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Turnover, Membrane Insertion, and Degradation of Sodium Channels in Rabbit Urinary Bladder

Abstract. *Noise analysis of rabbit bladder revealed two components: Lorentzian noise, arising from interaction of amiloride with the Na^+ channel, and flicker noise (1/f, where f is frequency), as in other biological membranes. Hydrostatic pressure, which causes exchange between intracellular vesicular membrane and apical membrane, increases the number but not the single-channel current of the amiloride-sensitive channels. Flicker noise arises from degraded channels that have lost amiloride sensitivity and Na^+ to K^+ selectivity. The degraded channels were selectively removed by washing the mucosal surface. These results imply channel turnover by intracellular synthesis, transfer from vesicular to apical membrane, degradation, and elimination.*

Most proteins are subject to molecular turnover, which serves at least two functions: regulation of protein concentration by the balance between rates of synthesis and degradation of the protein and elimination of damaged or defective protein molecules (*1*). Among ion permeation channels, turnover has been demonstrated only for the acetylcholine receptor and gap junction (*2*). Yet turnover would seem to be essential for regulating hormonally controlled channels and for channel repair in epithelia exposed to fluids with varying composition and damaging chemicals (for example, urine). We now provide electrophysiological evidence for turnover of Na^+ channels in rabbit urinary bladder.

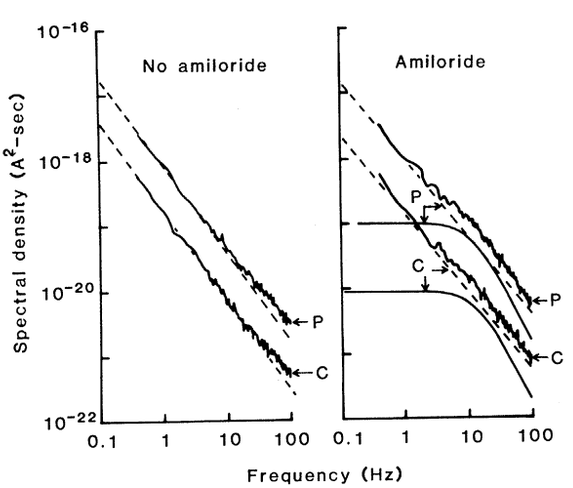

Fig. 1. Power density spectrum of a rabbit bladder, with (right) or without (left) amiloride (1.4 μM), and before (*C*) or after (*P*) punching. Data (wiggly curves) were fitted to the equation $S(f) = B/f^{\alpha}$ without amiloride, $S(f) = B/f^{\alpha} + S_o/[1 + (f/f_c)^2]$ with amiloride. The B/f^{α} component (1/*f* noise; dashed lines) is essentially the same with and without amiloride, whereas punching increases B but not α. Punching increases S_o but not f_c of the $S_o/[1 + (f/f_c)^2]$ component (Lorentzian noise; solid smooth curves).

Rabbit bladder maintains Na^+ gradients between urine and blood by active Na^+ absorption, which is regulated by aldosterone and can be inhibited partly but not entirely by the drug amiloride (*3*). The epithelial cells contain vesicles, which move between cytoplasm and the apical (lumen-facing) membrane and thereby accommodate the membrane area to fluctuations in bladder volume (*4*). Movement of vesicles depends on microfilaments and can be produced experimentally by rapid, repeated application of hydrostatic pressure to the apical surface ("punching") (*5*). This procedure increases short-circuit current (I_{sc}) and transepithelial conductance (G_t), suggesting that Na^+ entry channels are being transferred from vesicular to apical membrane.

We studied channels by the technique of noise analysis, which measures fluctuations in I_{sc} due to reversible blocking of channels by amiloride. Following the method of Lindemann and Van Driessche (*6*), we assume N statistically independent channels, each with single-channel current i. Combination of amiloride (A) and receptor (R) to form a blocked channel (AR) is described by

$$A + R \underset{K_{10}}{\overset{K_{01}}{\rightleftarrows}} AR$$

where K_{01} and K_{10} are rate constants. The residual amiloride-sensitive current (I_A') at amiloride concentration A, is

$$I_A' = iNK_{10}/(K_{01}A + K_{10}) \quad (1)$$

Fluctuations in I_A' yield a power density spectrum $S(f)$ (f, frequency in hertz) with a single time constant; that is, a Lorentzian spectrum $S(f) = S_o/[1 + (f/f_c)^2]$. The corner frequency f_c and plateau value S_o are

$$f_c = (K_{01}A + K_{10})/2\pi \quad (2)$$

$$S_o = 4iaI_A' K_{01}A/(K_{01}A + K_{10})^2$$
$$= 4Ni^2aK_{01}AK_{10}/(K_{01}A + K_{10})^3 \quad (3)$$

where a is membrane area.

We first determined S_o and f_c at five amiloride concentrations. Linear dependence of f_c on A yielded K_{01} and K_{10} by Eq. 2. Values of i and N were then determined from Eqs. 1 and 3 for each A value, and all five values were averaged (*7*).

Rabbit bladder epithelium, stripped of underlying muscle, was mounted between identical solutions at 37°C in chambers (area, 2 cm^2) designed to eliminate edge damage [see (*3*) for details of methods]. Values of I_{sc} and G_t were

normalized to transepithelial capacitance [determined as in (*5*)] as a measure of real membrane area (1 μF capacitance ≈ 1 cm^2 of area). Punching consisted of draining both chambers (volume, 15 ml each), then rapidly filling and emptying the mucosal chamber ten times to compress the epithelium against its supporting nylon mesh. The dose-response curve for inhibition of I_{sc} by amiloride followed Michaelis-Menten kinetics with a Michaelis constant (K_m) of 0.25 ± 0.11 μ*M* (mean ± standard error; $n = 3$). Average values of the total amiloride-sensitive current (I_A) and amiloride-insensitive or leak current (I_L) were, respectively, 0.72 ± 0.12 μA/μF ($n = 8$) and 0.61 ± 0.12 μA/μF ($n = 8$) for control bladders.

The protocol in studying channel transfer was to determine values of i and N before and after punching. Figure 1 illustrates the measured power density spectra (PDS). In the absence of amiloride, the PDS was of the form $S(f) = B/f^{\alpha}$, with $\alpha = 1.26 \pm 0.05$ ($n = 8$) and B (the intensity at 1 Hz) = (5.2 ± 1.4) × 10^{-19} A^2-sec ($n = 8$). A similar noise component with an α of 1 to 2 has been observed in many other biological membranes and is termed 1/*f* noise or flicker noise, but its origin is not understood (*8*). Addition of amiloride yielded an additional, Lorentzian, component. We fitted the total spectrum by a nonlinear least-squares algorithm to the equation $S(f) = B/f^{\alpha} + S_o/[1 + (f/f_c)^2]$; B and α were found to have the same values as in the absence of amiloride. Thus, amiloride did not affect 1/*f* noise.

The corner frequency f_c increased linearly with amiloride concentration as predicted by Eq. 2, yielding K_{01} = 52 ± 7 sec^{-1} μM^{-1} ($n = 3$) and K_{10} = 12 ± 1 sec^{-1} ($n = 3$). This corresponds to a mean lifetime ($1/K_{10}$) of 86 msec for the amiloride-blocked channel. The plateau value S_o varied biphasically with amiloride concentration as predicted by Eq. 3, yielding $i = 0.64 \pm 0.06$ pA ($n = 3$), $N = (2.0 \pm 0.1) \times 10^6$ channels per microfarad ($n = 3$). N is of the order of only 40 channels per cell at an external Na^+ concentration of 137 m*M*. (Since Na^+ itself is a channel blocker, channel density is higher at lower Na^+ concentration.)

Punching increased both I_A (by 11.9 ± 6.4 times; $n = 6$) and I_L (by 2.5 ± 0.8 times; $n = 6$), without affecting K_m for amiloride inhibition of I_A (0.30 ± 0.04 μ*M*; $n = 11$). The effect of punching on noise spectra (Fig. 1) was to increase the plateau value S_o of Lorentzian noise and the intensity B of 1/*f* noise by 12.6 ± 5.1 times ($n = 6$) and 3.3 ± 1.1 times ($n = 6$), respectively. These factors are approximately the same as the factors by which I_A and I_L, respectively, increase. This correspondence between the increases in S_o and I_A arises because both are proportional to the channel number N, which increases by the same factor. There was no change in α, f_c, or calculated values of i, K_{01}, and K_{10}. Thus, the increase in I_A by an order of magnitude due to punching arises solely from an increase in density of the amiloride-sensitive channel (S), with no change in single-channel properties. This effect of punching, together with previous morphological and electrophysiological evidence (*4, 5*), supports the view that S channels are synthesized intracellularly, stored in vesicular membrane, and transferred from vesicular to apical membrane.

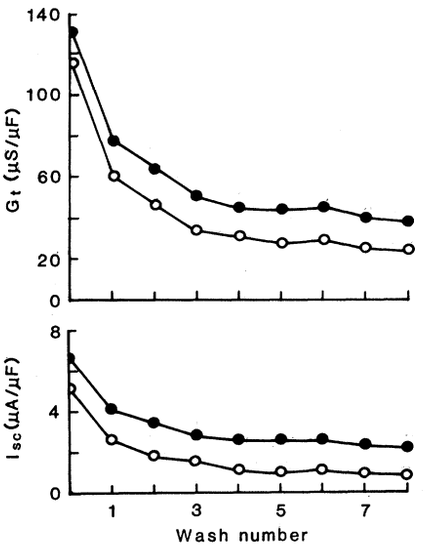

Fig. 2. Effect of eight consecutive 200-ml washes of the mucosal surface on G_t and I_{sc}, in the presence of (○) and absence (●) of amiloride (1.4 μ*M*). The difference between the curves with and without amiloride is the amiloride-sensitive G_A and I_A. These are unaffected by washing, which decreases only the amiloride-insensitive G_L and I_L.

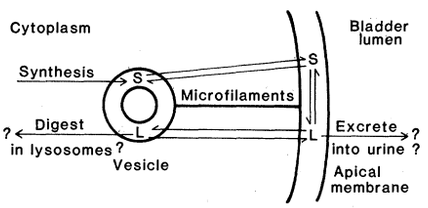

Fig. 3. Model of Na^+ channel turnover, membrane insertion, and degradation. The cytoplasm contains vesicles connected to each other and to the apical membrane by microfilaments. Fresh Na^+-selective amiloride-sensitive channels (*S*) are synthesized in the cell and transferred to vesicular membrane. Contact between vesicular and apical membrane permits channels to equilibrate between the two membrane pools. In the apical membrane the S channels become degraded into nonselective amiloride-insensitive leak channels (*L*), which are eventually destroyed (whether by excretion into urine or by intracellular digestion in lysosomes is unknown).

The correspondence between the increase in the intensity B of 1/*f* noise and the increase in I_L, and a linear correlation between B and I_L measured in different bladders, suggest that 1/*f* noise might arise from the amiloride-insensitive leak pathway L. A direct test of this hypothesis requires a means to vary this pathway in a single bladder. We found that washing the bladder's mucosal surface reduces I_L and offers such a means. To wash either surface of the bladder, we completely changed the contents of the chamber at that surface by injecting 200 ml of fresh solution isovolumically while removing solution at the same rate. Successive washes of the mucosal surface reduced both I_{sc} and G_t to asymptotic values (Fig. 2). This effect was due entirely to a reduction in the amiloride-insensitive components of I_{sc} (that is, I_L) and of G_t (that is, G_L); I_A and the amiloride-sensitive component of G_t (that is, G_A) were unaffected. Washes of the serosal surface were without effect on I_{sc} and G_t (*9*). Noise spectra showed that α, f_c, and S_o were unaffected by mucosal washing but that B decreased by approximately the same percentage as did I_L (by 62 ± 4 percent and 55 ± 6 percent, respectively; $n = 8$). This confirms that 1/*f* noise arises from L pathways.

Several observations imply that the amiloride-insensitive current I_L arises from a degraded form of the channel producing the amiloride-sensitive current I_A:

1) The ratio of the densities of S to L channels is greater in vesicles than in apical membrane, as punching increases the ratio I_A/I_L from about 1 in unpunched bladders to about 9 for punched bladders. Yet apical membrane is completely exchangeable with cytoplasmic vesicular membrane (*5*). Hence S channels are lost or L channels are gained, or both, in the apical membrane.

2) In five preparations we observed loss of I_A and approximately equal gains of I_L spontaneously or after voltage shocks.

3) The selectivity ratio P_{Na}/P_K is about 30 for the S channel in vesicular membrane (*5*), 2.6 to 9 for the S channel in unpunched apical membrane (*10*), and 0.7 for the L pathway [(*10*) and present results]. This suggests that the channels lose Na^+ to K^+ selectivity as they lose amiloride sensitivity. However, we find that both G_A and G_L are blocked by serotonin, an indication that S and L channels share a serotonin binding site.

Thus, channel turnover involves synthesis, insertion into apical membrane

via vesicular membrane, and degradation and elimination (Fig. 3). Continual degradation of channels ensures that they will not persist after disappearance of the physiological stimulus for their synthesis (aldosterone). It remains unknown whether degradation is enzymatic or accelerated by agents in urine, and whether degraded channels are then broken down intracellularly like other proteins involved in turnover or discarded into urine as suggested by the effect of washing on I_L.

Vesicular membrane may be a common vehicle for transferring hydrophobic channels from intracellular sites of synthesis to the plasma membrane. If so, the scheme of Fig. 3 may be relevant to other transport processes that involve vesicular delivery systems (*11*).

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7. A low-noise voltage clamp based on a dual matched pair of JFET amplifiers (*6*) [D. D. F. Loo, *Biophys. J.* **41**, 400 (Abstr.) (1983)] held transepithelial voltage to zero. The a-c component of the current was amplified to a sensitivity of at least 10^8 V/A, cut off at 100 Hz, and sampled every 5 msec to 12-bit resolution. The digitized signal was divided into 64 records of 512 words each, and the power density spectrum for each record was computed by a fast-Fourier-transform program. Spectra of 64 records were averaged.
8. Examples include myelinated nerve [A. A. Verveen and H. E. Derksen, *Kybernetik* **2**, 152 (1965)], squid axon [H. M. Fishman, L. E. Moore, D. J. M. Poussart, *J. Membr. Biol.* **24**, 305 (1975)], frog skin (*6*), *Necturus* gallbladder [H. Gögelein and W. Van Driessche, *J. Membr. Biol.* **60**, 187 (1981)], toad urinary bladder [J. H. Y. Li, L. G. Palmer, I. S. Edelman, B. Lindemann, *ibid.* **64**, 77 (1982)], and rabbit colon [W. Zeiske, N. K. Wills, W. Van Driessche, *Biochim. Biophys. Acta* **688**, 201 (1982)]. For discussion, see B. Neumcke [*Biophys. Struct. Mech.* **4**, 179 (1978)].
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Human Milk Kills Parasitic Intestinal Protozoa

Abstract. Giardia lamblia, *a common pathogenic intestinal parasite of humans, was rapidly killed by exposure to normal human milk in vitro. The killing did not depend on secretory immunoglobulin A.* Entamoeba histolytica, *the dysentery amoeba, was also killed by normal human milk.* Giardia*-cidal activity cochromatographed with an unusual lipase that is present in the milk of humans but not of lower mammals. Human milk may play a protective role in infants exposed to this parasite.*

Giardia lamblia is a major cause of waterborne enteric disease in the United States. Giardiasis may be debilitating, with severe diarrhea and malabsorption. Alternatively, symptoms may be moderate to mild or absent. Infection with this parasitic protozoan is especially prevalent in children and may cause failure to thrive. Some infections are resolved "spontaneously" within a few days, while others cause symptoms for years despite the presence of circulating or secretory antibodies (*1, 2*). Thus, nonimmune factors may influence the incidence, duration, and severity of giardiasis. Such factors are most likely to be present in the site colonized by this flagellate: the upper small intestine. They may be produced by intestinal tissue, secreted into intestinal fluid, or ingested (by breast-fed babies). Normal human milk (NHM) contains a number of antibacterial proteins, some of which are present in other mucosal secretions (*3*). In an animal model, infant mice were protected from *Giardia muris* infection only by milk from previously infected mice (*4*). To our knowledge, however, there has until now been no information concerning antiparasitic factors in *human* milk. We report here that NHM kills *Giardia lamblia* independently of secretory immunoglobulin A (S-IgA).

Giardia lamblia trophozoites were rapidly killed by low concentrations of NHM: 50 percent were killed in less than 30 minutes with 3 percent milk, in approximately 60 minutes with 1 percent milk, and in 280 minutes with 0.3 percent milk. At the lowest concentration a lag was followed by rapid killing (Fig. 1A). The lag was not reduced by first incubating the milk in assay medium for 1 or 3 hours. Milk (1 percent) from six other

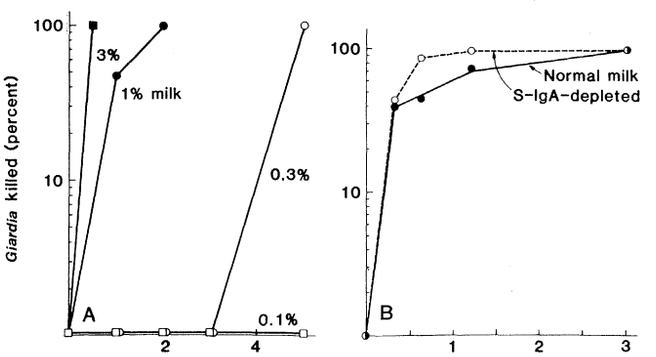

Fig. 1 (A). Kinetics of killing of *Giardia lamblia* trophozoites by NHM. Logarithmic-phase *Giardia lamblia* (ATTC 30888) trophozoites were grown in TP-S-1 growth medium (*6*). Nonattached parasites (including any dead organisms) were removed with the culture medium. Substrate-attached trophozoites were detached by incubation in cold, protein-free maintenance medium (*12*) and washed once in this medium and once in TP-S-1 without serum by centrifugation for 10 minutes at 2000*g* and 10°C. The parasites (5000 per milliliter) were exposed to various concentrations of filter-sterilized milk in serum-free TP-S-1 medium at 35.5° ± 0.5°C for 30 to 300 minutes. Killing was arrested and assayed by chilling the incubation mixtures and diluting them with complete TP-S-1 medium containing serum and melted agarose. This mixture was cooled to solidify it. Individual surviving parasites formed visible colonies after 5 to 6 days' incubation at 35.5°C (*13*). The percentage of parasites killed was calculated as 100 minus the percentage surviving, relative to controls lacking milk. (B) Killing of *G. lamblia* by NHM depleted of S-IgA. Trophozoites were incubated for 3 hours with various concentrations of normal milk or milk depleted (> 99 percent) of S-IgA. The percentage of parasites killed was determined as in (A). No killing was observed with control column buffer.