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# Complex response of epithelial cells to inhibition of Na<sup>+</sup> transport by amiloride

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FISHER, RICHARD S., AND JERRY W. LOCKARD. *Complex response of epithelial cells to inhibition of Na<sup>+</sup> transport by amiloride*. *Am. J. Physiol.* 254 (Cell Physiol. 23): C297-C303, 1988.—When toad urinary bladder or frog skin epithelia are treated with amiloride, short-circuit current ( $I_{sc}$ ), which represents the net active transepithelial Na<sup>+</sup> transport rate from the apical to basolateral surface, decreases rapidly (2–5 s) to ~15–20% of control values and then slowly, over several minutes, continues falling toward zero. The contribution of this second phase of the decline is dependent on the transporting condition of the tissue before administration of amiloride. Attenuation of the second phase was observed if tissues were subjected to a period of transport inhibition. Tissues preincubated in 0 Na<sup>+</sup> Ringer solution on the apical surface were returned to control Na<sup>+</sup> Ringer, which caused an ~25% increase of  $I_{sc}$  above control values. Immediate reapplication of amiloride caused  $I_{sc}$  to decrease more rapidly than the previous exposure to values near zero, substantially reducing or eliminating the secondary slow decline. After long-term reincubation of tissues in control, 100 mM Na<sup>+</sup> solution, another treatment with amiloride indicated that the magnitude of the secondary decline increased in frog skin but not in urinary bladder epithelia. We conclude that the effect of amiloride is complex and may cause additional effects besides simply blocking entry of Na<sup>+</sup> into the apical membrane channel, and we suggest that regulatory mechanisms may be invoked in response to transport inhibition.

sodium; ion transport; channels; electrophysiology; cytoskeleton; short-circuit current

STUDIES OF THE MECHANISMS of ion transport across cell membranes have identified various intracellular regulatory processes invoked in response to perturbations of the steady-state transport rates. For epithelial tissues such as toad urinary bladder and frog skin, it is well established that the rate-limiting step in active, transepithelial Na<sup>+</sup> transport from the apical to basolateral cell surface is at the apical membrane where Na<sup>+</sup> enters passively down its electrochemical gradient (16). Compounds that inhibit Na<sup>+</sup> entry at this site, such as the diuretic amiloride, are often used to characterize the properties of these pathways. Amiloride has been especially useful in electrophysiological studies because inhibition is rapid and reversible, and the drug is highly specific for conductive Na<sup>+</sup> channels in epithelia at concentrations of 0.1 mM or below (4).

Many studies of active Na<sup>+</sup> transport by epithelia have employed the classical short-circuit current ( $I_{sc}$ ) technique devised by Ussing and Zerahn (22). In toad urinary bladder and frog skin,  $I_{sc}$  is virtually identical to the net,

active Na<sup>+</sup> transport from the apical to basolateral bathing solution (16). Because amiloride binding is rapid, occurring within milliseconds (1, 12), it would be expected that  $I_{sc}$  should decrease to zero within seconds of amiloride application. This, however, is not observed in these tissues. In general, amiloride causes a rapid decrease of  $I_{sc}$ , followed by a slow decline over several minutes, often leaving what appears to be a finite amiloride-insensitive Na<sup>+</sup> current. Here we demonstrate that the response of epithelial cells to inhibition of Na<sup>+</sup> transport with amiloride is complex, and the second phase of the decline of  $I_{sc}$  may be modified by prior inhibition of Na<sup>+</sup> transport in epithelia. This "memory" of prior incubation conditions may last from several minutes to hours after reestablishing transporting conditions. Indeed, the secondary slow decline of  $I_{sc}$  after amiloride may be indicative of a cellular regulatory process invoked in response to inhibition of Na<sup>+</sup> entry into epithelial cells. We suggest that indiscriminate use of inhibitors of transport as simple tools that have a single, acute effect on cell membranes may be unwarranted, since epithelial cells appear to respond differently to amiloride after periods of Na<sup>+</sup> transport inhibition.

## MATERIALS AND METHODS

Experiments were performed using isolated frog skin and toad urinary bladders. Frogs (*Rana pipiens pipiens*) were stored unfed in water at room temperature (Nasco, WI). After decapitation, ventral skin was isolated and mounted horizontally as a flat sheet in Lucite chambers that permitted constant flow of Ringer solution over the mucosal and serosal surfaces. Tissues were positioned over a stainless steel screen, and the serosal solution was perfused with ~10 cm subpressure, which also stabilized the tissue. Toads (*Bufo marinus*) were maintained in plastic tanks or moistened hardwood sanichips at room temperature (National Reagents, Bridgeport, CT). After double pithing, urinary bladders were removed, moderately stretched, and mounted in the same fashion as frog skins. We observed no macroscopic oscillations of  $I_{sc}$  with this arrangement. The chamber was sealed with silicon grease and minimal pressure to prevent edge damage. It was designed to permit complete exchange of apical bathing solution in <400 ms. Area of the tissue exposed was 0.3 × 1.25 cm<sup>2</sup> oriented in a rectangular slot similar to that described previously (9). Apical solution was exchanged by gravity feed and suction. The solution inlet ports were located ~1 mm from each tissue surface.

A large-bore inlet tube (PE-205) positioned at one end of the slot on the mucosal side was used to deliver an additional bolus of solution when extremely rapid exchange was required for amiloride application.

Transepithelial voltage ( $V_T$ ), was measured using 3 M KCl-agar bridges positioned 0.5 mm from the tissue in the center of the slot in both chamber halves and connected to Ag-AgCl wires. Tissues were maintained at  $V_T = 0$  mV with a voltage clamp, and  $I_{sc}$  was recorded with a strip chart and digitized at 30 points/s with a computer for later analysis (CS9000, IBM Instruments, Danbury, CT). Ag-AgCl wires ran the length of the slot in both chamber halves to pass current with uniform density and were positioned 3-4 mm from the epithelial surface.

**Solutions.** Control  $\text{Na}^+$  toad Ringer solution contained the following (in mM): 50  $\text{Na}^+$ , 60 tetramethylammonium (TMA), 110  $\text{Cl}^-$ , 2.5  $\text{K}^+$ , 1.0  $\text{Ca}^{2+}$ , and 2.5  $\text{HCO}_3^-$ , pH 8.2. In some studies, TMA was eliminated and 110 mM  $\text{Na}^+$  was used. No difference was observed. Control  $\text{Na}^+$  frog Ringer solution contained 100 mM NaCl. Sodium-free (0  $\text{Na}^+$ ) Ringer solution consisted of complete TMA or *N*-methyl-D-glucamine for  $\text{Na}^+$  substitution. Colchicine (Sigma Chemical, St. Louis, MO) was dissolved in ethanol and added to Ringer solution to a final concentration of 0.01 mM colchicine and 0.09% ethanol. Cytochalasin B (Sigma) was dissolved in dimethyl sulfoxide (DMSO) and its final concentration was 0.04 mM cytochalasin in 0.08% DMSO.

## RESULTS

**Toad urinary bladder: changes of  $I_{sc}$  after amiloride.** Typically a biphasic decrease of  $I_{sc}$  in response to amiloride is observed (Fig. 1, solid line; also cf. Refs. 18 and 19).  $I_{sc}$  decreases within seconds to <10-15% of control values (Table 1, control) and then slowly decreases to-

ward zero over several minutes (18, 19). The current after amiloride still represented primarily a net  $\text{Na}^+$  current, since apical  $\text{Na}^+$  removal during the amiloride-induced decline of  $I_{sc}$  caused a decrease of current toward zero as shown in Fig. 2. Similar observations were described previously for isolated frog skin and are discussed below (19). Unlike previous workers, we could not describe the response uniquely as the sum of two exponentials, but we observed various time courses often described simply with two linear regions. Consequently, the initial decline was complete in a few seconds, whereas the secondary decrease of  $I_{sc}$  persisted over several minutes. Rate constants for amiloride binding to epithelia indicate that inhibition of transport should occur in milliseconds, thus it seemed possible that the secondary slow decline of  $I_{sc}$  represented a regulatory response of the cell to inhibition of  $\text{Na}^+$  transport. In the present study, we were interested in determining whether this phase of the response could be modified by various maneuvers.

It was unknown whether the secondary decrease of  $I_{sc}$ , which required several minutes, was attributable to amiloride per se or to a time-dependent effect of transport inhibition. To test this directly, we examined whether long-term inhibition of  $\text{Na}^+$  entry altered the tail of the  $I_{sc}$  response to amiloride. The protocol generally used throughout this study was as follows. After the first application of amiloride, tissues were washed with drug-free Ringer solution and were bathed in a  $\text{Na}^+$ -free (0  $\text{Na}^+$ ) apical solution. About 30 min later, control  $\text{Na}^+$  Ringer was returned to the apical chamber, and after  $I_{sc}$  reached its peak, tissues were reexposed to amiloride. Two typical examples of the response of  $I_{sc}$  after returning  $\text{Na}^+$  to the apical bath of toad urinary bladders are shown in Fig. 3. Most often after 30-60 min of 0  $\text{Na}^+$  in the apical bath (upper panel), returning to the control,

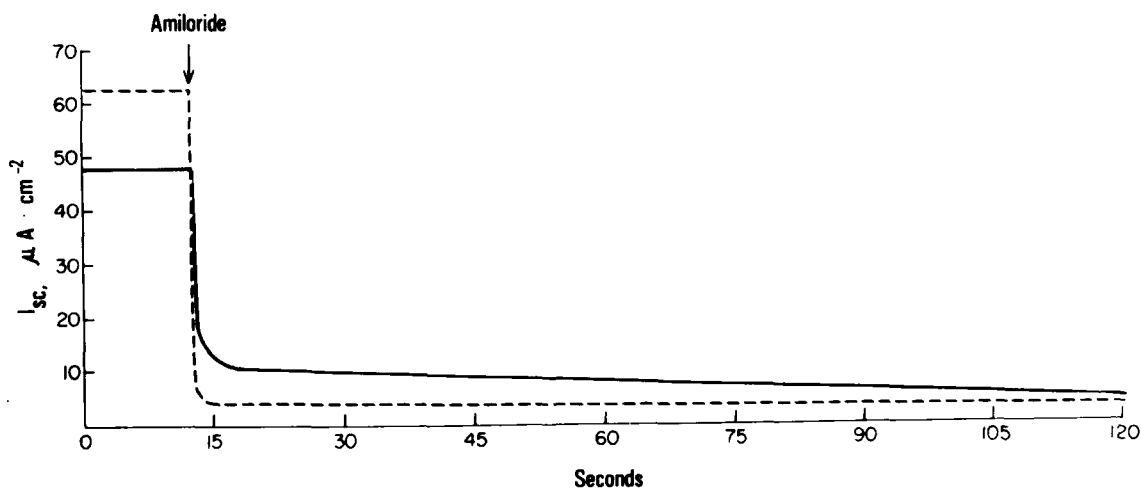


FIG. 1. Amiloride inhibition of short-circuit current ( $I_{sc}$ ) in toad urinary bladder. Amiloride ( $10^{-4}$  M) was added to apical bath at  $\downarrow$ . Points were acquired and plotted at 33-ms intervals. Both responses were from a single tissue and were superimposed for comparison. First, tissue was preincubated in control  $\text{Na}^+$  Ringer. Amiloride treatment (—) was followed by wash with amiloride-free, 0  $\text{Na}^+$  solution for 30 min. Then, immediately after returning control (100  $\text{Na}^+$  Ringer) to apical bath, amiloride was added again (- - - -).

TABLE 1. Response of  $I_{sc}$  to  $10^{-4}$  M amiloride in toad bladder

Ringer Solution	$I_{sc}$ , $\mu\text{A}/\text{cm}^2$						$\Delta I_{sc}$
	0 s	0.4 s	1 s	2 s	30 s	100 s	
Control	44.4±4.3	12.1±2.0	8.8±1.7	7.3±1.6	4.3±1.4	3.2±1.2	2.7±0.4
0 Na <sup>+</sup>	55.4±6.1	4.2±1.0	2.0±0.9	1.5±0.9	0.9±0.9	0.8±0.9	0.3±0.1

Values are means ± SE; n = 9.  $\Delta I_{sc}$ , difference between short-circuit current ( $I_{sc}$ ) measured 100 s after amiloride and  $I_{sc}$  measured when tissues were bathed in 0 Na<sup>+</sup> Ringer solution.

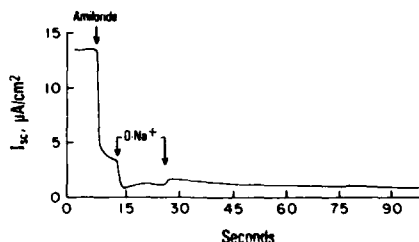


FIG. 2. Apical Na<sup>+</sup> removal during decline of short-circuit current ( $I_{sc}$ ). Apical bathing solution was exchanged for Ringer solution containing tetramethylammonium for Na<sup>+</sup> substitution.

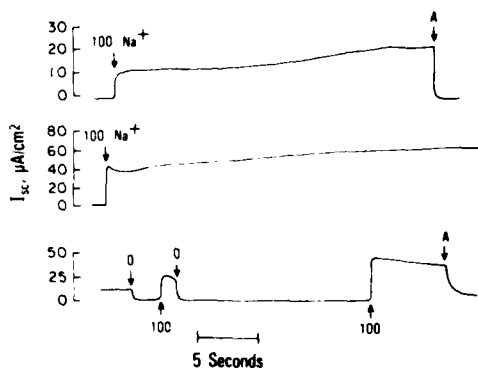


FIG. 3. Typical responses of urinary bladder (top and middle) and frog skin (bottom) to changes of apical Na<sup>+</sup> concentration.  $I_{sc}$ , short-circuit current. See text for details.

100 Na<sup>+</sup> Ringer solution, caused a step increase of  $I_{sc}$  to values near control and then over the next 1–2 min (70 ± 16 s) the current increased further. Alternatively, we observed a transient decrease of  $I_{sc}$  after the initial step followed by a similar slow increase over the next 2–3 min (Fig. 3, middle; see DISCUSSION).

Average values of  $I_{sc}$  in response to amiloride for toad urinary bladder are summarized in Table 1. On returning to control Na<sup>+</sup> solution (after 0 Na<sup>+</sup> pretreatment),  $I_{sc}$  increased ~25% above control (Table 1, 0 s after amiloride). An example of the subsequent amiloride treatment is also shown in Fig. 1 (dashed line). Although initially  $I_{sc}$  was greater than control, it decreased rapidly to stable values within seconds and little or no further decline was observed over the next several minutes. These data were normalized for the first 23-s period after amiloride and were replotted as shown in Fig. 4A, assuming that the magnitude of the net transepithelial Na<sup>+</sup> current could be described by the difference between the control  $I_{sc}$  and the value measured when the tissue was bathed in 0 Na<sup>+</sup>. Clearly, after incubating in 0 Na<sup>+</sup>, inhibition of  $I_{sc}$  approached 100% <3 s after exposure to amiloride, whereas the control Na<sup>+</sup> preincubation resulted in an

~85% inhibition of  $I_{sc}$ . The average difference between these two curves for the nine tissues studied is plotted in Fig. 4B. Without exception, the response to amiloride was faster after preincubation in 0 Na<sup>+</sup>. The difference averaged ~20% at 400 ms and then decreased monotonically over the next 90 s to ~8%. Because the  $I_{sc}$  in the presence of amiloride (after 0 Na<sup>+</sup> preincubation) was constant and near zero, the difference plotted in this figure primarily reflects the slow response observed after initial application of amiloride. On average, control  $I_{sc}$  decreased from 44.4 to 4.3  $\mu\text{A}/\text{cm}^2$  and over the next 70 s declined to 3.2  $\mu\text{A}/\text{cm}^2$  (Table 1). This was 2.7  $\mu\text{A}$  greater than the  $I_{sc}$  measured while the tissues were bathed in 0 Na<sup>+</sup>. Alternatively, after 0 Na<sup>+</sup> preincubation, despite greater base-line currents,  $I_{sc}$  decreased to significantly lower values after 30 s (0.9  $\mu\text{A}/\text{cm}^2$ ) and remained constant thereafter. This value was similar to the current measured while the tissues were bathed in 0 Na<sup>+</sup>, which indicates that indeed transepithelial Na<sup>+</sup> transport was rapidly eliminated (Table 1, right).

It was possible that the more rapid response was due to previous treatment with amiloride irrespective of whether current was inhibited by the preincubation with 0 Na<sup>+</sup>. However, in another group of studies, we found that the increased speed of the response to amiloride could be attributed directly to Na<sup>+</sup> removal and not to a nonspecific effect of amiloride treatment (cf. Table 2). In contrast to the above studies, tissues were preincubated with 0 Na<sup>+</sup> before the first amiloride exposure. Control  $V_T$  and  $I_{sc}$  averaged 53.8 ± 7.6 mV and 11.4 ± 1.7  $\mu\text{A}/\text{cm}^2$  (n = 6), respectively. After 30–45 min in apical 0 Na<sup>+</sup>, tissues were returned to control Ringer solution, which increased  $I_{sc}$  78% above control to 20.3  $\mu\text{A}/\text{cm}^2$  (Table 2, top). This time, amiloride treatment caused  $I_{sc}$  to decrease in 90 s to 3.8  $\mu\text{A}/\text{cm}^2$ . After washing amiloride for 10–15 min,  $I_{sc}$  returned near the value measured directly after 0 Na<sup>+</sup> preincubation, and when exposed to amiloride a second time,  $I_{sc}$  declined at a rate and to final values similar to the first exposure. Thus prior inhibition with either protocol results in a similar time course for the current response to amiloride. To determine whether the response was reversible, tissues were incubated for an additional 3–4 h in control Ringer and then were exposed a third and fourth time to amiloride. Clearly, the speed of the response was similar even after this long transport period. Thus, in toad urinary bladder, even though the  $I_{sc}$  returns to control after washing out the amiloride, there is some irreversible change in the response of the cell to amiloride that is apparently detectable only in the rate of amiloride inhibition.

In view of the possibility that the slow phase of the  $I_{sc}$

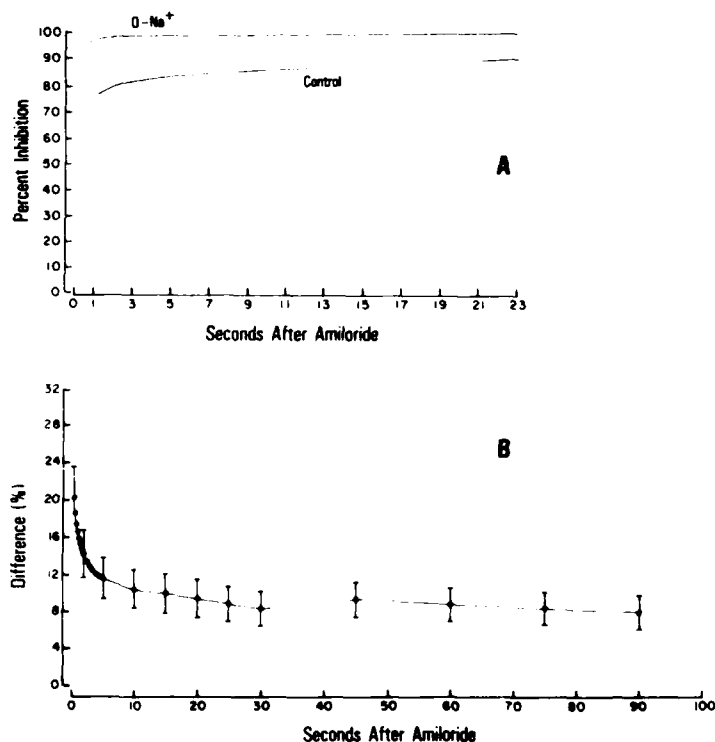


FIG. 4. Normalized plot of response of short-circuit current ( $I_{sc}$ ) from toad urinary bladder to  $10^{-4}$  M amiloride. A: percent inhibition of  $I_{sc}$  is shown vs. time after amiloride. Data from Fig. 1 was replotted as a percentage of change in  $I_{sc}$  measured for 1st 30-s period after application of amiloride. B: difference between 2 curves in A for each tissue at corresponding times after amiloride were averaged and plotted. ( $n = 9$ ). First point plotted is 400 ms after amiloride to eliminate inconsistencies in chamber mixing. Values are means  $\pm$  SE,  $n = 9$  (after 30 s,  $n = 7$ ).

TABLE 2. Repetitive exposures of urinary bladder to  $10^{-4}$  M amiloride on  $I_{sc}$ .

Preincubation Solution	$I_{sc}$ , $\mu\text{A}/\text{cm}^2$					
	0 s	0.4 s	1 s	2 s	30 s	90 s
1. 0 Na <sup>+</sup>	20.3 $\pm$ 2.7	7.1 $\pm$ 0.9	5.9 $\pm$ 0.7	5.2 $\pm$ 0.7	4.1 $\pm$ 0.5	3.8 $\pm$ 0.5
2. Amiloride wash	16.5 $\pm$ 2.1	5.3 $\pm$ 0.6	4.4 $\pm$ 0.5	4.1 $\pm$ 0.5	3.6 $\pm$ 0.5	3.6 $\pm$ 0.5
3. Ringer	16.6 $\pm$ 3.5	5.9 $\pm$ 1.0	4.6 $\pm$ 0.6	4.1 $\pm$ 0.4	3.3 $\pm$ 0.2	3.2 $\pm$ 0.2
4. Amiloride wash	15.3 $\pm$ 3.1	4.4 $\pm$ 0.6	3.6 $\pm$ 0.3	3.4 $\pm$ 0.2	3.1 $\pm$ 0.2	3.1 $\pm$ 0.2

Values are means  $\pm$  SE;  $n = 6$ . Initial control short-circuit current ( $I_{sc}$ ) ( $11.4 \pm 1.7$ ) was measured before incubating tissues in 0 Na<sup>+</sup> for 30-60 min and was followed by 2 successive exposures to amiloride (cf. 1 and 2). After 3- to 4-h incubation in Ringer solution, tissues were treated again with amiloride (3). After washing, they were subjected to a final application (4).  $I_{sc}$  in 0 Na<sup>+</sup> was  $1.4 \pm 0.3 \mu\text{A}/\text{cm}^2$ .

response of toad bladder to amiloride could involve recruitment of conductive sites into the apical membrane (see below), and because the cytoskeleton modulates recruitment of membrane proteins from cytoplasmic vesicles (14, 15, 20, 21), we examined the effects of cytoskeletal disruption by cytochalasin B and colchicine on the response of  $I_{sc}$  to amiloride. Tissues were incubated in chambers under short-circuit conditions for at least 3 h with one of these two agents. Then the same protocol as above was performed, i.e., incubation with amiloride, 30 min in 0 Na<sup>+</sup>, return to control, and a second amiloride exposure. The control parameters (Table 3) indicate that colchicine had no deleterious effect on  $V_T$  or  $I_{sc}$ , and after 0 Na<sup>+</sup> pretreatment  $I_{sc}$  increased similar to untreated tissues. Long-term incubation of tissues with cytochalasin yielded lower values of  $V_T$  and  $I_{sc}$  (cf. Table 3), and measurement of these parameters 30-60 min after mounting tissues in chambers indicated that the values were, as a group, lower than those described above. In addition, although not systematically studied, when these tissues were removed from the chambers after

TABLE 3. Effect of cytoskeletal agents on transepithelial  $I_{sc}$  and  $V_T$ .

Preincubation Solution	$V_T$ , mV	$I_{sc}$ , $\mu\text{A}/\text{cm}^2$
Cytochalasin*		
Control	27.0 $\pm$ 5.5	11.2 $\pm$ 1.5
0 Na <sup>+</sup>	27.8 $\pm$ 7.3	13.0 $\pm$ 1.7
Colchicine		
Control	47.5 $\pm$ 6.6	18.9 $\pm$ 3.5
0 Na <sup>+</sup>	52.7 $\pm$ 9.7	24.4 $\pm$ 4.8

Values are means  $\pm$  SE;  $n = 10$  for cytochalasin and 9 for colchicine. Values measured after 3-h incubation with agent before addition of  $10^{-4}$  M amiloride to apical bath. \* Control parameters for paired hemibladders ( $n = 4$ ): untreated, transepithelial voltage ( $V_T$ ) =  $91.8 \pm 9.3$  mV, short-circuit current ( $I_{sc}$ ) =  $32.8 \pm 10.2 \mu\text{A}/\text{cm}^2$ ; cytochalasin treated,  $V_T = 31.3 \pm 11.0$  mV,  $I_{sc} = 14.9 \pm 2.7 \mu\text{A}/\text{cm}^2$ .

completion of the experiment, they appeared less resilient when stretched compared with untreated tissues. Finally, the increase of  $I_{sc}$  after 0 Na<sup>+</sup> was attenuated.

If cytoskeletal components were required for the slow secondary decline of  $I_{sc}$ , it would be expected that the

difference between inhibition curves would be eliminated (cf. Fig. 4B). This, however, was not observed. As shown in Fig. 5, tissues treated with colchicine or cytochalasin responded more rapidly to the second amiloride treatment in a similar fashion to control tissues with the difference at 90 s averaging 7.6 and 5.4%, respectively.

**Frog skin: changes of  $I_{sc}$  after amiloride.** We repeated several of the studies described above using isolated frog skin. A similar protocol was used, i.e., amiloride, 0 Na<sup>+</sup>, control, amiloride. Unlike toad bladder, the response of frog skin to 100 mM Na<sup>+</sup> after a 30- to 60-min period in 0 Na<sup>+</sup> was consistent. As shown in Fig. 3 (bottom),  $I_{sc}$  increased rapidly to a peak and slowly declined thereafter in a manner similar to previous observations (11, 17). Although not systematically examined, it appeared that the magnitude of the increase of  $I_{sc}$  depended on the time in 0 Na<sup>+</sup> so, as above, tissues were routinely preincubated in 0 Na<sup>+</sup> for 30–60 min. Qualitatively similar results were observed for frog skin as for toad bladder (cf. Table 4), where  $I_{sc}$  increased from an average control value of 26.3 to 40.9  $\mu\text{A}/\text{cm}^2$  after 0 Na<sup>+</sup>. The initial decline of  $I_{sc}$  was significantly slower and more variable in frog skin attributable in part to the unstirred layer provided by the stratum corneum. This represented a significant problem for the first few seconds of the response, since the thickness of this dead cell layer and its permeability to amiloride were unknown. Still, despite values of  $I_{sc}$  averaging 55% more than control, within 5 s  $I_{sc}$  decreased to lower values after 0 Na<sup>+</sup>, and at 90 s  $I_{sc}$  averaged 2.1 compared with 2.9  $\mu\text{A}/\text{cm}^2$  for control tissues. These data were normalized and are summarized as the difference in percent inhibition curves in Fig. 6 (●). The difference decreased with time to an average value of 7.2% at 90 s. However, in contrast to toad bladder, the response in frog skin was reversible. After the first two amiloride treatments, tissues were incubated in control Ringer solution for ~2 h and then the same sequence of solution

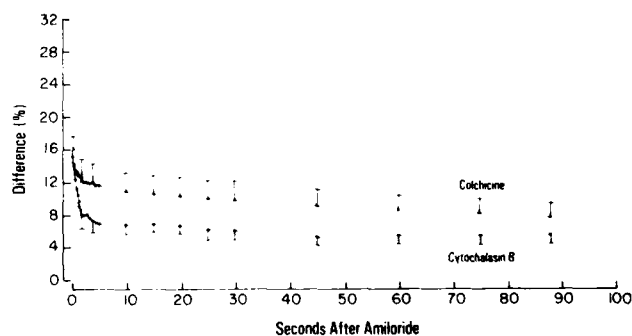


FIG. 5. Effect of cytochalasin B and colchicine on the difference plot for toad urinary bladder (cf. Fig. 4). Values plotted are means  $\pm$  SE. All points are significantly different from zero.

TABLE 4. Response of  $I_{sc}$  to  $10^{-4}$  M amiloride in frog skin

Preincubation Solution	$I_{sc}$ , $\mu\text{A}/\text{cm}^2$						$\Delta I_{sc}$
	0 s	2 s	5 s	10 s	30 s	90 s	
Control	26.3 $\pm$ 3.1	7.1 $\pm$ 0.9	5.2 $\pm$ 0.6	4.4 $\pm$ 0.5	3.6 $\pm$ 0.4	2.9 $\pm$ 0.3	2.3 $\pm$ 0.2
0 Na <sup>+</sup>	40.9 $\pm$ 3.9	8.9 $\pm$ 1.8	5.0 $\pm$ 1.0	3.5 $\pm$ 0.6	2.4 $\pm$ 0.4	2.1 $\pm$ 0.3	1.5 $\pm$ 0.2

Values are means  $\pm$  SE;  $n = 21$ .  $\Delta I_{sc}$ , difference between short-circuit current ( $I_{sc}$ ) measured 90 s after amiloride and  $I_{sc}$  measured when tissues were bathed in 0 Na<sup>+</sup> Ringer solution.

changes was performed. In this group of tissues, control  $I_{sc}$  averaged 29.9 and increased to 51.3  $\mu\text{A}/\text{cm}^2$  after 0 Na<sup>+</sup> (Table 5). Characteristically, the response time was decreased after 0 Na<sup>+</sup> inhibition (compare 2.2 with 3.8  $\mu\text{A}/\text{cm}^2$  at 30 s in Table 5). After the second exposure to amiloride, control Ringer solution was perfused in the apical bath for 135 min. By this time,  $I_{sc}$  had returned to control levels (28.8  $\mu\text{A}/\text{cm}^2$ ) and application of amiloride caused  $I_{sc}$  to decrease with a time course similar to the first exposure. In fact, exposures 3 and 4 were indistinguishable from exposures 1 and 2, respectively (cf. Table 5 and Fig. 6).

## DISCUSSION

Amiloride has been a useful tool for determining a number of kinetic and electrical parameters of the conductive channels of tight Na<sup>+</sup> transporting epithelia. In general, it has been assumed that the drug is a specific, reversible inhibitor of Na<sup>+</sup> transport in frog skin and toad urinary bladder. However, given the rate constants for amiloride binding, a number of observations indicate that the inhibitory properties of this drug are complex. First, after an initial rapid decline of  $I_{sc}$ , a secondary slow phase of decline in the  $I_{sc}$  over a period of several minutes representing 10–20% of the initial current under control conditions is observed. Rabito et al. (19) could modify the magnitude of this decline by changes of extracellular Ca<sup>2+</sup> concentration (19). Ca<sup>2+</sup> removal increased the contribution of the slow phase to ~30% of the initial current. Second, washout of  $10^{-4}$  M amiloride in these tissues requires long periods of time, usually ~10–15 min. Again, this is far greater than expected from the measured off-rate constants. Moreover, the washout kinetics do not appear to be simple. In some tissues amiloride washes out in <2 min while in others the washout period is prolonged, lasting 10–20 min. Indeed, in frog skin, we occasionally did not observe an immediate increase of  $I_{sc}$  on removing amiloride but a gradual increase over an ~20-min period. This effect was observed consistently when a supramaximal concentration of  $10^{-3}$  M amiloride was used. Although the cause of this is unknown, it is possible that residual amounts of amiloride nonspecifically bound or accumulated intracellularly may be responsible for the prolonged inhibition (5, 6, 8). Finally, the type of inhibition of Na<sup>+</sup> transport by amiloride is not clearly established and varies depending on the tissue and the laboratory where investigated (4).

During the course of these studies, it appeared that these epithelia do not respond to perturbations of baseline transport rates simply. Often observed but unreported transient changes in electrical parameters are common after perturbations of transport. For example,

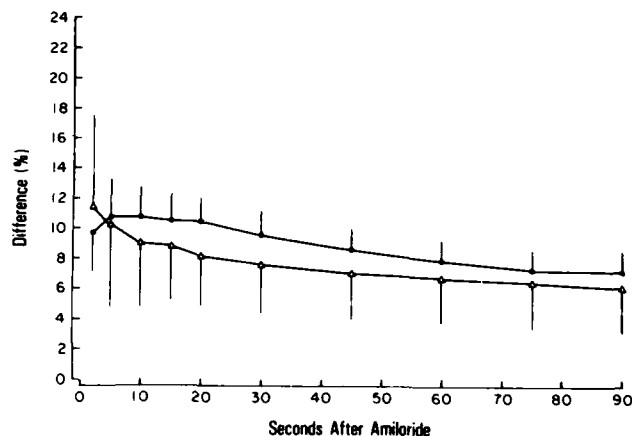


FIG. 6. Reversibility of short-circuit current response of frog skin to  $10^{-4}$  M amiloride. Difference plot derived from 1st and 2nd exposures (●) is indistinguishable from difference plot from 3rd and 4th exposures (Δ).

TABLE 5. Repetitive exposures of frog skin to  $10^{-4}$  M amiloride on  $I_{sc}$

Preincubation Solution	0 s	30 s	90 s
1. Control	29.9±3.2	3.8±0.4	3.0±0.3
2. 0 Na <sup>+</sup>	51.3±4.1	2.2±0.3	1.8±0.2
3. Control	28.8±3.3	3.2±0.4	2.6±0.3
4. 0 Na <sup>+</sup>	47.3±5.4	2.1±0.2	1.9±0.2

Values are means ± SE;  $n = 15$ .

changing from open to short-circuit conditions causes a transient increase of  $I_{sc}$  followed by a long-term (often lasting 1–2 h) decline to steady-state values (unpublished observations). In addition, we observed rather complex responses of  $I_{sc}$  after returning to control Ringer solution from 0 Na<sup>+</sup>. Most inconsistent were the responses of toad bladder where delays ranging from seconds to minutes were observed before the  $I_{sc}$  increased to peak values. Similarly, the magnitude of the increase of  $I_{sc}$  after 0 Na<sup>+</sup> varied from ~25% to >75% above control values. Variable responses of tissues to these maneuvers indicate to us that assessing macroscopic kinetic parameters from simple plots of  $I_{sc}$  vs. Na<sup>+</sup> concentration may be complicated by these time-dependent changes.

The object of this report was to examine the nature of the response of typical Na<sup>+</sup> transporting epithelia, frog skin, and toad urinary bladder to maximal inhibitory concentrations of amiloride. We found that we could modify the second, slower phase of the response by preincubation of the tissue in solutions lacking Na<sup>+</sup>. Consequently, we conclude that this slower decline may represent a cellular response to Na<sup>+</sup> transport inhibition. In other studies, we did not observe any difference in the rapidity of inhibition of  $I_{sc}$  in response to Na<sup>+</sup> removal after preincubation in 0 Na<sup>+</sup> solutions (unpublished observations). This was expected, since the current remaining during the second phase of decline after amiloride was carried by Na<sup>+</sup>. Thus it is clear that the cellular response to amiloride depends not only on the content of the bathing solution but on the history of transporting conditions.

The mechanism of this complex response is unknown,

but it has been suggested that the secondary decline of  $I_{sc}$  may represent a second population of channels with heterogeneous rate constants for amiloride or possibly diffusion of amiloride to deeper conductive sites in the apical membrane. Both of these ideas seem unlikely. First, current fluctuation analysis with amiloride indicates that amiloride binds to a single population of channels with homogeneous rate constants (1, 12). Second, assuming a reasonable diffusion coefficient for amiloride, one must postulate some interaction of amiloride with the cell membranes, which prevents rapid diffusional access to sites within the epithelium. Indeed, the secondary decline may represent intracellular effects of amiloride, since it has been demonstrated that the drug can enter cells (5, 8).

It is also possible that the slow decline may be attributed to a regulatory process of the cell in response to inhibition of Na<sup>+</sup> entry. For example, hyperpolarization of the cells or inhibition of a Na<sup>+</sup>-H<sup>+</sup> exchanger in the apical membrane by Na<sup>+</sup> removal could lead to changes of the intracellular pH or Ca<sup>2+</sup> concentration, which secondarily lead to changes of the cellular response to amiloride. At present, however, the extent of coupling between Na<sup>+</sup> and H<sup>+</sup> movements across the apical membrane is controversial (3, 13). Another interesting possibility relates to recent studies of current fluctuation analysis using amiloride or an uncharged analogue, 6-chloro-3,5-diaminopyrazine-2-carboxamide, in frog skin which indicate that these compounds induce a prompt autoregulatory response which manifests itself as an increase of open channel density as the concentration of Na<sup>+</sup> channel blocker increases (1). Thus the slow decline of  $I_{sc}$  after amiloride possibly involves this autoregulatory process invoked by inhibition of Na<sup>+</sup> transport. For example, amiloride may lead to an increase of apical membrane Na<sup>+</sup> channel density, possibly by causing insertion of new transport sites into the apical membranes of epithelial cells (7) or by recruiting inactive sites from a closed, nonconducting state. (1, 2, 12). In this context, we recently examined Na<sup>+</sup> channel density with noise analysis after tissues were preincubated in 0 Na<sup>+</sup>. Immediately after returning to control Na<sup>+</sup> Ringer solution in the apical bath, Na<sup>+</sup> channel density was increased substantially (10). Thus channel density was greater under the same conditions that diminished the contribution of the slow phase of the response of  $I_{sc}$  to amiloride. However, channel insertion (or recruitment) per se does not explain the slow decrease of current toward zero after amiloride, since new channels should be blocked immediately by the drug given the rate constants for amiloride binding. The slow decrease of  $I_{sc}$  therefore may indicate a change in the binding properties or accessibility of these new sites with time. If, after recruitment, these channels were initially conductive to Na<sup>+</sup> but insensitive to amiloride (yielding a finite  $I_{sc}$ ), then the slowly diminishing current might represent a balance between the rate of recruiting channels vs. the rate of developing amiloride sensitivity. With the assumption that the secondary decline of  $I_{sc}$  can be attributed to changes of channel density, it seems unlikely that channels are inserted into the apical membrane,

since cytoskeletal disruption did not affect this response in toad urinary bladder (see RESULTS).

Regardless of the mechanism involved in the response to amiloride, it may be concluded that the response of Na<sup>+</sup> transporting "tight" epithelia to inhibition is complex and may involve a regulatory response that lasts from several minutes to hours. This complicates electrophysiological studies in which it is assumed that the only effect of amiloride is blockage of Na<sup>+</sup> entry into the channel. This may be particularly important in studies where tissues are repeatedly pulsed with amiloride as a simple tool for increasing the apical membrane resistance of tight epithelia.

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