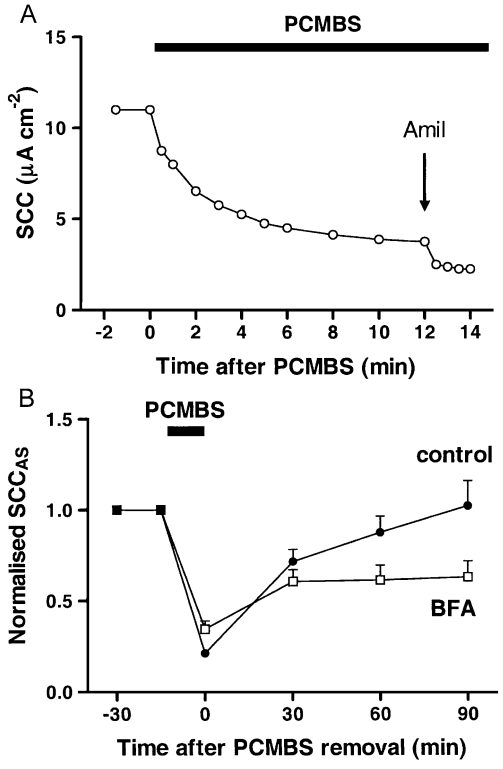

Fig. 5. Recovery of Na^+ transport after *p*-chloromercuribenzenesulphonic acid (PCMBS) treatment. Hemibladders were mounted in Ussing chambers and left for at least 4 h to allow a maximal amiloride-sensitive SCC to develop (bathing fluid $[Na^+] = 150$ mM). PCMBS (2.5 mM) was then added to the apical bathing solution, left for 14 min and removed by three successive replacements of the bathing solution. The amiloride-sensitive SCC was determined immediately prior to PCMBS removal and at 30-min intervals thereafter for 90 min. The black bars denote the duration of PCMBS treatment. Some hemibladders were pre-incubated for 90 min with brefeldin A (BFA, 5 mM; $n = 6$). Untreated hemibladders acted as controls ($n = 7$). Values are means ± S.E.M. of amiloride-sensitive SCC normalised by comparison with the SCC 30 min prior to PCMBS treatment. Amiloride-sensitive SCC increased significantly after PCMBS treatment, and this recovery was partially sensitive to brefeldin A ($P < 0.05$, ANOVA).

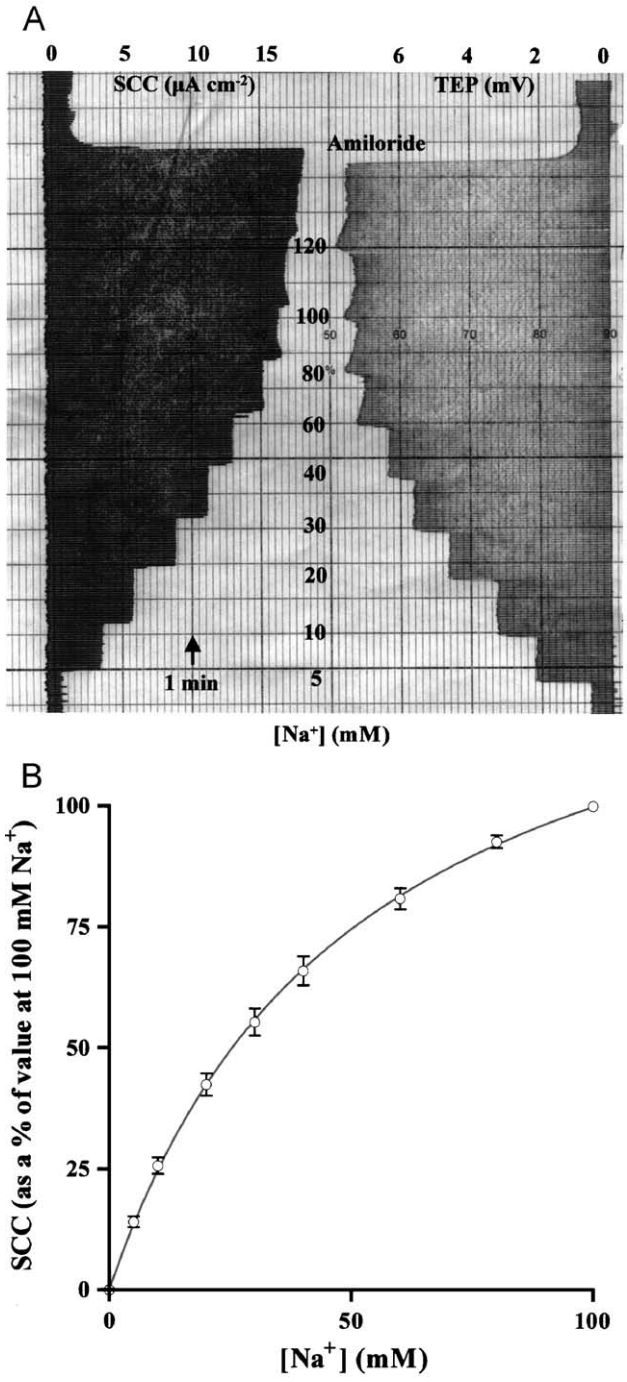

Fig. 6. Relationship between bathing fluid Na^+ concentration and total SCC. Hemibladders were allowed to equilibrate for 1 h in nominally Na^+-free Krebs' solution (with the Na^+ replaced by *N*-methyl-D-glucamine). Aliquots of standard Na^+-containing Krebs' solution were then added at 90-s intervals to achieve the indicated Na^+ concentrations. The composition of the bathing solutions was altered symmetrically. (A) Effect of stepwise changes in Na^+ concentration on SCC (left) and transepithelial potential difference (TEP) (right) in a typical experiment. At the end of the experiment amiloride (10 μM) was added to the apical reservoir. The SCC and TEP traces are out of phase by 30 s as a result of the pen alignment on the chart recorder. (B) Dependence of SCC on Na^+ concentration. SCC is expressed as a percentage of the SCC at 100 mM Na^+. Values are means ± S.E.M. ($n = 8$). The data were fitted to the Michaelis–Menten equation ($R^2 = 0.99$). Calculated values for SCC_{max} and EC_{50} were 151 ± 2% and 50.9 ± 1.2 mM Na^+, respectively.

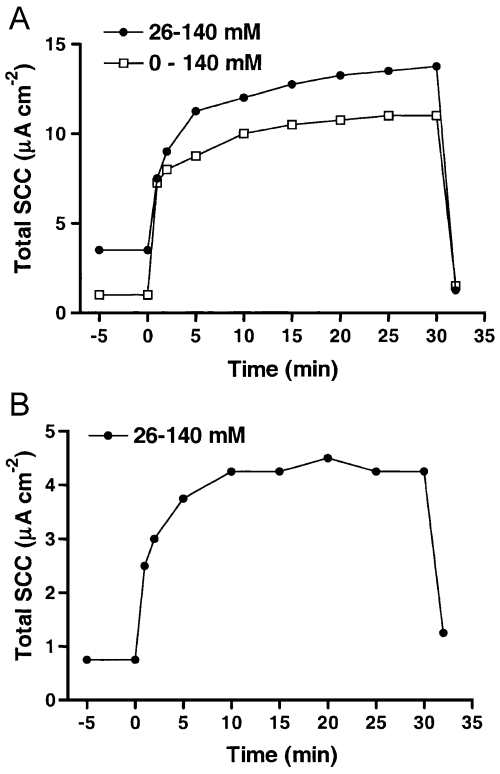

Fig. 7. Effect of an abrupt increase in bathing fluid Na^+ concentration on total SCC. Hemibladders were allowed to equilibrate for 1 h in Krebs' solution containing either 0 or 26 mM Na^+ (with the Na^+ replaced by *N*-methyl-D-glucamine). The Na^+ concentration was lowered either on both surfaces (A), or only on the apical surface (B). At time zero, the Na^+ concentration in both reservoirs was raised to 140 mM. After a further 30 min, amiloride (10 μM) was added to the apical bathing solution. Values are means from two experiments.

3.4. Changes in Na^+ transport in response to increases in Na^+ concentration in the bathing fluid

The changes in total short-circuit current and transepithelial potential difference occurring in response to stepwise additions of Na^+ were determined following exposure of the bladder to zero-Na^+ conditions for 1 h. The osmolarity of the bathing solutions remained constant, as Na^+ in the solutions was replaced by *N*-methyl-D-glucamine. The result from a typical experiment is shown in Fig. 6A. The most striking feature of this result is that the stepwise increases in both short-circuit current and transepithelial potential difference become smaller with repeated additions; in other words, Na^+ transport saturates as the Na^+ concentration increases. The overall results for eight replications of this experiment are shown in Fig. 6B. The hyperbolic curve was generated by fitting the data to the Michaelis–Menten equation. The calculated EC_{50} for Na^+ was 50.9 ± 1.2 mM.

The effect of an abrupt large increase in bathing fluid Na^+ concentration is shown in Fig. 7. The purpose of such experiments was to investigate whether the short-circuit current transiently peaked above its steady state value in response to an increased Na^+ concentration, as has been observed in the frog skin by Fuchs et al. (1977). In fact, a rise in bathing fluid Na^+ concentration, on either the apical surface alone or on both surfaces, produced a rise in the total short-circuit current to a new steady state over a 5-min period with no initial spike above the steady state value.

3.5. Visualisation of apical membrane endocytotic vesicles

The results presented above suggest that an elevation in apical Na^+ concentration triggers an endocytotic response

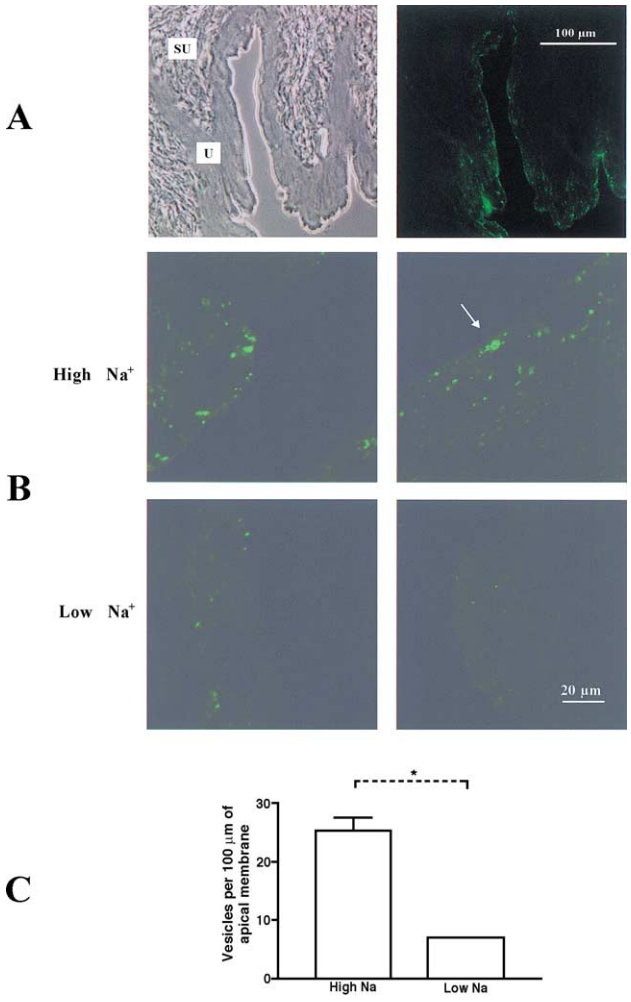

Fig. 8. Apical uptake of fluorescein isothiocyanate (FITC)–dextran in response to a rise in apical Na^+ concentration. (A) Images of a section of bladder tissue that had been subjected to an increase in apical Na^+ concentration in the presence of FITC–dextran. FITC uptake is shown (right panel) together with a phase contrast image (left panel) showing the urothelium (U) and sub-urothelium (SU). (B) Confocal images of the apical surface of the urothelium. Images depict preparations exposed to an increased apical Na^+ concentration (high Na^+) or control preparations (low Na^+). The arrow highlights a population of vesicles beneath the apical membrane. (C) Quantitation of FITC-labelled vesicles in a 10-μm zone beneath the apical membrane in preparations exposed to either high or low Na^+. Values are means ± S.E.M. of sub-apical vesicles per 100-μm length of apical membrane in 10 randomly selected areas of urothelium. * $P < 0.01$, comparison by unpaired Student's *t*-test.

that is responsible for the removal of epithelial Na^+ channels from the apical membrane. In order to establish whether a high-Na^+ challenge does indeed lead to the generation of a population of endocytotic vesicles, we investigated the effect of an increase in apical Na^+ concentration on the uptake of FITC–dextran, a fluid phase marker. We found that an increase in apical Na^+ concentration from 26 to 140 mM resulted in the uptake of FITC into a sub-apical zone (Fig. 8). When visualised by confocal microscopy, the pattern of FITC-staining was clearly vesicular. The number of FITC-labelled vesicles within a 10 μm-deep zone of the apical surface was 25 ± 2 per 100 μm of apical membrane. In contrast, the number of FITC-labelled sub-apical vesicles in control sections, that had been exposed to the same concentration of FITC–dextran for the same time period but in the absence of an increase in Na^+ concentration, was 7 ± 2 per 100 μm.

4. Discussion

The density of functional epithelial Na^+ channels at the apical surface of the rabbit bladder epithelium must depend on the balance between delivery of channels via vesicle fusion and removal by endocytosis or degradation. It has been shown previously (Lewis and Clausen, 1991) that epithelial Na^+ channels are sensitive to urinary enzymes, and that their degradation occurs in three steps: loss of response to amiloride, loss of ionic selectivity and lastly loss of the functional channel from the membrane. In our experiments, we remove the bladder from contact with these enzymes and mount the tissue in an enzyme-free solution. Under these conditions, it is likely that the constitutive delivery of epithelial Na^+ channels to the apical membrane continues for a time until cellular regulatory processes reduce it. In our experiments, we found a clear, time-dependent increase in both short-circuit current and in the proportion of the Na^+ transport which is inhibitable with amiloride, indicating that epithelial Na^+ channel density in the apical membrane of the urothelium increases over a period of several hours.

The rapid effect of brefeldin A on the spontaneous rise in short-circuit current is likely to be a consequence of its ability to cause a rapid inhibition of anterograde vesicular transport. Brefeldin A inhibits a guanine nucleotide exchange factor, which catalyses the exchange of guanine nucleotides bound to ADP-ribosylation factor (ARF); ARF in turn is involved in the generation of transport vesicles on the secretory pathway (Donaldson et al., 1992; Helms and Rothman, 1992). In contrast, the short-circuit current continues to rise almost as rapidly as normal for about 3 h after inhibition of protein synthesis by cycloheximide, presumably because epithelial Na^+ channels already on the secretory pathway are still being delivered to the plasma membrane. The observation that it takes 3 h to deplete the secretory pathway of epithelial Na^+ channels is consistent with previous estimates of the rates of constitutive protein transport to the plasma membrane (Daniels and Edwardson, 1988).

Lewis and Diamond (1976) showed that aldosterone increased Na^+ transport across rabbit bladders, and Burton et al. (2000) demonstrated increases in epithelial Na^+ channel activity over several hours in response to agents that increase intracellular cAMP levels. Stimulation of Na^+ transport by both of these mechanisms was shown to be dependent on the insertion of new epithelial Na^+ channels into the apical plasma membrane. This paper demonstrates for the first time the effect of simultaneous activation of both signalling systems in this tissue. The result is a synergistic interaction resulting in a potentiation of the maximum current generated and a reduction in the time taken for this response to occur. The physiological significance of this effect awaits the identification of the hormone responsible for cAMP elevation, which appears not to be vasopressin (Burton et al., 2000).

When the Na^+ concentration in the apical bathing reservoir was decreased from 150 to 26 mM, the short-circuit current initially fell to zero, and then returned almost to its original value within 20 min. This result would be expected of an epithelium that actively transports only Na^+ ions, as has been suggested by tracer flux studies (Lewis and Diamond, 1976), and also supports the notion that apical epithelial Na^+ channel activity responds to changes in Na^+ concentration in the bathing solution. When the bathing fluid Na^+ concentration was returned to its original level the new short-circuit current plateau greatly exceeded that prior to the reduction, thus confirming that channel activity had increased under low-Na^+ conditions.

PCMBS was used as a tool to distinguish between feedback inhibition and self-inhibition of Na^+ transport. As classified by Lindemann (1984), self-inhibition of apical Na^+ entry is believed to reflect a direct inhibitory interaction between the channel and external Na^+. Such a mechanism was first demonstrated in the frog skin (Fuchs et al., 1977). After a step increase in external Na^+ concentration, the short-circuit current and apical Na^+ permeability were shown to be transiently larger than their steady-state values. The decrease to a steady-state occurred within seconds, and before any change in intracellular Na^+ activity was recorded. It was proposed that external Na^+ acts as an inhibitory allosteric effector of the Na^+ channel. A similar phenomenon has been described in *Xenopus* oocytes expressing bovine epithelial Na^+ channels (Kroll et al., 1991) and in rat cortical collecting duct cells (Palmer et al., 1998). In contrast, feedback inhibition (a concept first proposed by MacRobbie and Ussing, 1961) is a reduction in apical Na^+ channel activity in response to a rise in intracellular Na^+ activity and has been demonstrated in many cell types (reviewed by Turnheim, 1991). In recent years, the regulation of apical Na^+ entry by intracellular Na^+ has been studied in detail in salivary duct cells. It was demonstrated that the amiloride-sensitive Na^+ conductance

in mouse mandibular salivary duct cells decreased as the intracellular Na^+ concentration increased (Komwatana et al., 1996). Noise analysis revealed that the decline in whole-cell Na^+ current was not accompanied by a change in single channel conductance, suggesting that a reduction in epithelial Na^+ channel density at the apical membrane was involved. It was subsequently shown that cytosolic application of amiloride analogues blocked feedback inhibition of Na^+ channels by cytosolic Na^+, indicating that these agents act upon a previously unrecognised intracellular Na^+ receptor (Komwatana et al., 1998). A downstream target of this receptor is Nedd4, a ubiquitin-protein ligase that binds to the cytoplasmic C-terminal PY motifs of β- and γ-epithelial Na^+ channel subunits. In response to Nedd4-mediated ubiquitination, apical epithelial Na^+ channel activity is reduced (Dinudom et al., 1998), and both anti-Nedd4 antibodies and a Nedd-4 fusion protein are able to block the inhibition of epithelial Na^+ channel activity by increased cytosolic Na^+.

Eaton (1981) demonstrated that a sustained reduction in apical Na^+ entry into the urothelial cells of the rabbit bladder, by treatment with amiloride, decreased intracellular Na^+ activity in this tissue over a 30-min period. PCMBS addition to the rabbit bladder would be expected to cause a similar reduction in intracellular Na^+ activity, permitting an investigation of the effects of a reduction in intracellular Na^+ concentration in the absence of a change in external Na^+ concentration. We found that PCMBS inhibited the short-circuit current across the rabbit urinary bladder, without an initial stimulatory effect. This result is in contrast to the response of the frog skin, where PCMBS produces an initial stimulation most likely by preventing self-inhibition, perhaps through interference with an allosteric effector site for external Na^+ (Fuchs et al., 1977). On removal of PCMBS, the amiloride-sensitive short-circuit current recovered rapidly. In light of the irreversible nature of PCMBS action, the most likely explanation for the recovery in short-circuit current is the insertion of epithelial Na^+ channels into the apical plasma membrane. Furthermore, the ability of brefeldin A to inhibit the late phase but not the early phase of the recovery suggests that the epithelial Na^+ channels are delivered initially from an endosomal compartment and then later from the secretory pathway. As the extracellular Na^+ concentration was unchanged in this experiment, it seems likely that epithelial Na^+ channel activity was being modulated by feedback regulation, rather than self-inhibition.

When the bathing fluid Na^+ concentration was increased in a stepwise fashion from 0 to 120 mM, the short-circuit current saturated. The EC_{50} for short-circuit current saturation was 51 mM, which corresponds closely with the urinary Na^+ concentration of rabbits fed on a normal diet (Lewis and Diamond, 1976). The important feature of the saturation in short-circuit current with bathing fluid Na^+ concentration is that it occurred over a concentration range in which single channel permeability remains constant, as determined by noise analysis (Lewis et al., 1984). The implication is that a rise in external Na^+ concentration, acting either directly (self-inhibition) or through a rise in intracellular Na^+ (feedback inhibition), inhibits apical channel activity.

In frog skins, where self-inhibition of Na^+ entry has been demonstrated (Fuchs et al., 1977), the short-circuit current responds to an abrupt increase in external Na^+ concentration by transiently spiking above the new steady state value. This was not demonstrated in the rabbit urinary bladder. However, such a demonstration is dependent on the rapid implementation of a concentration change since in the frog skin the duration of the short-circuit current spike above the new steady state lasted only seconds. Fuchs et al. (1977) achieved rapid concentration changes using a fast-flow chamber design. As this design was not employed in this study, self-inhibition cannot be excluded as a contributing mechanism for the saturation of short-circuit current across the rabbit urinary bladder.

An increase in the extracellular Na^+ concentration from 26 to 140 mM was accompanied by a stimulation in endocytotic activity at the apical membrane, as assessed using FITC–dextran as a fluid-phase marker. Many FITC-labelled vesicles were visualised in a sub-apical zone 30 min after the increase in apical Na^+ concentration. Although we have presented no direct evidence for the presence of epithelial Na^+ channels in the endocytotic vesicles, the results described above, together with previous work (Lewis and De Moura, 1984), strongly suggest that this sub-apical vesicular pool contains epithelial Na^+ channels that have been removed from the plasma membrane. This membrane compartment might also represent the pool from which epithelial Na^+ channels are recycled to the plasma membrane in response to manipulations such as treatment of the epithelium with PCMBS.

This study has demonstrated that Na^+ transport across the urinary bladder epithelium responds in a dynamic manner to changes in urinary composition that occur under physiological conditions. A major mechanism by which the bladder regulates its transepithelial Na^+ transport is through modulation of the transport of epithelial Na^+ channels into and out of its apical surface. The molecular details underlying the flow of membranes and proteins within cells are now emerging, and it is already clear that the intracellular transport of epithelial Na^+ channels involves specific interactions with the membrane fusion protein, syntaxin (Qi et al., 1999; Saxena et al., 1999), and with components of clathrin-coated pits (Shimkets et al., 1997). The potential involvement of proteins such as these in epithelial Na^+ channel processing in the urinary bladder epithelium now needs to be investigated.

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