

Coupling of active ion transport and aerobic respiratory rate in isolated renal tubules

(active transport/mitochondrial nicotinamide adenine dinucleotide/adenosine triphosphate/cytoplasmic signal)

ROBERT S. BALABAN*, LAZARO J. MANDEL, STEPHEN P. SOLTOFF, AND JANET M. STOREY†

Department of Physiology, Duke University Medical Center, Durham, North Carolina 27710

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ABSTRACT We report the results of studies in which the cytoplasmic coupling between Na^+, K^+ -ATPase activity (presumably a measure of active transport) and the mitochondrial respiratory rate was investigated in a tubule suspension from the rabbit kidney cortex. Simultaneous measurements of the redox state of mitochondrial nicotinamide adenine dinucleotide (NAD) (performed fluorometrically), the cellular ATP and ADP concentrations, and the oxygen consumption rate (Q_{O_2}) were made under conditions known to alter the Na^+, K^+ -ATPase turnover. Ouabain (25 μM) caused: (i) a 54% inhibition of Q_{O_2} , (ii) a net reduction of NAD, and (iii) a 30% increase in the ATP/ADP ratio. The addition of K^+ (5 mM) to K^+ -depleted tubules caused: (i) an initial 127% stimulation of Q_{O_2} followed by a new steady-state Q_{O_2} 50% above control, (ii) an initial large oxidation of NAD followed by a new steady state more oxidized than the control level, and (iii) a 47% decrease in the cellular ATP/ADP ratio. These data indicate that the cellular ATP and ADP concentrations or the ATP/ADP ratio may be part of the coupling mechanism linking Na^+, K^+ -ATPase turnover and the aerobic metabolic rate in kidney.

A basic question in cellular physiology concerns the mechanism whereby the rate of aerobic conversion of energy is coupled to the rate of active ion transport. A linear relationship between the rate of oxygen consumption and active sodium transport has been observed in numerous epithelia such as frog skin (1), toad bladder (2), and kidney (3, 4), indicating a close link between these two processes. Because Na^+, K^+ -ATPase appears to be either directly or indirectly involved in most active sodium transport processes (5, 6), it is generally accepted that ATP is the most likely energy source for sodium transport. However, it is still unclear how changes in transport rate elicit corresponding alterations in aerobic respiration. Attempts to answer this question have focused on the role of the cellular concentrations of ATP, ADP, and orthophosphate (P_i), or a combination of these variables to express a phosphate potential (7, 8) or energy charge (9), in controlling the mitochondrial respiratory rate. In theory, an increase in active ion transport would cause an increase in the rate of ATP hydrolysis, which would be expected to elicit a decrease in the cellular concentration of ATP and an increase in the concentration of the ATP hydrolysis products, ADP and P_i . In a model suggested by Whittam and coworkers (10, 11), such an increase in ADP and P_i concentrations could serve as a cytoplasmic feedback signal resulting in an acceleration of the mitochondrial respiratory rate, as described for isolated mitochondria by Chance and Williams (12).

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The same feedback signal operating in reverse would be expected to reduce the respiratory rate when the active transport rate decreased.

Different methods for determining whether this type of model is applicable to sodium-transporting tissue have produced conflicting results. Attempts to demonstrate changes in the cellular ATP and ADP concentrations in response to perturbations of active transport rate have failed in the amphibian urinary bladder (13) and in mammalian whole kidney (14) and kidney slices (15, 16).

In contrast, noninvasive microfluorometric monitoring of the redox state of mitochondrial NAD in the bullfrog kidney (17, 18), rat kidney (19), and avian salt gland (20) have demonstrated that the appropriate mitochondrial "state" transitions, as described by Chance and Williams (12), do occur in response to changes in active ion transport. That is, inhibition of active cellular transport caused the reduction of NAD, consistent with a mitochondrial transition to a less active or resting state, which would be expected to be accompanied by an increase in cellular ATP concentration and decrease in cellular ADP and P_i concentrations. Conversely, stimulation of active transport caused the oxidation of NAD, consistent with a mitochondrial transition from a less to a more active state, a decrease in cellular ATP concentration, and an increase in cellular ADP and P_i concentrations. Although these measurements are sensitive and reliable due to their noninvasive nature, they present only indirect evidence favoring the Whittam model, because other intermediates between active transport and the mitochondria could account for the observed state transitions.

We present here experiments performed on an isolated cortical tubule suspension from the rabbit (21), which is ideally suited to test directly the role of ATP and ADP concentrations and the ATP/ADP ratio in the coupling of cellular respiration to cellular ion transport. This preparation contains little glomerular contamination, is not oxygen deficient, and appears to perform transepithelial transport, as indicated by open tubular lumina and the 70% maximal inhibition of oxygen consumption by ouabain. These characteristics of the kidney tubule preparation overcome many of the problems of previous preparations used to study the relationship between active transport and metabolism. The initiation of these experiments was also aided by the development of an optical chamber that allowed the simultaneous monitoring of oxygen consumption, mitochondrial redox state (optically), and extracellular ion

* To whom reprint requests should be addressed at present address: University of Oxford, Nuffield Department of Clinical Biochemistry, Radcliffe Infirmary, Oxford OX2 6HE, England.

† Present address: Department of Biology, Carleton University, Ottawa, Ontario, Canada K1S 5B6.

concentration and allowed rapid ejection of sample aliquots for biochemical analysis (21).

MATERIALS AND METHODS

The preparation of the rabbit kidney cortical tubule suspension and the description of the chamber used for simultaneously obtaining tissue samples and monitoring oxygen consumption and the redox state of NAD (fluorometrically) have been published (21).

Fluorometric Measurements. The basic methods used for the fluorometric measurements have been described in detail (22, 23). Briefly, the incident light on the chamber was obtained from a sharply filtered (366-nm peak) mercury arc lamp. Light was collected from the opposite side of the chamber containing the cell suspension by a $\times 3.8$ microscope objective focused near the center of the suspension. This objective collected both transmitted and emitted light. The collected light was focused on a beam splitter, where 95% of the light was transmitted to a photomultiplier tube equipped with a filter transmitting 450-nm light. This photomultiplier tube, therefore, recorded the fluorescent emission from reduced NAD (NADH). The other 5% of the excitation and emitted light was diverted through a filter (366-nm peak transmittance) to a photomultiplier tube that recorded only the transmitted excitation light. The excitation light signal was subtracted electronically from the fluorescence signal to compensate for changes in the intensity of the arc lamp and for changes in scattering of the preparation, and to indicate movement artifacts (22). A net reduction of mitochondrial NAD (i.e., an increase in the ratio of reduced to oxidized NAD) corresponds to an increase in 450-nm fluorescence, whereas a net oxidation of NAD (i.e., a decrease in the ratio of reduced to oxidized NAD) causes a decrease in 450-nm fluorescence. Signals are reported as percent change from the baseline tissue fluorescence. The qualitative changes in fluorescence that occurred in response to each experimental perturbation were extremely reproducible. No attempt was made to quantitate the ratio of reduced to oxidized NAD fluorometrically in these experiments because the maximally oxidized NAD level is not presently obtainable in this preparation without optical artifacts and, also, because of the variable fluorescence quenching of different tubule suspension concentrations.

Determination of ATP, ADP, and Protein. Tubule suspension samples were ejected from the chamber in precise 0.4-ml volumes at appropriate times into equal volumes of ice-cold 6% perchloric acid/1 mM EDTA and were mixed by using a Vortex mixer.

ATP and ADP were determined with the fluorometric enzymatic assay of Lowry and Passonneau (24). Protein content of each sample was determined (21) by the methods of Lowry *et al.* (25). Two or three samples were obtained under each experimental condition and their values were averaged to obtain a single determination.

Initial experiments attempting to measure intracellular P_i failed because the tubules were found to be unstable in phosphate-free medium. Therefore, no determinations of P_i were made in these studies.

The standard incubation medium for these experiments had the following composition (in mM): NaCl (115), NaHCO_3 (25), NaH_2PO_4 (4), CaCl_2 (2.3), KCl (5), MgSO_4 (0.1), glucose (5), lactate (4), alanine (1), and 40,000 M_r dextran (6%). The solution osmolarity was adjusted to 295 milliosmolar. The suspension was bubbled with 95% O_2 /5% CO_2 to maintain the pH at 7.4. All experiments were performed at 37°C after equilibrating the cells for 45 min at this temperature. The pre-equilibration time was selected from time course studies which revealed that

this time interval was sufficient to stabilize the ATP per mg of protein values after the transition from the temporary storage temperature (5°C) to the experimental temperature (37°C). In some experiments the tubules were pre-equilibrated in K^+ -free medium in which K^+ was replaced by Na^+ in the standard medium. Wherever possible the data are expressed as the mean \pm SEM.

RESULTS

The stability of the tubule suspension over the usual 5- to 10-min experimental time course (after the 45-min pre-equilibration) was tested in initial experiments. An example of such an experiment is shown in Fig. 1. All of the three measured variables—NADH fluorescence, oxygen consumption, and the ATP/ADP ratio—remained constant in these experiments to an oxygen partial pressure, P_{O_2} , of less than 10 mm Hg (1 mm Hg = 133 Pa). Anoxia ensued below this P_{O_2} , causing a large increase in NADH fluorescence while oxygen consumption and the ATP/ADP ratio decreased dramatically. Similar results were obtained in K^+ -depleted tubules (see below). Arrows on the NADH fluorescence trace of Fig. 1 and subsequent figures indicate the time at which tissue samples were ejected from the chamber for biochemical determinations. Notice that no optical or oxygen electrode artifacts were elicited by the sampling process.

Effects of Na^+, K^+ -ATPase Inhibition. Ouabain was used to probe the effects that Na^+, K^+ -ATPase inhibition (i.e., inhibition of active transport) had on the measured metabolic parameters of the tubule suspension. Ouabain was previously found to inhibit as much as 70% of the oxygen consumption (Q_{O_2}) in this preparation, with half-maximal inhibition at 8.4 μM (21). A representative experiment is shown in Fig. 2, demonstrating that addition of 25 μM ouabain inhibited oxygen consumption by 51%, increased the ATP/ADP ratio by 44%, and simultaneously increased NADH fluorescence by 40%. Subsequent anoxia abolished oxygen consumption, greatly lowered the ATP/ADP ratio, and caused another large increase

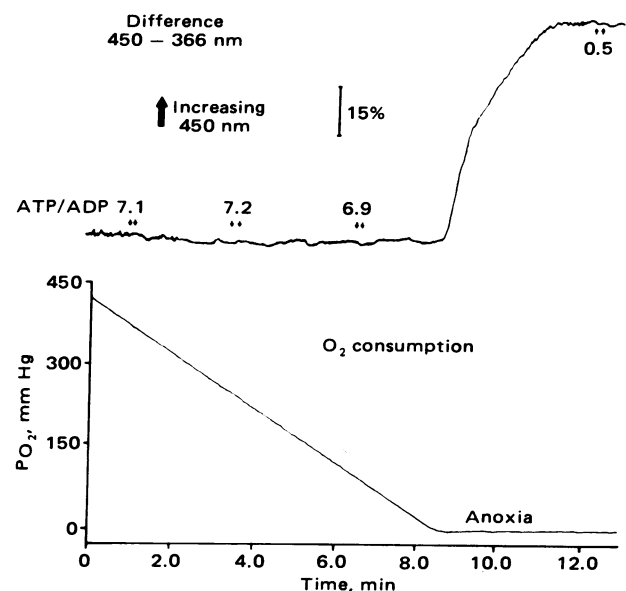


FIG. 1. Stability of NADH fluorescence, oxygen consumption (Q_{O_2}), and the ATP/ADP ratio as a function of time and bath P_{O_2} in a kidney cortical tubule suspension from the rabbit. The Q_{O_2} of this preparation was 38.4 nmol/mg protein per min. Arrows in this figure and all subsequent figures indicate the time at which tissue samples were ejected from the chamber. The ATP/ADP values indicated are the means of the two samples obtained at each point.

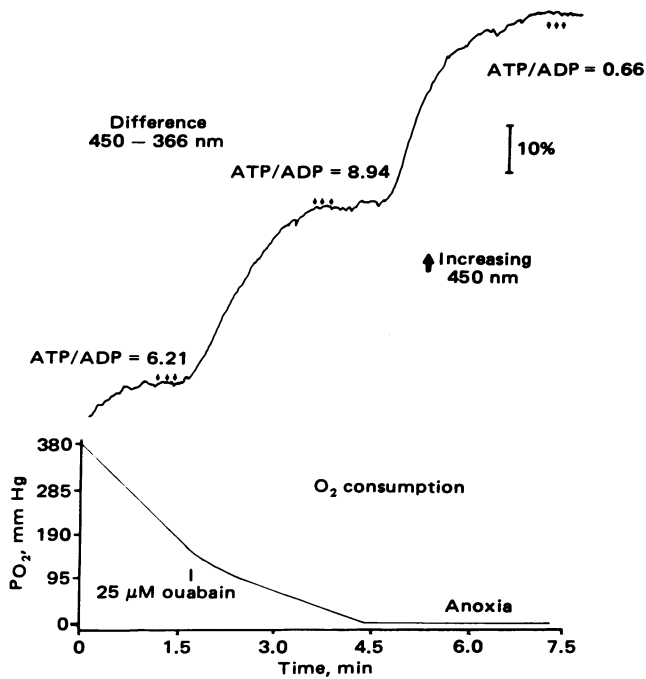


FIG. 2. Effect of 25 μM ouabain on NADH fluorescence, oxygen consumption, and the ATP/ADP ratio of a rabbit kidney cortical tubule suspension. The Q_{O_2} was 40 nmol/mg of protein per min during the control period and 19.6 nmol/mg of protein per min after ouabain addition. The ATP/ADP ratio values indicated are the means of the three samples obtained at each point.

in NADH fluorescence. The results from nine similar experiments are averaged in Table 1. Addition of ouabain (25 μM) increased cellular ATP and decreased ADP to elicit an average increase in the ATP/ADP ratio of 30%. The other effects of 25 μM ouabain were a 54% decrease in oxygen consumption and a 30–40% increase in NADH fluorescence. The effect of ouabain on NADH fluorescence was completely inhibited by allowing the suspension to become anoxic before the addition of ouabain.

Addition of the nonfluorescent mitochondrial uncoupling agent 1799 (DuPont) (1 μM) to five suspensions caused the oxidation of NAD and the stimulation of oxygen consumption (110 \pm 8.4%), both of which are characteristic effects of a mitochondrial uncoupling agent (Fig. 3). The presence of 1799 also completely inhibited the effects of ouabain on oxygen consumption and NADH fluorescence.

Effects of Increasing Na^+ , K^+ -ATPase Activity. Na^+ , K^+ -ATPase activity (i.e., active transport) was stimulated by adding 5 mM KCl to K^+ -depleted tubules. The K^+ -depleted tubules were obtained by washing the suspension three times in K^+ -free Ringer's solution and pre-equilibrating the tubules in the

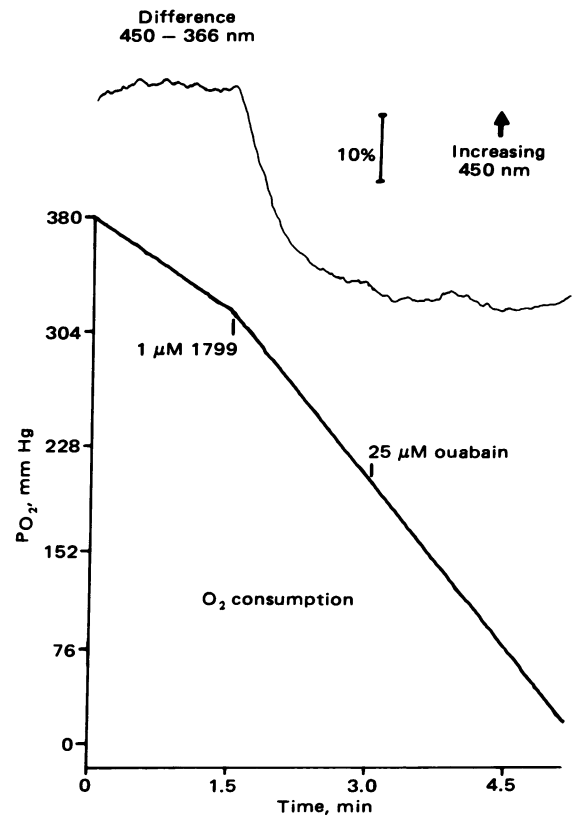


FIG. 3. Effect of the uncoupler 1799 (1 μM) on NADH fluorescence and oxygen consumption of a rabbit tubule suspension. The Q_{O_2} was 34.2 nmol/mg of protein per min during the control period and 67.1 nmol/mg of protein per min after the addition of 1799. The subsequent addition of 25 μM ouabain had no effect on either NADH fluorescence or Q_{O_2} .

K^+ -free medium for 45 min. The extracellular K^+ concentration after the pre-equilibration period was determined to be approximately 0.3 mM by a K^+ -sensitive electrode.

Illustrated in Fig. 4 is a representative experiment showing the effects of K^+ addition on the NADH fluorescence, oxygen consumption rate, and the ATP/ADP ratio of K^+ -depleted tubules. Two phases of response are seen in the fluorescence and Q_{O_2} traces. Oxygen consumption was initially stimulated by 127% and thereafter achieved a new steady-state level 50% higher than control. The NADH fluorescence also followed a biphasic pattern, initially decreasing dramatically to a minimum and returning thereafter to a new steady-state below the initial fluorescence level. As the arrows in Fig. 4 indicate, the tissue samples after K^+ addition were obtained during the interval of maximal stimulation of oxygen consumption and near the minimum in the fluorescence response. In this particular

Table 1. Effects of ouabain (25 μM) on various metabolic parameters of the cortical tubule suspension

Experimental condition	ATP, nmol/mg protein	ADP, nmol/mg protein	ATP/ADP	Q_{O_2} , nmol/mg protein per min	NADH fluorescence, % Δ
Control	6.5 (\pm 0.3)	1.3 (\pm 0.1)	5.8 (\pm 0.8)	35.0 (\pm 8.4)	
Ouabain	6.8 (\pm 0.24)	1.0 (\pm 0.1)	7.4 (\pm 0.9)	18.1 (\pm 4.3)	
Paired % Δ^*	+6.4 (\pm 2.0)	-19.2 (\pm 3.4)	+29.8 (\pm 5.0)	-54.2 (\pm 1.5)	+30 to +40
P % $\Delta = 0$	<0.02	<0.001	<0.001	<0.001	

* This represents the mean (\pm SEM) of the % change observed in the individual experiments after ouabain exposure ($n = 9$).

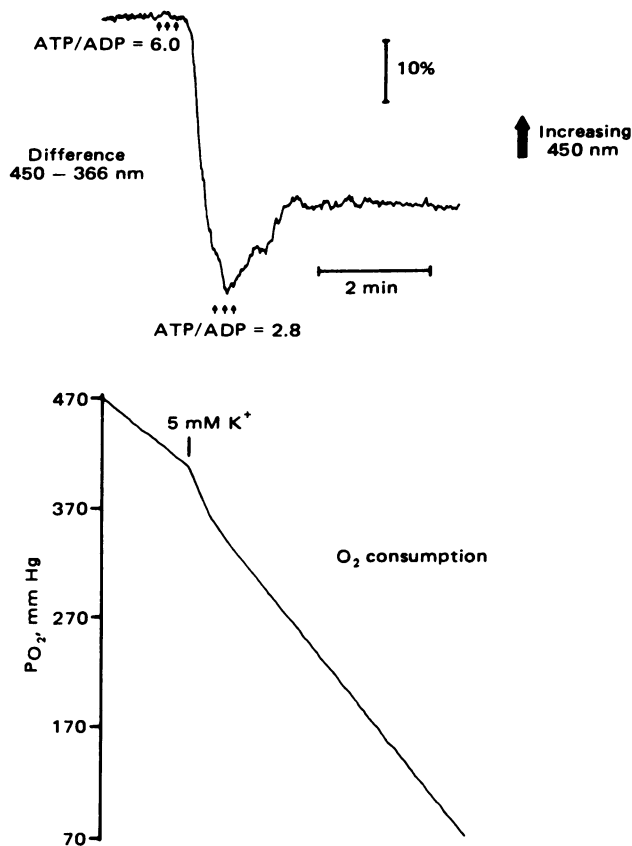


FIG. 4. Effect of 5 mM K⁺ on NADH fluorescence, oxygen consumption, and the ATP/ADP ratio in a K⁺-depleted rabbit cortical tubule suspension. The Q_{O₂} during the control period was 9.4 nmol/mg of protein per min, 23.2 nmol/mg of protein per min during the initial period after the addition of K⁺, and 14.9 nmol/mg of protein per min during the steady state after this initial rapid increase in Q_{O₂}. The ATP/ADP values indicated are the means of three samples obtained at each point.

experiment the fluorescence decreased 43% at the minimum and the ATP/ADP ratio decreased 53% upon K⁺ addition.

The averages of eight such experiments are shown in Table 2. Addition of 5 mM K⁺ to the K⁺-free medium is seen to decrease cellular ATP and increase ADP to result in a 47% reduction of the ATP/ADP ratio. The Q_{O₂} increased greater than 2-fold and NAD became oxidized by 35–45% at the minimum fluorescence.

These effects of K⁺ addition require a functioning Na⁺,K⁺-ATPase and coupled respiring mitochondria. Prior addition of

ouabain, anoxia, or the presence of the uncoupler 1799 abolished the redox and oxygen consumption responses to the added K⁺.

Experiments with an extracellular K⁺-sensitive electrode have shown that the addition of K⁺ to these K⁺-depleted tubules causes a K⁺ uptake by the cells that is ouabain sensitive, is dependent on aerobic metabolism, and exhibits an initial rapid uptake phase followed by a slower rate of K⁺ uptake (unpublished data).

DISCUSSION

These results demonstrate that the cellular ATP and ADP concentrations change as a function of active transport rate in an epithelial tissue. As such, the results strongly suggest that the intracellular adenosine phosphates or the ATP/ADP ratio is part of the feedback signal linking active transport and oxidative metabolism, as suggested by Whittam and coworkers (10, 11). The observed changes in the other measured metabolic parameters as a function of active transport rate are also consistent with this feedback model.

The cortical tubule suspension proved to be an ideal tissue for these studies for the following reasons: (i) The major function of the renal cortex is active transport, which is supported by a large aerobic metabolic rate (3, 4). (ii) The tubular preparation used consisted almost entirely of viable epithelial cells (21). (iii) The use of a homogenous preparation allowed sampling under control and experimental conditions using *internal* controls from the same suspension.

A decrease in Na⁺,K⁺-ATPase activity, elicited by ouabain, was associated with a decrease in oxygen consumption, as previously reported (21), and a net reduction of mitochondrial NAD. This suggests that the tubular mitochondria experienced an active-to-resting transition or, more precisely, an active-to-less-active transition. As previously mentioned, similar transitions have also been observed in the isolated perfused bullfrog kidney (17, 18) and rat kidney (19) in response to ouabain. The demonstration that cellular ATP increases and ADP decreases, resulting in a 30% increase for the ATP/ADP ratio under these conditions, strongly suggests that these intracellular changes are part of the signal causing the mitochondrial transitions. These types of transitions, as a function of the extra mitochondrial ADP or the ATP/ADP ratio, have been amply documented in isolated mitochondria (12).

Conversely, stimulation of Na⁺,K⁺-ATPase activity by the introduction of K⁺ to tubules pre-equilibrated in K⁺-free medium was associated with a two-phased increase in the respiratory rate: an initial rapid respiration (127% stimulation), followed by a new steady state 50% above that of control. This respiratory rate behavior was accompanied by a large rapid net oxidation of mitochondrial NAD followed by a new steady state

Table 2. Effects of addition of 5 mM K⁺ on various metabolic parameters of the cortical tubule suspension pre-equilibrated in K⁺-free medium

Experimental condition	ATP, nmol/mg protein	ADP, nmol/mg protein	ATP/ADP	Q _{O₂} (rapid phase), nmol/mg protein per min	NADH fluorescence, % Δ
K ⁺ -free	4.9 (±0.4)	0.84 (±0.07)	6.4 (±1.0)	8.2 (±0.8)	
5 mM K	3.9 (±0.1)	1.30 (±0.08)	3.1 (±0.2)	18.9 (±1.4)	
Paired % Δ*	-22.4 (±3.5)	+50.0 (±8.0)	-46.6 (±4.6)	+129.7 (±8.7)	-35 to -45
P % Δ = 0	<0.001	<0.001	<0.001	<0.001	

* This represents the mean (±SEM) of the % change observed in the individual experiments after addition of K⁺ (n = 7).

that was more oxidized than control. These responses would be expected from a resting-to-active transition in the cellular mitochondria. Again, on the basis of previous work on isolated mitochondria (12), the signal causing this state transition is likely to be the measured decrease in the cellular ATP/ADP ratio.

The transient nature of the oxygen consumption and NAD redox state responses upon the introduction of K^+ is probably due to an initial burst of Na^+, K^+ -ATPase activity stimulated by the addition of extracellular K^+ and the high cellular Na^+ concentration reached after the 45-min incubation in K^+ -free medium (26). Thus, it appears that the Na^+, K^+ -ATPase is initially strongly stimulated until the intracellular Na^+ concentration is reduced and a new steady state is established.

A comparison between Table 1 and Table 2 indicates that the ATP level was lower in the K^+ -depleted cells than in control cells. In addition, the oxygen consumption after the reintroduction of K^+ was also considerably lower than the control oxygen consumption shown in Table 1. It is likely that this difference is due to the presence of damaged cells in the "stressful" K^+ -depleted state. To investigate this possibility, we analyzed the supernatant obtained after the centrifugation (50 \times g, 5 min) of tubule suspensions (after a 45-min incubation at 37°C) for ATP, ADP, and protein content under control and K^+ -free medium conditions. A significant amount of ADP (2.2 nmol/ml of supernatant) and protein (1.2 mg/ml of supernatant) was found in the supernatant from the K^+ -depleted tubules; no ATP was detectable. In the supernatant from normal tubules there was no detectable ATP or ADP, but a small amount of protein (0.4 mg/ml of supernatant). These findings suggest that some cell lysis occurs in the K^+ -depleted state, which may depress the ATP and oxygen consumption per mg of protein as well as the ATP/ADP values under these conditions. However, due to the internal controls used in this study, the observed changes obtained upon the reintroduction of K^+ are valid measures of the metabolic transitions occurring in the tubules.

By using the uncoupler 1799, further evidence was found that cellular ATP and ADP concentrations are involved in the cytoplasmic signal that couples the mitochondrial redox state and respiratory rate with Na^+, K^+ -ATPase activity. The 1799 should uncouple the influence of ATP and ADP on the mitochondrial redox state and respiratory rate. When 1799 was added prior to the modification of Na^+, K^+ -ATPase activity, no effect of this modification was observed on the mitochondrial redox state or on the respiratory rate.

In conclusion, these findings in functioning isolated kidney tubules provide direct evidence supporting the notion that the cytoplasmic signal between Na^+, K^+ -ATPase activity and the

aerobic conversion of energy involves the cellular concentrations of ATP and ADP.

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