Hormonal Control of Apical Membrane Na Transport in Epithelia

Studies with Fluctuation Analysis

SANDY I. HELMAN, THOMAS C. COX, and WILLY VAN DRIESSCHE

From the Laboratorium voor Fysiologie, Campus Gasthuisberg, Leuven, Belgium; and the Department of Physiology and Biophysics, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

ABSTRACT To study the mechanisms by which antidiuretic hormone and prostaglandins regulate Na transport at the apical membranes of the cells of anuran tissues, studies were done with fluctuation analysis. Epithelia of frog skin (*Rana pipiens*) were treated with vasopressin alone, or treated with vasopressin after inhibition of Na transport by indomethacin. The tissues were bathed symmetrically with a Cl-HCO_3 Ringer solution and short-circuited continuously. In this experimental circumstance, the amiloride-induced current noise power density spectra were of the Lorentzian type with little or no $1/f$ noise, provided that "scraped" skins were used for study. Despite large changes of Na transport, especially in epithelia treated with indomethacin and vasopressin, the single-channel Na current remained essentially unchanged, whereas the density of amiloride-inhibitable, electrically conductive Na channels was increased by vasopressin and decreased by indomethacin.

INTRODUCTION

There is compelling evidence that apical membrane Na permeability of the cells of tight epithelia is controlled by hormones that alter the intracellular concentration of cyclic 3′5′-adenosine monophosphate. The action of antidiuretic hormone (ADH), which stimulates active transepithelial Na transport in anuran tissues such as frog skin and toad urinary bladder, has been well documented (Handler and Orloff, 1973; Andreoli and Schafer, 1976). More recently, the prostaglandins have been identified as important regulators of Na transport owing to their profound influence on transepithelial Na transport (Hall et al., 1976; Stokes and Kokko, 1977; Orloff and Zusman, 1978; Els and Helman, 1981). Thus,

Address reprint requests to Dr. Sandy I. Helman, Dept. of Physiology and Biophysics, University of Illinois at Urbana-Champaign, 524 Burrill Hall, 407 South Goodwin Ave., Urbana, IL 61801.

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/83/08/0201/20 $1.00 201
Volume 82 August 1983 201–220

endogenously produced prostaglandins and exogenous ADH act in concert to modulate the rate of transepithelial Na transport.

The mechanism of hormonal action involves changes of apical membrane permeability to Na. In isolated frog skin the change of P_{Na} is manifest electrophysiologically as a change in apical membrane slope conductance with little or no change of the electrophysiological parameters of the basolateral membranes (Nagel, 1978; Els and Helman, 1981). ADH increases, whereas indomethacin, an inhibitor of prostaglandin biosynthesis, decreases the apical membrane Na conductance, both acting presumably via alteration of intracellular cyclic AMP (Hall et al., 1976; Els and Helman, 1981). To investigate further the possible mechanisms of change of the P_{Na} and apical membrane conductance, we turned to studies of fluctuation analysis of frog skin as described by Lindemann and Van Driessche (1977) for K-depolarized epithelia; in the present studies, however, we used nondepolarized epithelia where the data of the present studies could be compared directly with the previous electrophysiological studies of Els and Helman (1981).

As will be shown, the data derived from fluctuation analysis could be interpreted according to a two-state model to indicate that changes of apical membrane Na transport caused by indomethacin and/or ADH + theophylline occur primarily, if not solely, by alteration of the density of electrically conductive Na channels with little or no change of the single-channel conductance to Na.

Preliminary reports of this work have appeared elsewhere (Helman et al., 1981*a*, *b*).

GLOSSARY

I_{sc} short-circuit current ($V_T = 0$) defined as a positive current from apical to basolateral solution ($\mu A/cm^2$)

I_{Na} macroscopic Na current at the apical membrane and equal to the I_{sc} at the steady state ($\mu A/cm^2$)

I_{Na}^{A} macroscopic I_{Na} when amiloride is present in the apical solution to inhibit the I_{sc} ($I_{Na}^{A} < I_{Na}$)

i_{Na} single-channel Na current at the apical membrane in the absence of amiloride

i_{Na}^{A} single-channel Na current at the apical membrane when amiloride is present in the apical solution

A amiloride

f_c corner frequency (Hz) of the Lorentzian power density spectrum at the half-power value of the low-frequency plateau, $S_o/2$

fR_o slope resistance of the apical membrane expressed as a fraction of the transcellular resistance ($R_o/R_o + R_i$)

E_i Thévenin emf of the basolateral membranes (slope formalism of electrical equivalent circuit analysis)

k_{01} association rate constant of the Na channel/amiloride receptor reaction

k_{10} dissociation rate constant of the Na channel/amiloride receptor reaction
$K_{½}$ equilibrium constant (k_{10}/k_{01})
$K_{½}^{app}$ apparent $K_{½}$ (μM); defined as the [A] that causes 50% inhibition of the I_{sc} (see text)
N^A density of amiloride-inhibitable, electrically conductive Na channels (channels/cm^2); ($N^A = N_o^A + N_1^A$)
N_o^A density of open channels in the presence of A ($N_o^A = N^A \cdot P_o$)
N_1^A density of closed channels in the presence of A ($N_1^A = N^A \cdot P_1$)
P_o probability of a channel being open in the presence of A
P_1 probability of a channel being closed in the presence of A
PDS power density spectrum
R_i slope resistance of the basolateral membrane of the cells
R_o slope resistance of the apical membrane of the cells
S_o low-frequency plateau of the Lorentzian spectrum (A^2/Hz·cm^2)
V_T transepithelial voltage (mV)
V_o^{sc} apical membrane voltage referenced to the apical solution with tissues short-circuited

METHODS

Abdominal skins of *Rana pipiens* (Nasco, Oshkosh, WI) were used in all studies. To reduce diffusion delays imposed by the corium to applied drugs or hormones, we used tissue preparations to be referred to as "scraped skins." With tissues pinned to a cured Sylgard 184 plate (Dow Corning Corp., Midland, MI), the tela subcutanea and most of the underlying connective tissue was removed by gentle scraping with the edge of a scalpel blade. Compared with intact skins, where maximal ouabain and K depolarization of the basolateral membranes occurs in ~10–15 min (Fisher, 1979; Helman et al., 1979), similar effects are realized in ~1–3 min when scraped skins are used in similar studies (unpublished observations).

To carry out noise analysis of the epithelium, scraped skins were placed in chambers (0.72 cm^2, 0.6 ml chamber volume) as described by Helman and Miller (1971). The tissues were bathed with a Ringer solution containing (mM): 100 NaCl, 2.4 $KHCO_3$, and 2.0 $CaCl_2$. They were short-circuited continuously for ~1–2 h to permit the short-circuit current, I_{sc}, to stabilize. Vasopressin (Pitressin; Parke, Davis, & Co., Detroit, MI), theophylline, and/or indomethacin (both from Sigma Chemical Co., St. Louis, MO) were added to the basolateral solutions at concentrations of 30 mU/ml, 1 mM, and 5 μM, respectively. Amiloride (Merck Sharp & Dohme Research Laboratories, West Point, PA), 0.5–12 μM, was added to the apical solutions to inhibit the I_{sc} and to induce the fluctuations of the I_{sc}. All studies were done at room temperature with air-saturated Ringer solution at a pH of 8.1.

Electrical Measurements

The methods and materials were identical to those described previously by the laboratory of Van Driessche (Van Driessche and Zeiske, 1980). In brief, a low-noise voltage clamp was used to short-circuit the tissues. After separation of DC and AC components of the I_{sc} by a high-pass RC filter (0.053 Hz, characteristic

frequency), the AC noise was amplified, and after passage through a Butterworth-type low-pass filter (48 db/octave; model 852; Wavetek, San Diego, CA), the signal was digitized. 40 time-domain sweeps were accumulated at sampling rates of 2 ms and each sweep was transformed to the frequency domain (FFT) for subsequent calculation of the current noise power density spectrum (PDS). Approximately 3 min were required to accumulate data for determination of the PDS. A Lorentzian function was fit by nonlinear least-squares regression analysis to the PDS as described previously (Van Driessche and Zeiske, 1980), from which the power at the low-frequency plateau, S_o, and corner frequency, f_c, were determined. In most cases, the PDS were, apart from the amplifier noise, ideally fit to a single Lorentzian (see Fig. 1). In relatively few cases, $1/f$ noise appeared at low frequencies (see Fig. 2), and this was taken into account in the analysis of the amiloride-induced noise-fitting of the data to $1/f$ and Lorentzian components of the PDS. The macroscopic I_{sc} (DC value) was read to within 0.01 $\mu A/cm^2$ from a digital meter.

Determination of the $K_{1/2}^{app}$

To calculate the i_{Na}^{A} and N^A (see Results), it was necessary to determine the equilibrium constant, $K_{1/2}$. Two methods were used. First, the association, k_{01}, and dissociation, k_{10}, rate constants were determined from the slope and intercept of the relationship between [A] and $2\pi f_c$, assuming a two-state model (open-closed) of the Na channel of the form:

$$[A] + [N_o^A] \underset{k_{10}}{\overset{k_{01}}{\rightleftharpoons}} [AN_1^A], \tag{1}$$

from which $K_{1/2} = k_{01}/k_{10}$ could be calculated. Second, $K_{1/2}^{app}$ was determined from the inhibition of I_{sc} caused by step increases of [A], though we recognized that the $K_{1/2}^{app}$ is an overestimate of the actual $K_{1/2}$ (see below and Discussion). As will be shown, the absolute values of k_{10}, being near zero, were rather uncertain, and thus an alternative method was necessary for estimation of $K_{1/2}$ and hence calculation of N^A.

All values are reported as mean ± SEM (N).

RESULTS

Power Density Spectra of Control and Treated Tissues

For skins bathed with identical Ringer solutions at their apical and basolateral surfaces, amiloride-induced power density spectra were recorded and single Lorentzian functions were fit to the data points as shown in Figs. 1 and 2. Over a range of [A] between 0.5 and 12 μM, the PDS showed a low-frequency plateau, and at higher frequencies, the slope was −2. When epithelia were treated with indomethacin and then with ADH + theophylline, the changes of peak-to-peak noise (Fig. 1*A*) were readily observed in the time-domain records, and as shown in Fig. 1*B*, their corresponding PDS were of the Lorentzian type. Indomethacin caused, in addition to inhibition of the I_{sc}, a decrease of the S_o, whereas further treatment of the tissue with ADH + theophylline caused an increase of the S_o and I_{sc} with little or no change of the f_c. Whereas most

spectra showed ideal Lorentzian behavior, we did observe in some cases low-frequency $1/f$ noise (Fig. 2). Also shown in Fig. 2 are the [A] dose-dependent changes of the S_o and f_c, namely, a decrease of S_o and an increase of f_c consequent to increases of [A].

Two-State Model

Data have been analyzed according to a two-state model where the Na channel is either fully open or fully closed by amiloride (Lindemann and Van Driessche, 1977). Accordingly, the fluctuations arise from changes of the single-channel current, i_{Na}, as amiloride associates or dissociates from the Na channel with rate constants k_{01} and k_{10}. Thus, according to

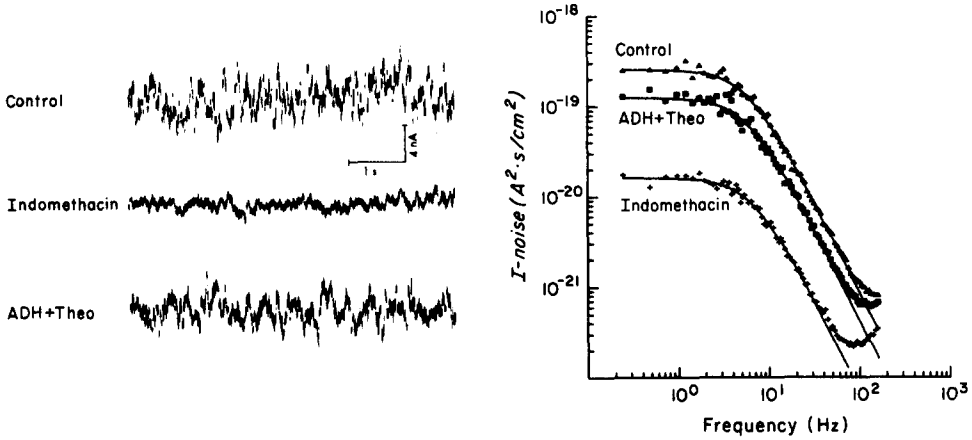

FIGURE 1. Time-domain records and corresponding PDS of a control epithelium treated first with indomethacin to inhibit the I_{sc} and then with ADH + theophylline (in the presence of indomethacin) to restimulate the I_{sc}. The plateau value S_o of the PDS decreased from 25.4 to 1.62×10^{-20} $A^2 \cdot s/cm^2$ after indomethacin and returned to 12.5×10^{-20} $A^2 \cdot s/cm^2$ after ADH + theophylline treatment of the epithelium. The corner frequency, 6.08 Hz, remained constant.

Lindemann and Van Driessche (1977):

$$2\pi f_c = k_{01}[A] + k_{10} \tag{2}$$

and

$$K_{1/2} = k_{10}/k_{01}. \tag{3}$$

It follows from the law of mass action that

$$P_o = \left[1 + \frac{A}{K_{1/2}}\right]^{-1} \tag{4}$$

and

$$P_1 = 1 - P_o, \tag{5}$$

where P_o is the probability of a channel being open. With 2 μM amiloride in the apical solution and a $K_{1/2}^{app}$ of $\sim$0.2 μM, $P_1 = 0.909$ and $P_o = 0.091$. Thus, where [A] $\gg K_{1/2}$, uncertainties in the value of $K_{1/2}$ cause relatively minor uncertainties of the value of P_1, whereas those of P_o are highly dependent on the validity of the $K_{1/2}$.

The single-channel current, i_{Na}^{A}, and the density of amiloride-inhibitable, electrically conductive channels are given by:

$$i_{Na}^{A} = (S_o \cdot 2\pi f_c)/(4I_{Na}^{A} P_1) \tag{6}$$

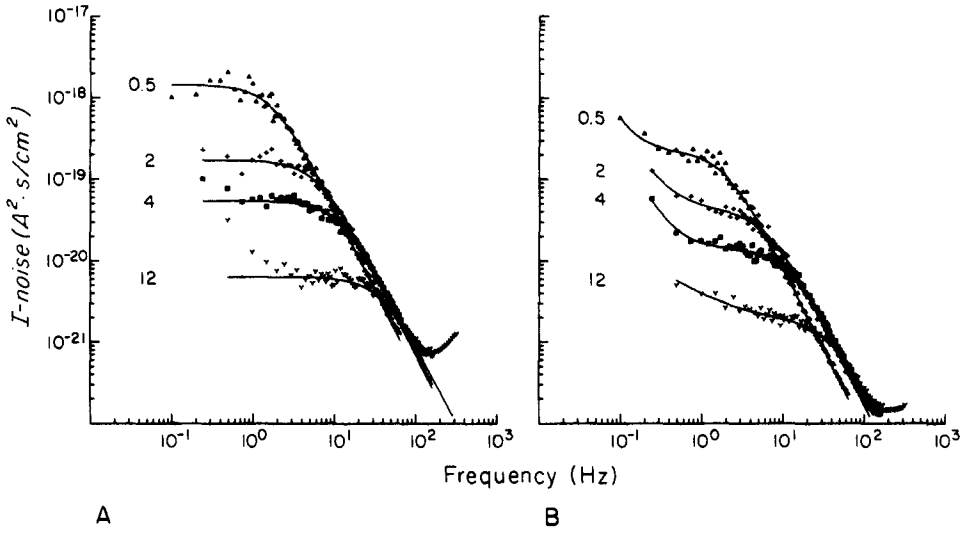

FIGURE 2. PDS of epithelia exposed to various amiloride concentrations between 0.5 and 12 μM. Representative spectra are shown at 0.5, 2, 4, and 12 μM with f_c at 1.74, 6.44, 12.2, and 39.7 Hz in *A* and 1.89, 6.33, 12.3, and 35.6 Hz in *B*. Low-frequency $1/f$ noise was observed in relatively few spectra.

and

$$N^{A} = I_{Na}^{A}/(i_{Na}^{A} \cdot P_o). \tag{7a}$$

As will be shown below, the values of P_o calculated from the microscopic rate constants were uncertain. Thus, we turned to measurement of the $K_{1/2}^{app}$ that allowed us to estimate the P_o and hence the N^A according to Eq. 7*a*.

We also chose to calculate the N^A from the I_{Na} (no amiloride) and the i_{Na}^{A} assuming that the i_{Na}^{A} and N^A were voltage, current, and amiloride concentration independent:

$$N^{A} = I_{Na}/i_{Na}^{A}. \tag{7b}$$

Both methods of calculation of the N^A gave similar results (see also Discussion).

k_{01}, k_{10}, and $K_{1/2}^{app}$

In preliminary studies, control and treated epithelia were exposed to amiloride at concentrations between 0.5 and 12 μM, from which the PDS yielded the relationship between [A] and $2\pi f_c$. As shown in Fig. 3, the relationship was strictly linear, in accordance with Eq. 2. Whereas the k_{01} estimated from the slope was precise, the k_{10} at the ordinate intercept was in absolute value close to zero and thus imprecise. Hence, it was impossible

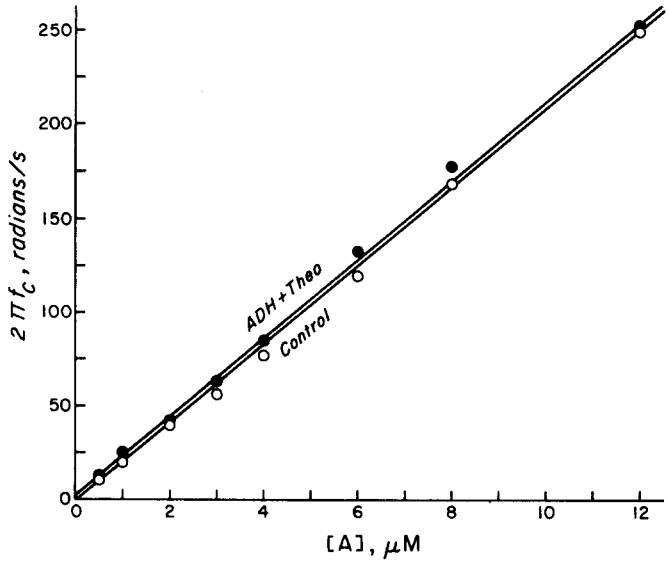

FIGURE 3. Relationship between [A] and $2\pi f_c$. Paired determinations taken from control and ADH + theophylline-treated states of an epithelium are shown. Note the small but consistent increase of f_c at all [A] after ADH + theophylline. Note also the low values of k_{01} of the intercept at the ordinate.

to obtain reasonably valid estimates of the $K_{1/2}$. Despite this, we observed in four paired studies that treatment of epithelia with ADH + theophylline for ~1 h caused an increase of the k_{10} (see Table I) without a change of the k_{01}, which led us to suspect that ADH + theophylline causes an increase of the $K_{1/2}$ (see below).

As shown in Table II, the mean k_{01} was unchanged by treatment of epithelia with indomethacin (~2 h) and/or ADH + theophylline. The k_{10}'s were not, on average, significantly different from zero, although it was apparent, as noted above, that k_{10} was increased by ADH + theophylline.

To circumvent this problem in the estimation of the $K_{1/2}$, we turned to studies of the [A] dependency of the I_{sc} to estimate the $K_{1/2}^{app}$ according to

TABLE I

Effects on ADH + Theophylline on k_{01} and k_{10}

	k_{01}	k_{10}	k_{10}/k_{01}
	radians/s·μM	*radians/s*	*μM*
Control	15.8	2.55	0.161
ADH + theophylline	17.0	6.26	0.368
Control	17.5	2.00	0.114
ADH + theophylline	17.0	3.83	0.225
Control	17.8	1.01	0.057
ADH + theophylline	17.9	4.25	0.237
Control	20.8	−1.79	—
ADH + theophylline	21.3	3.00	0.141
Mean ± SE			
Control	17.9±1.1	0.94±0.96	
ADH + theophylline	18.3±1.0	4.34±0.69	
Experimental/control	1.02±0.02 (4)	2.85 (3)	2.81 (3)

the following rearranged Michaelis-Menten equation (Engel, 1977; and see Discussion):

$$\Delta I_{sc} = -K_{1/2}^{app}\left(\frac{\Delta I_{sc}}{[A]}\right) + \Delta I_{sc}^{max}. \quad (8)$$

The I_{sc} was determined at [A] ranging between 0.5 and 50 μM, from which the $K_{1/2}^{app}$ was estimated from the linear slopes, as shown in Fig. 4. For the typical examples shown, ADH + theophylline caused the $K_{1/2}^{app}$ to increase from 0.111 to 0.342 μM. Indomethacin caused a relatively small increase of the $K_{1/2}^{app}$ from 0.156 to 0.238 μM, whereas ADH + theophylline caused a large further increase of the $K_{1/2}^{app}$ to 0.813 μM. The results of all studies are shown in Tables III and IV. For the two groups of studies described below, the mean $K_{1/2}^{app}$'s were 0.207 and 0.269 μM. Indomethacin caused no consistent change of the $K_{1/2}^{app}$, whereas ADH + theophylline caused increases of the mean $K_{1/2}^{app}$ to 0.468 and 0.614 μM. Thus, in accordance with the data above, ADH + theophylline appeared to cause an increase of the $K_{1/2}$ that could be attributed to an increase of the k_{10}.

TABLE II

Values of k_{01} and k_{10} under Various Experimental Conditions

	k_{01}	k_{10}
	radians/s·μM	*radians/s*
Control (9)	16.4±0.9	0.7±0.5
Indomethacin (4)	16.2±1.7	0.2±1.4
ADH + theophylline (4)	18.3±1.0	4.3±0.7
Indo + ADH + theophylline (3)	15.9±1.2	0.3±1.2

ADH + Theophylline and Indomethacin on i_{Na}^{A} and N^{A}

After a control period with 2 μM amiloride in the apical solution, during which several spectra were obtained, the tissues were treated with either ADH + theophylline (group I) or with indomethacin followed by ADH + theophylline (group II). Spectra were obtained at intervals of ~5 min until the I_{Na}^{A} appeared to reach a near-stable value. For group I studies, amiloride was removed from the apical solution for measurement of the I_{Na} and subsequent determination of the experimental $K_{1/2}^{app}$ (ADH + theophylline). For group II studies, the $K_{1/2}^{app}$ and I_{Na} were determined after indomethacin treatment of the epithelia. Thereafter, 2 μM amiloride

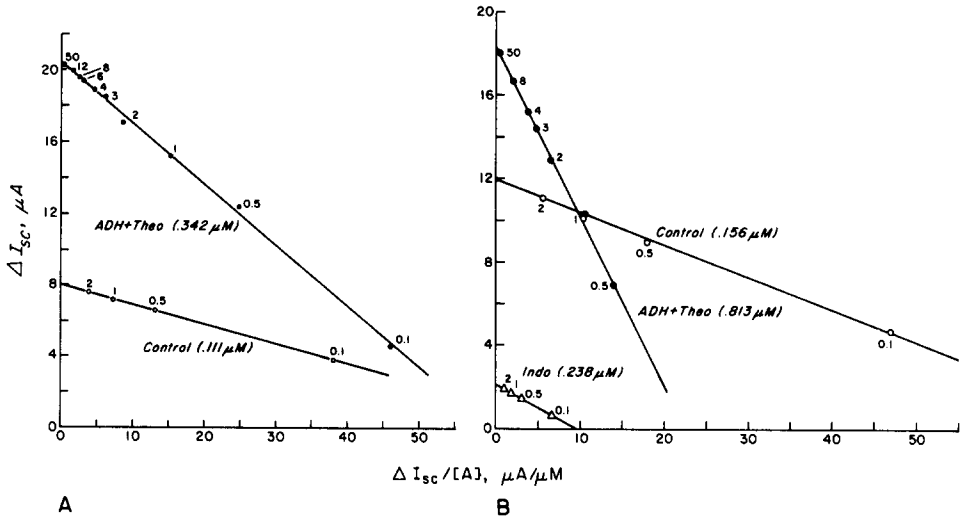

FIGURE 4. Determination of the $K_{1/2}^{app}$ of control and treated epithelia. Data were plotted in the form of an Edie-Hofstee plot (Engel, 1977) according to Eq. 8. Values of ΔI_{sc} are in microamperes (nominal chamber area of 0.72 cm^2). Each data point is labeled with corresponding [A] in micromolar. Note large increases of $K_{1/2}^{app}$ after ADH + theophylline treatment alone of the epithelium or after indomethacin.

was returned to the apical solution, and with indomethacin still present in the basolateral solution, the epithelia were treated further with ADH + theophylline. Again, the I_{Na} and $K_{1/2}^{app}$ were redetermined after the combined treatments of indomethacin and ADH + theophylline. The results of typical experiments are shown in Fig. 5.

When epithelia were treated only with ADH + theophylline (Fig. 5*A*), the $I_{sc} \equiv I_{Na}^{A}$ (2 μM amiloride) increased after a delay of several minutes, together with the S_o reaching new plateaus in ~30–40 min. The corner frequency remained unchanged, as would be expected if $k_{10} \ll k_{01}$ [A]. When epithelia were treated with indomethacin, the $I_{sc} = I_{Na}^{A}$ decreased markedly, falling to low values in ~100–150 min. The S_o decreased in

parallel with the I_{sc} in the absence of a change of f_c. After determination of the I_{Na} and $K_{1/2}^{app}$, restimulation of the I_{Na}^{A} by ADH + theophylline was again accompanied by a parallel increase of S_o with no measurable change of the f_c.

A summary of all data is shown in Tables III and IV, together with the calculated values of i_{Na}^{A} and N^{A}. I_{Na}, in the absence of amiloride, was increased by ADH + theophylline (group I) in six of seven tissues, with stimulation (experimental/control [E/C]) of I_{Na} ranging between 1.05

TABLE III

Effects of ADH + Theophylline on i_{Na}^{A} and N^{A}

Skin		I_{Na}	$K_{1/2}^{app}$	[A] = 2 μM I_{Na}^{A}	f_c	$S_o \times 10^{20}$	i_{Na}^{A}	$N^{A} \times 10^{-6}$, channels/cm²	
		μA/cm²	μM	μA/cm²	Hz	A²·s/cm²	pA/channel	$I_{Na}^{A}/(i_{Na}^{A} \cdot P_o)$	I_{Na}/i_{Na}^{A}
1	Control	16.8	0.221	1.32	7.1	6.2	0.59	22.6	28.5
	ADH + theophylline	24.6	0.698	3.50	7.1	14.5	0.62	21.7	39.7
2	Control	9.5	0.154	0.97	6.3	5.3	0.59	23.2	16.1
	ADH + theophylline	12.8	0.283	1.88	5.6	8.7	0.47	32.5	27.2
3	Control	36.7	0.320	5.40	5.8	38.4	0.75	52.2	46.9
	ADH + theophylline	45.3	0.572	9.33	5.7	48.9	0.60	69.6	75.5
4	Control	11.6	0.110	0.99	6.2	5.1	0.53	36.3	21.9
	ADH + theophylline	30.6	0.342	4.22	5.6	16.6	0.41	71.4	74.6
5	Control	13.7	0.247	1.39	5.3	9.5	0.64	19.8	21.4
	ADH + theophylline	21.6	0.437	4.94	5.4	26.1	0.55	50.5	39.3
6	Control	23.3	0.240	4.10	4.5	18.6	0.36	106.4	64.7
	ADH + theophylline	24.4	0.417	7.76	4.3	38.7	0.41	110.6	59.5
7	Control	8.8	0.159	0.50	5.4	3.7	0.68	10.1	12.9
	ADH + theophylline	12.0	0.526	2.07	5.1	9.7	0.47	21.0	25.5
Mean ± SE									
	Control	17.2	0.207	2.10	5.8	12.4	0.59	38.7	30.4
		±3.8	±0.027	±0.71	±0.3	±4.7	±0.05	±12.4	±7.1
	ADH + theophylline	24.5	0.468	4.81	5.5	23.3	0.50	53.9	48.8
		±1.5	±0.054	±1.06	±0.3	±8.8	±0.03	±12.3	±8.0
	Experimental/control	1.52	2.39	2.88	0.96	2.28	0.87	1.62	1.83
		±0.20	±0.29	±0.41	±0.02	±0.26	±0.06	±0.22	±0.29

and 2.64. With 2 μM apical amiloride the I_{Na}^{A} was similarly increased by ADH + theophylline, although in every case the increase of I_{Na}^{A} was larger than the increases of I_{Na} (E/C), ranging between 1.73 and 4.26. This difference in the response of the I_{Na} and I_{Na}^{A} to ADH + theophylline could at least in part be attributed to the increase of $K_{1/2}$ caused by ADH + theophylline, since 2 μM amiloride was constant during measurement of the I_{Na}^{A} response to ADH + theophylline.

Whereas the S_o showed increases parallel to those of I_{sc}, the f_c remained unchanged by ADH + theophylline. As noted above, this would be expected if $k_{10} \ll k_{10}$ [A].

The control i^A_{Na} was calculated to average 0.59 pA/channel. The i^A_{Na} was not changed consistently or significantly by ADH + theophylline, although in five of seven tissues, there may have been a small decrease of the i^A_{Na}. In view of the relative certainty of these estimates of i^A_{Na} (see Discussion) and because of increases of the I_{Na}, the density of apical membrane Na channels must have increased. The control N^A calculated from $I^A_{Na}/(i^A_{Na} \cdot P_o)$ averaged $38.7 \times 10^6/cm^2$, and when calculated from I_{Na}/i^A_{Na}, N^A averaged $30.4 \times 10^6/cm^2$. After ADH + theophylline, the

TABLE IV

Effects of Indomethacin and ADH + Theophylline on i^A_{Na} and N^A

Skin		I_{Na}	$K^{app}_{1/2}$	[A] = 2 ±μM I^A_{Na}	f_c	$S_0 \times 10^{20}$	i^A_{Na}	$N^A \times 10^{-6}$, channels/cm²	
		μA/cm²	μM	μA/cm²	Hz	A²·s/cm²	pA/channel	$I^A_{Na}/(i^A_{Na} \cdot P_0)$	I_{Na}/i^A_{Na}
1	Control	37.8	0.231	5.42	4.7	35.0	0.53	98.3	71.3
	Indomethacin	9.2	0.284	1.06	5.0	7.4	0.63	13.5	14.6
	ADH + theophylline	19.0	0.481	3.64	4.6	24.1	0.59	31.6	32.2
2	Control	18.3	0.219	2.11	6.5	9.4	0.50	42.5	36.6
	Indomethacin	4.6	0.285	0.36	6.8	2.1	0.72	4.2	6.4
	ADH + theophylline	15.3	0.586	1.74	6.3	10.8	0.80	9.6	19.1
3	Control	17.6	0.150	1.67	5.8	8.0	0.47	48.8	37.5
	Indomethacin	3.1	0.238	0.38	6.4	1.7	0.51	6.9	6.1
	ADH + theophylline	25.7	0.813	5.19	6.1	23.1	0.60	30.0	42.8
4	Control	26.0	0.302	3.83	6.6	14.5	0.45	64.7	57.8
	Indomethacin	8.2	0.395	1.03	6.5	6.0	0.72	8.7	11.4
	ADH + theophylline	20.7	0.531	2.97	6.3	14.3	0.60	23.5	34.5
5	Control	31.1	0.436	5.56	5.8	25.4	0.51	61.2	61.0
	Indomethacin	2.8	0.341	0.43	6.6	1.6	0.46	6.5	6.1
	ADH + theophylline	19.7	0.659	3.22	5.8	12.5	0.47	27.7	41.9
Mean ± SE									
	Control	26.2	0.269	3.72	5.9	18.5	0.49	63.1	52.8
		±3.8	±0.048	±0.81	±0.3	±5.1	±0.01	±9.7	±6.8
	Indomethacin	5.5	0.309	0.65	6.3	3.8	0.61	8.0	8.9
		±1.3	±0.027	±0.16	±0.3	±1.2	±0.05	±1.6	±1.7
	ADH + theophylline	20.1	0.614	3.35	5.8	17.0	0.61	24.5	34.1
		±1.7	±0.058	±0.56	±0.3	±2.8	±0.05	±4.0	±4.3
Experimental/control									
	Indomethacin/control	0.215	1.241	0.188	1.07	0.225	1.24	0.124	0.168
		±0.038	±0.13	±0.032	±0.03	±0.056	±0.12	±0.009	±0.019
	ADH + theophylline/ indomethacin	4.65	2.09	6.46	0.93	6.44	1.02	3.19	4.43
		±1.26	±0.35	±1.97	±0.02	±2.02	±0.06	±0.46	±1.04

mean N^A increased to 53.9 and $48.8 \times 10^6/cm^2$, respectively, for both methods of calculation with paired mean increases of 62 and 83%.

A similar analysis was carried out for five tissues treated first with indomethacin and then with ADH + theophylline (group II). The data are shown in Table IV. In this group the changes of I_{Na} and I^A_{Na} were considerably larger than observed with ADH + theophylline (group I). Indomethacin caused the mean I_{Na} to decrease from 26.2 to 5.5 $\mu A/cm^2$

and ADH + theophylline restimulated the mean I_{Na} to 20.1 $\mu A/cm^2$. I^A_{Na} (2 μM amiloride) was decreased from a mean of 3.72 to 0.65 $\mu A/cm^2$ by indomethacin, and after ADH + theophylline the mean I^A_{Na} was increased to 3.35 $\mu A/cm^2$. In part, the increase of I^A_{Na} by ADH + theophylline was due to an increase of $K_{1/2}$ from a mean of 0.309 to 0.614 μM.

No significant changes were observed in the values of f_c, but the S_o changed markedly, falling from a mean of 18.5×10^{-20} to 3.8×10^{-20} $A^2 \cdot s/cm^2$ after indomethacin and returning to 17.0×10^{-20} $A^2 \cdot s/cm^2$ after restimulation of the I_{sc} by ADH + theophylline. Despite the large changes of I_{Na}, I^A_{Na}, and S_o, the i^A_{Na} remained essentially constant with a mean control value of 0.49 pA/channel. Thus, the changes of Na transport caused by indomethacin and ADH + theophylline must have occurred via alterations of the density of Na-conducting channels at the

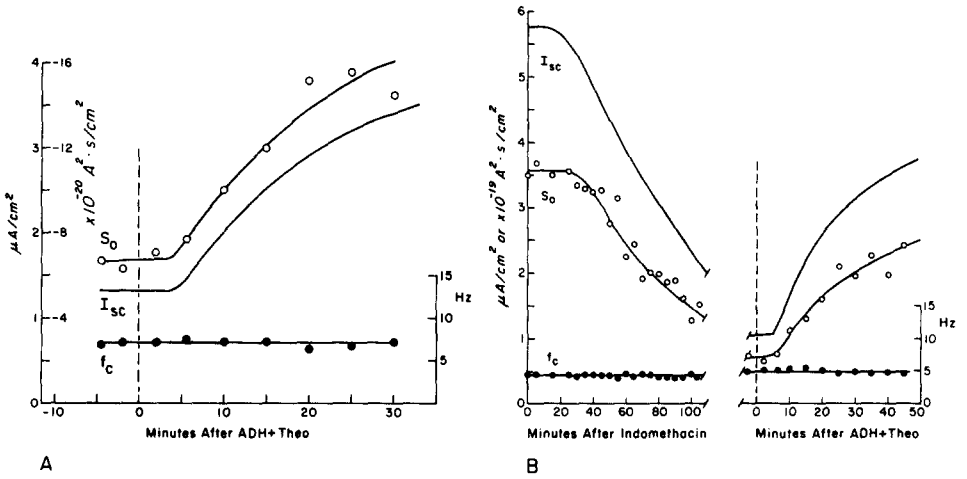

FIGURE 5. Changes of S_o and I_{sc} (I^A_{Na}) caused by ADH + theophylline or indomethacin followed by ADH + theophylline.

apical membranes of the cells. Control N^A calculated from $I^A_{Na}/(i^A_{Na} \cdot P_o)$ averaged 63.1×10^6, and the N^A calculated from I_{Na}/i^A_{Na} averaged 52.8 $\times 10^6$ channels/cm^2. After indomethacin, the mean N^A's decreased to 8.0 and $8.9 \times 10^6/cm^2$. An analysis of variance indicated that no significant differences of i^A_{Na} existed between treatment groups, whereas the differences of N^A were significant (Tukey multiple range test) at $P < 0.01$. Thus, it seemed clear that, despite the uncertainty of the values of $K_{1/2}$ and P_o, the principal changes in apical membrane permeability to Na occur via alteration of the density of electrically conductive, amiloride-inhibitable Na channels.

DISCUSSION

The principal aim of the present work was to evaluate with fluctuation (or noise) analysis the mechanisms by which alterations of cyclic AMP

metabolism in frog skin, by ADH and prostaglandins, change the apical membrane permeability to Na. Indeed, it would seem clear that the principal mechanism of action involves a change of the density of electrically conductive Na channels in the apical membrane of the cells during both the inhibitory and stimulatory phases of active transepithelial Na transport. To the extent that large changes of channel number density occur over relatively short periods of time, recruitment of Na channels seems highly likely, with cyclic AMP involved in this process. This conclusion is in essential agreement with Li et al. (1982), who studied the effects of oxytocin on toad urinary bladder depolarized by high serosal K concentrations.

Whereas it is clear that antidiuretic hormone and the prostaglandins exert their effects on Na transport in a number of target epithelia, the mechanism(s) of their action appears complicated by other factors that are not yet well understood. For example, in frog skin, vasopressin causes a sustained increase of the I_{sc} after a delay of a few minutes, whereas in toad urinary bladder the I_{sc} is highly transient, reaching a peak in a few minutes and declining thereafter to control values (Finn and Krug, 1973; Macchia and Helman, 1979). Transient stimulation by ADH of transepithelial Na transport has also been observed in isolated renal cortical collecting tubules (Frindt and Burg, 1972). Whereas prostaglandins appear to stimulate Na transport in frog skin, they appear to inhibit Na transport in cortical collecting tubules of rabbits (Stokes and Kokko, 1977). In contrast to the transient stimulation by vasopressin of the I_{sc} in toad urinary bladder bathed symmetrically with identical Ringer solutions, vasopressin and oxytocin cause a sustained increase of the I_{sc} of bladder depolarized by high serosal potassium concentrations (Finn and Krug, 1973; Li et al., 1982). The reasons for such differences of responses are not known, and in this regard it is presently impossible to present a coherent picture of how vasopressin and the prostaglandins interact in the regulation of transepithelial Na transport. Accordingly, it would seem prudent to avoid generalizations from the results of studies of any particular epithelium. In frog skin, it would seem clear that vasopressin and the prostaglandins are synergistic, stimulating Na transport by acting ultimately and primarily, if not solely, at the apical membranes to increase the density of the Na channels and hence leading to a sustained Na entry into the cells, with the rate of Na entry determined by the existing electrochemical potential differences. In this regard, the results of the present studies are in accordance with previous electrophysiological studies of the frog skin (Els and Helman, 1981).

Electrophysiological Considerations

We chose specifically to study the frog skin with fluctuation analysis because its electrophysiology was known, especially under conditions that were essentially the same as those used in the present studies. Indeed, with skins bathed symmetrically with a Cl-HCO_3 Ringer solution, both

vasopressin and prostaglandins cause an increase of the apical membrane slope conductance with little or no change of the basolateral membrane emf (slope formalism, Thévenin emf; see Helman and Thompson, 1982) or its slope conductance at physiologically negative intracellular voltages that average between −80 and −100 mV for epithelia in the short-circuited state (Helman and Fisher, 1977; Helman et al., 1979; Els and Helman, 1981). It has been suggested that potassium depolarization of the basolateral membranes yields an epithelial preparation where the basolateral membrane is removed as an important barrier that possesses negligible electrical resistance and voltage (Fuchs et al., 1977). It has not been possible to confirm this assumption in frog skin (Fisher, 1979; Nagel, 1979; Fisher et al., 1980; D. Benos, personal communication), so the contribution of sulfate-Ringer-bathed, K-depolarized basolateral membranes to the rate of Na entry at the apical membrane of the cells is unresolved. K depolarization apparently also causes a substantial increase of intracellular cyclic AMP (Cuthbert and Wilson, 1981), thereby causing an important perturbation of the system being studied. Accordingly, we made studies of noise analysis of the frog skin under the usual control conditions where tissues are bathed in vitro symmetrically with identical Ringer solution at the apical and basolateral surfaces of the tissues.

Under these conditions, the amiloride-induced current noise PDS of control and experimentally treated epithelia were near ideal, showing Lorentzian type spectra. Compared with previous studies of K-depolarized skin mounted in a rapid-flow chamber (Lindemann and Van Driessche, 1977), low-frequency noise amplitudes ($1/f$) were much smaller (W. Van Driessche, personal communication). Whether this was due to differences of chamber design or to scraping away the tela subcutanea and underlying corium is unknown, but it was clear that single Lorentzian-type PDS were readily observed in epithelia bathed symmetrically and short-circuited. The amiloride-induced noise under all control and experimental conditions was concentration dependent, with the plateau varying inversely with the [A] and the corner frequency increasing linearly with [A], in accordance with the expectations of a two-state model of amiloride binding to Na channels. Analysis of the [A] − $2\pi f_c$ plots indicated clearly that the association rate constant k_{01} remained unchanged despite large changes of apical membrane Na transport caused by indomethacin and/or ADH + theophylline. We also observed from noise analysis and the [A] dependency of the I_{sc} that ADH + theophylline caused significant increases of the equilibrium constant, $K_{½}$, which confirmed the finding of Cuthbert and Shum (1974). In view of the constancy of the k_{01}, we inferred that ADH + theophylline caused an increase of the amiloride receptor complex dissociation rate constant, k_{10}. Why this occurs is unknown, but it could be due to changes of cytoplasmic ionic composition or other factors that play a role in apical membrane structure and function. Nothing is yet known of such mechanisms and their relationship, if any, to apical membrane function.

Determination of the $K_{1/2}$ is complicated by the existence of the basolateral membrane and hence creates some uncertainty in the calculation of the magnitudes of the i_{Na}^{A} and N^{A}. Under ideal (single-barrier model) circumstances, the $K_{1/2}$ can be evaluated from the ΔI_{sc} caused by amiloride with the $K_{1/2}$ estimated at the [A] that causes 50% inhibition of the I_{sc}. However, in the face of two series membranes, both electrically active, the I_{sc} in frog skin is given by

$$I_{sc} = E_i/(R_o + R_i), \tag{9}$$

at the usual negative intracellular voltages, where E_i is the Thévenin emf of the basolateral membrane, R_o and R_i are the slope resistances determined from $\Delta V/\Delta I$, and the fractional transcellular resistance is $fR_o = R_o/(R_o + R_i) = \Delta V_o/\Delta V_T$. Hence, if amiloride acts alone to increase the R_o (or decrease apical membrane slope conductance, G_o), the I_{sc} will be determined not only by changes of the R_o but will in part depend on the R_i and E_i. Assuming in the simplest case that E_i and R_i are constant, and assuming moreover that the G_o – [A] relationship follows Michaelis-Menten kinetics, it can be shown that

$$\Delta I_{sc} = -\frac{\Delta I_{sc}}{[A]}\,(K_{1/2}/fR_o) + \Delta I_{sc}^{max}, \tag{10}$$

so that the apparent equilibrium constant, $K_{1/2}^{app}$, will overestimate the actual $K_{1/2}$:

$$K_{1/2}^{app} = K_{1/2}/fR_o. \tag{11}$$

In frog skin (*R. pipiens*), the fR_o averages ~0.75–0.85, and thus the $K_{1/2}^{app}$'s determined in this way are probably overestimated by ~15–25%. A more direct approach to this problem would be to evaluate directly the ΔG_o caused by amiloride, but this requires intracellular microelectrode techniques that were not available to us in Leuven at the time these studies were done. Thus, it can be shown that

$$\Delta G_o = -\left(\frac{\Delta G_o}{[A]}\right) K_{1/2} + \Delta G_o^{max}, \tag{12}$$

and indeed this has been verified experimentally (S. I. Helman, unpublished observations). Thus, for the purpose of evaluation of the present studies, we have taken this uncertainty into account in the interpretation of the data, as shown below.

With regard to the calculation of the i_{Na}^{A}, the P_1 is underestimated. With a $K_{1/2}^{app}$ of ~0.2 μM and a [A] of 2 μM, the P_1 is 0.909. Assuming a worst possible case where the fR_o is 0.5, the $K_{1/2}$ would be 0.1 μM and the P_1 would be 0.952. Accordingly, at the very most, the i_{Na}^{A} would be overestimated by ~5%, therefore yielding good estimates of the i_{Na}^{A} under all experimental conditions. On the other hand, the values of $P_o = 1 - P_1$ are subject to considerable uncertainty in value of $K_{1/2}$. Under comparable

conditions, the P_o taken from the 0.2 μM value of $K_{1/2}^{app}$ is 0.0909, whereas the actual P_o at maximum uncertainty would be 0.048, yielding an ~90% underestimate in the values of N^A. In an attempt to verify that such errors did not compromise the interpretation of the data, we calculated the N^A in two additional ways that avoided the uncertainties in value of $K_{1/2}$. First, we assumed that the i_{Na}^A was constant at all [A] so that the i_{Na}^A was the same both in the presence and absence of amiloride in the apical solution. Thus, $N = I_{Na}/i_{Na}^A$. Comparison of the values of N^A (see Tables III and IV) calculated in this way showed relatively minor differences, especially in view of the large changes of I_{Na} and relative constancy of the i_{Na}^A.

The N^A was calculated also with data of the mean values given in Tables II, III, and IV using Eq. 2 of Van Driessche and Lindemann (1979).

$$i_{Na}^A = \frac{S_o(2\pi f_c)^2}{4I_{Na}^A(k_{01}[A])}. \tag{13}$$

Since the k_{01} could be determined with precision (Table II), a mean value of i_{Na}^A could be calculated for control and experimental conditions by taking corresponding mean values of S_o, f_c, and I_{Na}^A (Tables III and IV). As all values were fairly certain, the i_{Na}^A so calculated should also be a rather good estimate of the mean single-channel Na current. As shown in Table V, the mean i_{Na}^A was 0.6 and 0.52 pA/channel for both groups of studies, and these values compare remarkably well with those of the mean i_{Na}^A given in Tables III and IV. Whereas in paired studies (Table III) ADH + theophylline caused no statistically significant decrease of the i_{Na}^A, the mean i_{Na}^A (Table V) appeared to decrease from 0.6 to 0.4 pA/channel. Similarly, for the indomethacin- and ADH + theophylline-treated epithelia, no significant changes of i_{Na}^A were observed upon perturbation of the I_{sc} (Table IV); however, as shown in Table V, indomethacin appeared to cause an increase of the mean i_{Na}^A from 0.52 to 0.71 pA/channel, whereas ADH + theophylline caused a reduction of i_{Na}^A to 0.53 pA/channel.These changes of i_{Na}^A, if real, put in the perspective of the larges changes of I_{Na}^A and I_{Na}, are relatively minor, but in fact could be important in the overall evaluation of the mechanisms involved. Despite our inability to resolve this, the N^A calculated from the mean I_{Na} and i_{Na}^A showed large increases of N^A, from 28.9 to 62.0 $\times$ 10^6 channels/cm^2 after ADH + theophylline alone, whereas indomethacin caused the N^A to decrease from 50.4 $\times$ 10^6 to 7.8 $\times$ 10^6 channels/cm^2 and to increase to 38.0 $\times$ 10^6 channels/cm^2 after ADH + theophylline (Table V). These values are remarkably similar to those reported in Tables III and IV, but here we relied only on the estimates of k_{01} of the [A] $-$ $2\pi f_c$ plots.

Electrochemical Potential Difference and Single-Channel Conductance

In general, and for the short-circuited frog skin epithelium, the apical membrane voltage, V_o^{sc}, is given by (Helman and Fisher, 1977; Helman, 1979):

$$V_o^{sc} = E_o(1 - fR_o) - E_i(fR_o), \tag{14}$$

where the E_o and E_i are the Thévenin equivalent emfs of apical and basolateral membranes, respectively. At high values of fR_o, especially with amiloride in the apical solution, the fR_o approaches unity and thus the $V_o^{sc} \rightarrow -E_i$. Accordingly, the apical membrane voltage is determined primarily by the E_i of the basolateral membrane and hence the i_{Na}^A is subject to variation with E_i, if and when changes of E_i occur. In this regard, vasopressin and indomethacin have little or no consistent effect on the E_i (Els and Helman, 1981), and thus, despite changes of R_o and N^A, the i_{Na}^A was not subjected to a significant change of potential difference across the apical membrane. Therefore, under the circumstances of the present studies, with the V_o^{sc} essentially constant, the i_{Na}^A was similarly essentially constant, given the uncertainties stated above. Although the V_o^{sc} were not measured directly in the present studies, they were probably in the vicinity of −90 to −120 mV with 2 μM [A] in the apical solution during measurement of the I_{Na}^A (Helman and Fisher, 1977; unpublished observations).

It is known that amiloride causes a substantial decrease of intracellular $[Na]_c$ in the epithelium of frog skin to relatively low values of ~5 mM or

TABLE V

Calculation of i_{Na}^A and N^A from Mean Data of Tables II, III, and IV

	i_{Na}^A	N^A
	pA/channel	*× 10^{-6} channels/cm²*
Control	0.60	28.9
ADH + theophylline	0.40	62.0
Control	0.52	50.4
Indomethacin	0.71	7.8
ADH + theophylline	0.53	38.0

less (Rick et al., 1978). Thus, a significant chemical potential difference exists together with the V_o^{sc} to provide the driving force for Na entry into the cells (Helman, 1979). It is unknown to what extent, if any, the $[Na]_c$ is changed by indomethacin and/or ADH + theophylline with a [A] of 2 μM. However, assuming there is simple diffusion and an electrochemical potential difference of ~180 mV (V_o^{sc} = 100 mV, ξ_{Na}^o = 90 mV), the Na flux ratio is ~1,000:1, and hence Na entry is dictated virtually alone by the apical solution $[Na]_o$ of 100 mM and the V_o^{sc}. Thus, in the face of a large electrochemical potential difference driving Na into the cells, physiological changes of intracellular $[Na]_c$ would not be expected to cause significant changes of the i_{Na}^A. Hence, it would seem safe to conclude that the relative constancy of the i_{Na}^A reflects a lack of major effects of ADH + theophylline and indomethacin on the single-channel Na current at the negative apical membrane voltages, although minor effects cannot be ruled out completely.

Given that intracellular $[Na]_c$ is not known with certainty, it is impossible to determine with precision the single-channel conductance. As a rough

approximation, the single-channel "chord" conductance according to Hodgkin and Huxley (1952) can be calculated from

$$g^{A}_{Na} = \frac{i^{A}_{Na}}{(-V^{sc}_{o} + \xi^{o}_{Na})} = \frac{0.6 \times 10^{-12}}{180 \times 10^{-3}}. \quad (15)$$

Thus, g^{A}_{Na} is ~3.3 pS/channel at 100 mM $[Na]_o$ and at a V^{sc}_{o} of about −100 mV.

The corresponding "slope" conductance ($\Delta I/\Delta V$) according to Thévenin's theorem (Thévenin, 1883; Chua, 1969; Helman and Thompson, 1982) at these negative voltages is given by:

$$G^{A}_{Na} = \frac{i^{A}_{Na}}{-V^{sc}_{o}} = \frac{0.6 \times 10^{-12}}{100 \times 10^{-3}} \quad (16)$$

and is in the vicinity of 6 pS/channel.

S.I.H. and T.C.C. are especially grateful to Drs. W. Van Driessche and R. Casteels and the Laboratory of Physiology in Leuven for providing facilities for conduct of the present work and for the hospitality extended to us during our visit (January 1981). We are most grateful to Mrs. Lieve Jansens-De Handschutter for excellent technical assistance.
Supported in part by U.S. Public Health Service grants AM16663 and GM07357.

Received for publication 20 September 1982 and in revised form 26 April 1983.

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