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SODIUM-DEPENDENT REGULATION OF EPITHELIAL SODIUM CHANNEL DENSITIES IN FROG SKIN; A ROLE FOR THE CYTOSKELETON

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SUMMARY

1. A weak electroneutral sodium channel blocker 6-chloro-3,5-diamino-pyrazine-2-carboxamide was used to perform noise analysis on isolated epithelium from *Rana fuscigula* to determine the cellular mechanism underlying autoregulation of Na^+ channel densities in response to a reduction in the mucosal Na^+ concentration.

2. The inherent transport rates of these tissues were generally lower than in other frog skins. The macroscopic sodium current, I_{Na}, averaged 10·71 $\mu A/cm^2$ and was mainly determined by the number of open channels (N_0) which averaged 21·6 million/cm^2. The calculated mean channel open probability (β') was 0·38, and corresponded very closely to values previously determined by patch clamp.

3. Reducing the mucosal Na^+ from 110 to 10 mM caused large increases in the open channel density, which stabilized the Na^+ transport rate. N_0 increased from a mean value of 26·6 to 64·3 million/cm^2 within 2 min.

4. Autoregulatory changes were induced primarily by increasing β' by about 60% and to a lesser extent by an increase in N_T, the total number of open and closed channels.

5. We also examined the role of the cytoskeleton in the regulation of Na^+ channel densities. Colchicine treatment, which disrupted microtubules, had no apparent effect on the ability of the tissues to autoregulate their Na^+ channel densities.

6. The integrity of the microfilaments were essential for autoregulatory changes in N_0. After we had disrupted the microfilaments with cytochalasin B, we observed a marked reduction in the ability of the tissues to increase N_0.

7. The mean N_0 did not increase in response to a drop in mucosal Na^+ despite the fact that β' increased by 69%. We, therefore, assumed that cytochalasin B did not affect Na^+ channels already present in the membrane but interfered with recruitment of new channels. Significantly, we did not observe any increase in N_T.

8. In kidney and other tight epithelia, microfilaments are responsible for regulating the delivery of newly synthesized membrane proteins. We believe that our results with cytochalasin-treated tissues support the theory that autoregulatory changes in N_0 are also regulated by the recruitment of channels from a cytoplasmic pool.

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INTRODUCTION

Regulation of the rate of Na^+ entry through Na^+-selective channels in apical membranes of Na^+-reabsorbing epithelia is important in the control of sodium homeostasis. Not surprisingly, apical membrane Na^+ permeability is critically regulated by extrinsic and intrinsic (homocellular) mechanisms. Extrinsic mechanisms are mediated by factors such as neural and hormonal influences (see reviews by Wills & Zweifach, 1987; Els & Helman, 1989; Smith & Benos, 1991) while the intrinsic or autoregulatory mechanisms reside within the cells and are important for primary cell functions like regulation of intracellular ionic composition (Schultz, 1985). Intrinsic regulation of Na^+ channels is governed by various factors including changes in the composition of the extracellular fluid, in cell volume, in cell metabolism or, the essence of this study, in Na^+ transport load (Zeiske & Van Driessche, 1984; Sariban-Sohraby & Benos, 1986). Several lines of evidence have clearly demonstrated an inverse relationship between the concentration of mucosal Na^+ and the apical membrane Na^+ permeability in frog skin (Fuchs, Larsen & Lindemann, 1977) and many other tight epithelia (reviewed by Wills & Zweifach, 1987; Garty & Benos, 1988; Smith & Benos, 1991).

Unfortunately, the mechanisms whereby mucosal Na^+ concentration modulate channel activity and regulate Na^+ transport remain disputable. Effects of mucosal Na^+ on membrane Na^+ channels have mainly been described in terms of changes in channel saturation and channel densities but the results have often been at variance. For example, in various epithelia noise analysis and patch clamp results have demonstrated that changes in mucosal Na^+ modify apical membrane permeability primarily by regulating the number of active Na^+ channels without affecting channel conductance (Van Driessche & Lindemann, 1979; Lewis, Ifshin, Loo & Diamond, 1984; Ling & Eaton, 1989). On the other hand, using patch clamp, Palmer & Frindt (1988) failed to observe any change in the number of active channels by external Na^+ in rat cortical collecting tubules. Various factors including differences in species, methods and conditions have been advanced to explain the contradictions.

In the present study we examined the effects of a reduction in mucosal Na^+ on the autoregulation of Na^+ channels in apical membranes of isolated frog skin epithelium with blocker-induced noise analysis. The use of potent channel blockers in some previous investigations may give cause for concern, specifically with regard to difficulties in measuring off-rate coefficients and determining autoregulatory changes in channel densities (Helman, Cox & Van Driessche, 1983; Abramcheck, Van Driessche & Helman, 1985). We performed noise analysis with the relatively weak electroneutral Na^+ channel blocker CDPC (6-chloro-3,5-diamino-pyrazine-2-carboxamide) which provided excellent noise signals at concentrations in a range well below its equilibrium blocker coefficient K_B (Helman & Baxendale, 1990), allowing analysis on tissues transporting Na^+ near their spontaneous rates. These conditions were particularly conducive to determining single-channel currents, channel densities and, more pertinently, also channel open probabilities (Helman & Baxendale, 1990). Our results with noise analysis confirmed a recent finding by Ling & Eaton (1989) with patch clamp that a reduction in mucosal Na^+ causes an increase in channel open probability. Furthermore, we resolved that increases in open channel density were also brought about by increases in total channel density.

In addition, we investigated the role of the cytoskeleton in the autoregulation of Na^+ channels. Although there is recent interest in the direct role of the cytoskeleton in the regulation of epithelial Na^+ channels (cf. Prat, Ausiello & Cantiello, 1991), we performed studies primarily to examine the cellular mechanisms underlying increases in open channel density. In particular, we considered the idea that newly activated Na^+ channels were recruited into apical membranes from a cytoplasmic pool of vesicles (Lewis *et al.* 1984) by a process mediated by the cytoskeleton (Valenti, Hugon & Bourguet, 1988). A preliminary report of some of our results has been presented in abstract form (Els & Chou, 1991).

METHODS

Tissue and experimental preparation

Rana fuscigula were kept at room temperature in distilled water with access to a dry surface and fed with meal worms twice a week. Abdominal skins were dissected from pithed frogs. Isolated epithelia were prepared from these according to a collagenase method similar to that described by Fisher, Erlij & Helman (1980). Tissues were mounted in a continuous flow chamber designed to conduct current–noise analysis under transepithelial voltage clamp conditions (Abramcheck *et al.* 1985). Mucosal and serosal compartments in the chambers (volumes of 0·6 ml) were continuously perfused with frog Ringer solution by gravity from reservoirs at rates of ~ 9 ml/min. The exposed area of tissue was 0·75 cm^2.

The transepithelial potential difference was clamped to 0 mV with a low-noise voltage clamp (W. Van Driessche, K. U. Leuven personal communication) which was also used for recording noise spectra. Transepithelial conductance (G_t) was determined by recording the current changes in response to brief 3 mV unipolar pulses which were applied every 15 s (Fig. 1).

Blocker-induced noise analysis

The short-circuit current (I_{sc}) and G_t were allowed to reach steady-state levels during control and experimental periods before performing noise analysis according to the staircase protocol described by Helman & Baxendale (1990). Microscopic fluctuations in I_{sc} were induced by sequential introduction of 10 to 50 μM of the sodium channel blocker, CDPC (6-chloro-3,5-diamino-pyrazine-2-carboxamide), into the mucosal solution. Following noise analysis, the blocker was completely washed out from the mucosal solution and the I_{sc} allowed to stabilize (Fig. 2). Data acquisition for power density spectral analysis was similar to that described previously (Van Driessche & Erlij, 1983; Abramcheck *et al.* 1985). We used a fundamental frequency of 0·5 Hz and the final power density spectra (PDS) were derived at each blocker concentration by collecting 50 or 60 sweeps of data which were transformed to the frequency domain with a fast Fourier transformation. The spectra were merged and displayed in a double-logarithmic representation. PDS were fitted by non-linear least-squares regression analysis to $1/f^{\alpha}$ and Lorentzian components to determine the low frequency plateau value (S_o) and radian frequency, $2\pi f_c$, of the Lorentzian.

We assumed a simple three-state model where channels are distributed between closed, open and blocked states and where blocker interacts primarily if not solely with open states of the channel (Helman & Baxendale, 1990):

$$N_c \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} N_o \overset{k_{ob}[B]}{\underset{k_{bo}}{\rightleftharpoons}} N_b.$$

The on- (k_{ob}) and off- (k_{bo}) rate coefficients of blocker interaction with the open state of the channel were determined from the slope and intercept of the rate–concentration plots according to $2\pi f_c = k_{ob}[B] + k_{bo}$ (Fig. 3*B*). K_B, the equilibrium blocker coefficient of open channels, was calculated from the quotient k_{bo}/k_{ob}. Mean single-channel Na^+ currents (i_{Na}) were calculated at each [B] according to eqn (1), and estimation of values for i_{Na} in the absence of blocker were obtained by extrapolation to zero [B]. Open channel density, N_o, was calculated as I_{Na}/i_{Na}. Note that the subscript (,B) included with the usual notations indicates that a measurement was made in the presence of blocker.

$$i_{Na,B} = [S_o(2\pi f_c)^2]/(4\, I_{Na}\, k_{ob}[B]). \quad (1)$$

Open channel probability in the absence of blocker (β'), defined by $\beta/(\beta+\alpha)$, was determined by

first calculating apparent open probabilities at each blocker concentration ($\beta'_{,B}$) with eqn (2) (Helman & Baxendale, 1990) and then extrapolating values to zero blocker concentration by linear regression in the $\beta'_{,B}$–[B] relationship (see Fig. 3*C*):

$$\beta'_{,B} = [1-(N_{o,B}/N_o)]/(N_{o,B}/N_o)([B]/K_B)$$
$$= (N_o/N_{o,B}-1)/([B]/K_B). \quad (2)$$

The total channel density (N_T), the sum of electrically active open and closed channels in the absence of blocker, was calculated from the quotient N_o/β'.

Time course protocol

In order to determine time-dependent changes in single-channel current and channel densities caused by a reduction in mucosal sodium concentration, experiments were performed according to a protocol described elsewhere (Els & Helman, 1991). Firstly, noise analysis was performed to obtain steady-state values of k_{ob}. Thereafter, with 20 μM CDPC constantly present in the mucosal solution, PDS were measured every 4 min to calculate S_o, and f_c. These values were then used to calculate values for $i_{Na,B}$ and $N_{o,B}$ during periods in 110 and 20 mM Na^+. The results are illustrated in Fig. 4.

Solutions

Collagenase Type II (Worthington), was used at a final concentration of 50 U/ml Ringer solution to isolate the epithelium. All tissues were bathed in Cl^{-1}–HCO_3^- Ringer solution which contained (mM) 110 NaCl, 2·4 $KHCO_3$ and 2·0 $CaCl_2$ and was equilibrated with air. The initial pH was ~ 8·0. Low-Na^+ Ringer solutions were prepared by isosmolar replacement of NaCl with tetramethylammonium chloride. Experimental changes in Na^+ concentrations were made in the mucosal solution alone, maintaining the Na^+ concentration in the serosal solution at 110 mM. CDPC (6-chloro-3,5-diamino-pyrazine-2-carboxamide) was purchased from Aldrich Chemical Company (Milwaukee, WI, USA) and made up to the desired concentrations in the mucosal Ringer solution immediately prior to noise analysis. Amiloride was donated by Merck, Sharp and Dohme.

For the depolymerizing drug studies, both surfaces of the tissues were perfused with Ringer solution containing either 100 μM colchicine (Sigma, St Louis, USA) to disrupt microtubules or cytochalasin B to disrupt microfilaments. A stock solution of cytochalasin B (Sigma, St Louis) was prepared by dissolving (2 mg/ml) in dimethyl sulphoxide (DMSO) and stored at −4 °C in the dark. The final concentrations of cytochalasin B and DMSO in Ringer solution were 10 μg/ml (21 μM) and 0·5% respectively. Control perfusion with 0·5% DMSO alone showed no significant effect on the Na^+ transport rate. Tissues were treated in colchicine for 3 h and for about 2 h in cytochalasin B before the experiments continued. The I_{sc} was monitored continuously during the depolymerizing periods and the experiments were only started when the I_{sc} had reached a stable value.

All experiments were carried out at room temperature (~ 17–22 °C). Statistical data are presented as means ± S.E.M. Student's *t* test, on paired data, was used to determine the significance ($P < 0·05$) of differences between means.

RESULTS

Macroscopic current kinetics

An acute reduction of the Na^+ concentration in the mucosal solution produced a transient decrease in the rate of Na^+ entry into the cells. Within seconds of reducing the mucosal Na^+ concentration to 20 mM or lower, the I_{sc} dropped to a minimum value followed by a relatively slow return to a new but lower steady-state level (Figs 1 and 2). The slow component for the relaxation in I_{sc} could be fitted by a single exponential, typically with time constants of about 9 min for tissues exposed to 20 mM Na^+. After 20 min recovery, levels of I_{sc} averaged about 24% or 35% below original levels in 20 and 10 mM Na^+ respectively (Table 2). Concurrently, after changing the mucosal Na^+ to 20 mM, the transepithelial conductance increased by 39% from a mean 436 ± 51 to 581 ± 40 μS.

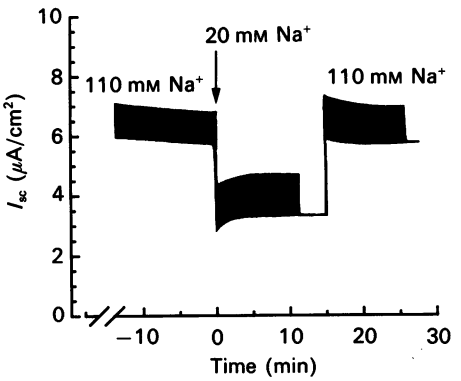

Fig. 1. Demonstration of autoregulation of the I_{sc} in isolated epithelium. The continuous line is the recording of the I_{sc} while the upward deflections are responses to a 3 mV pulse applied every 15 s, which were used to calculate the transepithelial conductance. Initially the tissue was exposed to 110 mM Na^+ Ringer solution on both sides. Replacing the mucosal solution with isosmolar Ringer solution containing 20 mM Na^+ caused a rapid inhibition of the I_{sc} followed by a slow recovery towards a new steady state. During the relaxation in I_{sc} there was a gradual increase in transepithelial conductance, which after 10 min had increased from 375 to 460 μS. A return to 110 mM Na^+ Ringer solution reversed the macroscopic changes and the conductance slowly decreased to a value of 410 μS after 10 min.

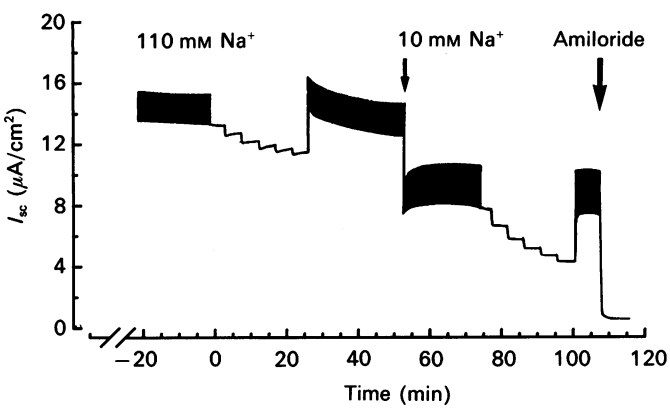

Fig. 2. Strip chart recording of an experiment to determine changes in Na^+ channel densities. Tissues perfused with Ringer solutions containing consecutively 110 mM and 10 mM Na^+ in the mucosal compartment, were exposed to a staircase increase of CDPC concentrations (10–50 μM). The PDS were measured during periods when the pulse generator was turned off. After noise analysis had been completed, the CDPC was washed out of the solution and the I_{sc} was allowed to stabilize. Amiloride, 100 μM, was added to the mucosal solution at the end of each experiment to determine the amiloride-sensitive I_{sc}, that averaged near 1 $\mu A/cm^2$. After 15 min in 10 mM Na^+ the transepithelial conductance had increased from 750 to 920 μS.

Results with noise analysis: steady-state control data

Noise analysis of apical membrane Na^+ channels was carried out during control and experimental periods according to protocols shown in Fig. 2. CDPC caused relatively small, graded inhibitions of I_{Na} not exceeding about 25% (Figs 2 and 5)

and hence, analyses were done under conditions close to spontaneous rates of apical Na^+ entry (Helman & Baxendale, 1990). Introduction of CDPC induced a single Lorentzian in the PDS, whose f_c varied linearly as a function of [B], allowing estimation of the on- and off-rate coefficients for blocker interaction with the Na^+ channel (Fig. 3*B*). Values for f_c ranged between about 25 and 90 Hz. The plateau

TABLE 1. Summary of steady-state values of epithelial Na^+ channels in 110 mM NaCl-Ringer solution

I_{Na} ($\mu A/cm^2$)	i_{Na} (pA)	N_o ($10^6/cm^2$)	β'	N_T ($10^6/cm^2$)	k_{ob} (rad/(s μM))	k_{bo} (rad/s)	K_B (μM)
10·71±0·98	0·59±0·03	21·6±2·2	0·38±0·02	65·6±8·7	4·73±0·16	171·7±6·8	37·1±1·3

Values expressed as means±S.E.M. ($n = 31$).

value (S_0) of the Lorentzian displayed the expected biphasic relationship with [CDPC] (Fig. 3*A*).

Variability of channel kinetics among species is an important consideration when speculating on transport mechanisms. With this in mind we begin by presenting the steady-state control data, the first reported for this species. The means of steady-state values collected during control periods from different groups of experiments are summarized in Table 1. The I_{Na} averaged 10·71 $\mu A/cm^2$, significantly lower than mean values for frog skin from many other species under similar conditions (see Helman & Kizer, 1990). Often skins with low steady-state control currents were unsuited for our experiments, since experimental conditions and time-dependent transients reduced the I_{sc} to such low values (1 or 2 $\mu A/cm^2$) that fluctuation analysis became unreliable. Accordingly, there were very few skins with steady-state currents large enough to examine the effects of very low mucosal Na^+ (5 or 10 mM) on the channel kinetics.

Epithelial Na^+ channels are generally low conductance channels, with single-channel conductances ranging from about 3 to 20 pS (Garty & Benos, 1988; Els & Helman, 1989). In agreement, i_{Na} averaged 0·59 pA, a value comparable to previous results with patch clamp and noise analysis (Helman & Baxendale, 1990; Helman & Kizer, 1990). We calculated that, assuming a mean apical membrane voltage of 85 mV and intracellular Na^+ concentration of 14·5 mM (Els & Helman, 1991), single-channel conductance would be about 4·3 pS. Since the value of the mean i_{Na} was not significantly different from other studies on frog skin, the low range for I_{Na} could be explained in terms of the relatively small number of open Na^+ channels, which averaged 21·6 million/cm^2 in the presence of 110 mM NaCl. Channel open probability averaged 0·38, a value similar to that measured with patch clamp (Palmer & Frindt, 1988) and with noise analysis (Helman & Baxendale, 1990) and showed no correlation with I_{Na}. Total channel densities that ranged between 17 and 179 million/cm^2 paralleled differences in N_o.

The blocker rate coefficients varied widely. k_{ob} averaged 4·73 rad/(s μM), significantly lower than the 7 rad/(s μM) determined with CDPC for *R. pipiens* (Els & Helman, 1991) but higher than in skins from other species (see review by Helman & Kizer, 1990). k_{bo} averaged 171·7 rad/s again slightly lower than in *R. pipiens* where

the off-rate coefficients averaged about 200 rad/s. The reasons for the variability of rate coefficients among tissues of the same and also of different species are unknown. It may indicate that the blocker binding site can be regulated but, membrane potentials, structural and other factors may also be responsible (see Helman & Kizer, 1990).

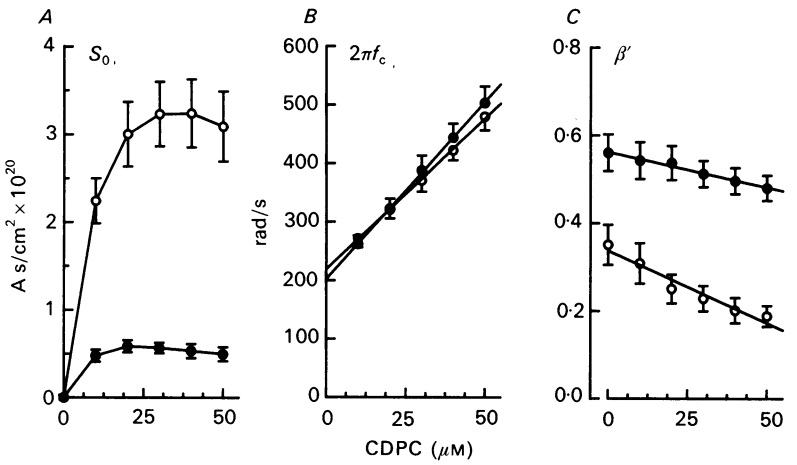

Fig. 3. CDPC-dependent changes of S_0, $2\pi f_c$ and β' in tissues perfused with 110 mM Na^+ (○) and 10 mM Na^+ (●) in the mucosal solutions. Mean changes of these parameters are summarized in Table 2. Apparent open probabilities (β'_B) were calculated according to eqn (2), and open probability (β') in the absence of blocker was measured by extrapolation to the ordinate.

Reduction in mucosal sodium concentration increases channel densities

Summarized in Fig. 3 are the changes of S_0 and $2\pi f_c$ of the CDPC-induced Lorentzians caused by a reduction in mucosal Na^+ to 10 mM. A large reduction in the mean value of S_0 was caused by the drop in mucosal Na^+ (Fig. 3*A*) while it had little effect on the on- and off-rate coefficients (Fig. 3*B*). Thus, k_{ob} did not change significantly while k_{bo} decreased by only 7%, this being the main reason why the K_B reduced from about 41 to 36 μM (Table 2).

We examined the effects of reducing the mucosal Na^+ concentration on two groups of skins. Effects of a reduction to 10 mM was examined on a group of eight epithelia, selected on the basis of their inherent higher transport rates. The compensatory changes in this group were the largest and best illustrated effects of reducing mucosal Na^+ concentration. Unfortunately the intrinsic transport rates and channel densities of the majority of frog skins were much lower than in this sample. Since our depolymerizing studies and time-related transients further reduced the I_{sc} to even lower levels, it became infeasible to examine subsequently with noise analysis effects in 10 mM Na^+. Consequently, we were bound in all other experiments to study the effects of reducing mucosal Na^+ to 20 mM. For comparison with our other data, we also summarized in Table 2 effects on Na^+ channels of reducing mucosal Na^+ to

20 mM. While the magnitude of changes in channel densities were smaller than in the previous group, the pattern of changes were similar.

Decreasing the mucosal sodium concentration lowers the electrochemical driving force for Na^+ across the apical membrane, resulting in a lower transport rate. After 20 min in 10 mM Na^+, and in compensation for the decrease in i_{Na}, the mean number

TABLE 2. Effects of reduced mucosal Na^+ concentration on epithelial Na^+ channels

	Control (mM)	Experiment (mM)	E/C
	110	10	10/110
	110	20	20/110
I_{Na} (μA/cm^2)			
	14·22 ± 1·60	9·47 ± 1·03	0·65 ± 0·02
	10·69 ± 1·04	8·14 ± 0·93	0·76 ± 0·04
i_{Na} (pA)			
	0·57 ± 0·04	0·16 ± 0·01	0·28 ± 0·02
	0·65 ± 0·04	0·30 ± 0·02	0·47 ± 0·01
N_o (10^6/cm^2)			
	26·6 ± 4·6	64·3 ± 11·0	2·58 ± 0·37
	17·8 ± 2·3	29·4 ± 4·3	1·65 ± 0·10
β'			
	0·35 ± 0·04	0·55 ± 0·04	1·63 ± 0·16
	0·40 ± 0·03	0·61 ± 0·03	1·55 ± 0·08
N_T (10^6/cm^2)			
	74·8 ± 13·8	120·7 ± 20·6	1·81 ± 0·30
	49·2 ± 12·5	57·5 ± 14·7	1·24 ± 0·10
k_{ob} (rad/(s μM))			
	5·26 ± 0·30	5·96 ± 0·57	1·12 ± 0·07*
	5·13 ± 0·13	5·52 ± 0·11	1·08 ± 0·02*
k_{bo} (rad/s)			
	215·4 ± 14·8	204·6 ± 12·7	0·93 ± 0·01
	193·9 ± 8·4	186·4 ± 8·4	0·96 ± 0·02
K_B (μM)			
	41·2 ± 2·9	35·8 ± 3·6	0·84 ± 0·06
	38·6 ± 1·9	34·4 ± 1·7	0·90 ± 0·02

The three columns contain values from paired experiments where the mucosal Ringer solution was reduced from 110 mM Na^+ to 10 mM (upper rows; $n = 8$) and to 20 mM (lower rows, $n = 14$) respectively. All values are means ± S.E.M. Non-significant changes ($P < 0·05$) assessed on a paired basis are indicated by asterisks.

of open channels had increased markedly by 158% from near 27 to 64 million/cm^2, being the main mechanism responsible for the relaxation of the I_{Na} towards the original level (refer also to Fig. 4). Increases of open channel density may arise by recruitment of channels from closed into open states (a change of open channel probability, β'), and/or by increase of the total channel density (Els & Helman, 1991). Foremost, we determined that the reduction in mucosal Na^+ to either 10 or 20 mM consistently increased channel open probability. In 10 mM Na^+, β' increased by 63% from a mean of 0·35 to 0·55 (Fig. 3*C*). This mechanism alone could not account for the large increase in N_o. We also calculated that, concurrently with the increase in N_o, N_T had increased significantly by an average of 81% from 74 to 120

million/cm^2. Hence, both mechanisms are used by these cells to stabilize the Na^+ transport rate. The changes were readily reversible on return to 110 mM Na^+ (data not shown).

Reduction in mucosal sodium causes rapid changes in N_0

We also performed an experiment to determine the time course of changes in N_0 induced by reducing the mucosal Na^+ to 20 mM. The control data for this group of six skins was as follows: I_{Na} averaged $16{\cdot}17 \pm 3{\cdot}20$ μA/cm^2; mean i_{Na} was $0{\cdot}56 \pm 0{\cdot}03$ pA; N_0 averaged $29{\cdot}0 \pm 6{\cdot}2$ million/cm^2; mean β' was $0{\cdot}28 \pm 0{\cdot}02$ and the mean N_T was $109{\cdot}2 \pm 16{\cdot}3$ million/cm^2.

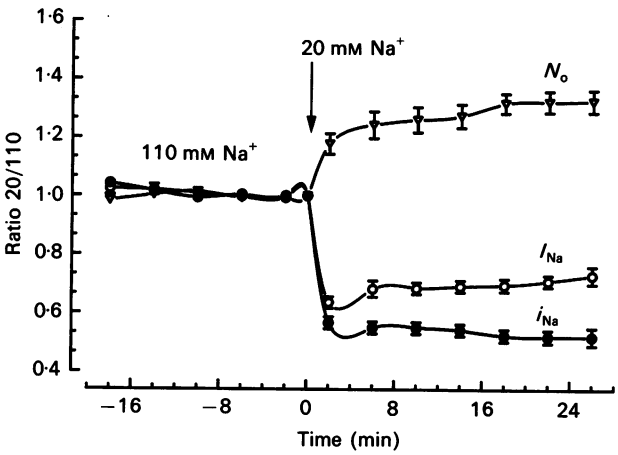

Fig. 4. Responses of I_{Na}, N_0, and i_{Na} to a reduction in mucosal Na^+ from 110 mM to 20 mM as determined with the time-course protocol. Data are expressed as values in 20 mM Na^+/values in 110 mM Na^+ (20/110). The control values for this group of tissues appear in the text. Noise analysis was performed every 4 min and each analysis took about 2·5 min to complete.

The results illustrated in Fig. 4 were consistent with our initial observations (Table 2). A drop in mucosal Na^+ decreased i_{Na} by about 57% within 2 min and continued to decline at a lesser rate for the next 20 min. Despite the reduction in i_{Na}, the I_{Na} returned to within 30% of the original value because of a concurrent increase in N_0. Of interest was the rate at which new open channels were recruited. We determined that an increase in N_0 took place within the first 2 min. This ruled out any possibility that new open channels were recruited by synthesis. After the initial increase, N_0 continued to increase steadily for the next 20 min, approaching a new steady state.

Effects of depolymerizing the cytoskeleton on autoregulation of sodium channel densities

The rapid recruitment of new active channels in response to changes in the external Na^+ concentration may be from quiescent apical channels or from a pool residing in some cytoplasmic site. To test the latter hypothesis we also investigated

whether the cytoskeleton was involved in the autoregulation of channel densities in response to a change in mucosal Na^+ to 20 mM. It has become established that the cytoskeleton plays a central role in regulation of epithelial cell membrane function by continually inserting and removing selected components. Best known is the role of the cytoskeleton in regulating the water channels by vasopressin (Pearl & Taylor, 1985).

TABLE 3. Effects of reduced mucosal Na^+ concentration on epithelial Na^+ channels after treatment with 100 μM colchicine

	110 mM	20 mM	20/110
I_{Na} ($\mu A/cm^2$)	9·89 ± 1·42	6·89 ± 1·09	0·71 ± 0·02
i_{Na} (pA)	0·55 ± 0·04	0·26 ± 0·02	0·48 ± 0·03
N_o ($10^6/cm^2$)	19·3 ± 3·0	27·4 ± 4·5	1·50 ± 0·15
β'	0·46 ± 0·04	0·61 ± 0·03	1·40 ± 0·15
N_T ($10^6/cm^2$)	49·5 ± 15·1	50·1 ± 9·0	1·26 ± 0·20*
k_{ob} (rad/(s μM))	3·98 ± 0·17	4·39 ± 0·14	1·12 ± 0·03
k_{bo} (rad/s)	122·6 ± 6·1	121·1 ± 6·4	0·99 ± 0·03*
K_B (μM)	31·6 ± 2·4	27·7 ± 1·7	0·89 ± 0·05*

Values are means ± S.E.M. ($n = 8$). Non-significant changes ($P < 0·05$) assessed on a paired basis are indicated by asterisks.

Colchicine did not alter effects of reduced mucosal sodium concentration

Autoregulatory changes in channel densities, induced by a reduction in mucosal Na^+ concentration to 20 mM, were unaffected by depolymerization of the microtubules with 100 μM colchicine for three hours (Table 3). The manoeuvre decreased the i_{Na} to 0·26 pA, a decrease by nearly identical proportions as previously (Table 2). Despite the reduction in entry rate, the I_{Na} recovered to within 30% of earlier steady-state levels after 20 min due mainly to a 50% increase in the number of open channels. The change in open channel density was primarily brought about by a 40% increase in the channel open probability, since N_T did not change significantly from control values.

Cytochalasin B blocks the increase in open channel density

Within five minutes of placing isolated epithelia in cytochalasin B, the I_{sc} dropped by approximately 2 to 5 $\mu A/cm^2$ and thereafter, continued to decline at a slower rate to a new steady-state level after about ninety minutes. Concurrently the transepithelial conductance increased by between 50 and 100% (Fig. 5). The drug treatment caused very little change to the properties of the channels. This becomes apparent when comparing the values in 110 mM Na^+ from two groups with nearly identical macroscopic transport rates (Tables 2 and 4). The main difference was that i_{Na} averaged 0·42 pA in cytochalasin-treated tissues, about 30% lower than mean values in 110 mM Na^+. Consequently, the mean N_o was correspondingly slightly higher than in the untreated group.

The ability of the tissues to increase their membrane Na^+ permeability in response to a reduction in rate of Na^+ entry was diminished by depolymerization of the microfilaments (Fig. 6). After cytochalasin B treatment and in response to a

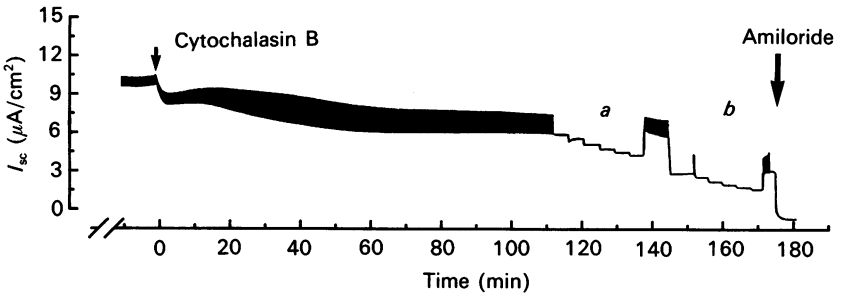

Fig. 5. A strip chart recording of an experiment to determine autoregulatory changes in channel densities after the tissue had been treated in cytochalasin B (10 μg/ml) for nearly two hours. Noise analysis was performed with tissues exposed to normal Ringer solution containing 110 mM Na^+ during period *a* and again when tissues were exposed to Ringer solution containing 20 mM Na^+ in the mucosal solution during period *b*. Note the absence of any relaxation in the I_{sc} immediately prior to period *b*. During control steady-state conditions the mean transepithelial conductance was 260 μS. This increased to 430 μS immediately prior to period *a*.

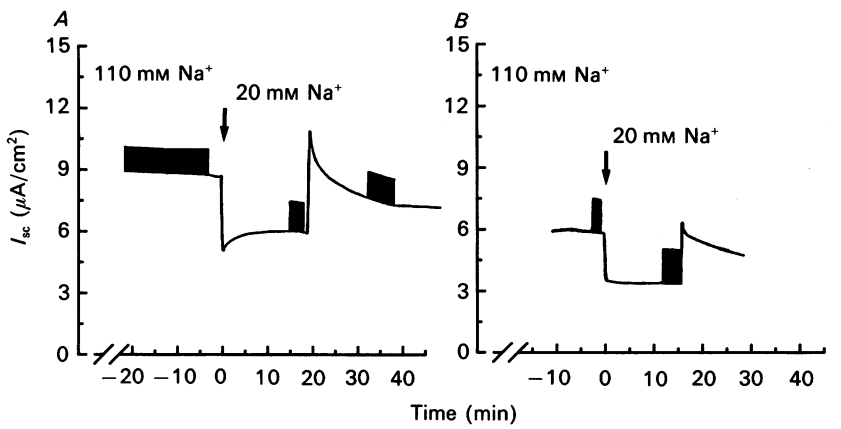

Fig. 6. Disruption of microfilaments with cytochalasin B prevents autoregulatory relaxation of the Na^+ transport rate. The recording in panel *A* demonstrates the usual response of the I_{sc} when we drop the mucosal Na^+ concentration from 110 to 20 mM. After about 20 min the tissue was returned to normal 110 mM Na^+ Ringer solution and the changes in I_{sc} and transepithelial conductance were reversed. Notice the big overshoot of the I_{sc} followed by a gradual return towards original levels. The recording in panel *B* is from the same tissue but after treatment with cytochalasin B to prevent polymerization of the microfilaments. Note there is little sign of any relaxation of the I_{sc} in response to a drop in mucosal Na^+ to 20 mM. The overshoot is also much reduced upon return to 110 mM Na^+.

reduction in mucosal Na^+ to 20 mM, the I_{Na} recovered to within 37% of previous levels compared to a recovery within 24% without the drug treatment (Tables 2 and 4). Noise analysis demonstrated that, unlike before, this was as a result of an inability by the tissues to autoregulate channel densities. Significantly, a reduction

in mucosal Na^+ to 20 mM did not produce the expected increase in N_0 which remained unchanged at control values near 24 million/cm^2. The mean open probability still increased from 0·43 to 0·67 despite treatment with cytochalasin. Accordingly, N_T did not increase, but actually reduced slightly but not significantly, from about 60 to 47 million/cm^2. We confirmed these results in two skins with significantly higher transport rates and channel densities. Subjecting these cytochalasin-treated tissues to an even greater reduction in mucosal Na^+ (110 to 10 mM Na^+), failed to produce any increase in N_0 or N_T (data not shown).

DISCUSSION

A change in the rate of Na^+ entry into cells of Na^+ transporting epithelia may lead to regulatory adjustments in Na^+ permeability. Two main hypotheses have been advanced to explain the mechanisms whereby the cells accomplish the modification; change in channel conductance and change in the number of active Na^+ channels. Effects of increasing external Na^+ up to 100 mM have been described in terms of channel saturation in rat cortical collecting tubule (Palmer & Frindt, 1988) and in A6 Na^+ channels reconstituted in planar lipid bilayers (Olans, Sariban-Sohraby & Benos, 1984). On the other hand, experiments using current fluctuation analysis on frog skin and rabbit urinary bladder (Van Driessche & Lindemann, 1979; Lewis *et al.* 1984) found no evidence for single-channel current saturation in response to changes in external Na^+. These authors concluded that Na^+ permeability was modified primarily by regulating the number of active channels. However, not all results are consistent with this idea. Palmer & Frindt (1988) failed to observe any change in the number of active channels in response to an increase in external Na^+ concentration in rat cortical collecting tubules. A number of factors, including differences in species, methods and conditions have been advanced to explain the contradictions.

We investigated effects of a reduction in mucosal Na^+ on Na^+ permeability with CDPC-induced noise analysis. By avoiding the use of potent Na^+ channel blockers to induce channel fluctuations, we could study changes in channel densities under conditions close to spontaneous rates of Na^+ absorption (Helman & Baxendale, 1990). In this manner we also avoided certain problems sometimes associated with the use of potent Na^+ channel blockers, specifically as regards to difficulties in measuring off-rate coefficients and determining autoregulatory changes in channel densities (Helman *et al.* 1983; Abramcheck *et al.* 1985; Helman & Kizer, 1990). Our results clearly showed that the reduction in mucosal Na^+ mainly induced an increase in the number of conducting Na^+ channels (Table 2). Hence, despite the decrease in i_{Na}, the macroscopic transport rate relaxed towards previous levels. Our data also supports recent results with the patch clamp. Ling & Eaton (1989), using cell-attached patches from A6 cells, demonstrated that decreasing the Na^+ concentration in the bath to 5 mM activated 'new' channels in the patch membranes and did not change the channel conductance. This phenomenon whereby cells adjust to a reduction in apical Na^+ entry rate by inducing an increase in the number of open channels, was referred to as autoregulation of channel densities by Abramcheck *et al.* (1985). They suggested that autoregulatory changes in channel densities might well be a major factor in regulating Na^+ permeability.

The autoregulatory increases in N_0 were generated within two to three minutes of

changing to low Na^+ (Fig. 4). This time course is consistent with effects of decreasing luminal Na^+ observed with patch clamp in A6 cells (Ling & Eaton, 1989). The rapid rate at which changes occurred rule out that the channels were recruited by the synthesis of new channels. It is also currently unknown how the changes are mediated. Since recent evidence argues against a direct interaction of Na^+ with the membrane channels it is likely that the changes were mediated via some intracellular mediator (Schultz, 1985; Ling & Eaton, 1989). There is ample evidence suggesting that intracellular Na^+ performs this role (Van Driessche & Erlij, 1983; Abramcheck *et al.* 1985) either directly or indirectly via changes in, for example, intracellular hydrogen or calcium ions (Palmer & Frindt, 1987).

The underlying mechanisms for autoregulation of channel densities are unknown. Frog skins which had been exposed to 10 or 5 mM Na^+ for two or three hours contained markedly higher values of N_0 and N_T compared to values in skins bathed in normal Ringer solution (Els & Helman, 1991). This prompted the suggestion that in *R. pipiens* autoregulatory changes in N_0 occurred primarily by changes in N_T. Along similar lines, inhibition of apical Na^+ entry by the use of mucosal Na^+ channel blockers leads to large increases in channel densities and transient return of the I_{sc} towards control levels (Abramcheck *et al.* 1985; Helman & Baxendale, 1990). Since open probability remained constant during inhibition of Na^+ entry, it was inferred that the autoregulatory changes in N_0 occurred via long time-constant changes of N_T. This was clearly not the situation in our experiments. A reduction in the mucosal Na^+ concentration induced relatively small changes of N_T, varying between 24% and 81% in 20 and 10 mM Na^+ respectively. These small changes could not alone account for the large increases in N_0. Clearly other factors, like the increase in open probability are equally important.

Palmer & Frindt (1988) found with patch clamp that the open probability of the channels in rat collecting duct was independent of changes in external Na^+. Using noise analysis, Helman and Baxendale (1990) also found that a reduction in apical Na^+ entry by Na^+ channel blockers did not change β' in *R. pipiens*. Nevertheless, the latter authors emphasized the notion that changes of open probability may still, and in some unknown manner, be an important mechanism in the regulation of the rate of Na^+ entry. In support of this view, but in contrast with the previous observations, our data show quite clearly that autoregulatory increases in N_0 were accompanied by parallel changes in open probability. Despite large spontaneous variability in open probability amongst skins, β' consistently increased by 55–60% when the mucosal Na^+ concentration was lowered. In support of our own observations, Ling & Eaton (1989) reported that in cell-attached patches they consistently observed a relative increase in open probability by a reduction in the luminal Na^+ concentration. The conflicting results with patch clamp may be attributed to differences in experimental procedures. Palmer & Frindt (1988) only changed the Na^+ concentrations in the pipette (direct effect on the channels) whereas Ling & Eaton (1989) changed Na^+ in the bathing solution outside the patch pipette. Our results imply that, contrary to previous beliefs (reviewed by Els & Helman, 1989) and at least in epithelium from *R. fuscigula*, changes of channel open probability is an important mechanism in the autoregulation of channel densities in response to a reduction in mucosal Na^+.

The reason why autoregulatory changes in open channel densities in *R. fuscigula* do not occur primarily by changes in N_T is not known. Species and tissue variability

could well account for differences in cellular strategies to compensate for a reduction in Na^+ transport rate. The low innate Na^+ transport rates in our tissues were primarily the result of the small number of open channels (P. H. Russel and W. J. Els, unpublished data). While mean open probability did not differ markedly from those in other frog skins, values of about 17 million/cm^2 for N_o and 60 million/cm^2 for N_T were about 3 to 4 times less than in other species (see Helman & Kizer, 1990). Since the inherent pool of channels in our frog skins was relatively small

TABLE 4. Effects of reduced mucosal Na^+ concentration on epithelial Na^+ channels after treatment with 10 μg/ml cytochalasin B

	110 mM	20 mM	20/110
I_{Na} (μA/cm^2)	10·10 ± 1·07	6·32 ± 1·07	0·63 ± 0·05
i_{Na} (pA)	0·42 ± 0·03	0·27 ± 0·02	0·65 ± 0·04
N_o (10^6/cm^2)	24·3 ± 2·4	23·5 ± 2·4	0·99 ± 0·12*
β'	0·43 ± 0·04	0·67 ± 0·03	1·69 ± 0·17
N_T (10^6/cm^2)	59·5 ± 8·3	46·6 ± 10·1	0·89 ± 0·28*
k_{ob} (rad/(s μM))	4·01 ± 0·25	4·24 ± 0·25	1·06 ± 0·04*
k_{bo} (rad/s)	120·8 ± 5·3	129·0 ± 4·8	1·08 ± 0·04*
K_B (μM)	30·7 ± 1·6	31·1 ± 1·7	1·03 ± 0·06*

Values are means ± S.E.M. ($n = 9$). Non-significant changes ($P < 0·05$) assessed on a paired basis are indicated by asterisks.

it was not surprising that we observed only small changes of N_T in response to a reduction in mucosal Na^+. This is unlike the situation in *R. pipiens* where a reduction in Na^+ uptake by mucosal amiloride increased Na^+ channel densities by an order of magnitude or more (Abramcheck *et al.* 1985; Helman & Baxendale, 1990). In fact, cells in tight epithelia are capable of responding to non-hormonal influences by eliciting changes of channel densities comparable to, or greater than, those elicited by hormones (Els & Helman, 1991). In this regard, it was also apparent that the moderate capacity of our tissues to autoregulate the channel densities was reflected in the long time-constant relaxation of the I_{Na}. After 20 min in low (20 mM) Na^+ we found that the I_{Na} had recovered on average to within 24% of the original level. At this stage the transport rate had reached an apparent steady state. Although they did not do paired experiments, Els & Helman (1991) found that in *R. pipiens* after about two hours in 10 or 5 mM Na^+ the average value of I_{Na} was not significantly different from that exposed to 100 mM Na^+.

Effects of disrupting the cytoskeleton on autoregulation of channel densities

We investigated the role of the cytoskeleton in regulation of channel densities to see if an increase in N_o might occur via recruitment from subapical membrane storage vesicles containing Na^+ channels (Garty & Edelman, 1983; Lewis *et al.* 1984). Alternatively, channels may be acquired from a pool of quiescent channels already present in the membrane (Li, Palmer, Edelman & Lindemann, 1982). In epithelial cells cytoskeletal proteins mediate important membrane functions. The function and composition of the apical membranes may be rapidly modulated by the exocytotic insertion and endocytotic removal of specific membrane components. Specifically, in

the renal collecting duct and urinary bladder the cytoskeleton mediates the rapid insertion of water channels and proton pumps into their plasma membranes in response to environmental changes (Stetson & Steinmetz, 1983; Brown, 1989).

Microtubules are often responsible for sorting and guiding membrane-bound traffic in certain polarized epithelial cells. Hence, disruption of microtubules with depolymerizing drugs blocked the transfer of proteins bound for the apical membrane in Madin–Darby canine kidney cells (Parczyk, Haase & Kondor-Kock, 1989) and markedly interfered with the delivery of water channels to the apical membrane in amphibian urinary bladder (Valenti *et al.* 1988). In our experiments prior treatment of the tissue for 3 h with colchicine had negligible effects on the macroscopic Na^+ transport rate and also did not affect the autoregulation of channel densities by a reduction in mucosal Na^+ concentration (Table 3). Apparently, at least within the time frame of our experiments, the integrity of the microtubules is not essential for regulation of apical Na^+ entry. This result is also consistent with previous observations where in the amphibian urinary bladder disruption of the microtubules did not interfere with Na^+ transport (Pearl & Taylor, 1985). We naturally cannot rule out the probability that microtubules are involved in the delivery of channels to the apical membrane. Autoregulation might only involve channels already present in or at the apical membrane. Parallel immunocytochemical studies showed that our results could not be attributed to colchicine resistance of microtubules. After 3 h incubation in colchicine, the microtubules in most of the epithelial cells disappeared or was severely altered by the treatment (Chou & Els, 1991).

Microfilaments have long been suspected to play an important role in regulation of water and solute transport in tight epithelia. Disruption of the actin cytoskeleton inhibits and antidiuretic hormone-induced increase in water permeability in toad urinary bladder and other tissues (Pearl & Taylor, 1985). Less is known about its role in solute transport but disruption of the cytoskeleton with cytochalasins reduced volume regulation in *Necturus* gall-bladder (Foskett & Spring, 1985) and rabbit proximal tubules (Linshaw, 1989), probably by mechanisms which prevented the activation of KCl transport out of the cells. More recently it was reported that microfilament disruption severely inhibits Na^+ reabsorption in perfused proximal tubules from rats (Kellerman, Clark, Hoilien, Linas & Molitoris, 1990).

Our results demonstrated that microfilaments appear to be involved in the regulation of channel densities. Treating the tissues with cytochalasin B for two hours, markedly decreased the relaxation of I_{Na} in response to a reduction in mucosal Na^+ (Figs 5 and 6). The data summarized in Table 4 indicated that the lack of recovery could be attributed to the absence of an autoregulatory increase in N_0 to compensate for the decrease in Na^+ entry. Significantly, treatment with cytochalasin B did not affect the change of β' but prevented an increase of N_T. These results imply that depolymerization of microfilaments did not affect channels already present in the membrane but apparently prevented the recruitment of new channels to increase the total number of channels. Although direct proof is lacking, the data suggest that autoregulatory activation of Na^+ channels also occurs by recruitment from cytoplasmic stores. A similar mechanism has been suggested by Garty & Edelman (1983) and Lewis *et al.* (1984) whereby, in urinary bladder, new Na^+ channels could be recruited from intracellular stores in response to physiological signals. Foskett &

Spring (1985) also suggested that during volume regulation in *Necturus* gall-bladder, microfilaments allow cytoplasmic vesicles with ion channels to fuse with the cell membrane. This system is in fact widespread among epithelia, enabling them to respond rapidly to external stimuli by selective exocytosis of intracellular vesicles to the membrane (Brown, 1989). Still, other hypotheses for the recruitment of Na^+ channels, or interpretation of the results, are equally tenable. Patch clamp studies have revealed a close association between Na^+ channels in A6 cells and cytoskeletal proteins, leaving open the possibility that the cytoskeleton may play an important direct role in modulating transport by influencing the properties of the channels. Prat *et al.* (1991) demonstrated that in cell-attached and excised membrane patches, disruption of microfilaments with cytochalasin *D* (but not cytochalasin *B*), enhanced Na^+ channel activity.

In summary, in *R. fuscigula* autoregulatory changes in Na^+ channel densities, induced by a reduction in Na^+ transport load, came about through recruitment of channels from closed states (increase in open probability) and through increase of the total channel pool. This is the first demonstration on a paired basis with noise analysis that a reduction in mucosal Na^+ activates Na^+ channels primarily by increasing the open probability. The autoregulatory changes in channel densities were moderate, possibly reflecting variance among species or differences in the functional states of the tissues. This leaves open the possibility that the degree of autoregulation would also depend on the size of the epithelial Na^+ channel pool, i.e. the functional state. While we have no definite proof, our results support the possibility that changes in N_T occurred by recruitment from a cytoplasmic pool. Clearly morphological and other evidence is necessary to determine the cellular mechanism whereby autoregulatory changes in channel densities occur in tight epithelia.

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