ELSEVIER

Biochimica et Biophysica Acta 1194 (1994) 303–314

BBA Biochimica et Biophysica Acta

Short-circuit current overshoot in epithelial sodium channels following apical sodium jump

Stefan Machlup [a,*], T. Hoshiko [b]

[a] *Department of Physics, Case Western Reserve University, Cleveland, OH 44106, USA*
[b] *Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH 44106, USA*

Received 7 March 1994

Abstract

Following a jump in the sodium concentration of the solution bathing the apical surface of frog skin, the inward sodium current rises rapidly to a peak and then falls to a steady-state plateau. Lindemann suggested that this fall is due to rapid closing (in 2 to 3 s) of Na channels. However, the lack of a corresponding corner frequency in the sodium-noise spectrum indicates a much slower closing. We propose a compartmental mechanism for the overshoot: the inward Na current causes Na to accumulate in the intracellular region adjacent to the sodium channel – a virtual compartment – thereby decreasing the outside/inside [Na] ratio. As that ratio falls with rising [Na] in the virtual compartment, the force driving the current falls. The predictions of such a model have been curve-fitted to the time-course of the current overshoot. The differential equation describing the rate of change of [Na] in the virtual compartment has several time constants: a filling time for the compartment, a leakage time for escape of Na into the larger intracellular space, a mixing time in the apical bathing solution, and, of course, the channel-closing time. This curve fitting shows that channel closing becomes important only in the tail of the overshoot (> 15 s) with mean open times in a range from 7 s to 3 min. Similarly, the time-course of the current after washout of apical [Na] was fitted using the same differential equation, with the channel-closing time replaced with a channel-opening time. Other phenomena explainable by this compartmental model but not by fast channel closing include the open-circuit-potential overshoot, current overshoot through nystatin channels, and the less-than-59-mV-per-decade slopes of semilog plots of open-circuit potential vs. [Na].

Key words: Sodium ion channel; Sodium ion current; Sodium ion regulation; Apical membrane; Kinetics

1. Introduction

The regulation of epithelial [Na] uptake has been studied extensively in isolated amphibian epithelia. Ussing [1] showed that active Na uptake saturates as apical [Na] is increased. The saturation effect was attributed by Kirschner [2] to an active carrier-transport mechanism. However, the Na permeability barrier was shown to be located in the apical membrane (for review see Hoshiko Ref. [3]), whereas active transport was thought to occur in the basolateral border. This suggested that a decreasing apical Na permeability with increasing apical [Na] was responsible for the current saturation. To test this possibility, Lindemann et al. [4] and Fuchs et al. [5] devised the apical-sodium-jump experiment. They observed a fast rise to a peak followed by a slower decay (dubbed 'recline') to a steady state. The shape of the decay curve implied at least two characteristic times: a fast-fall time of 1–2 s and a slow tail of a few minutes. Lindemann and Gebhardt [6] hypothesized that the decay was due to channel-closing in response to high [Na].

But the channel-closing hypothesis for current overshoot posed a paradox: the observed sodium-noise spectrum did not give any hint of a corresponding corner frequency. The sodium current fluctuates, even in the absence of any channel blocker, indicating that the sodium channels open and close spontaneously (Van Driessche and Borghgraef Ref. [7]). If the opening and closing events represent random transitions between a closed and an open state of the channel,

* Corresponding author. Fax: +1 (216) 3684671. E-mail: sxm9@pop.cwru.edu.

SSDI 0005-2736(94)00140-K

then the power spectrum of the current fluctuations should be Lorentzian [8]

$$S_o/(1+\omega^2/\omega_c^2)$$

with corner frequency $\omega_c/2\pi$ separating the high-frequency inverse-square behavior from the low-frequency plateau S_o. A mean decay time of 2 s should give a corner frequency of $(2\pi \cdot 2\ \mathrm{s})^{-1} = 0.08$ Hz. But even well below that frequency, the measured power spectra of sodium noise still look inverse-square, with no sign of a plateau [9–11]. The noise is really sodium noise, because it disappears when another cation is substituted. This absence of the expected corner is even more dramatic in urinary bladder. Gebhardt and Lindemann [12] reported a time constant for the current decay in frog bladder of 50 ms, corresponding to a corner frequency at 3 Hz. In toad urinary bladder, Na noise spectra show no sign of a plateau down to 0.1 Hz ([13], Fig. 4a). Nevertheless, we know from blocker noise measurements [14] that Na-channel density decreases with increased apical [Na], so that channel closing must play a role. Steady-state-current measurements show apical sodium permeability decreasing with increasing apical [Na] with 50%-inhibition constants in the 5 to 33 mM range [15,16].

A challenging feature of the work of the Lindemann group is the measurement on the same piece of skin of the time-course of both short-circuit current and open-circuit voltage following the Na jump. The two decay curves have very similar shapes. That suggests an explanation based on what Lindemann has called 'sodium loading', an increase in [Na] in some intracellular compartment during the decay. The diffusion potential associated with the applied Na gradient (high outside, low inside) would decrease as the inside loads up with Na. A compartmental mechanism has the feature that its rate constants do not show up as corners in the fluctuation spectrum. Simply put, the concept of 'filling a compartment' has no small-signal equivalent, resolving the paradox [17]. (On the other hand, compartment concentrations can affect the amplitude of other noise mechanisms.) In this paper we show that a compartmental model can quantitatively explain the overshoot response to a jump in apical [Na], as well as the fall in current after apical [Na] is suddenly washed out. The tail of the overshoot curves – the asymptotic approach to the steady state – represents channel closing, with mean closing times in a range from 10 s to 4 min, corresponding to corner frequencies well below the range of practical noise measurements, i.e., well below 0.05 Hz.

Varying the incubation time in low (0.5 mM) [Na] before the [Na] jump affects the height of the current peak attained [18]. The longer the incubation, the higher the peak. This is what our compartmental model predicts. Overshoot response following a jump in apical [Na] is also exhibited by nystatin-treated frog skin [19], but is not easily attributable to channel closing. Another phenomenon exhibited by epithelia, the shallow slope of the semilog plot of open-circuit voltage versus log[Na] [20] can be explained by intracellular [Na] increasing with apical [Na], but not by channel closing.

The sodium-loading hypothesis was tested by two experiments by Lindemann and Gebhardt [6], and dismissed as implausible. Their argument is dealt with in Section 5 (Discussion) and shown to be inconclusive.

Section 2 of this paper presents some innovations in the technique of the 'overshoot' experiment, which gave us some current records well suited for curve fitting. Section 3 analyzes a simple one-compartment model and derives the time-course of the short-circuit current it predicts. In Section 4 the results of curve-fitting short-circuit-current overshoot data with these theoretical curves are presented, and the parameter values are discussed. Of the various channel parameters, the channel-closing rate constant (= 1/mean open time) has perhaps the greatest experimental uncertainty, but is less than 0.1 per s at 120 mM Na, sometimes as low as 0.004 per s. That corresponds to a mean open time in a range from 10 s to more than 200 s. Channel closing is not the mechanism of the fast decay.

With the new interpretation of the early decay of short-circuit current, the experiments here reported give an estimate of the closing times of epithelial sodium channels in response to high [Na].

Preliminary results were reported in Abstracts of the 10th International Biophysics Congress, Vancouver, B.C., August 1990, p. 326.

2. Methods

Isolated abdominal skins of *Rana pipiens* were mounted using a modified Helman-type chamber [22]. The corium surface was glued to lucite rings and the apical surface sealed against another lucite ring with grease (Lubriseal, A.H. Thomas). The exposed area was 1 cm in diameter. Agar-3 M-KCl bridges and Ag-AgCl electrodes were used to connect to a four-electrode voltage-clamp system. Solutions used were 120 mequiv./l Na/K mixtures containing 1 mequiv./l Ca with sulfate as the anion, and buffered to pH 8.0 with 5 mM THAM.

Skins were 'depolarized' with 120 mM basolateral potassium in order to allow a more complete voltage clamp of the apical membrane [23]. After a 15-min equilibration in 0.1 mM $[Na]_{apic}$, the replacement cation being potassium, the apical surface was suddenly exposed to 120 mM [Na]. The apical chamber was drained just before the new solution was injected. A 1-MΩ resistor shunting the skin was used to avoid any possi-

ble open-circuit condition due to this step. Injection of about 20–30 ml was accomplished within less than 2 s with a 50-ml syringe fitted with a short piece of large-diameter tubing. The excess fluid simply overflowed from the open chamber. Baffles served to prevent splashing. Slow, constant perfusion (gravity-fed) of the appropriate solution into each chamber was maintained before and after the injection. The short-circuit current, negative with low apical [Na], changed sign (became positive) shortly after this sudden exposure to increased sodium concentration, rose to a peak in a fraction of a second, then fell, rapidly at first, more and more slowly later.

The short-circuit current was digitized at 5-ms or 10-ms intervals for 10 or 20 s before the [Na] jump and then followed for up to 5 min after the [Na] jump using CLAMPEX or FETCHEX (Axon Instruments). In addition, the current was monitored on a strip-chart recorder (Leeds & Northrup, Speedomax Model W) modified to record continuously. Full-scale response time of this recorder is 1 s. The data were compressed by averaging successive points up to a maximum of 256 consecutive points (to give one averaged point), starting a new average whenever the next value deviated by more than 2% from the 'going' average.

The rapidity of the concentration change resulting from this procedure was tested using a chloride electrode. A lucite ring with a sintered Ag-AgCl electrode embedded in its center replaced the frog-skin mounting ring in the chamber. The solutions used were 0.12 mM KCl in 120 mM KNO_3 before and 120 mM KCl after, respectively. The potential was digitized at 100-μs intervals using FETCHEX (Axon Instruments). The potential change was found to be complete in less than 3 ms. Since the exposed surface of the sensor was only 2 mm in diameter, the time to cover the total area of skin normally exposed may be somewhat longer, but most probably under 10 ms, the sampling interval used in tracking current responses of the skin.

3. Theory: apical membrane kinetics

3.1. Model of the sodium channel

Assume that most of the electrochemical potential difference driving the sodium current occurs across the selectivity barrier. Then the responsible structure is the amiloride-inhibitable apical sodium channel. Further assume that the channel is ohmic. Call its conductance g, and assume g proportional to the Na concentration in the channel [14]. The significance of $[Na]_{chan}$ in a structure with perhaps only one or two binding sites is discussed below.

The driving voltage is the Nernst potential associated with the Na concentration ratio across the channel,

$[Na]_{prox}/[Na]_{dist}$.

In most of our experiments the apical [Na] starts at 0.1 mM and ends up at 120 mM after the jump. The intracellular [Na] is subject to biological regulation and may be thought of as unchanged by the jump, say, at 12 mM. So we meet a problem of definition. It takes a few milliseconds after the sodium jump before $[Na]_{prox}$ becomes equal to $[Na]_{apic}$ by diffusion of Na through the unstirred layer. At the distal end of the channel, however, $[Na]_{dist}$ may be different from $[Na]_{intracellular}$ as long as current is flowing. In order to give meaning to the Nernst potential we need to think about a finite volume of solution characterized by $[Na]_{dist}$, so we postulate that the channel empties into an intracellular compartment or subcompartment having a virtual volume V of well-mixed solution. This region, anatomically not well defined, may have somewhat restricted access, since it may accommodate the cellular machinery for servicing channels. The assumption that the Nernst potential involves the [Na] ratio only at the apical membrane implies that the basolateral membrane potential and resistance have been eliminated by potassium depolarization [23].

Early in the history of this curve-fitting project [24], the assumption was made that most of the length of the channel quickly comes to equilibrium at $[Na]_{apic}$. How else can we explain the rapid rise of positive (inward) current a few tenths of a second after the Na jump has reversed the current? By the same logic, during the negative-current steady state preceding the jump, the sodium moving from the intracellular compartment into the channel keeps the channel in equilibrium with $[Na]_{comp}$. To talk about a Na concentration in the channel, which contains no Na ion much of the time and only one or two the rest of the time, does require a statistical outlook. It is the price we pay for assigning the channel a conductance.

For clarity of exposition, the derivation of the differential equation which is the equation of continuity for sodium in the compartment is presented in three stages. The first stage assumes that, during positive current, $[Na]_{chan}$ is equal to $[Na]_{apic}$, which is equal to its final value $[Na]_{after}$. So the initial focus is on the decay of the current from its positive peak, as though the rise were instantaneous. The second stage, in the section called 'finite rise time', looks quantitatively at the effect of diffusion delay in the first second. The third stage brings in channel closing, after noting that it was not needed to fit the data for the first few seconds.

3.2. Construction of a differential equation

The selectivity of the apical channel results in a sodium diffusion potential between its apical (prox-

imal) end and its intracellular (distal) end, a potential whose magnitude is

$$(RT/F)\ \log_e([\mathrm{Na}]_{\mathrm{prox}}/[\mathrm{Na}]_{\mathrm{dist}})$$

After the [Na] of the apical bathing solution is stepped to, say, 120 mM, the polarity of this potential is such as to drive Na^+ current into the cell, from the sodium-enriched apical end to the more dilute (perhaps 12 mM) [Na] solution in the intracellular fluid. Assuming the current carriers are all Na ions, the channel current driven by this Nernst potential is

$$I = g(RT/F)\log_e([\mathrm{Na}]_{\mathrm{prox}}/[\mathrm{Na}]_{\mathrm{dist}}) \quad (1)$$

where the channel conductance g would be written

$$g = g_o[\mathrm{Na}]_{\mathrm{prox}} \quad (1a)$$

because of the assumption of a quick equilibrium of $[\mathrm{Na}]_{\mathrm{chan}}$ with $[\mathrm{Na}]_{\mathrm{prox}}$. Recall that the distal 'compartment' is defined in such a way that $[\mathrm{Na}]_{\mathrm{comp}} = [\mathrm{Na}]_{\mathrm{dist}}$.

The number of moles of Na^+ brought into the compartment per unit time by this current is I/F. This amount of Na transport would increase the number of moles per liter in a compartment of volume V at a rate I/FV. The current leaving the compartment is carried by all the ions present, since cytoplasmic ion movement should show little selectivity. The Na fraction will be small. To write a continuity equation for Na we have to consider diffusion of Na out of the compartment into the larger intracellular space. Our assumption is that the intracellular space is large enough that its [Na], call it $[\mathrm{Na}]_{\mathrm{intra}}$, remains constant for a few minutes following the jump. The rate of equilibration of the compartment due to diffusion is assumed proportional to the concentration difference. Introducing the time constant τ for this diffusive leakage process, the continuity equation can be written

$$\mathrm{d}[\mathrm{Na}]_{\mathrm{comp}}/\mathrm{d}t = I/FV - (1/\tau)([\mathrm{Na}]_{\mathrm{comp}} - [\mathrm{Na}]_{\mathrm{intra}}) \quad (2)$$

To streamline the notation we use dimensionless notation for the Na concentration in the compartment and define

$$u(t) = [\mathrm{Na}]_{\mathrm{comp}}/[\mathrm{Na}]_{\mathrm{after}}$$

with the Na concentration in the apical solution after the jump called

$$[\mathrm{Na}]_{\mathrm{after}} = 120\ \mathrm{mM}.$$

Setting $[\mathrm{Na}]_{\mathrm{apic}}$ equal to $[\mathrm{Na}]_{\mathrm{after}}$ and using Eq. [1a] in Eq. [1] for the current $I(t)$, we obtain the nonlinear differential equation

$$\mathrm{d}u/\mathrm{d}t = (1/\sigma)y(t) - (1/\tau)(u - u_{\mathrm{intra}}) \quad (3)$$

where

$$y(t) = \log(1/u) \quad (3a)$$

is a dimensionless function proportional to the channel current $I(t)$ (see Eq. [1]) and

$$\sigma = V(F^2/g_o)RT$$

is another constant having dimensions of time. Call σ the 'filling time' of the compartment, since it is proportional to the compartment volume V. The way the function $y(t)$ (= the dimensionless current) decays from its peak according to this nonlinear differential equation is nothing like the sum of two or three falling exponentials. It does have a horizontal asymptote as the compartment concentration approaches its steady-state value.

What might be a reasonable filling time σ? A sodium channel might have a conductance of 0.4 pS at 12 mM Na. That gives g_o. Arbitrarily taking a (rather small) value for the compartment volume V of 0.2 μm^3 per channel and setting $RT/F = 25$ mV gives $\sigma = 23$ s. The differential equation is so nonlinear that filling times that long can make the current fall by one-third its peak value in about 2 s.

In the experiments, the current becomes positive (more [Na] outside than inside) about 0.2 s after the jump, so the conduction term in Eq. [3] (the first term on the right) brings sodium into the compartment. As soon as [Na] in the compartment becomes greater than in the larger intracellular space, the diffusive leakage term (the second term) becomes negative (Na flowing out). Computer solutions of this differential equation can be fit rather well to the observed time-course of the current decay from its peak value, for a good 10 or 20 s. Much beyond that time, channel closing has to be taken into account.

To compare the dimensionless current $y(t)$ with the measured current for curve fitting, one has to multiply $y(t)$ times RT/F to make it a voltage, times $g_o[\mathrm{Na}]_{\mathrm{after}}$ to make it a single-channel current (see Eq. [1a]), and times the number of channels in the skin sample to make it the total current. That multiplicative constant, which has dimensions of current, we call G:

$$\text{measured current} = Gy(t) \quad (4)$$
$$= (\text{No. of channels in sample})(RT/F)g_o[\mathrm{Na}]_{\mathrm{after}}y(t)$$

So G is a measure of the conductance of the skin per mM [Na].

'Curve fitting' means guessing a set of starting parameters and solving the differential equation (Eq. [3]) for the function $u(t)$, then using Eq. [3a] to plot the function $y(t)$. This is multiplied by the parameter G and compared with the experimental current record to improve the guess and go around again, seeking convergence.

3.3. Finite rise time of the Na jump

Of course, the jump in the apical [Na] is not instantaneous. The rise time for $[\mathrm{Na}]_{\mathrm{prox}}$ to approach its

steady-state value $[Na]_{after}$ (= 120 mM) can be a sizeable fraction of a second [25]. Cotton and Reuss [26] have shown that the time delay in the mixing of the newly added liquid with the old bulk solution is not negligible. Then the Na ions have to diffuse through an unstirred layer including the stratum corneum before they get to the channel. The mathematics of diffusion into a region bounded by a fixed wall (the apical membrane) is well-known. If mixing at the 'source' (in the bulk solution) gives a concentration build-up proportional to

$$q(t) = 1 - \exp(-\beta t) \quad (5)$$

with mixing time $1/\beta$, then the build-up of concentration from zero at the membrane obeys Eq. 4.29 of Crank (Ref. [27], page 49) with diffusion-delay time constant r proportional to the square of the unstirred-layer thickness and inversely proportional to the diffusivity of the ion. The six experimental curves discussed below had best-fit diffusion-delay times ρ of less than 1 ms, i.e., essentially zero on the scale of our $1/\beta$ values, which were on the order of a tenth of a second. Accordingly we could cut down the number of parameters by one without sacrificing goodness of fit. Letting ρ go to zero makes Crank's equation go to Eq. [5], with only the one time constant, the mixing time $1/\beta$. The solutions of diffusion equations can be expressed as sums of exponentials, like the mixing equation (Eq. [5]), so our curve-fitting results do not mean that the time to diffuse through the stratum corneum is less than 1 ms. What parades as mixing time here is, in fact, diffusion delay. We know that it cannot be mixing delay from experiments with the chloride electrode and no skin, which yield mixing times of a few milliseconds.

To be sure, the apical [Na] builds up not from zero but from some small value like $[Na]_{before}$ = 0.1 mM. Let r_o be the ratio of the apical [Na] before and after the sodium jump:

$$r_o = [Na]_{before}/[Na]_{after} \quad (6)$$

In our experiments this was usually 0.1 mM/120 mM. The fraction $f(t)$ of the buildup attained at time t can be written

$$[Na]_{prox}/[Na]_{after} = f(t) = r_o + (1 - r_o)q(t) \quad (7)$$

with $q(t)$ given by Eq. [5]. The differential equation for $u(t)$ may then be approximated by writing Eq. [3] with

$$y(t) = f(t) \log[f(t)/u(t)] \quad (8)$$

as the dimensionless channel current replacing Eq. [3a]. As before, Eq. [4] makes that into the measured current. The symbol r_o in Eq. [6] should not be confused with $u(0)$, the ratio of the [Na] in the compartment before the jump to the [Na] outside after.

3.4. Channel closing with increasing [Na]

To compare $y(t)$ with observed values of current through an area of skin containing possibly thousands of channels, one needs to multiply the dimemensionless channel current $y(t)$ by the number of open channels. In order to preserve the per-channel meaning of the constant σ, multiply $y(t)$ by the constant G of Eq. [4] and by the fraction of channels remaining open as the apical [Na] increases. Postulating an exponential approach to the steady state, i.e., a first-order mass-action law, the open fraction can be written

$$F_{open}(t) = w + (1 - w) \exp(-t/\gamma) \quad (9)$$

Here w is the fraction of channels remaining open in the steady state, or you may think of $(1 - w)$ as the fraction closing as a result of the increase in apical [Na]. A fraction $1/e$ of that closing occurs in the time γ; i.e., the probability that a channel will close in a short time Δt is $\Delta t/\gamma$. Our method of estimating w is discussed below. To take channel closing into account, the correct $y(t)$ dimensionless-current function to replace Eq. [3a] and Eq. [8] is

$$y(t) = F_{open}(t)\, f(t) \log[f(t)/u(t)] \quad (10)$$

Curve fitting with such a large number of parameters may seem unconvincing at first. But notice that of the four time constants σ, τ, $1/\beta$, and γ, the time constant $1/\beta$ governs principally the very beginning of the time-course, the 1-s build-up to the peak. The time constant γ governs principally the long-time tail. Methods of estimating w, $u(0)$, and u_{intra} are discussed below, leaving us with five free parameters to be determined by curve fitting: the four time constants, and the specific conductance G.

4. Results

4.1. Na jump from 0.1 mM to 120 mM

4.1.1. Curve fitting

Can this theory adequately describe the short-circuit-current records? The theory is based on a 1-compartment model, a very rough approximation to what a Na^+ encounters as it emerges from the channel into the intracellular space. But the time constants appearing in the theory are supposed to describe real events – like the rate of channel closing. So curve-fitting the theory to the experimental data is our way to learn something about channel kinetics.

One parameter is fit by eye. Time 'zero' is adjusted by imposing the recorded points on the theoretical curve on the computer screen. The other parameters are fit using a Marquardt nonlinear least-squares tech-

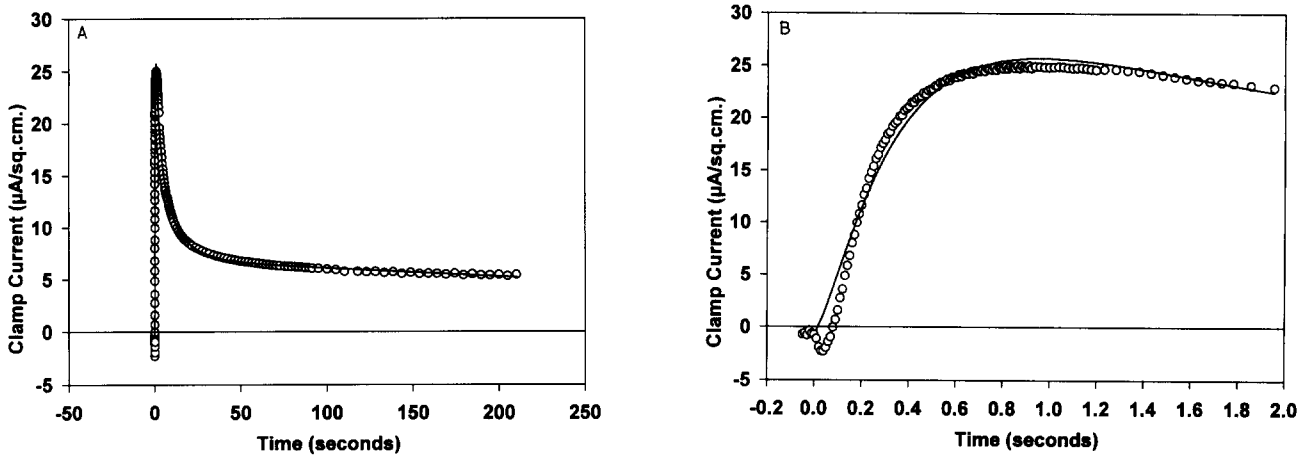

Fig. 1. (A) The entire record, 215 s after the Na jump. (B) The first 2 s only. The theoretical curves (solid line) fit the experimental points (circles) with $\chi^2 = 0.352$. Parameter values: $\sigma = 30.5$ s, $\tau = 27.9$ s, $G = 36.4$, $1/\gamma = 0.004$ s^{-1}, $\beta = 2.5$ s^{-1}, $u_{\text{intra}} = 0.084$. Expt. No. 287f01a.

nique, as described by Bevington's [30] CURFIT program.

This is not a trivial computational task, because there is no 'formula' for the fitting function $y(t)$. Recall that the $y(t)$ function – the dimensionless channel current – is obtained by solving the differential equation (Eqs. [3] and [10]) numerically. For each set of values of the parameters a_i, a table of the $y(t)$ function is stored. To calculate the derivative of $y(t)$ with respect to a_j the solution of the differential equation is repeated with $a_j + \Delta a_j$ replacing a_j. The two $y(t)$ tables are subtracted and the differences divided by Δa_j. So if a record has been compressed, say, to 100 points and we are fitting five parameters, then at each of the 100 points a 5-vector is stored whose components are the five derivatives. The 100 points are not, in general, uniformly spaced. To fit them well seems to require at least 200 points of $y(t)$, computed with a Runge-Kutta method.

One data point which is actually known to better accuracy than the others is the pre-jump steady-state value of the short-circuit current (negative!), here called Baseline. We use it to determine two other numbers: a good starting value for the [Na] in the compartment, and a good value for the intracellular [Na]. Eq. [1] gives the first, and the steady-state form of Eq. [3], $du/dt = 0$, gives the second. Thus, setting

$$f_0 \log(f_0/u_0) = \text{Baseline}/G = y(0) \qquad (11)$$

with $f_0 = f(0)$ and $u_0 = u(0)$ denoting the pre-jump values, gives $u(0)$. Then the steady-state condition

$$u_{\text{intra}} = u(0) - (\tau/\sigma)(\text{Baseline})$$

gives u_{intra}. Even a 2-s-long record gives a rather good value of the 'specific-conductance' parameter G, allowing the conversion of a measured Baseline current into dimensionless channel current $y(0)$. So we know the right-hand side of Eq. [11]. On the left-hand side we

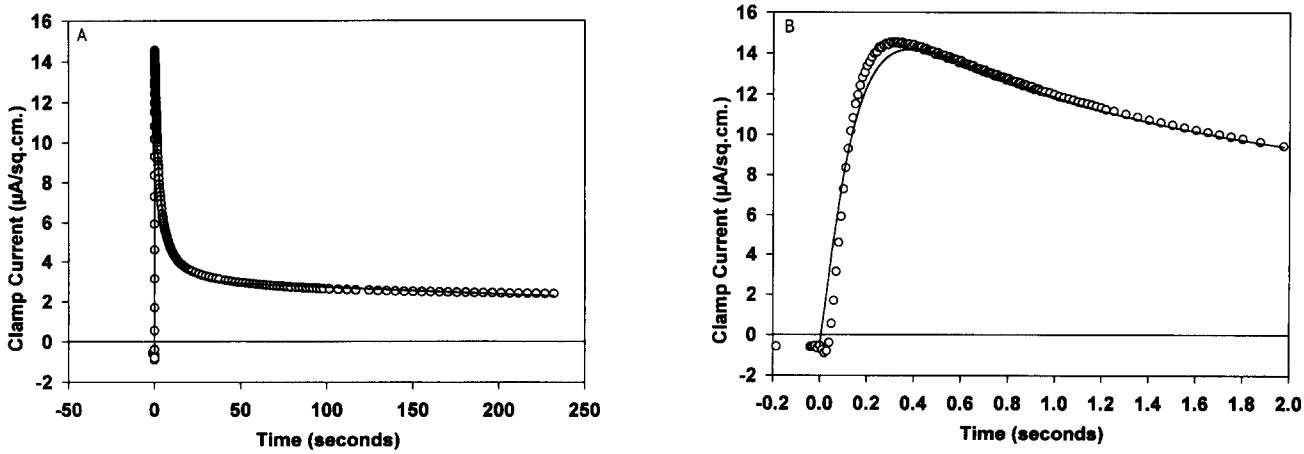

Fig. 2. (A) The entire record, 232 s after the Na jump. (B) The first 2 s only. The theoretical curves (solid line) fit the experimental points (circles) with $\chi^2 = 0.1$. Parameter values: $\sigma = 21.0$ s, $\tau = 21.9$ s, $G = 18.5$, $1/\gamma = 0.003$ s^{-1}, $\beta = 7.6$ s^{-1}, $u_{\text{intra}} = 0.143$. Expt. No. 290f02a.

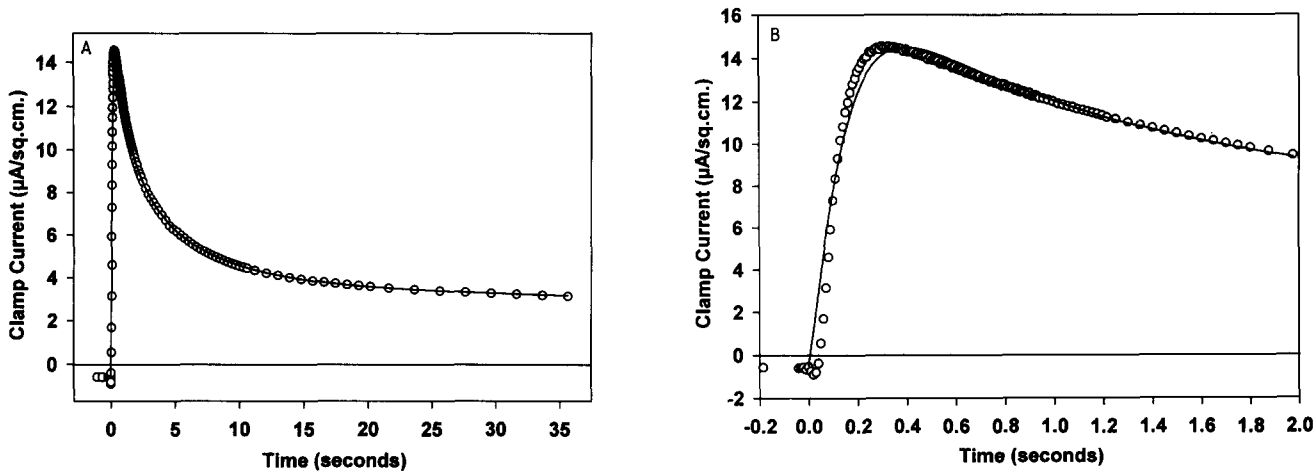

Fig. 3. Same record as Fig. 2, but the curve fitting used only the first 36 s after the Na jump in order to improve the fit to the early points. (A) The first 36 s. (B) The first 2 s only. The theoretical curves (solid line) fit the experimental points (circles) with $\chi^2 = 0.34$. Parameter values: $\sigma = 17.1$ s, $\tau = 15.1$ s, $G = 19.8$, $1/\gamma = 0.01\ s^{-1}$, $\beta = 6.9\ s^{-1}$, $u_{intra} = 0.118$. Compare with Fig. 2. values to appreciate how very rough the theory really is. Expt. No. 290f02a.

know $f(0) = 1/1200$. A typical result is $u(0) - u_{intra} = -0.04$. That translates into a concentration difference of 0.04×120 mM = 4.8 mM between the compartment and the larger intracellular space. Na is flowing into the apical bathing solution, down the concentration gradient; hence the minus sign. Here we need estimates of G, σ, and τ to solve for u_{intra}. These turn out to be rather insensitive to $u(0)$ and u_{intra}. Some values of u_{intra} came out a bit smaller than our initial estimate, as we might have expected from Rick [28]. Corresponding to the data in Fig. 1 we obtained a pre-jump $[Na]_{comp}$ of 3.5 mM and a $[Na]_{intra}$ of 12.4 mM.

The new solution-changing technique described in Section 2 enabled rapid, relatively low-noise solution change and resulted in more reliable data. Details previously obscured by noise are now resolved. A few of our records show the current going to a negative maximum about 0.1 s after the Na jump, before changing sign and going to the much larger positive maximum. We reported this observation in an abstract [21], but our explanation was quite wrong, indeed, illogical. It postulated (mistakenly) the [Na] in the channel to be a weighted average of the apical and compartmental [Na] values, independent of current direction. It is now clear to us that the 'volume' of the channel is so small (occupancy probability so low) that the channel [Na] must come very quickly into equilibrium with the [Na] on the side the Na current flows *from*. We have concluded that the initial negative 'dip' of the (negative) current must be an experimental artifact. Because the entire negative dip lasts only during the first 0.2 s of a current record which is typically 200 s long, the effect on the parameter values of disregarding the dip in curve fitting is indeed slight (Figs. 2 and 3).

4.1.2. *The fraction w of open channels*

Records less than 20 s long could be fit well by assuming all channels are open: $F_{open} = 1$. In other words, 20 s was not long enough to show channel closing, or the mean open time γ is much longer than

Table 1
'Best-fit' parameters for overshoot experiments on three frogs

Expt. No.	σ (s)	τ (s)	G	$1/\gamma$ (s^{-1})	β (s^{-1})	u_{intra}	χ^2	Dur (s)
287f01a	30.5	27.9	36.4	0.0035	2.5	0.0840	0.352	215
287f05x	24.4	34.2	42.5	0.0032	2.1	0.167	0.385	287
287f07a	3.1	8.3	41.7	0.0430	0.9	0.166	0.197	249
289s06a	4.7	2.9	30.2	0.125	7.3	0.0390	0.359	160
289s07a	2.7	3.6	41.1	0.119	3.2	0.0422	0.258	160
289s10a	4.2	2.5	27.5	0.144	9.0	0.0289	0.293	161
290f02a	21.0	21.9	18.5	0.0031	7.6	0.143	0.10	237

All jumps are from 0.1 mM to 120 mM $[Na]_{apic}$. The theory uses $[Na]_{chan}$ = the greater of $[Na]_{apic}$ and $[Na]_{comp}$ to take account of the sign (+ or −) of the sodium current.

Note that Experiments 287f01, 05, and 07 were done about half an hour apart on the same piece of skin. The great discrepancy (look at the 07 parameters!) is not bad curve fitting. The skin really changed during an open-circuit period in Experiment 06, a failed experiment which kept an open-circuit-voltage record instead of a short-circuit-current record.

20 s. For such short records, the value of w, the equilibrium fraction of channels open at the new sodium concentration, is irrelevant. For long records, records which might be useful for estimating γ, an independent estimate of w is highly desirable. To be sure, one could let w be one of the free parameters and fit it with the Marquardt method. The result is a rather uncertain w with a very uncertain γ. But our six 'best' records go out 200 to 300 s after the Na jump, and the current is settling down, falling more and more slowly. When no digital records exist there is always the strip-chart (analog) record, usually set to move 1 inch in 5 min. Call the last available current value the 'steady-state' value; then w is the ratio of that equilibrium value to the peak value the current would have reached right after the Na jump if the 'filling time' σ had been infinite and the channels had not closed. That means Eq. [10] reverts back to Eq. [3a], $y = \log(1/u)$, and all we are interested in is the pre-jump value. From fitting short records ($F_{open} = 1$) we had good estimates of the specific conductance G. Our long records now give us estimates of w.

What values of w might we expect? The 50% concentration K_M for channel closing ought to be around 15 mM [Na] [31]. After a jump to 120 mM Na, the equilibrium fraction of open channels w should have come out $1/(1 + 120\ \text{mM}/15\ \text{mM}) = 11\%$, according to that mass-action-law argument. For the record shown in Fig. 1, the value of w came out 13%. That corresponds to a K_M of 18 mM, well within the 5–33-mM range cited earlier. That $w = 13\%$ value was used for all seven experiments of Table 1. Values of the fitted parameters were not very sensitive to the value of w.

4.1.3. The mean open time γ

Table 1 shows the 'best-fit' parameters for seven records using skin from three frogs. In the column labeled $1/\gamma$, Greek γ is the mean closing time for a sodium channel brought suddenly from 0.1 mM to 120 mM $[Na]_{apic}$. Frog No. 289 is so different from the other two that the average closing rate for the three frogs is $0.06 \pm 0.05\ s^{-1}$, corresponding to a closing time of 16 s. But for frog No. 287 the average closing rate is $0.017 \pm 0.017\ s^{-1}$, corresponding to a closing time of 60 s, and frog No. 290 closes in an average of 3 min. What we can say is that the measurements are reproducible to about 20%, but the frog-to-frog variation covers more than a decade.

Curve-fitting different lengths of the record of any one experiment gives somewhat different values of $1/\gamma$. For example, in changing from 110 s to 215 s of the same record, the best-fit value of $1/\gamma$ decreased by 40%. That seems to imply that the open fraction $F_{open}(t)$ may not be quite the decaying exponential we hypothesized.

The fact remains that none of the closing times are nearly as short as the 2-s or 3-s fall-off times of the current from its peak, so channel closing can not be the 'overshoot mechanism'. In A6 membranes incorporated into lipid bilayers [32] the reported $1/\gamma$ is 0.1 s^{-1}, i.e., a 10-s closing time.

4.1.4. The filling time σ

The parameter σ, which is a measure of the volume V of the intracellular 'compartment' into which the Na channel empties (see Eq. [3]), is the one that determines how fast the current starts to decay from its peak. The record of Fig. 1. is fitted with a σ value of 31 s, corresponding to a compartment volume V of 0.26 μm^3. Noting from the record that the $1/e$ decay time of the current to its steady-state value is around 3 s, the 31-s filling time seems long. The great discrepancy between 3 s and 31 s reflects the nonlinearity. The decay time of the theoretical curve turns out to be rather insensitive to changes in σ. A volume V of 0.26 μm^3, on the other hand, seems small when considered as a fraction of the perhaps 500 μm^3 volume of an epithelial cell. It may be useful to calculate how thick a layer that volume corresponds to. Suppose there are four channels per μm^2 of apical membrane [33]. The product $0.26\ \mu m^3 \times 4/\mu m^2$ gives a layer thickness of 1 μm. Note that both compartment volume and channel density have considerable dispersion, i.e., can vary greatly from frog to frog.

4.2. Downward [Na] jump from 120 mM to 0.1 mM

We can now ask whether the theory of Section 3, designed to explain the overshoot following an upward jump in apical [Na], also predicts the time-course of the current after a sudden downward jump – and which parameters we should expect to be the same.

Three parameters for each skin ought to be the same whether $[Na]_{apic}$ is changed from 0.1 mM to 120 mM or vice versa. The specific conductance of the channels given by the parameter G should be the same, since we assumed it to be the same for both directions of the current. The characteristic time τ for diffusive leakage between the 'compartment' and the larger intracellular space was taken to be independent of sign, so one might assume it would be the same for both signs of the Na jump. The sodium concentration of the larger intracellular space, $u_{intra} \times 120$ mM, should not have changed much in the minutes between the two experiments. (See comment below, however!)

On the other hand, instead of channel closing we now have channel opening, and that might have a very different characteristic time γ. As for the solution mixing, instead of Na ions being washed in, they are now being washed out, so one should not expect the mixing time $1/\beta$ to be the same. The compartment filling time σ is a measure of compartment volume,

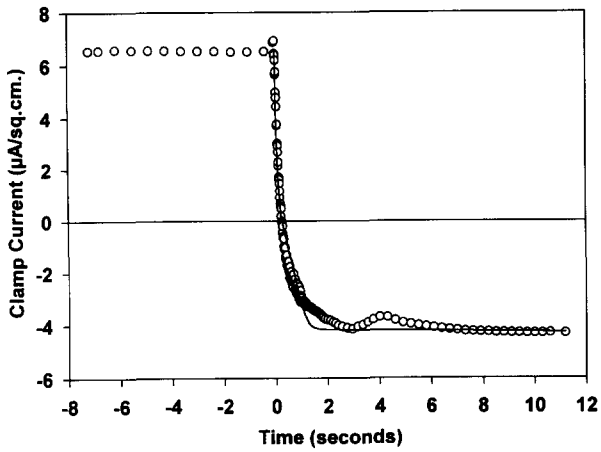

Fig. 4. Clamp current following downward Na jump. The theoretical curves (solid line) fit the experimental points (circles) with $\chi^2 = 0.08$ over 12 s following the jump. Reasonable curve fitting was not possible over longer times, indicating the limitations of a one-compartment model. The parameter values: $\tau = 21.9$ s, $G = 18.5$, $u_{intra} = 0.143$ were taken from the curve fit to the 290f02a upward Na jump immediately following this downward jump. Three parameters were varied for best fit: $\sigma = 160$ s, $1/\gamma = 0.006$ s^{-1}, $\beta = 6.2$ s^{-1}with $F_{open}(0)$ chosen to match the initial ('baseline') current. Note that γ is now a mean closed time rather than a mean open time; it is well over 2 min. Expt. No. 290f02.

and we were tempted to think of it as an anatomical characteristic. But the compartment has no walls; it is a mathematical fiction. There is no reason it should be the same for washout as for washin. So there are three new time constants to be determined by curve fitting. (Again, the millisecond-scale ρ has been elided and set equal to zero.) It should be noted that the mixing time $1/\beta$ affects only the first few seconds of the sudden current fall, while the channel-opening time constant γ affects only the long-time part, the negative-current 'plateau'.

Fig. 4 shows the result of such curve fitting with a 3-parameter fit. The theoretical curve is made to agree with the initial (pre-jump steady-state) current by fitting the initial value of F_{open}. Studying the records it is clear that F_{open} has not had time to go all the way down to its high-[Na] equilibrium value $w = 13\%$, since closing times γ are long. The good fits of the theoretical curves to the time-course of the current during the first few seconds of low apical [Na] lend further support to the theory (Table 2).

But now, a comment about long records ($\gg 20$ s). They all show the current slowly going to a less negative value, i.e., having an upward slope at long times. But we know that more channels are opening, and estimate opening times to be on the order of minutes. Our theory, assuming a constant u_{intra}, does not explain the observed long-time behavior! Apparently the negative current is slowly draining the (large) intracellular space of sodium, reducing u_{intra} and hence the sodium gradient responsible for the negative current. Another way of saying that is, we really need a two-compartment theory in order to accomodate the fall in intracellular [Na].

5. Discussion

5.1. Rival theories

The theory of Section 3 leading up to the differential equation (Eq. [3]) is the theory of the overshoot mechanism that has been called 'sodium loading'. The sodium gradient drives the (positive) sodium ions through the channels into the cell. As sodium accumulates in the cell, that gradient decays, resulting in the overshoot. The theory is a one-compartment theory, stripped down to bare essentials.

This mechanism is proposed to supplant Lindemann's 'fast channel-closing' mechanism. To be sure, channel closing occurs. The jargon is 'sodium self-inhibition'. The question is, how fast do the channels close in high [Na]?

To escape the paradox posed by the absence of a corner frequency in the sodium-noise spectrum, this paper proposes that the 'filling time' of a 'compartment' distal to the Na channel is short enough and the mean open time of the channel long enough that compartment filling dominates as the mechanism of the early current decay. Some readers might consider it incumbent on the theory to show that it disagrees dramatically with the data if that inequality is inverted, i.e., if the mean open time is short and the filling time

Table 2
Parameters fitting jumps from 120 mM [Na] down to 0.1 mM

Expt. No.	σ (s)	τ (s)	G	$1/\gamma$ (s^{-1})	β (s^{-1})	u_{intra}	χ^2	Dur (s)	w
287f01	81.1	27.8	36.4	0.309	0.76	0.084	0.331	2.5	0.39
287f07	28.4	8.3	41.7	0.0032	5.2	0.166	0.047	25.3	0.07
290f02	160	21.9	18.5	0.006	6.2	0.143	0.076	12.0	0.21

Only three parameters are varied: σ, $1/\gamma$, and β. The others – τ, G, and u_{intra} – are taken from the upward-jump fits of Table 1. The washout experiment 287f01 was done immediately before the overshoot experiment 01a of Table 1, etc.

is long. Perhaps the paradox has some other resolution! Can the data be fitted with a mean open time of, say, 2 s?

The answer turns out to be no, not if the fraction w of open channels in equilibrium at 120 mM $[Na]_{apic}$ is to have a value between 11% and 35%, corresponding to reported K_M values. The predicted long-time plateau is much too low, about one-third of the observed current. A good fit to the record of Fig. 1 would require a w value near 60%. Is such a large value possible? A value of $w = 0.6$ corresponds to a channel-closing K_M of 180 mM Na, well outside the reported 5–33-mM range.

One might ask whether embellishments to the theory might get around this contradiction. The idea that there might be more than one 'closed' state of the channel has been proposed [34]. With two closed states there are two γ time constants and two w equilibrium-open-fractions. Another way to say that is that there are two closing-rate constants k_{o1}, k_{o2} and two opening-rate constants k_{1o}, k_{2o}. A rather general 3-state model was treated by Frehland, Hoshiko and Machlup [35], and their Eq. [9] gives the formula for the two corner frequencies of the two Lorentzians adding to form the noise spectrum:

$$\omega = \sum \pm \left(\Sigma^2 - k_{1o}k_{2o} - k_{1o}k_{o2} - k_{2o}k_{o1}\right)^{1/2} \qquad (12)$$

with

$$\sum = \tfrac{1}{2}(k_{o1} + k_{1o} + k_{o2} + k_{2o})$$

Since we are trying to account for the absence of an observed corner at low frequencies, our interest is in ω_{hi}, Eq. [12] with the plus sign, the higher of the two corners. Now suppose that one of the four rate constants is much bigger than the other three; then inspection shows that ω_{hi} is approximately equal to that one dominant rate. For this class of models, if there is no corner in the noise spectrum above $\omega = 0.3\ s^{-1}$ then that fast rate constant is not greater than $0.3\ s^{-1}$. The nonexistence of such a corner therefore eliminates this class of alternative mechanisms.

A simulation of the 'overshoot' experiment with a computer algorithm was done by Mikulecky, Huf, and Thomas [36] using the Virginia school's technique of network thermodynamics, and yields current-vs.-time curves of the right shape. Network parameters do not, however, correspond to any specific overshoot (negative feedback) mechanism.

5.2. Lindemann's evidence against 'sodium-loading'

Two experiments were performed by Lindemann and Gebhardt [6] essentially to eliminate sodium loading as the overshoot mechanism. (1) Current–voltage measurements using a current-pulse technique 8 s after the jump showed an increase in series resistance, ascribed to the apical membrane ([6], Fig. 5). The resistance increase reported is, however, not sufficient to account for the observed current fall-off. If, as these authors suggest, the resistance increase reflects channel closing, then channel closing does not explain the overshoot. (2) Open-circuit voltage following several seconds of drawing forward current was not appreciably dependent on the strength of that current. A doubling of forward current should, one might argue, double the amount of sodium transported into the cell from the apical bathing solution, with concomitant change in Nernst potential if Na collects inside. No estimate of the magnitude of the expected effect was given, however. The reported insensitivity to current history of the steady-state open-circuit voltage would be consistent with rather high levels of [Na] in an intracellular compartment, as well as with rapid diffusion of Na out of such a compartment. The theory of Section 3 above takes account of both conductive increase and diffusive decrease of the [Na] of the intracellular compartment immediately distal to the Na channel.

The mechanism of the resistance increase (experiment No. 1 above) has not been resolved in this paper. The technique is not without ambiguities. Passing a short current pulse while measuring the concomitant voltage change is likely to leave changed values of [Na] in the intracellular compartment. Some frogs show channel-closing rates 20 times as fast as others. (See our No. 289 in Table 1.) With a γ of 8 s, 63% of the channel closing would already have taken place 8 s after the jump.

5.3. Channel opening-closing vs. channel birth-death

The picture of apical Na channels is of protein molecules embedded in the lipid wall of the membrane. Such channels are surely dynamic, i.e., they appear and disappear randomly. The role of the apical [Na] in affecting the rates of channel birth and death is not known, but it can not be discounted. Death cannot properly be called a state of the channel; it is irreversible. For the noise spectrum, it makes a difference whether a channel closes and reopens, or whether it dies and another one is born. In the former there is a correlation between the closing and the opening; in the latter there is none. The probability that a new channel appear in a short time Δt is independent of whether an old one has just died or not. On the other hand, a channel can open only if it is closed. The birth-death process has only one time constant, call it the mean lifetime. The birth rate affects only the power level of the noise spectrum, not its shape. The obvious complication is that between birth and death a channel can close and open many times, and it can only close if it

has been born. The simplification is that the power spectra of the two processes simply add. The corner of the opening-closing process is the sum of the two rates [8], while the corner of the birth-death process is just the death rate. If the death rate is very different from that closing-plus-opening-rate sum, a separate Lorentzian (an additional corner) should appear in the noise spectrum. Again, the fact that no corner shows up tells us that all three of those rates are well below 0.3 s^{-1}.

5.4. Predictions and postdictions

The theory set forth in Section 3 above was developed to predict the time-course of short-circuit current. Yet it can be useful in the interpretation of a long-standing puzzle having to do with steady-state potentials investigated in this laboratory. Lindley and Hoshiko [20] measured open-circuit potentials as a function of apical [Na] and found that the limiting slope of the voltage vs. log[Na] curves (which were indeed nice and straight) was considerably less than the thermodynamically predicted 59 mV per decade when total cation concentration was high (120 mM). Of course, 'open circuit' does not mean that no currents flow. The measuring technique allowed time (15 min) for the establishment of a steady state after each change of apical [Na]. In the Lindemann jargon, it allowed time for 'sodium loading', i.e., increasing [Na] in the intracellular space, lowering the Nernst potential more the greater the apical [Na]. The same line of reasoning explains the time-course of the open-circuit voltage after the apical-[Na] jump.

We have found that skins of some species of frog usually do not show an overshoot: *Rana catesbeiana, Rana clamatans, Rana esculenta, Rana temporaria*. Skins of these frogs treated with various drugs (novobiocin, BIG), however, show overshoot much like the *R. pipiens*. The intention here is not to report these occasional experiments, but rather to explain them. All it takes is a long filling time σ and a short leakage time τ to suppress overshoot. On the other hand, the explanation of why cold inhibits overshoot [37] is probably not that simple.

The experiment with nystatin channels [19] showed current overshoot behavior with potassium jump as well as sodium jump in the apical bathing solution. On the one hand this was not surprising, since nystatin channels are not sodium selective. On the other hand, there is no way to explain those overshoots as channel closing. It seems unlikely that nystatin channels close in response to either high [Na] or high [K]. No attempt has been made to fit those current records with theoretical curves, but the cation-loading hypothesis would seem to provide the only reasonable explanation.

List of symbols

g = g_o[Na] = channel conductance, assumes that [Na] in the channel is equal to the apical [Na] for positive current and to the compartment [Na] for negative current

RT/F = 25 mV

V = volume of the virtual compartment (below Eq. [3])

σ = compartment filling time = VF^2/g_oRT

$u(t)$ = dimensionless sodium concentration in the virtual compartment (below Eq. [2])

τ = leakage time characterizing diffusive flow of Na out of the compartment into the surrounding intracellular space

u_{intra} = dimensionless sodium concentration in that intracellular space, assumed constant during the overshoot

$1/\beta$ = characteristic time for mixing new solution in the apical bath (Eq. [5])

ρ = characteristic time for diffusion of Na through the stratum corneum (very short, set = 0 in the analysis)

r_o = before : after ratio of apical sodium concentration = 0.1 mM/120 mM (Eq. [6])

$f(t)$ = theoretical fraction of apical sodium concentration buildup attained at time t after the jump (Eq. [7])

$y(t)$ = dimensionless sodium current, Eqs. [3a], [8], [10]

γ = characteristic time for channel closing (Eq. [9])

w = fraction of channels remaining open in equilibrium at the high apical [Na] after the jump (120 mM) (Eq. [9])

$F_{open}(t)$ = theoretical fraction of channels open at time t after the jump (Eq. [9])

G = measure of skin conductance at 120 mM apical sodium concentration (Eq. [4])

References

[1] Ussing, H.H. (1948) Cold Spring Harbor Symp. Quant. Biol. 13, 193–200.
[2] Kirschner, L.B. (1955) J. Cell. Comp. Physiol. 45, 61–87.
[3] Hoshiko, T. (1961) Biophys. Physiol. Pharmacol. Actions, 31–47 (Am. Assoc. Adv. Sci.).
[4] Lindemann, B., Gebhardt, U. and Fuchs, W. (1972) T.-I.-T.J. Life Sci. 2, 15–26.
[5] Fuchs, W., Gebhardt, U. and Lindemann, B. (1972) Biomembranes 3, 483–498; Fuchs, W., Gebhardt, U. and Lindemann, B. (1972) in Passive Permeability of Cell Membranes (Kreuzer, F. and Siegers, J.F.G., eds.), Plenum Press, New York.
[6] Lindemann, B. and Gebhardt, U. (1973) in Transport Mechanism in Epithelia (Ussing, H.H. and Thorn, N.A., eds.), Munksgaard, Copenhagen.

[7] VanDriessche, W. and Borghgraef, R. (1975) Arch. Int. Physiol. Biochim. 83, 140–142.
[8] Machlup, S. (1954) J. Appl. Phys. 25, 341–343.
[9] Hoshiko, T. (1975) J. Gen. Physiol. 66, 16a.
[10] Hoshiko, T. (1984) Curr. Top. Membr. Transp. 20, 3–26 (in particular p. 11).
[11] Lindemann, B. (1984) Annu. Rev. Physiol. 46, 497–515.
[12] Gebhardt, U. and Lindemann, B. (1974) Pflügers Arch. 347, 9–18.
[13] Li, K.Y., Palmer, L.G., Edelman, I.S. and Lindemann, B. (1982) J. Membr. Biol. 64, 77–89.
[14] VanDriessche, W. and Lindemann, B. (1979) Nature 282, 519–520.
[15] Fuchs, W., Larsen, E.H. and Lindemann, B. (1977) J. Physiol. 267, 137–166.
[16] Garty, H. and Benos, D.J. (1988) Physiol. Rev. 68, 309–363.
[17] Hoshiko, T. and Machlup, S. (1988) FASEB J. 2, A750.
[18] Machlup, S. and Hoshiko, T. (1991) Biophys. J. 59, 648A.
[19] Hoshiko, T., Hoshiko, K.L. and Machlup, S. (1985) J. Gen. Physiol. 86, 41a.
[20] Lindley, B.D. and T. Hoshiko, T. (1964) J. Gen. Physiol. 47, 749–771.
[21] Machlup, S. and Hoshiko, T. (1992) Biophys. J. 61, A535.
[22] Helman, S.I. and Miller, D.A. (1971) Science 173, 146–148.
[23] Klemperer, G., Garcia-Diaz, J.F., Nagel, W. and Essig, A. (1986) J. Membr. Biol. 90, 89–96.
[24] Machlup, S. and Hoshiko, T. (1990) Biophys. J. 57, 88a.
[25] Kidder, G.W., III, Cereijido, M. and Curran, P.F. (1964) Am. J. Physiol. 207, 935–940.
[26] Cotton, C.U. and Reuss, L. (1989) J. Gen. Physiol. 93, 631–647.
[27] Crank, J. (1956) The Mathematics of Diffusion, Oxford University Press, Oxford.
[28] Rick, R. (1984) J. Membr. Biol. 78, 129–145; Rick, R., Dörge, A., Von Arnim, E. and Thurau, K. (1978) J. Membr. Biol. 39, 313–331.
[29] Hill, T.L. and Kedem, O. (1966) J. Theoret. Biol. 10, 399–441.
[30] Bevington, P.R. (1969) Data Reduction and Error Analysis for the Physical Sciences, McGraw Hill, New York.
[31] Wills, N.K. and Zweifach, A. (1987) Biochim. Biophys. Acta 906, 1–31.
[32] Olans, L., Sariban-Soraby, S. and Benos, D.J. (1984) Biophys. J. 46, 831–835.
[33] Hoshiko, T., Grossman, R.A. and Machlup, S. (1988) Biochim. Biophys. Acta 942, 186–198.
[34] Lindemann, B. (1986) Proc. Int. Union Physiol. Sci. 30th Congress (Vancouver) 16, 343.
[35] Frehland, E., Hoshiko, T. and Machlup, S. (1983) Biochim. Biophys. Acta 732, 636–646.
[36] Mikulecky, D.C., Huf, E.G. and Thomas, S.R. (1979) Biophys. J. 25, 87–106.
[37] Hoshiko, T., Ambigapathy, R. and Machlup, S. (1989) Biophys. J. 55, 159a.