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Activation of epithelial Na^+ channel activity in the rabbit urinary bladder by cAMP

Timothy J. Burton*, Sohier Elneil, Carl P. Nelson, Douglas R. Ferguson

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, UK

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Abstract

The rabbit urinary bladder actively absorbs Na^+ from the urine. The rate-limiting step in this process is the diffusion of Na^+ across the apical membrane of bladder epithelial cells, mediated by amiloride-sensitive epithelial Na^+ channels. We have investigated the effects of cAMP on epithelial Na^+ channel activity in the rabbit bladder by measuring the amiloride-sensitive short-circuit current across bladders mounted in Ussing chambers. Three agents that raise intracellular cAMP levels (forskolin, dibutyryl-cAMP and 3-isobutyl-1-methylxanthine (IBMX)) increased the amiloride-sensitive short-circuit current relative to control preparations. The forskolin-induced increase in amiloride-sensitive short-circuit current was significantly inhibited by the vesicle fusion inhibitor brefeldin A and the protein synthesis inhibitor cycloheximide. These findings, together with the magnitude and protracted time course of the cAMP effects, suggests that cAMP stimulates the insertion of new Na^+ channels into the apical membrane of the rabbit bladder epithelium.

Keywords: Epithelial Na^+ channel; cAMP; Urinary bladder

1. Introduction

The epithelial Na^+ channel, characterized by its sensitivity to the diuretic drug amiloride, is the rate-limiting step in Na^+ absorption across a number of epithelia including the distal nephron, colon, lung and urinary bladder. Active Na^+ absorption across the mammalian urinary bladder was first demonstrated in the rabbit by Lewis and Diamond (1976). They suggested that this active Na^+ absorption was necessary to maintain ion gradients established by the kidney. We have since proposed an alternative role for Na^+ absorption in the mammalian bladder (Ferguson et al., 1997), namely that epithelial Na^+ channels in the rabbit bladder are mechanosensitive and may have a sensory role in detecting distension of the bladder wall.

Many factors are now known to regulate epithelial Na^+ channel activity and this is currently an area of intense research (review by Garty and Palmer, 1997). The ability of cAMP to regulate epithelial Na^+ channel activity was highlighted in studies on the action of anti-diuretic hormone on amphibian epithelia and mammalian collecting tubules (Handler and Orloff, 1981). The binding of anti-diuretic hormone to vasopressin V_2 receptors and subsequent activation of adenylate cyclase results in increased epithelial Na^+ channel activity and Na^+ absorption in these tissues.

Two alternative mechanisms for a cAMP-mediated increase in epithelial Na^+ channel activity have been proposed (review by Smith, 1999). Firstly, a direct phosphorylation of the epithelial Na^+ channel or an associated regulatory protein activating quiescent channels pre-existing in the membrane. Secondly, that phosphorylation of vesicle trafficking or cytoskeletal proteins results in the insertion of new channels into the cell membrane. In agreement with the latter mechanism, Erlij et al. (1999) recently showed that the forskolin-stimulated increase in Na^+ conductance in toad kidney cells (A6 cells) was accompanied by changes in membrane capacitance suggesting the insertion of channels from an intracellular pool.

* Corresponding author. Tel.: +44-1223-334065; fax: +44-1223-334040.

E-mail address: tjb23@hermes.cam.ac.uk (T.J. Burton).

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In this paper, we provide the first demonstration that the magnitude of epithelial Na^+ channel activity in the epithelium of the rabbit urinary bladder is enhanced by cAMP. We show that epithelial Na^+ channel activity (as measured by amiloride-sensitive short-circuit current) is increased in response to three agents that raise intracellular cAMP levels: forskolin (an activator of adenylate cyclase) dibutyryl-cAMP (a membrane permeable cAMP analogue) and 3-isobutyl-1-methylxanthine (IBMX) (a phosphodiesterase inhibitor). We present data using brefeldin A (an inhibitor of vesicular trafficking) that suggests the increase in epithelial Na^+ channel activity is due to insertion of new channels from an intracellular pool. In addition, we show that the effects of forskolin are blocked by the protein synthesis inhibitor cycloheximide suggesting that cAMP may stimulate the synthesis of new channel subunits in this tissue. With relevance to the cycloheximide effect, we are interested to note that a putative cAMP response element has recently been described in the α-subunit of the mouse epithelial Na^+ channel gene (Dagenais et al., 1999).

We do not believe that these results are of relevance to the action of anti-diuretic hormone, as this hormone has been shown to have no effect on the short-circuit current across the rabbit urinary bladder (Lewis and Diamond, 1976). At present, we have not identified an endogenous mediator that raises cAMP levels in this tissue and this remains an area of ongoing research.

2. Methods

2.1. Animals

Dutch rabbits of either gender were used in these experiments. The animals weighed less than 1 kg on purchase. Rabbits were killed by intravenous administration of sodium pentobarbitone (200 mg ml^{-1}). The urinary bladder was identified, dissected free and washed in cold Krebs' solution. The bladder was opened vertically from the urethral orifice to the fundus and then divided into two equal halves in the plane of the original incision. The epithelial lining of the bladder (the urothelium) was separated from the underlying detrusor muscle by injecting cold Krebs' solution into the suburothelial plane and cutting the muscle away.

2.2. Ussing chamber: measurement of short-circuit current

Muscle-stripped hemibladders were mounted to act as a diaphragm of 0.2 cm^2 between two halves of an Ussing chamber. Oxygenated Krebs' solution (20 cm^3) maintained at 37°C by means of thermostatted water jackets, was circulated on each side of the bladder.

A DVC-1000 dual voltage clamp (World Precision Instruments, Sarasota, FL, USA) was used to measure the

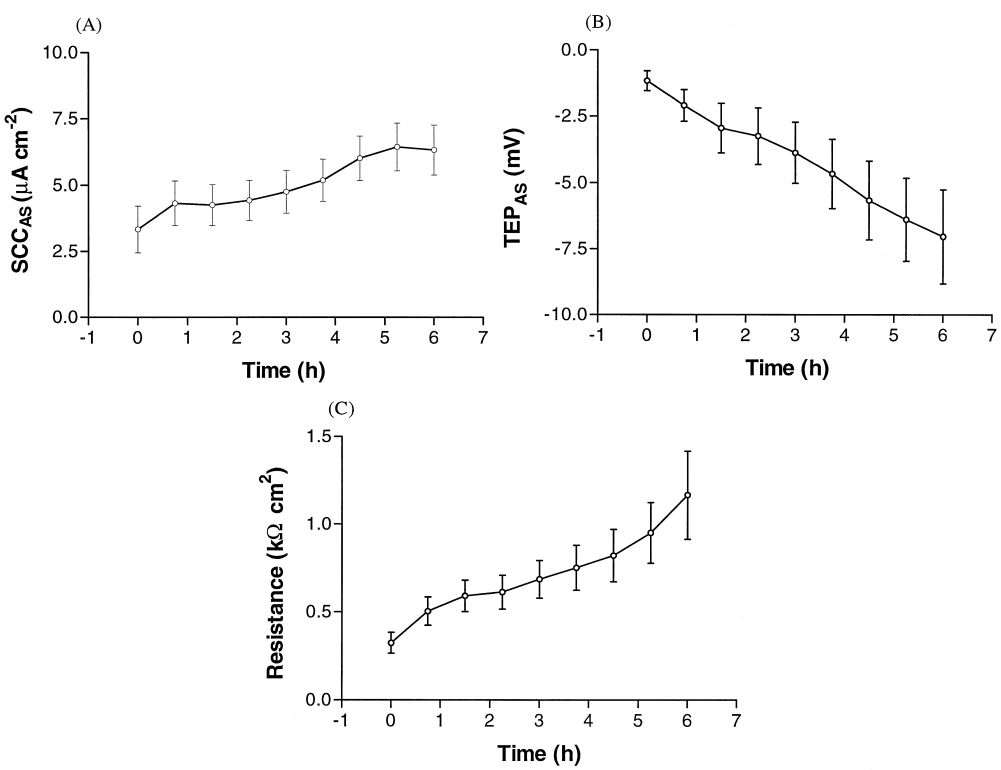

Fig. 1. (A) Amiloride-sensitive short-circuit current (SCC_{AS}). (B) Amiloride-sensitive transepithelial potential difference (TEP_{AS}). (C) Transepithelial resistance. These values are shown over a 6-h period for 20 control hemibladders.

transepithelial potential difference and short-circuit current. Matched voltage-measuring calomel electrodes (type SR4, Russell) were connected via 3 M KCl-agar bridges to the bathing solutions on either side of the bladder. To measure short-circuit current, the transepithelial potential difference was clamped to zero by passing current through Ag–AgCl electrodes connected to the bathing solutions via KCl-agar bridges. Dual measurements of short-circuit current and transepithelial potential difference were obtained using the timer function on the DVC-1000 by clamping the voltage to zero for 1 s at a frequency of 12 min^{-1}. Output, in terms of either a short-circuit current or transepithelial potential difference, was recorded on a Kipp and Zonen flat bed recorder.

2.3. Amiloride-sensitive short-circuit current

The amiloride-sensitive short-circuit current was calculated as the decrease in short-circuit current, 2 min after the application of 10 μM amiloride (a maximal dose) to the apical bathing solution. Measurements of amiloride-sensitive short-circuit current were taken 30 min after mounting the bladder in the Ussing chamber and at 45 min intervals thereafter. After each amiloride response, the Krebs' solutions were replaced.

2.4. Drug additions

The effects of forskolin, dibutyryl-cAMP and IBMX on amiloride-sensitive short-circuit current were determined by the addition of the drug to both apical and basolateral bathing solutions after the first amiloride response. Repeat drug additions were given each time the bathing solutions were replaced. In all experiments, the second half of the bladder acted as a control preparation.

The effects of brefeldin A and cycloheximide on forskolin-induced amiloride-sensitive short-circuit current were determined by repeat additions of the drugs to both bathing solutions each time the forskolin was added.

2.5. Reagents

The composition of the Krebs' solution was (in mM): NaCl 123.9, $NaHCO_3$ 26.0, KCl 5.0, $CaCl_2$ 1.1, $MgSO_4$ 1.3, KH_2PO_4 1.4 and glucose 10.0. The solution was oxygenated with 95% O_2 and 5% CO_2 and pH adjusted to 7.4.

Forskolin, dibutyryl-cAMP and IBMX were dissolved in ethanol. Solutions of amiloride and cycloheximide were made up in distilled water. Brefeldin A was dissolved in methanol. Solvent controls were performed where necessary.

All reagents were obtained from Sigma (Poole, UK).

2.6. Statistics

Results are expressed as means ± S.E.M. Statistical significance of differences in amiloride-sensitive short-circuit current between drug-treated and control preparations was determined using paired two-tailed Student's *t*-tests. Statistical testing of the effects of brefeldin A and cycloheximide on forskolin-stimulated amiloride-sensitive short-cir-

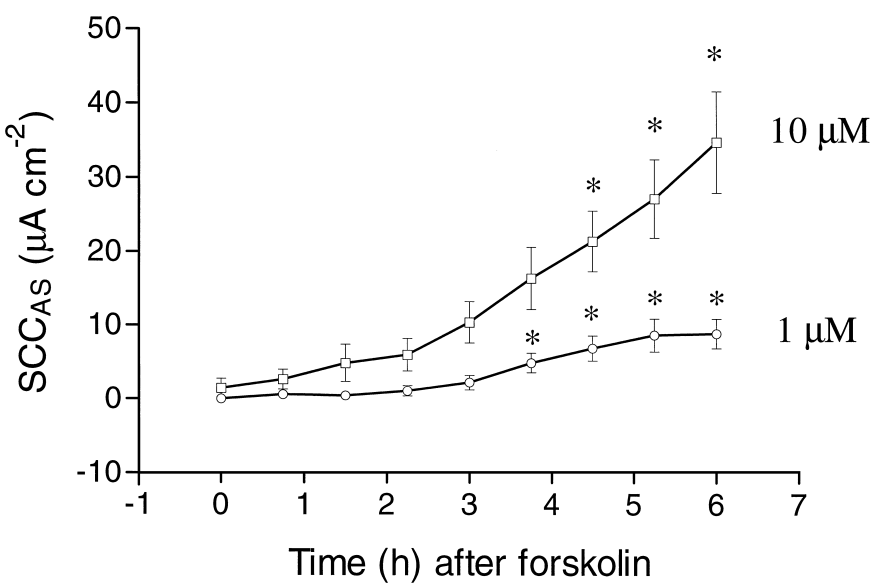

Fig. 2. Time-dependent increase in SCC_{AS} produced by forskolin. This is given as the additional SCC_{AS} in forskolin-treated bladders compared to control bladders for the 6-h period after forskolin treatment. The effects of 1 μM ($n = 5$) and 10 μM ($n = 5$) forskolin are shown. Values are given as means ± S.E.M. (* denotes $P < 0.05$, *t*-test).

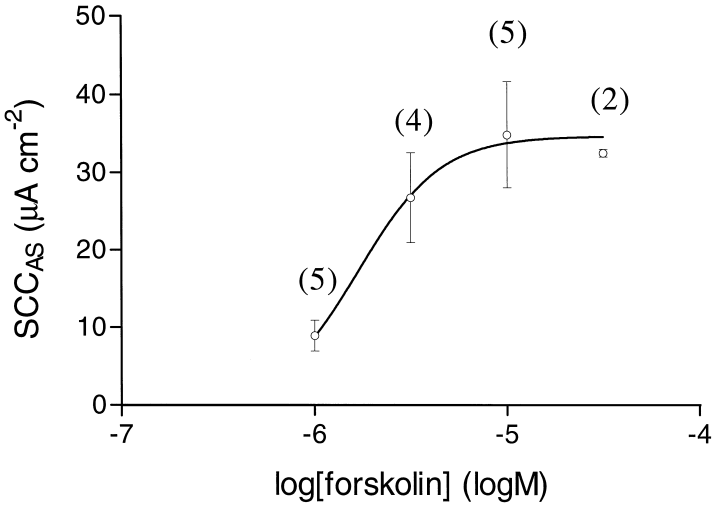

Fig. 3. Log dose–response curve for stimulation of SCC_{AS} by forskolin. The SCC_{AS} induced 6-h after treatment with different concentrations of forskolin is shown (*n* values are given in brackets). The curve was generated by fitting the data to the four parameter logistic equation. The EC_{50} is 1.7 μM and the Hill slope is 2.0.

cuit current was performed using a two-way analysis of variance test.

3. Results

3.1. Short-circuit current and transepithelial potential difference

When mounted in Ussing chambers, rabbit urinary bladders displayed a mucosa-negative transepithelial potential difference that varied from 0.5 to 50 mV and a short-circuit current that varied from 1.5 to 20.0 μA cm^{-2} in the absence of drug treatment. The larger degree of variation in transepithelial potential difference measurements reflects the variable degree of edge damage, which occurs unavoidably when the bladder is clamped in the Ussing chamber. It is for this reason that short-circuit current measurements have been used throughout.

It has been shown using tracer flux measurements (Lewis and Diamond, 1976) that active Na^+ absorption from the urine accounts for all the short-circuit current in the rabbit bladder.

3.2. Inhibition of short-circuit current by amiloride

A large proportion of the short-circuit current was inhibited by the epithelial Na^+ channel blocker amiloride.

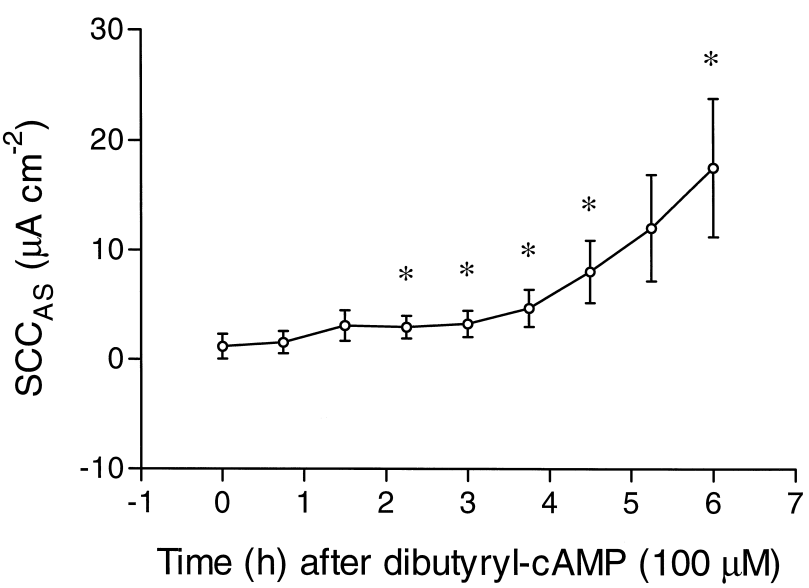

Fig. 4. Time-dependent increase in SCC_{AS} produced by dibutyryl-cAMP This is given as the additional SCC_{AS} in bladders treated with 100 μM dibutyryl-cAMP compared to controls. Values are given as means ± S.E.M. for six preparations (* denotes $P < 0.05$, *t*-test).

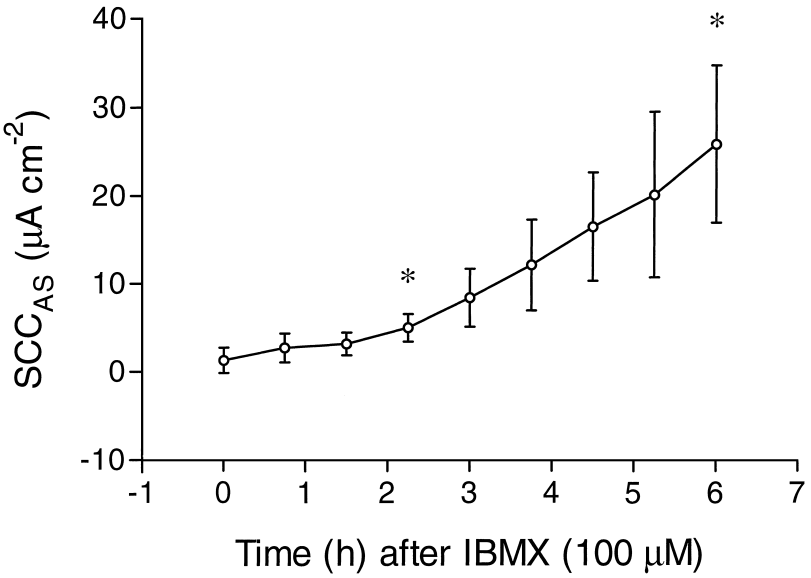

Fig. 5. Time-dependent increase in SCC_{AS} produced by IBMX. This is given as the additional SCC_{AS} in bladders treated with 100 μM IBMX compared to controls. Values are given as means ± S.E.M. for five preparations (* denotes $P < 0.05$, *t*-test).

The ability of this diuretic drug to block the apical Na^+ entrance pathway in Na^+-absorbing epithelia was first demonstrated by Bentley (1968) in the toad bladder. It has been suggested that the amiloride-insensitive component of the short-circuit current in the rabbit bladder can be accounted for by degraded Na^+ channels that have lost their amiloride sensitivity (Lewis and Clausen, 1991).

Amiloride produced a dose-dependent inhibition of short-circuit current across the rabbit bladder when applied to the apical bathing solution. The IC_{50} for this effect was 3×10^{-7} M. Amiloride had a maximum effect at 10^{-5} M and was without effect when added to the basolateral bathing solution. The effect of amiloride was rapidly reversed by replacement of the apical Krebs' solution.

Fig. 1(A) shows the amiloride-sensitive short-circuit current for control hemibladders. A gradual upward trend in the amiloride-sensitive short-circuit current was seen with time, but this was minimal when compared to preparations treated with cellular cAMP-raising drugs. Fig. 1(B) and (C) show, respectively, the amiloride-sensitive transepithelial potential difference and transepithelial resistance for control hemibladders. The increase in resistance with

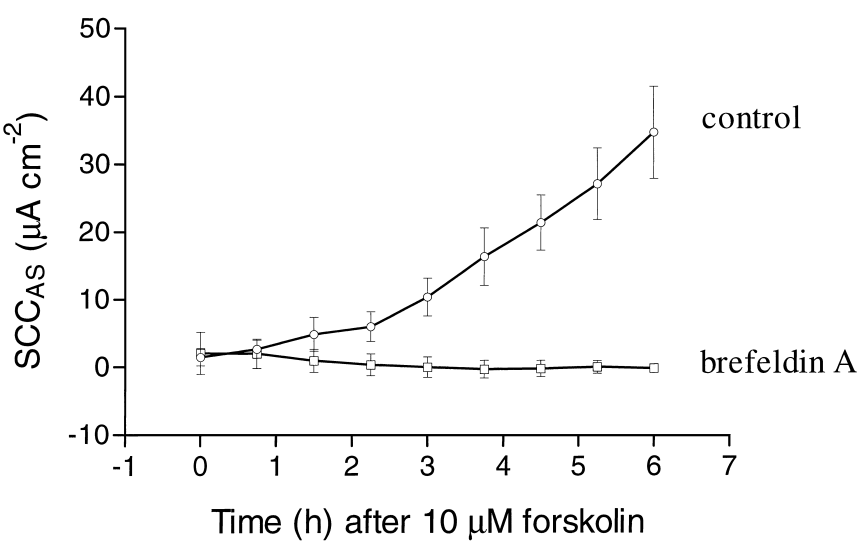

Fig. 6. Inhibition of forskolin-stimulated SCC_{AS} by brefeldin A. The SCC_{AS} produced by treatment with 10 μM forskolin is shown in the presence (□) and absence (○) of 5 μM brefeldin A. Values are given as means ± S.E.M. for five preparations. Brefeldin A produces a significant inhibition of the forskolin-stimulated current ($P < 0.01$, two-way analysis of variance).

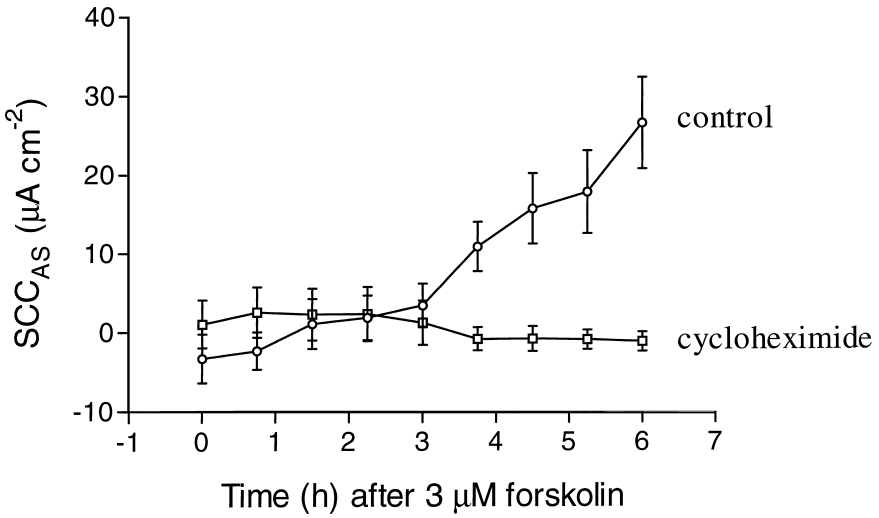

Fig. 7. Inhibition of forskolin-stimulated SCC_{AS} by cycloheximide. The additional SCC_{AS} produced by treatment with 3 μM forskolin is shown in the presence (□) and absence (○) of 100 μM cycloheximide. Values are given as means ± S.E.M. for five preparations. Cycloheximide produces a significant inhibition of the forskolin-stimulated current ($P < 0.01$, two-way analysis of variance).

time reflects an improvement in the seal between tissue and chamber.

3.3. Activation of amiloride-sensitive short-circuit current by agents that raise intracellular cAMP levels

Fig. 2 shows a dose-dependent increase in amiloride-sensitive short-circuit current produced by forskolin. The addition of forskolin at concentrations of 1 μM and 10 μM significantly increased the amiloride-sensitive short-circuit current relative to control preparations. Six hours after these forskolin additions, the amiloride-sensitive short-circuit current increased by 8.9 ± 2.0 and 35.8 ± 8.7 μA cm^{-2} ($P < 0.05$), respectively. This represents a 2.8 ± 0.5-fold and 7.4 ± 1.3-fold increase in amiloride-sensitive short-circuit current relative to controls. The forskolin-induced activation of amiloride-sensitive short-circuit current did not become significant until at least 3.75 h after the addition.

Fig. 3 shows a log dose response curve for the activation of amiloride-sensitive short-circuit current by forskolin. Each point on the curve shows the additional amiloride-sensitive short-circuit current in preparations treated for 6 h with different forskolin concentrations compared to controls. The EC_{50} for forskolin-induced activation of amiloride-sensitive short-circuit current is 1.7 μM.

The membrane-permeable cAMP analogue, dibutyryl-cAMP, produced a similar time-dependent increase in amiloride-sensitive short-circuit current (Fig. 4). Dibutyryl cAMP (100 μM) significantly increased the amiloride-sensitive short-circuit current by 17.5 ± 6.3 μA cm^{-2} ($P < 0.05$), 6 h after addition. Relative to control preparations, this denotes a 4.1 ± 0.9-fold increase.

The phosphodiesterase inhibitor, IBMX, also significantly increased the amiloride-sensitive short-circuit current (Fig. 5). Six hours after the addition of 100 μM IBMX, the amiloride-sensitive short-circuit current was increased by 25.7 ± 8.9 μA cm^{-2} ($P < 0.05$), denoting a 5.0 ± 0.6-fold increase compared to controls.

3.4. Inhibition of forskolin-stimulated amiloride-sensitive short-circuit current by brefeldin A and cycloheximide

Fig. 6 shows the effect of 10 μM forskolin on the amiloride-sensitive short-circuit current in the presence and absence of 5 μM brefeldin A. Brefeldin A significantly inhibited the increase in amiloride-sensitive short-circuit current induced by a maximal dose of forskolin ($P < 0.01$, as determined by a two-way analysis of variance test).

Cycloheximide also inhibited the forskolin-induced amiloride-sensitive short-circuit current (Fig. 7). The effect of 3 μM forskolin is significantly reduced in the presence of 100 μM cycloheximide ($P < 0.01$, as determined by a two-way analysis of variance test).

4. Discussion

The experiments presented in this paper demonstrate that the amiloride-sensitive short-circuit current across the rabbit urinary bladder is significantly increased by three agents that raise intracellular cAMP levels. An increase in amiloride-sensitive short-circuit current signifies a change in the Na^+-transporting capabilities of the apical membrane of the rabbit bladder epithelium, as this constitutes the rate-limiting step for amiloride-sensitive Na^+ transport (Lewis and Wills, 1983). Possible explanations include an

increase in the number of apical amiloride-sensitive channels or an increase in the opening probability or single channel conductance of pre-existing amiloride-sensitive channels.

Several lines of evidence suggest that the cAMP-induced increase in epithelial Na^+ channel activity is due to the insertion of new channels. Firstly, cAMP-induced activation of epithelial Na^+ channel activity shows a protracted time course. The effect has not reached a maximum 6 h after treatment with either forskolin, dibutyryl-cAMP or IBMX. This is in contrast to the effects of anti-diuretic hormone on Na^+ channel activity in amphibian epithelia, which develop over a 5–20-min period and are believed to be due, at least in part, to a protein kinase A mediated increase in channel open probability (Handler and Orloff, 1981). We have observed, however, (unpublished observations) that the stimulatory effects of cAMP-raising drugs occur more rapidly in bladders treated with aldosterone.

A second reason to suppose that the increase in amiloride-sensitive short-circuit current is due to insertion of new channels is the magnitude of the cAMP effect. There is a more than seven-fold increase in amiloride-sensitive short-circuit current in response to 10 μM forskolin. To explain this solely in terms of an increase in channel open probability would be to assume that the open probability of unstimulated channels was very low. Although patch clamp studies have revealed substantial variability in open probability values of epithelial Na^+ channels, the majority of channels investigated have an open probability in the range of 0.3–0.5 (review by Garty and Palmer, 1997).

Thirdly, the forskolin-induced increase in epithelial Na^+ channel activity is inhibited by the vesicular fusion inhibitor brefeldin A. This fungal metabolite has been widely used as a pharmacological tool to block anterograde vesicular transport of newly synthesized proteins. The molecular target of brefeldin A is a guanine-nucleotide exchange factor which has a role in the uncoating of Golgi-derived vesicles prior to vesicle fusion (Donaldson et al., 1992). Brefeldin A has been used previously to investigate the mechanism of cAMP-induced activation of epithelial Na^+ channels. Kleyman et al. (1994) showed that the forskolin-stimulated increase in Na^+ conductance in A6 cells was inhibited by brefeldin A. They therefore proposed that the effect of forskolin in these cells was to stimulate the recruitment of channels to the cell surface from an intracellular pool.

It is known that the Na^+-transporting epithelial cells of the rabbit urinary bladder possess a population of vesicles beneath the apical membrane. Lewis and De Moura (1982, 1984) showed that certain stimuli, namely hypo-osmotic challenge and hydrostatic pressure gradients, resulted in the translocation of vesicles from this sub-apical pool to the apical plasma membrane. In response to these stimuli, increases in apical membrane capacitance were accompanied by increases in amiloride-sensitive short-circuit current suggesting the presence of Na^+ channels within the vesicles. It is possible that the effect of raising cAMP levels in the epithelial cells is to promote the translocation of channels from the subapical pool to the apical plasma membrane. A similar phenomenon has been demonstrated in rat skeletal muscle. Li and Sperelakis (1993) suggested that the insulin-induced hyperpolarization seen in this tissue is due to translocation of Na–K pumps from intracellular pools to the sarcolemma. Notably, this effect reached a peak after only 15 min. The much more protracted time course of the cAMP effects in the rabbit bladder epithelium may therefore suggest an action on channel synthesis de novo, as opposed simply to channel translocation. Our finding that the stimulatory effect of forskolin is inhibited by cycloheximide would support this hypothesis. To our knowledge, this is the first demonstration that a protein synthesis inhibitor can inhibit cAMP-induced activation of the epithelial Na^+ channel. The ability of cAMP to up- or down-regulate gene transcription is currently an area of intense research (review by De Cesare et al., 1999).

At present, we do not understand the precise relevance of these findings to urinary bladder physiology. An important question to address is the nature of the endogenous mediator that raises cAMP levels in the epithelial cells of the rabbit urinary bladder and this is currently one focus of our research.

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