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Autoregulation of Apical Membrane Na^+ Permeability of Tight Epithelia

Noise Analysis with Amiloride and CGS 4270

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ABSTRACT Noise analysis of the Na^+ channels of the apical membranes of frog skin bathed symmetrically in a Cl-HCO_3 Ringer solution was done with amiloride and CGS 4270. Tissues were studied in their control states and after inhibition of transepithelial Na^+ transport (I_{sc}) by addition of quinine or quinidine to the apical solution. A critical examination of the amiloride-induced noise indicated that the single channel Na^+ currents (i_{Na}) were decreased by quinine and quinidine, probably because of depolarization of apical membrane voltage. Despite considerable statistical uncertainty in the methods of estimation of the Na^+ channel density with amiloride-induced noise (N^A, see text), the striking observation was a large increase of N^A with amiloride inhibition of the rate of Na^+ entry into the cells. N^A was increased to 406% of control, whereas I_{sc} was inhibited to 8.6% of control by 6 μM amiloride. Studies were done also with the Na^+ channel blocker CGS 4270. Noise analysis with this compound was advantageous, permitting i_{Na}^{CGS} and N^{CGS} to be measured in individual tissues with a relatively small inhibition of I_{sc}. As with amiloride, inhibition of I_{sc} with CGS 4270 caused large increases of the Na^+ channel density (~200% at ~35% inhibition of the I_{sc}). Quinine and quinidine caused an ~50% increase of Na^+ channel density while inhibiting i_{Na} by ~60–70%. As inhibition of Na^+ entry leads to an increase of Na^+ channel density, a mechanism of autoregulation appears to be a major factor in adjusting the apical membrane Na^+ permeability of the cells.

INTRODUCTION

The mechanism(s) of regulation of the permeability to Na^+ (P_{Na}) of the apical membranes of epithelial cells remains a subject of importance and controversy. Although the existence of hormonal influences are well documented (see review by Andreoli and Schafer, 1976; and, for example, Orloff and Handler, 1967;

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Els and Helman, 1981; Helman et al., 1983), it has been suggested that changes of intracellular and extracellular concentrations of Na^+, H^+, and Ca^{++} are also determinants of P_{Na} (see reviews by Schultz, 1981; Roos and Boron, 1981; Windhager and Taylor, 1983). To our knowledge, it has so far been impossible in studies of intact epithelial cells to design experiments where any single factor can be varied by itself. Thus, it has been difficult to define which factor or factors govern the P_{Na} to the exclusion of others that may also contribute to the regulation of P_{Na}.

In the present studies, we adopted the methods of fluctuation analysis described by Lindemann and Van Driessche (1977) in an attempt to measure the single channel Na^+ currents and Na^+ channel densities of the apical membranes of the epithelial cells of frog skin. Our original purpose was to determine what, if any, effects quinine and quinidine exerted on these parameters of the apical membranes since it had been suggested that by virtue of increases of cytosolic Ca^{++} activity, P_{Na} was decreased, which led to inhibition of transepithelial Na^+ transport (Taylor and Windhager, 1979). However, electrophysiological studies with intracellular microelectrode techniques indicated that inhibition of apical membrane Na^+ entry into the cells was due primarily to depolarization of apical membrane voltage (Abramcheck, 1984).

The scope of this project was expanded when it was observed that the Na^+ channel density was increased markedly by inhibition of Na^+ entry by amiloride (autoregulation). Because of the statistical uncertainties in the analysis of the amiloride-induced noise data, we turned to studies of the weak amiloride-like Na^+ channel inhibitor CGS 4270 (Benos and Watthey, 1981). Similar results were obtained with this compound under experimental conditions that circumvented the uncertainties inherent in the analysis of amiloride-induced noise. We concluded from these studies that a mechanism of autoregulation exists at the apical membrane of the cells that senses changes of the rate of Na^+ entry and adjusts the Na^+ channel density in an attempt to maintain a constant rate of Na^+ entry.

Because of this phenomenon, analysis of the possible direct effects of quinine and quinidine on Na^+ channel density was complicated. The Na^+ channel density (in the absence of amiloride or CGS 4270) appeared either to remain unchanged or to increase upon treatment of epithelia with either quinine or quinidine. Preliminary reports of this work have been presented (Abramcheck et al., 1983; Helman and Van Driessche, 1984).

MATERIALS AND METHODS

Abdominal skins of *Rana pipiens* (Nasco Biologicals, Fort Atkinson, WI; Lemberger, Oshkosh, WI) were studied as described in detail elsewhere (Helman et al., 1983). After scraping away the majority of the unstirred layers of the corium, the tissues were placed between chambers (0.72 cm^2), short-circuited, and allowed to equilibrate for 2–3 h while bathed symmetrically in a Ringer solution containing 100 mM NaCl, 2.4 mM $KHCO_3$, and 2.0 mM $CaCl_2$. After completion of the studies with amiloride, the chambers were modified for use in the CGS 4270 studies (see Fig. 1) to permit continuous flow of solutions between and during periods of data acquisition of the power density spectra (PDS). This not only facilitated exchanges of solution but avoided the usual flushing

transients that occur after the flow of the bathing solutions is stopped. Background spectra indicated that there was less $1/f$ noise during continuous flow than during stopped flow.

Several groups of studies were done with amiloride- and CGS 4270–induced noise and the protocols of these experiments are described in detail in the Results. In all studies, PDS were measured during control and experimental periods at several increasing concentrations of the current noise inducer (amiloride or CGS 4270) added to the apical solution. Amiloride (Merck, Sharp & Dohme Research Laboratories, West Point, PA) was used at concentrations of 0.88, 1.76, 2.64, 3.52, 4.4, and 5.28 μM, and CGS 4270 [3-(3-amino-1,2,4-oxadiazol-5-yl)-5-chloro-2,6-pyrazinediamine; Ciba-Geigy, Pharmaceuticals Division, Ardsley, NY] was used at concentrations of 25, 50, 75, 100, 125, and 150 μM. For convenience in referring to the amiloride concentrations, nominal values of 1–6 μM

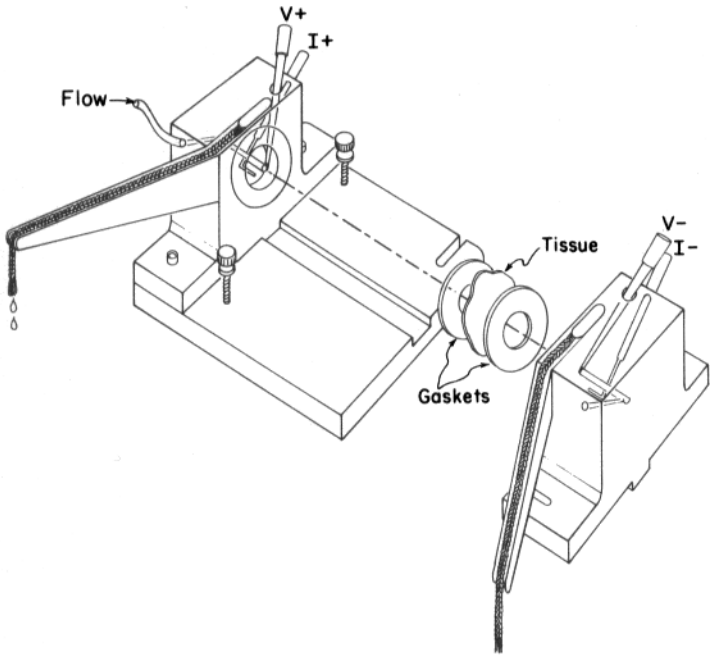

FIGURE 1. Modification of chambers (Helman and Miller, 1971) to permit continuous flow of solution. The chamber volume is ~0.6 ml. Fluid enters the chambers by gravity feed via polyethylene tubing. Spillways containing candle wicking allow the solution to leave the chamber. Fluid is not recirculated. Low-resistance bridges for current and voltage are connected via Ag/AgCl wires to the voltage clamp.

will be used below, although the actual concentrations are those given above. At each concentration of the noise inducer and after inhibition of the macroscopic I_{sc}, the current noise fluctuations were digitized on line by computer and the data were transformed (FFT) for calculation of the PDS. Lorentzian components of the PDS were calculated using a nonlinear curve-fitting routine (Van Driessche and Zeiske, 1980) and were characterized by their low-frequency plateaus (S_o) and corner frequencies (f_c).

Collection of data for amiloride-induced noise was identical to that described previously (Helman et al., 1983). Because the corner frequencies of CGS 4270-induced noise are considerably higher than those for amiloride (50–120 Hz at 25–150 μM CGS 4270), the current fluctuations of the I_{sc} were sampled in sweeps of 2 s duration at slightly greater than 1 kHz at cutoff frequencies of 480 Hz (model 752A brickwall filter, Wavetek

Rockland, Inc., Rockleigh, NJ). This provided a frequency resolution of 0.5 Hz, from which $1/f$ and Lorentzian components of the PDS could be measured. 20 sweeps of data (40 s) were accumulated at each [CGS 4270], providing an adequate signal-to-noise ratio.

Notation

Specific notation is given in the text. In general, i_{Na} and N will refer to the single channel Na^+ current and Na^+ channel density in units of picoamperes and millions of channels per square centimeter, respectively. Superscripts A and CGS will designate values measured in the presence of amiloride or CGS 4270. The appearance of a bar above a symbol will indicate the mean value of the tissue population sampled. For example, I_{sc} is the value of the short-circuit current in the absence of amiloride or CGS 4270 and is taken to be identical to the rate of Na^+ entry, I_{Na}. I_{sc}^{A} and I_{sc}^{CGS} are the values of the short-circuit current in the presence of a specific concentration of amiloride ([A]) or CGS 4270 ([CGS]), respectively. $\overline{I_{sc}}$ and $\overline{I_{sc}^{A}}$ are the mean values of n observations (tissues) of the short-circuit current measured in the absence or presence of amiloride, respectively. The same notations will be used for all parameters, including the low-frequency plateau of the Lorentzian function (S_o), corner frequency ($2\pi f_c$), microscopic rate constants (k_{01} and k_{10}), the equilibrium constant ($K_{1/2}$) as defined by the ratio k_{10}/k_{01}, and the apical membrane voltage of short-circuited tissues (V_o^{sc}).

Quinine and quinidine (Sigma Chemical Co., St. Louis, MO) were added to the apical solution and were used at a concentration of 5×10^{-4} M.

Statistical data are reported either as means ± SEM or as means ± 95% confidence interval (see Results). All studies were done at room temperature.

RESULTS

Experimental Protocols: Amiloride-induced Noise

Within 20 min, both quinine and quinidine cause inhibition of the short-circuit current and hence Na^+ entry at the apical membranes of the cells (Abramcheck, 1984). The I_{sc} values of 16 epithelia bathed symmetrically in the control Ringer solution averaged 22.93 $\mu A/cm^2$ (see Table I). Amiloride was added to the apical solution at graded concentrations with data acquisition at each concentration of amiloride ([A]) to determine the current-noise PDS. At 6 μM amiloride, I_{sc} was inhibited by ~90%. After washout of amiloride from the apical solution and a return of I_{sc} to its pre-experimental control value, tissues were treated with either quinine or quinidine for 20 min and subjected again to amiloride inhibition of I_{sc} for determination of the amiloride-induced PDS. As shown in Table I for control studies, the pre-experimental control values of I_{sc} were essentially the same as the original control values of I_{sc}. Quinine and quinidine caused I_{sc} to be inhibited to 48.6 and 51.0% of control, respectively, and amiloride at 6 μM inhibited the I_{sc} of quinine- and quinidine-treated epithelia by ~85–90%.

Because amiloride at the concentrations necessary for noise analysis causes a substantial inhibition of I_{sc}, we carried out two additional groups of experiments to be referred to as chronic amiloride studies. During both control and experimental periods, the tissues were bathed chronically with 2 μM amiloride in the apical solution. Hence, changes of the PDS and the effects of quinine and quinidine could be assessed from the changes of I_{sc} from their already inhibited

states of Na^+ entry. Thus, in both the control and chronic amiloride studies, it was important to keep in mind that the data accumulated were derived from tissues where Na^+ transport was markedly inhibited by amiloride from their more usual rates of transepithelial Na^+ transport.

Amiloride-induced Current Noise

QUININE: CONTROL AND CHRONIC AMILORIDE STUDIES Amiloride-induced current noise was measured in control periods and after quinine inhibition of the tissues. Summarized in panels *A*, *B*, and *C* of Fig. 2 are the short-circuit currents (I_{sc}^A), low-frequency plateaus (S_o^A), and corner frequencies ($2\pi f_c$), where the superscript A refers to parameter values measured with amiloride inhibition of Na^+ transport. Similar data are shown in Fig. 3 for tissues treated chronically

TABLE I

Changes of I_{sc} Caused by Amiloride in Control and in Quinine- or Quinidine-treated Epithelia

	I_{sc}: control period		I_{sc}: experimental period			
	Control	6 μM amiloride	Pre-experimental control	Quinine	6 μM amiloride	Quinine/control
	$\mu A/cm^2$	$\mu A/cm^2$	$\mu A/cm^2$	$\mu A/cm^2$	$\mu A/cm^2$	
Control studies						
(n = 10)	22.93±3.21	1.67±0.24	23.96±3.50	10.75±1.31	1.63±0.25	0.486±0.040
				Quinidine		Quinidine/control
(n = 6)	22.93±2.34	2.49±0.34	24.18±2.87	11.94±1.25	1.45±0.29	0.510±0.048
2 μM Chronic amiloride studies						
				Quinine		Quinine/control
(n = 12)	4.42±0.60	2.40±0.37	5.54±0.63	4.25±0.57	2.22±0.35	0.794±0.052
				Quinidine		Quinidine/control
(n = 11)	3.11±0.40	1.64±0.21	3.97±0.56	2.87±0.45	1.15±0.19	0.768±0.075

Values are mean ± SEM.

with amiloride. As in previous studies, the corner frequency plots ([A] vs. $2\pi f_c$) were linear in both control and experimental conditions of the tissues. This linearity is compatible with the two-state model of open-closed kinetics of amiloride binding to the Na^+ channels (Lindemann and Van Driessche, 1977), and hence the microscopic rate constants for amiloride can be calculated from the slope and intercept at the ordinate of the corner frequency plots:

$$2\pi f_c^A = k_{01}^A [A] + k_{10}^A, \quad (1)$$

where k_{01}^A and k_{10}^A are the microscopic association and dissociation rate constants, respectively.

Provided that valid estimates of k_{01}^A and k_{10}^A can be obtained with this method, the equilibrium constant ($K_{1/2}^A$) for amiloride can be calculated from the quotient

k^A_{10}/k^A_{01}, leading ultimately to the calculation of the single channel Na^+ current, i^A_{Na}, and the Na^+ channel density, N^A. According to Lindemann and Van Driessche:

$$i^A_{Na} = S^A_o(2\pi f^A_c)^2/(4I^A_{Na}k^A_{01}[A]) \quad \text{(method A);} \tag{2a}$$

$$= (S^A_o\ 2\pi f^A_c)/(4I^A_{Na}P_1) \quad \text{(method B),} \tag{2b}$$

and we will refer below to the calculation of i^A_{Na} with methods A and B as defined by Eqs. 2*a* and 2*b*.

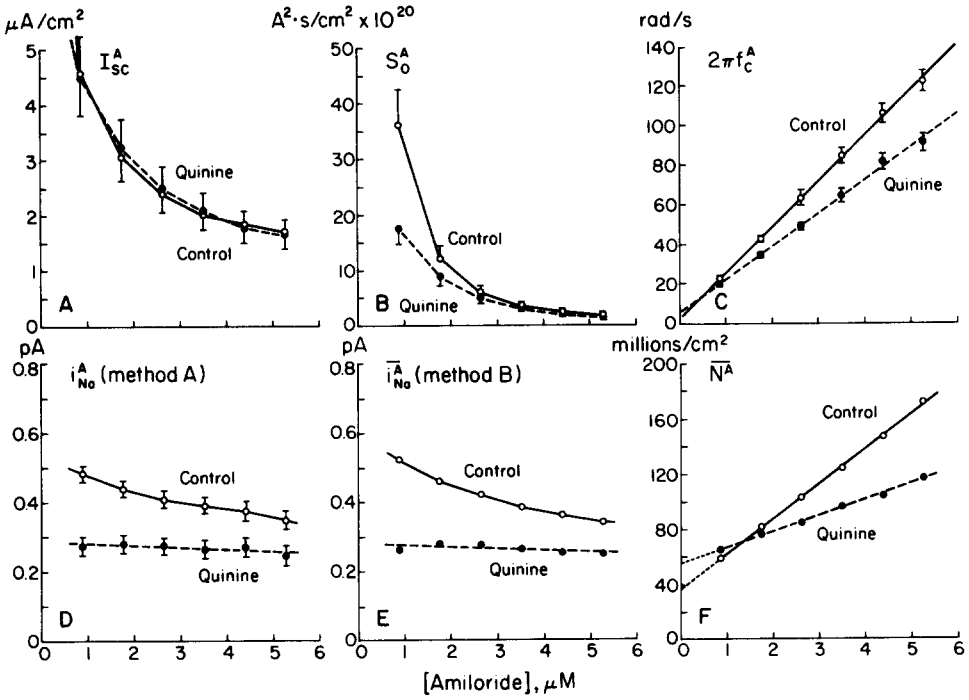

FIGURE 2. Changes of I^A_{sc}, S^A_o, $2\pi f^A_c$ caused by amiloride in control tissues and tissues treated with quinine. i^A_{Na} was calculated with method A, and $\overline{i^A_{Na}}$ was calculated with method B (see text). Compare values of i^A_{Na} and $\overline{i^A_{Na}}$. N^A was calculated with the values of $\overline{i^A_{Na}}$. Values in panels *A–C* are means ± SEM.

$$N^A = I^A_{Na}/(i^A_{Na}\ P_o), \tag{3}$$

where the probabilities of the channel being open (P_o) or closed (P_1) are:

$$P_o = (1 + [A]/K^A_{1/2})^{-1} \tag{4}$$

and

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

Summarized in Tables IIA and IIB are the values of k^A_{01} and k^A_{10} calculated according to Eq. 1 for 39 individual epithelia. For each tissue, the ±95%

confidence interval was determined according to the statistical analysis outlined by Kleinbaum and Kupper (1978). In general, the uncertainty of the k_{01}^{A} as specified by the ±95% confidence interval averaged about ±13.4% of the least-squares value of k_{01}^{A}. The mean $\overline{k_{01}^{A}}$ values of control tissues (23.0 ± 0.7 [n = 16]; Table IIA) and of tissues treated chronically with 2 μM amiloride (21.9 ± 1.2 [n = 23]; Table IIB) were essentially the same. In control epithelia, quinine and quinidine caused a small but significant decrease of the k_{01}^{A} (see Table IIA), whereas in the tissues treated chronically with amiloride, there was no consistent

TABLE IIA

Values of Amiloride k_{01}^{A} and k_{10}^{A} of Control Studies

	k_{01}^{A}		k_{10}^{A}	
	Control	Quinine	Control	Quinine
	rad/s·μM		*rad/s*	
	24.2±3.1	22.3±3.6	0.17±10.67	−1.34±12.27
	24.8±3.0	18.1±3.8	−1.17±10.28	10.10±12.87
	26.5±3.9	21.0±4.5	4.31±13.21	3.23±15.43
	26.7±2.3	15.3±5.0	0.96±7.80	7.79±17.19
	27.8±3.5	16.1±4.9	6.37±12.17	11.67±16.93
	19.6±1.2	15.1±1.1	5.92±4.05	4.37±3.83
	20.5±2.0	13.8±1.5	2.25±6.74	3.31±5.13
	19.8±2.2	15.4±1.0	2.28±7.44	3.03±3.50
	22.8±1.6	16.0±1.2	−0.25±5.56	5.12±4.17
	19.8±1.1	15.5±2.0	0.22±3.75	4.72±6.97
Mean ± SEM (10)	23.3±1.0	16.9±0.9	2.11±0.83	5.20±1.19
	Control	Quinidine	Control	Quinidine
	22.9±1.7	18.2±2.2	5.70±5.90	9.34±7.65
	22.1±2.3	19.4±2.9	7.12±8.01	2.35±9.77
	23.6±1.5	17.6±2.1	1.05±5.09	4.44±7.19
	24.3±5.4	18.7±3.5	7.41±18.61	9.72±11.88
	21.8±3.0	17.9±2.6	13.66±10.13	7.58±8.78
	20.9±1.3	16.6±1.8	0.29±4.31	3.64±6.01
Mean ± SEM (6)	22.6±0.5	18.1±0.4	5.87±1.99	6.18±1.27
Combined mean ± SEM (16)	23.0±0.7		3.52±0.99	

Individual values are given together with their ±95% confidence intervals.

change of the k_{01}^{A} caused by either quinine or quinidine. Thus, within the uncertainties given by the ±95% confidence intervals of k_{01}, the i_{Na}^{A} of individual epithelia could be calculated with method A of Eq. 2. The results of these calculations are shown in Fig. 2*D* for control tissues. At [A] between 1 and 6 μM, the i_{Na}^{A} values of tissues treated with quinine were significantly smaller than the control values of i_{Na}^{A}. With increases of [A], the i_{Na}^{A} of control tissues (0.48 ± 0.02 pA at 1 μM amiloride) fell progressively to 0.35 ± 0.03 pA at 6 μM amiloride. After the tissues were treated with quinine, the i_{Na}^{A} was 0.27 ± 0.03 pA at 1 μM amiloride and remained unchanged at the higher [A].

Similar calculations of the i^{A}_{Na} (method A) were done for the tissues treated chronically with amiloride. As shown in Fig. 3*D*, the mean i^{A}_{Na} of control and quinine-treated tissues at 2 μM amiloride were 0.52 ± 0.06 and 0.41 ± 0.04 pA, respectively. With increases of [A] from 2 to 6 μM, the i^{A}_{Na} of the quinine-treated tissues remained unchanged.

TABLE IIB

Values of Amiloride k^{A}_{01} and k^{A}_{10} of Chronic Amiloride Studies

	k^{A}_{01}		k^{A}_{10}	
	Control	Quinine	Control	Quinine
	rad/s·μM		*rad/s*	
	19.6±5.7	23.7±1.9	22.37±21.4	9.80±7.06
	21.2±3.5	23.1±6.4	22.12±12.87	15.46±23.95
	33.0±4.7	26.8±2.1	10.43±17.44	8.55±8.00
	35.6±4.1	23.6±4.1	3.27±15.39	20.73±15.26
	32.6±7.7	25.7±3.4	8.55±28.83	12.06±12.84
	28.5±8.3	21.5±2.9	−4.27±30.92	20.48±10.94
	18.2±3.7	15.3±3.6	−1.16±13.73	7.98±13.39
	18.3±2.6	18.8±1.2	2.19±9.63	−1.07±4.65
	15.4±2.1	16.9±2.8	10.15±7.81	0.79±10.49
	20.8±0.7	18.9±2.6	−0.88±2.62	2.36±9.87
	19.5±1.6	15.3±4.2	3.33±5.89	9.65±15.70
	19.4±2.7	17.0±3.6	2.79±10.22	6.92±13.55
Mean ± SEM (12)	23.5±2.0	20.6±1.2	6.57±2.48	9.48±2.02
	Control	Quinidine	Control	Quinidine
	23.1±1.7	14.9±4.2	9.55±6.42	29.28±15.85
	24.2±5.8	20.9±6.4	13.95±21.50	9.93±23.85
	18.3±1.8	15.7±5.2	27.52±6.79	17.09±19.39
	20.9±8.7	20.1±5.2	15.71±32.28	2.39±19.52
	23.6±4.1	21.4±6.5	9.93±15.39	3.52±24.32
	17.9±3.3	17.2±2.7	8.75±12.41	9.30±10.16
	20.6±2.3	17.0±2.5	1.77±11.19	12.99±9.30
	17.7±1.7	18.6±2.2	6.18±6.15	3.15±8.21
	19.7±1.1	19.2±4.5	3.14±4.07	4.10±16.89
	18.1±1.4	19.1±3.3	0.83±5.05	−2.15±12.17
	16.5±2.7	18.1±1.2	5.88±10.19	3.00±4.61
Mean ± SEM (11)	20.1±0.8	18.4±0.6	9.38±2.30	8.42±2.67
Combined mean ± SEM (23)	21.9±1.2		7.92±1.69	

Individual values are given together with their ±95% confidence intervals.

Examination of the individual values of k^{A}_{10} as shown in Tables IIA and IIB indicated that it was not possible to obtain valid estimates of the k^{A}_{10} of individual experiments with reasonable certainty. Indeed, some tissues gave negative values of k^{A}_{10}. Given the known potency of amiloride in inhibiting Na^+ entry, it is not surprising that values of k^{A}_{10} near zero were observed, and moreover, given the steep slope of the amiloride corner frequency plots, relatively small random

errors in estimation of the f_c led to great uncertainty in the estimates of the values of k_{10}^A. Thus, it was not possible to obtain an estimate of the N^A of individual epithelia, as this required calculation of $K_{1/2}^A$, P_o, and P_1 (Eqs. 3–5).

We chose, of necessity, to attempt a crude measure of the Na^+ channel densities by assuming that the influence of small random errors in the measurement of f_c could be minimized by calculation of tissue population mean values of $\overline{k_{10}^A}$ and $\overline{k_{01}^A}$ and hence $\overline{K_{1/2}^A}$. For the corner frequency plots shown in Figs. 2*C* and 3*C*, we calculated the mean ±95% confidence interval of $\overline{k_{01}^A}$ and $\overline{k_{10}^A}$ using the mean values of $\overline{2\pi f_c^A}$. These values are summarized in Table III. For control tissues and tissues treated with quinine and quinidine, the $\overline{k_{01}^A}$ values were the same as

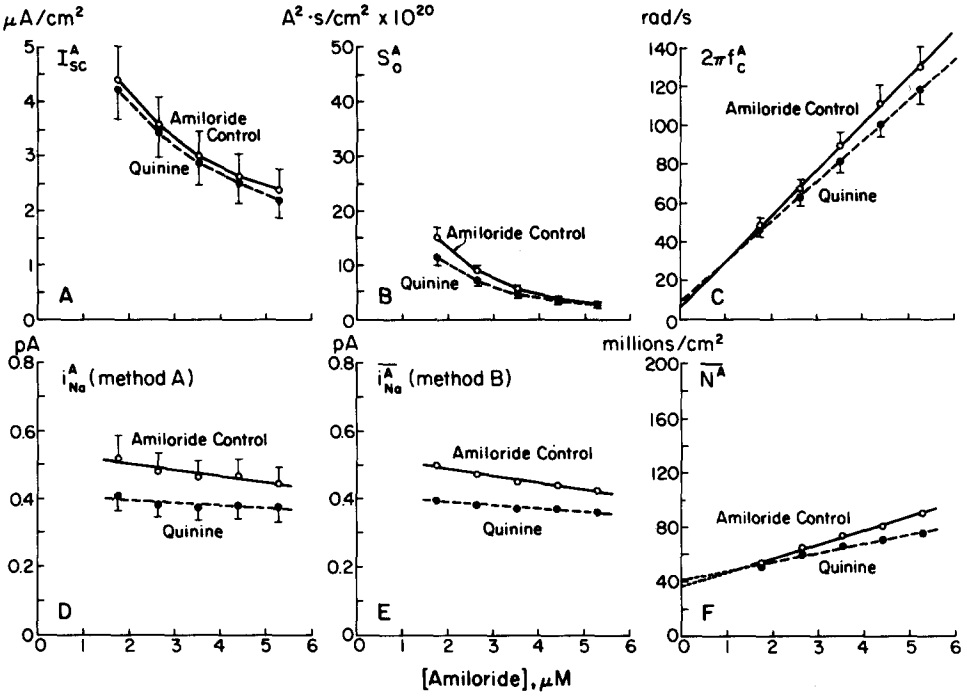

FIGURE 3. Changes of I_{sc}^A, S_o^A, $2\pi f_c^A$ caused by amiloride in control tissues and tissues treated chronically with 2 μM amiloride. See legend of Fig. 2 and text.

given above and both quinine and quinidine caused small decreases of $\overline{k_{01}^A}$. No significant change of $\overline{k_{01}^A}$ occurred after quinine or quinidine treatment of tissues exposed chronically to amiloride. The $\overline{k_{10}^A}$ was 3.52 rad/s in control tissues, and with the ±95% confidence intervals shown in Table III, it was not possible to determine if quinine or quinidine caused changes of $\overline{k_{10}^A}$. In the absence of an alternative method of estimating $K_{1/2}^A$, and recognizing clearly the uncertainties involved, we calculated $\overline{K_{1/2}^A}$. The values of $\overline{K_{1/2}^A}$ for control and quinine- and quinidine-treated epithelia were 0.15, 0.31, and 0.34 μM, respectively. For tissues treated chronically with amiloride, the amiloride control and quinine- and quinidine-treated values of $\overline{K_{1/2}^A}$ were 0.36, 0.46, and 0.46 μM, respectively.

On the premise that these values of $\overline{K^A_{1/2}}$ reflected to at least a first approximation the equilibrium constants for amiloride, we calculated a tissue population mean $\overline{i^A_{Na}}$ (method B) at every [A], using also the mean values of $\overline{I^A_{sc}}$, $\overline{S^A_o}$, and $\overline{2\pi f^A_c}$ given in panels *A*, *B*, and *C* of Figs. 2 and 3. The results of these calculations are shown in Figs. 2*E* and 3*E*. Remarkably, the values of $\overline{i^A_{Na}}$ (method B) were similar to those of i^A_{Na} (method A). As a criterion of acceptability of $\overline{K^A_{1/2}}$, we examined, in all groups of studies, the similarity of the values of i^A_{Na} and $\overline{i^A_{Na}}$. As the values of i^A_{Na} (method A) were reasonably certain, we assumed that an identity in the values of i^A_{Na} and $\overline{i^A_{Na}}$ was sufficient to permit calculation of the $\overline{N^A}$. In this way, we attempted to obtain a measure of N^A and its dependence on [A].

The $\overline{N^A}$ values calculated with Eq. 3 using the $\overline{I^A_{sc}}$, $\overline{i^A_{Na}}$, and P_o are summarized in Figs. 2*F* and 3*F* for the control tissues and tissues treated chronically with amiloride. In all groups, $\overline{N^A}$ increased with increasing [A]. At 1 μM amiloride,

TABLE III

Estimates of the Tissue Population Mean of $\overline{k^A_{01}}$, $\overline{k^A_{10}}$, and $\overline{K^A_{1/2}}$ of Control and Chronic Amiloride-treated Skins

	$\overline{k^A_{01}}$	$\overline{k^A_{10}}$	$\overline{K^A_{1/2}}$
	rad/s·μM	*rad/s*	*μM*
Control studies			
Control	23.0±1.2	3.52±3.99	0.15
Quinine	16.9±1.4	5.20±4.68	0.31
Quinidine	18.1±1.4	6.18±4.87	0.34
Chronic amiloride studies			
Amiloride Control	21.8±1.6	7.92±5.94	0.36
Quinine	20.6±0.9	9.48±3.43	0.46
Quinidine	18.4±2.1	8.42±7.68	0.46

Values are given together with their ±95% confidence intervals (see text).

$\overline{N^A}$ was in the range of 40–50 million channels/cm^2. At 6 μM amiloride, $\overline{N^A}$ was increased markedly to ~180 million/cm^2, whereas smaller but marked changes of $\overline{N^A}$ were observed in quinine-treated tissues. Despite the uncertainties involved in arriving at these data, and given the differences in I_{sc} and other parameter values among the groups of tissues, it seemed reasonably certain, at least qualitatively, that Na^+ channel density varied markedly with increases of amiloride concentration with relative constancy of the single channel Na^+ currents. However, in view of this dependency of $\overline{N^A}$ on [A], it was not at all obvious what effect, if any, quinine alone exerted on the $\overline{N^A}$ of the apical membrane of the cells (see below).

QUINIDINE: CONTROL AND CHRONIC AMILORIDE STUDIES Studies identical to those above were done with quinidine. Summary values of $\overline{I^A_{sc}}$, $\overline{S^A_o}$, and $\overline{2\pi f^A_c}$ are given in the upper panels of Fig. 4 for control and quinidine-treated tissues and in the lower panels for the chronic amiloride control and quinidine-treated states of these tissues. The corner frequency plots again showed the linearity

between the $2\pi f_c^A$ and [A] in all tissues, and the i_{Na}^A values of individual tissues were calculated with the values of k_{01}^A (method A). The individual values of k_{01}^A and k_{10}^A are summarized in Tables IIA and IIB and $\overline{k_{01}^A}$, $\overline{k_{10}^A}$, and $\overline{K_{1/2}^A}$ are summarized in Table III.

In previous electrophysiological studies, quinidine, unlike quinine, caused either a decrease of the electromotive force (E_i) of the basolateral membranes of the cells (group I) or little or no change of the E_i (group II) (Abramcheck, 1984). Quinine in every tissue depolarized E_i. Accordingly, in the presence of amiloride, the apical membrane voltage of short-circuited epithelia (V_o^{sc}) was either depolar-

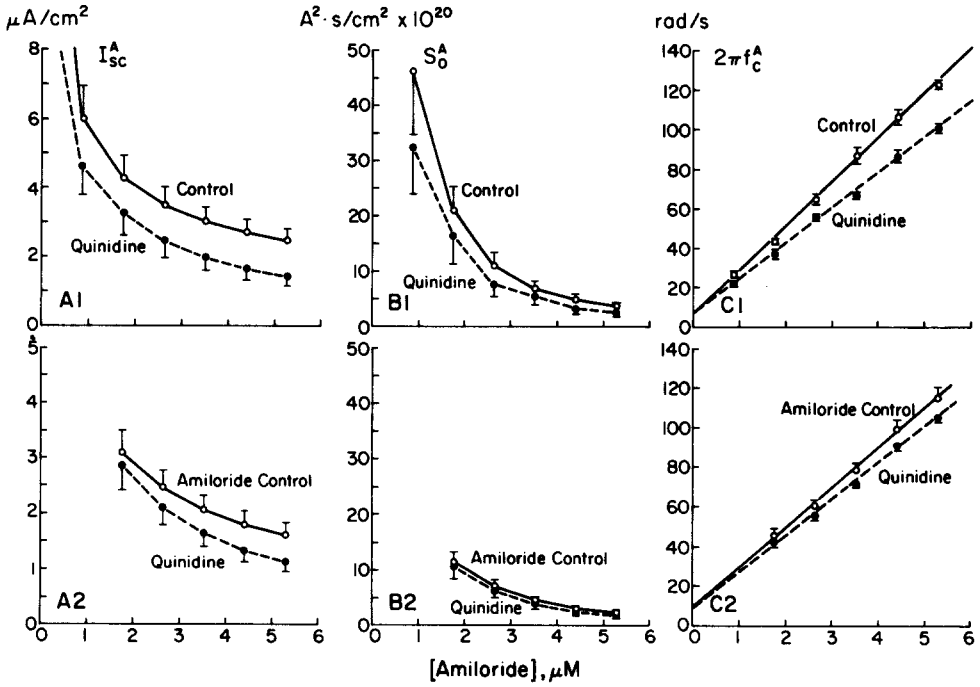

FIGURE 4. Changes of I_{sc}^A, S_o^A, and $2\pi f_c^A$ caused by amiloride in control tissues treated with quinidine (panels *A1–C1*) and in chronic amiloride tissues treated with quinidine (panels *A2–C2*).

ized or remained essentially unchanged after treatment of the tissues with quinidine, and thus we expected to observe a similar variability in response of i_{Na} to quinidine but not to quinine.

Inspection of the data of six control tissues showed that the i_{Na}^A values (method A) of three tissues were decreased by quinidine (group I, panel *A1*, Fig. 5) and in three tissues, i_{Na}^A was either unchanged or increased by quinidine (group II, panel *A2*, Fig. 5). Of 11 tissues treated chronically with 2 μM amiloride, 4 tissues (group I) showed decreases of i_{Na}^A after quinidine (panel *A1*, Fig. 6), whereas the i_{Na}^A values of tissues of group II (panel *A2*, Fig. 6) were not changed by quinidine.

Although the reasons for this difference are not known, these observations were consistent with the previous electrophysiological studies, where decreases of apical membrane voltage, when they occurred, were expected to cause decreases of i_{Na}^{A}.

To determine the Na^+ channel densities, we turned again to the criteria outlined above and compared the mean values of i_{Na}^{A} calculated with method A with those of $\overline{i_{Na}^{A}}$ calculated with method B. Inspection of the data revealed that, in general, the values of i_{Na}^{A} and $\overline{i_{Na}^{A}}$ were similar at all [A]. However, as can be seen in panels *A2* and *B2* of Fig. 5 and panels *A1* and *B1* of Fig. 6, systematic deviations of i_{Na}^{A} and $\overline{i_{Na}^{A}}$ were observed in some groups of tissues, leading to a systematic error in the estimation of $\overline{N^A}$. Nevertheless, while taking such errors

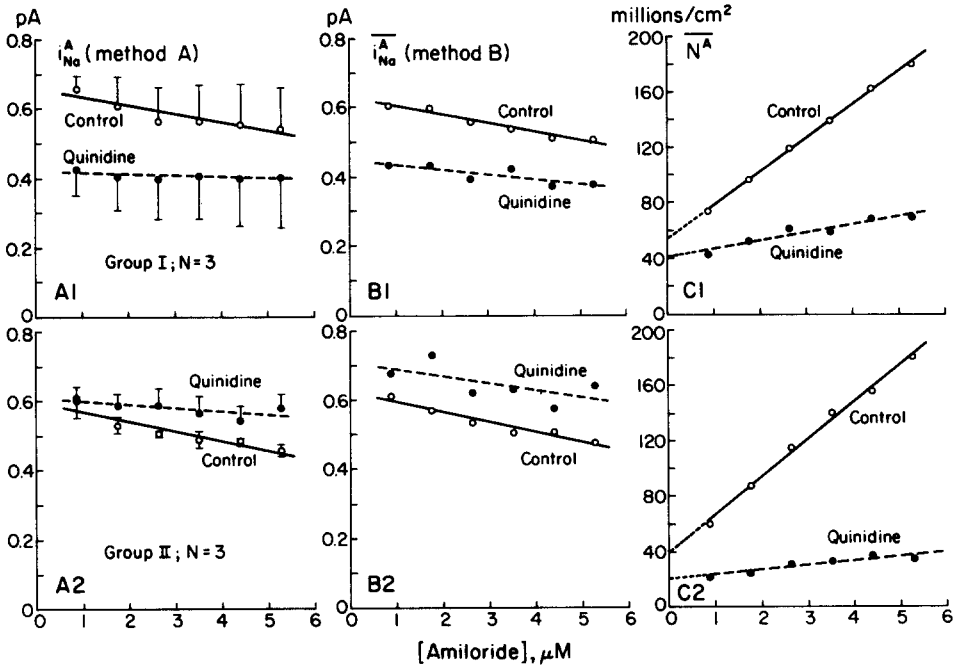

FIGURE 5. Changes of i_{Na}^{A}, $\overline{i_{Na}^{A}}$, and $\overline{N^A}$ in control tissues and control tissues treated with quinidine. Data are grouped (I and II) according to the response of i_{Na}^{A}.

into account, the $\overline{N^A}$ of the control tissues showed again large increases of $\overline{N^A}$ with increasing [A] (see Fig. 5). After quinidine, the changes of $\overline{N^A}$ caused by amiloride were markedly reduced. For the tissues treated chronically with amiloride, the $\overline{N^A}$ of the group II tissues was increased by [A] and a similar calculation for the group I tissues was not done since $\overline{i_{Na}^{A}} \neq i_{Na}^{A}$. After quinidine, the changes of $\overline{N^A}$ with increasing [A] were virtually absent. Thus, as in the quinine studies, because of the sensitivity of $\overline{N^A}$ to [A], it was not possible in the presence of amiloride to determine what effects quinidine alone exerted on the $\overline{N^A}$. It was apparent, however, that with tissues treated chronically with 2 μM

amiloride, the N^A at 2 μM amiloride remained unchanged by either quinine or quinidine (see panel *F* of Fig. 3 and panel *C2* of Fig. 6).

ESTIMATES OF THE $\bar{N}$ AND $\bar{i}_{Na}$ OF NON-AMILORIDE-INHIBITED TISSUES The ultimate goal of the above studies was to obtain estimates of the single channel Na^+ currents and Na^+ channel densities in the absence of a Na^+ channel noise-inducing agent such as amiloride. It is known, however, that amiloride inhibition of apical membrane permeability causes significant hyperpolarization of the apical membrane voltage concurrent with the inhibition of I_{sc} that occurs primarily in the range of [A] between 0 and 1 μM. Thus, given the electrodiffusive nature of

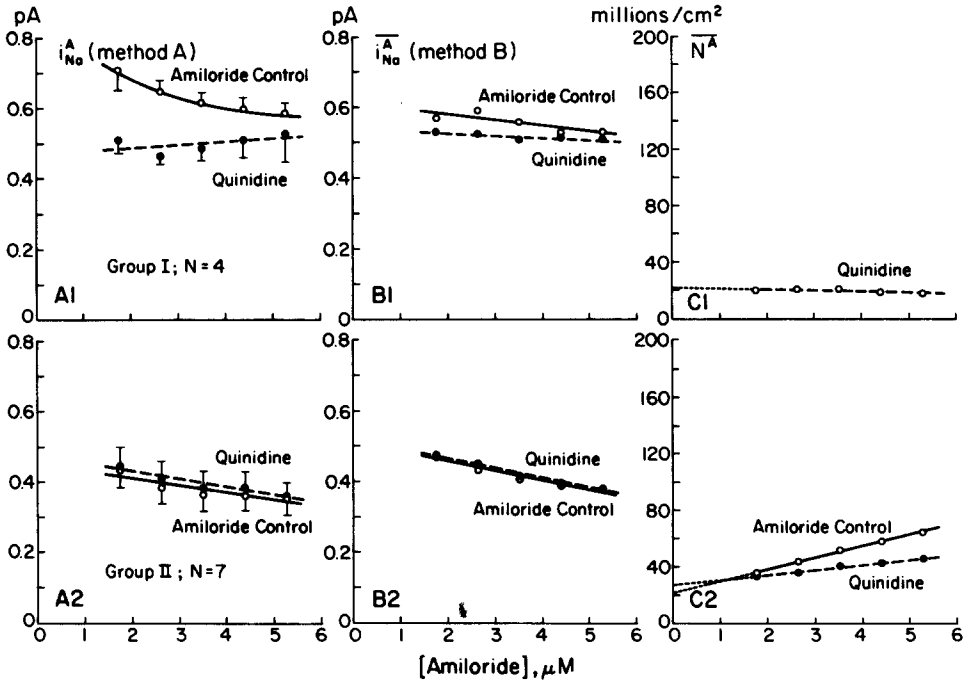

FIGURE 6. Changes of i^A_{Na}, $\bar{i}^A_{Na}$, and $\bar{N}^A$ in chronic amiloride control tissues and tissues treated with quinidine. Data are grouped (I and II) according to the response of i^A_{Na}.

Na^+ entry, i^A_{Na} cannot be extrapolated directly to the zero-amiloride condition of the tissues, and moreover, it is not known whether the single channel density is voltage and/or current independent. In this regard, we chose to obtain estimates of $\bar{N}$ (zero-amiloride condition), assuming at first that $\bar{N}$ was voltage independent. To test this assumption, we compared the calculated changes of i_{Na} (no amiloride) caused by quinine and quinidine with the changes of V^{sc}_o that had been measured previously (Abramcheck, 1984).

Shown in Fig. 7 are the values of $\bar{N}^A$ measured in control epithelia and epithelia treated with quinine or quinidine. As the [A] vs. $\bar{N}^A$ relationship was linear in all groups, the zero-amiloride intercept at the ordinate, $\bar{N}$, was measured

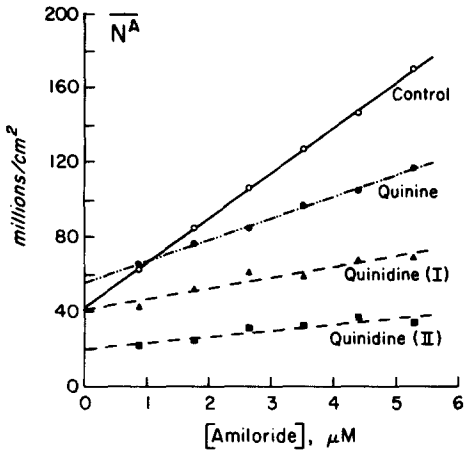

FIGURE 7. Changes of $\overline{N^A}$ in control and quinine- and quinidine-treated tissues with increases of amiloride concentration. The $\bar{N}$ at zero amiloride was estimated from the extrapolated value at the ordinate (see text).

by linear regression extrapolation of the $\overline{N^A}$. The control value was 42.0 million/cm^2. After quinine, $\bar{N}$ was 55.3 million/cm^2, and after quinidine, $\bar{N}$ was 40.7 and 19.9 million/cm^2 for group I and group II quinidine-treated tissues. Given the uncertainties involved in obtaining these estimates of $\bar{N}$, the apparent differences from control should not be taken seriously. If these differences are real, one may surmise that the changes of $\bar{N}$ caused by quinine and quinidine are in the range of about ±50% of the control value, and such an inference would be compatible with the electrophysiological studies reported previously (Abramcheck, 1984).

From the $\bar{I}_{sc}$ and $\bar{N}$ values, we calculated the zero-amiloride $\overline{i_{Na}}$, and, as shown in Table IV, both quinine and quinidine (group I) appeared to cause a large decrease of the $\overline{i_{Na}}$. The control $\overline{i_{Na}}$ was 0.546 pA. After quinine, $\overline{i_{Na}}$ was decreased to 35.5% of control or 0.194 pA. After quinidine, $\overline{i_{Na}}$ was decreased to 52.4% of control or 0.286 pA in group I tissues, although in group II tissues the $\overline{i_{Na}}$ increased to 112.8% of control or 0.616 pA. Comparison of the values of V_o^{sc} measured previously indicated that the changes of $\overline{V_o^{sc}}$ (see Table IV) were essentially the same as the changes of $\overline{i_{Na}}$. Thus, we conclude that the $\overline{i_{Na}}$ is

TABLE IV

Values of Tissue Population $\bar{N}$, $\bar{I}_{sc}$, $\overline{i_{Na}}$, and $\overline{V_o^{sc}}$ of Control and Quinine- or Quinidine-treated Skins

					Experimental/control	
	$\bar{N}$	$\bar{I}_{sc}$	$\overline{i_{Na}}$	$\overline{V_o^{sc}}$	$\overline{i_{Na}}$	$\overline{V_o^{sc}}$
	millions/cm²	*μA/cm²*	*pA*	*mV*		
Control	42.0	22.93	0.546	−94.3	—	—
Quinine	55.3	10.75	0.194	−36.1	0.355	0.383
Quinidine I	40.7	11.62	0.286	−42.3	0.524	0.449
Quinidine II	19.9	12.25	0.616	−80.6	1.128	0.855

Values of V_o^{sc} are taken from Abramcheck (1984).

decreased by depolarization of apical membrane voltage. Given the uncertainties in the estimates of $\bar{N}$, it was not possible to arrive at a firm estimate of any changes of $\bar{N}$ caused by quinine and quinidine, so we eventually turned to studies of CGS 4270–induced noise of the apical Na^+ channels (see below).

Perhaps the most interesting phenomenon observed in these studies was the apparent increase of the Na^+ channel density upon inhibition of Na^+ entry by amiloride. We replotted the data of Fig. 7 as a percent of the control value at zero amiloride concentration. At 6 μM amiloride, as shown in Fig. 8*A*, the Na^+ channel density was increased to 405.6% of its zero-amiloride control value. In tissues treated with either quinine or quinidine, the increases of channel density were smaller, but were still relatively large. The channel density was increased by 6 μM amiloride to 211.9% of control in quinine-treated tissues and to 170.2 and 171.5% of control in quinidine-treated tissues of group I and group II, respectively.

We also plotted the changes of single channel current as shown in Fig. 8*B* as a percent of control of the zero-amiloride value of i_{Na} given in Table IV. We expected to observe a change of $\overline{i_{Na}}$ in control tissues of ~10–20% between 0 and 1 μM amiloride because of the hyperpolarization of V_o^{sc}. This was not observed, probably because of the uncertainties inherent in the method and assumptions of the calculations. Put in perspective, the consistent error involved is in the range of ~10–20% if we assume that i_{Na} is directly proportional to V_o^{sc}. In control tissues, the V_o^{sc} usually averages about −80 to −90 mV, and when tissues are treated with 100 μM amiloride, the V_o^{sc} averages about −100 to −120 mV. With increasing [A], $\overline{i_{Na}^A}$ tended to decrease by ~20% at 6 μM. Whether this is due to changes of V_o^{sc} or to changes of the single channel conductance is unknown. Resolution of this question will require a paired analysis of V_o^{sc} and i_{Na}^A, and at the moment this is impractical.

After quinine or quinidine (group II), $\overline{i_{Na}^A}$ increased at 1–2 μM amiloride, and at higher [A], $\overline{i_{Na}^A}$ tended to decrease as in the control tissues. Again, it is not known whether this is due to changes of V_o^{sc} or single channel conductance, although the increases of $\overline{i_{Na}^A}$ at lower [A] are probably due in part to hyperpolarization of the V_o^{sc} upon inhibition of apical membrane Na^+ entry by amiloride.

CGS 4270–induced Current Noise

The most interesting compound for Na^+ channel noise analysis that we have studied so far is CGS 4270. We used this compound to circumvent the difficulties in measuring the microscopic rate constants and hence the Na^+ channel densities. This compound was described by Benos and Watthey (1981) as a weak, reversible, amiloride-like Na^+ channel blocker. In solution, CGS 4270, unlike amiloride, is uncharged with a pK_a of <2 and is thus pH insensitive at physiological pH. Preliminary studies of CGS 4270–induced current noise (Helman and Van Driessche, 1984) indicated that the $K_{1/2}^{CGS}$ was ~100 μM as estimated from the corner frequencies and the microscopic rate constants k_{01}^{CGS} and k_{10}^{CGS}. In addition to the ease of determination of the $K_{1/2}^{CGS}$, studies with CGS 4270 offered the additional advantage that inhibition of the I_{sc} from control was relatively small, at least in comparison with studies with amiloride, so that measures of the i_{Na}^{CGS}

and N^{CGS} could be made at Na^+ transport rates reasonably close to those encountered spontaneously. Accordingly, studies were done to assess the effects of quinine and quinidine on i_{Na} and N using CGS 4270 as the noise inducer of the Na^+ channels.

EXPERIMENTAL PROTOCOLS: CGS 4270 The experimental protocols were virtually the same as those described above. After the I_{sc} of the control period had stabilized, the tissues were exposed to CGS 4270 at concentrations between 25 and 150 μM. The control I_{sc} of 12 tissues averaged 21.00 ± 1.52 $\mu A/cm^2$, and at 150 μM CGS 4270, the I_{sc} was decreased to 13.41 ± 0.88 (12) $\mu A/cm^2$ or to 65.0 ± 3.0% of control (see Table V). After washout of CGS 4270 from the apical solution and return of the I_{sc} to its pre-experimental control value of 19.91 ± 1.08 (12) $\mu A/cm^2$, the tissues were treated with either quinine or quinidine

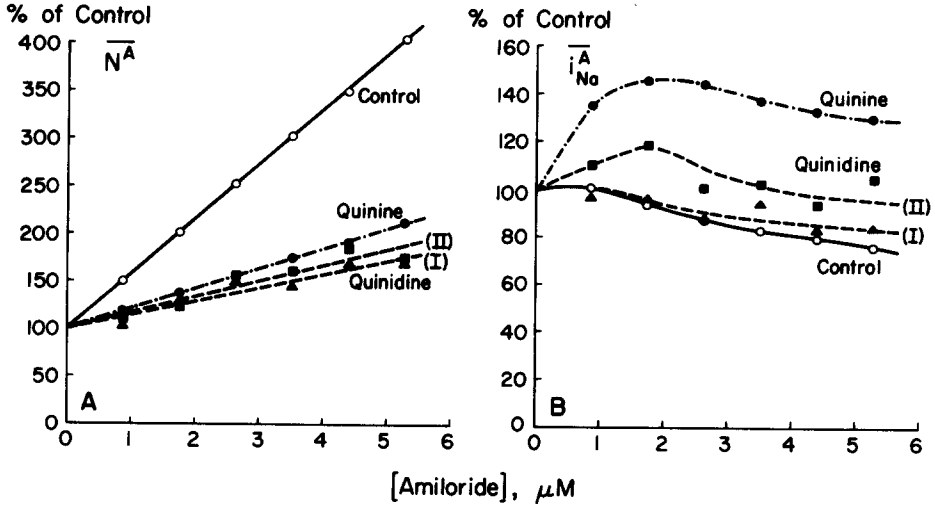

FIGURE 8. Changes of $\overline{N^A}$ and $\overline{i^A_{Na}}$ as a percent of control (zero amiloride) caused by amiloride.

for 20 min. After quinine, the I_{sc} was decreased to 11.53 ± 1.55 (7) $\mu A/cm^2$, and in the presence of 150 μM CGS 4270, the I_{sc}^{CGS} was reduced further to 8.88 ± 1.15 (7) $\mu A/cm^2$ or to 78.4 ± 4.3% of its quinine control value (Table V). Quinidine caused the I_{sc} to decrease to 10.72 ± 1.08 (5) from its pre-experimental control value of 20.29 ± 1.24 (5) $\mu A/cm^2$. In the presence of 150 μM CGS 4270, the I_{sc}^{CGS} was reduced further to 61.8 ± 5.8% of its quinidine control value or to 6.51 ± 0.66 (5) $\mu A/cm^2$.

Shown in Fig. 9 are representative examples of the changes of I_{sc} of control and quinine- or quinidine-treated tissues in response to increases of concentration of CGS 4270 ([CGS]). Two features of the records are noteworthy. First, the records appeared "scalloped" and showed transient decreases of the I_{sc} at each concentration of CGS 4270. During the relative plateau periods at the end of each period, data were accumulated for determination of the PDS (~40 s).

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TABLE V

Changes of I_{sc}: Caused by CGS 4270 in Control and in Quinine- or Quinidine-treated Epithelia

	I_{sc}: Control period			I_{sc}: Experimental period			
Quinine studies	Control	150 μM CGS	150 μM/control	Pre-experimental control	Quinine	150 μM CGS	150 μM/quinine
	$\mu A/cm^2$	$\mu A/cm^2$	$\mu A/cm^2$	$\mu A/cm^2$	$\mu A/cm^2$	$\mu A/cm^2$	
	14.51	8.74	0.602	17.00	10.09	7.15	0.709
	15.58	8.52	0.547	15.68	8.05	6.81	0.846
	17.94	12.46	0.695	18.71	12.10	7.19	0.594
	26.80	17.64	0.658	25.50	18.75	14.11	0.753
	20.30	13.90	0.685	20.50	11.77	9.37	0.796
	24.20	17.58	0.726	25.60	13.84	11.75	0.849
	13.27	11.42	0.861	14.43	6.10	5.76	0.944
Mean ± SEM (7)	18.94±1.92	12.89±1.42	0.682±0.038	19.63±1.70	11.53±1.55	8.88±1.15	0.784±0.043
Quinidine studies					Quinidine		150 μM/quinidine
	25.00	14.46	0.578	19.12	10.36	5.46	0.527
	26.10	17.12	0.656	23.60	13.10	8.35	0.637
	27.90	13.25	0.475	21.50	13.11	6.22	0.474
	16.27	12.32	0.757	16.25	7.52	4.83	0.642
	24.10	13.53	0.561	21.00	9.49	7.67	0.808
Mean ± SEM (5)	23.87±2.00	14.14±0.82	0.605±0.048	20.29±1.24	10.72±1.08	6.51±0.66	0.618±0.058
Combined mean ± SEM (12)	21.00±1.52	13.41±0.88	0.650±0.030	19.91±1.08			

Second, upon complete washout of CGS 4270 from the apical solution, the I_{sc} increased to peak values above control, returning slowly thereafter to control values in ~20–30 min. These records were obtained with the continuous flow chambers described in Methods. Similar results have been obtained more recently with amiloride, where flushing transients previously obscured the observation of such phenomena. As evidenced by the rather small changes of the I_{sc} at these high concentrations of CGS 4270, this compound compared with amiloride is a poor inhibitor of Na^+ entry.

POWER DENSITY SPECTRA: CGS 4270 Shown in Fig. 10 is a typical PDS of CGS 4270–induced current noise. In the absence of CGS 4270, the PDS

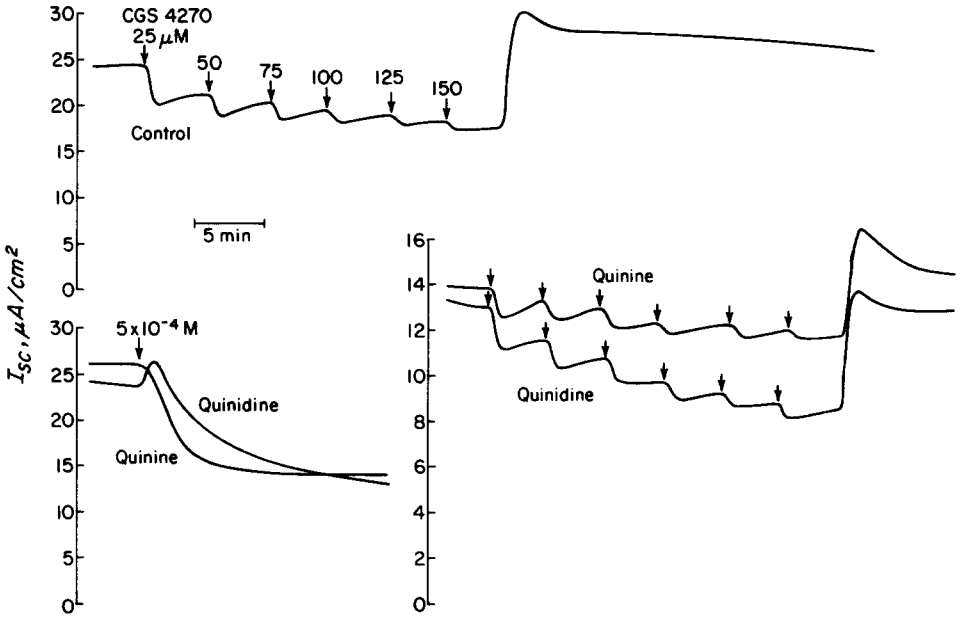

FIGURE 9. Typical responses of I_{sc} to increases of CGS 4270 concentration. Note "scalloped" appearance of the records and overshoots above control when CGS 4270 was flushed completely out of the apical solution. Quinine caused inhibition of I_{sc} within ~5–10 min, whereas quinidine caused inhibition of I_{sc} after a relatively small stimulation (Abramcheck, 1984).

contained its usual low-frequency $1/f$ noise with amplifier noise appearing at the higher frequencies. However, when CGS 4270 inhibited Na^+ entry, a single Lorentzian component was observed with corner frequencies (f_c^{CGS}) ranging between ~50 and 120 Hz at [CGS] between 25 and 150 μM. A summary of the short-circuit currents (I_{sc}^{CGS}), low-frequency plateaus (S_o^{CGS}), and corner frequencies ($2\pi f_c^{CGS}$) of control and quinine- or quinidine-treated epithelia is shown in Fig. 11. Not surprisingly, the S_o^{CGS} values were decreased markedly upon inhibition of I_{sc}^{CGS}. In contrast to amiloride, the k_{01}^{CGS} and k_{10}^{CGS} could be measured with reasonable certainty in each individual tissue. As shown in Table VI, k_{01}^{CGS} averaged 2.95 ± 0.10 rad/s·μM, whereas k_{10}^{CGS} averaged 304.3 ± 5.03 rad/s. As

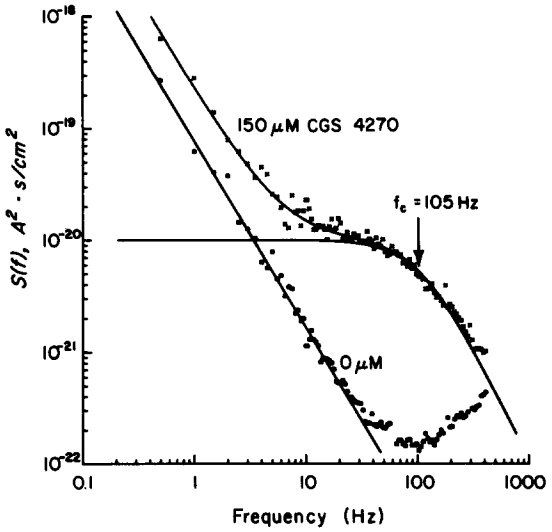

FIGURE 10. Power density spectra in the absence (0 μM) and presence (150 μM) of CGS 4270.

there was no difference of the corner frequencies between control and quinine-treated tissues or between control and quinidine-treated tissues, $K_{1/2}^{CGS}$ was unchanged by these drugs. $K_{1/2}^{CGS}$ ranged between 79.9 and 133.1 μM, averaging 104.5 ± 4.3 μM in 12 tissues.

CHANGES OF i_{Na}^{CGS} AND N^{CGS} The observed changes of I_{sc} caused by CGS 4270 are plotted in Fig. 12 as a percent of control. Clearly, inhibition of the I_{sc} was less than expected if in fact CGS 4270 inhibited 50% of the Na^+ channels at a [CGS] of 104.5 μM. Accordingly, such differences between the observed and expected changes of I_{sc} could be explained either by increases of channel density and/or by increases of i_{Na} when Na^+ entry is inhibited by CGS 4270.

Shown in Figs. 13 and 14 are the results of typical experiments where i_{Na}^{CGS} and N^{CGS} were calculated according to Eqs. 2 and 3. For the experiments shown, the

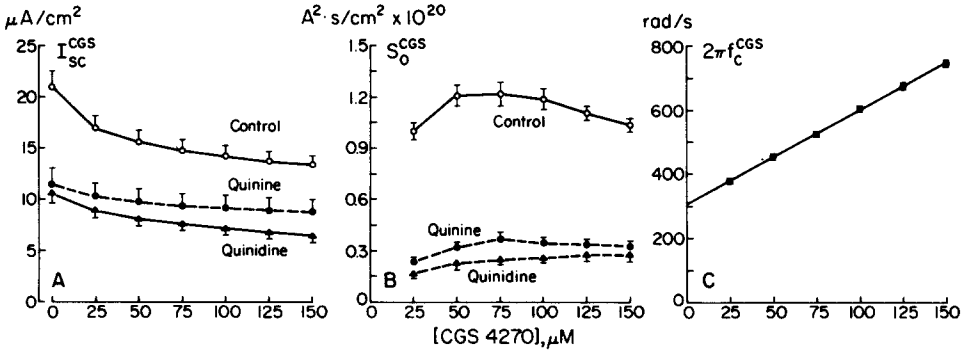

FIGURE 11. Changes of I_{sc}^{CGS}, S_o^{CGS}, and $2\pi f_c^{CGS}$ caused by CGS 4270 of control and quinine- and quinidine-treated tissues.

TABLE VI

Values of k_{01}^{CGS}, k_{10}^{CGS}, and $K_{1/2}^{CGS}$

	k_{01}^{CGS}	k_{10}^{CGS}	$K_{1/2}^{CGS}$
	rad/s·μM	*rad/s*	*μM*
	2.53±0.39	286.5±37.6	113.2
	2.99±0.39	295.0±38.0	98.7
	3.21±0.37	321.4±36.3	100.1
	2.73±0.28	293.6±28.2	107.6
	3.45±0.41	276.5±41.8	79.9
	3.30±0.31	319.0±30.0	96.7
	3.12±0.45	326.2±43.7	104.6
	3.17±0.38	305.9±36.6	96.5
	3.16±0.43	302.4±42.2	95.7
	2.92±0.30	288.2±29.4	98.7
	2.49±0.49	331.5±44.3	133.1
	2.36±0.34	306.1±34.4	129.7
Mean ± SEM (12)	2.95±0.10	304.3±5.0	104.5±4.3

Individual values are given together with their ±95% confidence intervals.

i_{Na}^{CGS} values of control and quinine- or quinidine-treated tissues were essentially constant and independent of the [CGS]. The i_{Na}^{CGS} values of all tissues treated with quinidine were reduced markedly below control. Accordingly, they appeared to correspond to those of the group I quinidine-treated tissues described above. In control and quinine-treated tissues, N^{CGS} was increased markedly with increasing [CGS], and in this regard, these results are similar to those of the amiloride-induced noise studies. The large decrease of i_{Na}^{CGS} caused by quinine and quinidine (group I tissues) was also compatible with the previously observed depolarization of the apical membrane voltage. After the tissues were treated

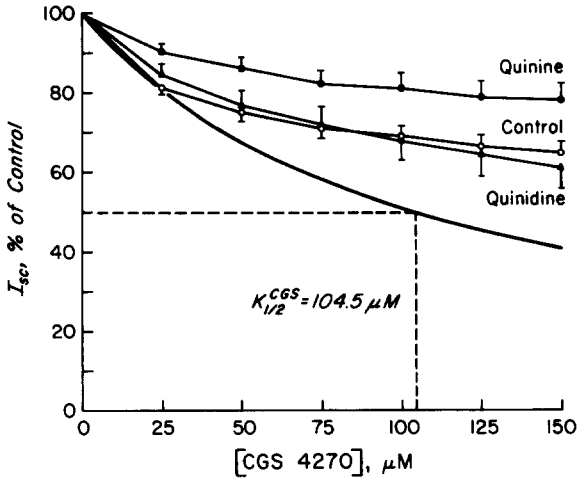

FIGURE 12. Inhibition of I_{sc} (percent of control) caused by CGS 4270. The solid line was drawn assuming a $K_{1/2}^{CGS}$ of 104.5 μM and assuming that Na^+ channel density and single channel current were constant.

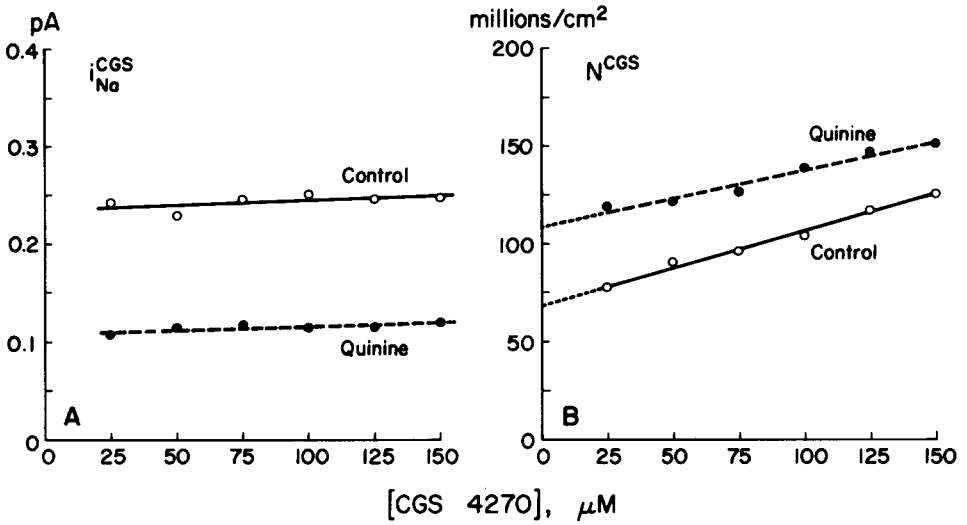

FIGURE 13. Changes of i_{Na}^{CGS} and N^{CGS} caused by quinine. Note also increases of N^{CGS} with increasing [CGS 4270].

with quinidine, the N^{CGS} appeared to remain essentially constant and independent of the [CGS].

We extrapolated the data of the [CGS]-N^{CGS} relationship to zero [CGS] so as to obtain an estimate of the channel density (N) of the uninhibited state of the tissue. This seemed justifiable since, especially at the lower [CGS], the changes of I_{sc} from control were relatively small. The single channel Na^+ current (i_{Na}) was estimated from the quotient I_{sc}/N. A summary of these parameters is given

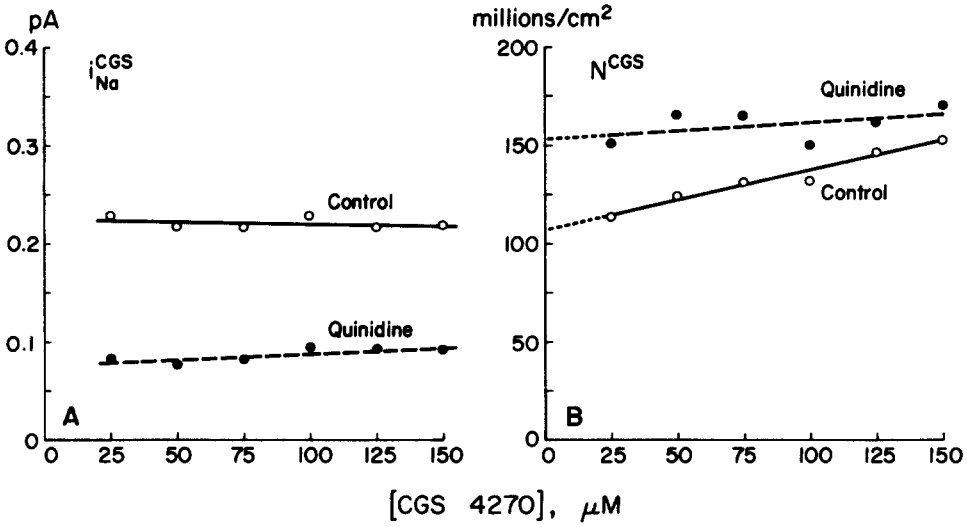

FIGURE 14. Changes of i_{Na}^{CGS} and N^{CGS} caused by quinidine. Note constancy of N^{CGS} of quinidine-treated tissue.

TABLE VII

Changes of i_{Na}^{CGS} and N^{CGS} Caused by Quinine and Quinidine

	i_{Na}^{CGS}			N^{CGS}		
Quinine studies	Control	Quinine	Quinine/control	Control	Quinine	Quinine/control
	pA	*pA*		*millions/cm²*	*millions/cm²*	
	0.556	0.159	0.286	26.1	63.3	2.425
	0.569	0.177	0.311	27.4	45.4	1.657
	0.262	0.111	0.424	68.6	109.0	1.589
	0.257	0.117	0.455	104.1	159.4	1.531
	0.287	0.114	0.397	70.7	103.1	1.458
	0.295	0.125	0.424	82.1	110.8	1.350
	0.294	0.132	0.449	45.2	46.4	1.027
Mean ± SEM (7)	0.360±0.053	0.134±0.010	0.392±0.025	60.6±11.0	91.1±15.7	1.577±0.162
Quinidine studies	Control	Quinidine	Quinidine/control	Control	Quinidine	Quinidine/Control
	0.242	0.082	0.339	103.4	126.9	1.227
	0.327	0.110	0.336	79.9	119.3	1.493
	0.261	0.086	0.330	107.0	153.4	1.434
	0.523	0.113	0.216	31.1	66.7	2.145
	0.253	0.064	0.253	95.1	147.8	1.554
Mean ± SEM (5)	0.321±0.053	0.091±0.009	0.295±0.025	83.3±13.9	122.8±15.4	1.571±0.154

in Table VII for tissues treated with either quinine or quinidine. Quinine caused the i_{Na} to decrease on average to 39.2 ± 2.5% of its control value of 0.360 ± 0.053 pA, whereas quinidine in group I tissues caused i_{Na} to decrease to 29.5 ± 2.5% of its control value of 0.321 ± 0.053 pA. Correspondingly, the Na^+ channel densities of both groups of tissues were increased on the average to 157% of their control values of 60.6 ± 11.0 and 83.3 ± 13.9 million/cm^2, respectively. These estimates of Na^+ channel density are in the range of those measured in the amiloride-induced noise studies reported above. As can also be seen in Table VII, the increase of N caused by quinine or quinidine was highly variable, ranging from 102.7 to 242.5% of control. In view of the previous electrophysiological studies, the variability in increase of N probably reflects a tissue-to-tissue variability in response to quinine and quinidine.

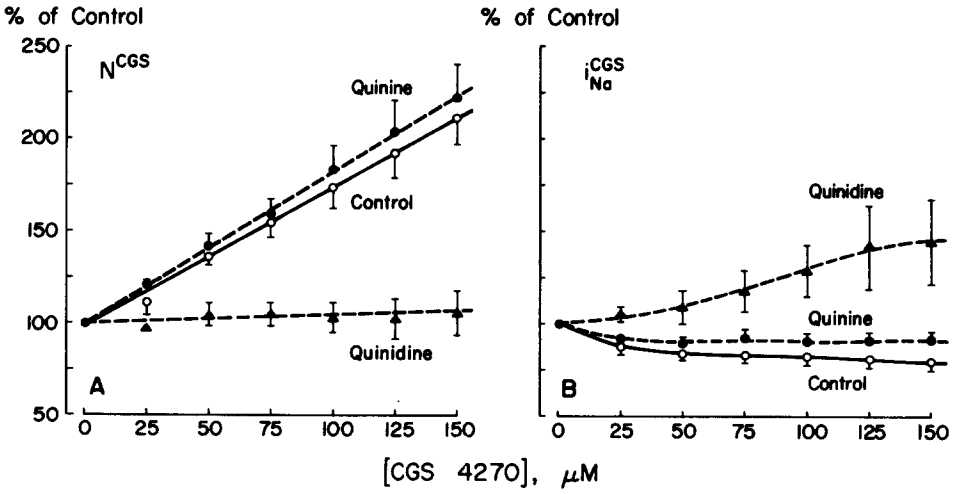

FIGURE 15. Changes of N^{CGS} and i_{Na}^{CGS} as a percent of control (zero CGS 4270). Note increases of N^{CGS} of control and quinine-treated tissues (autoregulation) and its absence in tissues treated with quinidine.

The CGS 4270–dependent increases of N^{CGS} and the changes of i_{Na}^{CGS} are shown in Fig. 15, where the data are plotted as a percent of the control values given in Table VII. In control and quinine-treated tissues, N was increased to 212.0 ± 15.1 and 222.1 ± 18.1% of control at 150 μM CGS 4270, at a time when the I_{sc} was reduced to 65.0 ± 3.0 and 78.4 ± 4.3% of control, respectively. Interestingly, in the quinidine-treated group I tissues, N remained unchanged with inhibition of Na^+ entry by CGS 4270. The changes of i_{Na}^{CGS} were relatively small compared with the changes of N^{CGS}. In control and quinine-treated tissues, i_{Na}^{CGS} was decreased from control by ~10–20%, whereas in quinidine-treated tissues, i_{Na}^{CGS} was increased by ~40% at 150 μM CGS 4270. The reason for these changes of i_{Na}^{CGS} and the difference in response in quinine- and quinidine-treated tissues is unknown and may reflect either changes of V_o^{sc} and/or changes of the single channel conductance of the Na^+ channels.

DISCUSSION

By virtue of the ability to measure single channel currents and channel densities, noise analysis of the Na^+ channels of epithelial tissues represents an important methodological advance in attempting to resolve the mechanisms by which the permeability to Na^+ of the apical membranes of cells is regulated. Indeed, it is known that transepithelial Na^+ transport can vary markedly among individuals of a population of skins, and various drugs and hormones alter the spontaneous rates of Na^+ transport. There is much compelling evidence to indicate that apical membrane permeability to Na^+ varies markedly, and this variability of P_{Na} plays a determinant role in transepithelial Na^+ transport.

It is also clear that the basolateral membranes of the cells play a major role in the regulation of transepithelial Na^+ transport. Indeed, by virtue of the electrical coupling of apical and basolateral membrane voltages, changes of basolateral membrane ionic permeability or conductance can alter apical membrane voltage so as to change the electrochemical potential difference for entry of Na^+ into the cells at their apical membranes (for review, see Helman, 1979). In this regard, quinine and quinidine cause a marked depolarization of the apical membrane voltage through changes of basolateral membrane ionic permeability (Abramcheck, 1984). Therefore, inhibition of apical membrane electrodiffusive Na^+ entry would be expected to occur independently of any possible alteration of the P_{Na} of the apical membranes of the cells and can be explained by a mere change of membrane voltage. Although measurements of the slope resistance of the apical membranes had indicated a highly variable, but relatively small, if any, consistent response to quinine and quinidine, it was not possible from the electrophysiological studies to include or exclude changes of Na^+ channel density and/or changes of single channel conductance. Thus, the present studies were initiated to attempt to measure the i_{Na} and N of the apical membrane of the cells in control and drug-treated states of the tissue.

Following the methods of Lindemann and Van Driessche (1977), we used amiloride-induced noise analysis. However, inspection of the data revealed that channel density varied with the amiloride concentrations used to induce the Na^+ channel fluctuations. Although the single channel currents could be measured with reasonable certainty (method A), and decreases of i_{Na} by quinine and quinidine were observed as expected, it was not possible, because of the uncertainty of measurement of the dissociation rate constant k_{10}^A, to obtain estimates of the channel densities of individual tissues. We attempted to use tissue population mean values to measure $\overline{K_{1/2}^A}$ so as to factor out small random errors in the measurement of f_c^A. However, given the steep slope of the corner frequency plots and the low values of k_{10}^A, it was not possible to obtain reasonable confidence intervals of the values of k_{10}^A. Thus, we turned to an alternative criterion, which required that the mean i_{Na}^A of individual epithelia (method A) be the same as the $\overline{i_{Na}^A}$ (method B). We assumed that if i_{Na}^A was the same or nearly the same as $\overline{i_{Na}^A}$, $\overline{N^A}$ could be calculated using $\overline{K_{1/2}^A}$. We found that despite relatively small or no decreases of i_{Na}^A in control and quinine- or quinidine-treated tissues, the Na^+ channel density increased enormously with inhibition of Na^+ entry by amiloride. Although such a finding may have been fortuitous, similar observations were

made again with CGS 4270–induced noise, where the uncertainties encountered with amiloride were circumvented. Because the Na^+ channel densities varied with increasing amiloride concentration, and because of the nature of the assumptions required for the analysis, it was not possible to determine whether quinine or quinidine caused significant changes of Na^+ channel density.

Because of the problems encountered with amiloride, we turned to studies with the inhibitor CGS 4270. In contrast to amiloride, this weak Na^+ channel inhibitor permitted studies to be done with a relatively small inhibition of the I_{sc}, yielding power density spectra containing a Lorentzian component with corner frequencies in the range of 50–120 Hz at [CGS 4270] between 50 and 150 μM. The linearity of the corner frequency plots allowed the microscopic rate constants to be measured with reasonable certainty. Hence i_{Na}^{CGS} and N^{CGS} were calculated for each epithelium and, as in the amiloride studies, the N^{CGS} was observed to increase markedly with inhibition of Na^+ entry, particularly in the control and quinine-treated tissues. This "autoregulation" occurred despite the constancy of the apical solution concentrations of Na^+, K^+, Ca^{++}, and pH, and thus the mode of sensing the changes of the rate of Na^+ entry probably occurs within the cells with the feedback response mediated within the cytosol (see below). How this occurs is unknown, but, given the magnitudes of change of N, it is clear that this type of regulation is a major factor in the adjustment of P_{Na} of the apical cellular membranes.

From the changes of i_{Na} and N estimated at zero [CGS 4270], we concluded that both quinine and quinidine cause a highly variable increase of the Na^+ channel density and hence P_{Na}. To the extent that Na^+ entry is reduced by these drugs, it may be, at least in part, that the increase of N occurs secondarily to the decreases of the rate of Na^+ entry into the cells. Thus, it remains unclear whether quinine or quinidine, acting purportedly through increases of cytosolic Ca^{++}, inhibits P_{Na} in the absence of a reduced rate of Na^+ entry. To the extent that Na^+ entry is reduced by depolarization of V_o^{sc}, the increases of N caused by autoregulation may overcompensate the decreases of N caused by increased cytosolic Ca^{++} activity so that P_{Na} appears either to remain unchanged or to be increased.

Stimulation of transepithelial Na^+ transport by various agents, including amiloride at very low concentrations, has been reported (Cuthbert and Maetz, 1972; Garcia-Romeu, 1974; Zeiske and Lindemann, 1974; Li and de Sousa, 1979; Benos, 1982; Thurman and Higgins, 1982; Li and Lindemann, 1983). Whether this is due to direct effects of these agents at the apical membrane or to a secondary effect of autoregulation acting via inhibition of Na^+ entry is unclear. It is certainly possible that a small inhibition of Na^+ entry sets into effect a feedback mechanism that overcompensates for inhibition of Na^+ entry, which results in an increase of P_{Na} with a net stimulation of Na^+ entry.

There has also been considerable debate over the nature of the Na^+ entry site and the mechanism of interaction, if any, of amiloride and Na^+ on the process of Na^+ entry into the cells. Since Kirschner's (1955) observation of the Na^+ saturability of Na^+ entry, various schemes have been proposed to explain the nonlinear relationship between apical solution [Na^+] and the rate of Na^+ entry

(see reviews by Schultz, 1981; Lindemann, 1984). The action of amiloride has been investigated extensively, especially with regard to its possible interaction with Na^+. It has been concluded at various times that amiloride and Na^+ in various tissues behave either as competitive or noncompetitive inhibitors of the Na^+ channels (see review by Benos, 1982). To our knowledge, however, the range of [A] over which this interaction has been investigated has been limited to high [A], greater than the apparent $K^A_{1/2}$. In our laboratory, we have not been able to fit [A]-I_{sc} data to any linearization of the Michaelis-Menten equation over ranges of [A] that encompass $K^A_{1/2}$, especially at [A] < $K^A_{1/2}$ (unpublished observations). We now believe that our difficulties with this are most likely attributable to autoregulation. Because of changes of Na^+ channel density with changing [A], the fundamental assumption of constancy of P_{Na} is violated. In this regard, it would be difficult at best to decide whether Na^+ and amiloride are in fact competitive or noncompetitive inhibitors of the Na^+ entry process. Thus, it remains to be proven whether or not Na^+ acts as an inhibitor of the Na^+ channels. Self-inhibition by Na^+ of the Na^+ channels has been suggested as a modulator of the P_{Na} (see review by Lindemann, 1984), but there appears to be no direct evidence to support this idea to the exclusion of other mechanisms mediated via alterations of the cytosolic environment. Thus, the hypotheses of Na^+ self-inhibition or autoregulation can equally well be invoked to explain changes of apparent channel density. However, it is unlikely that the results of the present studies can be ascribed to Na^+ self-inhibition of Na^+ entry. In this regard, we observed consistently the "scalloped" appearance of the I_{sc} records, which, according to our analysis, was due to increasing Na^+ channel density. Moreover, upon washout of CGS 4270 from the apical solution, I_{sc} overshot its control value and returned slowly (10–20 min) toward its original control value. Such time-dependent phenomena would not be expected if Na^+ self-inhibition alone were responsible for the apparent changes of Na^+ channel density. In more recent studies with amiloride, similar time-dependent changes of the I_{sc} records have been observed, and we presume that, as with CGS 4270, the transient increases of the I_{sc} are due to a secondary increase of Na^+ channel density.

There has been considerable discussion recently about the insertion (and removal) of vesicles into apical membranes of epithelial cells where subapical vesicles may represent storage sites for ion-specific channels (see, for example, Minsky and Chlapowski, 1978; Wade et al., 1981; Gluck et al., 1982; Lewis and de Moura, 1982; Pumplin and Fambrough, 1982; Loo et al., 1983; Stetson and Steinmetz, 1983). It is enticing to speculate that such stored channels are made available to the apical membranes when Na^+ entry is reduced, as in the present studies, subserving in part an autoregulatory mechanism of Na^+ entry and thus transepithelial Na^+ transport.

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