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Studies of Sodium Channels in Rabbit Urinary Bladder by Noise Analysis

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Summary. Sodium channels in rabbit urinary bladder were studied by noise analysis. There are two components of short-circuit current (I_{sc}) and correspondingly two components of apical Na^+ entry, one amiloride-sensitive (termed I_A and the A channel, respectively) and one amiloride-insensitive (I_L and the leak pathway, respectively). The leak pathway gives rise to *1/f* noise, while the A channel in the presence of amiloride gives rise to Lorentzian noise. A two-state model of the A channel accounts well for how the corner frequency and plateau value of Lorentzian noise vary with amiloride concentration. The single-channel current is 0.64 pA, and the conducting channel density is on the order of 40 copies per cell. Triamterene blocks the A channel alone, and increasing external Na^+ decreases the number but not the single-channel permeability of the A channel. Hydrostatic pressure pulses ("punching") increase the number of both pathways. Repeated washing of the mucosal surface removes most of the leak pathway without affecting the A channel.

Properties of the A channel revealed by noise analysis of various tight epithelia are compared, and the mechanism of *1/f* noise is discussed. It is suggested that the A channel is synthesized intracellularly, stored in intracellular vesicles, transferred with or from vesicular membrane into apical membrane under the action of microfilaments, and degraded into the leak pathway, which is washed out into urine or destroyed. The A channel starts with $P_{Na}/P_K \sim 30$ and loses selectivity in stages until P_{Na}/P_K reaches the free-solution mobility ratio (~0.7) for the leak pathway. This turnover cycle functions as a mechanism of repair and regulation for Na^+ channels, analogous to the repair and regulation of most intracellular proteins by turnover. Vesicular delivery of membrane channels may be operating in several other epithelia.

Key Words Na^+ channels · channel turnover · fluctuation analysis · tight epithelium · mammalian urinary bladder

Introduction

At all levels from the molecular to the whole-animal, biological systems face the problem of repairing damage inflicted by environmental agents. Visible injuries to tissues are patched, and damage to one class of molecules, DNA, is repaired by very specific molecular mechanisms (Lindahl, 1982). However, most molecular damage is instead anticipated and repaired by turnover of the entire molecular pool, such that old copies whether damaged or intact are constantly being replaced by new intact copies. Since the discovery of molecular turnover by Schoenheimer (1942), it has been shown that each type of protein or lipid turns over (is degraded and synthesized) at a characteristic rate (Siekevitz, 1972; Goldberg & St. John, 1976; Hershko & Ciechanover, 1982). Turnover of proteins serves at least two other functions besides repair: it generates free amino acids when needed; and it permits regulation of the level of each protein in response to physiological signals. For example, continual breakdown of a hormonally regulated protein is one way of ensuring that the protein not remain active after the physiological need and hormonal signal had disappeared.

To date, the sole cell membrane ion permeation channel for which molecular turnover has been demonstrated is the acetylcholine receptor (Pumplin & Fambrough, 1982). Turnover of the channels at the gap junction is also apparent from the results of Dahl, Azarnia and Werner (1981) and Flagg-Newton, Dahl and Loewenstein (1981). Yet one might expect turnover of channels to be a widespread phenomenon, especially in two situations. The first is in membranes at high risk of damage because of chronic exposure to fluids that have variable composition and that regularly contain toxic or damaging solutes. The urinary tract and biliary tree come immediately to mind. The second situation is channels under hormonal control.

The present paper will provide electrophysiological evidence for turnover of a channel that fits both of the above situations: the aldosterone-regulated sodium channel in the apical membrane of rab-

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bit urinary bladder. It appears that recently synthesized channels are first stored in the membrane of intracellular vesicles, then inserted with the help of microfilaments into the apical membrane, where the Na^+-selective channels are degraded into a channel of low selectivity and eventually eliminated. This cycle may be relevant to other epithelia in which vesicles and/or microfilaments have been implicated in channel insertion, such as gastric mucosa, amphibian and turtle urinary bladders, and intestinal secretory cells . A by-product of this study was the discovery that $1/f$ noise, which has been observed in many membranes but whose origin is poorly understood, arises specifically from degraded channels in rabbit urinary bladder. Preliminary accounts of these results have appeared (Loo, Lewis & Diamond, 1982; Lewis, Ifshin, Loo & Diamond, 1983; Loo, Lewis, Ifshin & Diamond, 1983).

As background, three sets of relevant observations about rabbit bladder will be summarized:

First, only part of the short-circuit current (I_{sc}) of rabbit bladder is sensitive to amiloride. Lewis and Wills (1981) showed that amiloride-inhibited Na^+ entry into the epithelial cells from the lumen involves a pathway with high selectivity for Na^+ over K^+, while amiloride-insensitive entry scarcely discriminates between Na^+ and K^+.

Second, the cytoplasm of the transporting cells is full of vesicles that are joined to each other and to the apical membrane by a network of microfilaments (Porter, Kenyon & Badenhausen, 1967; Minsky & Chlapowski, 1978). Reversible transfer of membrane between vesicles and apical membrane contributes to the adjustment in apical membrane area required for the bladder to accommodate the large changes of lumen volume associated with filling and emptying (Lewis & de Moura, 1982). Stretch or luminal pressure are probably the physiological signals for this transfer, which depends on microfilaments.

Finally, pressure-driven fusion of the vesicles with the apical membrane causes transfer of amiloride-sensitive conductance from vesicles to apical membrane (Lewis & de Moura, 1982, 1984). The P_{Na}/P_K ratio of the newly inserted conductance is several times higher than that of the amiloride-sensitive conductance previously in the apical membrane. This observation begs the question of the relation between the vesicular and the apical amiloride-sensitive channels.

Theory

Our experimental method for characterizing the amiloride-sensitive channel involves noise analysis. From statistical analysis of the fluctuations in I_{sc} arising from interaction of the diuretic amiloride and the channel, one can extract the single-channel current i and the channel number N (Lindemann & Van Driessche, 1977). We begin by formulating the theory and defining symbols.

The essence of the method is as follows. We assume a population of N identical channels, each behaving independently of the others, having a current i, and existing in only two states: open (conducting) or closed (nonconducting). The probabilities of being open and closed are respectively p and $q = 1 - p$. Hence the total current measured macroscopically is

$$I = ipN. \tag{1}$$

Current fluctuations arising from spontaneous opening and closing of the amiloride-sensitive channel have not yet been detected in rabbit urinary bladder or in other epithelia. Instead, following Lindemann and Van Driessche (1977), we study the fluctuations induced artificially by the reversible channel-blocker amiloride, which causes a channel to fluctuate randomly between open and closed states. As did Lindemann and Van Driessche, we assume a pseudo first-order reaction between amiloride (symbol A) and an unblocked channel (symbol R) to form a channel blocked by amiloride:

$$A + R \underset{K_{10}}{\overset{K_{01}}{\rightleftharpoons}} AR \tag{2}$$

where K_{01} and K_{10} are the association and dissociation rate constants, respectively. If the amiloride concentration A greatly exceeds the number of channels $N(= R + AR)$, as seems likely, the chemical reaction rate τ, given by the sum of the forwards and backwards rate constants, becomes

$$\tau = (K_{01}A + K_{10})^{-1} \tag{3}$$

At equilibrium, $K_{01}(A)(R) = K_{10}(AR)$. Hence the probabilities p or q that the channel is open or blocked are respectively

$$p = R/N = K_{10}/(K_{01}A + K_{10}) \tag{4}$$

$$q = AR/N = K_{01}A/(K_{01}A + K_{10}). \tag{5}$$

The mean lifetimes of the blocked or open channel are respectively $(K_{01}A)^{-1}$ and $(K_{10})^{-1}$. K_{10}/K_{01} is the Michaelis-Menton constant. The macroscopic current (in units of amperes) is given by

$$I = ipN = iNK_{10}/(K_{01}A + K_{10}). \tag{6}$$

Since the variance in current for a single channel is proportional to the product of p and q (Neher & Stevens, 1977), then the variance of the fluctuations in macroscopic current for all N channels is

$$\sigma I^2 = Npqi^2 = Iqi = IiK_{01}A/(K_{01}A + K_{10}). \quad (7)$$

For the reaction of Eq. (3) the single-sided power spectral density $S(f)$ of the current fluctuations, which is the frequency contribution to the variance, is described by a Lorentzian spectrum, i.e., one with a single time constant:

$$S(f) = S_o/[1 + (f/f_c)^2] \quad (8)$$

where f is frequency, S_o is termed the plateau value (asymptotic value of $S(f)$ at low f), and f_c is termed the corner frequency. Examples of such spectra will be shown in Figs. 3, 5, and 7. f_c is simply the chemical rate τ of the reaction of Eq. (3), reexpressed as an angular frequency:

$$f_c = 1/2\pi\tau = (1/2\pi)(K_{01}A + K_{10}). \quad (9)$$

The plateau value S_o is proportional to the variance of the current fluctuations σI^2 (Bendat & Piersol, 1971):

$$S_o = 4a\sigma I^2 = 4iaI(K_{01}A)/(K_{01}A + K_{10})^2 \quad (10)$$

where a is the membrane area.

Of the three unknowns to be determined, i and N are assumed independent of amiloride concentration A, while p is assumed to vary with concentration according to Eq. (4). To determine these unknowns, we measure the power spectral density $S(f)$ at each of 3–6 amiloride concentrations and fit $S(f)$ to Eq. (3) to extract the corner frequency f_c and plateau value S_o at each concentration. This procedure in effect yields the rate τ and the variance of the fluctuations σI^2 at each concentration [*cf.* Eqs. (9) and (10)]. Linear regression of $2\pi f_c$ on A yields the rate constants K_{01} and K_{10} as the slope and intercept, respectively. At each amiloride concentration the single-channel current i is then calculated from S_o by Eq. (10), and the channel number N is calculated from the macroscopic current I by Eq. (6). The final values for i and N are the averages of the values calculated for various amiloride concentrations.

The validity of this model is tested by comparing the observed dependences of f_c and S_o on A with the predicted dependences. According to Eq. (9), f_c should increase linearly with A. Substitution of Eq. (6) into Eq. (10) yields the dependence of S_o on A:

$$S_o = 4Ni^2aK_{01}K_{10}A/(K_{01}A + K_{10})^3. \quad (11)$$

This predicts that S_o should vary biphasically with amiloride concentrations, rising from 0 at very low A to a maximum at A equal to one-half of the Michaelis-Menten constant $K_m(= K_{10}/K_{01})$, and declining again towards 0 at high A. We confirmed the predictions of both Eqs. (9) and (11) (*see* Fig. 6).

Materials and Methods

Dissection, Chambers, and Solutions

The urinary bladder of a male New Zealand white rabbit (4–5 lbs) was stripped of its underlying muscle layer and mounted in temperature-controlled (37°C) modified Ussing chambers designed to eliminate edge damage (Lewis & Diamond, 1976). Fluid volume in each chamber was 15 ml, and exposed membrane area was 2 cm^2. Both sides of the epithelium were bathed in a solution of composition 111.2 mM NaCl, 25 mM $NaHCO_3$, 5.8 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 11.1 mM glucose buffered at pH 7.4. Except for 3.5-min periods when noise spectra were being recorded, solutions were constantly stirred by magnetic spin bars and gassed with 95% O_2/5% CO_2.

Measurement of PD, I_{sc}, and Resistance

Open-circuit voltage (V_T), transepithelial resistance (R_T) or conductance (G_T), and I_{sc} were continuously monitored and stored on a laboratory computer. V_T was sensed by Ag/AgCl electrodes placed close to and on opposite sides of the epithelium and connected to a current/voltage clamp. Current-passing Ag/AgCl electrodes were placed in the rear of each half chamber and were also connected to the voltage clamp. Voltage and current outputs from the clamp were connected to an *A/D* converter interfaced to a small laboratory computer. R_T was calculated from Ohm's law and the measured transepithelial voltage response (ΔV_T) to a computer-controlled transepithelial current pulse (ΔI). I_{sc} was calculated by dividing the measured V_T by the calculated R_T. Epithelial capacitance was determined by the method of Lewis and de Moura (1982). R_T and I_{sc} are always normalized to capacitance as a measure of real membrane area (1 μF ≅ 1 cm^2 of apical membrane; *see* Clausen, Lewis & Diamond, 1979, and Lewis & de Moura, 1982).

Punching

Intracellular vesicles in the superficial epithelial cell layer can be inserted into the apical membrane by stretching the bladder (Lewis & de Moura, 1982). Simple stretching mimics the natural expansion of the bladder during filling with urine but has two disadvantages for fluctuation analysis. First, since the muscle layers have been removed, the epithelium is frequently damaged by stretching. Second, the increases in I_{sc} with stretch are small. We therefore used a method called "punching" (Lewis & de Moura, 1982), which is less destructive and yields much larger increases in I_{sc}. In this method, we first removed the solution

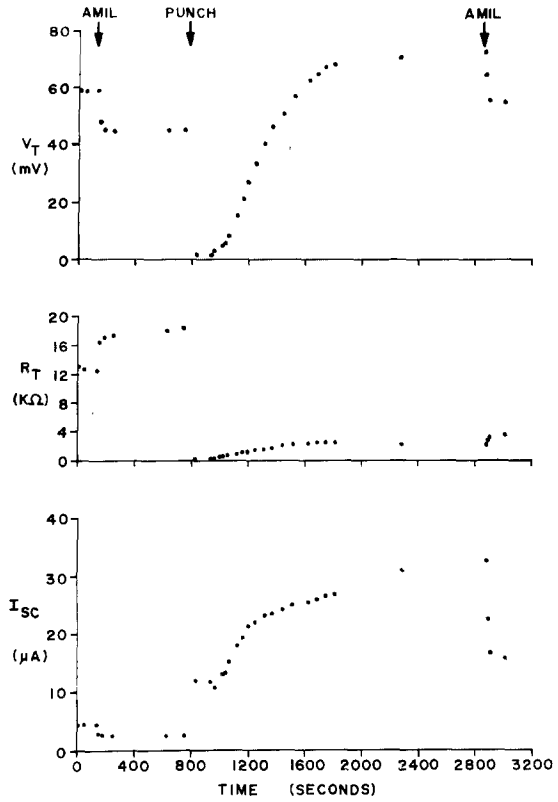

Fig. 1. Effect of punching on the transepithelial voltage (V_T), resistance (R_T), and short-circuit current (I_{sc}) of rabbit urinary bladder. Amiloride was added to a concentration of 1.4 μM at the first arrow, removed with punching, and added again at the last arrow. Note that punching reduces V_T and R_T transiently, but at steady state increases V_T, R_T and I_{sc}

from both mucosal and serosal chambers. Next, we applied a rapid series of hydrostatic pressure pulses by rapidly filling and emptying (10 times) the mucosal chamber with solution. This procedure compressed the epithelium against a nylon mesh. Finally, both chambers were refilled with bathing solution. As previously reported (Lewis & de Moura, 1982), this procedure caused an initial drop in V_T and R_T, followed by a rise in V_T, R_T, and I_{sc} to new steady-state levels within 10–30 min after the punch (*see* Fig. 1 as an example). There is no measurable capacitance change after a punch (Lewis & de Moura, 1982). This suggests that the pressure pulse drives apical membrane and cytoplasmic vesicles into temporary contact, permitting equilibration of ion permeation channels between these two membrane pools, but that the apical membrane rebounds after the pressure pulse and the vesicular membrane returns to the cytoplasm (Lewis & de Moura, 1982).

Washing

The mucosal or serosal face of the epithelium was washed by slow perfusion. Fresh solution was injected into the chamber at the solution surface via a syringe needle, and solution was simultaneously withdrawn by vacuum suction connected to another syringe needle with its tip also at the solution surface. We found that 200 ml of perfused solution was required to restore I_{sc} to its pre-amiloride value after inhibition of I_{sc} by addition of amiloride to the mucosal solution.

Noise Measurements

The voltage clamp used for fluctuation analysis was based on a dual matched pair of JFET amplifiers, Siliconix 2N5515 (*see* Loo,[1] 1983), and was similar in design to the one described by Van Driessche and Lindemann (1978). The contribution of instrumentation noise could be neglected because it was two orders of magnitude below the current fluctuations observed when the inputs of the amplifiers were connected to a dummy network whose electrical parameters resembled the passive electrical equivalent characteristics of the epithelium and the measuring electrode. The amplified fluctuations in I_{sc} had a sensitivity of 10^6 V/Amp. This signal was then amplified by a variable-gain instrumentation amplifier (PARC 113). The gain was selected so that the maximum output was between ±4 volts to minimize the noise contribution from the subsequent anti-aliasing filter (LP 120, Unigon). The filter had a maximum dynamic range of ±5 volts and high frequency roll-off of 120 dB/octave, and the cutoff was set at 100 Hz. The output of the filter was connected differentially to a Nicolet 2090-III digital oscilloscope with disk and *I/O* port. Digitization was set at 5 msec/pt in the long-sweep mode, with the result that 32,768 sequential points were collected and stored on Nicolet disk. The 32,768 points were divided into 64 nonoverlapping blocks of 512 points, and each block was analyzed by a 512-point Fast Fourier Transform program (Cooley & Nolan, 1979) on a North Star Horizon II computer. Each power spectral density (PSD) studied contained 256 values and was the mean of 64 blocks.

I_{sc} is composed of both amiloride-sensitive and amiloride-insensitive components. The interaction of amiloride with the amiloride-sensitive channel resulted in a Lorentzian spectrum [Eq. (8)] for the power spectral density (PSD), as already discussed in the Theory section. In addition, we observed in all spectra a $1/f$ noise component of the form B/f^{α}, where B and α are constants (*see* Results). Hence we fitted the total power spectral density $S(f)$ (both in the presence and absence of amiloride) by the equation

$$S(f) = B/f^{\alpha} + S_o/[1 + (f/f_c)^2] \tag{12}$$

using a derivative-free nonlinear least-squares algorithm (Brown & Dennis, 1972; Clausen, et al., 1979) to estimate the four parameters B, α, f_c, and S_o. In the absence of amiloride f_c and S_o were poorly determined parameters because of the absence of a Lorentzian component: f_c was very large and S_o small when we attempted to fit a Lorentzian component in the absence of amiloride. Since each PSD consisted of 256 points but most of the points were in the frequency range 55–100 Hz, this concentration of points biased the fit towards this frequency range, whereas fits in the lower frequency range 0.8–55 Hz are important for the parameters to be estimated accurately. Hence prior to parameter estimation we deleted all points above 80 Hz and portions of the spectra at 55–80 Hz in order to avoid aliasing artifacts, biasing of

[1] Loo, D.D.F. A simple and low-cost voltage clamp amplifier for fluctuation analysis in epithelia. *Pfluegers Arch.* (*submitted*)

the fit in this frequency range, as well as to eliminate errors due to 60-Hz contamination. The lowest frequency value at 0.4 Hz was also deleted to avoid low-frequency contamination. Thus, the remaining PSD data set used for the fitting routine contained 60–100 points. As the fitting criterion, we assumed convergence to a minimum when successive iterations during the minimization resulted in less than 1% change in the parameter values. To assure a "unique" fit to S_o, f_c, α, and B, we employed the method of Clausen et al. (1979), which consisted of providing different initial parameter estimates and then confirming that the same set of best-fit values was determined for each case.

Results

This study is based on results from 17 bladders. We first describe macroscopic studies of inhibition of I_{sc} by amiloride and triamterene. We then describe the characterization of these channels by means of fluctuation analysis applied to the action of amiloride, and more briefly of the other agents and of Na^+ itself, in normal (i.e., unpunched and unwashed) bladders. Next, we study the channels in the vesicular membrane as revealed by punching. Finally we describe the reduction of I_{sc} by washing.

Macroscopic Studies

Step-wise addition of amiloride to the mucosal solution caused step-wise decrease in I_{sc}, V_T, and G_T, as reported by Lewis and Diamond (1976). The decreases in I_{sc} and V_T reached minimum asymptotic values. The dose/response curve for amiloride (Fig. 2) followed Michaelis-Menten kinetics with K_m = 0.25 ± 0.1 μM (n = 3). Hence the amiloride-insensitive current I_L was defined operationally as the residual short-circuit current at an amiloride concentration of 100 μM, while the amiloride-sensitive current I_A was defined as the decrease in I_{sc} due to 100 μM amiloride. The ratio of I_{sc} to I_L varied considerably among bladders and was on the order of 1.

Triamterene has been reported to be a specific blocker of the amiloride-sensitive Na^+ current in several tight epithelia, including frog skin (Hoshiko & Van Driessche, 1981) and hen coprodeum (Christensen & Bindslev, 1982). Unlike amiloride, triamterene is relatively insoluble in physiological saline. We made a stock solution of nominally 1 mM triamterene in aliquots to the mucosal solution. At nominal concentrations of 5–80 μM triamterene reversibly inhibited I_A in three bladders (*cf.* Fig. 6*b* for effect on S_o) but did not affect I_L.

These effects of amiloride and triamterene suggest the existence of two mechanisms for Na^+ entry, one blocked and the other not blocked by these drugs.

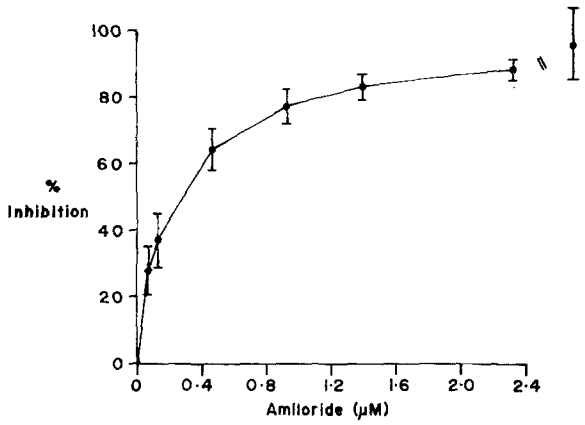

Fig. 2. Macroscopic dose/response curve for inhibition of I_{sc} by amiloride. To reduce scatter arising from bladder differences in I_A/I_L, ordinate values in each bladder were normalized to the maximal inhibition observed in that bladder at 100 μM amiloride. Points are means, bars are SEM's, for 11 bladders

Fluctuation Analysis in Normal Bladder

In the absence of amiloride the power spectral density (PSD) varied inversely with frequency (Fig. 3). Hence the relationship is of the form $S(f) = B/f^{\alpha}$, where B is called the intensity and equals the magnitude of current fluctuations at 1 Hz. For eight bladders the average values and standard errors of B and α were $B = 5.2 \pm 1.4 \times 10^{-19}$ A² sec, $\alpha = 1.26 \pm 0.05$. A similar noise component with α around 1 has been observed in numerous other biological and nonbiological membranes (*see* Discussion for references) and is termed *1/f* noise or flicker noise. We noticed that variation among bladders in the intensity B of *1/f* noise was correlated with variation among bladders in the magnitude of the amiloride-insensitive current I_L (Fig. 4*a*). This observation provided the first indication that *1/f* noise in rabbit bladder arises from the amiloride-insensitive pathway, a conclusion that will be supported by four other observations.

We routinely used an amiloride concentration of 1.4 μM to test the effect of amiloride on the PSD. This concentration was chosen because it yields 85% inhibition of I_A and good separation of *1/f* noise from any observable Lorentzian noise component due to amiloride-induced fluctuations (*see* following results). In about half of all preparations 1.4 μM elicited no measurable change in PSD, because I_A/I_L was too low and the amiloride-induced noise was obscured by *1/f* noise (*cf.* Fig. 4*b*). The remaining discussion is based on seven bladders in which I_A/I_L exceeded 2 and an effect of 1.4 μM amiloride on the PSD was detectable.

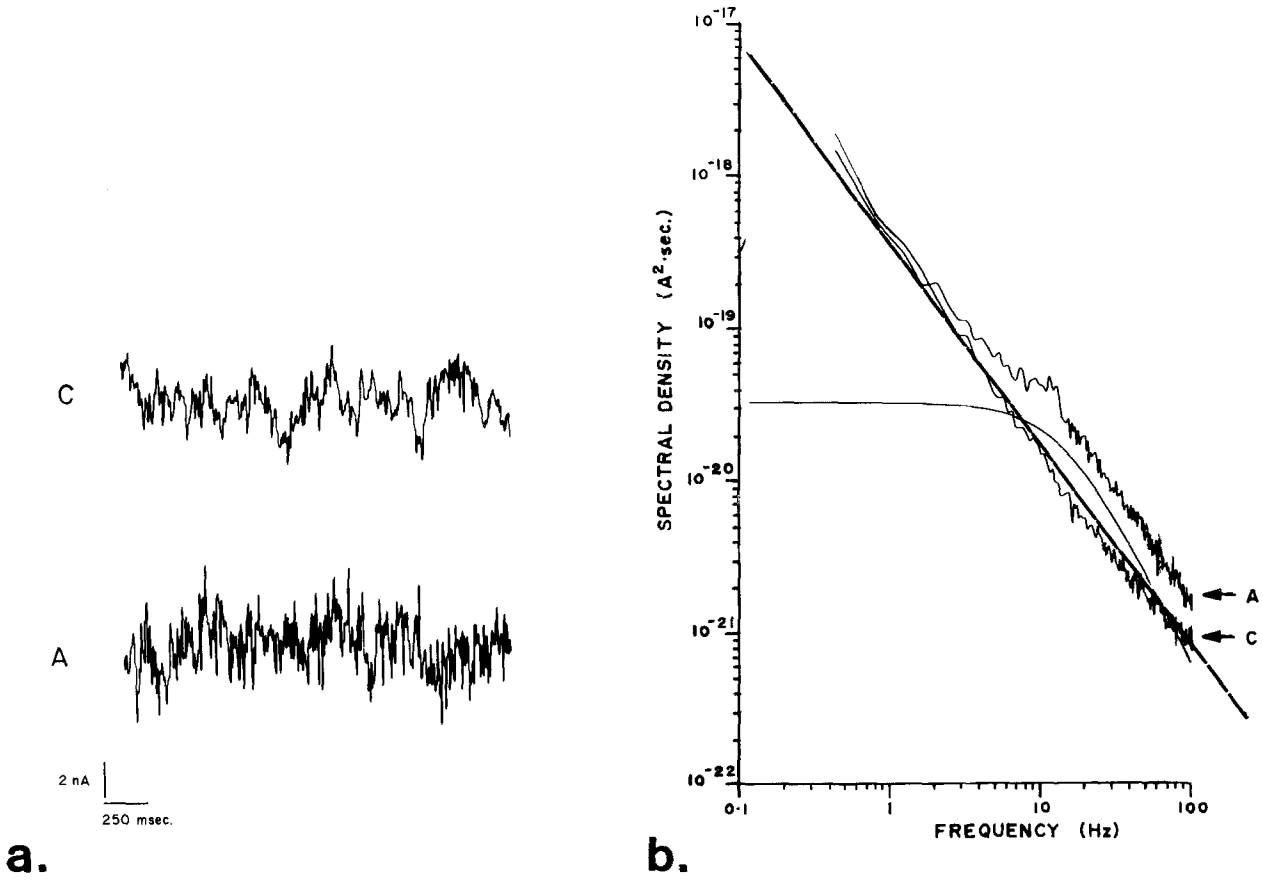

Fig. 3. Current fluctuations (*a*) and power spectral density (*b*), in the absence (control = *C*) and presence (*A*) of 1.4 μM amiloride. Wavy lines are experimental data, smooth curves (*b*) are best-fit curves. The control spectrum was fit to $S(f) = B/f^{\alpha}$, yielding $B = 38 \times 10^{-20}$ A^2 sec, $\alpha = 1.43$. The amiloride spectrum was fit to $S(f) = B/f + S_o/[1 + (f/f_c)^2]$, yielding $B = 34 \times 10^{-20}$ A^2 sec, $\alpha = 1.33$, $S_o = 3.3 \times 10^{-20}$ A^2 sec, $f_c = 13.5$ Hz. The dashed line gives the B/f^{α} component, while the smooth solid line gives the $S_o/[1 + (f/f_c)^2]$ component. The latter component appears after amiloride, while the former component is unchanged by amiloride

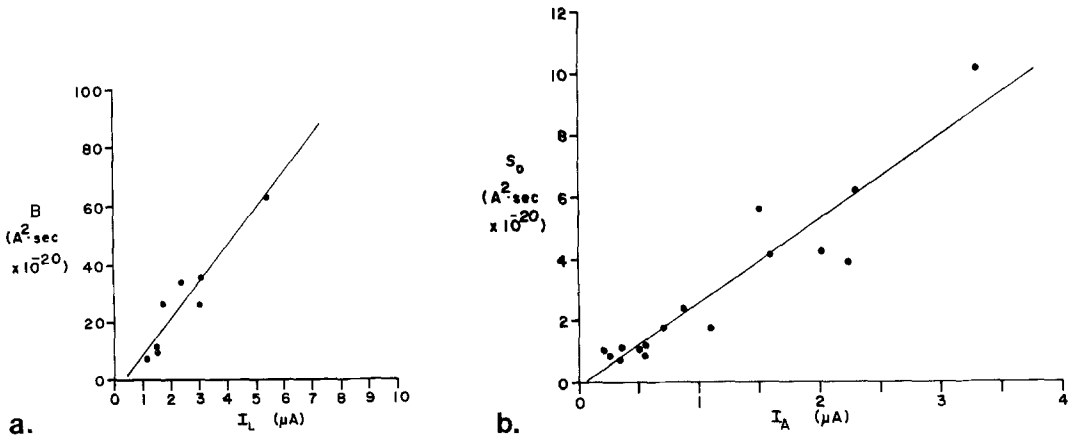

Fig. 4. (*a*): Correlation between the current I_L and the intensity of B if $1/f$ noise among eight bladders. The slope is 12.5×10^{-20} A^2 sec/μA. The correlation suggests that $1/f$ noise arises from the amiloride-insensitive pathway. (*b*): Correlation between the current I_A (actually, the decrease in I_{sc} due to 1.4 μM amiloride) and the plateau value S_o of Lorentzian noise among 16 bladders. The correlation is because Lorentzian noise arises from the amiloride-sensitive channel

In the absence of amiloride the measured peak-to-peak variations in current were ca. 7 nA/μF. With 1.4 μM amiloride this increased to ca. 9 nA/μF, and the variance of the fluctuations also increased. As illustrated in Fig. 3, the effect of 1.4 μM amiloride was to add a Lorentzian component to the $1/f$ noise observed in the absence of amiloride. B and α for $1/f$ noise were essentially unchanged by amiloride, as demonstrated by paired experiments (Table 2). Best-fit values for the Lorentzian component $S_f = S_o/[1 + (f/f_c)^2]$ were f_c = 12.2 ± 1.0 Hz, S_o = 0.96 ± 0.19 × 10^{-20} A^2 sec. This variation in S_o, which expressed variation in intensity of the Lorentzian component, was directly related to the magnitude of the amiloride-sensitive current I_A (Fig. 4*b*). This is why an effect of amiloride on PSD could not be detected in bladders with a low value of I_A relative to I_L. In addition, this linear relationship between S_o and I_A (Fig. 4*b*) indicates that the amiloride binding kinetics and single channel currents are constants among these preparations, and that the variability in I_A is directly correlated with a variation in channel number N [see Eqs. (6) and (10)] and not with a variation in i. If i were the variable, then one would observe S_o proportional to I_A^2.

In three bladders we measured PSD at 3–6 different amiloride concentrations [A] in order to extract the single-channel current i and channel density N and also to test the validity of the amiloride binding model described in the Theory section. Figure 5 illustrates how PSD changes with [A]. Figure 6*a* (upper) shows that the corner frequency f_c increases linearly with [A], as predicted by Eq. (9). The slope and y-intercept yield K_{01} = 52 ± 7 sec^{-1} μM^{-1}, K_{10} = 11.6 ± 1.1 sec^{-1}, respectively. This corresponds to a mean life-time of $1/K_{10}$ = 86 msec for the amiloride-blocked channel. The ratio K_{10}/K_{01}(=11.6/52 = 0.22 μM), the apparent dissociation constant, should be equivalent to the macroscopic Michaelis-Menten constant K_m; the agreement with our directly determined K_m value of 0.25 μM (from the variation in I_A with [A] shown in Fig. 2) is excellent. (Table 1 shows that this agreement is maintained after K_m has decreased due to a decrease in [Na^+], and Table 2 shows that the agreement is preserved when channel number is increased by punching). Fig. 6*a* (lower) shows, as predicted by Eq. (11), that the plateau value S_o depends biphasically on [A], falling towards zero at low and high concentrations and reaching a maximum at an intermediate concentration of about 0.13 μM. This abscissa value of the maximum in S_o is about half K_m (0.24 μM), as predicted. Thus, the theory developed from the reaction of Eq. (3) accounts for the effect of amiloride on the noise spectrum.

The single-channel current i was then calculated as described in the Theory section, yielding 0.64 ± 0.1 pA (n = 3). The channel density N varied among preparations depending on their I_A value,

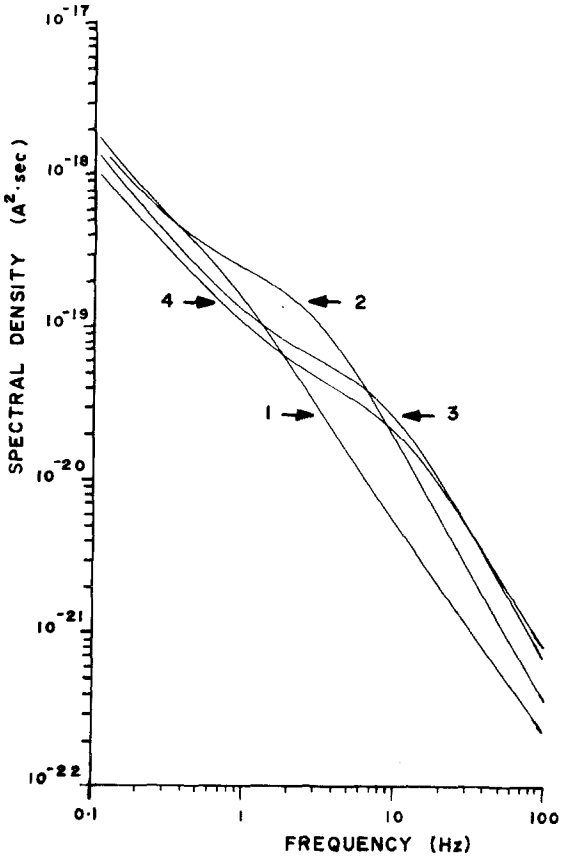

Fig. 5. Best fits of the equation $S(f) = B/f^{\alpha} + S_o/[1 + (f/f_c)^2]$ to PSD for a bladder at four amiloride concentrations

Table 1. Effect of mucosal Na^+ concentration on the amiloride-sensitive channel

Na^+ (mM)	i (pA)	N ($\mu F^{-1} \times 10^6$)	I_A ($\mu A/\mu F$)	K_{01} (μM^{-1} sec^{-1})	K_{10} (sec^{-1})	K_{10}/K_{01} (μM)	K_m (μM)
134	0.71 ± 0.06	5.6 ± 1.9	5.9 ± 1.9	46.0 ± 1.5	13.2 ± 0.4	0.33 ± 0.07	0.37 ± 0.1
30	0.18 ± 0.03	13.8 ± 4.1	2.0 ± 0.6	55.5 ± 10.5	9.8 ± 1.9	0.20 ± 0.06	0.23 ± 0.07

Values are mean ± SEM for four bladders.

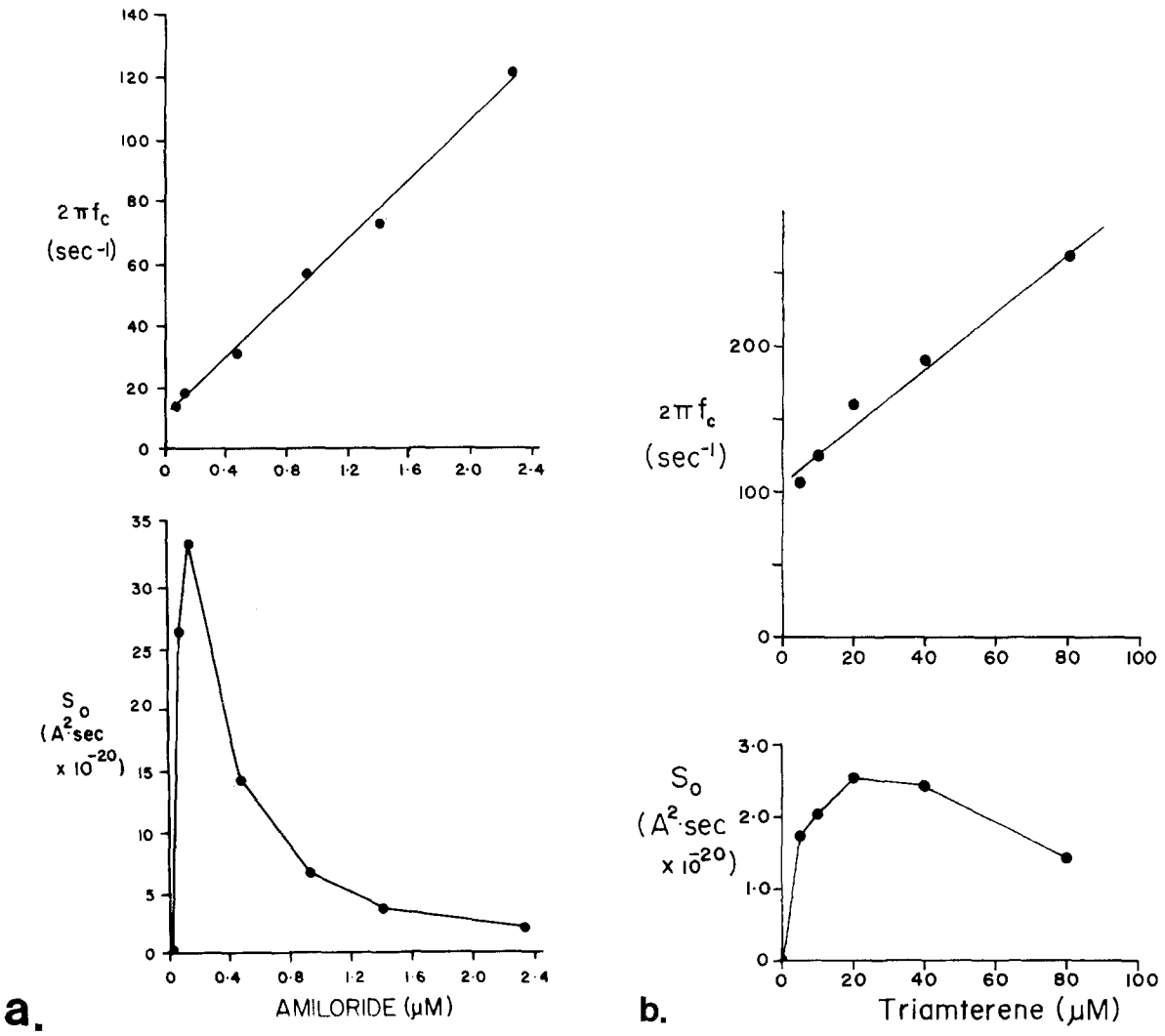

Fig. 6. Corner frequency f_c (upper) and plateau value S_o (lower) for Lorentzian noise in a bladder, plotted against amiloride concentration (*a*) or triamterene concentration (*b*)

with a mean of 2.4×10^6 channels/μF. Equating 1 μF capacitance with about 1 cm^2 real membrane area, this means about one conducting channel per 40 μm^2 of membrane, or about 40 channels per cell! At our experimental Na^+ concentration of 137 mM there are in addition more channels blocked by Na^+, but this is still a remarkably small number of copies per cell.

We also used noise analysis to examine cursorily the effects of two other agents: triamterene and Na^+.

Triamterene resembled amiloride in its effect on the PSD: it added a Lorentzian component without affecting the underlying *l/f* noise. Just as for amiloride, f_c increased linearly and S_o increased biphasically with triamterene concentration (Fig. 6*b*). The apparent value of the ratio K_{10}/K_{01} calculated from this concentration dependence was 41 ± 9 μM ($n = 3$), in fair agreement with the apparent value of the macroscopic K_m, 54 ± 12 μM ($n = 3$). We have not attempted to extract i and N because of the uncertainty about the actual triamterene concentration. However, the similar effects of triamterene and amiloride on the PSD support the conclusion drawn from their similar effects on the macroscopic current (inhibition of I_A but not of I_L): both appear to be specific blockers of the same channel.

Decreasing mucosal Na^+ concentration increases the permeability of the apical membrane to Na^+ in frog skin (Van Driessche & Lindemann, 1979), toad urinary bladder (Li, Palmer, Edelman & Lindemann, 1982), and hen coprodeum (Christen-

sen & Bindslev, 1982), because Na^+ itself blocks the channels, reducing their number. We confirmed this interpretation for rabbit urinary bladder. We decreased mucosal [Na^+] from 137 to 30 mM by replacement with K^+, then measured fluctuations induced by step-wise addition of amiloride and calculated i and N as discussed above for normal conditions of [Na^+] = 137 mM. In five paired experiments (Table 1) the channel number N increased 2.3 times at the lower Na^+ concentration, meaning that Na^+ itself is a channel blocker. The single-channel current i decreased from 0.71 to 0.18 pA, as expected from calculations using the constant-field equation and assuming unchanged single-channel permeability (*see* Discussion). The decrease in single-channel current is simply due to the decreased [Na^+]. Thus, with increasing [Na^+] the single-channel permeability is constant, single-channel current increases as the concentration of the conducting species increases, but channel number decreases. The net result is a saturation of Na^+ transport with increasing [Na^+], as demonstrated for rabbit bladder (Fig. 4 of Lewis & Diamond, 1976) and numerous other epithelia.

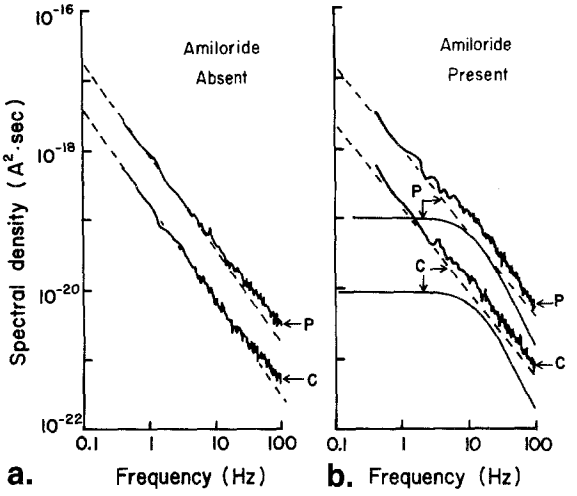

Fig. 7. Effect of 1.4 μM amiloride on PSD before (C = control) and after (P) punching. Data (wiggly curves) were fitted by $S(f) = B/f^{\alpha} + S_o/[1 + (f/f_c)^2]$ with and without amiloride (*see* Methods). The B/f^{α} component (= $1/f$ noise, dashed lines) is unaffected by amiloride, while B but not α increases with punching. Punching increases S_o of the $S_o/[1 + (f/f_c)^2]$ component (= Lorentzian noise, solid smooth curve) by an order of magnitude, but f_c is unaffected

Effect of Punching

Lewis and de Moura (1982) reported that punching increases I_A by an order of magnitude. We confirmed this finding and also observed a smaller increase in I_L. In our experiments punching increased I_A by 11.9 ± 6.4 times (n = 6), I_L by 2.5 ± 0.8 times (n = 6). The amiloride dose/response curve yielded the same Michaelis-Menten constant after punching (0.30 ± 0.04 μM, n = 11) as before punching (0.25 ± 0.1 μM, n = 3).

Does punching increase I_A by increasing channel number or single-channel currents? This question can be answered by means of noise analysis. (Noise analysis does not at present suffice to answer the corresponding question for I_L and the amiloride-insensitive pathway, as we do not have a model permitting extraction of i and N from $1/f$ noise). Figure 7 illustrates the effect of punching on PSD in one bladder. It is obvious that punching increased the $1/f$ noise observed in the absence of amiloride (Fig. 7*a*) and the $1/f$ component observed in the presence of amiloride (dashed lines, Fig. 7*b*), but increased even more the Lorentzian component observed in the presence of amiloride (solid curves, Fig. 7*b*). The factor by which punching increased the intensity B of $1/f$ noise in this experiment, 5.0, was close to the factor of 4.2 by which the current I_L increased. The factor by which punching increased the plateau value S_o of Lorentzian noise, 11.1, was close to the factor of 9.4 by which the current I_A increased. It is apparent from Fig. 7*b* that the corner frequency of the Lorentzian component was unaffected by punching.

Table 2 summarizes results from three paired experiments using 1.4 μM amiloride to study the effect of punching on the best-fit parameters B, α, S_o, and f_c. The exponent α of $1/f$ noise and the corner frequency f_c of Lorentzian noise were unchanged by punching. The intensity B of $1/f$ noise and the current I_L increased by about the same factor (8.3 and 8.8 times, respectively).

Table 3 compares single-channel properties of punched and unpunched preparations as extracted from noise analysis. Punching does not alter the single-channel current i, association and dissociation rate constants K_{01} and K_{10} for amiloride binding, or microscopic (K_{10}/K_{01}) or macroscopic (K_m) Michaelis-Menten constant. The order-of-magnitude increase in I_A is due entirely to a corresponding increase in N. Thus, punching increases the number of amiloride-sensitive channels without affecting their properties.

Effect of Washing

When the mucosal surface of the bladder was washed, I_{sc} and G_T decreased for a few minutes before reaching a new steady state. Since V_T in-

creased, washing removed proportionately slightly more conductance than current. Washing the serosal surface had no effect. We obtained these results of washing in all 12 bladders tested.

Figure 8 illustrates an experiment performed to determine whether washing removes amiloride-sensitive or amiloride-insensitive pathways or both. A bladder was punched to increase I_{sc} and G_T. Amiloride was then added at 1.4 μM to the mucosal solution to resolve I_{sc} into I_A and I_L, whereupon the mucosal chamber was perfused with 200 ml to remove the amiloride and wash the mucosal surface. This procedure was repeated eight times. Figure 8 shows that G_T and I_{sc} decreased to minimum asymptotic values after four or five washes and were unaffected thereafter by several more washes. The decrease in G_T was by 70%, decrease in I_{sc} by 67%, increase in V_T by 5.4% (from −51.5 to −54.3 mV, mucosa negative). Despite this large decrease in total I_{sc}, the change in I_{sc} caused by 1.4 μM amiloride (difference between two curves of Fig. 8*b*), representing about 85% of the total amiloride-sensitive current I_A, remained unchanged at 1.58 μA/μF. Similarly, the change in G_T caused by 1.4 μM amiloride (difference between two curves of Fig. 8*a*) was unchanged with washing. Thus, the effect of washing is solely on the amiloride-insensitive pathway. When one corrects the lower I_{sc} curve (■) of Fig. 8 for the residual 15% of I_A that persists at 1.4 μM amiloride, one finds that I_L was reduced almost 90% by washing (dashed line, Fig. 8).

The co-variation of $1/f$ noise and I_L among different bladders (Fig. 4*a*), and their increase by the same factor after punching (Fig. 7 and Table 2), suggested that $1/f$ noise arises from the amiloride-insensitive pathway. The progressive elimination of most of this pathway by washing permits a stronger test of this conclusion. Figure 9 illustrates an experiment in which PSD in the presence of 1.4 μM amiloride was determined after each of a series of washes and best-fit parameters were calculated. The Lorentzian noise component (S_o and f_c) remained unchanged, as did the exponent α of the $1/f$ component. The only parameter affected was the intensity B of $1/f$ noise, which decreased linearly with the decrease in the I_{sc} measured in the presence of 1.4 μM amiloride (top of Fig. 9). This residual I_{sc} in the presence of 1.4 μM amiloride approximates the amiloride-insensitive current I_L but is slightly greater than I_L, because 1.4 μM amiloride abolishes only 85% of I_A. (Similarly, the nonzero abscissa intercept of the *B-vs.*-I_{sc} line equals the 15% of I_A that is not abolished by 1.4 μM amiloride.)

The slope of the *B-vs.*-I_{sc} line of Fig. 9 is 15.2 × 10^{-20} A^2 sec/μA. This is virtually the same as the slope (12.5 × 10^{-20} A^2 sec/μA) of Fig. 4*a*, which plots B *vs.* the amiloride-insensitive current I_L for eight bladders. Thus, variations in the amiloride-insensitive pathway cause the same change in intensity of $1/f$ noise, whether those variations arise from washing or from natural variation among individual bladders.

Discussion

We shall consider in turn: the properties of the amiloride-sensitive channel (abbreviated A channel) in various tight epithelia; the P_{Na}/P_K ratio, susceptibility to washing, and $1/f$ noise of the amiloride-insen-

Table 2. Effects of punching on best-fit parameters of noise spectra

Condition	B (A^2 sec × 10^{-20})	α	S_o (A^2 sec × 10^{-20})	f_c (Hz)	I_L (μA/μF)	I_A (μA/μF)
Control	17.1 ± 5.1	1.50 ± 0.13				
Control +1.4 μM amiloride	14.7 ± 4.4	1.26 ± 0.06	0.81 ± 0.2	12.3 ± 1.2	1.28 ± 0.3	1.54 ± 0.4
Punched	67.2 ± 40.9	1.17 ± 0.13				
Punched +1.4 μM amiloride	47.6 ± 21.8	1.14 ± 0.05	6.76 ± 2.4	12.6 ± 0.4	3.43 ± 1.5	13.5 ± 4.5
Increase with punching	3.2×	—	8.3×	—	2.7×	8.8×

Values are mean ± SEM for three bladders.

Table 3. Effect of punching on the amiloride-sensitive channel

Condition	i (pA)	N (μF^{-1} × 10^6)	I_A (μA/μF)	K_{01} (μM^{-1} sec^{-1})	K_{10} (sec^{-1})	K_{10}/K_{01} (μM)	K_m (μM)
Control (n = 3)	0.64 ± 0.10	2.0 ± 0.1	0.72 ± 0.2	52.1 ± 7.3	11.6 ± 1.1	0.24 ± 0.05	0.25 ± 0.1
Punched (n = 3)	0.74 ± 0.09	18.6 ± 9.3	5.0 ± 1.3	43.9 ± 2.9	13.2 ± 0.8	0.32 ± 0.04	0.30 ± 0.04
Increase with punching	—	9.3×	7.0×	—	—	—	—

sitive pathway (abbreviated leak pathway; probably but not yet provenly a channel); the relation between the A channel and the leak pathway; and a proposed turnover cycle of channels.

The Amiloride-Sensitive Channel

Noise analysis has now been used to study the amiloride-sensitive Na^+ entry channel in five tight epithelia: frog skin (Lindemann & Van Driessche, 1977; Van Driessche & Lindemann, 1979; Hoshiko & Van Driessche, 1981), toad urinary bladder (Van Driessche & Hegel, 1978; Li et al., 1982; Palmer, Li, Lindemann & Edelman, 1982), rabbit descending colon (Zeiske, Wills & Van Driessche, 1982), hen coprodeum (Christensen & Bindslev, 1982), and rabbit urinary bladder (present study). Table 4 summarizes properties of the channel in these epithelia. The following generalizations emerge:

1. The channel has not been observed to exhibit spontaneous current fluctuations, unlike the K^+ channels in the apical membrane of frog skin (Van Driessche & Zeiske, 1980), *Necturus* and toad gallbladder (Gögelein & Van Driessche, 1981), and rabbit descending colon (Wills, Zeiske & Van Driessche, 1982) or the basolateral K^+ channels found in tadpole skin and rabbit descending colon (Van Driessche, Wills, Hillyard & Zeiske, 1982). Instead, fluctuations have to be induced with amiloride or triamterene.

2. A simple 2-state model (open and closed) fits the results adequately.

3. Single-channel currents (i) reported in different epithelia are not strictly comparable because of different bathing or intracellular ionic compositions

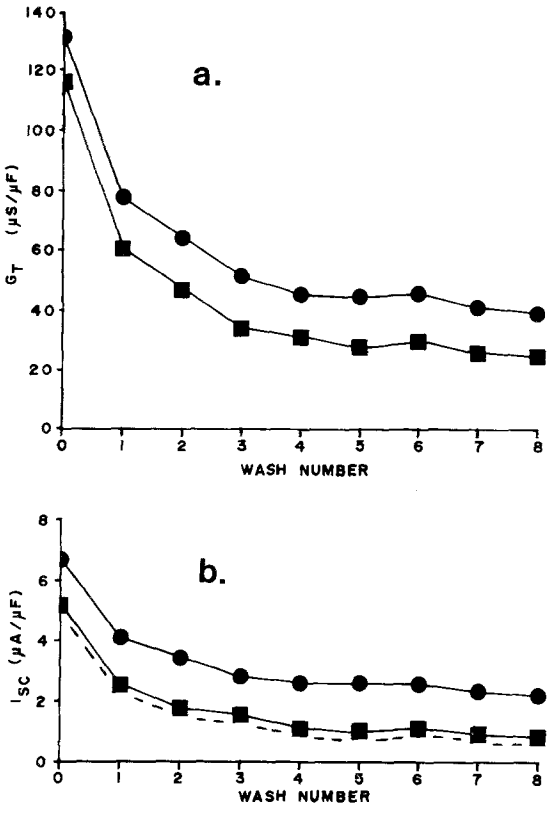

Fig. 8. Effect of eight successive 200-ml washes of the mucosal surface on G_T (*a*) and I_{sc} (*b*). Measurements after each wash were first made without amiloride (●), then with 1.4 μM amiloride (■). The difference between points ● and ■ at each wash number is a measure of the amiloride-sensitive conductance or current, which remains constant, meaning that washing removes only the amiloride-insensitive pathway. The dashed curve is the leak current I_L, calculated by correcting the points for the residual 15% of I_A that persists at 1.4 μM amiloride

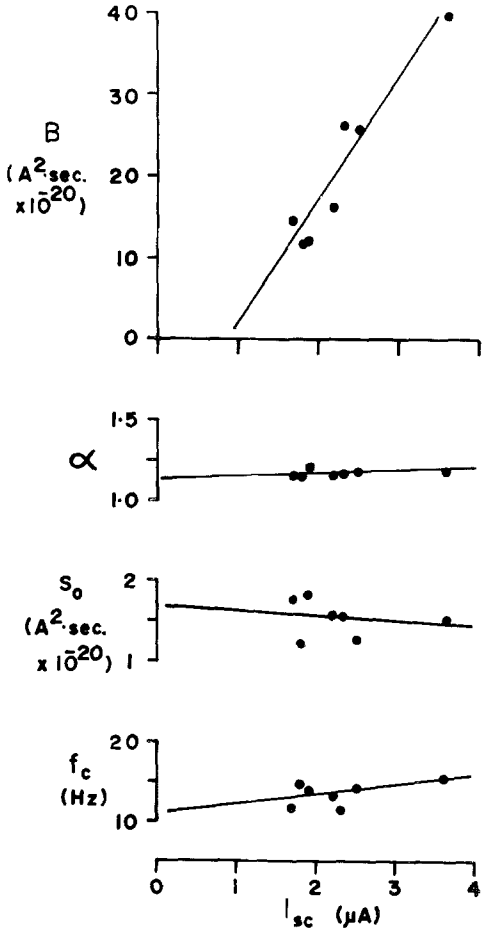

Fig. 9. I_{sc} and noise spectra in the presence of 1.4 μM amiloride were measured after each of a series of consecutive washes of a bladder's mucosal surface (*cf.* Fig. 8). The figure shows the best-fit parameters B, α, S_o, and f_c as a function of I_{sc}, and the best-fit straight lines through these points. Only the intensity B of $1/f$ noise is significantly correlated with I_{sc} and hence with washing. The x-intercept of B-*vs.*-I_{sc} is the small portion of the amiloride-sensitive current not abolished by 1.4 μM amiloride

Table 4. Comparison of amiloride-sensitive Na^+ channel among tight epithelia

Tissue	Depolarized preparations	Spontaneous current fluctuations	2-state model adequate for amiloride blockage?	i (pA)	γ (pS)	P_i^{Na} (cm/sec)	$[Na^+]_o$ decreases N?	Aldosterone increases I_A?	Triamterene decreases I_A?
Rabbit urinary bladder[a]	No	No	Yes	0.64 (37°C)	5.8	3.7×10^{-14}	Yes	Yes	Yes
Frog skin[b]	Yes	No	Yes	0.3–0.46 (20°C)	5.5		Yes	Yes	Yes
Frog skin[c]	No	No	Yes	0.59 (20°C?)			Yes		Yes
Toad urinary bladder[d]	Yes	No	Yes	0.18 (~20°C)			Yes	Yes	
Rabbit colon[e,f]	No	No	Yes	0.4 (37°C)	3.8	2.1×10^{-14}		Yes	
Hen coprodeum[g]	No	No	Yes	0.3 (37°C)	4		Yes	Yes	Yes

References: [a] Present study. [b] Lindemann & Van Driessche, 1977; Van Driessche & Lindemann, 1979. [c] Helman, Cox & Van Driessche, 1981; Hoshiko & Van Driessche, 1981. [d] Li et al., 1982. [e] Zeiske, Wills & Van Driessche, 1982. [f] N. Wills (*unpublished observations*). [g] Christensen & Bindslev, 1982. [e,f] Data not corrected for attenuation factor which will increase i, γ, and P_i^{Na} by roughly 60%.

and lack of normalization to actual surface area in preparations other than rabbit bladder. With this reservation, the i values are at least similar: 0.15–0.4 pA in the four other epithelia, 0.64 pA in rabbit bladder.

4. From i one can calculate the single-channel chord conductance γ by the equation

$$\gamma = i/(E_{Na} + V_a^{sc}) \tag{13}$$

where E_{Na} is the Nernst potential for Na^+ at the apical membrane and V_a^{sc} is the apical membrane potential under short-circuit conditions. From data in Wills and Lewis (1980) for rabbit bladder at 37°C one estimates V_a^{sc} as 52 mV, E_{Na} (for Na^+ activity of 104 mM = a_o^{Na} in the mucosal solution and 7 mM = a_i^{Na} intracellularly) as 72 mV. From our i value of 0.64 pA, Eq. (13) yields ~5.8 pS, close to the values of 3–5.5 pS found for the channel in other tight epithelia.

Comparison of γ among epithelia is strictly justified only if they have the same chemical and electrical gradients. It is more meaningful to calculate the single-channel permeability P_i^{Na}. If the channel obeys the constant-field equation, the following form of the equation relates current to permeability:

$$i_{Na} = (F^2/RT)P_i^{Na}V_a^{sc} (a_o^{Na} - a_i^{Na}e^{V_a^{sc}F/RT})/(1 - e^{V_a^{sc}F/RT}). \tag{14}$$

Substituting V_a^{sc}, a_o^{Na} as above, taking i_{Na} as 0.64 pA, assuming for simplicity one channel per cm^2 of tissue, and with RT and F having their usual meaning, we calculate $P_i^{Na} = 3.1 \times 10^{-14}$ cm/sec for rabbit bladder.

5. An increase in extracellular $[Na^+]$ reduces channel density in frog skin (Van Driessche & Lindemann, 1979), toad urinary bladder (Li et al., 1982), hen coprodeum (Christensen & Bindslev, 1982), and rabbit urinary bladder (present study). In rabbit bladder N increases 2.3-fold when $[Na^+]_o$ is reduced from 137 to 30 mM (a_o^{Na} reduced from 104 to 23 mM). Substituting a_o^{Na} = 23 mM and the measured single-channel current i_{Na} = 0.18 pA into Eq. (14) yields $P_i^{Na} = 3.7 \times 10^{-14}$ cm/sec, not significantly different from the value of 3.1×10^{-14} cm/sec at a_o^{Na} = 104 mM. Thus, external $[Na^+]$ affects channel density but not single-channel permeability.

6. Amiloride has been shown to block the Na^+ channel in all tight epithelia studied to date. Triamterene has been tested and shown to be a blocker in frog skin (Hoshiko & Van Driessche, 1981), hen coprodeum (Christensen & Bindslev, 1982), and rabbit urinary bladder (present study).

The Amiloride-Insensitive Pathway

P_{Na}/P_K

The apparent electromotive force of this pathway, hence its ratio P_{Na}/P_K, can be estimated by a method similar to that devised by Yonath and Civan (1971) for estimating the so-called E_{Na}.[2] The method

[2] E_{Na} is the transepithelial potential which must be applied across an epithelium to equal the chemical driving force for Na^+ entry across the apical membrane. E_{Na} has been shown to be equal to the sum of the electromotive force (emf) on Na^+ at the apical membrane, the emf at the basolateral membrane, and a term to account for an apical leak pathway (Lewis et al., 1978).

rests on the observation that washing removes only the leak pathway, which Lewis and Wills (1981) localized to the apical membrane.

By an argument similar to that used by Lewis, Wills and Eaton (1978), one can show that the inverse slope of a graph of G_T *vs.* I_{sc} as a function of washing is given by the equation

$$(\text{slope})^{-1} = E_L^a + E^b + (R^b/R_A^a)(E_L^a - E_A^a) \qquad (15)$$

where E_L^a and E_A^a are the apical membrane emf's associated with the leak pathway and the A channel, respectively, R_b is the basolateral membrane resistance, and R_A^a is the apical membrane resistance associated with the A channel.

Two arguments indicate that the term $(R^b/R_A^a)(E_L^a - E_A^a)$ in Eq. (15) can be neglected. First, R^b/R_A^a is usually less than 0.1 (Lewis & Wills, 1983). Second, Fig. 10 shows that the inverse slope of G_T *vs.* I_{sc} is essentially the same in the absence of amiloride (48.1 mV) as in the presence of 1.4 μM amiloride (47.9 mV). Since 1.4 μM amiloride increases R_A^a nearly 10-fold, this observation confirms that the term $(R^b/R_A^a)(E_L^a - E_A^a)$ contributes negligibly to the slope.

Thus, Eq. (15) reduces to: $(\text{slope})^{-1} = E_L^a + E^b$. Substituting $(\text{slope})^{-1} = 48$ mV from Fig. 10 and $E^b = 53$ mV from Lewis and Wills (1983) yields $E_L^a = -5$ mV (cell interior negative to mucosal solution), implying that P_K slightly exceeds P_{Na}. Inserting $E_L^a = -5$ mV with published values of intracellular ion activities (Wills & Lewis, 1980) into the constant field equation yields $P_{Na}/P_K = 0.7$ for the leak pathway, a value near the free-solution mobility ratio.

Effect of Washing

Washing by intracellular perfusion causes loss of K^+ channel conductance in squid axon (Chandler & Meves, 1970; Almers & Armstrong, 1980) and *Myxicola* axon (Schauf, 1982), and of Ca^{++} channel conductance in rat dorsal root ganglion neurons (Kostyuk, Veselovsky & Fedulova, 1981), *Limnea stagnalis* nerve cell bodies (Byerly & Hagiwara, 1982), and bovine chromaffin cells (Fenwick, Marty & Neher, 1982). In these examples the effect of washing might depend on loss of a cytoplasmic constituent necessary for channel conduction (or perhaps stabilization), rather than of the channel itself. For example, the effect in squid axon depends on replacement of internal K^+ with impermeant cations and is prevented by perfusion with K^+ or Cs^+. The effect in rat dorsal root ganglion neurons is prevented by perfusion with cAMP, ATP, and Mg^{++}. In *Myxicola* axons K^+ conductance disappears with internal perfusion but not with internal

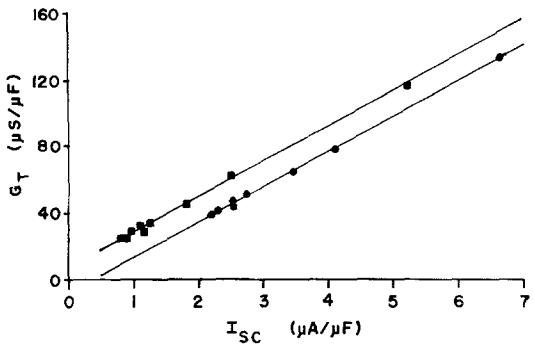

Fig. 10. Correlation between G_T and I_{sc} as a function of washing the mucosal surface, in the presence (■) or absence (●) of 1.4 μM amiloride. Data from Fig. 8 (*see* text for discussion)

dialysis, implying that the effective agent removed has a molecular weight over 5,000.

In our experiments the apical surface of the bladder under *in vivo* conditions is in contact with urine, which we replace by Ringer's solution. It seems unlikely that the loss of amiloride-insensitive conductance with washing is due to absence of a stabilizing factor present in urine. More likely, washing removes the leak protein itself in our experiments. Our guess is that the protein responsible for this conductance dissociates into the mucosal solution and that an equilibrium exists between dissociated and inserted protein.

Origin of 1/f Noise

Five observations suggest that *1/f* noise in rabbit bladder arises from the leak pathway. These observations are that: (1) Variation among individual bladders in intensity of *1/f* noise is closely correlated with variation in the amiloride-insensitive conductance G_L (Fig. 4*a*). (2) Amiloride has no effect on the intensity of *1/f* noise. (3) Punching increases G_L and the intensity of *1/f* noise by the same factor (Table 2). (4) Washing decreases G_L and the intensity of *1/f* noise by the same factor (Fig. 9). (5) In bladders of old rabbits with bladder stones, the amiloride-sensitive current and conductance and the intensity of the Lorentzian component are elevated by a factor of up to 26, while *1/f* noise and I_L remain the same as in normal bladders (Loo & Diamond, 1983).

1/f noise has been studied in numerous biological and artificial membranes. In several nerve preparations both *1/f* noise and a Lorentzian component are associated with K^+ transport, and the exponent α is close to 1.0 (myelinated nerve: Verveen & Derksen, 1965; lobster axon: Poussart, 1971; squid axon: Fishman, Moore & Poussart, 1975). *1/f* noise

has also been observed in four epithelia besides rabbit urinary bladder, with α between 1.7 and 2.0 (frog skin: Lindemann & Van Driessche, 1977; *Necturus* gallbladder: Gögelein & Van Driessche, 1981; toad urinary bladder: Li et al., 1982; rabbit descending colon: Zeiske et al., 1982). The origin of $1/f$ noise is unknown in these other epithelia. Our results for rabbit bladder suggest that $1/f$ noise without Lorentzian noise arises from one channel while Lorentzian noise without $1/f$ noise arises from another channel.

As in membranes where the origin of $1/f$ noise has been identified (e.g., originating in K^+ transport in nerve), the mechanism responsible for it is uncertain (Radeka, 1969; Hooge, 1972; Verveen & DeFelice, 1974; Neumcke, 1978). Different mechanisms may be responsible in different membranes, since in principle a $1/f$ noise spectrum could arise from any physical process with a continuous distribution of time constants. Our observation that the intensity B of $1/f$ noise in rabbit bladder is proportional to I_L suggests that $1/f$ noise in this tissue does not arise from channel resistance, as we would expect B to vary as I_L^2 for such a mechanism. A possible mechanism stems from the observation of Lewis and Wills (1981) that P_{Na}/P_K changes greatly during degradation of the A channel. If degradation occurs stepwise and the degraded channels are heterogeneous, opening and closing of each channel might yield a different Lorentzian, and a sum of many different Lorentzians could approximate $1/f$ noise.

Relation between the Amiloride-Sensitive Channel and Amiloride-Insensitive Pathway

Six observations suggest that the leak pathway may arise by degradation of the A channel:

1. In five rabbit bladders we have observed a loss of amiloride-sensitive conductance G_A and equal gain of G_L, either spontaneously or else as a result of voltage shocks. This suggests conversion of the A channel to the peak pathway.

2. If the leak pathway were not a degraded version of something else in the process of being lost, it would be difficult to understand why it is washed out so easily, or why the apical membrane of a Na^+-transporting epithelium should be burdened with a nonselective Na^+-and-K^+ pathway at all.

3. The proteolytic enzyme trypsin irreversibly reduces G_A, indicating the susceptibility of the A channel to degradation (S.A. Lewis, *unpublished observation*). Could its natural degradation be by proteolytic enzymes in urine or in the cytoplasm?

4. The density of A channels is higher in membrane of the intracellular vesicles than in apical membrane, even though channels eventually equilibrate between the two membrane pools (Lewis & de Moura, 1982). This suggests that in the steady state A channels undergo degradation in the apical membrane.

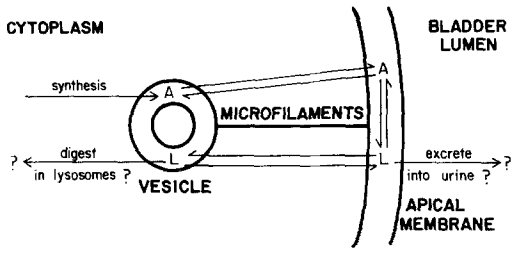

Fig. 11. Model of Na^+ channel turnover, membrane insertion, and degradation. The cytoplasm contains vesicles connected to each other and to the apical membrane by microfilaments. Fresh Na^+-selective amiloride-sensitive channels (*A*) are synthesized in the cell and transferred to vesicular membrane. Contact between vesicular and apical membrane permits channels to equilibrate between the two membrane pools. In the apical membrane the A 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)

5. The ratio of I_A to I_L is around 1 in unpunched bladders but around 6 for the I_{sc} added by punching. This suggests that in the apical membrane A channels are lost or leak pathways are produced, or both processes occur.

6. P_{Na}/P_K is 30 for I_A in freshly punched membranes (Lewis & de Moura, 1982), 2.6–9 for I_A in unpunched membranes (Lewis & Wills, 1981), and 0.7 (present paper) or 0.4 (Lewis & Wills, 1981) for the leak pathway. This implies that the A channels lose Na^+/K^+ selectivity along with amiloride sensitivity as they become degraded.

A Proposed Turnover Cycle of Channels

The evidence of the preceding section, suggesting degrading of A channels to leak pathways, will now be integrated with morphological information into a model of channel turnover (Fig. 11; *see also* Lewis & deMoura, 1984).

Morphological studies have shown that the distinctive thick regions of the apical membrane of mammalian urinary bladder are synthesized in the Golgi apparatus apparently as membrane vesicles, which are released into the cytoplasm (Hicks, 1966; Severs & Hicks, 1979). Vesicular membrane is inserted into the apical membrane when the bladder distends with urine, and microfilaments play a role in the insertion process (Minsky & Chlapowski, 1978; Lewis & de Moura, 1982). When the bladder empties, vesicles pinch off the apical membrane and

are withdrawn into the cytoplasm again, as shown by appearance in cytoplasmic vesicles of ferritin added to the bladder lumen (Porter, Kenyon & Badenhausen, 1967). Finally, the vesicles are destroyed within cytoplasmic vacuoles (Porter et al., 1965, 1967). It is unknown how many cycles of insertion and withdrawal a vesicle experiences during its lifetime.

A channels are synthesized under the control of aldosterone. The electrophysiological evidence of the present paper is consistent with the previous interpretations of Lewis and de Moura (1982) and Lewis and Wills (1981) as follows:

1. The A channels are first transferred to the cytoplasmic vesicles.

2. The channels are then transferred to the apical membrane by either of two mechanisms depending on the circumstances: incorporation of vesicular membrane into apical membrane, with a consequent rise in apical capacitance and area (during bladder distension); or contact between vesicular and apical membrane and equilibration of vesicles between these two membrane pools, without change in apical capacitance and area (during punching).

3. A channels are degraded into leak pathways. We do not know whether the degradation occurs while the channels are in the apical membrane or in the vesicles. If the former, the degradation could be promoted by agents in urine.

4. The leak channels are eliminated, either into urine (as suggested by their disappearance with washing) or else by being broken down intracellularly like other proteins involved in turnover.

It is obvious that numerous major features of the scheme of Fig. 11 remain unresolved. Is the site of A channel degradation and leak pathway destruction confined to the apical membrane? Is the degradation spontaneous, enzymatic, or dependent on urinary constituents? How are leak pathways destroyed? Are A channels and vesicular membrane synthesized at the same intracellular site? Is the change in P_{Na}/P_K, from 30 in fresh A channels to 2.6–9 in older A channels to 0.7 in leak channels, graded or stepwise? What is the molecular mechanism of this change in P_{Na}/P_K, and is it related to the molecular mechanism by which amiloride sensitivity is lost?

The scheme of Fig. 11 may be relevant to other epithelia in which insertion of membrane channels by vesicles is suggested by morphological evidence, capacitance changes, or inhibition of transport by colchicine or cytochalasin B. The most spectacular example is acid secretion by gastric mucosa (Diamond & Machen, 1983). Other examples are acid secretion by turtle urinary bladder (Arruda, Sabatini, Mola & Dytko, 1980), ADH-stimulated water permeability in toad urinary bladder (Taylor, Mamelak, Reaven & Maffly, 1973; Wade, Stetson & Lewis, 1981), and intestinal secretion (Notis, Orellana & Field, 1981). More generally, many membrane transport proteins and channels are likely to have a hydrophobic exterior and hence to pose problems for translocation from an intracellular site of synthesis through the cytoplasm to the plasma membrane. Vesicular membrane is a promising candidate as a delivery system.

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