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Topical Review

The Beginning of Fluctuation Analysis of Epithelial Ion Transport

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Spontaneous fluctuations of membrane voltage and membrane current can provide insight into transport mechanisms and the mechanisms of transport control. For the past few years, the analysis of such fluctuations has also been applied with increasing success to problems of epithelial ion transport. In the epithelia studied, shot-noise controlled by first-order relaxation processes was found to be prominent. One of the elementary concepts needed for the analysis of such processes is that of fluctuating equilibrium reactions.

Restless Equilibrium

Nonperiodic chemical steady states and chemical equilibria are recognized by the constancy of reactant concentrations, but a close look at these concentrations reveals that they are not quite constant. As long predicted by statistical mechanics, measurements of high precision do not yield exactly reproducible values even in equilibrium situations but show the concentrations to fluctuate randomly around their equilibrium values, which are found by averaging over many observations. Thus, it is not so much the constancy of concentrations but the absence of systematic displacements of the mean concentrations which may serve for recognition of nonperiodic steady states and of equilibria.

The spontaneous fluctuations are understood as a basic consequence of the discontinuous "granular" nature of matter viewed as a many-body system (e.g., van Kampen, 1976). Their amplitude- and frequency-distribution carry information on system properties which can be retrieved by a statistical analysis. It is important to realize that the fluctuations are observed in macroscopic systems and that their standard deviation σ does not decrease when the system is made larger; on the contrary, it increases with system size. The frequency range of the fluctuations is determined by the rate constants of the reactions. Therefore, frequency analysis of randomly fluctuating equilibrium systems may in principle yield the same information as macroscopic relaxation experiments (fluctuation-dissipation theorem), as was convincingly demonstrated for transport processes through membranes (e.g., Zingsheim & Neher, 1974; Kolb & Bamberg, 1977). Consequently, to analyze for rate constants we need not perturb the equilibrium by small and fast (and expensive) step changes, because small perturbations occur spontaneously, although randomly, at temperatures above 0 °K.

From this theoretical concept arises some of the fascination which "noise analysis" has to offer. The experiment runs itself; the principle task of the experimenter – apart from observing – is merely to leave the system alone, to shield it from its surroundings. Another attractive aspect is that no fortune need be spent to make this possible: the most costly part of the equipment is a small nonspecialized computer which will already be available in most laboratories. Furthermore, the results are rewarding in that numerical values are found not only for rate constants but also for such interesting quantities as single channel transport rates and channel densities. (It goes without saying that these numbers – derived from kinetic data – depend on the model concepts used in their computation.) From here the analysis proceeds to describe the force-dependence of flow through single channels and the control mechanisms which may modify single-channel properties and particularly channel densities.

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Shots Make It Easy

Since the pioneering work of Verveen and Derksen (1965) many ion transporting systems of biological membranes were found to show unexpectedly intense current fluctuations, explained by thousands of ionic charges moving through a channel in correlated fashion, i.e., in a brief burst or "shot". Clearly such channels are not permanently open, and it appears that their staccato performance has to do with the control of permeabilities by membrane voltage, chemical effectors, etc.

In the fundamental two-state model a channel is either open (conducting) or fully closed, and these functional states convert into each other by an equilibrium reaction. Interference with this reaction changes the macroscopic permeability. The reaction may be thought of as a stochastic chopping process which generates a random sequence of "microscopic" pulses of net current (the "shots") of amplitude i. The pulse height will depend on the single-channel permeability and the electrochemical driving force acting on the permeant species. The superposition of many such pulses produced by N channels per membrane area is the macroscopic current density I:

$$I = iNP_o \tag{1}$$

where P_o, the probability of a channel to be in the open state, may be obtained from the law of mass action. The intensity of current fluctuations superimposed on this macroscopic (mean) current may be expressed by the variance per cm^2

$$\sigma_I^2 = Ni^2 P_o P_1 = IiP_1 \tag{2}$$

in which $P_1 = 1 - P_o$ is the probability to find a channel in the closed state. Thus, the variance increases linearly with the system size (N), while the "signal-to-noise ratio" (I/σ_I) grows proportionally to $\sqrt{N}$.

(It is easy to see that in a more general n-state model, in which all states conduct current to different degrees, the macroscopic current is given by

$$I = N \cdot i(a_o P_o + a_1 P_1 \ldots + a_n P_n) \tag{1a}$$

in which $a_o, \ldots a_n$ indicate dimensionless fractions of i and $P_o, \ldots P_n$ the probabilities of the states. If – to give an example – the n states are in series, this model will produce microscopic current pulses of amplitude $i(a_o - a_1)$, $i(a_1 - a_2), \ldots i(a_{n-1} - a_n)$.)

The statistical analysis of time series fluctuations requires constancy of system parameters. This means at least steady-state conditions for the shot-controlling reaction and the flow of the transported species. In the analysis of shot-noise arising in ion-transporting membranes it is typically the chopping reaction which is near equilibrium, while for the transported ionic species a mere steady state is of advantage. In fact, the more this steady state deviates from the electrochemical equilibrium, the larger will the microscopic current i be, and the more easily will the shot-noise be detected and analyzed (*see* Eq. (2)). Thus, the valve-effect of spontaneously switching channels acts like a molecular amplifier, a rather fortuitous property which has, of course, promoted the application of fluctuation analysis to ion transporting systems.

It will be shown below that, in cases where spontaneous switching does not occur or occurs at inconveniently low frequencies, a reversibly reacting channel-blocker added to the bathing medium can serve as an "extrinsic chopper". Current fluctuations from permanently open channels are much smaller, their analysis will require more technical and theoretical sophistication (e.g., Läuger, 1978).

The Ubiquitous Lorentzian

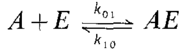
The shot-controlling reactions will often be of first order or second order, in which case the differential equations that described the response to small perturbations have exponential solutions. In the frequency domain exponential solutions are represented by so-called Lorentzians. These are rather common components of power density spectra of spontaneously fluctuating systems, where small perturbations are the rule. Lorentzian functions have the algebraic form

$$G(f) = G_o/(1 + f^2/f_c^2) \tag{3}$$

where $G(f)$ is the power density at frequency f, G_o the plateau power density at frequencies $f \ll f_c$ and f_c the corner frequency at $G(f) = G_o/2$. Thus, a Lorentzian power density spectrum (PDS) is recognized by a plateau at low frequencies followed at high frequencies by a rolloff with $1/f^2$, i.e., a slope of -2 in the double logarithmic plot. The integral of the PDS is the variance of the fluctuations and can be computed as $\sigma^2 = G_o \cdot f_c \cdot \pi/2$.

The appearance of a single Lorentzian component in a current PDS indicates that a linear first-order differential equation determines the microscopic and macroscopic response to small perturbations. Therefore, the time dependence of the shot-controlling process or reaction-generating power in the frequency band of the Lorentzian will be described by the single relaxation time constant, τ, of an exponential solution. τ depends on the reaction rate constants and concentrations, and by its concentration dependence the rate constants may be determined (this kind of rate analysis being a common procedure in kinetics).

To give an example, for the bimolecular, second-order reaction



the reaction rate (sum of the decay rates of all components) for small perturbations is

$$1/\tau = 2\pi f_c = k_{01}[A] + k_{01}[E(A)] + k_{10}. \qquad (4)$$

A linear dependence of $1/\tau$ on $[A]$ in a large range of $[A]$ would show that $[E]$ is negligible with respect to $[A]$ (i.e., that the reaction is of pseudo-first order) and would permit the estimation of k_{01} and k_{10}. Rate analysis is usually followed by an evaluation of the Lorentzian plateau, G_o. For a two-state "open-fully closed" system controlled by one reaction the plateau power density per cm^2 will be

$$G_o = 4N \cdot i^2 \cdot P_o \cdot P_1 \cdot \tau = 4I \cdot i \cdot P_1 \cdot \tau = 4\sigma_I^2 \tau. \qquad (5)$$

This relationship shows that the determination of I, τ (with f_c) and G_o already allows us to compute the shot-amplitude i if a reasonable theoretical estimate of P_1 can be provided. The splitting of the product $i \cdot P_1$ in Eq. (2) or (5) into its factors is a step where the model-dependence of the analysis becomes apparent, often by the introduction of an assumption about the value of P_1. Once i has been determined, N can be obtained from Eq. (1).

Two Series Membranes and a Shunt

The channel noise recorded from epithelia will not differ in principle from that of other membranes. Merely some technical considerations are peculiar for the noise analysis of these tissues:

1) *Area:* epithelia offer a large membrane area, and this is of advantage because – as we have seen from Eq. (2) – the fluctuation amplitude increases with the size of the system ($N \cdot cm^2$). However, a large area means a large capacitance which limits tolerable voltage clamp performance to low frequencies, and a large area is more sensitive to microphonic artifacts.

2) *Location of the source:* epithelial transport occurs through at least two membranes in series, and through the paracellular pathway which shunts these membranes. The experimenter must, of course, find out in which of these structural elements an observed noise component arises. Early attempts at epithelial noise analysis aimed at analyzing active transport (of the laterobasal membrane). Their conclusions were somewhat misleading, mainly because noise-generating passive transport steps through the apical series membrane were not taken into account.

In this context a pitfall should be mentioned which might be called "current-dependent contralateral noise power." When in short-circuited tight amphibian epithelia an apical current blocker like Cs or amiloride is applied, the mean short-circuit current is decreased rather rapidly. The current noise power may be observed to drop accordingly, but this does by no means show that the noise source resides in the apical membrane. Rather, the noise power of a laterobasal source would decrease equally promptly in response to a driving force dependent drop of its mean current (*see* Eqs. (2) and (5)).

3) *Macroscopic current:* many epithelia contain cells of differing transport function in parallel. Together with the paracellular shunt these cells constitute a complex mosaic membrane which supports intraepithelial current loops under so-called open circuit conditions. The loop-currents must be taken into account when transcellular "macroscopic" currents enter into the analysis (Eqs. (2) and (5)). Even under voltage-clamp conditions, loop-current may contribute to the transcellular current when the solution series resistance is a significant fraction of the total resistance seen by the voltage-sensing amplifier. The effect is particularly important in the analysis of leaky epithelia.

4) *Series impedance:* the spectral presentation (the PDS) of current noise arising in one of the membranes may be changed by the series membrane. This influence of the second membrane's impedance must be accounted for quantitatively at each frequency if it is not possible to shunt out one of the membranes for the duration of the experiment.

5) *Driving force:* the amplitude of shot-effects arising in one of the membranes can provide insight into the translocation mechanism when studied as a function of ionic concentrations and membrane voltage. For this purpose intracellular concentrations and voltages must be estimated, and this is difficult to do reliably with microelectrodes when the epithelial cells are small. Shunting of one membrane provides an alternative, but noise generation by the shunting pathway must be considered.

Lorentzian Shot-Noise from Frog Skin

The interesting paper by John R. Segal (1972) first described electrical fluctuations arising in an epithelium. Voltage fluctuations were recorded across frog skin at nine frequencies (0.025 to 12.5 Hz) with and without constant current passing through the tissue. NaCl-Ringer was used at the apical and corial side. Only a small area of $0.32\ cm^2$ was exposed, a trick which reduces microphonic artifacts. No attempt was made to eliminate one of the two functional mem-

branes. Without external current, the power density spectrum (PDS) of the voltage fluctuations was found to decrease towards high frequencies with a slope of -1.6 to -2 as is typical for Lorentzian shot-noise (Eq. (3)). At increased temperature (32 °C) the spectral slope at low frequencies decreased to values closer to -1. The corner frequency of this quasi-Lorentz spectrum indicated a shot-controlling random process with a relaxation time constant of about 0.6 sec. Surprisingly, the fluctuations became less intense when the tissue was depolarized by inward current, a measure which is expected to increase net Na uptake. The fluctuations disappeared when active transport was inhibited by ouabain, but re-appeared when in the presence of ouabain constant current was passed through the epithelium.

The interpretation given for these observations was that the fluctuations may arise from a fluctuating energy supply or directly from the active transport machinery, by binding of Na^+ to a carrier molecule which will then pass "across the skin" (*ibid.*, p. 1386), a notion which perhaps we should not take too seriously. The lesson from this pioneering paper is that ion transport noise can be recorded from tight epithelia, and that its PDS can resemble a Lorentzian.

There is widespread agreement that in the granular cells of tight amphibian epithelia Na ions pass passively through the apical membrane and are subsequently moved "uphill" into the interstitial space by ouabain-sensitive pump units residing in the inward facing "laterobasal" membranes (Koefoed-Johnsen & Ussing, 1958). Thus active transport, by keeping the cytosol Na level low, creates an electrochemical gradient across the apical membrane which allows net Na-uptake into the cell. It also creates a K-gradient across the laterobasal membrane. When the pump is blocked both gradients are diminished. A simultaneous attenuation of the fluctuations does not prove that they arise from the pump itself. The fluctuations may just as well arise from passive Na movement through the apical or passive K movement through the laterobasal membrane. The passive origin of the fluctuations becomes more likely when we hear that they reappear – in the presence of ouabain – during passage of constant current, since a ouabain-blocked pump cannot *a priori* be expected to conduct current.

Segal's observation that the voltage noise increases when outward current flows and decreases when inward current flows through the tissue is intriguing. This behavior would not be expected if Na transport were the only noise source, because both active and passive Na transport increases when depolarizing inward current is passed. It would be important to check whether the higher shot-noise level found on hyperpolarization is caused not by Na transport but by Cl transport. Indeed, Hoshiko (1978) has shown that with Cl-free solutions the power density does not decrease on depolarization, but increases as expected for a pure Na-transport system. Today there is indirect evidence suggesting that the density of apical Cl channels and the density of apical Na channels may change with membrane voltage. On hyperpolarization Cl channels appear to become more numerous (Hviid Larsen and Kristensen, 1978; Hviid Larson, 1980) while Na channels appear to decrease in number (Cuthbert & Shum, 1976). Thus for Na and Cl the channel densities possibly change together with the driving forces for the net ion flows which they support. Noise analysis will provide a more direct way of showing such voltage-dependent changes in channel density.

In Toad Bladder One Shot Moves Several Thousand Ions

Strandberg and Hammer (1975) deal with voltage fluctuations arising during active transport of Na ions in another tight amphibian epithelium, the toad urinary bladder. In this study, which used chambers of 3 cm^2 of geometrical membrane area, severe problems with microphonics were encountered, which could be overcome only by multifold shockmounting and sound-deadening. (The smooth muscle contractions of the tissue were not eliminated by depolarization or block, nor were bulk tissue movements arrested by the rather effective combination of a small hydrostatic gradient and a filter paper support.) No attempt was made to shunt out one of the membranes.

The method used (voltage fluctuations were obtained at 3 frequencies only) did not permit the construction of complete power density spectra. However, above 7 Hz the computed power current density decreased by the factor ($1/f^2$), indicating a Lorentzian (Eq. (3)). Thus, shot-noise originating from a first-order relaxation process seems to prevail in this preparation. The ionic charge transported per shot was estimated to 5000 and the mean shot duration to 20 msec at a channel density of 3 per μm^2 of membrane area. The shot effects were attributed to "active transport channels," a formulation which may just mean that they arise in the cellular transepithelial pathway.

Strandberg and Hammer first combined noise analysis with impedance spectra derived from the epithelial preparations. They passed up the chance, however, to use the impedance spectra for a separate estimate of the electrical properties of the two membranes in series, and the equivalent network employed – a highly lumped parameter circuit – did not en-

courage them to think of the two membranes as separate noise sources. Surprising is their finding that the computed macroscopic "pump current" became smaller when the epithelium was short-circuited. Nevertheless, the main result that the transepithelial Na uptake of toad bladder is accompanied by shot-noise and that each shot comprises thousands of ionic charges is of great significance.

The Shots Need Not Arise from Active Transport

Van Driessche and Borghgraef (1975) recorded open-circuit voltage noise from frog skin epithelium under much improved technical conditions. With the aid of a dedicated Fourier analyzer, 1000 frequencies could be monitored simultaneously. The power density spectra were of the $(1/f^2)$ type without a low frequency plateau when NaCl Ringers was used on both sides of the tissue. Flattening of the low-frequency part could be achieved by decreasing the apical Na concentration or by replacing Cl by gluconate. The authors conclude that the voltage spectrum contains a Lorentzian component arising in the apical membrane by the saturable passive Na uptake. A relaxation time of 0.4 sec was computed from the corner frequency. In the presence of Cl or at high apical Na concentrations the low frequency plateau of this Lorentz spectrum can be obscured by additive diffusional noise.

Later research has shown that Van Driessche and Borghgraef were quite right in suggesting that the Na-dependent Lorentzian noise source is located in the apical membrane. It is difficult, of course, to reach such a conclusion from the mere observation that the voltage PDS responds to changes in the apical Na concentration. This is because the granular cells of frog skin mounted open circuit in a chamber are partly shunted by several cellular and extracellular pathways (paracellular shunt, damaged edge areas, mitochondria-rich cells, glands). Therefore, supracellular loop-current must be expected to pass the granular cells in the inward direction even under overall open-circuit conditions. According to the principle of "current dependent contralateral noise power" mentioned on p. 3, current noise sources in either membrane may deliver less power when the transcellular current is lowered by decreasing the apical Na-concentration (Eq. (5)). More important than the anatomical location may be the functional origin of the noise. Here, Van Driessche and Borghgraef were the first to explicitly consider the possibility that the shot noise observed in tight amphibian epithelia need not be directly dependent on the activity of the ion pump.

We know today that the ouabain-sensitive Na/K-pump moves Na at least partly in exchange for K, and only few (certainly less than 10) Na ions per pump cycle (per ATP split). Thus, the "shot" i of each pump molecule cannot be larger than 10 charges. Above it was shown that the variance of fluctuations (Eq. (2)) depends on the first power of N but the second power of i. Therefore, shots comprising only 10 charges or less produce a small level of shot noise which will be hard to detect. I may add that if larger shots should be recorded from ion pumps, they are likely to reflect the kinetics of pump modulating mechanisms acting on many pump units at once. In this case their study will reveal properties of these control mechanisms rather than of the active transport process itself (*see also* Segal, 1972, p. 1389).

Current Clamp or Voltage Clamp

The results so far described were obtained from analyzing voltage fluctuations which occurred under current-clamp conditions. In principle such fluctuations, when recorded transepithelially, contain additive, uncorrelated voltage displacements from the apical and laterobasal membrane. The voltage displacements of each membrane will be attenuated with increasing intrinisc frequency by the factor $(1+(2\pi fRC)^2)^{-1}$, where R and C are resistance and capacitance of the membrane of origin. Therefore, the voltage power density spectrum of an epithelium may contain two Lorentzians (one from each membrane, if the two time constants are not equal) which arise from first-order impedance effects and do not signify shot-noise. The situation is recognized when the time constants of the two membranes or the complete impedance spectra are obtained. To correct the voltage PDS of one of the membranes for its impedance effect, the PDs must be divided at each frequency by the squared modulus of the impedance of this membrane, the result being a current power density spectrum.

Without net current passing the cell, a transport-modifying drug in contact with one membrane will affect the voltage power spectrum of only this membrane. However, as mentioned in previous sections, epithelia are, in principle, mosaic membranes, supporting small but supracellular current loops. Thus, a net current may flow transcellularly even when extrinsic current sources are switched off. Then a drug in contact with one membrane may affect the voltage PDS of the contralateral membrane by changing the chemical or electrical component of the driving force across the contralateral membrane, and this may easily cause misinterpretations.

A technical advantage of voltage noise is the rather high sensitivity which may be obtained when two parallel voltage amplifiers are used and their outputs cross-correlated. While cross-correlation of current noise is equally possible, it does not help in voltage-clamp situations where the unwanted noise does not arise in the current amplifier but in the voltage sensing input stage of the feedback amplifier. Here the best solution known today is to design low-noise input stages. They should be adapted to the impedance of the membrane which is expected in the frequency range of interest (DeFelice, Wanke & Conti, 1975). Examples for such designs are given in Van Driessche and Lindemann (1978).

Current noise measured during voltage clamp is band-limited by the membrane impedance because mainly the voltage sources of the clamp amplifier input stage generate more output noise at high frequencies, where the membrane impedance is small. This noise from the input stage is fed back to the preparation and

is added to the current fluctuations recorded from it. On the whole, the membrane capacitance determines the bandwidth which can be increased by reducing the membrane area.

The recording of current-rather than voltage-fluctuations seems attractive in that the shot amplitude (Eqs. (2) and (5)) is itself a current. Conversion of voltage noise to current noise by means of the impedance spectrum becomes unnecessary. However, this advantage is restricted to the use of single-membrane preparations. For the evaluation of current noise from epithelia with two series-membranes impedance spectra are again required, this time to account for the "series impedance effects." These effects may become apparent in the current PDS in the form of depressed low or high frequency power or both. Peaking of the spectrum at intermediate frequencies is possible (Lindemann & DeFelice, 1980[1]). Corrections can be applied after analyzing the impedance spectrum in terms of RC parameters of each membrane (e.g., Gögelein & Van Driessche, 1979). It is probably easier, however, to eliminate one membrane experimentally by shunting it with high conductance pathways.

The current noise output of voltage clamped leaky epithelia can be depressed by the solution series resistance R_s seen by the voltage-sensing clamp amplifier. The presence of R_s attenuates G_o in the PDS and causes i to be underestimated. R_s tends to be increasingly dominating at high frequencies, where the membrane impedances become small. The situation may be dealt with by placing the voltage electrodes very close to the epithelium, by feed-back compensation of R_s or by data correction which is best based on impedance spectra. Where R_s is left uncompensated the current loop between cellular- and shunt-pathway cannot be completely suppressed by the voltage clamp. As mentioned before, the "macroscopic current" passing the cells will then be the sum of an extrinsic and an intraepithelial component, and this sum must be used in the computation of N and i.

Patch Clamp through the Str. Corneum

Hoshiko and his colleagues have studied ion transport noise in the epidermis of the norther *Rana pipiens*. Large and small areas were used to obtain voltage and current noise spectra as well as admittance spectra over a wide range of frequencies. The voltage PDS obtained with large areas (0.05 to 10 Hz) in Na-sulfate Ringer's is quasi-Lorentzian with a slanted plateau and a low corner-frequency (about 0.2 Hz), as previously described by Segal (1972) and Van Driessche and Borghgraef (1975). The current PDS have a similar shape. On clamping from 0 mV to outside negative transepithelial voltages the current spectral power decreased, while Segal (1972) found the opposite behavior of voltage noise when using Cl-containing solutions. Amiloride depressed the low frequency power and caused a new noise component to appear at higher frequencies. This phenomenon was not studied in detail. No attempt was made to shunt out one of the series membranes, nor was the admittance spectrum used to estimate the time constants of the two membranes (Hoshiko & Moore, 1978; Hoshiko, 1978).

[1] Lindemann, B., DeFelice, L.J. 1980. Membrane impedance effects in power density spectra of electrical fluctuations arising in epithelia (*in preparation*).

To study the frequency band 5 to 1000 Hz, a patch voltage clamp of the apical membrane was attempted (Hoshiko, 1978; Hoshiko & Moore, 1978). For this purpose the str. corneum was covered with fluorosilicone oil through which a double-pipette of 100 μm tip diameter was pushed onto the outer epidermal surface. One barrel was used for voltage sensing and superfusion with different solutions, the second, parallel one for feedback of clamp current through the str. corneum. The area of apical membrane of the str. granulosum which was effectively clamped was estimated to 0.001 cm^2, i.e., about 12-times the area of the pipette opening. Above 50 Hz the open-circuit voltage PDS was found to be white (plateau at 10^{-13} V^2/Hz). Under voltage clamp the admittance spectrum showed a pronounced peak at about 100 Hz which was independent of clamp voltage but depended on Na being present in the outer solution. A peak was also apparent in the current PDS, and here it was seen to shift to higher frequencies on depolarization. The high-frequency rolloff of this peak was close to $1/f^2$.

The 100-Hz peaking of the current PDS and the admittance spectrum is an interesting phenomenon not previously observed with these preparations. The authors tend to explain it by resonance phenomena in the apical membrane, perhaps related to the excitation response of this membrane (Finkelstein, 1964). This would account for the dependence on apical Na, but would require a strong voltage dependence of the admittance peak which was not observed. It is also noteworthy that these admittance peaks were not found with large areas (e.g., Smith, 1971). An equivalent inductance of 0.761 H·cm^2 was calculated (Hoshiko, 1978).

Saturation of Current: Limitation in i or N_o?

The macroscopic Na flux and Na current passing the apical membrane of frog skin epithelium is not a linear function of the apical Na concentration, $(Na)_o$, but saturates when $(Na)_o$ is increased. Since the discovery of this phenomenon by Ussing (1949) and Kirschner (1955), several attempts were made to find an explanation. Cereijido, Herrera, Flanigan and Curran (1964) concluded that the saturation is not caused by a decrease of the force driving Na through the apical membrane but by a decrease of the apical Na permeability, P_{Na}. Considering that P_{Na} is a product of the single-channel permeability and the number of conducting channels, this result leads to two new questions: (i) is it the single-channel permeability which decreases or the density (N_o) of conducting channels? and (ii) if the channel density decreases, which parameter (concentrations, membrane potential)

controls the channel density directly? Fuchs, Hviid Larsen and Lindemann (1977) have observed that the current saturation is restricted to the steady state. In the transient state following a step increase of $(Na)_o$ the current can be much larger than the saturation curve predicts. This result was taken to show that a limitation in the translocation rate of the Na channels themselves is not responsible for the saturation. A decrease in the number of conducting channels remained to explain the current saturation. The new idea thus introduced was that at high apical Na concentrations (Ringer's) most Na channels are closed and only a small fraction conducts current. The relaxation time constant for a change in the number of conducting channels in response to a step change of $(Na)_o$ was found to be in the order of seconds. As the cellular Na activity, estimated with current-voltage curves, changed little in this time and membrane voltage was kept approximately constant, binding of Na to apical receptor-groups followed by a conformational change of the channel protein was held responsible for channel closure (Fuchs et al., 1977; *see also* Lindemann, 1978). Some chemicals like PCMPS (parachlormercuriphenylsulfonate) and BIG (benzimidazolylguanidin) were seen to increase P_{Na} by suppression of this Na-selfinhibition effect (Dick & Lindemann, 1975; Zeiske & Lindemann, 1974).

From these results a number of predictions for the fluctuation behavior of the apical membrane can be derived:

(a) The PDS of Na-current fluctuations should contain one Lorentzian indicating the relaxation time constant of the Na-selfinhibition effect. (More Lorentzians may be found if additional channel blocking occurs, effected for instance by protoplasmic Na or Ca ions.)

(b) The density of conducting channels (N_o), as computed from power density spectra, should be found smaller at higher $(Na)_o$.

(c) The computed single-channel currents should not saturate in the $(Na)_o$ range where the macroscopic current saturates.

(d) PCMPS and BIG should not increase the single-channel currents, but the density of conducting channels.

All of these predictions have meanwhile been investigated and to some extent verified by noise analysis. Prediction a does in retrospect provide a theoretical basis for the quasi-Lorentzian PDS found in the presence of apical Na by Segal (1972), Strandberg and Hammer (1975), Van Driessche and Borghgraef (1975) and Hoshiko (1978). Unfortunately, these presumed "Na-channel spectra" very seldomly show a truely horizontal plateau at low frequencies, but merely a decrease in slope to values scattering around −1. Effects like concentration polarization may be involved (*See* Neumcke, 1975), but the difficulty of obtaining reliable low-frequency data has so far prevented a convincing analysis of the phenomenon. The corner frequency of below 1 Hz is in the range expected from the macroscopic P_{Na}-relaxation data and the shift of the PDS obtained when changing $(Na)_o$ is as expected from the Na-selfinhibition mechanism (Lindemann & Van Driessche, 1978). A quantitative evaluation of the Na-channel spectrum itself in terms of channel densities and single channel currents has not yet been possible. These parameters could be obtained, however, by evaluation of the fluctuations caused by the intermittent blocking action of amiloride.

A Use for Random Blockers

The amidino-pyrazine diuretic amiloride is known as a reversible blocker of apical Na uptake in tight amphibian epithelia (Ehrlich & Crabbé, 1968). At the molecular level the reversible action of such drugs may be envisaged as a random block-unblock sequence experienced by individual Na channels. This intermittent channel closure causes an easily recognized fluctuation component to appear in the short-circuit current of frog skin epithelium. The PDS is Lorentzian. When the blocker concentration is increased, the plateau-power density decreases and the corner frequency increases. Plotted against the amiloride concentration, the corner frequencies yield linear relationships (in the concentration range 2–12 µM) from which the on- and off-rate constants of the blocking process can be estimated by means of Eq. (4) (Lindemann & Van Driessche, 1977). Mean blocking times of 100 to 300 msec were found for amiloride itself (room temperature). Analogues of amiloride (very kindly provided by Dr. E. J. Cragoe, Jr., Merck, Sharp & Dohme) were found to have shortened blocking times if modified at position 6 or 5 of the pyrazine ring (Li & Lindemann, 1979). It appears likely, therefore, that the amiloride molecule attaches itself to the Na channel through ligands of the pyrazine ring. The shift of corner frequency with drug concentration and the dependence of corner frequency on drug structure shows that the amiloride induced fluctuations are of apical origin. A contralateral noise source would respond to the change in transcellular current primarily by a shift of its Lorentzian plateau (provided its rate-constants are not voltage-dependent).

Rate of Na-Translocation through Single Channels

In order to interprete the shot amplitudes generated by amiloride blockage, membrane voltage and Na

concentrations at both sides of the apical membrane have to be known. In our experiments membrane voltage was controlled by a transepithelial voltage clamp while a high K concentration was present at the serosal side. Impedance spectra have meanwhile shown that high serosal K shunts the laterobasal membrane so much that its resistance becomes negligible (Warncke & Lindemann, 1979 and *unpublished*). Fuchs et al. (1977) have used the same K-depolarization method and estimated the voltage error at the apical membrane to less than 10 mV. By means of current-voltage curves, Fuchs et al. estimated the cellular Na activity (at the apical membrane) to values of about 1/10 the activity in the apical solution if the pump is not inhibited. In the presence of amiloride the cellular Na activity should be even smaller and the apical resistance even more dominating. We may expect, therefore, that in the K-depolarized, short-circuited epithelia the membrane potentials are not too far from 0 mV and that Na translocation is driven by the apical Na gradient which at small cellular Na activity is governed by $(\text{Na})_o$. When one of the membranes in series is to be shunted, a noise contribution from the shunt pathway thus introduced must always be considered. In the case of K shunting a possible shot-noise contribution from laterobasal K-channels will be small as long as K is close to equilibrium, but will increase with the macroscopic current.

Evaluation of the plateaus of the amiloride PDS (Eq. (5)) yielded for $(\text{Na})_0 = 60$ mM shot amplitudes of $i \approx 0.2$ pA which, based on a two-state model, were interpreted as single-channel Na currents. Their value corresponds to Na translocation rates of more than 10^6 Na ions per second through single open channels. Lindemann and Van Driessche (1977), following Armstrong (1975*a, b*), have argued that this high translocation rate is in keeping with a pore-mechanism but excludes Na transfer via mobile carriers, from which lower rates are expected. While better evidence for or against a pore mechanism is certainly desirable, it remains noteworthy that the apical Na channels show a high translocation rate at the very modest driving force specified above.

The dependence of single-channel currents on the apical Na concentration was found to be linear up to $(\text{Na})_o = 60$ mM, although the macroscopic Na current saturated in this concentration range (Van Driessche & Lindemann, 1979). This result verifies prediction *c*, and shows directly that the current saturation is not due to a limitation in the translocation rate of the Na channels.

The voltage-dependence of the apical single channel Na currents has not yet been investigated. From the macroscopic results of Fuchs et al. (1977), a curvilinear relationship conforming to the constant-field equation would be expected for a near-instantaneous single-channel $i(V)$ curve. Time-series noise-analysis, of course, will provide primarily steady-state relationships.

Apical Na-Channel Densities

When Eq. (5) is applied to plateau values of amiloride PDS the parameter "N" includes all channels accessible to amiloride at the given Na and amiloride concentration, i.e., those not blocked (N_o) or blocked by amiloride (N_2), but not fraction N_1 blocked by the Na-selfinhibition effect. By performing this evaluation at different $(\text{Na})_o$, it became apparent that the amiloride-accessible fraction $N_o + N_2$ increased when the amiloride concentration was made larger, but decreased when at the same amiloride concentration $(\text{Na})_o$ was made larger (Lindemann & Van Driessche, 1977; Van Driessche & Lindemann, 1979). This behavior verifies prediction *b* and shows that the Na-selfinhibition effect is essentially a decrease in the density of open channels. The result is compatible with the view that Na and amiloride act as competitive blockers. A competition between Na and amiloride was first noted in dose-response curves by Cuthbert and Shum (1974), but the channel density data suggest more specifically that competition between two blockers is involved. This means that in the conservative relationship

$$N = N_o + N_1 + N_2$$

a simultaneous block ($N_{1,2}$) by Na and amiloride is excluded. N can be estimated by extrapolation to infinite amiloride concentration. Proceeding in this way, we computed for *Rana ridibunda* total apical Na channel densities of no more than $50/\mu\text{m}^2$ of geometrical chamber area (Lindemann & Van Driessche, 1977).

Chen's Shortcut in Kinetics

If Na and amiloride are competitive blockers, a double-Lorentzian PDS will be expected, indicating two relaxation time constants of which the smaller one belongs to the observed "amiloride PDS" and the larger one to the "Na-PDS". Then a plot of $(1/\tau)_{\text{high}}$ against the amiloride concentration must be evaluated by an expression which is more complicated than Eq. (4), containing four rate constants and all concentrations of the three-state system. The expression (eigenvalues of the determinant of the reduced relaxation rate matrix) has been derived (Lindemann & Van Driessche, 1978), but it was shown experimentally that

– owing to the small on-rate constant of the Na block – Eq. (4) is in error by no more than 1 Hz (Van Driessche & Lindemann, 1979). The plateaus of the double-Lorentzian were derived analytically by the eigenfunction method (Lindemann & Van Driessche, 1978), but a more elegant way is provided by the matrix method of Yi-Der Chen (1975). Here the power spectrum is directly obtained from the relaxation rate matrix of the differential equations of the means. The advantage is that computer solutions for a large variety of kinetic problems can be obtained elegantly by using matrix notation in the program and that power spectral data can be analyzed by inverting the algebraic procedure. Since based on linear or linearized differential equations, the method will always yield solutions in the form of one Lorentzian or a set of superimposed Lorentzians. However, the combination of only a few Lorentzians can already result in power density spectra which look decidedly non-Lorentzian.

Regulation by Channel Density

Once rate analysis of corner frequencies has provided information on the time behavior of the blocking process, the amplitude information of the PDS will be used to compute i and N. The estimation of these parameters opens the way for a subsequent investigation of the mechanisms which control permeability. In the case of amiloride-blockable Na channels, a local control of permeability appears to be effected by $(Na)_o$, which somehow decreases the density of open channels. The cellular Na or Ca activity may have a similar effect, but as yet noise analysis has not shown this directly. As mentioned above, the $(Na)_o$-selfinhibition effect is to a large extent abolished by P_{Na}-increasing chemical agents like PCMPS and BIG. In accordance with prediction d our results show that these agents increase the density of open channels but do not increase the single channel current (Li & Lindemann, 1980). The apical Na-permeability of amiloride-sensitive tight amphibian epithelia is controlled by several hormones, particularly by the antidiuretic hormone (ADH) and by aldosterone. Working with the urinary bladder of *Bufo marinus*, we have made a first attempt at elucidating the ADH effect and found that the stimulation of P_{Na} effected by ADH involves an increase in the total channel density (N) but not an increase of single-channel currents (Li et al., 1979). Presently, no case is known where a change in permeability is effected by a graded change of single-channel currents.

Apical K-Channels

The gallbladder of the toad was the first leaky epithelium subjected to noise analysis. Van Driessche and Gögelein (1978) found its apical membrane to contain spontaneously switching K channels. Their Lorentzian shows a corner frequency of about 4 Hz which stays constant when the K concentration of either solution is changed. The channels can be blocked by tetraethylammonium (TEA) added to the mucosal solution, and this is in contrast to the K channels of squid axon, which are blocked by TEA only from the protoplasmic side. Ouabain also suppresses the K fluctuations, probably by a secondary decrease of the driving force for passive K flow across the apical membrane. The same suppression was observed with metabolic inhibitors, but the Lorentzian re-appeared when a large K gradient was applied across the epithelium. When the mucosal K concentration of unpoisoned gallbladders was increased, the fluctuation amplitude decreased, then increased again at higher K concentrations. This interesting behavior suggests that the noise power has a minimum where the apical Nernst potential for K equals the membrane potential, as expected from theory.

The mechanism of the spontaneous switching behavior is not yet known. Since a change of corner frequency could not be effected by changing K concentrations, these or the membrane potential are not likely to be responsible. An increase in corner frequency was observed only during partial pump inhibition by ouabain. It would be interesting to check, therefore, whether a protoplasmic constituent (like Ca?) which increases its concentration when the Na/K pump is blocked, is instrumental in the spontaneous switching. Channel densities and single-channel currents were evaluated for *necturus* gallbladder (Gögelein & Van Driessche, 1979) after correction for series impedance effects.

K channels with somewhat different properties were found in the apical epidermal membrane of the frog species *Rana temporaria*. In parallel to the Na-transport channels, this membrane contains a passive K-specific pathway which can be reversibly blocked by H^+, Rb^+, Cs^+ or Ba^{++} present in the apical solution (Hirschmann & Nagel, 1978; Zeiske & Van Driessche, 1979*a*). In short-circuited epithelia the inward K-current increases with the apical K concentration, $(K)_o$, but saturates at a few $\mu A/cm^2$. It seems to be strongly dependent on the apical membrane potential and disappears when this potential is reduced by increasing the interstitial K concentration.

Noise analysis has shown that these K channels also change randomly between an open and closed state (Van Driessche & Zeiske, 1978; 1980). The cor-

ner frequency is about 80 Hz and not affected by the apical K concentration. Like an increase of the interstitial K concentration, an increase of apical Na uptake (removal of amiloride) decreases the Lorentzian plateau, showing that the fluctuations arise in the cellular pathway and that i_K may be partly driven by the apical membrane potential. Addition of apical Cs depresses corner frequencies and plateaus, as expected from a high-rate competitive blocker. In contrast to Cs, apical Ba was found to be a low-rate competitive blocker. Ba induces a second Lorentzian of a lower corner frequency which increases with the Ba concentration (Zeiske & Van Driessche, 1979*b*).

The mechanism of the spontaneous switching of these apical K channels is not yet clear. One possibility would be that an intermittent block by Ca ions is involved. Another interesting aspect of the K-kinetics is the saturation of the macroscopic K-current at high apical K-concentrations. Noise analysis could show whether effects like an adjustment of channel density are responsible, or whether i_K itself saturates. Interestingly, G_o is reported to increase superproportionally with $(K)_o$, an observation which would rather point to nonsaturating single-channel currents.

Can Frogs be Wrong?

The physiological significance of the apical K channels of *R. temporia* is uncertain. The epidermis of *R. esculenta* is reported to have few such channels, if any, and nonmoulting epithelia of similar function, like the urinary bladder of *B. marinus,* have none at all (Van Driessche & Zeiske, 1979). Concluding that the spontaneously switching K channels actually reside in the apical membranes of granular cells, Van Driessche and Zeiske (1978) touch on the question whether these channels are there by mistake or by design. The K channels could be left over from the premoult period in which their cells were not yet linked by zonulae occludentes and presumably completely surrounded by a highly K-permeable "laterobasal" membrane. In this case the continued presence of K channels in the apical membrane might be a logistic error of granular cells which otherwise conform to the Koefoed-Johnsen/Ussing scheme. Could a greater compliment be paid to the two physiologists?

Conclusion

Noise analysis is increasingly used to investigate transfer and control mechanisms of epithelial ion transport. The early results with tight and leaky epithelia are restricted to those processes of passive Na and K transport which generate shot-noise by channel switching in the presence of ionic gradients build-up by active transport. At least the amiloride-blockable Na channels show the high rate of translocation at modest driving forces which is in keeping with a pore mechanism. Where investigated, the regulation of permeability appears to involve a change of channel density rather than a graded change of single-channel properties. For the future, noise analysis of paracellular pathways, of anion transport, of nonswitching membrane channels, of transport noise in electrochemical equilibrium, and of the active ion transport process itself may be expected.

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