Effect of Sodium on Amiloride- and Triamterene-induced Current Fluctuations in Isolated Frog Skin

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ABSTRACT The apparent association constants of two agents, amiloride and triamterene, that block the Na-selective channel of apical membrane of frog skin are shown to decrease as the Na concentration is increased in the apical bathing solution in isolated skin of the frog, Rana temporaria, Rana esculenta, and Rana pipiens. These results were obtained in "normally polarized" skins. These effects were independent of the anion used (chloride or methylsulfate) or the cation used as the Na substitute (Tris, DDA, or K ion). When NaCl was replaced with mannitol, the Na effect on the amiloride association rate constant persisted, which shows that ionic strength was not critically involved. The amiloride corner frequency was unaffected when the clamp potential was altered from +100 to -60 mV. The Na dependence was greatly attenuated or absent when the serosal surface was bathed in 120 mM K Ringer's, an effect that appears to be attributable to some pharmacological effect of high serosal K. A previously described three-state model is used to analyze the inhibitory effect of Na on the blocker association rate constant.

INTRODUCTION

Epithelial Na transport is controlled primarily by modulation of apical Na permeability (for review, see Lindemann, 1984). In turn, apical Na permeability is mediated by Na-selective channels in the apical surface of the outermost epithelial cell layer. Hormonal stimulation of Na transport, e.g., caused by aldosterone and vasopressin, involves an increase in the density of the Na-selective channels. These results were obtained through analysis of fluctuations in the short-circuit current induced by amiloride, which in low concentrations is a specific blocker of Na-selective channels. Such an analysis is heavily dependent upon the specific model used to interpret the fluctuations. Thus far, most of the data have been interpretable in terms of the simplest model, a two-state blocking model in which the fluctuations are assumed to arise from a random association-dissociation of blocker and channel site. This model predicts that the steady state current inhibition kinetics of a blocker such as amiloride should exhibit classic Michaelis-Menten-type behavior. In experiments using a "K-depolarized" frog
skin, a preparation in which the basolateral membrane is K-depolarized to reduce the basolateral membrane potential and resistance, Van Driessche and Lindemann (1979) found the amiloride association rate constant to be independent of the Na concentration. (Although the dissociation rate appeared to be Na dependent, the error in the measurement was deemed large enough to discount such dependence.) This finding is in accord with the assumptions behind the two-state model, which ignore the possibility that the association rate constant and the amiloride inhibition constant, $K_A$ (i.e., the amiloride concentration required to inhibit the amiloride-sensitive short-circuit current by 50%), might be dependent on the Na concentration. However, reports of amiloride inhibition of the short-circuit current show that the apparent amiloride inhibition constant is Na dependent (Benos et al., 1979), and force us to go beyond the assumptions of the simple two-state model.

In the present report, it is shown that the apparent association rate constant of blocker (amiloride or triamterene) decreases as the apical Na concentration is increased, but that the dissociation rate constant is Na independent. These results were obtained in "normally polarized" skins bathed in Na-containing solutions at the corium surface. A three-state model (Frehland et al., 1983) is used to interpret these data.

**METHODS**

Abdominal skins of frog (*Rana temporaria, Rana esculenta*, and *Rana pipiens*) were mounted in an Ussing-type chamber described previously (Van Driessche and Zeiske, 1980) or a modified Helman-type chamber (Helman and Miller, 1971). In the Helman chamber, the corium surface was glued to Lucite rings with cyano-acrylate tissue glue, while the apical surface was sealed with silicone grease and partially polymerized Sylgard against either Lucite or silicone rubber rings. The exposed area ranged from 0.4 to 1.0 cm in diameter. The actual area in any given experiment was estimated by staining the corium surface after the experiment and measuring the stained area with a caliper. 3 M KCl/agar bridges were used to connect Ag/AgCl electrodes to the bathing solutions in a four-electrode voltage clamp (Van Driessche and Lindemann, 1978). The corium bathing solution was usually (in mM/liter): 120 NaCl, 5 KCl, 0.5 CaSO$_4$, and 5 Tris-Cl, pH 7.4. Other solutions of various compositions were used as indicated with each protocol and were changed using a syringe. Reagent-grade chemicals were used. Tris, bis[2-hydroxyethyl]-dimethylammonium (DDA; 11337, Eastman Kodak Co., Rochester, NY), and K were used as Na substitutes. Amiloride was a gift from Merck, Sharp & Dohme, West Point, PA, and triamterene was a gift from R.I.T.T., Genval, Belgium. An air-lift bubbling system was used with the Ussing-type chamber, but frequent solution changes with the Helman-type chamber obviated the need for aeration. Bubbling was stopped during the measurement of fluctuations. Spectral analysis was carried out using either a digital magnetic tape method (Van Driessche and Zeiske, 1980) or a dedicated dual microprocessor (Hoshiko and Van Driessche, 1981; Zeiske et al., 1982; Van Driessche and Gullentops, 1982; Van Driessche and Erlj, 1983).

The spectra were fitted as the sum of a Lorentzian component caused by the action of the blocking agent plus a low-frequency component, using the procedures described previously (Van Driessche and Zeiske, 1980). The Lorentz function is characterized by two parameters, the plateau or zero-frequency value, $S_0$, and the corner frequency, $f_c$. For the simplest two-state model of amiloride blocking of the apical Na channel, the corner frequency is a linear function of the amiloride concentration (Lindemann and Van
Driessche, 1977). The intercept of a plot of corner frequency (in radians per second) vs. the amiloride concentration is the rate constant for dissociation of the blocker from a membrane binding site, and the slope is the apparent association rate constant. The validity of the simple two-state model in the interpretation of these types of experiments has been discussed previously (Hoshiko, 1984).

RESULTS

Time Course of Corner Frequency Estimates

The dose-response curve of the short-circuit current vs. amiloride (or other blocker) concentration can be obtained reliably only if the doses are applied in increasing amounts in rapid succession. The resulting scalloped curve is used to obtain the current data for each concentration of blocker, even though a steady state had not yet been reached (cf. Cuthbert, 1976; Van Driessche and Erlij, 1983). If each dose is allowed to remain until a quasi-steady state is reached, the resulting data typically do not exhibit Michaelis-Menten kinetics. Thus, only a minute or so is usually allowable for each dose. On the other hand, the fluctuation measurement requires a significantly longer time, especially if the corner frequency is low, as in the case of amiloride. For example, to estimate a corner frequency of 1 Hz reliably requires several frequency points below that value, preferably at least one decade, e.g., down to ~0.1 Hz. This means that each spectral estimate requires at least 10 s to permit observation of the lowest frequency. Since the averaging of 32–64 spectra is required, ~5–10 min may be needed at the lowest amiloride concentrations. In other words, the first measurable spectrum can be taken only long after the current is measured for the dose-response experiment. Thus, to compare the results of fluctuation experiments with those from dose-response experiments, it is necessary to demonstrate that kinetic estimates by fluctuation analysis are stable over this time.

In order to investigate this question, triamterene was used as the blocker, since the triamterene corner frequency is higher than that of amiloride. Both the association and dissociation rate constants for triamterene are usually higher than those for amiloride. Thus, the spectrum could be obtained with a shorter measurement period than with amiloride. Nonetheless, a minimum time of ~3 min was required for the current to settle before the first spectrum could be obtained. 10 replications were performed in five R. temporaria skins bathed on the corium surface in 120 mM NaCl Ringer’s and on the apical surface in 120 mM NaCl, pH 6.0, with 5 mM MES buffer. The triamterene concentration was 8 µM. At ~3-min intervals for ~30 min, the apical solution was changed and 60 spectra were averaged. The means, standard deviations, and a table of analysis of variance for these five skins are shown in Table 1. It is clear that almost all of the variance arises from the variability among skins rather than from within a given preparation. A representative time course is shown in Fig. 1, together with the concomitant short-circuit current and plateau values.

These results show that the corner frequency does not appear to change between 3 and 30 min after application of the blocker and that the major source of variability is among frogs. Since drift in the corner frequency with time is minimal, it appears to be preferable to repeat the experiment on a significant
number of skins rather than repeating measurements on individual skins. More importantly, fluctuation measurements appear to be stable in time in the period immediately after minimal stabilization of the short-circuit current. This is the time when the current is usually measured in a dose-response experiment.

Effect of Apical Na on Corner Frequency of Triamterene-induced Fluctuations

In a series of experiments using triamterene (Hoshiko and Van Driessche, 1981), we found that the corner frequency increased as the apical Na concentration was decreased, and that this was due to an increase in the on rate constant for association of triamterene with some inhibitory binding site. In these earlier experiments, the Na concentrations were changed in increasing order and not randomly, which left open the possibility of confusing a time effect with an Na effect. In order to demonstrate an Na effect on the corner frequency for once and for all, an experiment was performed in which the order of presentation was varied. Three different apical Na concentrations were used: 120, 40, and 20 mM. The six possible permutations of the order of presentation of the three Na concentrations were used on six skins (R. temporaria). The pH was again 6, the triamterene concentration was 8 μM, and the Na ion was replaced by Tris. At each Na concentration, the apical solution was changed five times and five spectra (each the average of 60 instantaneous spectra) were obtained. Table II shows the means and standard deviations at the three Na concentrations for 56 skins, together with a table of analysis of variance. Here we see that the Na effect is highly significant. A similar analysis (which is omitted) of the short-circuit currents and plateaus also showed significant effects of the Na concentration. The present result confirms the effect reported previously (Hoshiko and Van

\[\text{Table I} \]

| Skin | Average ± SD | Range |
|------|--------------|-------|
| 1    | 20.84±1.03   | 3.08  |
| 2    | 25.80±2.42   | 9.52  |
| 3    | 22.49±1.46   | 4.72  |
| 4    | 19.91±2.82   | 9.29  |
| 5    | 22.29±1.14   | 4.13  |

Analysis of variance table

| Source of variation | Sums of squares | Degrees of freedom | Mean squares | F ratio |
|---------------------|----------------|--------------------|--------------|---------|
| Replication         | 17.14          | 9                  | 1.90         | —       |
| Frogs               | 240.37         | 4                  | 60.09        | 15.6    |
| Replication × frog  | 139.1          | 36                 | 3.86         | —       |
| Total               | 396.6          | 49                 | —            | —       |

Corner frequencies were obtained in triamterene (8 μM, pH 6). Each value is the average of 10 replications of spectra obtained over a 30–40-min period. 60 spectra were averaged for each replication. The range refers to the difference between the maximum and minimum values.
Driessche, 1981) that the corner frequency caused by triamterene increases with decreasing Na concentration. It was further shown in that report that the Na effect was on the association rate constant. In the next section, it is shown that the amiloride association rate constant also exhibits a similar dependence on Na. In these experiments, no effort was made to titrate the fluctuations with graded doses of triamterene. However, it is probably safe to assume a linear relationship between the corner frequency and the triamterene concentration as observed previously and since. If so, and if the previously obtained value of the intercept can be used, it is possible to calculate the slope and hence estimate a value for the association rate constant at the three Na concentrations. These calculations were made and the association rate constants are shown plotted as a function of
the Na concentration in Fig. 2 (plusses, upper solid curve). The significance of this plot is considered in the Discussion.

**Effect of Na on the Amiloride Association Rate Constant**

The effects of apical Na concentration on the amiloride association and dissociation rate constants were examined in five skins from *R. temporaria*. Direct comparisons were made of the effects of 120 vs. 10 mM apical Na on the association and dissociation rate constants for amiloride. In these experiments, the corium solution composition was 120 mM NaCl, 2.5 mM KHCO₃, 1 mM CaCl₂, and 5 mM glucose. The solutions were bubbled with air and the pH was ~8. The low-Na solution contained 10 mM NaCl, 190 mM mannitol plus the other components as in the corium bathing solution. Table III shows the results. The intercept of the $f_c$ vs. [amiloride] line, which estimates the dissociation rate constant, was close to the origin at [Na] = 110 mM, and did not merely fail to increase in [Na] = 10 mM, but may actually have fallen. (The uncertainty of the intercept is such that some intercepts are actually negative.) In contrast, the slope that estimates the association rate constant clearly increases at [Na] = 10 mM, as shown by the $t$ test as given in Table III. Average amiloride association rate constants at these two concentrations from six *R. temporaria* skins are plotted in Fig. 2 (×'s, lower solid line).

**TABLE II**

*Effect of Apical Na on Corner Frequency*

| Skin | [Na] = 120 mM | 40 mM | 20 mM |
|------|--------------|-------|-------|
| 1    | 21.82        | 25.76 | 32.68 |
| 2    | 22.88        | 26.80 | 29.22 |
| 3    | 23.72        | 26.74 | 27.64 |
| 4    | 23.60        | 27.60 | 26.26 |
| 5    | 23.58        | 29.36 | 35.72 |
| 6    | 25.16        | 29.74 | 36.20 |
| Mean | 23.46        | 27.67 | 31.29 |
| SD   | 1.10         | 1.58  | 4.21  |

| Source of variation | Sums of squares | Degrees of freedom | Mean squares | F ratio |
|---------------------|-----------------|--------------------|--------------|---------|
| (a) Replication     | 37.6            | 4                  | 9.4          |         |
| (b) Frogs           | 289.4           | 5                  | 57.9         |         |
| Replication × frog  | 64.5            | 20                 | 3.2          |         |
| (c) Na              | 916.6           | 2                  | 458.3        | 18.6    |
| Na × replication    | 14.4            | 8                  | 1.8          |         |
| Na × frog           | 245.8           | 10                 | 24.6         |         |
| $a × b × c$         | 74.4            | 40                 | 1.9          |         |
| Total               | 1642.7          | 89                 |              |         |

Each value is the average of five replications of corner frequencies in triamterene (8 µM, pH 6). 60 spectra were averaged for each replication.
Factors That Do Not Account for the Na Effect

Anion. Replacement of chloride ion by methylsulfate does not abolish the effect of Na on the association rate constant. This is demonstrated by the data shown in Figs. 3 and 5, in which chloride was replaced by methylsulfate. The slope of each line represents the association rate constant for amiloride. The average values obtained from three skins at three Na concentrations are plotted in Fig. 4 (X’s, upper solid curve) as a function of Na concentration.

Replacement cation for Na. In most of the present experiments, Tris was used as the replacement cation. DDA⁺ can also be used as an Na replacement, and the estimated association constants for triamterene in one representative experiment are plotted vs. the Na concentration in Fig. 2 (diamonds, upper...
TABLE III

Effect of [Na] on Amiloride Association and Dissociation Rate Constants

| Skin | Association rate constant [Na] = 120 | Association rate constant [Na] = 10 | Dissociation rate constant [Na] = 120 | Dissociation rate constant [Na] = 10 |
|------|-------------------------------------|-------------------------------------|---------------------------------------|---------------------------------------|
|      | radians/s·μM                       | radians/s                           | radians/s                             | radians/s                             |
| 1    | 4.19                               | 9.0                                 | 4.66                                  | 4.00                                  |
| 2    | 6.99                               | 25.7                                | 1.95                                  | -5.52                                 |
| 3    | 9.03                               | 18.8                                | 3.05                                  | 4.90                                  |
| 4    | 8.65                               | 20.9                                | 0.45                                  | -0.23                                 |
| 5    | 6.57                               | 15.2                                | 0.84                                  | 0.49                                  |

Mean: 7.09 17.9 2.19 0.73
Mean difference: -10.8 1.46
Variance: 5.28 2.48
Student's t (d.f. = 4): 4.71 0.93
Significance: $P > 0.01$ No

Corner frequencies were obtained in amiloride at 0.5, 1.0, 1.5, 2.0, 3.0, and 4.0 μM, pH 7.8, in *R. temporaria*. Linear correlation coefficients of the plot of $f_c$ vs. [amiloride] were above 0.99 except in two cases at 0.97. 60 spectra were averaged for each replication.

Dashed curve. K was used as the Na substitute with similar results in the experiments shown in Figs. 3 and 5. In other words, the Na effect seems to be specific to Na.

**Ionic strength.** In the above experiments, ionic strength was maintained by using Tris, DDA, or K as the Na substitute. If NaCl is removed and replaced with mannitol, the Na effect on the association rate constant persists, as illustrated in Fig. 6. Thus, the effect of removal of Na is unaffected by changes in ionic strength.

![Figure 3](image-url)  
**Figure 3.** Corner frequency vs. amiloride concentration in *R. temporaria* using Na-methylsulfate at 10, 40, and 120 mM, pH 7.8. K was used to substitute for Na. NaMeSO₄ (mM): 10 (x), 40 (Φ), 120 (+).
Figure 4. The amiloride association rate constant, or "on rate constant," vs. apical Na concentration determined with methylsulfate as the anion in both basolateral and apical solutions and with either Na or K as the major basolateral cation. (X) Association rate constants from five skins, with Na in the basolateral solution. The Na inhibition constant is 114 mM. (O) Association rate constants from seven skins; basolateral [K] was either 120 or 80 mM. Data are slightly displaced along the x axis to avoid overlap. Na now has no discernible effect.

Species. The Na effect has been observed not only in R. temporaria but also in both R. pipiens (Fig. 5) and R. esculenta. The R. pipiens result is interesting in that amiloride is said to inhibit noncompetitively (Benos et al., 1979). The average values of the association rate constants for three skins from R. esculenta are shown in Fig. 2 (squares, bottom dashed curve).

Figure 5. Corner frequency vs. amiloride concentration in R. pipiens using NaMeSO₃, pH 7.8, with K as the Na substitute. NaMeSO₃ (mM): 10 (X), 20 (O), 120 (+).
In 1979, Van Driessche and Lindemann reported that in *R. esculenta* bathed in 109 meq/liter K sulfate on the corium surface, the amiloride association rate constant was unchanged over a range of apical Na concentrations from 11 to 109 meq/liter. We found also that in skins bathed on the corium surface in high concentrations of K, the association rate constant was relatively less dependent on the apical Na concentration. This is illustrated in Fig. 4 (diamonds; lower dashed curve), where the anion was methylsulfate. In contrast to the results reported in skins bathed in Na on the corium surface, in which Na had no significant effect on the dissociation rate constant, Van Driessche and Lindemann (1979) reported an increase in the dissociation rate constant with increasing apical Na concentration. Perhaps this is due to our use of triamterene, which has a higher dissociation rate constant than amiloride, which resulted in better precision in our estimation of the intercept.

**Effect of Clamp Voltage**

Basolateral K was used by Lindemann and Van Driessche (1977) in order to depolarize the basolateral membrane and reduce its resistance (cf. Palmer, 1984). The rationale was to allow a more effective or complete control of the apical membrane potential and to ensure that the fluctuations being measured do arise at the apical membrane. A possible criticism of the present experiments, which were performed primarily with normally polarized basolateral membrane ([K]_i = 2.5–5 mM), is that the effect of apical Na could have been mediated by changes in the apical clamp potential, since the intracellular potential may change when the apical Na concentration is changed. To check this possibility, an experiment...
TABLE IV
Effect of Clamp Potential on \( f_c \)

| Clamp potential (mV) | \( n \) | Corner frequency ± SEM (Hz) |
|----------------------|-------|----------------------------|
| 100                  | 4     | 3.98±0.68                  |
| 80                   | 5     | 3.83±0.49                  |
| 60                   | 6     | 3.98±0.52                  |
| 40                   | 7     | 3.61±0.41                  |
| 20                   | 7     | 3.56±0.36                  |
| 0                    | 7     | 3.59±0.33                  |
| -20                  | 7     | 3.59±0.37                  |
| -40                  | 7     | 3.84±0.45                  |
| -60                  | 4     | 3.94±0.72                  |

Each value is the average of \( n \) replications of corner frequencies in amiloride (1 \( \mu \)M, pH 7.8, [Na] = 20). 60 spectra were averaged for each replication.

was performed in which the basolateral potential was purposely forced to extreme values. To accomplish this, the skin clamp potential was varied over a wide range and the corner frequencies were measured. In these experiments, the corner frequency was found to change very little. In seven skins, with 120 NaCl Ringer’s on the corium surface and 20 NaCl, 1 \( \mu \)M amiloride on the apical surface, the clamp voltage was varied between +100 and -60 mV in 20-mV steps, for a total change in clamp potential of 160 mV. The average corner frequencies are shown in Table IV and are plotted in Fig. 7. The points could be fitted with a straight line with a slope of 0.00085 (Hz/mV), whose standard deviation was 0.0013. The \( F \) test (Bevington, 1969, p. 196) showed that the slope was essentially zero. Thus, the corner frequency was independent of the clamp voltage over this

**Figure 7.** Corner frequency vs. clamp potential in *R. temporaria* skin in 1 \( \mu \)M amiloride and apical [NaCl] = 20 mM, plus mannitol, pH 7.8. Each voltage step was maintained for 3–5 min.
range. Similar results were obtained with the apical surface bathed in 120 meq/liter Na. Palmer (1983) has reported that amiloride inhibition of toad bladder conductance was voltage dependent, but there was no mention of recovery after these large voltages. Perhaps species difference and/or the large voltage range accounts for the effect.

For a sixfold change in apical Na concentration from 120 to 20 mM, the maximum change in apical membrane potential that can be expected is about \((RT/F) \ln 6\) = 45 mV. Even if only half the change in clamp voltage occurred across the apical membrane, it would have been almost twice the 45-mV change expected as a maximum from the change in Na concentration. In other words, the large change in clamp potential should have affected the apical membrane potential even more than the Na concentration change could have done. There was no change in corner frequency (with changes in clamp voltage) large enough to account for the effects of apical Na concentration. From this, we conclude that the Na effect on the association rate constant cannot be attributed to an effect on apical membrane potential.

Recently, an interesting confirmation was reported by Palmer (1985). He obtained the on and off rate constants for amiloride in toad bladder from the current relaxation time constants after a step in the clamp voltage. He reported an Na dependence of the on rate constant such that when the apical \([Na]\) was decreased 10-fold, the on rate constant increased almost 2-fold, despite the fact that the serosal surface was exposed to KCl-sucrose. A further confirmation was reported by Lindemann and Warncke (1985).

**DISCUSSION**

The amiloride and triamterene corner frequencies increase as the Na concentration is decreased, as shown conclusively in Tables II and III. This effect of Na is due primarily and probably exclusively to an effect on the association rate constant, both for amiloride (Table III) and triamterene (Table I in Hoshiko and Van Driessche, 1981). The Na effect is independent of the anion, of the replacement cation used in the apical solution, and of the ionic strength of the apical bathing solution. The Na effect has been observed in skins of three species of *Rana*: *temporaria*, *esculenta*, and *pipiens*. This effect is reduced or abolished when the basolateral membrane is exposed to high concentrations of K (Fig. 4). In high K, the corner frequency becomes relatively independent of the apical Na concentration, a finding reported previously by Van Driessche and Lindemann (1979).

These observations will be discussed first in terms of possible criticisms of the measurements and then in regard to how the Na effect might relate to previous observations on the steady state kinetics of blocking agents. Finally, possible molecular mechanisms for the Na effect are considered.

*Is the Na Effect an Indirect Effect Caused by Changes in Cell [Na] or Apical Membrane Potential?*

In order to establish the Na effect, we have used fluctuation analysis and estimation of the corner frequency of the spectrum of blocker-induced fluctua-
tions. Several possible criticisms of these measurements are as follows. (a) The long-term presence of blocker necessary for the steady state measurement of fluctuations may lead to a redistribution of ions. Rick et al. (1978) showed that the intracellular Na content decreased in skins inhibited by apical amiloride. This may explain the short-duration exposures of blocker required to obtain data exhibiting Michaelis-Menten behavior in short-circuit current measurements. (b) The Na effect on the association rate constant of blocker is not observed when the basolateral surface is bathed in a high K concentration. Thus, the Na effect is suggested to be an artifact caused by the intrusion of fluctuations from other than an apical membrane source induced by the blocker. (c) The Na effect is indirect, because of a change in the apical membrane voltage (Linde mann, 1984). Changing the apical Na concentration may lead to a change in the apical potential since it is the total potential across the whole skin that is clamped and not that across the apical membrane. These possibilities are discussed below.

Stability of the corner frequency measurement. The possibility that some consequence of the long duration required for the fluctuation measurement was the cause of the Na effect was examined by following the time course of the corner frequency after the application of triamterene. As shown above, the corner frequencies obtained between 3 and 30 min after exposure to triamterene were remarkably constant, regardless of the apical Na concentration. 3 min is about the time required for the short-circuit current to reach a quasi-steady state and corresponds to what might be expected for a skin Na content of 10–30 neq/cm² at the current densities observed. 3 min is also the time at which the current is usually read for gauging inhibition. It is the further drift in the current that usually spoils the expected Michaelis-Menten behavior. Nevertheless, the corner frequency was virtually unchanged for a good half hour, despite any drift in current. Exposure to the inhibitors cyanide or ouabain in concentrations and for times that are effective in increasing intracellular Na leave the corner frequency unaffected, although the short-circuit current and plateau were greatly depressed (Hoshiko et al., 1984). These observations would argue that the Na effect is not due to intracellular Na concentration changes. It is possible that the long-term effects of amiloride are on other structures, perhaps even intracellularly, since Benos et al. (1983) have shown that amiloride can penetrate the erythrocyte membrane. There are long-term effects on the short-circuit current and the apparent amiloride inhibition constant, $K_A$, but the fluctuation kinetics appear unaffected and the spectral parameters can be estimated reliably.

Fluctuations may arise from other structures (e.g., basolateral membrane) in the skin. This proposition was analyzed in another report (Hoshiko, 1984) and rejected. Perhaps the most compelling experimental observation for the idea that the blocker-induced fluctuations arise at the apical border is the correlation of the effects with the blocker concentration. Nystatin, which should also render the basolateral membrane more permeable, leaves the corner frequency unaffected when the basolateral cation is Na (Hoshiko et al., 1984).

The Na effect is due to a failure to clamp the apical membrane voltage. As indicated in Fig. 7 and Table IV, the corner frequency is virtually independent of the overall potential at which the skin is clamped. The range of voltages used far exceeded what might be predicted for the maximum potential change
attributable to changes in the Na gradient. Thus, it would appear that the corner frequency is independent of the apical membrane potential, and the Na effect is not an indirect effect caused by changes in apical membrane potential.

**Mechanism of the Na Effect and the Use of Basolateral K**

Since the Na effect is suppressed by basolateral K, two possible explanations should be considered: either that the association rate constant is voltage dependent, or that changes in intracellular Na are responsible. Neither of these two explanations seems satisfactory, as discussed above.

Originally, the use of K to "depolarize" the basolateral membrane and reduce its resistance was offered as a solution to the problem of voltage-clamping the multiple-membrane epithelium (Fuchs et al., 1977; Palmer, 1984). The assumption that "the basolateral membrane is removed as an important barrier that possesses negligible electrical resistance and voltage" (Helman et al., 1983) is controversial. As shown above, the corner frequency is independent of wide variations in the clamp potential, variations large enough to match the conceivable changes predictable for the apical Na changes involved. This argues against a simple voltage effect. Since inhibitors in concentrations known to result in significant changes in intracellular Na also fail to change the corner frequency (Hoshiko et al., 1984), the second possibility seems to be ruled out. On the other hand, basolateral K has pharmacological effects. For example, basolateral K is known to stimulate frog skin. Share and Ussing (1965) showed that basolateral K stimulates the skin glands and enhances osmotic water flow in toad skin. Thus, basolateral K has been used as a method of stimulating the hydro-osmotic response of amphibian epithelia (Grosso et al., 1982). In response to basolateral K, the frog skin corium appears to release prostaglandin-like material (Hall et al., 1977), which in turn stimulates cAMP production in the epithelium. Basolateral K results in the stimulation of intracellular cAMP levels (Cuthbert and Wilson, 1981) and the enhancement of the overshoot response to apical Na (Hoshiko and Machlup, 1983a, b). Basolateral K clearly has effects on more than just the electrical properties of the basolateral membrane. As such, this maneuver confounds the fluctuation experiment and represents the introduction of unknown variables. The mechanism by which basolateral K reduces the Na effect on the association rate constant remains unclear, but it may involve cAMP.

**Mechanism of Na Inhibition of Blocking**

In the past, the mechanism of interaction between amiloride and Na has been interpreted in terms of classical enzyme kinetics. For example, Benos et al. (1979) determined $K_A$, the amiloride concentration required to inhibit the short-circuit current by 50%, in the presence of increasing concentrations of Na in the apical bathing solution. In the case of R. temporaria skin and toad bladder, the apparent $K_A$ decreased as the Na concentration decreased. On the other hand, the apparent $K_A$ increased as the Na concentration decreased in bullfrog and R. pipiens. The present results show that the blocker association rate increased as the Na concentration decreased for both R. temporaria and R. pipiens skins. In other words, as the Na concentration decreased, blocker inhibition became more
effective. This means that the inhibition constant should decrease (i.e., less amiloride is needed to inhibit) as the Na concentration decreases. The source of this discrepancy may lie in the manner in which the apparent $K_A$ values were calculated. Although it is not explicitly stated, a comparison of the data for bullfrog listed in Table I of Benos et al. (1979) and the log dose-response curve (Fig. 1, p. 312) from which they derive would indicate that no correction was made for any baseline amiloride-insensitive current. Such a current may become significant when a concentration gradient is imposed across the epithelium. The error would increase the apparent $K_A$ for the low Na concentrations and hence may account for the discrepancy. Another possibility is seasonal variation. The data on 50% current inhibition may have involved summer frogs, whereas the present data were obtained in the fall and winter. This could mean that biochemical changes, induced presumably by hormonal effects dependent on the season, alter the response of the apical channels to Na, and that the properties of the apical Na-selective channel are subject to hormonal modulation. The essential observation is that the association rate constant is a function of the Na concentration.

In order to account for the inhibitory effect of Na on the association rate constant, a kinetic model requires that Na make Na conduction less sensitive to amiloride. If Na conduction were mediated by two states, one made insensitive to amiloride by Na, the requirement is met. One simple assumption is that a conducting, “Na-occupied” channel cannot be blocked by amiloride, and that only a conducting, “empty” channel is blockable. Thus, a minimal set of three states will suffice (Frehland et al., 1983), as illustrated in Fig. 8. In this way, the apparent association rate constant for amiloride blockage of Na conduction becomes a function of the Na concentration. The corner frequency for this model is

$$2\pi f_c = \omega_{\text{blocking}} = k_+ + kA/(1 + k_1S/k_-),$$

where the rate constants ($k$) are as defined in Fig. 8. $A$ is the amiloride or blocker concentration and $S$ is the Na concentration. The apparent association constant ($k_+$) for the amiloride is therefore

$$k_+ = k_{(\text{true})}/(1 + k_1S/k_-).$$

$k_{(\text{true})}$ is the unsubscripted rate constant in Fig. 8 (upper right). The data in Table II are for the corner frequencies obtained at a triamterene concentration of 8 $\mu$M. However, we do have estimates from previous work of the $k_-$ intercept, which was independent of [Na]. Using a mean value for $k_-$ of 15 radians/s, it is possible to estimate $k_+$ from the corner frequencies in Table II. The $k_+$ values estimated as indicated are plotted in Fig. 2 (plusses, upper solid curve). A nonlinear least-squares method was used to estimate the two parameters, the Na-free triamterene association constant, $k_{(\text{true})}$, and the Na “inhibition constant,” $(k_+/k_1)$. According to the model, $(k_+/k_1)$ is the Na concentration needed to halve the effective association rate constant for blocker. These estimated parameters were used to generate the lines shown.

In $R.\, tempora$ skin, the values for the Na inhibition constant, $(k_+/k_1)$, range
from 54 to 290 mM for triamterene (pH 6.0) and from 62 to 114 for amiloride (pH 7.8). No attempts were made to test for quantitative differences among species or blocking agent. Although it is not shown, the $k_+$ from the table in our earlier article (Hoshiko and Van Driessche, 1981) has been determined to give a value of $(k_-/k_1)$ of 190 mM. This means that the apparent association rate constant, “$k_+$,” when estimated in 120 mM Na, may be only 30–70% of the Na-independent value, $k_{(true)}$. Since $(k_-/k_1)$ was estimated using the corner frequency at a single triamterene concentration and an assumed value for the intercept (15 radians/s), the Na inhibition constant may be thought to be subject to a large error. However, the corner frequencies in 8 μM triamterene are so high that the error in the estimated $k_-$ would have to have been enormous to affect the results significantly. Parenthetically, the Na inhibition constant, $(k_-/k_1)$, should not be confused with the apparent Na “self-inhibition” constant, which is the Na concentration at which the Na current is half its maximal value as the apical Na concentration is increased. Instead, the new Na inhibition constant $(k_-/k_1)$ is a measure of Na’s interference with the ability of amiloride or triamterene to inhibit Na transport at the apical border. One possible interpretation of this kind of Na effect is that the presence of Na ion serves to prohibit blocker access to an Na-selective site. Other Na substitutes as diverse as Tris, K, arginine (unpublished data), and DDA do not exert such an effect; i.e., it is specific for Na. Basolateral K may act to reduce the Na effect just described, perhaps by opening up the channel entrance site to allow easier access for the blocker.

The experiments reported here were not designed to test the model; the model was devised to account plausibly for the Na effect. Although the proposed model is consistent with the observed effect, the fit is not particularly good. The statistical variability of the Na inhibition constant $(k_-/k_1)$ is unknown and it is not clear, for example, whether the value for triamterene is different from that for amiloride. The two experiments were done at different pH’s and cannot be compared. Other kinds of mechanisms might be responsible for the Na effect, such as mechanisms that go beyond the molecular properties of the channel itself but involve intracellular regulatory factors. For example, it is possible that apical Na alters an intracellular parameter, which in turn affects the triamterene and amiloride association rate constants. Although a change in intracellular Na has been tentatively ruled out, other poorly buffered variables may serve, such as intracellular Ca or pH. An apical proton-Na exchange process (Drewnowska and Biber, 1984) might lead to rapid alkalinization of the cell, which in turn may

![Figure 8](image-url)
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