peroxide perfusion did not alter neuromuscular transmission or the processes involved in excitation-contraction coupling for brief time intervals. Second, autoregulation of blood flow, manifested by the presence of flow transients after brief periods of arterial occlusion, was present in each experiment both before and after hydrogen peroxide administration (*10*). This showed that hydrogen peroxide did not interfere with integrated physiological regulation of blood flow. Third, recovery of muscle function after hydrogen peroxide perfusion was tested after a rest period of 100 minutes in two experiments. Muscle oxygen consumption was 96 and 88 percent of control values while twitch tension amplitude returned to 83 and 75 percent of preinfusion values. The cut sections of the muscle were red and appeared similar to the contralateral control muscle, suggesting that return of myoglobin to its functional ferrous state was associated with return to normal muscle function. This may be attributable to metmyoglobin reductase activity, which is generally correlated with muscle myoglobin content (*11*). Finally, the effect of hydrogen peroxide on respiration of isolated muscle mitochondria was tested. Rat hindlimb muscle mitochondria were prepared by the method of Makinen and Lee (*12*). Respiratory control indices and P : O ratios were determined with glutamate and malate substrates at hydrogen peroxide concentrations ranging from 0 to 0.7 m*M*. Hydrogen peroxide did not adversely affect these indices of mitochondrial oxidative phosphorylation at a concentration twice that present in the perfusate used in the studies in vivo. Hence, I conclude that the effect of hydrogen peroxide was specific for muscle myoglobin rather than for the mitochondrial cytochrome system. This interpretation is supported by studies of pigeon breast muscle fiber bundles, where maximal oxygen consumption was unaffected by a variety of chemical interventions that rendered myoglobin nonfunctional (*4*).

Myoglobin in vivo may act as an oxygen store, as an agent involved in enhancing oxygen flux through tissue, or possibly as an oxygen buffer, maintaining cell PO_2 constant when there are changes in oxygen supply and demand. Myoglobin may have been carrying out any of these functions in the present study. At the same time, these experiments do not establish that the exercising muscle was hypoxic in the absence of functional myoglobin. This question requires additional study with probes of intracellular mitochondrial oxidation state or measurement of an appropriate biochemical correlate of cellular hypoxia. Nonetheless, I conclude that functional myoglobin plays an important role in maintenance of oxygen consumption and tension generation in exercising skeletal muscle.

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References and Notes

1. J. B. Wittenberg, *Physiol. Rev.* **50**, 559 (1970).
2. F. Kreuzer, *Respir. Physiol.* **9**, 1 (1970).
3. J. deKoning, L. J. C. Hoofd, F. Kreuzer, *Pfluegers Arch.* **389**, 211 (1981).
4. B. A. Wittenberg, J. B. Wittenberg, P. R. B. Caldwell, *J. Biol. Chem.* **250**, 9038 (1975).
5. R. P. Cole, B. A. Wittenberg, P. R. B. Caldwell, *Am. J. Physiol.* **234**, H567 (1978).
6. M. Tamura, N. Oshino, B. Chance, I. A. Silver, *Arch. Biochem. Biophys.* **191**, 8 (1978).
7. W. N. Stainsby, J. T. Fales, J. L. Lilienthal, Jr., *Bull. Johns Hopkins Hosp.* **99**, 249 (1956).
8. W. N. Stainsby, *Am. J. Physiol.* **219**, 435 (1970).
9. K. Mészáros, B. Chance, H. Holtzer, *J. Mol. Cell. Cardiol.* **12**, 965 (1980).
10. W. N. Stainsby, *Am. J. Physiol.* **202**, 273 (1962).
11. L. Hagler, R. I. Coppes, Jr., E. W. Askew, A. L. Hecker, R. H. Herman, *J. Lab. Clin. Invest.* **95**, 222 (1980).
12. M. W. Makinen and C. Lee, *Arch. Biochem. Biophys.* **126**, 75 (1968).
13. I thank P. R. B. Caldwell and W. N. Stainsby for helpful discussions and suggestions. I wish to acknowledge the expert assistance of N. M. McWethy and B. A. Brent. The mitochondrial studies were done in the laboratory of J. Wittenberg and B. Wittenberg at the Albert Einstein College of Medicine. This work was supported by grants NHLBI-HL24994 and NHLBI-HL07018 and by the Joe and Emily Lowe Foundation and was performed while I was a Parker B. Francis Foundation fellow in pulmonary medicine.

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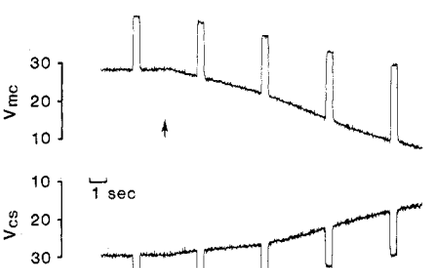
Sodium Transport Inhibition by Amiloride Reduces Basolateral Membrane Potassium Conductance in Tight Epithelia

Abstract. *In toad and frog urinary bladder, electrophysiological data suggest that inhibition of transepithelial sodium transport by mucosal amiloride results in a decrease in basolateral membrane conductance. These results were confirmed by showing that amiloride addition caused a decrease in basolateral membrane potassium permeability.*

The transport properties of tight epithelia (*1*) arise largely because the apical membrane is primarily sodium-selective, whereas the basolateral membrane is primarily potassium-selective (*2*). Investigators usually assume that inhibition of sodium transport—either by removal of sodium from, or by addition of the diuretic amiloride to, the mucosal medium—has no effect on the conductive properties of the basolateral membrane (*3, 4*). It has been shown in toad urinary bladder, however, that inhibition of transepithelial sodium transport by either means causes a decrease in steady-state basolateral membrane conductance (*5, 6*). Furthermore, there is evidence in other tight epithelia that basolateral membrane conductance is correlated with the rate of sodium transport (*7, 8*). To test the hypothesis that basolateral membrane conductance is sensitive to changes in transepithelial sodium transport, we used two methods to evaluate the permeability of the cell membranes: (invasive) electrophysiological measurements were corroborated by (noninvasive) cell volume measurements. We present new evidence that in toad and frog urinary bladder, basolateral membrane conductance decreases through a decrease in potassium permeability when



R_t	8.6	8.8	9.4	10.6	12.3
R_a	10.7	10.8	12.3	16.7	27.6
R_b	5.9	6.8	7.7	9.8	14.8
R_a/R_b	1.8	1.6	1.6	1.7	1.7

Fig. 1. Effects of mucosal amiloride on open-circuit cell membrane potentials and resistances in toad urinary bladder. The record begins with the microelectrode in a cell recording base-line apical (V_{mc}) and basolateral (V_{cs}) membrane potentials and potential deflections caused by intermittent transepithelial constant-current pulses. At the arrow, the mucosal superfusate was switched from Ringer solution to Ringer plus amiloride ($10^{-4}M$). In the tabular portion, transepithelial (R_t), apical membrane (R_a), and basolateral membrane (R_b) resistances and the ratio of cell membrane resistances are listed for each period in which a constant-current pulse was applied. R_a and R_b were calculated by using the assumption that paracellular resistance (R_s) is given by R_t (*18*) in the steady state after amiloride addition. From the values of R_t [$= (R_a + R_b)R_s/(R_a + R_b + R_s)$; see (*3, 5, 6*)], R_a/R_b, and R_s, we may calculate R_a and R_b

$$R_a = [(R_a/R_b)R_sR_t]/\alpha \text{ and } R_b = (R_sR_t)/\alpha \text{ where } \alpha = [(R_a/R_b) + 1]\,[R_s - R_t]$$

[see (*10*)]. Potentials are in millivolts and resistances are in kilohms times square centimeters.

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Table 1. Effect of increased serosal potassium and mucosal amiloride on cell volume regulation and effect of amiloride on isosmotic potassium-induced cell swelling. Change in cell volume is expressed as percentage of original volume; values are means ± S.E. *N*, number of observations (see legends to Figs. 2 and 3).

Condition	*N*	Rate of change of cell volume (percent/min)
Volume-regulatory shrinking		
Control	4	18.6 ± 4.3
Serosal potassium (25 m*M*)	3	1.5 ± 0.4
Amiloride	3	< 0.5
Isosmotic potassium swelling		
Control	3	10.2 ± 1.7
Amiloride	3	2.1 ± 0.4

sodium transport is inhibited with amiloride.

Cell membrane potential differences and membrane potential deflections that result from the passage of transepithelial current were monitored before and after the rapid addition of amiloride ($10^{-4}M$) to the mucosal bathing solution. Conventional intracellular microelectrode techniques were used in these studies (*9, 10*). As observed previously (*5, 6, 9, 10*), amiloride causes a sudden decrease in both cell membrane potentials (Fig. 1). A new observation, however, is that as the current-induced potential deflection across the apical membrane increases, so does that across the basolateral membrane; the net effect is that their ratio does not change, or even decreases. Since this ratio is equal to the ratio of membrane resistances (*11*), the resistance of the basolateral membrane must rise with approximately the same time course as that of the apical membrane during transport inhibition. In nine tissues, at a time when the total transepithelial resistance had increased by 44 ± 15 (standard error) percent, the ratio of resistances did not change significantly from a mean value of 1.5 ± 0.2 (S.E.) (change, 0.04 ± 0.17). In contrast, steady-state values for the ratio of resistances have been reported as 1.4 to 1.7 under control conditions and 4.5 to 5.5 after amiloride inhibition (*3, 5, 6*). When impalements in four tissues were maintained for longer times, the ratio of resistances remained unchanged for 1 to 2 minutes after transport inhibition by amiloride, and only then changed to high values. In other tissues, a rapid increase in this ratio has been reported (*7, 12*), but such an increase does not preclude an increase in the resistance of the basolateral membrane.

Since the basolateral membrane in amphibian urinary bladder is mainly potassium-conductive (*2, 13*), and since inhibition of sodium transport by amiloride does not affect cell potassium content (*14*), it is probable that the increase in membrane resistance is due to a decrease in potassium conductance. We have tested this hypothesis by using changes in epithelial cell volume as an index of membrane permeability.

Cell volume was measured in excised frog urinary bladders (*15*) with a light microscopic–optical sectioning technique (*16*). We first studied the effect of alterations in serosal potassium concentration on cell volume regulation by the epithelial cells. When the solution bathing the basolateral surface is made hyposmotic, the cells rapidly swell, but then spontaneously regulate their volume despite the continued exposure to the hyposmotic bathing medium (*17*). Control volumes were regained within 2 to 3 minutes after osmotic swelling (Fig. 2). Mean rates of volume regulation are shown in Table 1. Diminishing the basolateral membrane potassium gradient by raising serosal potassium concentration from 2.5 to 25 m*M* decreased the rate of

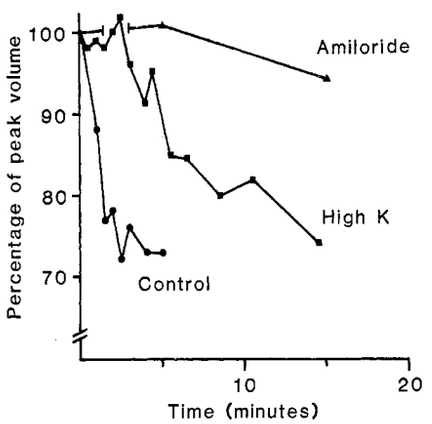

Fig. 2. Examples of the effects of a diminished basolateral potassium gradient or mucosal amiloride on the rate of cell volume regulation by frog urinary bladder epithelial cells. The data are plotted as percentages of the peak volume reached following osmotic swelling caused by the exposure of the tissue to a hyposmotic serosal Ringer (solution osmolality was reduced from 220 to 149 mosmole/kg through the removal of NaCl). The period required to reach the peak volume, equally rapid for all experiments, was 2 to 3 minutes. The cells reached a peak of 133 ± 1.5 percent of their original volume. Individual results from three cells representing different tissues are shown. *Control*, no additional treatment; *High K*, 25 m*M* potassium replacing an equal amount of sodium on the serosal side; *Amiloride*, mucosal Ringer containing $10^{-4}M$ amiloride to inhibit sodium transport. Rates of volume regulation (Table 1) for control and high-potassium experiments were calculated as the least-squares fit to the data lying between peak and control volumes. In the amiloride experiments there were no significant changes in volume.

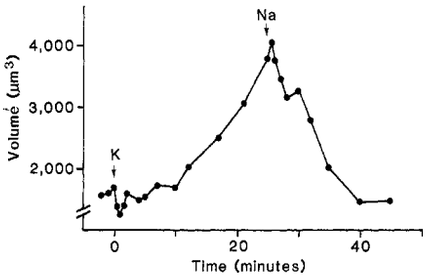

Fig. 3. The effect of replacing serosal sodium with potassium on the volume of a frog urinary bladder cell. At the first arrow, the serosal perfusate was switched from Ringer to one in which KCl replaced all NaCl. At the second arrow, the serosal perfusate was switched back to Ringer. The mean rates of swelling in Table 1 were calculated from a least-squares fit of the data to the swelling phase that follows the initial volume loss and recovery.

cell volume regulation (Fig. 2 and Table 1); this action had no effect on cell volume in the absence of an osmotic gradient. Therefore, volume regulation probably results from an increase in basolateral membrane potassium permeability and a net flux of potassium and water from the cell. Presumably, chloride is the major accompanying anion. The effect of amiloride on volume regulation was tested by adding the drug to the mucosal medium (final concentration, $10^{-4}M$) and measuring the volume response of the cells to a serosal hyposmotic gradient. Amiloride abolished volume regulation under these conditions, a result consistent with a marked inhibition of basolateral potassium permeability (Fig. 2).

In another approach, we examined the effects of amiloride on cell swelling induced by the isomolar replacement of sodium by potassium (Fig. 3). After the replacement of sodium, the cells initially shrink, recover their volume, and then swell at a constant rate. Table 1 gives the mean swelling rate under these conditions. Since an anion must accompany potassium during swelling, these results also indicate a significant anion (probably chloride) permeability. When the cells are exposed to amiloride on the mucosal side before replacement of serosal sodium with potassium, the rate of swelling is reduced to about one-fifth of its control value (Table 1). These results are again compatible with a reduction in basolateral membrane permeability to potassium during inhibition of sodium transport by amiloride.

A simple hypothesis explaining these data is that a single potassium pathway is responsible for both base-line potassium conductance and volume regulation and that the conductance of this pathway is modulated in some manner by sodium

transport. The electrophysiological results provide evidence that both apical and basolateral membrane conductance are coupled to sodium transport. Since replacement of serosal sodium with potassium causes membrane depolarization (*13*), potassium movement during isosmotic potassium-induced cell swelling is probably conductive. Thus, it is likely that the effects of amiloride on this process and on the electrophysiological properties of the basolateral membrane reflect a single pathway for potassium. Since amiloride also inhibits cell volume regulation, it is likely that the volume-regulatory potassium movements also occur through this pathway.

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References and Notes

1. Tight epithelia (urinary bladder, frog skin, renal distal tubule) have transepithelial resistances greater than 500 ohm-cm^2 and potentials greater than 30 mV; in leaky epithelia (gallbladder, proximal tubule), both values are considerably smaller.
2. V. Koefoed-Johnsen and H. H. Ussing, *Acta Physiol. Scand.* **43**, 298 (1958); D. E. Leb, T. Hoshiko, B. D. Lindley, *J. Gen. Physiol.* **48**, 527 (1965).
3. L. Reuss and A. L. Finn, *J. Gen. Physiol.* **64**, 1 (1974).
4. S. A. Lewis, D. C. Eaton, J. Diamond, *J. Membr. Biol.* **28**, 41 (1976); S. M. Thompson, Y. Suzuki, S. G. Schultz, in *Ion Transport in Epithelia*, S. G. Schultz, Ed. (Raven, New York, 1981), p. 47.
5. L. Reuss and A. L. Finn, *Biophys. J.* **15**, 71 (1975).
6. J. Narvarte and A. L. Finn, *J. Gen. Physiol.* **75**, 323 (1980).
7. E. Frömter and B. Gebler, *Pfluegers Arch.* **371**, 99 (1977).
8. W. Nagel and J. Crabbé, *ibid.* **385**, 181 (1980); J. Warncke and B. Lindemann, in *Physiology of Non-Excitable Cells*, J. Salánki, Ed. (Pergamon, New York, 1981), p. 129.
9. Urinary bladders from toads (*Bufo marinus*) were perfused with Ringer solution (composition in m*M*: NaCl, 109; KCl, 2.5; $NaHCO_3$, 2.4; and $CaCl_2$, 0.9; gassed with room air; *p*H about 8.4) on both sides. Electrodes contacted each chamber and cells were impaled with microelectrodes having tip resistances of 18 to 25 megohms and tip potentials of less than 8 mV (*3, 5, 6, 10*). For a discussion of the validity of microelectrode impalements in toad urinary bladder, see (*6*) and A. L. Finn, C. W. Davis, J. Narvarte, in *Ion Transport in Epithelia*, S. G. Schultz, Ed. (Raven, New York, 1981), p. 61.
10. C. W. Davis and A. L. Finn, *J. Gen. Physiol.*, in press.
11. From Kirchhoff's current law, the amount of current entering and exiting the cell must be equal. The ratio of the voltage deflections ($\Delta V_{mc}/\Delta V_{cs}$) across the membranes is therefore equal to the ratio of membrane resistances, even though the absolute current is not known.
12. S. G. Schultz, R. A. Frizzell, H. N. Nellans, *J. Membr. Biol.* **33**, 351 (1977).
13. H. S. Frazier and A. Leaf, *J. Gen. Physiol.* **46**, 491 (1963).
14. R. Rick, A. Dörge, E. von Arnim, K. Thurau, *J. Membr. Biol.* **39**, 313 (1978); W. Nagel, J. F. Garcia-Diaz, W. M. Armstrong, *ibid.* **61**, 127 (1981); B. A. Robinson and A. D. C. Macknight, *ibid.* **26**, 269 (1976).
15. Urinary bladders from bullfrogs rather than toads were selected for the cell volume study because of their superior optical qualities. The transepithelial electrical characteristics of the two tissues are similar (*2*), and preliminary intracellular electrophysiological measurements also indicate a similarity in cell membrane potentials, the ratio of membrane resistances, and in the effects of amiloride on these parameters: thus, $V_{mc} = 41 \pm 8$ mV; $V_{cs} = 49 \pm 13$; the ratio of apical to basolateral membrane resistance = 2.0 ± 0.8; and transepithelial resistance = 2382 ± 689 ohm-cm^2. After amiloride addition, when transepithelial resistance had increased by 61 ± 19 percent, the resistance ratio had changed by -0.18 ± 0.04 (five observations in three tissues).
16. K. R. Spring and A. Hope, *Science* **200**, 54 (1978); *J. Gen. Physiol.* **73**, 287 (1979); C. W. Davis and A. L. Finn, in *Membrane Biophysics: Structure and Function in Epithelia*, M. A. Dinno, Ed. (Liss, New York, 1981), p. 25. Briefly, cell volume was determined in urinary bladders from frogs (*Rana catesbaena*) mounted in a micro-Ussing chamber. The microscope was focused on the apical surface, and the focal plane was advanced in discrete 1-μm steps through the cell. Cross-sectional areas of these optical sections were obtained by tracing the cell perimeter from stored video images. Cell volume was calculated from the cross-sectional area of each section and the distance between sections.
17. For a review of cell volume regulation, see E. K. Hoffman, in *Transport of Ions and Water in Animals*, B. L. Gupta, R. B. Moreton, L. Oschman, B. J. Wall, Eds. (Pergamon, Oxford, 1977), p. 43.
18. H. H. Ussing and E. Windhager, *Acta Physiol. Scand.* **61**, 484 (1964).
19. Supported by grants AM17854 and AM25483 from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases.

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A New Tumor-Promoting Agent, Dihydroteleocidin B, Markedly Enhances Chemically Induced Malignant Cell Transformation

Abstract. *Teleocidin, which was isolated from mycelia of Streptomyces, is a potent tumor promoter in mouse skin. The catalytically hydrogenated compound dihydroteleocidin B markedly enhanced malignant cell transformation induced by 3-methylcholanthrene or ultraviolet radiation. Dihydroteleocidin B was at least 100 times more effective in enhancing transformation than 12-O-tetradecanoyl phorbol-13-acetate, the strongest promoter known until now, whereas both promoters showed equal capacities to induce early membrane effects and DNA synthesis.*

The induction of cancer in humans is thought to follow complex interactions between initiators and factors that influence tumor development (*1, 2*). Among such factors are tumor promoters, compounds that are weakly carcinogenic but which markedly enhance the yield of tumors when applied after a low dose of an initiating carcinogen (*2, 3*). In recent years the biological and biochemical effects of promoting agents have been examined extensively with various cell systems. However, the mechanism by which promoting agents convert initiated cells into visible tumors and their etiological significance are still not known.

We report here that dihydroteleocidin B (DHTB), a catalytically hydrogenated compound of teleocidin, which was isolated from mycelia of Streptomyces (*4, 5*), is unusually potent in enhancing 3-methylcholanthrene (MCA)–induced malignant transformation and in inducing morphological alterations in BALB 3T3 cells. DHTB is at least 100 times stronger in enhancing transformation than 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), the strongest promoting agent known until now. On the other hand, DHTB and TPA inhibited the binding of epidermal growth factor and [^{3}H]phorboldibutyrate to cell surface receptors, increased 2-deoxyglucose uptake, and stimulated DNA synthesis in G_0-arrested cells to the same extent, suggesting that the early induction of biochemical responses is not directly related to the enhancement of cell transformation.

Malignant transformation of A31-1-1 cells was assayed by scoring transformation foci as reported previously with BALB 3T3–A31 subclones (*6, 7*). Cells treated with a low dose of MCA (1 μg/ml) alone had very low transformation frequencies (5×10^{-6}). Subsequent treatment with as little as 0.1 ng of DHTB per milliliter for 2 weeks (beginning the fourth day after the removal of MCA) markedly increased the transformation frequency, which was proportional to the concentration of DHTB (Fig. 1). This treatment was found to be optimal for maximizing the transformation frequencies. At the concentrations used (0.1 to 10 ng/ml), DHTB had no cytotoxic effects on the MCA-treated or untreated cells, whether DHTB was added to the culture immediately after cell seeding, immediately after MCA treatment, or 4 days after MCA treatment. TPA increased transformation frequencies only at high concentrations (10 to 1000 ng/ml), as reported for the enhancement of MCA-induced transformation of 10T1/2 cells (*8, 9*). Thus, DHTB is at least 100 times as effective as TPA in enhancing MCA-induced transformation, as shown by comparing concentrations of promoting agents giving the same transformation in A31-1-1 cells. Dose-response tests with various doses of MCA and 10 ng of DHTB or TPA per milliliter indicated that DHTB permits the use of 100-fold lower initiating doses of MCA to achieve the same transformation frequency. Similar marked enhance-

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