Activation of Epithelial Na Channels by Hormonal and Autoregulatory Mechanisms of Action

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ABSTRACT Methods of blocker-induced noise analysis were used to investigate the way in which forskolin and vasopressin stimulate Na transport at apical membranes of short-circuited frog skin transporting Na at spontaneous rates of transport. Experiments were done under conditions where the apical Ringer solution contained either 100 mM Na or a reduced Na concentration of 5 or 10 mM Na and buffered with either HCO_3 or HEPES. Reduction of apical solution Na concentration caused a large autoregulatory increase of Na channel density (N_T) similar in magnitude to that observed previously in response to blocker (amiloride) inhibition of apical membrane Na entry. Forskolin at 2.5 μM caused maximal and reversible large increases of N_T, which were larger than could be elicited by 30 mU/ml vasopressin. In both the absence and presence of the autoregulatory increase of N_T (caused by reduction of apical Na concentration), forskolin caused large increases of N_T. Although the fractional increases of N_T in response to forskolin were roughly similar, the absolute increases of N_T were considerably larger in those tissues studied at reduced Na concentration and where baseline values of N_T were markedly elevated by reduction of apical Na concentration. Because the effects on N_T were additive, it is likely that the cAMP-dependent and autoregulatory mechanisms that lead to changes of N_T are distinct. We speculate that autoregulation of N_T may involve change of the size of a cytosolic pool of Na-containing vesicles that are in dynamic balance with the apical membranes. cAMP-dependent regulation of N_T may involve change of the dynamic balance between vesicles and the apical membranes of these epithelial cells. Alternative hypotheses cannot at present be ruled out, but will require incorporation of the idea that regulation of N_T can occur both by hormonal and nonhormonal (autoregulatory) mechanisms of action.

INTRODUCTION

Considerable attention has been given to understanding the mechanism by which 3',5'-cyclic adenosine monophosphate (cAMP) is involved in the regulation of apical membrane Na^+ transport in epithelial cells. Changes of intracellular concentration of cAMP are known to alter apical membrane permeability to Na^+ (Orloff and Handler, 1962; Handler et al., 1965) and electrical conductance (Nagel, 1978; Els and Helman, 1981; Els and Mahlangu, 1987). When tight epithelia of frog skin are stimulated by oxytocin (Li et al., 1982) or vasopressin (Helman et al., 1983), the density of open Na^+ channels is increased as determined by blocker-induced (amiloride) noise analysis.

Because previous investigations were carried out in the presence of amiloride and consequently under conditions where the macroscopic rates of Na transport were markedly inhibited (Li et al., 1982; Helman et al., 1983), it became of interest to evaluate the response of tissues to cAMP at or near spontaneous rates of transepithelial Na transport. In this regard, we chose to use the weak Na channel blocker, CDPC (6-chloro-3,5-diamino-pyrazine-2-carboxamide), as CDPC-induced noise analysis of epithelial Na channels can be carried out with tissues transporting Na quite near their spontaneous rates of Na transport and under conditions where it is possible to determine not only single channel currents and channel densities but also channel open probabilities (Helman and Baxendale, 1990). Since inhibition of Na transport by 0.5 μM amiloride has been observed to cause about a sevenfold autoregulatory increase of open channel density (Helman and Baxendale, 1990), and since cAMP is believed to increase open channel density, it was of particular interest to know to what extent cAMP caused changes of channel density in the presence and absence of autoregulatory changes of Na transport.

In this paper we report the changes of apical membrane single channel currents and channel densities at normal and reduced apical solution Na^+ concentrations consequent to stimulation of epithelial sheets of frog skin by vasopressin and in particular by forskolin that acts directly on the catalytic subunit of adenylate cyclase to increase intracellular concentration of cAMP.

MATERIALS AND METHODS

Experiments were carried out with abdominal skins of Northern frogs (*Rana pipiens;* Kons Scientific Co., Inc., Germantown, WI) with methods described in detail elsewhere (Helman and Baxendale, 1990). The tissues were scraped free of ~90% of the corium and short-circuited continuously for 2–3 h to allow the short-circuit current (I_{sc}) to stabilize. A HCO_3-buffered Ringer solution containing (in mM): 100 NaCl, 2.4 $KHCO_3$, and 2 $CaCl_2$ bathed both apical and basolateral surfaces of the tissues. Apical and basolateral solutions were allowed to flow continuously and without recirculation through chambers designed especially for conduct of current–noise analysis under transepithelial voltage clamp conditions (Abramcheck et al., 1985). Except for 2-min periods of data collection for determination of power density spectra (PDS), a voltage command pulse of a few millivolts and of 2-s duration was used to follow the changes of transepithelial conductance as determined from the current deflections recorded on a strip chart recorder (see Fig. 1). Forskolin (stock solution of 10^{-2} M in ethanol; Sigma Chemical Co., St. Louis, MO) was used at final concentrations of 2.5 and 25 μM, and vasopressin (Sigma Chemical Co.) was used at a final concentration of 30 mU/ml of basolateral

solution. Apical solution Na$^+$ concentration was reduced by equimolar substitution of Na$^+$ with tetramethylammonium to either 10 or 5 mM. A HEPES-buffered Ringer solution containing (mM): 100 NaCl, 2.4 KCl, 2 CaCl$_2$, and 2 HEPES was used in some groups of experiments (see Results). The notations "100 Na," "10 Na," and "5 Na" will refer to the Na concentration in millimolar of the apical solution. All experiments were carried out at room temperature (22–27°C).

Blocker-induced Noise Analysis

Staircase protocol. Introduction of CDPC (catalog No. 27,788-6; Aldrich Chemical Co., Milwaukee, WI) into the apical solution causes the appearance of a Lorentzian component of the current–noise PDS whose corner frequency (f_c) increases linearly with increasing blocker concentration [B] (Baxendale and Helman, 1986; Helman and Baxendale, 1990). On- (k_{ob}) and off-rate (k_{bo}) coefficients of blocker interaction with the open state of the channel were measured from the slope and intercept of the [B]-$2\pi f_c$ relationship according to $2\pi f_c = k_{ob}$ [B] + k_{bo}, and the K_B's were calculated from the quotient k_{bo}/k_{ob}. CDPC was introduced into the apical solution in a staircase manner at increasing concentrations of 5, 10, 20, 30, 40, and 50 μM that gave f_c's between ~30 and 120 Hz. The low frequency plateau value of the Lorentzian (S_o), taken together with the $2\pi f_c$ (radians per second), the amiloride-sensitive macroscopic rates of Na$^+$ transport, I_{Na}^B, and the on-rate coefficients were used to calculate the single channel Na$^+$ currents, i_{Na}^B, at all [B] according to Eq. 1. Open channel density N_o^B was calculated as I_{Na}^B/i_{Na}^B.

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

The density of open + blocked states of the channels (N_{ob}) according to mass law action was calculated with Eq. 2:

$$N_{ob} = N_o^B(1 + [B]/K_B) \tag{2}$$

It should be emphasized that values of I_{Na}^B, i_{Na}^B, N_o^B, and N_{ob} were determined under conditions where blocker inhibits a fraction of the spontaneous rate of apical Na$^+$ entry (I_{Na}). At 50 μM CDPC or less, the fractional inhibition of the macroscopic I_{Na} is <20% (see Results) and is graded with increasing blocker concentration.

To obtain values of i_{Na} and open channel density, N_o, in the absence of blocker, the i_{Na}^B were extrapolated to zero [B] and the N_o was calculated as I_{Na}/i_{Na}. The superscript B notation will indicate that measurements were made in the presence of blocker.

Apparent open channel probabilities ("β'''") were calculated with Eq. 3 (Helman and Baxendale, 1990), and the channel open probability in the absence of blocker (β') was determined at the zero blocker concentration intercept at the ordinate of the [B]-"β'''" relationships (see Results). Total channel density, N_T, was calculated from the quotient N_o/β'.

$$\beta' = \frac{1 - (N_o^B/N_o)}{(N_o^B/N_o)([B]/K_B)} = \frac{N_o/N_o^B - 1}{[B]/K_B} \tag{3}$$

It should be noted that (a) linearity of the [B]-$2\pi f_c$ relationship is indicative of constancy of k_{ob} and k_{bo} for at least the 35–40 min required to obtain the PDS; (b) k_{ob} and k_{bo} do not vary with inhibition of I_{Na}^B by [CDPC] between 5 and 200 μM; and (c) k_{ob} and k_{bo} do not vary as a function of the spontaneous I_{Na} in epithelia bathed with 100 mM Na and HCO$_3$-buffered Ringer solution (Helman and Baxendale, 1990).

Time course protocol. To determine the time-dependent changes of channel densities and single channel currents, experiments referred to as time course experiments were done with a constant 20 μM CDPC in the apical solution during both control and experimental periods.

Prior to this, tissues were studied as described above with staircase protocol procedures that provided values of k_{ob} required for calculation of i_{Na}^B.

The time course control period consisted of a 30-min period during which PDS were measured at 5-min intervals. i_{Na}^B and N_o^B were calculated from the measured S_o, f_c, and I_{Na}^B, and the k_{ob} measured previously. It was established with staircase protocol experiments that the k_{ob} for CDPC was not changed by vasopressin or forskolin (see Results), thereby justifying the assumption made here in the calculation of i_{Na}^B. A similar insensitivity to vasopressin (Helman et al., 1983) and oxytocin (Li et al., 1982) of the amiloride k_{ob} has been reported. Data collection during experimental periods was continued at 5-min intervals (a) after addition of forskolin or vasopressin to the basolateral solution, (b) during return to the blocker control condition, and (c) during restimulation of Na^+ transport by either agent.

Tissue cAMP

Assay for tissue concentration of cAMP was carried out with isolated epithelia (split skins) where the corium with glands was separated completely from the epithelium (and verified by microscopic observation of the basolateral surface of the tissue) after incubation with collagenase (Fisher et al., 1980). Each isolated epithelium was divided into four to five pieces (1 cm^2 each) that permitted paired analysis of control and experimental values of cAMP. Experimental pieces of tissue were treated with either 2.5 or 25 μM forskolin for 20 and 60 min. One piece of tissue was allowed to recover to control conditions after further incubation in forskolin-free Ringer solution for 60–90 min. During all phases of these experiments, exceptional care was taken to assure that the tissues remained "self" short circuited while incubated in vials so as to avoid open circuiting of the tissues that may lead to changes of tissue cAMP unrelated to the action of forskolin.

All tissues were processed identically for determination of cAMP. After quickly siphoning off extracellular fluid and freezing on a dry ice–cooled aluminum plate (1–2 s), the tissues were dropped into 500 μl of boiling sodium acetate buffer (pH 6.2) containing 8 mM theophylline (or IBMX) for 2.5–3 min and stored thereafter when necessary at −80°C for subsequent analysis. The tissues were homogenized with a power-driven pestle, vortex mixed, and then sonicated briefly for subsequent radioimmunoassay using ^{125}I-cAMP.

cAMP concentrations were determined with the assistance of Ms. Tracey Mencio using a validated radioimmunoassay in the laboratory of Dr. Victor Ramirez of our department according to a procedure described previously (Kim and Ramirez, 1986). The sensitivity of the assay was 0.1 pmol/tube, and the inter- and intraassay coefficients of variation were 8–9% and 3–4% at 5 pmol/tube, respectively.

Statistical data are summarized as means ± SEM. Student's t test was used to determine the significance ($P < 0.05$) of differences between means and was done on a paired basis where possible.

Intracellular Na^+ Concentration

Intracellular Na^+ concentration was measured by the ^{24}Na isotope equilibration method of Stoddard and Helman (1985). Paired pieces of the same isolated epithelium were exposed to Ringer solution containing ~2 μCi ^{24}Na during a 60-min control incubation. Experimental pieces were treated thereafter with 2.5 μM forskolin for 20 or 60 min. Intracellular water (ICW) was determined after correction for extracellular water (including stratum corneum) and expressed as ICW per square centimeter or per milligram dry weight (DW) of stratified epithelial cells. The amount of intracellular Na^+ (A_{Na}) was calculated from the zero time intercept of cellular isotope content from washout curves carried out in the presence of 100 μM amiloride, 1 mM ouabain, and 1 mM furosemide to trap ^{24}Na within the cells. From the

half-time of washout of ^{24}Na under these conditions, the unidirectional basolateral membrane leak efflux rate of Na^+ (FJ_{Na}^{23*}) was calculated and expressed in microamperes per square centimeter.

RESULTS

Macroscopic Changes of I_{Na}

Stimulation of Na transport in tight epithelia in response to vasopressin and other agents is known to be highly variable among batches of tissues and the conditions of study. In general, stimulation of Na transport has been observed to be transient but reversible. In this regard, studies were carried out to evaluate the changes of single channel currents and open channel densities that contributed to the macroscopic changes of Na transport.

Summarized in Table I are the changes of I_{Na} caused by 2.5 μM forskolin and 30 mU/ml vasopressin. Within 15–20 min I_{Na} was maximally stimulated more by forskolin than by vasopressin, from mean control values of 20.69 and 26.89 μA/cm², respectively. During the following 60–90 min (experimental period 1), the I_{Na} decreased gradually (Fig. 1) but remained elevated above control values that were measured just before treatment of the tissues. Occasionally, as illustrated in Fig. 1 A, oscillation of the I_{Na} was observed.

The effects of forskolin and vasopressin on I_{Na} were reversible (Fig. 1, Table I). After allowing the tissues to recover to their unstimulated state, the tissues were challenged again with forskolin or vasopressin (experimental period 2). The recovery values of I_{Na} were invariably lower than those of the original control values, averaging 12.57 and 16.85 μA/cm² in tissues treated with forskolin and vasopressin, respectively. The I_{Na} appeared to be more sensitive to restimulation by forskolin and vasopressin, and this increased sensitivity is probably due to lower baseline values of the recovery I_{Na}.

Whereas the I_{Na} exhibited transient behavior (see Table I; Control, Max, and SS values of I_{Na}) during the first exposure to forskolin and vasopressin, such transient behavior of the I_{Na} was either absent or markedly reduced upon the second exposure to these agents despite larger fractional increases of the I_{Na}. In this regard, the tissues exhibited a "memory" effect with a time period much longer than could be attributed to changes of intracellular cAMP.

Staircase Protocol Noise Analysis

Tissues were studied with CDPC-induced noise analysis during control, first experimental, recovery, and second experimental periods as illustrated in the strip chart recordings of Fig. 1. After sequential exposure to increasing concentrations of CDPC, the blocker was washed completely from the apical solution. PDS were determined at each blocker concentration and values of I_{Na}^B, S_o, and radian frequency, $2\pi f_c$, were plotted for each experiment and summarized as shown in Fig. 2 (all data not shown for sake of clarity). CDPC caused a relatively small inhibition of the I_{Na} at concentrations between 5 and 50 μM CDPC (Fig. 2, A and E). Inhibition of I_{Na}^B due to mass law action of the blocker was smaller than expected due to an autoregulatory increase of channel density that accompanies blocker inhibition of Na transport (Helman and

TABLE I

Effect of Forskolin and Vasopressin on I_{Na}

	Control	Experimental period 1		Recovery	Experimental period 2	
		Max	SS		Max	SS
			$\mu A/cm^2$			
Forskolin	20.69 ± 2.07 (18)	39.99 ± 3.16 (18)	34.57 ± 2.80 (18)	12.57 ± 1.41 (18)	31.33 ± 3.17 (3)	28.30 ± 3.12 (3)
Vasopressin	26.89 ± 3.11 (13)	38.24 ± 5.52 (13)	32.92 ± 4.27 (13)	16.85 ± 1.84 (13)	31.10 ± 4.61 (9)	30.36 ± 4.44 (9)

	Max/Cont	SS/Cont	Rec /SS	Max/Rec	SS/Rec
Forskolin	2.19 ± 0.21	1.95 ± 0.23	0.39 ± 0.04	5.83 ± 2.33	5.20 ± 1.99
Vasopressin	1.40 ± 0.08	1.23 ± 0.06	0.55 ± 0.04	1.79 ± 0.19	1.76 ± 0.19

Epithelia were treated with either 2.5 μM forskolin or 30 mU/ml vasopressin with recovery (Rec) to the control (Cont) condition between experimental periods (1 and 2). Maximum (Max) and steady state (SS) refer to values of I_{Na} 15–20 min and 60–90 min after stimulation of I_{Na}, respectively. Values are means ± SEM.

Baxendale, 1990). This autoregulatory response of channel density was not investigated in the present series of studies, but its existence should be noted insofar as blocker-related autoregulatory increases of channel density are reflected in changes of macroscopic rates of Na transport[1] and evaluation of channel open probability (see below).

Single channel currents, i_{Na}^B, were observed to increase slightly with increase of the blocker concentration (Fig. 2, *D* and *H*). Forskolin more than vasopressin caused a reversible decrease of the i_{Na}^B regardless of the blocker concentration, and in general the i_{Na}^B values were greater in the recovery periods than during the control periods.
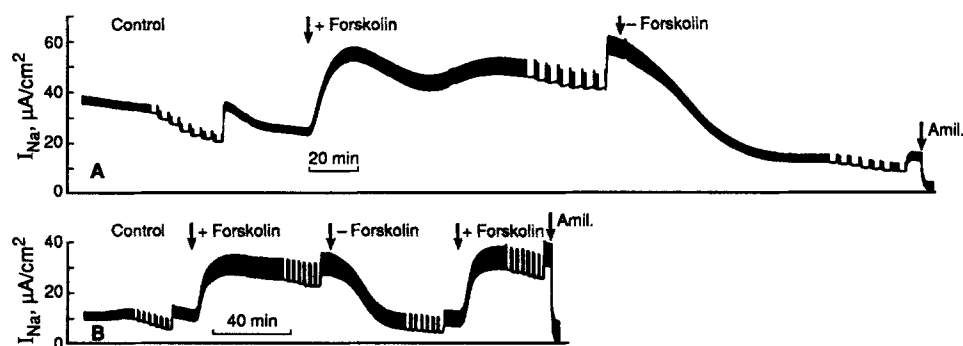

FIGURE 1.   Strip chart recordings showing changes of I_{Na} caused by forskolin (2.5 μM). During control and experimental periods, tissues were exposed to a staircase increase of CDPC concentration (5–50 μM) and PDS measured during periods when the voltage command pulse generator was turned off. Note the difference in time scale for the experiments shown in *A* and *B*. Amiloride (100 μM) was used at the end of each experiment to determine the amiloride-insensitive short-circuit current that normally averaged near 1 μA/cm². Mean changes of I_{Na} and other blocker concentration-dependent parameters are summarized in Fig. 2.

The rate–concentration plots ([B] vs. $2\pi f_c$; Fig. 2, *C* and *G*) were invariably linear yielding the k_{ob} and k_{bo} of CDPC interaction with the open channels. As indicated in Table II and Fig. 2, *C* and *G*, neither vasopressin nor forskolin caused a change of the on rate, k_{ob}. A selective and reversible increase of the off rate, k_{bo}, which was reflected in the changes of K_B summarized in Table II, was caused by vasopressin and forskolin.

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[1] The autoregulatory response of Na channel density to blocker inhibition of Na transport cannot be avoided with use of staircase protocols where tissues are subject to graded increases of [B] for periods of 30–40 min. Accordingly, the appearance of macroscopic changes of I_{Na}^B will vary not only with differences of K_B and β′ but also with the time constant and magnitude of the autoregulatory response of channel density to blocker inhibition of Na transport. To the extent that the time constant is in the range of 10–20 min (Helman and Baxendale, 1990) and may vary during the course of an experiment, and since the autoregulatory increases of open channel density are time dependent and are quantitatively similar in magnitude to the mass law related decreases of channel density, the macroscopic changes of I_{Na} in response to staircase increases of blocker concentration may not appear to be the same during all phases of an experiment.
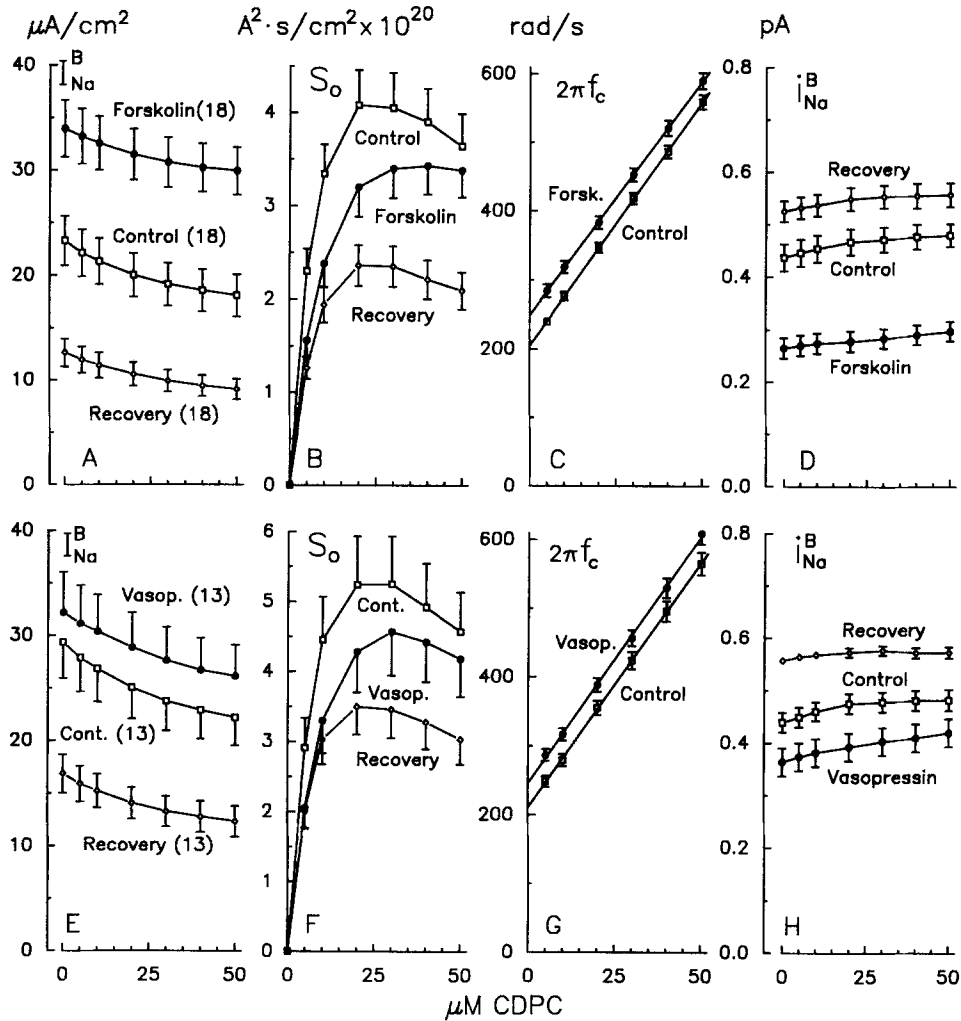
FIGURE 2.   Blocker-dependent changes of I_{Na}^B, S_o, $2\pi f_c$, and i_{Na}^B in control, experimental, and control periods. (A–D) 2.5 μM forskolin. (E–H) 30 mU/ml vasopressin. Note increase of k_{bo} and no change of k_{ob} (see also Table II). i_{Na} (no blocker) was determined by extrapolation of i_{Na}^B to zero [CDPC]. For the purpose of clarity, recovery period rate–concentration relationships (C and G) have been omitted (see Table I).

Single channel currents, i_{Na} (extrapolated to zero blocker concentration), were used to calculate the open channel density, N_o, with the corresponding I_{Na} measured before exposure of the tissues to CDPC. These values for the blocker-free state of the tissues are also summarized in Table II. i_{Na} averaged 0.44 pA and was decreased appreciably and reversibly by vasopressin and forskolin.

Control open channel density, N_o, averaged 60.1 and 67.8 million/cm^2 (Table II) in forskolin and vasopressin groups of experiments, respectively. The forskolin- and vasopressin-mediated changes of N_o were reversible. The recovery N_o were invariably

less in value than control values (25.2 and 30.3 million/cm²; Table II). The greater sensitivity to restimulation of Na transport by forskolin and vasopressin could be attributed to larger increases of N_o from lower baseline values of N_o (Table II).

The changes of N_o were due principally to changes of total channel density, N_T, and to a much lesser extent to changes of channel open probability. As indicated in Fig. 3, β' was determined by extrapolation of the [B]-"β'''" relationships to the

TABLE II

Effects of Forskolin and Vasopressin on Epithelial Na Channels
(100 mM Na, HCO_3-buffered Ringer Solution)

	Control Forskolin: $n = 18$ Vasopressin: $n = 13$	Experimental period 1 Forskolin: $n = 18$ Vasopressin: $n = 13$	Recovery Forskolin: $n = 17$ Vasopressin: $n = 13$	Experimental period 2 Forskolin: $n = 3$ Vasopressin: $n = 9$
I_{Na} (μA/cm²)				
Forskolin	23.26 ± 2.37	33.95 ± 2.72	12.61 ± 1.36	28.71 ± 3.32
Vasopressin	28.82 ± 3.42	32.21 ± 3.86	16.85 ± 1.84	30.69 ± 4.30
i_{Na} (pA)				
Forskolin	0.44 ± 0.03	0.27 ± 0.02	0.53 ± 0.02	0.39 ± 0.07
Vasopressin	0.44 ± 0.02	0.36 ± 0.03	0.56 ± 0.01	0.41 ± 0.02
N_o (10⁶/cm²)				
Forskolin	60.1 ± 8.4	148 ± 20	25.2 ± 3.1	80.8 ± 21.3
Vasopressin	67.8 ± 8.7	96.3 ± 13.7	30.3 ± 3.3	77.1 ± 11.7
β'				
Forskolin	0.46 ± 0.04	0.34 ± 0.03	0.49 ± 0.03	0.40 ± 0.06
Vasopressin	0.49 ± 0.03	0.44 ± 0.04*	0.44 ± 0.03ᵗ	0.50 ± 0.03
N_T (10⁶/cm²)				
Forskolin	141 ± 20	467 ± 62	57.0 ± 9.7	220 ± 69
Vasopressin	145 ± 21	216 ± 26	74.9 ± 6.9	158 ± 26
k_{ob} (rad/s · μM)				
Forskolin	6.96 ± 0.14	6.63 ± 0.14*	6.64 ± 0.18ᵗ	6.51 ± 0.18ˢ
Vasopressin	7.16 ± 0.26	7.17 ± 0.27*	7.13 ± 0.29ᵗ	7.07 ± 0.36ˢ
k_{bo} (rad/s)				
Forskolin	208 ± 6.2	253 ± 9.3	222 ± 6.3	220 ± 5.9
Vasopressin	211 ± 6.9	247 ± 8.2	221 ± 5.0	255 ± 8.1
K_B (μM)				
Forskolin	30.0 ± 1.0	38.5 ± 1.8	33.9 ± 1.4	34.3 ± 3.1
Vasopressin	29.7 ± 1.0	35.4 ± 2.3	31.7 ± 1.5	36.9 ± 2.2

Values are means ± SEM (n). Columns 1 and 3 are control (C) and recovery (R) periods, respectively, during which forskolin or vasopressin was absent from the basolateral solution. Columns 2 and 4 refer to experimental periods (E1 and E2) during which tissues were treated with forskolin or vasopressin. Nonsignificant changes ($P < 0.05$) assessed on a paired basis are indicated by *(E1/C), ᵗ(R/E1), and ˢ(E2/R).

blocker-free state of the tissues, thereby taking into account autoregulatory increases of N_T that give rise to apparent values of β' that underestimate the actual β' (Helman and Baxendale, 1990). When analyzed on a paired basis, forskolin caused a 21 and 28% decrease of β' from control and recovery baseline values of 0.46 and 0.49, respectively (Table II). The changes of β' caused by vasopressin were not significant. Accordingly, the increases of N_o caused by forskolin and vasopressin were due to

increases of N_T that are summarized in Table II. From control and recovery baseline values of 141 and 57 million/cm², forskolin caused N_T to increase 4.83 ± 0.89- and 7.18 ± 0.33-fold, respectively. Smaller increases of N_T were observed with vasopressin, where from control and recovery baseline values of 145 and 74.9 million/cm², N_T was increased 1.77 ± 0.23- and 2.27 ± 0.49-fold, respectively. In this regard, it appeared that 2.5 μM forskolin was a significantly more potent stimulator of Na transport than 30 mU/ml vasopressin.
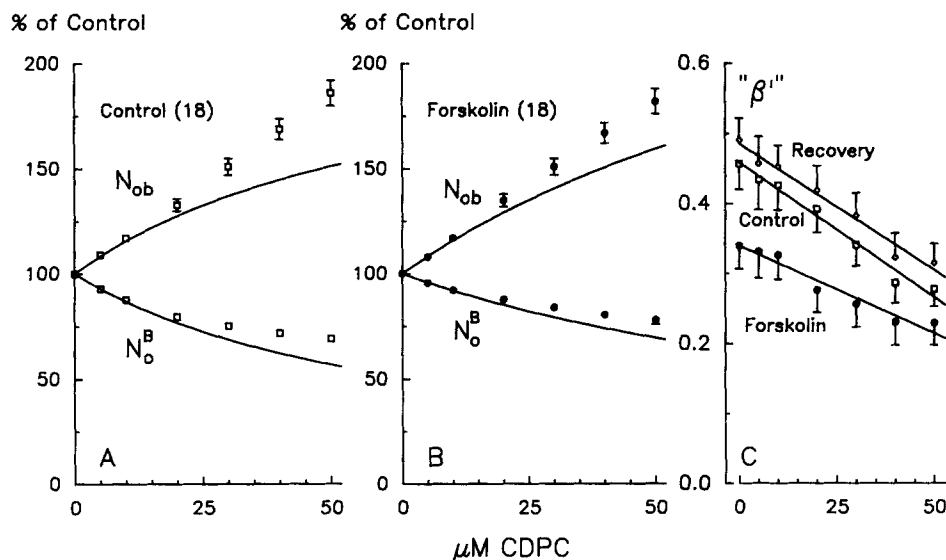

FIGURE 3.   Blocker-dependent changes of open channel density (N_o^B and open + blocked (N_{ob}) channel densities in control and forskolin-treated tissues. Data (A and B) were normalized to values of N_o (no blocker; see Table II). Apparent open probability ("β''', panel C) was calculated with Eq. 3. Open probability in the absence of blocker (β') was measured by extrapolation of "β''' to the ordinate (see Table II). Similar analysis was carried out for vasopressin-treated tissues (not shown). Solid lines in A and B were calculated for expected mass law changes of channel densities (assuming $N_T^B = N_T$) with mean β' of 0.46 and 0.34 for control and forskolin-treated tissues. Deviations from mass law behavior indicate autoregulatory increases of N_T. β' after recovery to the control condition averaged 0.49 and the changes of N_o^B and N_{ob} (not shown) were similar to those of A. Note that data were derived with a staircase protocol of increasing blocker concentration where autoregulatory processes in addition to mass law effects contribute to changes of channel densities (Helman and Baxendale, 1990).

Time Course Protocol Noise Analysis

Forskolin. Time course protocol experiments were done to evaluate the time-dependent changes of i_{Na}^B and N_o^B after treatment of tissues with 2.5 or 25 μM forskolin. In these experiments, 20 μM CDPC was present continuously in the apical solution, giving rise to PDS that were measured at 5-min intervals during control, experimental, and recovery periods. For the group of experiments ($n = 5$) illustrated in Fig. 4, the control I_{Na}^B was 12.49 ± 2.08 μA/cm², i_{Na}^B was 0.68 ± 0.06 pA, and N_o^B

was 19.8 ± 4.5 million/cm². N_o^B was maximally stimulated within 20 min by 2.5 μM (Fig. 4) or 25 μM forskolin (not shown), and N_o^B remained constant thereafter in experimental periods. For tissues treated with 2.5 μM forskolin, N_o^B began to decrease within 5 min after removal of forskolin from the basolateral solution and fell to recovery values less than control, but more slowly than was observed upon stimulation of Na transport. When tissues were treated with 25 μM forskolin, a similar pattern was observed except that return of the N_o^B to recovery values was delayed by ~15–20 min after removal of forskolin from the basolateral solution.

Despite a 10-fold increase of forskolin concentration (and a considerably larger increase of cAMP concentration; see below), the increase of N_o^B was not greater than that elicited by 2.5 μM forskolin at comparable spontaneous rates of I_{Na}. In four experiments (data not shown), epithelia were exposed in sequence to 2.5 and 25 μM
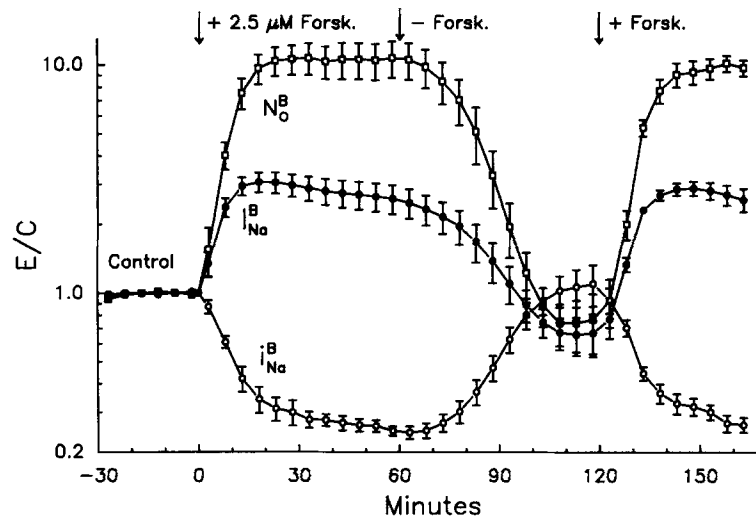

FIGURE 4.   Response of tissues to 2.5 μM forskolin as determined with time course protocols (see text). Data are expressed as experimental/control (E/C) (n = 5). See text for control values. Similar results were obtained with 25 μM forskolin.

forskolin with no additional changes of N_o^B, i_{Na}^B, or I_{Na}^B. Thus, maximal responses were elicited by 2.5 μM forskolin.

In experiments with forskolin, the transient changes of I_{Na}^B were due principally to time-dependent changes of i_{Na}^B. This is illustrated in Fig. 4 where, following the initial changes of I_{Na}^B, i_{Na}^B, and N_o^B that occurred within 15–20 min, the i_{Na}^B continued to fall with time and in parallel with the changes of I_{Na}^B while the N_o^B remained constant.

Vasopressin. 30 mU/ml vasopressin elicits a near maximal stimulation of Na transport in skins of *R. pipiens*. In contrast to experiments with forskolin, and as illustrated in Fig. 5, the response of N_o^B was transient upon first time stimulation with vasopressin. Notably, this transient of N_o^B was absent upon restimulation from the recovery state of the tissues. At comparable I_{Na}^B (control I_{Na}^B = 12.86 ± 1.75 μA/cm²; i_{Na}^B = 0.53 ± 0.02 pA; N_o^B = 24.8 ± 4.4 million/cm² [n = 5]) to experiments with
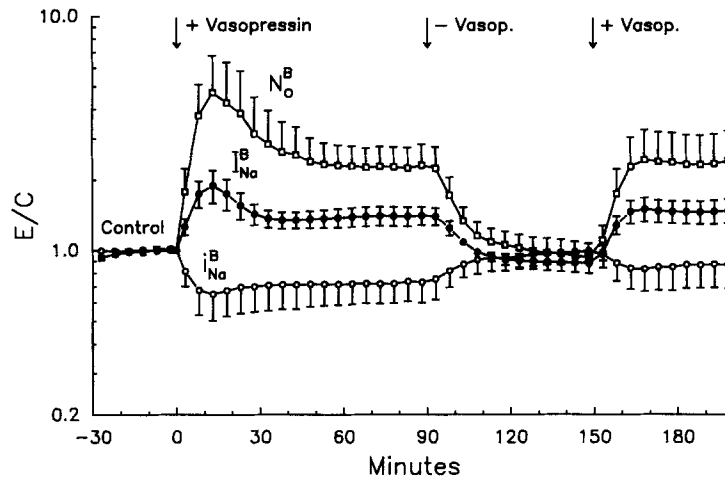
FIGURE 5.   Response of tissues to 30 mU/ml vasopressin as determined with time course protocols (see text). Data are expressed as experimental/control (E/C) $(n = 5)$. See text for control values.

forskolin, the magnitude of the N_o^B response to vasopressin was less than observed with forskolin (compare above), and the transient behavior of I_{Na}^B when observed was due principally to transient changes of N_o^B and not to i_{Na}^B.

Autoregulation of Channel Density

Acute reduction of the Na concentration of the apical solution is known to lead to a transient decrease of the short-circuit current and hence a transient decrease of the rate of Na entry into the cells not unlike the response of tissues to submaximal blocker inhibition of Na transport. As indicated in Table III and Fig. 6, reduction of apical Na concentration to 5 mM caused the i_{Na} to decrease to 0.067 pA and the N_o to increase to a mean of 302 million/cm^2 (compare with Table II). Thus, in spite of the

TABLE III

Effects of Forskolin on Epithelial Na Channels
(5 mM Na, HCO_3-buffered Ringer Solution)

	5 mM Na control	2.5 μM forskolin	Recovery
I_{Na} (μA/cm^2)	19.98 ± 1.80	29.67 ± 4.38	13.42 ± 2.17
i_{Na} (pA)	0.067 ± 0.004	0.054 ± 0.009	0.098 ± 0.011
N_o (10^6/cm^2)	302 ± 28	626 ± 100	156 ± 32
β'	0.66 ± 0.03	0.59 ± 0.06*	0.70 ± 0.05^i
N_T (10^6/cm^2)	464 ± 47	1093 ± 142	283 ± 63
k_{ob} (rad/s·μM)	7.57 ± 0.25	6.65 ± 0.36*	6.76 ± 0.40^i
k_{bo} (rad/s)	161 ± 6.8	207 ± 17.7	231 ± 12.2
K_B (μM)	21.4 ± 0.8	32.0 ± 3.4	35.3 ± 3.4

Apical solution contained 5 mM Na and was buffered with HCO_3. Values are means ± SEM $(n = 8)$. Nonsignificant changes $(P < 0.05)$ are indicated by *(forskolin/control) and ^{i}(recovery/forskolin).

fact that the macroscopic I_{Na} averaged 19.98 μA/cm^2 and was similar in value to that of tissues bathed with the 100 Na apical solution, it was apparent that the similarity of I_{Na} at reduced 5 Na was due both to a large decrease of i_{Na} to 15% and to a large increase of the N_o to ~502% of their values of 100 Na-bathed tissues. Channel open probability, β', averaged 0.66 (5 Na, Table III) and was elevated above values observed with 100 Na-bathed tissues (Table II). Total channel density, N_T, averaged 464 million/cm^2 (Table III) and was increased to ~330% of its value when tissues were studied with the 100 Na Ringer solution (compare with Table II).

Similar studies were carried out with tissues bathed with a HEPES-buffered Ringer solution and where apical solution Na concentration was either 100 or 10 mM Na. I_{Na} averaged 13.90 ± 1.84 (100 Na, n = 9) and 13.63 ± 2.53 (10 Na, n = 6) μA/cm^2. i_{Na}
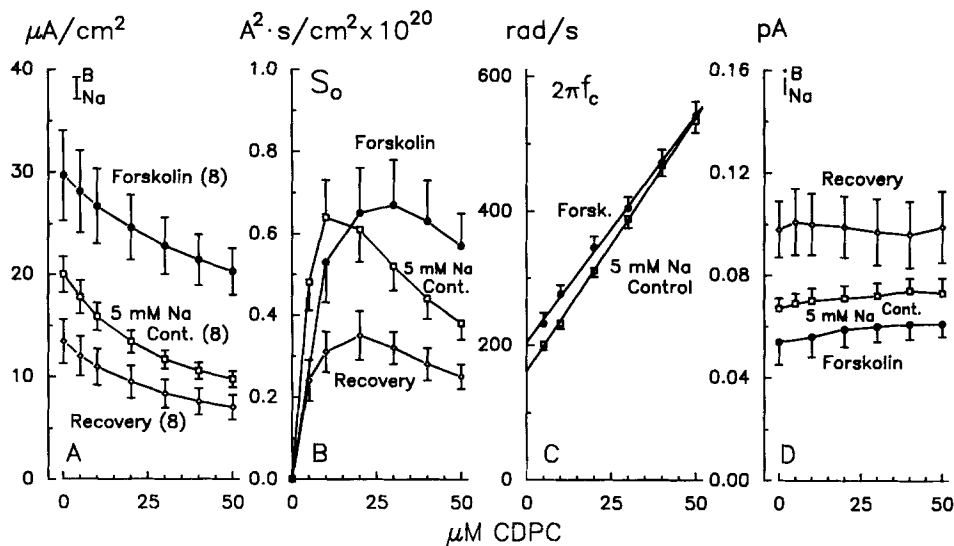

FIGURE 6. Epithelia were bathed with apical 5 mM Na (HCO$_3$-buffered) Ringer solution. Shown are blocker concentration-dependent changes of I_{Na}^B, S_o, $2\pi f_c$, and i_{Na}^B for epithelia in their control state, after treatment with 2.5 μM forskolin, and after recovery to their control state. Recovery data in C are omitted for clarity (see Table III).

was decreased from 0.37 ± 0.04 to 0.12 ± 0.01 pA, and N_o was increased from 43.3 ± 7.7 to 124 ± 33 million/cm^2 by reduction of apical Na concentration. β' averaged 0.50 ± 0.04 (100 Na) and 0.64 ± 0.08 (10 Na), and hence reduction of apical Na concentration caused the N_T to increase from 90.3 ± 14.7 (100 Na) to 228 ± 88 (10 Na) million/cm^2. The response to reduction of apical Na concentration was thus similar for tissues bathed with either the HCO$_3$- or HEPES-buffered Ringer solutions.

The k_{ob} of HEPES-buffered tissues averaged 7.46 ± 0.30 (100 Na) and 7.67 ± 0.42 (10 Na) and the k_{ob} of HCO$_3$-buffered tissues averaged 7.57 ± 0.25 (5 Na; compare 100 Na, Table II); thus, k_{ob} was unchanged by reduction of Na concentration. On the other hand, k_{bo} was decreased by reduction of Na concentration. For HEPES-buffered

tissues k_{bo} was decreased from 214 ± 12.4 (100 Na) to 173 ± 6.6 (10 Na), and for HCO_3-buffered tissues k_{bo} was decreased from 208 ± 6.2 (100 Na, Table II) to 161 ± 6.8 (5 Na, Table III). Accordingly, reduction of apical Na concentration caused a selective decrease of the k_{bo} that was accompanied by appreciable decreases of single channel currents and increases of channel densities.

It is evident that large autoregulatory increases of channel density occurred in response to the reduction in apical solution $[Na^+]$. This is probably due to decreased apical Na entry since reduction of apical Na entry by blocker inhibition of Na channels leads to an autoregulatory increase of channel density.

Response to Forskolin at Reduced Apical Na Concentration: 5 mM Na

Despite the fact that channel density was elevated markedly by reduction of apical solution Na concentration, the 5 Na tissues remained sensitive to stimulation of Na transport by forskolin. As illustrated in Fig. 6 and from data summarized in Table III, it was apparent that forskolin caused a reversible decrease of i_{Na}^B with recovery values
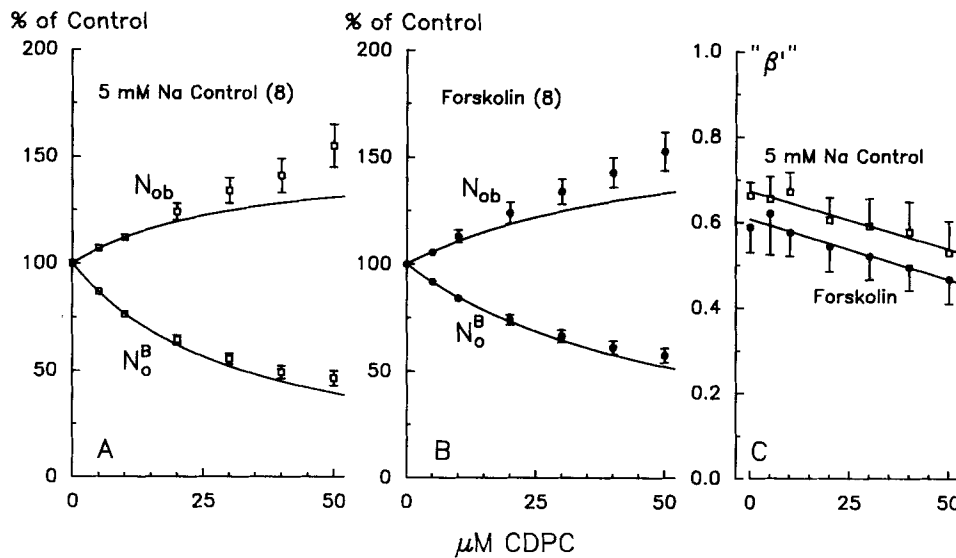

FIGURE 7. Blocker-dependent changes of channel densities in 5 mM Na control and 2.5 μM forskolin-treated tissues. "β'" values were extrapolated to the ordinate to give values of β' (Table III).

(0.098 pA) greater than controls (0.067 pA). As with 100 Na-bathed tissues, the recovery I_{Na} was less than control I_{Na}, and the recovery N_o (156 million/cm²) was considerably less in value than observed in the control (302 million/cm²) or forskolin experimental periods (626 million/cm²). Channel open probability, β', was not significantly changed from control in experimental (Fig. 7) or recovery periods and in this regard the response to forskolin was not the same as was observed with 100 Na

tissues (see Table II). From its elevated value of 464 million/cm^2, N_T was increased markedly to 1,093 million/cm^2 by 2.5 μM forskolin with recovery to 283 million/cm^2 after washout of forskolin from the basolateral solution.

Similar observations were made with tissues bathed with HEPES-buffered Ringer solution containing 10 Na. N_T was increased by forskolin from 228 $\pm$ 88 to 800 $\pm$ 233 with recovery to 211 $\pm$ 53 million/cm^2. β' was decreased reversibly by 12.5% from its control value of 0.64 $\pm$ 0.08. In comparable experiments done with 100 Na HEPES-buffered solution, the changes of β' were not significant (control = 0.50 $\pm$ 0.04), and N_T was increased by forskolin from 90.3 $\pm$ 14.7 to 235 $\pm$ 69 with recovery to 77.4 $\pm$ 17.9 million/cm^2.

Although the forskolin-related fractional increases of N_T at reduced Na concentration were not as large as those of 100 Na-bathed tissues, it should be noted, as emphasized in Table IV, that the absolute increases of channel density were considerably larger at lower apical Na concentration despite the elevated baseline values of N_T.

TABLE IV

Forskolin-mediated Changes of N_T at 100 mM and Reduced Apical Solution Na Concentration

Ringer solution	[Na]	$\overline{I_{Na}}$	$\overline{N_T}$		
			Control	Forskolin	Δ
	mM	*$\mu A/cm^2$*	*millions/cm²*		
HCO₃-buffered	100	23.26	141	467	326
	5	19.98	464	1,093	629
HEPES-buffered	100	13.90	90.3	235	145
	10	13.63	228	800	572

Data taken from Tables II, III, and text. Basolateral solution Na concentration was 100 mM in all experiments. Apical solution Na concentration was either 100, 10, or 5 mM.

As with 100 Na-bathed tissues, forskolin caused an increase of the off rate, k_{bo}. From reduced values of 161 $\pm$ 6.8 (5 Na, Table III) and 173 $\pm$ 6.6 (10 Na, HEPES) k_{bo} was increased by forskolin to 207 $\pm$ 17.7 and 283 $\pm$ 29.9, respectively. k_{bo} was partially reversible to 228 $\pm$ 17.6 (10 Na, HEPES) and was not reversible with 5 Na-bathed tissues (Table III). The on rate, k_{ob}, was not changed significantly by forskolin, indicating here, as above, that forskolin caused a selective change of the off rate of CDPC interaction with the open state of the channels.

Intracellular Na⁺ Concentration and cAMP (HCO₃-buffered Ringer)

Changes of intracellular Na⁺ concentration, [Na]$_c$, caused by 2.5 μM forskolin were measured in isolated epithelia bathed with 100 mM Na, HCO₃-buffered Ringer solution. Control [Na]$_c$ averaged 14.6 mM (Table V) and is the same as reported previously (Stoddard and Helman, 1985). Intracellular water averaged 4.10 μl/cm^2 and was not changed significantly by forskolin treatment for 20 or 60 min. Within 20 min [Na]$_c$ was increased to 29.7 mM, falling thereafter to 20.7 mM at 60 min. Taking

TABLE V

Effect of 2.5 μM Forskolin on Intracellular Na Content and Concentration

	ICW		A_{Na}	$[Na]_c$	$t_{1/2}$	FJ_{Na}^{23}*
	μl/mg DW	μl/cm²	neq/cm²	mM	min	μA/cm²
Control	2.66 ± 0.06 (6)	4.10 ± 0.31	59.8 ± 6.8 (5)	14.6	49.2 ± 14.1 (5)	1.59 ± 0.36
20' forskolin	2.79 ± 0.18	4.41 ± 0.29	130.8 ± 18.0	29.7	50.7 ± 5.2	2.91 ± 0.36
60' forskolin	2.90 ± 0.06	4.80 ± 0.19	99.2 ± 15.9	20.7	51.3 ± 7.9	2.21 ± 0.34
Experimental/control						
20'/control	1.05 ± 0.06‡	1.10 ± 0.10‡	2.21 ± 0.24	2.06	1.22 ± 0.19‡	1.91 ± 0.18
60'/control	1.06 ± 0.08‡	1.11 ± 0.07‡	1.67 ± 0.23	1.42	1.20 ± 0.18‡	1.45 ± 0.17

Values are means ± SEM; 2.5 μM forskolin. Intracellular water (ICW) was normalized per milligram dry weight (DW) and per square centimeter of isolated epithelium. Intracellular $[Na]_c$ was calculated from the quotient of the amount of intracellular Na (A_{Na}) and ICW measured in separate but paired halves of tissues from the same epithelium. The unidirectional Na efflux via leak pathway through basolateral membranes $(FJ_{Na}^{23}$*$)$ was determined by the method of Stoddard and Helman (1985) from the half-time $(t_{1/2})$ of washout of ^{24}Na from epithelia treated with 100 μM amiloride, 1 mM ouabain, and 1 mM furosemide. ‡Not significantly different from control $(P < 0.05)$.

the unidirectional basolateral membrane ^{24}Na efflux measured in the presence of amiloride and ouabain $(FJ_{Na}^{23}$*$)$ as an index of the "permeability" of the basolateral membranes, it was apparent that the efflux rate closely followed the changes of $[Na]_c$, indicating little or no change of permeability via electroneutral mechanisms of transport as suggested previously (Stoddard and Helman, 1985).

Isolated epithelia were assayed for changes of intracellular cAMP caused by either 2.5 or 25 μM forskolin. Control cAMP of these two groups of epithelia averaged 2.75 and 1.77 pmol/cm² (range ~1–6 pmol/cm²) or near 0.5 μM taking ICW to be 4.10 μl/cm² (Table VI). Whereas 2.5 and 25 μM forskolin caused a transient increase of cAMP as indicated by the values measured at 20 and 60 min, the increases of cAMP elicited by 25 μM forskolin were considerably larger, averaging 18.18 and 11.54 pmol/cm² at 20 and 60 min, respectively, as compared with mean values of 5.01 (20 min) and 4.32 (60 min) pmol/cm² observed after 2.5 μM forskolin. With recovery (washout of forskolin) to the control conditions, cAMP decreased to 2.44 (2.5 μM forskolin) and 3.43 (25 μM forskolin) pmol/cm². Such a decrease of cAMP is probably

TABLE VI

Assay for cAMP in Isolated Epithelia of Frog Skin

	Control	Experimental		Recovery
		20'	60'	
2.5 μM forskolin (16)	2.75 ± 0.26	5.01 ± 0.14	4.32 ± 0.09	2.44 ± 0.11
25.0 μM forskolin (17)	1.77 ± 0.13	18.18 ± 0.38	11.54 ± 0.58	3.43 ± 0.28
	20'/control		60'/control	Recovery/control
2.5 μM forskolin	1.99 ± 0.07		1.72 ± 0.04	0.98 ± 0.07
25.0 μM forskolin	10.99 ± 0.29		7.06 ± 0.36	2.07 ± 0.21

cAMP in units of picomoles per square centimeter.

attributable to phosphodiesterase degradation of intracellular cAMP since isolated epithelia are very impermeable to cAMP, as evidenced by insensitivity of epithelia to exposure to millimolar concentrations of extracellular cAMP. Noting, as indicated above, that the physiological response of the epithelia was at or near maximum with 2.5 μM forskolin, it was evident that changes of open channel density and N_T were sensitive to rather small changes of total intracellular cAMP (see Discussion).

DISCUSSION

cAMP-mediated hormonal regulation of Na^+ absorption by tight epithelia has been studied extensively in a variety of tissues and with a variety of techniques including blocker-induced noise analysis (Li et al., 1982; Helman et al., 1983). Compelling evidence supports the idea that cAMP, acting via protein kinase A, leads to an increase of apical membrane channel density, resulting in an increase of apical membrane Na entry and hence transepithelial Na absorption. The results of our present experiments are in accordance with this idea, as both vasopressin and forskolin caused large increases of channel density (N_T).

Autoregulation of N_T

It is perhaps less well appreciated that tight epithelial cells may respond to nonhormonal influences that are capable of eliciting changes of channel density comparable to, if not greater than, those achieved by cAMP alone. In this regard, the phenomenon of autoregulation of apical membrane Na channel density described by Abramcheck et al. (1985) was the first demonstration that inhibition of apical membrane Na entry could lead to a compensatory increase of channel density.[2] Submaximal inhibition of Na entry by amiloride or weaker channel blockers (CGS 4270, CDPC) gives rise to long time constant ($\sim$ 10–20 min) autoregulatory increases of N_T without a change of channel open probability (Helman and Baxendale, 1990). When Na entry is blocked by $\sim$ 80–90% (0.5 μM amiloride), the cells respond with an approximately sevenfold increase of N_T. Under such conditions of blocker inhibition of Na entry, apical membrane voltage is hyperpolarized and intracellular Na concentration is reduced. Since the autoregulatory response occurs over many minutes, it is difficult to know which factors are critical in sensing the decrease of Na entry and effecting increases of channel density, and it remained possible that the autoregulatory phenomenon could be attributed to a nonspecific effect of the blocker.

In the present series of experiments, we included studies of tissues that were exposed chronically to a reduced apical Na concentration of either 5 or 10 mM Na. It was observed here, as in previous experiments with blockers, that inhibition of Na

[2] In response to rapid millisecond range step changes of apical solution [Na⁺], Lindemann et al. (1972) described a Na⁺ related "recline" phenomenon with a relaxation rate of a few seconds at most. Since changes of Na channel density attributable to autoregulation occur with time constants of many minutes, it would be unlikely that the mechanisms of the recline and autoregulatory phenomena are related. In this regard, the recline phenomenon appears to reflect a change in the rate of Na entry in the absence of change of channel density.

entry by reduction of Na concentration gave rise to a large autoregulatory increase of N_T, thereby ruling out nonspecific effects of blockers and permitting a generalization of the idea that autoregulation of N_T is a response to a decrease of apical Na entry, regardless of how it is achieved.

At present it cannot be ruled out absolutely that the Na concentration-dependent autoregulatory changes of N_T are mediated by cAMP. It was nevertheless observed in all groups of tissues treated with vasopressin and forskolin that the tissues maintained an autoregulatory response to CDPC inhibition of Na entry despite elevated levels of tissue cAMP that caused maximal physiological increases of N_T. It seems, therefore, more likely that nonhormonal autoregulation of N_T is mediated via a non-cAMP mechanism (see below). Regardless of how autoregulation of apical Na entry is initiated, it remains to be explained how and why acute inhibition of Na entry is sensed and how the cells respond with an increase of N_T.

Single Channel Current/Conductance

Hormone-mediated effects of cAMP have been examined previously under conditions that assured minimal changes of intracellular voltage and intracellular Na concentration. In studies by Li et al. (1982), toad urinary bladders were depolarized by high basolateral solution K concentration that also decreases basolateral membrane resistance. In the presence of 1–4 μM amiloride that increases apical membrane resistance with a consequent decrease of apical Na entry, the fractional transcellular resistance is increased toward unity, thereby rendering the electrochemical potential difference driving Na into the cells rather insensitive to oxytocin stimulation of Na transport. Under these conditions, i_{Na}^B averaged 0.15 pA and was decreased slightly by oxytocin.

In nondepolarized preparations of frog skin studied in their amiloride-inhibited state (2 μM amiloride; Helman et al., 1983), treatment of the tissues with a combination of ADH and theophylline caused an increase of I_{Na}^B from 2.10 to 4.81 μA/cm^2 and a concurrent small decrease of the i_{Na}^B from 0.59 to 0.50 pA. The difference in baseline values of i_{Na}^B between the above studies of toad urinary bladder and those of frog skin are undoubtedly due predominantly to K$^+$ depolarization of the intracellular and hence apical membrane voltage driving Na into the cells (Tang et al., 1985). Regardless of baseline values, stimulation of Na transport caused relatively small decreases of i_{Na}^B due, most likely, to small increases of intracellular Na concentration and/or small decreases of apical membrane voltage. In this regard, amiloride is known to cause a baseline hyperpolarization of apical membrane voltage together with a decrease of intracellular Na concentration, thereby elevating i_{Na}^B above i_{Na} in non–K$^+$-depolarized tissues (Helman and Baxendale, 1990).

The present series of experiments were done with tissues transporting Na at their spontaneous rates of transport and where changes of apical membrane resistance and hence changes of fractional transcellular resistance are expected to cause significant changes of apical membrane voltage and intracellular Na concentration. Thus, it was not surprising to observe that vasopressin and forskolin caused significant and reversible changes of i_{Na} (Tables II and III) and i_{Na}^B (Figs. 4 and 5). In experiments with vasopressin, i_{Na} was decreased on average by 20–30%, and such findings are compatible with the changes of apical membrane voltage and fractional transcellular

resistances recorded in previous microelectrode experiments of *R. pipiens* (Els and Helman, 1981). The mean decreases of i_{Na} caused by forskolin were considerably larger ($\sim 40-70\%$), as were the changes in macroscopic Na transport and channel densities. Although intracellular voltages have not yet been measured in *R. pipiens* under the forskolin conditions of the present experiments, impedance analysis has indicated large decreases of fractional transcellular resistance upon treatment of tissues with 2.5 μM forskolin (Helman, S. I., and M. S. Awayda, personal communication) that would lead to large decreases of apical membrane voltage. Intracellular Na concentration was increased substantially by forskolin (14.6 to 29.7 mM) within 20 min, thereby reducing the chemical potential difference driving Na into the cells. At present, it would be reasonable to assume that decreases of apical membrane voltage are responsible for the decreases of i_{Na} which cannot be accounted for by decreases of the chemical potential difference. To the extent that recovery values of macroscopic I_{Na} and corresponding N_o were considerably less than control, it is not surprising that recovery i_{Na} values were larger than control i_{Na} values if indeed changes of fractional transcellular resistance dictate in large part changes of apical membrane voltage (Helman and Fisher, 1977; Nagel, 1978).

It is curious, however, as illustrated in Fig. 4, that the transient changes of macroscopic I_{Na} were observed to occur as a consequence of a time-dependent decrease of i_{Na}^B (20 to 60 min). During this time interval, intracellular Na concentration fell toward 20.7 mM from its value of 29.7 mM at 20 min, and hence the chemical potential difference increased with time. Most simply, it would be expected that i_{Na} would have also increased with time after the increase of chemical potential difference, assuming, of course, that all other factors are constant. In the absence of careful measurements of intracellular voltage under the conditions of these experiments, it remains possible that apical membrane voltage continues to depolarize more than the chemical potential difference is increased, thereby leading to a continuously falling electrochemical potential difference driving Na into the cells. During this time the channel density remains constant. In the absence of such a change of driving force, it also remains possible that single channel conductance is decreased under these maximal conditions of stimulation of Na transport by forskolin.

It may be noted that, regardless of the conditions of the experiments presented here, or previously, the single channel currents measured by blocker-induced analysis are similar in value to those observed by patch clamp (Helman and Van Driessche, 1990). When i_{Na} is normalized for the electrochemical potential difference, the single channel chord conductance is in the vicinity of 3.3 pS (Helman et al., 1983).

Vasopressin/Forskolin

During the course of our experiments, it became obvious that forskolin could elicit maximal stimulation of Na transport far greater than could be achieved with vasopressin. Indeed, increasing forskolin concentration 10-fold (2.5 to 25 μM) did not further stimulate Na transport despite a considerable further increase of tissue cAMP. Accordingly, our observations are consistent with the belief that the physiological response of Na transport to cAMP was saturated by 2.5 μM forskolin stimulation of the adenylate cyclase. Despite the transient nature of tissue levels of cAMP as

measured at 20 and 60 min after forskolin, the N_T remained constant at its elevated value, indicating additionally that the increases of cAMP were sufficiently above the concentration of cAMP required to saturate the mechanism involved in elevating N_T. It was of additional interest to note that the time required for channel density to begin to fall toward recovery values after removal of forskolin from the basolateral solution was greater (15–20 min) in tissues treated with 25 μM forskolin than in tissues treated with 2.5 μM forskolin (<5 min). We presume that the additional time delay in 25 μM forskolin-treated tissues reflects the time required for cAMP to fall to less than saturating levels of cAMP. Indeed, tissue cAMP (60 min after forskolin) was considerably larger in tissues treated with 25 μM forskolin (11.54 pmol/cm^2) than in tissues treated with 2.5 μM forskolin (4.32 pmol/cm^2). Such observations argue in favor of the idea that cAMP is directly involved in stimulation of Na transport and that maximal physiological responses are achieved with rather small increases of tissue levels of total cAMP.

It is impossible to know at present to what extent cAMP is compartmentalized within the cells. Our observations, nevertheless, are compatible with those of Johnsen and Nielsen (1984) who also observed stimulation of Na transport with a relatively small increase of tissue cAMP. Assuming homogeneous distribution of cAMP within cell water, mean cAMP concentration in control tissues was in the range of 0.43–0.67 μM. Schlondorff and Franki (1980) reported that 0.05 μM cAMP is sufficient to achieve half-maximal activation of cAMP-dependent protein kinase A derived from toad urinary bladder. Accordingly, it is not surprising that small increases of total tissue cAMP are sufficient to saturate the physiological response of N_T to cAMP.

It is of interest that tissue cAMP undergoes a transient increase upon stimulation with either 2.5 or 25 μM forskolin. Whether such transients (20–60 min) represent decreased activation of adenylate cyclase or increased degradation by phosphodies-terase is unknown for the conditions of the present experiments. Regardless, and as noted above, the physiological response to forskolin was saturated despite falling levels of cAMP.

In contrast to forskolin, the response of N_T to vasopressin was not saturated (Fig. 5). From maximal values of N_o^B, achieved ~15 min after vasopressin, N_o^B decreased thereafter to lesser but elevated values. It is possible that such transient behavior of channel density may be due at least in part to transient changes of subsaturating levels of cAMP. It is curious, however, that upon restimulation of the tissues from their recovery states that the transient response of channel densities was absent (Fig. 5). We know of no obvious explanation for this behavior. The response of tissues to vasopressin is more complex than the response to forskolin because of the involvement of the complex of regulatory G proteins that intervene between hormonal receptor and adenylate cyclase (Neer and Clapham, 1988). Whether the transient response of channel density to vasopressin stimulation of Na transport in frog skin involves G proteins and/or other factors remains to be determined.

CDPC Rate Coefficients

Blocker rate coefficients can vary with experimental manipulations of tissues (for review, see Helman and Kizer, 1990). In the present series of studies, forskolin and vasopressin caused a selective increase of the CDPC off rate, k_{bo}, at normal (100 Na)

and reduced apical Na concentrations, while reduction of Na concentration caused a decrease of the k_{bo}. How and why this occurs is presently unknown, and it remains of interest that the on rate, k_{ob}, appears insensitive to these experimental perturbations of the tissues. It should be stressed that the rate coefficients were measured during steady-state conditions, thereby leaving open the possibility of transient and/or time-dependent changes of the rate coefficients after stimulation of the tissues by vasopressin or forskolin. It should be emphasized that the determination of the i_{Na}^B in time course experiments relied upon the assumption of constancy of k_{ob} (but not k_{bo}). Accordingly, the actual time dependency of change of i_{Na}^B (and hence N_o^B) must await verification of this assumption.

The cAMP-related change or lack of change of rate coefficients is not unique to CDPC. Previous studies indicated that ADH + theophylline caused a selective increase of the amiloride k_{bo} with no change of k_{ob} (Helman et al., 1983). In K⁺-depolarized toad urinary bladder, Li et al. (1982) reported no change of the amiloride k_{ob} with a 40% increase of K_B^{amil} due presumably to increase of the amiloride k_{bo} which, when evaluated on a paired basis, was not significantly changed by oxytocin (Li et al., 1982). This inconsistency is probably due to difficulty in measurement of small values of k_{bo} that arise with potent channel blockers, like amiloride, as was argued previously (Abramcheck et al., 1985).

Channel Open Probability

The cAMP- and Na concentration–mediated changes of open channel density could be attributed to increases of the density of channels in the apical membrane and not to changes of channel open probability. Channel open probability averaged 0.46, 0.49, and 0.50 in the 100 Na experiments, and although the data are not paired, β' averaged 0.66 (5 Na) and 0.64 (10 Na) and so appeared to be elevated by reduction of apical Na concentration. Thus, if reduction of Na concentration causes an increase of β' and hence N_o, the increases of N_o due to change of β' are rather small compared with the increases of N_o attributable to change of N_T.

The effects of vasopressin and forskolin on β' were not consistent in all groups of tissues. Whereas vasopressin caused no significant change of β' (100 Na), forskolin caused a significant and reversible decrease of β' of ~20–25% (100 Na; Table II) that may possibly be due to the greater effect of forskolin in stimulating Na transport and elevating intracellular cAMP. In other groups of tissues, including 100 Na HEPES-buffered Ringer and those studied at reduced Na concentration, the decreases of β', if any, were not significant. Accordingly, at the steady states of Na transport the cAMP-mediated increases of open channel density were due to increases of N_T. It should be stressed here, as above, that if reduction of Na concentration or stimulation of Na transport by cAMP causes transient changes of β', such changes cannot be assessed at present and thus cannot be ruled out. In this regard, Palmer and Frindt (1988) observed no change of "open probability" (for review, see Helman and Kizer, 1990) upon reduction of intrapipette Na concentration in patch clamp experiments of rat cortical collecting tubules. Such observations taken with others (for review, see Helman and Kizer, 1990) provide compelling evidence against the Na self-inhibition hypothesis advanced by Fuchs et al. (1977).

Speculation on Mechanism

It has been suggested that hormonally mediated recruitment of channels may occur from a pool of inactive or quiescent channels already resident in the apical membrane (Li et al., 1982). More recent studies with Na^+ channel-containing vesicles derived from toad urinary bladder (Lester et al., 1988) indicated that neither cAMP nor cAMP-dependent protein kinase was involved directly in activation of Na^+ conductance, leaving open the possibility that changes of N_T may arise from the trafficking of channels between apical membranes and subapical membrane storage sites. The results of our own experiments do not favor either hypothesis. Our results, however, rule out the possibility that hormonal activation of apical membrane conductance by cAMP and autoregulatory increase of open channel density occur by way of an increase of channel open probability. Therefore, hormonal and autoregulatory stimulation of Na transport must be due to increase of the total pool of channels that fluctuate between closed and open states.

It was suggested above that autoregulatory and cAMP activation of N_T probably occurs via different mechanisms. If autoregulatory increases of N_T are mediated via cAMP, then the response of N_T to subsequent challenge with cAMP should be diminished. Indeed, this was not the case. As indicated in Table IV, the absolute increases of N_T were considerably larger in response to forskolin after the autoregulatory elevation of N_T, indicating that cAMP was capable of recruiting more channels to the apical membrane than was possible in the prior "nonautoregulated" state of the tissue. In this regard, we believe it is reasonable to speculate that autoregulation may involve an increase in the size of a pool of channel-containing cytosolic vesicles that are in dynamic balance with the apical membrane, while cAMP favors fusion of vesicles with the apical membrane, thereby accounting for the additive effects of autoregulation and cAMP in elevating N_T. Alternative hypotheses are tenable and must be considered.

It is remarkable that both hormonal and autoregulatory mechanisms of activation of Na channel density are capable of eliciting large increase of channel density and apparently by differing mechanisms of action. Regardless of the precise mechanisms involved, a change of luminal Na concentration, as occurs physiologically in renal distal tubules, may provide an important autoregulatory feedback mechanism that would tend to minimize and constrain changes of the rates of transepithelial Na reabsorption and yet allow comparable responses to antidiuretic hormone. The autoregulatory response to Na channel blockers is of obvious interest pharmacologically, insofar as diuretic therapy would be compromised by feedback stimulation of Na channel density.

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