ed with restriction endonuclease Sau 3AI, ligated to purified segments cleaved by Bam HI of coliphage Charon 30 (*2*), packaged in vitro (*3*), and used to infect *Escherichia coli* strain K802. The resulting plaques were screened for *T. pallidum* antigens by an in situ radioimmunoassay. Screening was done by a modification of the procedure of Towbin *et al.* (*4*). Nitrocellulose disks were placed over the phage plaques, and the disks were allowed to absorb protein for 10 to 30 minutes. Little protein was absorbed from the unlysed *E. coli* of the lawn. The nitrocellulose filters were then soaked for 10 minutes in 5 percent ovalbumin in 50 m*M* tris-HCl, *p*H 7.5, 150 m*M* NaCl, and 0.15 percent sodium azide (TSA–5 percent OA). The plaque blots were incubated overnight in either human secondary syphilitic serum or in normal human serum diluted 1:300 in TSA–1 percent OA, washed, exposed to ^{125}I-labeled *Staphylococcus aureus* protein A, and washed; autoradiographs were made as described (*4*). One plaque designated Tp3a, which gave a strong reaction with a secondary syphilitic serum (Fig. 1A), was chosen for further study. Phage from this plaque was diluted and replated on *E. coli* CSH 18. When screened with three different secondary syphilitic serums, all plaques showed radioactivity, whereas control plaques of the cloning vector, Charon 30, showed little or no reaction (data not shown). Secondary syphilitic serums reacted strongly with Tp3a plaques while serums from individuals without syphilis show no reaction with Tp3a plaques (Fig. 1B).

The polypeptide treponemal antigens of Tp3a were studied by electrophoretic transfer of total lysate proteins to nitrocellulose filters after sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (*4*) (Fig. 1C, lane 1). Tp3a encodes for at least seven clearly discernible polypeptides which have molecular weights of 46,000, 43,000, 38,000, 24,000, 23,000, 20,000, and 18,500 and react specifically with syphilitic serums. The molecular weights of these antigens correspond to those of antigenic proteins of *T. pallidum* (*5*). Identical or greater amounts of Charon 30 lysate proteins do not react with the syphilitic serums (Fig. 1C, lane 2). DNA isolated from Tp3a has been characterized by restriction endonuclease analysis (Fig. 2). Bacteriophage Tp3a contains a 16-kbp insert of *T. pallidum* DNA. The total molecular weight of the *T. pallidum* antigens is well within the coding capacity of this 16-kbp insert of cloned DNA.

There is a remote possibility that we have cloned a contaminating sequence of rabbit DNA because our original *T. pallidum* preparation was harvested from rabbit testicles. The treponemal origin of the Tp3a DNA was established on the basis of the antigenicity of its products with human syphilitic serums. Since secondary syphilitic serums do not react with protein blots of normal rabbit testicular tissue and normal rabbit serum (*5*), these serums were specific for detection of treponemal antigens. Further, two additional clones that produce Tp3a antigens were identified during a subsequent screening of 500 clones. Therefore, we have obtained three isolates having identical DNA sequences from a total of 750 clones, an indication that the genome size of the organism from which the DNA was cloned is the prokaryotic range of 10^8 to 10^9 daltons (*6*).

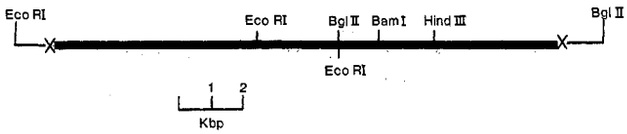

Fig. 2. Partial restriction endonuclease map of the *T. pallidum* DNA of clone Tp3a. The 16-kbp insert is circumscribed by Sau 3AI or Bam HI sites at the insert-vector junctions, indicated by X. The outer Eco RI site on the left is on the long Bam HI arm of Charon 30; the outermost Bgl II site on the right is on the short arm of Charon 30.

We expect that these and other cloned *T. pallidum* antigens will be the basis for new investigations of pathogenesis and immunity in experimental and human syphilis. In addition, antigens cloned in this manner should permit the development of treponemal tests exhibiting greater specificity than those presently in use. Hanff and Lovett have described the IgG (immunoglobulin G) antibody response of humans and rabbits to individual polypeptides of *T. pallidum* (*5*), and serums analyzed have included those obtained from patients and rabbits with immunity to reinfection. With unlimited amounts of cloned key treponemal antigens, it should be possible to duplicate through experimental vaccination the pattern of antibody to treponemal polypeptides seen in these immune states and test whether this antibody affords protection against the disease.

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Myoglobin Function in Exercising Skeletal Muscle

Abstract. *Short-term perfusion of the isolated dog gastrocnemius-plantaris muscle with hydrogen peroxide resulted in a decrease in steady-state muscle oxygen consumption and isometric tension generation. Hydrogen peroxide converted intracellular myoglobin to products incapable of combination with oxygen, but had no deleterious effect on neuromuscular transmission or on mitochondrial oxidative phosphorylation. It is concluded that functional intracellular myoglobin is important in maintaining oxygen consumption and tension generation in exercising skeletal muscle.*

Myoglobin is present in skeletal and cardiac muscle of mammals and birds in concentrations ranging from 0.05 to 5 mmole/kg and in the muscles and nerves of certain invertebrates (*1*). Notwithstanding its ubiquity, evidence is lacking with respect to its function. In the presence of an oxygen concentration gradient, myoglobin facilitates the transport of oxygen through solutions in physical systems (*1, 2*). DeKoning *et al.* (*3*) found that the flux of oxygen through sections of chicken gizzard was increased when myoglobin was present in its functional form. Wittenberg *et al.* (*4*) determined that chemical treatment of intracellular myoglobin that rendered it unreactive with oxygen resulted in a decrease in the steady-state respiration of pigeon breast muscle fiber bundles under conditions of hypoxia. In the isolated perfused heart, however, similar chemical treatments failed to produce this effect (*5, 6*). I report here that myoglobin is important

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Table 1. Comparison of control and hydrogen peroxide–treated groups.

Group	Resting QO_2 (μl/min-g)	Muscle weight (g)	PO_2 (mmHg)	PCO_2 (mmHg)	pH	Hematocrit (% by volume)	Mean arterial pressure (mmHg)
Control (N = 5)							
Before perfusion	3.3 ± 0.7		75 ± 10	51 ± 5	7.33 ± 0.04	44.6 ± 4.8	112 ± 19
After perfusion		60.5 ± 18.8	75 ± 8	47 ± 8	7.35 ± 0.05	42.7 ± 6.3	112 ± 17
Hydrogen peroxide (N = 7)							
Before perfusion	4.8 ± 2.1		77 ± 7	47 ± 8	7.34 ± 0.05	45.9 ± 9.6	103 ± 25
After perfusion		44.2 ± 8.1	77 ± 12	45 ± 10	7.35 ± 0.06	42.9 ± 9.7	94 ± 23

in the maintenance of both oxygen consumption and tension generation in exercising skeletal muscle.

Mongrel dogs (N = 12) were anesthetized with intravenous pentobarbital (30 mg/kg) and intubated, and then spontaneously breathed room air for the experiments. By using the technique of Stainsby and co-workers (*7, 8*), the arterial and venous blood supply of the gastrocnemius-plantaris muscle was isolated and the distal tendon was cut and attached to a strain gauge. Heparin (2200 U/kg) was administered and catheters placed for arterial and venous blood sampling and arterial pressure measurement. The muscle was stretched to optimal length (maximal twitch tension) and subjected to supramaximal 0.3-msec twitch stimuli at 3 Hz through the cut sciatic nerve. At this stimulation frequency, muscle oxygen consumption and isometric twitch tension amplitude can be sustained for more than 60 minutes without loss of function.

After 30 minutes of stimulation, the blood supply of five control dogs was interrupted, and the resting muscle was perfused with 400 ml of Ringer lactate solution containing 5 mM glucose and 30 mM $NaHCO_3$ and equilibrated with 14 percent O_2 and 6 percent CO_2 at 37°C. A second group of seven dogs received the modified Ringer lactate solution with 0.35 mM hydrogen peroxide added to render the muscle myoglobin nonfunctional. At this concentration, intracellular myoglobin was invariably converted from the ferrous form to higher oxidation states incapable of reversible combination with oxygen. The conversion was assessed by color change and has been analyzed spectroscopically in muscle in previous studies (*4–6, 9*). Small volumes of Ringer lactate were administered immediately before and after the solution containing hydrogen peroxide to prevent direct contact between hydrogen peroxide and blood. Perfusion pressure was kept below 100 mmHg during perfusate administration. The perfusate infusion rate averaged 0.74 ± 0.06 ml/min-g (mean ± standard deviation) in the control group and 0.76 ± 0.42 ml/min-g in the hydrogen peroxide–treated group. The hydrogen peroxide infusion rate was 300 ± 110 nmole/min-g. After administration of the perfusate solution, blood flow to the muscle was reestablished and 3-Hz twitch stimulation continued for an additional 30 minutes.

Twitch tension amplitude, muscle blood flow, venous and arterial blood O_2 content (Lex-O_2-Con TL, Lexington Instruments), arterial blood O_2 partial pressure (PO_2), CO_2 partial pressure (PCO_2), pH, hematocrit, and mean arterial pressure were measured at 10-minute intervals before and after perfusate administration. Steady-state muscle oxygen consumption was calculated from the product of muscle blood flow and difference in arterial and venous O_2 content. The animal was then killed and the muscle weight and color of cut sections of the muscle were compared with those of the contralateral unstimulated muscle. Data were compared by applying Student's unpaired t-test.

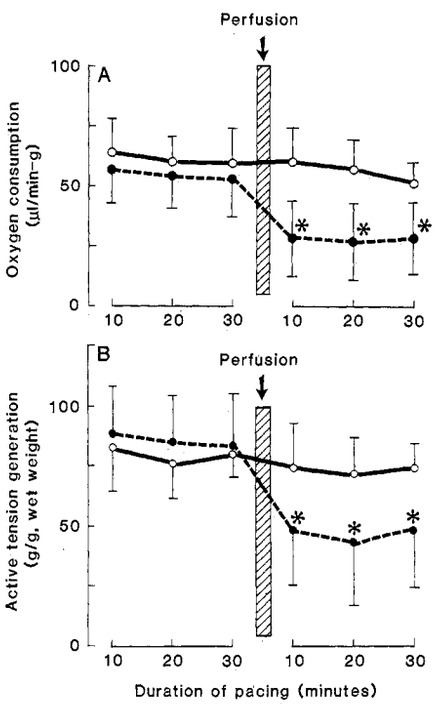

Fig. 1. (A) Relation of muscle oxygen consumption to duration of stimulation before and after perfusate administration. Means and standard deviations are shown; (○) control group (N = 5); (●) hydrogen peroxide group (N = 7); (*) significant at $P < .05$. In the hydrogen peroxide group, but not in the control group, muscle oxygen consumption decreased significantly. (B) Relation of muscle twitch tension generation to duration of stimulation before and after perfusate administration. Means and standard deviations are shown; symbols as above. In the hydrogen peroxide group, but not in the control group, muscle active tension generation decreased significantly.

The control and hydrogen peroxide–treated groups were similar before and after infusion with respect to arterial blood PO_2, pH, PCO_2, hematocrit, mean arterial pressure, muscle weight, and prestimulation resting muscle oxygen consumption (QO_2) (Table 1). Muscle oxygen consumption in the control group was unaffected by the Ringer's lactate solution, while oxygen consumption in the hydrogen peroxide–treated group decreased to 65 percent of the comparable preinfusion value after 30 minutes (Fig. 1A). Similarly, twitch tension amplitude remained unchanged in the control group but decreased to 54 percent of the preinfusion value at 30 minutes in the hydrogen peroxide–treated group (Fig. 1B). Thus, in the isometrically contracting dog gastrocnemius muscle, using oxygen at a rate approximately 15 times the resting value, loss of functional myoglobin was associated with reductions in both steady-state muscle oxygen consumption and twitch tension amplitude.

Hydrogen peroxide perfusion in the test group resulted in oxidation of intracellular myoglobin. Additional studies were performed to evaluate the specificity of this effect. First, a similar isolated gastrocnemius preparation was subjected to supramaximal high-frequency (20 to 150 Hz) stimulation through the sciatic nerve before and after administration of 0.35 mM hydrogen peroxide. Both the level of tetanic tension and the stimulation frequency at which sustained tetanic tension generation decreased were unchanged by hydrogen peroxide treatment. This showed that the hydrogen

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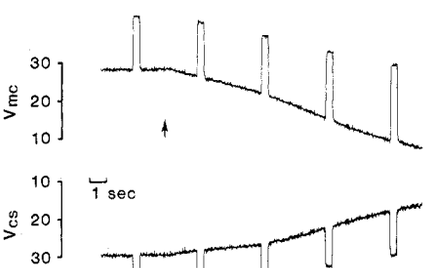
Sodium Transport Inhibition by Amiloride Reduces Basolateral Membrane Potassium Conductance in Tight Epithelia

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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