Sodium Flux Ratio through the Amiloride-sensitive Entry Pathway in Frog Skin

DALE J. BENOS, BARBARA A. HYDE, and RAMON LATORRE

From the Department of Physiology and Biophysics, Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT The sodium flux ratio of the amiloride-sensitive Na^+ channel in the apical membrane of in vitro *Rana catesbeiana* skin has been evaluated at different sodium concentrations and membrane potentials in sulfate Ringer solution. Amiloride-sensitive unidirectional influxes and effluxes were determined as the difference between bidirectional ^{22}Na and ^{24}Na fluxes simultaneously measured in the absence and presence of 10^{-4} M amiloride in the external bathing solution. Amiloride-sensitive Na^+ effluxes were induced by incorporation of cation-selective ionophores (amphotericin B or nystatin) into the normally Na^+-impermeable basolateral membrane. Apical membrane potentials (V_a) were measured with intracellular microelectrodes. We conclude that since the flux ratio exponent, n', is very close to 1, sodium movement through this channel can be explained by a free-diffusion model in which ions move independently. This result, however, does not necessarily preclude the possibility that this transport channel may contain one or more ion binding sites.

INTRODUCTION

Net sodium movement across the apical membrane of most electrically high resistance epithelia appears to be driven by the electrochemical potential gradient (Nagel, 1976; Helman and Fisher, 1977). This particular transport pathway is rate-limiting for the transport of Na^+ across the entire epithelium and is specifically inhibited by the diuretic drug amiloride (see Macknight et al., 1980, for a recent review). Single-site ion turnover numbers deduced from noise experiments are consistent with a channel- or pore-type mechanism (Lindemann and Van Driessche, 1977). Fuchs et al. (1977) and Van Driessche and Lindemann (1979) found that under their experimental conditions, Na^+ permeation through these channels could be adequately described by an electrodiffusion model in which the passive movement of Na^+ obeys independence.

Address reprint requests to Dr. D. J. Benos, Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, 45 Shattuck St., Boston, MA 02115. Dr. Hyde's present address is Dept. of Animal Science, University of Massachusetts, Amherst, MA.

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One necessary criterion of independent ion movement is that the relationship between unidirectional Na^+ efflux, J_o, and influx, J_i, obey the Ussing flux ratio equation (Ussing, 1949):

$$\frac{J_o}{J_i} = \frac{[Na]_i}{[Na]_o} \exp(V_a F/RT) = \exp[(V_a - V_{Na})F/RT], \quad (1)$$

where $[Na]_i$ and $[Na]_o$ are the intracellular and extracellular ion activities of Na^+, V_a is the apical membrane potential, V_{Na} is the sodium equilibrium potential, and R, T, and F have their usual meanings. Ussing and Zerahn (1951) first proposed that the ratio of the unidirectional fluxes in the active Na^+ transport pathways provided a measure of the effective driving force of the sodium pump in frog skin. However, the transmural flux ratio cannot really be used to conclude much about the apical Na^+ entry pathway since a significant fraction of these fluxes may traverse the skin via paracellular shunts (Fromter and Diamond, 1972; Helman and Miller, 1974). In fact, because of the relatively high degree of basolateral membrane impermeability to Na^+ (Koefoed-Johnson and Ussing, 1958; Labarca et al., 1977), the very small intracellular Na^+ transport compartment (Macknight and Leaf, 1978; Turnheim et al., 1978), and the magnitude of the electrochemical gradient across the apical membrane favoring Na^+ entry, a flux ratio analysis through amiloride-sensitive Na^+ sites is not an easy task. Palmer (1982) found a flux ratio exponent (n') equal to 1 in toad urinary bladder by using an expression derived by Hodgkin and Keynes (1955) that relates sodium conductances and fluxes at the equilibrium potential for Na^+. Inasmuch as n' was determined only at two sodium concentrations and a single membrane potential, data for other electrochemical potential gradients are needed.

The direction of net sodium movement across frog skin is, under most experimental conditions, from the outside to the inside. Flux measurements reveal a virtual identity between Na^+ influx across the apical membrane, net transepithelial Na^+ influx, and the short-circuit current (I_{sc}) in tight epithelia like frog skin, toad and rabbit colon, and toad urinary bladder (Macknight et al., 1980). Under most circumstances, amiloride, when added to the outside bathing solution, does not inhibit the efflux of Na^+ through the frog skin (Morel and Leblanc, 1975; Leblanc and Morel, 1975; O'Neil and Helman, 1976). On the other hand, Biber and Mullen (1976, 1977) found that the unidirectional Na^+ efflux exhibited saturation kinetics, was increased by ouabain, and was inhibited by amiloride at low $[Na^+]_i$. Wolff and Essig (1977), Dawson and Al-Awqati (1978), and Palmer et al. (1980) have also provided evidence that Na^+ can move across the apical membrane bidirectionally in toad urinary bladder under appropriate conditions.

In this paper we report a flux ratio analysis for the amiloride-sensitive Na^+ entry pathway in bulifrog skin epithelium. Our results show that the flux ratio of sodium flow through these apical membrane channels is very close to that predicted by Eq. 1 under a wide range of experimental conditions.

A preliminary account of this work has been published (Benos and Latorre, 1982).

METHODS

These experiments were done on abdominal skin obtained from the bullfrog, *Rana catesbeiana* (West Jersey Biological, Wenonah, NJ). Voltage-clamp techniques and chamber arrangement have been described in detail (Benos et al., 1979, 1980). Skins were initially symmetrically bathed with a chloride-Ringer solution containing 110 mM NaCl, 1.0 mM $CaCl_2$, 2.5 mM KCl, and 5.0 mM HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid) buffered to pH 7.4. After the transepithelial zero-current potential (V_T) and short-circuit current (I_{sc}) reached steady state (30–60 min), the serosal solution was replaced with a high-potassium sulfate Ringer containing either 5 μg/ml amphotericin B or 200 μg/ml nystatin. The compositions of the Ringer solutions used were as follows (mM): for the outside, 55 Na_2SO_4, 1.25 K_2SO_4, 1 $CaSO_4$, 55 mannitol, 5 HEPES, pH 7.4; for the inside, 5 Na_2SO_4, 50 K_2SO_4, 1 $CaSO_4$, 60 mannitol, 5 HEPES, pH 7.4. In some experiments, a chloride solution of the following composition was used for the inside solution (mM): 102.5 KCl, 10 NaCl, 1 $CaCl_2$, 5 HEPES, pH 7.4. An additional 45–60-min equilibration period was allowed before beginning the experiment. All experiments were conducted at room temperature (21°C). All chemicals were reagent grade. Amphotericin B and nystatin were obtained from Sigma Chemical Co. (St. Louis, MO) and Calbiochem-Behring Corp. (La Jolla, CA), respectively. Stock solutions of amphotericin B were made up in ethanol; those of nystatin were made up in dimethylsulfoxide (DMSO). The final concentrations of ethanol and DMSO themselves had no effect on either the electrical parameters or the radiotracer fluxes measured in this system. These solutions were made fresh on the morning of the experiment.

As substitution of KCl for NaCl in the serosal solution produces cell swelling (MacRobbie and Ussing, 1961; Palmer et al., 1980), experiments were performed to evaluate the influence of cell volume on the observed unidirectional influxes and effluxes of sodium. Fluxes were performed and compared in eight skins whose inner surfaces were bathed with a solution consisting of either 10 mM NaCl, 85 mM KCl, 30 mM sucrose, 1 mM $CaCl_2$, 5 mM HEPES, and 200 μg/ml nystatin, or a solution in which all of the chloride was replaced with sulfate (using mannitol to maintain osmolality). Sulfate Ringer is normally used to avoid cell swelling. The unidirectional $^{22}Na^+$ influxes and effluxes determined under either condition were statistically indistinguishable from the values reported in this paper. We conclude, therefore, that variations in cell volume do not affect the amiloride-sensitive Na^+ fluxes in this preparation.

Unidirectional $^{22}Na^+$ Flux Experiments

Depending upon whether a transepithelial influx or efflux experiment was to be performed, 3 μCi of ^{22}NaCl (New England Nuclear, Boston, MA) (final concentration of radioactivity, 0.25 μCi/ml) was added to the solution bathing one side of the skin (defined as the *cis* side). After a 30-min equilibration period under voltage-clamp conditions (see Tables II and III), 1-ml samples were withdrawn from the *trans* solution at 20-min intervals and replaced with 1 ml of nonradioactive solution. Two 10-μl samples were obtained from the *cis* solution, and fluxes were computed from the following equation:

$$J_{Na} = \frac{\Delta cpm}{(\text{area})\ (\text{time})\ (SA_c - SA_t^{t1/2})}, \quad (2)$$

where Δcpm are the counts per of $^{22}Na^+$ accumulated in the *trans* compartment during the flux period, SA_c is the specific activity of Na^+ in the *cis* compartment (in counts

per minute per mole Na^+), and $SA_t^{t1/2}$ is the specific activity of Na^+ in the *trans* compartment at the midpoint of the flux period. Fluxes were measured and averaged for at least three sequential flux periods per condition on the same piece of skin. Amiloride-sensitive fluxes were calculated as the difference between the flux measured in the absence and presence of 10^{-4} M amiloride in the outside bathing solution. Opposite unidirectional $^{22}Na^+$ effluxes were performed on different frog skins. Fluxes were performed on at least nine separate skins under each experimental condition.

In most experiments, however, the unidirectional fluxes of Na^+ across the frog skin epithelium were measured simultaneously, using ^{22}Na for the efflux and ^{24}Na (New England Nuclear) for the influx. The protocol for these double-label experiments was essentially the same as that for the single-label fluxes, except that 0.5-ml samples were taken from both solutions at every time point. These samples were immediately counted for ^{22}Na and ^{24}Na in a Packard Auto-Gamma 500c counter (Packard Instrument Co., Downers Grove, IL). These same samples were counted again after 2 wk (22.4 half-lives of ^{24}Na) to obtain only the ^{22}Na counts. Between 6 and 20 different frog skins were used under each experimental condition.

Electrical Measurements

The techniques for microelectrode manufacture and recording were essentially identical to those reported previously by Nagel (1976) and Helman and Fisher (1977). Briefly, microelectrodes (1.2 mm OD) were fashioned from fiber-filled borosilicate glass tubing and filled with 3 M KCl immediately before use. The tip resistance ranged from 10 to 40 MΩ when the microelectrode was immersed in the standard Na_2SO_4 Ringer solution. Electrical contact between the fluid in the microelectrode and one input of the potential-measuring amplifier was made with an Ag-AgCl wire. This intracellular potential-measuring circuit was completed via another Ag-AgCl wire immersed in 3 M KCl and connected to the solution bathing the external surface of the frog skin with a 3% agar-Ringer bridge. All potential measurements were referenced to the outside bathing solution. Microelectrode potential asymmetry (always <5 mV) was nulled using a voltage reference source. This nulled offset was always checked before and after a cell puncture and was found never to vary by more than ±3 mV even after the microelectrode was inside the cell for 4 or 5 h. The frog skin was transepithelially voltage-clamped as described above. All output signals were fed to a strip-chart recorder and to digital panel meters or an oscilloscope. Pulse commands of voltage could be superimposed upon baseline clamp potentials to permit steady-state values of voltage and current to be read 600 ms after command onset. The input stage of the microelectrode circuit had an impedance of 10^{13} Ω (AD 545; Analog Devices, Norwood, MA).

Bullfrog skins (exposed area = 0.72 cm^2) were mounted horizontally in a chamber designed to minimize edge damage. The outer surface of the tissue was oriented upward, and the inner surface of the skin was scraped with a scalpel to remove the bulk of the corial layer. The skin was transepithelially voltage-clamped to 0 mV, and a cell was impaled perpendicular to the skin surface by advancing the microelectrode directly from above. The outer and inner compartments (~0.4 ml each) were continuously perfused by gravity with normal Ringer solution equilibrated with 100% O_2 at rates of 5–10 and 2–3 ml/min, respectively. After a cell was punctured and a stable membrane potential was recorded, the inner solution was changed to one containing 10 mM Na^+, 100 mM K^+, and either 5 μg/ml amphotericin B or 200 μg/ml nystatin, all other components remaining as indicated earlier. An additional 45-min equilibration period ensued. After this, apical membrane potentials were recorded

under different experimental conditions. At the end of an experiment, the bathing solutions were returned to 110 mM Na^+ on both sides to test reversibility.

Data Analysis

Unless specified, all results are expressed as the mean value plus or minus one standard error of the mean. The statistical probability of significance between two population means was computed by the Student's t test if the population variances were the same; otherwise, the Fischer-Behrens test for significance was applied (Armitage, 1971). The variance of the ratio of the independently measured one-way Na^+ fluxes was computed according to standard statistical methods, assuming the distributions of fluxes were normal and that the deviations between the individual flux measurements and the mean value were small (Armitage, 1971; Boas, 1966). The flux ratio exponent, n' (Hodgkin and Keynes, 1955), was calculated from:

$$n' = \frac{RT}{(V_a - V_{Na})F} \ln(J^o_{Na}/J^i_{Na}). \quad (3)$$

Assumptions and Experimental Rationale

Most of our knowledge concerning the molecular characteristics of the amiloride-sensitive Na^+ entry channel has been derived from current fluctuation measurements made under conditions of elevated serosal potassium (Lindemann and Van Driessche, 1977; Van Driessche and Lindemann, 1979). Hence, we wanted to perform our experiments under comparable conditions. The most crucial problem in terms of data interpretation involves the assumptions made concerning the Na^+ electrochemical potential gradient across the apical membrane. Fisher and Helman (1981) reported that elevated serosal [K^+] does not result in an equivalence between transepithelial potential difference and the apical membrane potential, V_a, in chloride Ringer solution. Consequently, microelectrode measurements of V_a under all experimental conditions are essential.

We will assume in our analysis that the intracellular [Na^+] is equal to 10 mM under all experimental conditions. In other words, we are assuming that high serosal potassium together with the addition of cation-selective ionophores to the serosal side effectively eliminate the resistance of the basolateral membrane (see, for example, Fuchs et al., 1977; Palmer et al., 1980; Thompson et al., 1982). We argue that this assumption is reasonable in high serosal K^+ *sulfate* Ringer because the apical membrane potential is essentially equal to the transepithelial potential under all experimental conditions, and the steady-state voltage-divider ratio is 1, which indicates that the basolateral membrane resistance is essentially 0. Furthermore, an $[Na^+]_i$ = 10 mM is very close to the measured value of $[Na^+]_i$ (Rick et al., 1978; Nagel et al., 1981). However, the situation when Cl^- Ringer is used is not as straightforward as is the sulfate Ringer case (see Table II). First, we have always found a V_a of approximately −20 mV under high serosal K^+ conditions (see Table II). Thus, V_a is not equal to V_T, which indicates that in Cl^- Ringer there is significant basolateral resistance. When frog skins are bathed with symmetrical solutions of 110 mM NaCl Ringer, the voltage-divider ratio averages between 0.7 and 0.8. After increasing serosal [KCl] to 100 mM, this ratio first rises to 0.99 within 5 min and thereafter falls to 0.5 within 60 min, which suggests that the resistance of the apical membrane has become equal to that of the basolateral membrane. It is not apparent to us what the reasons are for the differences between $SO_4^=$ and Cl^- Ringer. One possible explanation may be that increasing the serosal KCl concentration not only decreases the basolateral resistance

but also induces a conductance in the apical membrane and/or shunt pathway (see Discussion and Table IV). The Na^+ flux ratio obtained from isotope measurements will therefore be interpreted only quantitatively for the more straightforward sulfate Ringer experimental conditions.

The final assumption we make is that 10^{-4} M amiloride inhibits only the Na^+-specific apical membrane channels and has no effect on parallel shunt pathways. Experimental support for the validity of this assumption is twofold. First, amiloride had no effect on unidirectional $^{22}Na^+$ efflux in the absence of serosally added ionophore (see Fig. 2), even if external [Na] is increased to 300 mM, a condition that

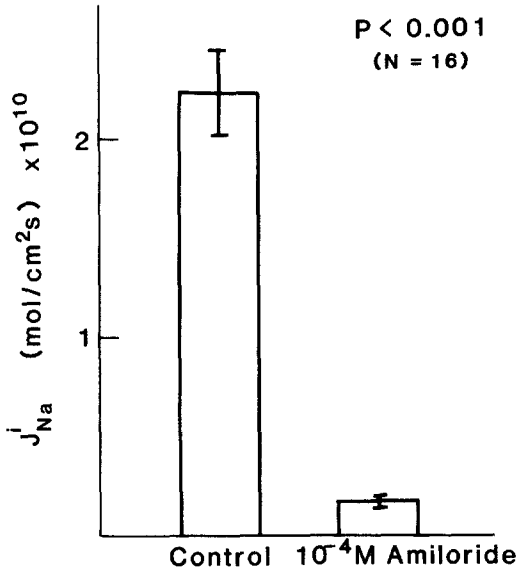

FIGURE 1. The effect of 10^{-4} M amiloride on J^i_{Na} in bullfrog skin epithelia treated with amphotericin B (5 μg/ml) or nystatin (200 μg/ml) and bathed in sulfate Ringer solutions. Ionophores were added to the serosal solution (high K^+ plus 10 mM Na^+ Ringer), the external [Na^+] was 110 mM, and the transepithelial potential difference was voltage-clamped to 0 mV. Similar results are obtained using chloride Ringer solutions.

greatly increases shunt pathway permeability. Second, no change in shunt resistance (estimated from an electrical equivalent circuit model of the frog skin epithelium) was noted after the addition of amiloride in the microelectrode experiments reported below.

RESULTS

Nystatin and Amphotericin B Induce an Amiloride-sensitive Na^+ Efflux

Fig. 1 shows the results of the measurement of the amiloride-sensitive component of Na^+ influx (J^i_{Na}) under conditions of 110 mM external Na^+ and with the basolateral membrane depolarized with 100 mM K^+ in the presence of amphotericin B or nystatin. Amiloride (10^{-4} M) decreased J^i_{Na} by 92%.

This inhibition is comparable to that produced by this concentration of drug in the absence of ionophore and when the serosal bathing solution contains high Na^+ and low K^+. Similar experiments have been performed in frog skins whose external surfaces have been bathed with solutions of different [Na^+] and/or skins transepithelially voltage-clamped to different membrane potentials. Hence, serosal inclusion of amphotericin B or nystatin does not induce changes in the amiloride sensitivity or in the absolute magnitude of Na^+ influx across in vitro bullfrog skin epithelium.

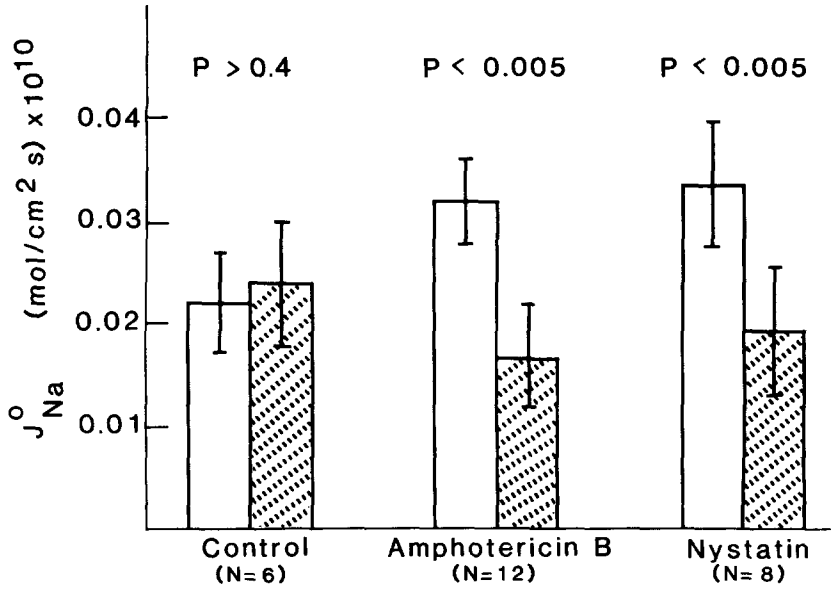

FIGURE 2. The effect of 10^{-4} M amiloride on sodium efflux in amphotericin B- or nystatin-treated bullfrog skin epithelia. The experimental conditions were the same as indicated in the legend to Fig. 1. Hatched bars represent data obtained in the presence of 10^{-4} M amiloride in the outer bathing solution.

The basolateral membrane impermeability to Na^+ has until now prevented flux ratio measurements of the amiloride-sensitive Na^+ channel in epithelia, but not measurements of n' (see Palmer, 1982). As shown below, the basolateral membrane permeability to Na^+ can be increased by incorporation of either nystatin or amphotericin B in this membrane. This allowed us to measure an amiloride-sensitive efflux of Na^+ through the apical membrane. Fig. 2 shows the results of experiments in which $^{22}Na^+$ efflux was measured in the presence and in the absence of 10^{-4} M amiloride and in the absence (control) and in the presence of amphotericin B or nystatin. It is evident that in the absence of added ionophore, there is no amiloride-sensitive component to $^{22}Na^+$ efflux. However, when either ionophore is present, a significant ($P < 0.005$) amiloride inhibition of Na^+ efflux can be measured. The magnitude of the amiloride-sensitive J^i_{Na} was 1.5 ± 0.4 and 1.4 ± 0.3 pmol cm^{-2} s^{-1} in the case of

amphotericin B or nystatin treatment, respectively. Fig. 3 summarizes a series of experiments designed to determine the dose dependency of the nystatin-induced, amiloride-sensitive J^{o}_{Na}. In this figure, the percent of J^{o}_{Na} inhibited by amiloride is plotted vs. serosal nystatin concentration. At nystatin concentrations of 50, 100, and 200 μg/ml, there was a significant ($P < 0.005$) amiloride-sensitive component to J^{o}_{Na}. The maximal effect of nystatin was attained at 100 μg/ml.

Additional experiments were designed to test further the idea that these ionophores do in fact increase basolateral membrane permeability to sodium.

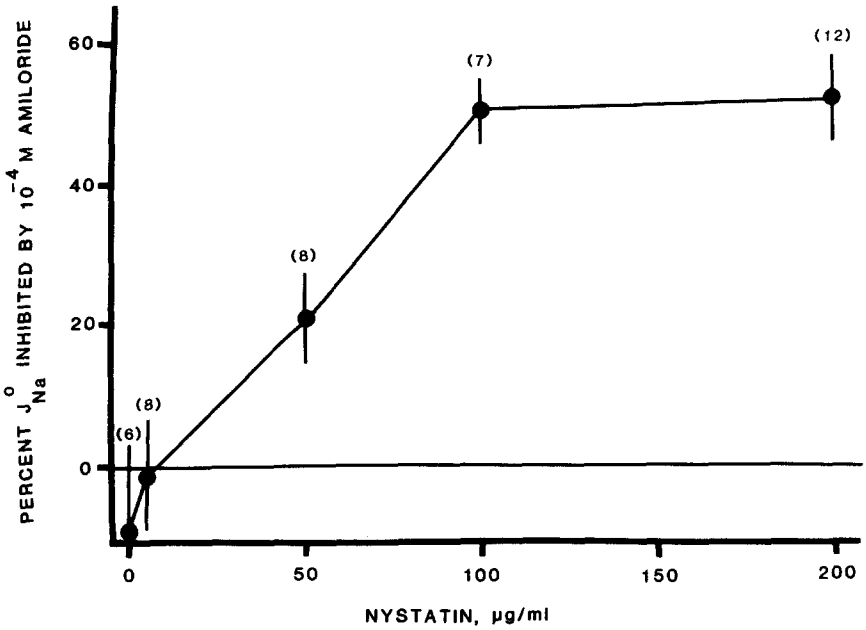

FIGURE 3. The effect of different concentrations of nystatin on amiloride-sensitive ^{22}Na efflux from high serosal potassium-treated bullfrog skin epithelia. The points represent the mean value of N experiments and the vertical lines represent 1 SEM. These experiments were performed in sulfate Ringer solution. Similar curves were measured under comparable conditions in chloride-containing solutions.

The distribution space of ^{22}Na within the frog skin epithelium was measured and compared with that of [^{3}H]mannitol in the presence and absence of serosally added nystatin. Mannitol is a known extracellular space marker in frog skin (Biber et al., 1972; Cala et al., 1978). These experiments were performed in corial-scraped pieces of frog skin; both isotopes were present in the serosal solution. These experiments were performed essentially as described earlier (Benos et al., 1980), the only differences being that a rapid-uptake chamber was used and equilibration time was reduced from 15 min to either 1 or 5 min. In the absence of nystatin, the distribution space of $^{22}Na^{+}$ was 28.0

± 2.6% (N = 15) greater than that of [^{3}H]mannitol. However, after pre-equilibrating the frog skin for 45 min in the presence of 200 μg/ml nystatin in the serosal solution, the ^{22}Na distribution space exceeded the [^{3}H]mannitol space by 66.7 ± 8.3% (N = 14). These results demonstrate that nystatin significantly ($P < 0.001$) increases the distribution of ^{22}Na within the epithelium (and presumably the transport compartment) of the frog skin.

Summary of Data

Table I presents a compilation of all simultaneous influx and efflux experiments performed when $[Na^+]_o$ was 110 mM and when the skins were transepithelially voltage-clamped to 0 mV. These data are presented to indicate the extent of skin-to-skin variation in the degree to which amiloride inhibits

TABLE I

AMILORIDE-SENSITIVE UNIDIRECTIONAL SODIUM FLUXES IN NYSTATIN-TREATED BULLFROG SKINS

	Amiloride sensitive fluxes				
Number	J^i_{Na}	J^s_{Na}	J_i/J_o	$\ln(J_i/J_o)$	n'
	mol cm^{-2} s^{-1} × 10^{11}				
1	9.10	1.00	9.09	2.207	0.92
2	11.9	0.52	22.8	3.125	1.30
3	9.60	0.60	15.9	2.769	1.15
4	3.61	0.16	22.7	3.122	1.30
5	7.27	0.63	11.5	2.446	1.02
6	1.87	0.14	13.4	2.599	1.08
7	1.50	0.28	5.36	1.680	0.70
8	8.00	0.80	10.0	2.300	0.96
9	13.6	0.91	15.0	2.707	1.13
10	15.7	0.94	16.8	2.819	1.18
11	8.83	0.68	13.0	2.564	1.11
Mean	8.27(±1.38)	0.60(±0.09)	14.14(±1.62)	2.576(±0.126)	1.08±0.05

$[Na^+]_o$ = 110 mM and V_T = 0 mV (sulfate Ringer).

each unidirectional flux. Each flux across a single tissue represents the average of three separate determinations; the variability between flux periods within the same animal was small (within 15% of the reported mean value). As expected, we found some difference in the absolute values of the fluxes between different frog skins. For example, the coefficients of variation for the amiloride-sensitive influxes and effluxes shown in Table I were 55.3 and 49.7%, respectively. Therefore, to obtain reliable estimates of the flux ratio through the Na^+ entry channel, all analyses were done from measurements of bidirectional fluxes performed simultaneously on the same piece of tissue.

Tables II and III summarize the results of all the flux data measured under the different experimental conditions tested. The potential difference across the apical membrane averaged −104.5 ± 3.1 mV (range: −83.0 to −129.0

mV; $N = 18$) when the frog skin was bathed with symmetrical NaCl solutions, and -91.3 ± 5.2 (range: -78.0 to -111.0 mV; $N = 6$) in sulfate Ringer. When the serosal solution was changed to one containing high potassium plus

TABLE II

FLUX RATIO ANALYSIS OF Na^+ MOVEMENT THROUGH AMILORIDE-SENSITIVE CHANNELS IN K^+-DEPOLARIZED BULLFROG SKIN EPITHELIUM (CHLORIDE MEDIUM)

$[Na]_o$	Clamp potential	Apical membrane potential	Transepithelial slope conductance	Amiloride-sensitive fluxes		$\ln(J_i/J_o)$
				J^i_{Na}	J^o_{Na}	
mM	*mV*	*mV*	*mS/cm²*	*mol cm⁻² s⁻¹ × 10¹⁰*		
3	0	−22	1.820±0.250	0.365±0.044	0.159±0.030	0.831±0.13
3	+50	+17	1.496±0.189	0.182±0.031	0.119±0.034	0.425±0.11
3	−50	−59	1.859±0.391	0.91±0.13	0.017±0.0045	3.98±0.23
10	0	−20	1.719±0.195	1.23±0.03	0.065±0.030	2.94±0.16
10 (double label)	0	−21	1.367±0.188	0.472±0.046	0.058±0.006	2.12±0.14
30	0	−22	2.074±0.239	2.07±0.36	0.53±0.022	3.66±0.40
110	0	−22	1.925±0.238	2.05±0.24	0.015±0.003	4.92±0.57
110 (double label)	0	−22	1.475±0.327	3.15±0.55	0.237±0.069	2.81±0.14
110	−50	−57	2.362±0.238	2.76±0.46	0.0082±0.0027	5.82±0.25
110	+100	+51	6.716±0.991	0.38±0.06	0.116±0.014	1.19±0.15
150	0	−23	2.181±0.308	1.24±0.33	0.041±0.005	3.40±0.17
300	0	−17	3.440±0.413	0.021±0.015	0.0005±0.0001	3.70±0.40
300	−50	−34	3.584±0.510	2.37±0.47	0.015±0.008	5.08±0.35

TABLE III

FLUX RATIO ANALYSIS OF Na^+ MOVEMENT THROUGH AMILORIDE-SENSITIVE CHANNELS IN K^+-DEPOLARIZED BULLFROG SKIN EPITHELIUM (SULFATE MEDIUM)

$[Na]_o$	Clamp potential	Transepithelial conductance	Amiloride-sensitive fluxes		$\ln(J_i/J_o)$	$\frac{F}{RT}(V_{Na} - V_a)$	n'
			J^i_{Na}	J^o_{Na}			
mM	*mV*	*mS/cm²*	*mol cm⁻² s⁻¹ × 10¹¹*				
3 (6)	0	0.197±0.039	0.169(±0.054)	0.502(±0.137)	−0.974(±0.297)	−1.205	0.81
3 (12)	+50	0.361±0.030	0.191(±0.056)	1.99(±0.36)	−2.130(±0.374)	−3.182	0.67
3 (18)	−50	0.140±0.009	2.21(±0.29)	0.65(±0.14)	1.219(±0.182)	0.772	1.56
10 (6)	0	0.308±0.057	3.46(±0.82)	2.92(±0.87)	0.185(±0.133)	0	—
30 (6)	0	0.310±0.109	4.38(±1.42)	1.34(±0.12)	0.944(±0.402)	1.099	0.86
110 (11)	0	0.460±0.014	8.27(±1.38)	0.63(±0.09)	2.576(±0.126)	2.399	1.074
110 (6)	−50	0.692±0.107	52.3(±16.6)	0.624(±0.092)	4.432(±0.197)	4.375	1.103
110 (6)	+100	1.443±0.268	0.496(±0.085)	2.00(±0.324)	−1.391(±0.212)	−1.553	0.90
150 (6)	0	0.732±0.139	6.87(±1.84)	0.374(±0.067)	2.817(±0.160)	2.708	1.06
150 (6)	+50	0.608±0.096	7.99(±1.00)	1.57(±0.59)	1.696(±0.246)	0.731	2.32
300 (20)	0	1.042±0.133	3.21(±0.32)	0.048(±0.010)	4.204(±0.214)	3.401	1.24
300 (6)	−50	1.166±0.204	1.68(±0.44)	0.009(±0.003)	5.271(±0.320)	5.375	0.98
300 (6)	+50	1.117±0.121	1.11(±0.18)	0.493(±0.146)	1.042(±0.245)	1.423	0.73

Note: V_a was measured to be within ±6 mV of the transepithelial clamp potential.
* Numbers of experiments are in parentheses.

ionophore, V_a fell to -21.7 ± 1.3 mV ($N = 4$) and -5.2 ± 0.9 mV ($N = 45$) in the case of Cl^- and SO_4 Ringer, respectively.

These data indicate that under conditions of high serosal KCl plus ionophore, V_a does not equal V_T. Furthermore, measurements of the steady-state voltage-divider ratio, i.e., the ratio of the apical membrane resistance to the total transcellular resistance in Cl^- Ringer, yield values of ~0.5. These data thus show that the apical membrane resistance constitutes only 50% of the

total transcellular resistance under these conditions. Surprisingly, the data suggest that the apical membrane is still rate-limiting for Na^+ transport in spite of a voltage-divider ratio of 0.5. Addition of 200 μg/ml nystatin to the *outside* bathing solution *after* preequilibration in high serosal KCl plus nystatin solution results in a dramatic increase in I_{sc} and transepithelial Na^+ flux (Fig. 4). If under these conditions the apical membrane were not rate-limiting for

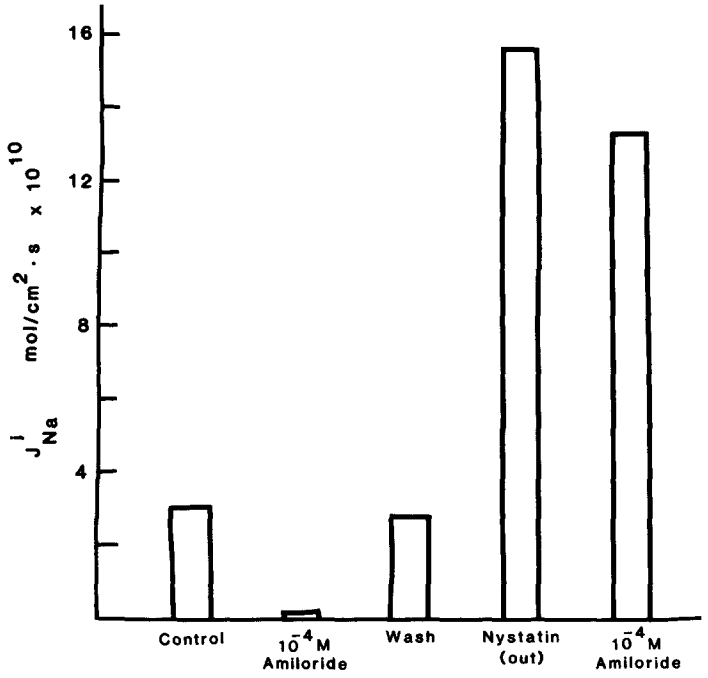

FIGURE 4. Representative experiment in which the transepithelial unidirectional ^{22}Na influx was measured across bullfrog skin bathed in chloride Ringer solution under different conditions. The frog skin was first equilibrated in high serosal $[K^+]$ plus 200 μg/ml nystatin in the serosal solution and 110 mM Na Ringer on the outside (control). The addition of 10^{-4} M amiloride externally inhibited the control flux by 95% (from 3.02 to 0.15 × 10^{-10} mol/cm^2 s). The outside surface of the frog skin was then thoroughly washed with drug-free Ringer, and J^i_{Na} was restored to near control level. Next, 200 μg/ml nystatin was added to the outside bathing solution; J^i_{Na} increased to 15.7 × 10^{-10} mol/cm^2 s. The addition of 10^{-4} M amiloride inhibited this flux by only 15.6%. Similar results have been measured when sulfate was used as the major anion.

transepithelial Na^+ influx, addition of nystatin to the apical membrane would not be expected to increase Na^+ current, contrary to what is observed. Comparable results were observed in sulfate Ringer solution (data not shown). Fig. 4 also shows that after nystatin treatment of the apical membrane, the amiloride-sensitive current has the same absolute magnitude as in the case without apical nystatin.

The measurement of a finite membrane potential in high serosal K^+-depolarized skins bathed in chloride medium is puzzling. Although microelectrode-tip potentials and resistances were comparable before and after cell puncture, we cannot exclude the development of a tip potential during impalement. We do not know why a tip potential would occur only in Cl^- medium. Nonetheless, the presence of a constant tip potential would not account for a voltage-divider ratio of <1. As pointed out by Boulpaep and Sackin (1979) and Essig (1982), the plasma membrane resistance ratio cannot be derived simply from the voltage-divider ratio if the paracellular conductance is large as compared with both the cellular and lateral interspace conductances. As shown in Table IV, high serosal [KCl] causes a much larger increase in transepithelial conductance than does elevated [K_2SO_4]. These experimental circumstances may induce a parallel ionic conductance pathway in this outer membrane. Because of the observed differences in transepithelial conductances in Cl^- and SO_4^- medium (Table IV), a Cl^--selective pathway may be a candidate. In some preliminary experiments measuring the rapid

TABLE IV

TRANSEPITHELIAL CONDUCTANCES OF BULLFROG SKIN EPITHELIUM AT 110 mM EXTERNAL SODIUM ION CONCENTRATION

Condition	Transepithelial slope conductance	
	Chloride Ringer	Sulfate Ringer
	mS/cm²	*mS/cm²*
110 mM Na^+, both sides	0.486±0.037 (39)	0.242±0.013 (30)
10 mM Na^+, 100 mM K^+, serosal side	1.664±0.224 (18)	0.460±0.016 (22)
10 mM Na^+, 100 mM K^+, 200 μg/ml nystatin, serosal side	1.925±0.238 (33)	0.460±0.014 (18)

uptake of ^{36}Cl across the outer surface of bullfrog skin epithelium, we found that with the skin short-circuited and bathed with 110 mM NaCl Ringer on both sides of the epithelium, there was no measurable apical ^{36}Cl uptake in 12 separate skins. In high serosal [KCl]- and 200 μg/ml nystatin-equilibrated skins, J^i_{Cl} (corrected for extracellular space trapping with [^3H] mannitol) was found to be 1.18 (±0.53) × 10^{10} mol/cm² s ($N = 7$).

Relationship between I_{sc} and External [Na^+]

Experiments were also performed in which the amiloride-sensitive I_{sc} was determined over a wide range of external [Na^+] concentrations (Fig. 5). It can be seen that at high [Na^+], i.e., >200 mM, the amiloride-sensitive I_{sc} is decreased as compared with that observed at 110 mM Na^+. Although hypertonic external solutions greatly increase tissue conductance, presumably by opening up the shunt pathway (Erlij and Martinez-Palomo, 1978), this decrease in amiloride-sensitive current has been corroborated in $^{22}Na^+$ influx

experiments. The decrease in I_{sc} observed at high $[Na^+]$ can be explained with the same type of arguments used by Van Driessche and Lindemann (1979) to explain saturation. Their results show that although the current through the open channel increases linearly with $[Na^+]_o$, the number of sodium channels open at any given time decreases with increasing $[Na^+]$. This decrease in the number of sodium channels as $[Na^+]_o$ is increased follows a single-site titration curve. Therefore, in the limit very high apical $[Na^+]$, I_{sc} must tend toward 0. However, this does not preclude the possibility that at higher external concentrations of sodium the channel conductance saturates (see Discussion).

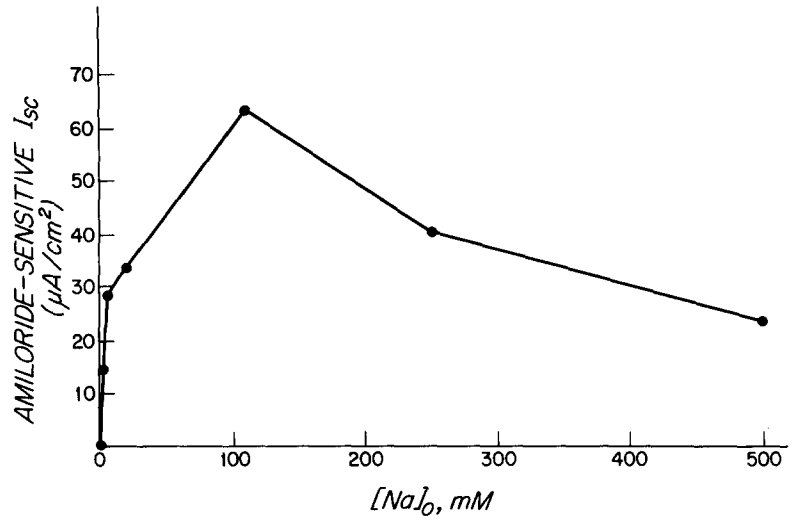

FIGURE 5. Amiloride-sensitive I_{sc} vs. $[Na^+]_o$ in bullfrog skin epithelium. The inside solution contained 110 mM Na^+ sulfate Ringer solution, whereas the external solution contained 2.5 mM K, 1 mM calcium gluconate, and 5 mM HEPES buffer, pH 7.3. The $[Na^+]_o$ was varied by stepwise addition of concentrated Na_2SO_4. At each $[Na^+]_o$, the I_{sc} was measured, 10^{-4} M amiloride was added, and I_{sc} was measured again within 3 min. The external surface of the skin was washed and the skin was allowed to re-equilibrate in 110 mM Na^+ Ringer. This was repeated for each $[Na^+]_o$ tested. In all cases, I_{sc} and V_T returned within 10% of their values at the beginning of the experiment. Similar results were obtained when the osmolality of the external solution was maintained at 600 mosmol at all $[Na^+]_o$ tested (0–300 mM) with either sucrose or choline chloride.

Na^+ Transport through the Apical Na^+ Channel Follows the Ussing Flux Ratio Equation

The natural logarithm of the observed ratio of J^i_{Na}/J^o_{Na} is plotted against the ratio predicted by the flux ratio equation (Eq. 1) in Fig. 6 (see also Table III). There is reasonable agreement between the observed and predicted ratios over the entire range of electrochemical potentials, assuming a flux ratio exponent, n', of 1. The correlation coefficient of the weighted linear regression for the data is 0.94. An analysis of variance of the regression slope indicates

that the confidence interval for the slope is ±0.123 (slope = 1.001) at a probability level of 0.001. Based on the data of Fig. 6, the probability that n' = 1 is 0.93. Another important observation is that the bidirectional amiloride-insensitive Na^+ fluxes determined under the conditions listed in Table I also

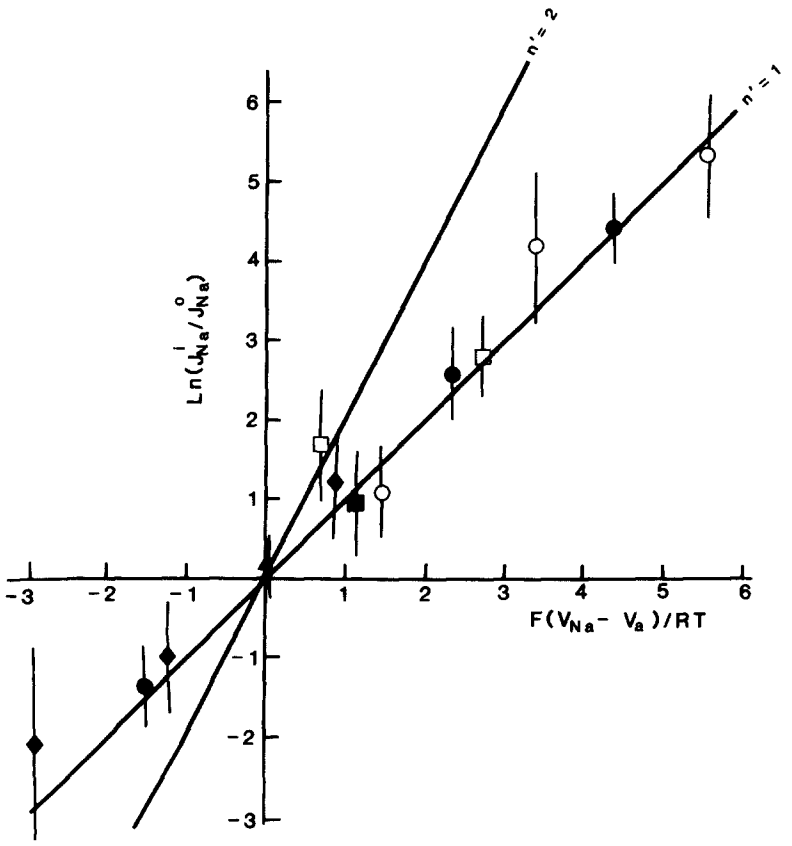

FIGURE 6. The natural logarithm of the observed amiloride-sensitive Na^+ flux ratio (J^i_{Na}/J^o_{Na}) across bullfrog skin epithelia vs. the predicted flux ratio at different electrochemical potentials. All points represent the mean values of 6–20 experiments of bidirectional fluxes performed simultaneously in the same tissues (see Table III), and vertical lines indicate 1 SD. The regression equation fitted to the data points by a weighted linear least-squares analysis is $y = 0.416 + 1.001x$ (correlation coefficient = 0.94). Symbol legends: (◆) 3 mM $[Na^+]_o$; (▲) 10 mM; (■) 30 mM; (●) 110 mM; (□) 150 mM; (○) 300 mM.

conform to the Ussing flux ratio equation. The measured flux ratio through this amiloride-insensitive pathway (J^i_{Na}/J^o_{Na} = 0.10 ± 0.03) is in excellent agreement with the flux ratio predicted by the Ussing equation (0.091). This finding is consistent with the idea that amiloride-insensitive Na^+ movements across the frog skin are diffusional in nature.

DISCUSSION

A central assumption in our studies is that the use of high serosal [K^+] together with amphotericin B or nystatin eliminates the resistance of the basolateral membranes to Na^+ so that in essence the preparation behaves as a single barrier. This assumption is necessary if the intracellular [Na^+] is taken to be equal to that in the serosal bathing solution (namely, 10 mM) and is unaffected by changes in [Na^+] in the outer solution. Yet the data presented in Table II show that in chloride medium an appreciable potential difference exists across the apical membrane under conditions of high serosal [K^+] plus ionophore. Furthermore, if high serosal K^+-ionophore medium reduces the resistance of the basolateral membrane, the voltage-divider ratio should theoretically approach a value of unity. Under these conditions V_a would equal V_T. Clearly, this is not the case for skins bathed in chloride Ringer solution and this makes these flux data difficult to interpret at present from the point of view of flux ratio analysis. Also, the data of Table IV and the rapid ^{36}Cl uptake measurements suggest that high serosal [KCl] is able to induce a Cl^- conductance somewhere within the apical surface of the frog skin epithelium, i.e., either in the membrane or in the paracellular pathway. We think this phenomenon is very interesting and deserves further study. However, for the present purposes, these data also demonstrate how conditions of high serosal [KCl] can make interpretation of the Na^+ flux ratio data in Cl^- medium difficult.[1] However, if the bathing solutions contain sulfate rather than chloride as the anion, V_a is very close to 0 mV and the voltage-divider ratio approaches 1. The same appears true for the rabbit colon, where, under comparable conditions, i.e., high serosal [K^+] in sulfate Ringer, V_a (but without ionophore) is near 0 mV and the voltage-divider ratio is near 1 (Thompson et al., 1982). We conclude, therefore, that in sulfate Ringer solution a flux ratio analysis for Na^+ flows through the amiloride-sensitive entry pathway is valid and yields important information concerning these Na^+-selective channels. The results of the present study show that the sodium flux ratio in amiloride-sensitive sodium channels in the apical membrane of the frog skin can be well explained with an $n' = 1$. We further show that n' is independent of the concentration of sodium in the apical solution in the range between 3 and 300 mM and is voltage independent in the range between −50 and +100 mV (apical or external side ground).

Measurements of unidirectional fluxes have been a traditional tool in membrane transport studies since Ussing (1949) pointed out that the flux ratio exponent, n', is related to specific structural properties of the transport pathway. More specifically, Eq. 3 has been useful in deciding whether an ion migrates through biological membranes by simple electrodiffusion. Since the

[1] It is worthwhile to note here that if the apical membrane potential measured in chloride medium is taken into account, the values of n' are much larger than 1 at all external Na^+ concentrations. This result does not agree with any reasonable pore model (e.g., multi-ion pore). For a multi-ion pore one would predict that as $[Na^+]_o$ increases pore occupancy and therefore n' increases (Hille and Schwarz, 1978). On the other hand, if V_a is taken to be 0, $n' = 1$ and the data agree with those obtained in SO_4^{2-} medium. This suggests to us that the measurements of V_a in Cl^- medium may be the consequence of artifact.

pioneering work of Hodgkin and Keynes (1955), Eq. 3 has been also used to decide whether a given ionic channel obeys the independence principle, or if it can contain several ions at a given time (Horowicz et al., 1968; Begenisich and De Weer, 1980; Begenisich and Busath, 1981; Fromm and Schultz, 1981).

Begenisich and Busath (1981) have found that the sodium channel of the squid giant axon follows the flux ratio equation with an $n' = 1$ and pointed out that there are at least four general types of pores in which a value of unity for n' is expected: first, ion channels through which Na ions pass independently of each other (Hodgkin, 1951); second, ion channels that can only be occupied by one ion at a time (Lauger, 1973; Hille, 1975); third, multi-ion channels with a single high-energy barrier (Hille and Schwarz, 1978); and fourth, multi-ion channels with almost all sites empty or almost all sites filled (Heckman, 1972; Hille and Schwarz, 1978).

The macroscopic Na^+ current passing through the frog skin epithelium is not a linear function of the apical concentration of Na^+ but saturates when the apical concentration of sodium is increased (Ussing, 1949; see also Fig. 5). In frog skin epithelium, the saturating current is rate-limited by the flow of current through the apical membrane (Helman and Fisher, 1977) and, therefore, it is the Na^+-selective entry step that saturates. The noise analysis done by Lindemann and Van Driessche (1977) has shown that the sodium transport through the apical membrane is channel-mediated, inasmuch as the elementary transport units are able to translocate $\sim 10^6$ sodium ions/s. The question arises, then, whether the observed current saturation is due to current flow saturation at constant channel density. Van Driessche and Lindemann (1979) have offered, however, the alternative explanation that current saturation is due to a regulatory process that decreases the number of transporting Na^+ channels as the apical $[Na^+]$ is increased. They further showed that in the presence of amiloride and at different apical sodium concentrations (between 0 and 60 mM) the current passing through the channel increases linearly with apical $[Na^+]$. The fact that the current saturation is restricted to the steady state has also been taken as evidence that saturation is not a consequence of a rate-limiting step in the transport of sodium through the apical membrane (Fuchs et al., 1977). Altogether, these results are compatible but do not yet prove that the Na^+ movement through this channel obeys the independence relation of Hodgkin and Huxley (1952). Our n' of 1 indicates that the Ussing (1949) flux ratio test for free diffusion is positive.

However, several questions remain unanswered. For example, we suggest that the apical $[Na^+]$ range tested by Van Driessche and Lindemann (1979) is not wide enough to be really certain that the detailed ion transport mechanism across the sodium channel is not of a more complex type. Any model of this channel must explain simultaneously its high degree of selectivity (e.g., Benos et al., 1980) and the process of ion transfer across it. Saturation of the single-channel conductance at higher $[Na^+]_o$ (i.e., >60 mM) (Van Driessche and Lindemann, 1979) cannot be discarded at present.

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