Blocker-related Changes of Channel Density

**Analysis of a Three-State Model for Apical Na Channels of Frog Skin**

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**ABSTRACT** Blocker-induced noise analysis of apical membrane Na channels of epithelia of frog skin was carried out with the electroneutral blocker (CDPC, 6-chloro-3,5-diamino-pyrazine-2-carboxamide) that permitted determination of the changes of single-channel Na currents and channel densities with minimal inhibition of the macroscopic rates of Na transport (Baxendale, L. M., and S. I. Helman. 1986. *Biophys. J.* 49:160a). Experiments were designed to resolve changes of channel densities due to mass law action (and hence the kinetic scheme of blocker interaction with the Na channel) and to autoregulation of Na channel densities that occur as a consequence of inhibition of Na transport. Mass law action changes of channel densities conformed to a kinetic scheme of closed, open, and blocked states where blocker interacts predominantly if not solely with open channels. Such behavior was best observed in "pulse" protocol experiments that minimized the time of exposure to blocker and thus minimized the contribution of much longer time constant autoregulatory influences on channel densities. Analysis of data derived from pulse, staircase, and other experimental protocols using both CDPC and amiloride as noise-inducing blockers and interpreted within the context of a three-state model revealed that Na channel open probability in the absence of blocker averaged near 0.5 with a wide range among tissues between 0.1 and 0.9.

**INTRODUCTION**

We address in this paper the issue of blocker-induced noise analysis of apical membrane Na channels in epithelia of frog skin with specific regard to the determination of single-channel Na currents and channel densities that together determine the rates of apical membrane Na entry and hence Na absorption by such tissues. For
this purpose we have used a relatively weak electroneutral Na channel blocker (CDPC, 6-chloro-3,5-diamino-pyrazine-2-carboxamide) that has permitted noise analysis with relatively small inhibition of the macroscopic rates of Na transport and at blocker concentrations in a range well below and above its $K_c^{CDPC}$ (Baxendale and Helman, 1986). It was thus possible to determine changes of single-channel currents and channel densities in the vicinity of the spontaneous rates of Na transport and to analyze for kinetic models that could explain the blocker-dependent changes of channel densities (open and blocked states). As will become evident, the blocker-dependent changes of channel density according to mass law action conformed rather well to a simple three-state model of closed, open, and blocked state kinetics in which the blocker interacts predominantly if not solely with open states of the Na channel. In addition to mass law–related changes of channel densities, blocker inhibition of apical Na entry led to secondary long time-constant autoregulation of channel densities (Abramcheck et al., 1985).

Similar studies were carried out with the more potent Na channel blocker amiloride. This however required knowledge of the $K_{b}^{Ami}$ and led to a new method for its determination (see Results). Microscopic $K_{b}^{Ami}$ averaged 61 nM and was less in value than $K_{b}^{Ami}$ measured macroscopically from changes of short-circuit current under similar conditions (Helman et al., 1983). Such findings are expected according to three-state kinetics where blocker interacts principally with open states of the epithelial Na channel. In all groups of studies mean Na channel open probability averaged near 0.5.

**GLOSSARY**

$I_{sc}$ short-circuit current; $\mu$A/cm$^2$

$[B]$ blocker concentration in apical solution; $\mu$M

$I_{Na}^{b}$, $I_{Na}^{o}$ amiloride inhibitable $I_{sc}$ in the absence or presence of blocker in the apical solution; $\mu$A/cm$^2$

$i_{Na}$, $i_{Na}^{b}$ single-channel Na current in the absence or presence of blocker; pA

$k_{ob}$ open to blocked state rate coefficient; radians/s.$\cdot$M

$k_{bo}$ blocked to open state rate coefficient; radians/s

$k_{eq}$ equilibrium blocker coefficient of open channels $= k_{bo}/k_{ob}$; $\mu$M

$\beta^{'}$ open probability in the absence of blocker $= \beta/(\beta + \alpha)$

$N_{o}$, $N_{o}^{b}$ open-channel density in the absence or presence of blocker

$N_{c}$, $N_{c}^{b}$ density of blocked open channels

$N_{cb}$ sum of open and blocked open-channel densities; $N_{ob} = N_{o}^{b} + N_{b}$

$N_{c}$, $N_{c}^{b}$ density of closed channels in the absence and presence of blocker

$N_{cb}$ density of blocked closed channels

$N_{T}$, $N_{T}^{b}$ total channel density in the absence or presence of blocker

$K_{b}^{eq}$ equilibrium blocker concentration of closed channels (see text)

$F$ relative equilibrium blocker coefficients $= K_{b}^{eq}/K_{b}$

$\nu_{c}$ corner frequency (Hz) of current noise PDS

$S_{c}$ low frequency plateau value of PDS
**MATERIALS AND METHODS**

Abdominal skins of *Rana pipiens* (Kons Scientific, Germantown, WI) were used in all experiments. After "scraping" away ~90% of the corium to reduce the unstirred layer at the basolateral surface, the tissues were short-circuited in continuous flow chambers (Abramcheck et al., 1985). Flow rates were ~8 ml/min through chamber volumes of 0.6 ml. Within 2–3 h of continuous short-circuiting, the short-circuit current $I_s$ stabilized to control values. The Ringer's solution contained in millimolar: 100 NaCl, 2.4 KHCO$_3$, and 2.0 CaCl$_2$ at a pH near 8.1.

**Noise Analysis and Theoretical Considerations**

Data acquisition for power density spectral analysis was identical to that described previously (Van Driessche and Zeiske, 1980; Abramcheck et al., 1985). In brief, current noise was digitized by computer and Fourier transformed to give current noise PDS that were fit by non-linear least-squares regression analysis to $1/f^p$ and Lorentzian components according to Eq. 1:

$$S(f) = \frac{S_0}{f^p} + \frac{S_0}{1 + (f/f_0)^2}$$

The PDS were derived from an average of 60 sweeps of data (2,048 points) of either 2- or 1.2-s duration resulting in excellent signal/noise ratio (see Fig. 2).

CDPC (Aldrich Chemical Co., Milwaukee, WI) (see Fig. 1) was used at concentrations between 5 and 200 #M. The relationship between $f_c$ and $[B]$ (corner frequency plots) was linear (see Results), from which the ON ($k_{ob}$) and OFF ($k_{bo}$) rate coefficients for blocker interaction with open channels were determined according to Eq. 2:

$$2\pi f_c = k_{ob} [B] + k_{bo}$$

In the absence of blocker, Lorentzian components were not detectable within a bandwidth of 0.5 and 1,000 Hz, and the PDS were characterized by $1/f^p$ noise at the lower frequencies (Fig. 2 A). When apical membranes were exposed to CDPC, a single Lorentzian appeared in the PDS as shown in Fig. 2 B and $C$. $f_c$ ranged between ~35 and >220 Hz at [CDPC] between 5 and 200 #M. Amiloride was used at $[B]$ between 0.5 and 10 #M. Lorentzian components of the PDS could not be resolved at amiloride concentrations <0.5 #M, leading therefore to the conduct of amiloride-induced noise analysis at amiloride concentrations considerably greater than the mean $K^{am} = 61$ nM (see Results).

From the values of $S_{oo}$, $f_c$, $k_{ob}$, and the macroscopic rates of apical Na entry $I_{Na}^m$, the single-channel Na current, $i_{Na}^m$, was calculated at each blocker concentration (Eq. 3) assuming in accordance with the observed linearity of the corner frequency plots that the rate coefficients reflected only fluctuations of the channels between open and blocked states.

$$i_{Na}^m = \frac{S_0(2\pi f_c)^2}{4I_{Na}^m k_{bo} [B]}$$

The density of open channels at any blocker concentration is by Eq. 4:

$$N_o^m = \frac{I_{Na}^m}{i_{Na}^m}$$

With channels fluctuating between open ($N^o_o$) and blocked states ($N^b_o$) the total pool of channels in open + blocked states ($N_{ob}$) at any $[B]$ is according to the Law of Mass Action:

$$N_{ob} = N^o_o (1 + [B]/K_b) = N^o_o + N^b_o$$

where $K_b = k_{bo}/k_{ob}$ of the open-state blocker reaction.
In the absence of a closed state (two-state model consisting of open and blocked states), $N_{ob}$ so calculated is the same as the total channel density, $N_T$, at any $[B]$. In the absence of "recruitment" of channels from any source and by any mechanism during blocker inhibition of $I_o$, $N_{ob}$ and hence $N_T$ would be expected to remain constant (independent of $[B]$). This however is not the case for experiments carried out with amiloride and CGS 4270 (Helman et al., 1983; Abramcheck et al., 1985), other Na channel blockers (Li and Lindemann, 1983; Li et al., 1985, 1987), nor indeed for CDPC (to be reported below). In principle, blocker-dependent increases of $N_{ob}$ can arise from recruitment of channels by mass law action from a closed state (three-state model, see below) at constant total channel density ($N_T - N^B_T$) provided that blocker binds preferentially to the open state of the channel and not to a closed state of the channel. Blocker-dependent increases of $N_{ob}$ can arise also from recruitment of new, stored, or dormant channels that lead to increases of $N^B_T > N_T$. Hence the general problem is to distinguish between blocker-dependent changes of channel density due directly to mass law action and changes of channel density due to "autoregulation" of channel density, namely, mechanisms other than those related directly to the rate coefficients governing the exchange of channels between closed, open, and blocked states of the channels.

**Mass Law Considerations**

Patch-clamp experiments indicate unequivocally that highly selective epithelial Na channels fluctuate spontaneously between closed and open states with rather long mean open and closed times of several to many seconds (Sariban-Sohraby et al., 1984; Helman et al., 1985; Palmer and Frindt, 1986; Eaton and Hamilton, 1988; Marunaka Y., and D. C. Eaton, personal communication). In the presence of blocker, channels are distributed among at least...
three states:

\[
\begin{align*}
N_c & \xrightarrow{a} \frac{\beta}{k_a} N_c & \xrightarrow{[B]k_a} N_b & \quad \text{Three-state} \\
N_c & \xrightarrow{a} N_c & \xrightarrow{[B]k_a} N_b & \quad \text{(Scheme 1)}
\end{align*}
\]

In the absence of blocker the open state probability, \(P\), is defined by \(\beta/(\beta + a)\). In the presence of blocker and its interaction with open states, channels are “recruited” from closed into open and blocked states. Blocker-dependent changes of open-channel density \(N_c\) and open + blocked channel density \(N_{ob}\) are given by the equations summarized in Table I. For purpose of reference, equations for two- and four-state models are also given in Table I, where it was assumed that \(N_c\) is constant at all \([B]\) \(N_c = N_c^0\). It should be noted that \(N_{ob}\) would remain constant for all \([B]\) if channels fluctuated alone between an open and blocked state (two-state and four-state model). As this does not occur under any circumstance, and in view of spontaneous fluctuations of channels between open and closed states as observed in patch-clamp records, a two-state model can be dismissed from consideration without further comment.

It has been suggested that amiloride binds equally well to both the open and closed state of the Na channel (Ilani et al., 1984) leading in principle to a four-state kinetic model when channels are exposed to blocker.

\[
\begin{align*}
N_{ob} & \xrightarrow{\gamma} \frac{\beta}{k_a} N_c & \xrightarrow{a} N_a & \xrightarrow{[B]k_a} N_b & \quad \text{Four-state} \\
N_{ob} & \xrightarrow{\gamma} N_c & \xrightarrow{a} N_a & \xrightarrow{[B]k_a} N_b & \quad \text{(Scheme 2)}
\end{align*}
\]

Assuming that blocker binds equally well to both open and closed states of the channel, \(N_{ob}\).
would be expected to remain constant and independent of \([B]\). As this is not the general observation for any Na channel blocker studied so far, it must be inferred that blocker binding to open states of the channel is preferred over blocker binding to closed states of the channel. \(^1\) Defining \(K_C^* = \delta/\gamma\) as the equilibrium coefficient of the blocker reaction with the closed state, and defining \(K_C^* / K_s = F\), we find that in general:

\[
N_{Bo} / N_o = [(1 + \beta[B] / K_s) + (\beta[B] / FK_B)(1 - \beta')^{-1}
\]

and

\[
N_{ob} / N_o = (N_{Bo} / N_o) \cdot (1 + [B] / K_B)
\]

If \(K_C^* \gg K_s\) (hence \(F \rightarrow \infty\)), Eq. 6 reduces to the three-state model where

\[
N_{Bo} / N_o = [1 + \beta'([B] / K_s)]^{-1}
\]

and

\[
N_{ob} / N_o = (N_{Bo} / N_o) \cdot (1 + [B] / K_B)
\]

Thus, according to mass law considerations, the blocker-dependent changes of \(N_{Bo}\) and \(N_{ob}\) as given by the above equations provide a basis for evaluation and interpretation of data with relatively simple models of kinetic interactions of blocker with Na channels. To the extent that changes of channel density due to autoregulatory mechanisms can be avoided or at least minimized, we proceeded to analyze and interpret data in accordance with the expectations of the above models.

**Autoregulatory Considerations**

It is well known that acute perturbation of Na transport leads to secondary long time-constant transients of the \(I_o\). The mechanisms involved are unknown. In experiments of blocker-

\(^1\) Patch-clamp experiments by Y. Marunaka and D. C. Eaton (personal communication) have examined the blocker interaction of CDPC and amiloride with apical Na channels derived from aldosterone-treated A6 epithelia grown on permeable supports. Their data (manuscript submitted) indicate that unlike amiloride, the existence of CDPC closed-blocked states is at best infrequent and that for all practical purposes, CDPC binds preferentially if not solely to open states of the channel, which is in agreement with the thesis of this paper.

It is curious that amiloride, unlike CDPC, causes the appearance of a high affinity closed-blocked state (Marunaka Y., and D. C., Eaton, manuscript submitted). At least four possibilities may be considered to explain this anomaly: (a) Marunaka and Eaton suggest that because the residency or occupancy time for CDPC in the channel is very short (according to our data ~ 5 ms for CDPC and ~1,000 ms for amiloride), the probability of transition of a blocked channel into a closed-blocked state would be relatively infrequent. (b) Amiloride with a \(pK_a\) near 8.7 exists in both cationic and electroneutral forms, thereby exposing the channels to different forms of the blocker with undoubtedly different rate coefficients for binding to open and perhaps closed channels. (c) It has come to our attention that amiloride-like impurities contaminate some commercially available preparations, which would lead to problems in assessing channel kinetics in the face of multiple forms of blocker interactions with the channels. (d) Amiloride at micromolar concentrations penetrates cells exerting influence on cellular enzymes that are involved in regulation of apical membrane permeability to Na and may alter channel kinetics. Such problems are exacerbated especially with amiloride where data must be accumulated over rather lengthy intervals of time and where the stability of the patch is questionable due either to spontaneous decay of patch stability and/or time-dependent changes of regulatory mechanisms influencing channel activity and its interactions with blocker. With high rate blockers like CDPC, such concerns are minimized and may at least in part account for the compatibility of noise and patch experiments, which indicates that blocker kinetics conform to a three-state model where blocker interacts almost exclusively if not solely with open channels.
induced noise analysis using a "staircase" protocol of increasing blocker concentration, the $I_c$ records appear "scalloped" with $I_c$ returning toward control values after step increases of blocker concentration. Long time-constant secondary transients of the $I_c$ may reflect time-dependent changes of rate coefficients and/or changes of the total pool of channels ($N_T$) involved in the blocker reaction. Indeed, such time-dependent changes of channel densities would add to those of $N_N^o$ and $N_{ob}$ due to the mass law expectations indicated above.

To the extent that $k_{ob}$ and $k_{bo}$ of the blocker reaction is independent of time of exposure of the channels to blocker (see below), autoregulatory influences on $N_N^o$ and $N_{ob}$ were expected to be manifest as changes of either $\beta$ and/or $N_T$.

**Kinetics of the Blocker Reaction**

Long time-constant transient autoregulation of channel density as indicated in Results, is of the order of 10–20 min. Such time constants cannot arise from mass law blocker interaction with the Na channels. If for example the epithelium is exposed to a step increase of blocker concentration, the channels will redistribute among closed, open, and blocked states with time constants related directly to the rate coefficients. For a step increase of [CDPC] from 0 to 5 μM where $f$ is ~40 Hz (see Results), the time constant for channel redistribution between open and blocked states is near 4 ms, which for all practical purposes is near instantaneous. Assuming equal mean open and closed times of 5 s for spontaneous fluctuations between closed and open states, the time constant for redistribution of channels between closed and open states is 2.5 s with a corner frequency of 0.06 Hz. Accordingly, equilibrium redistribution of channels after exposure to blocker would occur well within 30 s since it is dictated by the values of $\alpha$ and $\beta$. In the absence of evidence to the contrary, we assumed that it was most unlikely that mean open and closed times of Na channels are in the range of 5–10 min as would be necessary to explain long time-constant transients in the range of 10–20 min.

It should be recalled that spontaneous Lorentzians are not observed in the absence of blocker at frequencies >0.1 Hz. Such observations are in agreement with the idea that spontaneous fluctuations between open and closed states occur at a low rate and as such will have little or no influence on the blocker-induced Lorentzians that occur in a range of 30 to >200 Hz (at [CDPC] between 5 and 200 μM). Thus, it was not surprising that when using Na channel blockers like CDPC the relationship between $[B]$ and $2\pi f_c$ (Eq. 2) was linear, as would be expected when Lorentzian current noise arises solely from blocker-related fluctuations between open and blocked states, which leads directly to the determination of $k_{ob}$ and $k_{bo}$ of the open-state blocker reaction.

**Control Parameters**

Whereas blocker inhibition of Na transport leads to changes of channel densities and single-channel currents, it remains of particular interest to determine control parameters in the absence of blocker. With weak channel blockers like CDPC, where $I_c$ is perturbed minimally from its control value and where noise analysis is possible at $[B] \ll K_b$, extrapolation of blocker concentration-dependent changes of $I_{as}$ to zero blocker concentration provides a relatively certain method for determination of the control $I_{as}$ and hence the $N_o - I_{as}/I_{bo}$. It was moreover apparent, assuming for the moment an ideal three-state model, that the open probability $\beta'$ in the absence of blocker can be calculated at all $[B]$ after rearrangement of Eq. 8.

$$\beta' = \frac{1 - N_N^o / N_o}{(N_N^o / N_o) \left ([B] / K_b \right )}$$

Three-state

If $N_T = N_N^o$, $\beta'$ is expected to be constant at all $[B]$ with possible values between 0 and 1. As will be shown in Results, data derived from "pulse" experiments that minimized time of
exposure to the blocker were consistent with this idea. When, however, Na transport was inhibited for prolonged durations, apparent open probabilities, to be referred to as \( \beta' \), decreased with time of exposure to the blocker reflecting most likely time-dependent changes of \( N_\alpha' \). In such experiments, the changes of \( \beta' \) were extrapolated to the zero time and zero blocker concentration control state of the tissue to yield \( \beta' \) of the preblocker control state of the tissue.

In principle, regardless of time and/or other dependencies of the channel densities and single-channel currents, and provided that these parameters were well behaved and minimally perturbed by blocker from their control values, extrapolation to zero blocker concentration was assumed to provide reasonable control parameter values of the apical membrane Na channels, namely, \( \bar{I}_\text{Na}, N_\alpha, \) and \( \beta' \). It follows directly that total channel density in the absence of blocker is given by \( N_T = N_\alpha/\beta' \).

All experiments were carried out at room temperature (22–27°C), and statistical data are given as means ± SEM. Standard error bars not appearing in figures are within the size of the data points.

**RESULTS**

**Pulse Protocol**

The dependency of single-channel Na current (\( i_{\text{Na}} \)) and open-channel density (\( N_\alpha' \)) on blocker concentration was evaluated using two protocols, which will be referred to as pulse and staircase protocols. As illustrated in Fig. 3, tissues were subjected to pulses of CDPC (2 min) at concentrations between 5 and 50 \( \mu \text{M} \) or 10 and 200 \( \mu \text{M} \). It was evident that CDPC caused abrupt inhibition of the \( I_{\text{Na}} \) followed by a relatively small relaxation of the \( I_{\text{Na}} \) both during and after pulsing with CDPC. CDPC-induced current noise was measured during the latter 72 s of each pulse (60 sweeps x 1.2 s/sweep). \( i_{\text{Na}}' \) was taken as the mean over this time interval.
Between pulses, the $I_{\text{Na}}$ relaxed back toward control values. This phenomenon was explored in greater detail (see below).

Current noise spectra were obtained during the last 60 s of each pulse of CDPC and the data were summarized as shown in Fig. 4 and Table II. In 10 experiments CDPC pulses ranged between 5 and 50 μM (Fig. 4, A–D) and in a second group of 8 experiments between 10 and 200 μM (Fig. 4, E–H). The amiloride-sensitive macroscopic Na transport ($I_{\text{Na}}^B$) was inhibited progressively by increasing concentration of CDPC while $S_0$ showed the expected biphasic behavior with increasing [B]. The corner frequency plots ($2\pi f_c$ vs. [CDPC]) were linear up to 200 μM CDPC from which ON ($k_{o\alpha}$) and OFF ($k_{b\beta}$) rate coefficients were determined for individual experiments.

Figure 4. Summary data of pulse protocol experiments. 0–50 μM CDPC pulse experiments are shown in panels A–D. 10–200 μM CDPC pulse experiments are shown in E–H. Values are means ± SEM for all experiments. Analysis of individual experiments provided the data summarized in Table I.
| Protocol | ${I_{Na}}$ | $k_{ab}$ | $k_{ss}$ | $K_h$ | $i_{Na}$ | $N_v$ | $\beta'$ | $N_t$ |
|----------|------------|----------|----------|-------|----------|-------|--------|-------|
|          | $\mu A/cm^2$ | rad/s $\cdot \mu M$ | rad/s | $\mu M$ | $pA$ | millions/cm$^2$ | millions/cm$^2$ |       |
| Pulse    |            |          |          |       |       |       |        |       |
| 0–50 $\mu M$ CDPC (N = 10) | 16.24 ± 1.60 | 6.57 ± 0.32 | 206.9 ± 6.3 | 32.0 ± 1.7 | 0.53 ± 0.03 | 31.9 ± 4.1 | 0.58 ± 0.05 | 63.3 ± 11.9 |
|          | (9.15–23.78) | (4.59–8.12) | (174.3–237.0) | (26.6–45.5) | (0.42–0.65) | (19.7–53.1) | (0.27–0.77) | (25.5–140.0) |
| 0–200 $\mu M$ CDPC (N = 8) | 17.42 ± 2.30 | 6.27 ± 0.48 | 238.0 ± 6.9 | 40.2 ± 4.4 | 0.52 ± 0.05 | 36.7 ± 7.3 | 0.45 ± 0.04 | 80.1 ± 14.9 |
|          | (13.20–32.90) | (3.84–8.36) | (210.3–259.1) | (25.6–65.5) | (0.25–0.71) | (23.9–83.4) | (0.28–0.61) | (52.4–192.6) |
| Staircase |            |          |          |       |       |       |        |       |
| 0–50 $\mu M$ CDPC (N = 43) | 25.95 ± 1.45 | 7.53 ± 0.12 | 212.2 ± 5.7 | 28.2 ± 0.6 | 0.48 ± 0.01 | 56.5 ± 4.1 | 0.45 ± 0.03 | 151.5 ± 15.1 |
|          | (12.30–53.60) | (6.08–9.09) | (161.5–308.4) | (23.5–38.2) | (0.32–0.62) | (25.7–167) | (0.065–0.791) | (38.2–540.0) |

Values are means ± SEM (range).
and summarized as groups (Fig. 4). Summary values of $k_b$ and $k_{ob}$ are given in Table II together with the equilibrium constant $K_B$. Notably, the rate coefficients varied significantly among experiments. $K_B$ ranged spontaneously between ~26 and 65 μM indicating that the rate coefficients governing the blocker reactions are labile and so possibly regulated by hormonal and/or other cytosolic factors (Els, W.J., and S. I. Helman, submitted for publication).

Single-channel Na currents ($i_{Na}$) were calculated at all blocker concentrations (Fig. 4, D and H). Despite the wide range of $[B]$ and inhibition of $I_{Na}$, the $i_{Na}$ remained fairly constant, tending to increase slightly with increasing $[B]$. In each experiment the $i_{Na}$ were extrapolated to the ordinate at zero blocker concentration, yielding values of the $i_{Na}$ (absence of blocker). The $i_{Na}$ values averaged near 0.53 pA, ranged between 0.29 and 0.71 pA, and were similar to those values reported previously where amiloride and CGS 4270 were used as the noise-inducing blocker (Helman et al., 1983; Abramcheck et al., 1985).

Blocker-dependent changes of open-channel density ($N_o^b$) and open + blocked channel density ($N_{ob}$) were calculated with Eqs. 4 and 5, respectively. The control open-channel density in the absence of blocker ($N_o$) was calculated from the quotient $I_{Na}/i_{Na}$. With $i_{Na}$ averaging 16.24 μA/cm², $N_o$ averaged 31.9 x 10⁶ channels/cm² (Table II).

The blocker-dependent changes of $N_o^b$ and $N_{ob}$ are summarized in Fig. 5, A and C, where as shown $N_o^b$ and $N_{ob}$ were normalized to the values of $N_o$. $N_o^b$ was decreased while $N_{ob}$ was increased progressively from control $N_o$ with increasing blocker concentration. Clearly, $N_{ob}$ was increased markedly above control $N_o$, indicating that the pool of channels in open and blocked states was not constant and independent of $[B]$. Moreover, it was apparent, as pointed out previously for experiments with CGS 4270 (Abramcheck et al., 1985), that $N_o^b/N_o$ (and $i_{Na}^b/I_{Na}$) was >0.5 at $[B] = K_B$. This indicates that changes of $N_o^b$ did not follow either a two-state or a four-state model of blocker kinetics, where in the four-state model blocker interacts equally well with open and closed states of the channel.

An apparent open probability “$β$” was calculated at each $[B]$ with Eq. 7. The data are summarized as a function of $[B]$ as shown in Fig. 5, B and D. It should be emphasized that the apparent value “$β$” calculated in this manner presumes that total channel density (open + closed + blocked states) remains constant. Indeed, despite relatively large changes of $i_{Na}$ “$β$” remained essentially constant, tending to decrease slightly with increasing $[B]$. For each experiment, the “$β$” values were extrapolated to the origin at zero blocker concentration to yield values of $β$ in the absence of blocker. $β$ averaged 0.58 and 0.45 for the two groups of pulse protocol experiments, showing, however, a large range of values between 0.27 and 0.77 among tissues.

Assuming that $β$ remained constant at all $[B]$, the expected mass law behavior of $N_o^b$ and $N_{ob}$ according to three-state model kinetics was calculated and is illustrated by the solid lines drawn in A and C of Fig. 5. The curves fit the data remarkably well with small consistent deviations from ideal behavior at the higher $[B]$ (Fig. 5 C). Such deviations from ideal mass law behavior in these pulse experiments is due most likely to relatively small increases of $N_o^b$, as will be explored further below. To a rather good first approximation, however, the data derived from pulse experiments...
were consistent with channel density kinetics according to a simple three-state model in which blocker interacts predominantly if not solely with open channels. If we accept the idea that $N_{ob}^b$ remained essentially unchanged during brief periods of pulsing with CDPC, then the increases of $N_{ob}$ would reflect "recruitment" of channels from closed into open and blocked states, resulting thereby in the increase of $N_{ob}$. 

**Figure 5.** Blocker-dependent changes of $N_o^o$ (open channels in the presence blocker) and $N_{ob}$ (open + blocked channels in the presence of blocker) observed in pulse experiments are shown in A (5-50 μM CDPC) and C (10-200 μM CDPC). An apparent open probability "$\beta'$" was calculated at each blocker concentration as shown in B and D (see text). Extrapolation of the apparent "$\beta'$" to zero blocker concentration provided estimates of open probability in the absence of blocker ($\beta$). The solid lines shown in A and C were drawn using mean $\beta'$ of 0.58 and 0.45 assuming that $N_{ob}^b = N_{ob}$.
FIGURE 6. Staircase protocol. Typical experiments are shown in A where the $I_{Na}$ was inhibited by a staircase increase of apical solution CDPC concentration. After complete washout of CDPC and secondary relaxation of $I_{Na}$, the tissue was exposed to a staircase increase of apical solution amiloride concentration. (B) Similar experiments to those of A for CDPC staircase protocol. 0.5 μM amiloride was used to inhibit $I_{Na}$. Note secondary relaxation of $I_{Na}$. Current deflection reflects changes of $I_{Na}$ in response to voltage-clamp pulses of a few millivolts. Current noise spectra were measured after turning off the pulse generator.

Autoregulation of Apical Membrane Na Transport

It has been a consistent observation that inhibition of apical Na transport by blockers leads to a secondary transient return of the $I_{Na}$ towards its control value. This has been reported explicitly for studies done with CGS 4270 (Abramcheck et al., 1985) where the $I_{Na}$ records appeared “scalloped” and where, as shown in Fig. 6, similar patterns of scalloping were observed with CDPC and with amiloride at the lowest
concentrations of amiloride that permit blocker-induced noise analysis. With staircase protocols (Fig. 6), where tissues were continuously inhibited by CDPC and with progressively increasing blocker concentrations, complete washout of CDPC from the apical solution was prompt, showing a relatively large overshoot of the $I_{Na}$ and of a magnitude far larger than had ever been observed in the pulse protocol experiments (compare with Fig. 3). It was surmised, as suggested above, that prolonged inhibition of the $I_{Na}$ may have led to an increase of either $N_{T}$ and/or $\beta'$. Because $f_c$ remained constant during prolonged exposure to $[B]$ and because the corner frequency plots were linear regardless of the duration of exposure to blocker, it was most unlikely that the scalloped appearance of the $I_{Na}$ records during exposure to blocker could be attributed to changes of the blocker rate coefficients. Moreover, after washout of CDPC, the $I_{Na}$ relaxed toward control rather slowly, indicating that

![Graph showing changes of $I_{Na}$ after inhibitor exposure.](image)

**Figure 7.** Changes of $I_{Na}$ (expressed as a percentage of control) after inhibition by 20 and 50 $\mu$M CDPC. The dashed lines of A and B were used to extrapolate the spontaneous changes of $I_{Na}$ independent of those caused by CDPC. The difference between the observed $I_{Na}$ and the extrapolated values was plotted as shown in C and fit to a single exponential (solid line). The mean time constants were 10.9 and 19.0 min for tissues inhibited by 20 and 50 $\mu$M, respectively (see Table II).

Changes of either $N_{T}$ and/or $\beta'$ were involved in the post-CDPC autoregulatory decrease of $I_{Na}$.

To obtain a quantitative estimate of the time-dependent changes of the $I_{Na}$ secondary to inhibition by CDPC, two groups of experiments were done (Fig. 7). The $I_{Na}$ was monitored continuously to establish its baseline rate of change with time. At time zero, tissues were exposed to apical solution CDPC at concentrations of either 20 or 50 $\mu$M for 35 min. As with staircase protocols, washout of CDPC from the apical solution resulted in overshoot of the $I_{Na}$, followed thereafter by a rather slow return towards control values. The control values of $I_{Na}$ (0 time = 100%) and the values of $I_{Na}$ at 1 and 35 min after exposure to CDPC are summarized in Table III. To obtain an estimate of the time constant of secondary relaxation of the $I_{Na}$, the mean data shown in A and B of Fig. 7 were corrected for baseline drift and the
TABLE III

| [CDPC] | \(I_c\) control | \(I_c^*\) 1 min | \(I_c^*\) 35 min | \(\Delta I_c\) | \(\tau\) | \(I_c(\infty)\) |
|--------|-----------------|----------------|----------------|--------------|-------|----------------|
| 20 \(\mu\)M (7) | 13.76 ± 0.35 | 10.16 ± 0.39 | 11.74 ± 0.54 | 19.9 | 10.9 | 92.7 |
| 50 \(\mu\)M (9) | 16.17 ± 1.46 | 10.40 ± 1.07 | 13.79 ± 1.37 | 32.1 | 19.0 | 94.9 |

Values are means ± SEM.

mean percentage of control values were plotted as shown in C of Fig. 7. These data were fit to a single exponential with time constants of 10.9 and 19.0 min for tissues inhibited by 20 and 50 \(\mu\)M CDPC, respectively (Table II). Expressed as a percentage of control, the magnitudes of the exponentials were 19.9\% (20 \(\mu\)M CDPC) and 32.1\% (50 \(\mu\)M CDPC), and the values of \(I_c(\infty)\) at \(t = \infty\) were 92.7 and 94.9\%, respectively. Thus, within 35 min, autoregulatory mechanisms compensate for mass law inhibition of the apical Na channels.

Estimates of \(\beta^*\) from Microelectrode Experiments

Experiments were done to assess the time rate of change of the \(I_c\) and apical membrane voltage (\(V_a\)) in response to apical CDPC, which led to an alternative method for determination of \(\beta^*\). The experimental protocols are illustrated in Fig. 8 with data from typical experiments. \(V_a\) and \(I_c\), summarized in Table IV, were normalized to their control values (Fig. 8). 50 \(\mu\)M CDPC caused an abrupt inhibition of \(I_c\) with the expected hyperpolarization of \(V_a\) (Fig. 8 A). Within 1 min, \(I_c\) decreased on average from 34.7 to 25.0 \(\mu\)A/cm\(^2\), while \(V_a\) was hyperpolarized from its mean control value of -81.1 to -93.9 mV. During the following 9 min, both \(V_a\) and \(I_c\) relaxed towards control values averaging -88.6 mV and 27.5 \(\mu\)A/cm\(^2\), respectively, at 10 min after exposure to CDPC. Both \(I_c\) and \(V_a\) exhibited a scalloped appearance when

![Figure 8](image-url)
| Protocol                  | Time | \( I_0 \)  | \( \beta R_a \) | \( V_i \) | \( I^*_o/I_o \) | \( V_o/V^*_o \) | \( \beta \)   |
|--------------------------|------|-------------|-----------------|----------|-----------------|-----------------|------------|
| Pulse (10)               |      |             |                 |          |                 |                 |            |
| Control                  |      | 34.7 ± 4.8  | 0.812 ± 0.027   | -81.1 ± 5.2 | 0.705 ± 0.050 | 0.863 ± 0.016 | 0.379 ± 0.040 |
| 50 \( \mu \) M CDPC      | -1   | 25.0 ± 3.9  | 0.907 ± 0.021   | -95.9 ± 5.6 | 0.731 ± 0.705 | 0.851 ± 0.018 | 0.359 ± 0.041 |
|                          | 10   | 27.5 ± 4.4  | 0.884 ± 0.021   | -88.6 ± 5.6 |                 |                 |            |
| Washout CDPC             | -1   | 36.9 ± 5.2  | 0.792 ± 0.024   | -75.6 ± 5.0 |                 |                 |            |
| Staircase (10)           |      |             |                 |          |                 |                 |            |
| Control                  |      | 32.8 ± 3.4  | 0.817 ± 0.025   | -86.0 ± 5.2 |                 |                 |            |
| 5–50 \( \mu \) M CDPC   | -25  | 24.0 ± 2.9  | 0.888 ± 0.019   | -91.8 ± 5.5 |                 |                 |            |
| Washout CDPC             | -1   | 33.2 ± 3.6  | 0.789 ± 0.039   | -76.9 ± 5.7 | 0.706 ± 0.026 | 0.824 ± 0.034 | 0.419 ± 0.046 |

Values are means ± SEM. See text for calculation of \( \beta' \).
tissues were subjected to a staircase increase of [CDPC] between 5 and 50 μM (Fig. 8 B). As the initial changes of $V_a$ and $I_{Na}$ occurred rapidly (<1 min) relative to the secondary long time-constant transients, it was assumed in the following calculations that the initial changes of $V_a$ and $I_{Na}$ reflected changes of $N_{Na}^B$ and $i_{Na}$ at essentially constant $N_{Na}$.

Since $I_{Na} = (i_{Na} \cdot N_{Na})$ and $I_{Na}^B = (i_{Na}^B \cdot N_{Na}^B)$, Eq. 8 can be rewritten as:

$$
(I_{Na}^B / I_{Na}) \cdot (i_{Na}^B / i_{Na}) = [1 + \beta' ( [B] / K_B ) ]^{-1} \quad (11)
$$

At the negative $V_a$ reported in Table IV and with intracellular Na concentration averaging near 14 mM (Rick et al., 1978; Stoddard and Helman, 1985), it is readily shown that:

$$
i_{Na} / i_{Na}^B = V_a / V_a^B \quad (12)
$$

Substituting $V_a / V_a^B$ for $i_{Na} / i_{Na}^B$ in Eq. 11

$$
\beta' = 1 - \left[ (I_{Na}^B / I_{Na})(V_a / V_a^B) \right] (1 - \beta' ( [B] / K_B ) )^{-1} \quad (13)
$$

Mean ratios of $V_a / V_a^B$ and $I_{Na}^B / I_{Na}$ at 1 min after washin and washout of CDPC from the apical solution are reported in Table IV for the 10-min pulse experiments, and for washout of CDPC of the staircase protocol experiments. The mean values of $\beta'$ were 0.379 and 0.359 at the beginning and end, respectively, of the 10-min pulse experiments and 0.419 at the end of the 25-min staircase experiments (Table IV). Taking into account the spontaneous variability of $\beta'$ amongst tissues, these mean values of $\beta'$ are remarkably similar to those of the blocker noise pulse experiments (see above). The constancy of $\beta'$ near 0.37 would indicate that $\beta'$ remained unchanged during the inhibition of apical Na entry by 50 μM CDPC for 10–25 min. The inference from these experiments was that long time-constant transients of the $I_{Na}$ were due to time-dependent changes of $N_{Na}^B$ and not to changes of $\beta'$.

**Time Course Experiments**

To test this idea further, experiments were done as illustrated in Fig. 9. During a control period (not shown) epithelia were subjected to CDPC noise analysis for determination of the CDPC rate coefficients and hence $K_B$. Thereafter, each tissue was exposed to 5 μM CDPC for ~30 min before increasing apical [CDPC] abruptly from 5 to 50 μM. PDS were obtained at intervals of 2 min during the 5 μM CDPC control period and thereafter for 30 min after the change to 50 μM CDPC. This protocol permitted calculation of the changes of $i_{Na}^B$, $N_{Na}^B$, and $i_{Na}$ after abrupt inhibition of the macroscopic $I_{Na}^B$. As shown in A of Fig. 9, the $I_{Na}^B$ exhibited a secondary long time-constant transient similar to that reported above. The $i_{Na}^B$ increased abruptly upon inhibition of $I_{Na}^B$ and is due most likely to the concurrent hyperpolarization of $V_a$. The $i_{Na}^B$ also showed a secondary long time-constant transient which is associated with secondary depolarization of $V_a$ (see Fig. 8 A).

For perfect Goldman rectification (Goldman, 1943; Palmer, 1984) and assuming little or no change of intracellular Na concentration during abrupt changes of $V_a$, this approximation introduces at most a 2% error. At the extreme $V_a$ of −76.9 and −91.8 mV (see Table IV), $V_a / V_a^B$ is 0.838 and $i_{Na} / i_{Na}^B$ is 0.852.
The changes of $N_{a}^{b}$ and $N_{ob}$ are summarized in Fig. 9 B. Acute increase of [CDPC] from 5 to 50 µM caused within 2 min abrupt changes of $N_{a}^{b}$ and $N_{ob}$, followed thereafter by long time-constant increases of both $N_{a}^{b}$ and $N_{ob}$. Notably, and in comparison with data obtained in pulse experiments, the long time-constant changes of $N_{a}^{b}$ and $N_{ob}$ were relatively small during the first 2 min after step increase of blocker concentration. We take this as evidence in support of the idea that blocker-related changes of channel densities that occur acutely reflect primarily if not solely the mass action of the blocker to reduce open-channel density. Indeed, it appeared that changes of channel density due to mass action and due to autoregulatory influences could be separated because of the large difference in time constants associated with these processes.

If indeed $N_{a}^{b}$ increased progressively with time after inhibition of $I_{Na}$, then according to Eq. 10, the "$\beta$" was expected to underestimate $\beta$ (due to time-dependent increases of $N_{a}^{b}$). As shown in Fig. 9 C, "$\beta$" decreased continuously with time after step inhibition of $I_{Na}$. "$\beta$" was calculated with Eq. 16 below recognizing that $N_{a}^{b}$ were measured at 5 and 50 µM CDPC. At these concentrations of CDPC:

$$\frac{N_{a}^{5}}{N_{o}} = [1 + \beta'(5/K_{B})]^{-1}$$  (14)

and

$$\frac{N_{a}^{50}}{N_{o}} = [1 + \beta'(50/K_{B})]^{-1}$$  (15)

Hence

$$\beta'' = \frac{1 - (\frac{N_{a}^{50}}{N_{a}^{5}})}{[(\frac{N_{a}^{50}}{N_{a}^{5}}) \cdot 50/K_{B} - (5/K_{B})]}$$  (16)
Extrapolation of the "β" to the ordinate (zero [B] and also zero time) gave β' of 0.26 ± 0.03 for this group of experiments.

**Staircase Protocol**

Blocker-induced noise analysis of epithelia has to our knowledge been carried out exclusively using a staircase protocol (Fig. 6) of increasing blocker concentration. This protocol has the advantage of requiring less time to obtain corner frequency plots (as compared with a pulse protocol) but is disadvantageous insofar as the tissues are subjected to graded and prolonged inhibition of $I_{Na}$ with attendant autoregulatory changes of channel densities. Noting this complication especially with regard to assessing blocker-dependent changes of channel densities and hence determination of β', a summary of blocker-dependent changes of measured and calculated parameters for 43 tissues is shown in Fig. 10. $I_{Na}$ was decreased from a mean control of $26.05 ± 1.51$ to $19.80 ± 1.21 \mu A/cm^2$ at 50 µM CDPC or on average to 76.0% of...
the control value. In comparison, 50 μM CDPC in the pulse protocol noise experiments caused $I_{\text{Na}}$ to decrease on average to 61.4% of the control value. In staircase protocol experiments where the $I_{\text{Na}}$ was inhibited by CDPC for ~35 min (and at progressively increasing $[B]$) the long time-constant increases of $N_{\text{B}}$ would be expected to result in lesser inhibition of the $I_{\text{Na}}$ at 50 μM CDPC, as observed here, and as compared with results of the pulse protocol experiments. The corner frequency plots (Fig. 10 C) were linear in each experiment yielding ON- and OFF-rate coefficients in a range similar to those observed in the pulse protocol experiments (see Table II). $i_{\text{Na}}$ averaged 0.48 pA and was the same as in the pulse protocol experiments. The mean $i_{\text{Na}}$ remained essentially constant with increases of $[B]$ (Fig. 10 D) as would be expected with near constancy of $V_\text{m}$ (Fig. 8 B).

The mean $N_{\text{o}}$ of this group of tissues averaged 56.6 × 10⁶ channels/cm² at a mean $I_{\text{Na}}$ of 25.95 μA/cm² (Table I). In comparison with pulse protocol experiments (Fig. 5 A) and as summarized in Fig. 10 E, the blocker-dependent decreases of $N_{\text{B}}$ were less, and the increases of $N_{\text{ob}}$ were greater than those of pulse protocol experiments, especially at the higher $[B]$, where the $I_{\text{Na}}$ was inhibited for the longest periods of time.

Noting again that β', as calculated with Eq. 10, is subject to error owing to time-dependent increases of $N_{\text{T}}$, the "β" values shown in Fig. 10 F were extrapolated to the ordinate to obtain β'. β' averaged 0.45 (Table 1, Fig. 10 F). In the absence of time-dependent increases of $N_{\text{T}}$, the solid curves drawn in Fig. 10 E (using a mean β' of 0.45) would represent the expected mass action changes of $N_{\text{ob}}$ with increasing $[B]$. At the lower $[B]$ and for the shorter durations of inhibition of $I_{\text{Na}}$, the $N_{\text{ob}}$ and $N_{\text{ob}}$ followed rather closely such theoretical expectations for a three-state model. However, as indicated clearly in Fig. 10 E and in contrast to the results of pulse protocol experiments, significant deviations of $N_{\text{ob}}$ and $N_{\text{ob}}$ were observed in these staircase protocol experiments at the higher $[B]$ and during longer durations of inhibition of $I_{\text{Na}}$. Notably, similar behavior of blocker-dependent changes of $N_{\text{ob}}$ was reported for experiments done with CGS 4270 and amiloride (Abramcheck et al., 1985). Such phenomena are therefore unrelated to the specific characteristics of the blocker used to induce blocker noise.

$K_B$ of Amiloride

It has been impossible to determine with adequate certainty the microscopic $K_B$ for amiloride due to the very low value of its off-rate coefficient, $k_{bo}$ (Helman et al., 1983; Abramcheck et al., 1985). Because it was important to know the value of $K_B^\text{Amil}$ for the evaluation of blocker-dependent changes of channel densities, a new method was devised to determine $K_B^\text{Amil}$. It was assumed that any blocker of the Na channels would cause the same inhibition of the $I_{\text{Na}}$ when used at the same $B/K_B$. Thus for the same fractional inhibition of $I_{\text{Na}}$ by any pair of blockers, namely, CDPC and amiloride:

$$\frac{[\text{Amil}]}{K_B^\text{Amil}} = \frac{[\text{CDPC}]}{K_B^{\text{CDPC}}}$$

Since amiloride and CDPC concentrations are known and since $K_B^{\text{CDPC}}$ is determined easily and with certainty from corner frequency plots, $K_B^\text{Amil}$ could be calcu-
lated with Eq. 17 at the concentration of amiloride that gave the same fractional inhibition of $I_e$ that was caused by CDPC.

Calculation of $K_B^{Am}$ was done with data obtained in pulse protocol experiments. Epithelia were exposed first to CDPC pulses at concentrations between 10 and 200 $\mu$M yielding the empirical relationship between $B/K_B^{CDPC}$ and $I_e^{CDPC}/I_e$ (Fig. 11 A). This was followed by a second series of amiloride pulses at concentrations between 0.02 and 0.4 $\mu$M yielding the relationship between [Amiloride] and $I_e^{Amil}/I_e$ (Fig. 11 B). This range of [Amiloride] gave fractional inhibitions of $I_e$ similar to those encountered with CDPC. Knowing the $I_e^{Amil}/I_e$ at each amiloride concentration and knowing the $B/K_B$ at the same $I_e^{Amil}/I_e$ determined previously with CDPC, the $K_B^{Am}$ was calculated as a function of increasing [Amiloride] (Fig. 11 C). $K_B^{Am}$ was on average independent of $[B]$. In 12 such experiments (Table V), $K_B^{Am}$ ranged between 44 and 88 nM and averaged $61 \pm 4$ nM (pH ~ 8.1). Since the ON-rate coefficient $k_{on}^{Amil}$ averaged $18.7 \pm 0.6$ rad/s.$\mu$M (Table V), the OFF-rate coefficient $k_{off}^{Amil}$ was calculated to be 1.14 rad/s. It is thus not surprising that $k_{off}^{Amil}$ cannot be measured with certainty at the ordinate of corner frequency plots.\(^5\) For the groups of amiloride experiments

\(^5\)With a pK$_a$ of nearly 8.7, ~80% of amiloride is in its active cationic form, and the rate coefficients of the blocker reaction are voltage dependent (Palmer, 1984; Warmke and Lindemann, 1985). $k_{on}^{Amil}$ of the blocker reaction would therefore be expected to be of lower value in our studies as compared with K-depolarized epithelia where $V_e$ is markedly depolarized from its control value. This undoubtedly contributes to the difficulty in determination of $k_{on}^{Amil}$ from corner frequency plots of non-K-depolarized tissues as studied here and previously (Helman et al., 1983; Abramcheck et al., 1985). It should be noted also that $k_{on}^{Amil}$ and $k_{off}^{Amil}$ as reported here are not corrected either for voltage or pK$_a$. Accordingly, extrapolation of these values to other conditions must be done with caution.
reported below $k_{am}^{\text{amil}}$ extrapolated at the ordinate of the amiloride corner frequency plots averaged $4.96 \pm 1.31$ rad/s (Table VI) or about four times larger than the more likely value of 1.14.

Amiloride-dependent Changes of $i_{Na}$, $N_o$, and $N_{ab}$

Because of the widespread interest in amiloride as a Na channel blocker, it was of interest to evaluate the amiloride-dependent changes of $i_{Na}^B$ and the channel densities with the experimental protocols given in Fig. 6. The control blocker-dependent parameters were evaluated first with CDPC-induced noise. This was followed by either a staircase protocol of amiloride inhibition of $I_{Na}$ (Fig. 6 A) or a time course protocol inhibition of $I_{Na}$ (Fig. 6 B). To be noted in Fig. 6 B was the secondary long time-constant transient of the $I_{Na}$ upon exposure of tissues to 0.5 $\mu$M amiloride that was also apparent in the scalloped appearance of the $I_{Na}$ record of Fig. 6 A especially at the lower amiloride concentrations.

**Table V**

| Expt. No. | $k_{am}^{\text{amil}}$ (nM) |
|-----------|-----------------------------|
| 1         | 51 ± 2                      |
| 2         | 59 ± 3                      |
| 3         | 46 ± 2                      |
| 4         | 54 ± 4                      |
| 5         | 68 ± 1                      |
| 6         | 83 ± 1                      |
| 7         | 63 ± 1                      |
| 8         | 88 ± 2                      |
| 9         | 47 ± 1                      |
| 10        | 63 ± 2                      |
| 11        | 63 ± 1                      |
| 12        | 44 ± 1                      |
| Mean ± SEM| 61 ± 4                      |

The CDPC-derived control data are summarized in Table VI. Pre-amiloride control values of $I_{Na}$, $N_o$, and $i_{Na}$ are also summarized in Table VI. The pre-amiloride $I_{Na}$ was measured immediately before the addition of amiloride (0.5 $\mu$M) to the apical solution. $N_o$ was calculated from the quotient of the pre-amiloride $I_{Na}$ and the $i_{Na}$ determined with CDPC; these values served as controls for the $I_{Na}^B$ and $N_o$ measured with amiloride-induced noise. $I_{Na}^B$ was calculated with Eq. 3 at the respective concentrations of amiloride using the measured $k_{am}^{\text{amil}}$ that averaged $18.7 \pm 0.6$ rad/s $\cdot \mu$M (Table VI).

The blocker-dependent changes of $I_{Na}^B$, $i_{Na}^B$, $N_o^B$, and $N_{ab}$ observed in staircase protocol experiments are summarized in Fig. 12. As shown in Fig. 12 A, amiloride caused the $i_{Na}^B$ to increase on average by $\sim$ 30%. A similar hyperpolarization of $V_a$ has been observed consistently in microelectrode studies of apical membrane voltage in response to amiloride inhibition of Na entry (Helman and Fisher, 1977). $N_o^B$ decreased markedly from control at 0.5 $\mu$M amiloride and higher concentrations
### TABLE VI

**Summary Data for Amiloride Experiments**

| Protocol     | CDPC          | Pre-amiloride controls | Amiloride       |
|--------------|---------------|------------------------|----------------|
|              | $I_{na}$  | $k_{de}$ | $k_{nu}$ | $K_{a}$ | $i_{na}$ | $N_0$ | $\beta$ | $I_{na}$  | $N_0$ | $k_{ab}$ | $\beta$ | $K_{a}$ |
|              | $\mu A/cm^2$ | rad/s | $\mu M$ | $\mu M$ | $\times 10^6/cm^2$ | $\mu A/cm^2$ | $\times 10^6/cm^2$ | rad/s | $\mu M$ | rad/s | $\mu M$ | $\mu M$ |
| Staircase    | 28.47         | 6.66       | 193.8    | 29.5    | 0.508          | 67.1           | 0.44             | 28.80 | 68.1    | 18.7   | 4.96   | 0.273 |
| ($N = 7$)    | $\pm 6.11$   | $\pm 0.29$ | $\pm 5.3$ | $\pm 1.6$ | $\pm 0.052$  | $\pm 19.2$    | $\pm 0.09$      | $\pm 5.15$ | $\pm 16.3$ | $\pm 0.6$ | $\pm 1.31$ | $\pm 0.076$ |
| Time course  | 18.40         | 6.93       | 181.7    | 26.2    | 0.469          | 39.1           | 0.41             | 16.59 | 38.9    | —      | —      | —    |
| ($N = 5$)    | $\pm 1.35$   | $\pm 0.17$ | $\pm 6.8$ | $\pm 0.8$ | $\pm 0.012$  | $\pm 2.2$     | $\pm 0.10$      | $\pm 1.09$ | $\pm 3.7$ | —      | —      | —    |

Values are means ± SEM.

* Included in this table are values of $k_{de}$ and $K_a$ determined from the [amiloride]-2$\eta_f$, corner frequency plots. Although no negative values of $k_{ab}$ were observed in this group of experiments, the values of $k_{de}$ and $K_a$ so measured are considered to be unreliable (see text).
(Fig. 12 B). \( N_{ob} \) increased markedly above control averaging near 700% of control at 10 \( \mu M \) amiloride (Fig. 12 C). As with the CDPC staircase protocol experiments, the apparent "\( \beta' \)" fell progressively with increasing [\( B \)] (Fig. 12 D), with \( \beta' \) extrapolated to the ordinate averaging 0.45. The solid lines in B and C of Fig. 12 were drawn according to Eqs. 8 and 9 assuming that \( N_{T} \) remained unchanged from the control. As with CDPC staircase protocol experiments, marked deviation from ideal three-state model behavior was observed with deviation from ideal behavior being far greater than that observed with CDPC inhibition of \( I_{Na} \) and due most likely to the much larger inhibition of \( I_{Na} \) caused by amiloride. Indeed, at these high amiloride concentrations where \( [B]/K_{B} \) is greater than ~2, the \( N_{ob} \) would approach a "saturating" value with a limit equal to the \( N_{T} \) (absence of blocker). For a \( \beta' \) of 0.45, this value is 222% of control provided \( N_{T} \). Clearly the calculated \( N_{ob} \) far exceeds such an expectation, leading to the same conclusion as above, namely that increases of \( N_{T} \) during secondary long time-constant transients contribute to the pool of channels in open and blocked states.

Time course experiments with amiloride, summarized in Fig. 13, led again to the same conclusion. Acute inhibition of \( I_{Na} \) by 0.5 \( \mu M \) amiloride caused a similar increase of \( N_{ob} \) (compare with Fig. 12 A) that remained elevated above control for the 38-min experimental period (Fig. 13 A). \( N_{ob} \) showed an immediate decrease to ~22% of control followed by a secondary long time-constant increase of the \( N_{ob} \) (Fig. 13 B). \( N_{ob} \) increased markedly and progressively with time despite the constancy of amiloride concentration in the apical solution (Fig. 13 C). The apparent "\( \beta'' \)" fell progressively with time, and the \( \beta' \) extrapolated to the ordinate averaged 0.50. On the premise again that the secondary changes of \( N_{ob} \) and \( N_{ob} \) reflected long time-

![Figure 12](image-url)
min) that $N^b_{o}$ and $N^b_{ob}$ were not far different from values expected at 0.5 μM amiloride at a mean $\beta'$ of 0.5 in this group of experiments. Accordingly, we conclude that amiloride, like CDPC, caused changes of $N^b_{o}$ and $N^b_{ob}$ in accordance with a three-state model of closed, open, and blocked state kinetics, and, moreover, amiloride, like CDPC, binds predominantly if not solely to open channels.

FIGURE 13. Summary of time course experiments after exposure of tissues to 0.5 μM amiloride. Data are normalized to the pre-amiloride control values (Table V).

DISCUSSION

An ultimate description of Na absorption by epithelial cells will require detailed knowledge of the regulation of apical membrane channel density and channel kinetics. In this regard, we have turned to blocker-induced noise analysis of the highly selective Na channels of frog skin using the electroneutral Na channel blocker CDPC. Being electroneutral and thus insensitive to change of apical membrane voltage, and lacking a pK at physiological pHs of interest, CDPC, unlike amiloride and other numerous charged analogues, is advantageous in design and interpretation of data obtained from blocker-induced noise analysis. As CDPC-induced Lorentzian-type noise is easily measurable under conditions of minimal inhibition of the macroscopic rates of Na entry, its utility is obvious in assessing Na channel behavior at or very near normal spontaneous rates of Na transport. We have taken advantage of these properties of CDPC to study the changes of single-channel Na currents and Na channel densities that occur with inhibition of apical Na entry.

To our knowledge, no evidence has emerged to indicate that single-channel currents are different when measured with different blockers of the Na channels. Indeed, in the paired experiments using both CDPC and amiloride, the increases of $i_{Na}$ caused by amiloride were similar to those expected to result from the hyperpolarization of $V_a$. Moreover, taking into account the dependency of $i_{Na}$ on $V_a$, with $V_a$ varying between individual tissues and among species of tissues, the values of $i_{Na}$ are
remarkably similar to those of highly selective Na channels studied by patch clamp
(Palmer and Frindt, 1986; Eaton and Hamilton, 1988; Marunaka, Y., and D. C.
Eaton, personal communication). Accordingly, despite vast differences in rate coef-
ficients among blockers and the difference of inhibition of \( i_{Na} \) by blockers, it seems
reasonable to conclude that blockers like CDPC act as probes of the open Na chan-
nel with no measurable change of its intrinsic properties. In accordance with this
thesis is the consistent observation of linear corner frequency plots of CDPC, like
those of other blockers, which argues, moreover, in favor of the idea that the \( f_c \)
arises alone from blocker interaction with open channels being measurably unin-
fluenced by other kinetic reactions—notably spontaneous fluctuations between
open and closed states of the channel.

With minimal inhibition of the macroscopic rates of Na entry and with minimal
change of \( V_n \), extrapolation of the \( i_{Na} \) to zero blocker concentration gave values of
\( i_{Na} \), which when taken together with the \( I_{Na} \), gave values for the open channel den-
sity, \( N_o \). \( i_{Na} \), averaged near 0.5 pA with significant variability amongst tissues. This at
least in part reflects differences of apical membrane voltage and intracellular Na
concentration. Assuming a mean \( V_n \) of \(-80 \text{ mV} \) and cellular Na concentration of 14
mM, the single-channel Na conductance is in the vicinity of 3.8 pS (chord) or 6.3 pS
(slope). Such values are similar to those previously reported that were determined
by noise analysis (Helman et al., 1983), analysis of highly selective Na channels
reconstituted into lipid bilayers (Sariban-Sohraby et al., 1984), and by patch clamp
(Palmer and Frindt, 1986; Eaton and Hamilton, 1988; Marunaka, Y., and D. C.
Eaton, personal communication). Although not shown in detail, it was quite appar-
ent that a relationship existed between \( N_o \) and \( I_{Na} \) over large ranges of spontaneous
rates of Na transport encountered among tissues (see Tables II and VI). At a mean
\( I_{Na} \) of 25.95 \( \mu A/cm^2 \), \( N_o \) averaged 56.5 million open channels/cm\(^2\) or \( \sim \)56 channels/
cell, or about a single channel per 2 \( \mu m^2 \) of apical membrane area. At such Na
transport rates, it would thus be surprising to find more than one open channel in a
patch of apical membrane of 1-2 \( \mu m^2 \) unless channels existed in clusters.

**Blocker Concentration Dependence of Channel Densities**

Availability of CDPC permitted an analysis of the dependence of open-channel den-
sity and open + blocked channel density on blocker concentration in a range of
concentrations well below and above its \( K_B \), which averaged near 30 \( \mu M \). Without
exception open-channel density decreased while open + blocked channel density
increased from the control \( N_o \) with progressive increases of \( [B] \). Such behavior is
incompatible with the idea of a two-state model of open and blocked channels (see
Methods), and indeed some form of "recruitment" is necessary to explain blocker
concentration-dependent increases of \( N_{ob} \). Similar observations were made with
electroneutral CGS 4270, cationic amiloride, and triamterene as blockers (Baxen-
dale and Helman, 1986). Therefore, such phenomena did not seem attributable to
the specific noise-inducing blocker. If the blockers interacted equally well with open
and closed states of the channel as had been suggested (Ilani et al., 1984), then
according to the four-state scheme of Table I, \( N_{ob} \) would have remained independ-
ent of \([B]\), and \( N_{ob} \) would have decreased to 50% of the \( N_o \) at \([B]/K_B = 1.0 \). As such
behavior was never observed in our own experiments or in those of Marunaka and
Eaton (personal communication), we conclude that neither a two-state nor a four-state model of equal blocker interaction with open and closed channels satisfies the empirical observations.

Complicating the analysis and interpretation of the data is the existence of long time-constant transients of the $I_{Na}$ that appear after inhibition of Na entry. We attempted therefore to separate those changes of channel density due to mass law effect from those that occur by so-called autoregulatory influences, which at present are not understood but which clearly are quantitatively important in understanding regulation of apical membrane channel density. It became evident that mass law changes of channel density could be separated from those of autoregulatory influences by virtue of large differences in the time constants involved. This led in the design of the experiments to a pulse protocol that minimized time of inhibition of the $I_{Na}$ where blocker-dependent changes of channel density would be due principally to those occurring by mass law action. According to a three-state scheme where blocker interacts alone with open channels, $N_o^B$ was expected to decrease but to a lesser extent than would occur for two-state or four-state considerations ($N_o^B / N_o = 0.5$ at $B/K_B \approx 1.0$). Indeed, $N_o^B / N_o$ was consistently $>0.5$ even at $[B] \gg K_B$. Moreover, it was observed consistently here as in previous experiments that $N_{ob}$ increased with increasing $[B]$, indicating a blocker concentration-dependent recruitment of channels to open + blocked states. Hence, in pulse protocol experiments where autoregulatory influences on channel density are for all practical purposes absent, blocker-dependent recruitment of channels occurs from closed states of the channel. If so, it follows that blocker interacts primarily if not solely with open states of the channel. In this regard, as noted above, compelling evidence exists from experiments with amiloride that cationic blockers sense changes of membrane voltage and accordingly the suggestion has been made that blockers gain access to the open channels. This however does not preclude binding of blocker to the outside of the channel, but taken with the above observations it does suggest that such binding if it occurs is ineffective in altering the kinetics of the channels. If our view with regard to the long time-constant autoregulatory processes is correct, then it would be reasonable to believe that the acute blocker concentration dependence of channel densities can be ascribed to mass law action and is in accordance with a simple three-state model of blocker kinetics. This would explain the blocker-dependent changes of $N_o^B$ and $N_{ob}$ where recruitment of channels from closed into open and blocked states occurs at constant total channel density.

On the premise that $N_T$ remained constant in pulse protocol experiments, the open probability was calculated at all $[B]$. It was observed that for all practical purposes, $\beta'$ was essentially independent of $[B]$ despite relatively large changes of $I_{Na}$. In most groups of experiments, $\beta'$ averaged near 0.4, and this value is remarkably similar to that reported for apical Na channels of rat distal tubules studied by patch clamp (Palmer and Frindt, 1986). Notably, $\beta'$ varied widely among tissues ranging between ~0.1 and 0.9. A summary distribution of open probabilities is shown in Fig. 14, which emphasizes the idea that changes of open probability may in fact underlie an important mechanism by which epithelial cells regulate their Na entry. Rather little is yet known of the mechanism of control of open probability, although agents like benzoyleimidazole-2-guanidine, diphenylhydantoin, and especially MK-196
(Merck, Sharp & Dohme) appear to increase the open probability (unpublished observations).

In all groups of experiments we calculated the expected blocker-dependent changes of \( N_B^a \) and \( N_{ab} \) assuming \( N_T = N_T^a \) and \( \beta' \) were constant (independent of [B] and independent of duration of inhibition of apical Na entry). These curves were superimposed as solid lines on the empirical measurements of \( N_B^a \) and \( N_{ab} \). For pulse experiments, the empirical data conformed rather well with the expectation of this model, although as noted in the Results, small deviations from perfect behavior were observed at the higher [B]. Whether such deviations from ideal behavior indicate imperfection of the model or whether such deviations are due more likely to relatively small time-dependent increases of \( N_T^a \) cannot now be known with certainty. However, in view of the secondary transients of the \( I_{Na} \), even in pulse experiments, the simplest explanation was to assume that deviations from ideal behavior were due most likely to long time-constant autoregulatory increases of \( N_T^a \) and not to changes of \( \beta' \). Indeed, \( \beta' \) as determined in the microelectrode experiments indicated that open probability was not changed significantly by blocker inhibition of apical Na entry despite prolonged periods of inhibition of apical Na entry.

When staircase or time course protocols were used that necessarily involved prolonged durations of inhibition of apical Na entry, mean control values of \( \beta' \) were estimated from the extrapolated values of apparent "\( \beta' \)" to the zero time and zero blocker concentration control state of the tissues. Done in this way, mean \( \beta' \) were similar for all groups of tissues. It should be emphasized here as above that values of apparent "\( \beta' \)" calculated with Eq. 10 are subject to the assumption of constancy of \( N_T^a \). With prolonged inhibition of apical Na entry, such an assumption did not appear valid. Indeed, in those experiments where CDPC was increased abruptly from 5 to 50 \( \mu \)M, \( N_{ab} \) was increased abruptly at first because of mass action of CDPC. The secondary long time-constant increase of \( N_{ab} \) reflected likely increases of \( N_T^a \). Such behavior here as in other experimental groups with prolonged inhibition of apical Na entry would lead to time-dependent progressive decreases in calcu-
lation of the "β". Having established the falling trend of "β", the zero time and zero blocker concentration extrapolations of "β" at the ordinate should have provided reasonable estimates of the control open probability of the Na channels. From the results of both pulse and other protocols, we therefore conclude that both mass law action and autoregulatory processes contribute to changes of channel density where these processes are separable by differences in their time constants.

**Autoregulatory Processes**

Essentially nothing is known of the autoregulatory processes that influence apical membrane Na channel density nor the mechanisms that sense a change in the rate of apical Na entry. Our own experiments have so far been limited to blocker inhibition of Na entry. For this type of submaximal inhibition, autoregulatory processes are capable of returning the Na entry rate to near control values within 10–20 min so that the blocker does not appear to sustain mass law inhibition of Na entry. The reason for this appears to be a steady-state increase of the NT with little or no measurable difference of open probability. After prolonged inhibition of INa and where NT was increased, abrupt removal of CDPC from the apical solution resulted in an overshoot of the INa which thereafter relaxed slowly towards original control values. Such observations are in accordance with the hypothesis of long time-constant changes of NT and hence No after release of channels from steady-state blocker inhibition.

**Amiloride**

Determination of blocker-dependent changes of channel densities with amiloride is complicated by two factors. First, the threshold for detection of a Lorentzian component of the PDS occurs at ~0.5 μM amiloride, which causes about an 80% inhibition of the INa. Accordingly, blocker-induced noise analysis can be done only in tissues where INa is reduced markedly from the spontaneous rates of Na transport and where extrapolation to control conditions is not reliable. Second, whereas the amiloride ON-rate coefficient (kob) is satisfactorily determined from the slope of the corner frequency plots, the amiloride OFF-rate coefficient (kbo), owing to its very small value, is not measurable with sufficient precision to allow reasonable estimates of the KAn (Abramcheck et al., 1985; see footnote 3). Hence in the absence of a KAn determined this way, it was impossible to know the expected blocker-dependent mass law behavior of the channel densities.

We have in the measurement of the KAn taken advantage of the fact that KAn is readily measured together with the fractional inhibitions of the INa caused by CDPC and amiloride. The important assumption was that fractional inhibition of the INa would be identical at the same [B]/K for any blocker of the Na channels. KAn obtained this way averaged 61 nM for short-circuited epithelia bathed with Cl-HCO3 Ringer solution of a pH of ~8.1. For amiloride concentrations <0.4 μM, KAn was independent of amiloride concentration. The experiments were carried out with a pulse protocol so that the time of inhibition of INa was the same for CDPC as for amiloride.

Because of differences of pH and apical membrane voltage between and among various preparations, it is difficult to compare directly values of KAn reported by
ourselves and others. However, it is clear that $K_{Na}^{amil}$ measured macroscopically from changes of $I_{Na}$ are $>61 \text{ nM}$, usually falling into a range of a few hundred nanomolar amiloride. For example, in two groups of experiments reported by Helman et al. (1983), the apparent $K_{Na}^{amil}$ determined by analysis of Eadie-Hofstee plots (Engel, 1977; and Eq. 8 of Helman et al., 1983) averaged 207 and 269 nM. In such analyses the $\Delta I_{Na}/[B]$ is plotted against the $\Delta I_{Na}$ from which the slope yields the concentration of amiloride that causes a 50% reduction of the $I_{Na}$. If, however, inhibition of open channels is accompanied by recruitment of channels by mass law action from closed states at constant $N_T$, such macroscopic values of $K_{Na}^{amil}$ must by necessity overestimate the microscopic $K_{Na}$. It is easily shown for mass law action alone that:

$$\Delta I_{Na} = -(K_{Na}/\beta)(\Delta I_{Na}/[B]) + \Delta I_{Na}^{\text{max}}$$

where the slope of the line is given by the microscopic $K_{Na}$ divided by the open probability. If we assumed as a first approximation the absence of autoregulatory changes of $N_T$, then with a $K_{Na}^{amil}$ of 61 nM, $\beta$ for the two groups of experiments referred to above are in the vicinity of 0.31 and 0.25, respectively. With autoregulatory increases of $N_T$, these values are underestimates of the actual $\beta$.

The implication from such reasoning according to three-state model kinetics is manifest in the interpretation of $K_{Na}$ obtained macroscopically from changes of $I_{Na}$. Certainly, changes of macroscopic $K_{Na}$ will reflect changes of either microscopic $K_{Na}$ and/or changes of $\beta$. Thus it would seem prudent in the interpretation of changes of macroscopic values of $K_{Na}$ to keep in mind that open probability contributes importantly to its value and may at least in part be responsible for a change of macroscopic $K_{Na}$ when observed.

Epithelia were subjected to amiloride-induced noise analysis with both staircase and time course protocols. As expected, due to hyperpolarization of $V_a$, $I_{Na}$ was increased on average by $\sim 30\%$, while $I_{Na}^{amil}$ was inhibited markedly at $[\text{Amil}] > 0.5 \mu M$. At this drug concentration, $[\text{Amil}]/K_{Na}^{amil}$ was 7.57 and was increased to a $[B]/K_{Na}$ of 151.5 at 10 $\mu M$ amiloride. Obviously, it was not advantageous to investigate blocker-dependent changes of channel densities in this range of $B/K_{Na}$. Nevertheless, as indicated in Figs. 12 and 13, the expected changes of $N_o^b$ and $N_{ob}$ from mass law action were remarkably the same as expected for control open probabilities that averaged 0.45 and 0.50 for both groups of experiments at 0.5 $\mu M$, and for which time of inhibition of $I_{Na}$ was minimal. Autoregulatory changes of $N_o^b$ and $N_{ob}$ were also observed in both groups of experiments, although here, in comparison with CDPC, the time-dependent changes of channel densities were considerably larger, due most likely to the far greater inhibition of $I_{Na}$ over similar time intervals than those encountered with CDPC. Thus, in pragmatic terms, the behavior of the tissues was the same qualitatively despite quantitative differences in response to blocker inhibition of Na entry. If we accept the idea of a three-state model with $\beta$ of 0.45 for the experiments of Fig. 12, then as shown in Fig. 12 C, recruitment from closed into open and blocked states by mass action alone should have “saturated” the $N_{ob}$ at amiloride concentrations $>1-2 \mu M$. This however was not observed. Rather, $N_{ob}$ was increased markedly above expected values. This is attributable to autoregulatory processes where channels are recruited from other than closed states of the channel, possibly from dormant channels within apical membranes or from channel-con-
taining vesicles inserted into apical membranes from stored sites within the cytoplasm.

It should be emphasized again that the three-state model adopted here deals alone with mass law effects that occur on a time scale of seconds as dictated by the rate coefficients $\alpha, \beta, k_{da}$, and $k_{bo}$. Clearly, additional states of the Na channel must exist that influence $N_T$, thereby contributing to autoregulatory, hormonal, and other mechanisms of regulation of channel densities that must be capable of changing $N_T$. No unique insights into these additional states have emerged from the present investigation. Although open probability varies markedly among tissues, inhibition of $I_N$ by either CDPC or amiloride did not appear to affect $\beta'$, and thus the newly recruited channels would seem to possess the same $\beta'$ as the older channels. Accordingly, differences of open probability are not likely to be related to the age of the channels.

Supported by National Institutes of Health grants DK-16663 and DK-30824. Dr. Baxendale was an American Heart Association (Illinois affiliate) fellow during part of this project.

We are grateful to Nancy Suarez and John Waters for excellent technical assistance and to Dr. Alvin Essig and Dr. David Dawson for helpful suggestions during the preparation of this manuscript. We are also grateful to Dr. Yoshinori Marunaka and Dr. Douglas C. Eaton for allowing us to read a preprint of their manuscript (Marunaka, Y., and D. C. Eaton, manuscript submitted for publication).

Original version received 17 April 1989 and accepted version received 11 September 1989.

REFERENCES

Abramcheck, F. J., W. Van Driessche, and S. I. Helman. 1985. Autoregulation of apical membrane Na$^+$ permeability of tight epithelia. Noise analysis with amiloride and CGS 54270. Journal of General Physiology. 85:555–582.

Baxendale, L. M., and S. I. Helman. 1986. Sodium concentration dependence of apical membrane single channel Na$^+$ current and density of nondepolarized frog skin (three state model). Biophysical Journal. 49:106a. (Abstr.)

Eaton, D. C., and K. L. Hamilton. 1988. The amiloride-blockable sodium channel of epithelial tissue. In Ion Channels. Vol. 1. T. Narahashi, editor. Plenum Publishing Corp., New York. 251–282.

Engel, P. C. 1977. Enzyme Kinetics of the Steady State Approach. Chapman & Hall, London. 96 pp.

Goldman, D. E. 1943. Potential, impedance, and rectification in membranes. Journal of General Physiology. 69:571–604.

Helman, S. I., T. C. Cox, and W. Van Driessche. 1983. Hormonal control of apical membrane Na transport in epithelia. Studies with fluctuation analysis. Journal of General Physiology. 82:201–220.

Helman, S. I., and R. S. Fisher. 1977. Microelectrode studies of the active Na transport pathway of frog skin. Journal of General Physiology. 69:571–604.

Helman, S. I., B. M. Koeppen, K. W. Beyenbach, and L. M. Baxendale. 1985. Patch clamp studies of apical membranes of renal cortical collecting ducts. Pflügers Archiv. 405(Suppl. 1):S71–S7.

Ilani, A., S. Yachin, and D. Lichtstein. 1984. Comparison between bretylium and diphenylhydantooin interaction with mucosal sodium-channels. Biochimica et Biophysica Acta. 777:323–330.
Li, J. H-Y., E. J. Cragoe, Jr., and B. Lindemann. 1985. Structure-activity relationship of amiloride analogs as blockers of epithelial Na channels. I. Pyrazine-ring modifications. *Journal of Membrane Biology*. 83:45-56.

Li, J. H.-Y., E. J. Cragoe, Jr., and B. Lindemann. 1987. Structure-activity relationship of amiloride analogs as blockers of epithelial Na channels. II. Side-chain modifications. *Journal of Membrane Biology*. 95:171-185.

Li, J. H.-Y., and B. Lindemann. 1983. Competitive blocking of epithelial sodium channels by organic cations: the relationship between macroscopic and microscopic inhibition constants. *Journal of Membrane Biology*. 76:235-251.

Palmer, L. B. 1984. Voltage-dependent block by amiloride and other monovalent cations of apical Na channels in the toad urinary bladder. *Journal of Membrane Biology*. 80:153-165.

Palmer, L. G., and G. Frindt. 1986. Amiloride-sensitive Na channels from the apical membrane of the rat cortical collecting tubule. *Proceedings of the National Academy of Sciences*. 83:2767-2770.

Rick, R., A. Dörge, E. von Arnim, and K. Thurau. 1978. Electron microprobe analysis of frog skin epithelium: evidence for a syncitial sodium transport compartment. *Journal of Membrane Biology*. 39:313-331.

Sariban-Sohraby, S., R. Latorre, M. Burg, and D. Benos. 1984. Amiloride-sensitive epithelial Na⁺ channels reconstituted into planar lipid bilayer membranes. *Nature*. 308:80-82.

Stoddard, J. S., and S. I. Helman. 1985. Dependence of intracellular Na⁺ concentration on apical and basolateral membrane Na⁺ influx in frog skin. *American Journal of Physiology*. 249:F662-F671.

Warncke, J., and B. Lindemann. 1985. Voltage dependence of Na channel blockage by amiloride: relaxation effects in admittance spectra. *Journal of Membrane Biology*. 86:255-265.

Van Driessche, W., and W. Zeiske. 1980. Ba²⁺-induced conductance fluctuations of spontaneously fluctuating K⁺ channels in the apical membrane of frog skin (*Rana temporaria*). *Journal of Membrane Biology*. 56:31-42.