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The Signal Transduction Function for Oxidative Phosphorylation Is at Least Second Order in ADP*  

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To maintain ATP constant in the cell, mitochondria must sense cellular ATP utilization and transduce this demand to F$_{2}-$F$_{3}$-ATPase. In spite of a considerable research effort over the past three decades, no combination of signal(s) and kinetic function has emerged with the power to explain ATP homeostasis in all mammalian cells. We studied this signal transduction problem in intact human muscle using $^{31}$P NMR spectroscopy. We find that the apparent kinetic order of the transduction function of the signal cytosolic ADP concentration ([ADP]) is at least second order and not first order as has been assumed. We show that amplified mitochondrial sensitivity to cytosolic [ADP] harmonizes with in vitro kinetics of [ADP] stimulation of respiration and explains ATP homeostasis also in mouse liver and canine heart. This result may well be generalizable to all mammalian cells.

Prior work considered that mitochondria behave as a transducer with approximately first order response characteristics (1–4). This means that the response of mitochondrial oxidative phosphorylation (MOP) to a stimulus would follow an approximately hyperbolic relation according to a Michaelis-Menten kinetics of ADP-mediated signal transduction (1, 4). This was unfortunate, because earlier work on isolated mitochondria had shown that the response of MOP to changes in [ADP] is not hyperbolic (5, 6). Therefore, it remains possible that a higher order kinetic function for extramitochondrial [ADP] stimulation of MOP is responsible for the maintenance of energy balance in the mammalian cell.

Here, we studied cytosolic [ADP] transduction in an intact cellular system. We used a general and unbiased analysis to test the apparent kinetic order of the transduction function. The generality of the in vivo result is tested against published kinetics of ADP stimulation of MOP in various other systems, and its implications for understanding the biochemistry of mitochondrial and the integrative physiology of mitochondrial function in the cell are discussed.

**MATERIALS AND METHODS**

$^{31}$P NMR Spectroscopy of Intact Muscle—Phosphocreatine (PCr), P$_{i}$, and ATP $^{31}$P NMR resonances in well perfused human forearm flexor muscle of six consenting, healthy adult volunteers (five males and one female; age, 28–55 years) were measured using high time resolution (7 s) $^{31}$P NMR spectroscopy, and data acquisition and analysis methods developed in this laboratory (7, 8). $^{31}$P NMR spectra were collected using a CSI spectrometer operating at 2 tesla (General Electric). Different energy balance states were imposed by transcutaneous nerve stimulation (electric pulse duration, 0.2 ms; amplitude, 50–300 V), which resulted in recruitment of all motor units in the muscle (7). Average PCr, P$_{i}$, and ATP levels and intracellular pH (pH$_{i}$) in muscle fibers during 6 min of twitch contractions were studied over a 2-Hz range of twitch frequencies (0–2.2 Hz).

Total cytosolic ATP hydrolysis flux and glyco(gen)lytic ATP synthesis flux (in mmol ATP liter$^{-1}$ s$^{-1}$) were calculated at each twitch frequency from the measured time course of PCr and pH$_{i}$ during twitch contractions (8). MOP flux at steady state, $v_{MOP}$(in mmol ATP liter$^{-1}$ s$^{-1}$), was calculated as the difference between these fluxes. The concentrations of PCr, P$_{i}$, ADP, and pH$_{i}$ at each steady state were calculated assuming concentrations of ATP and total creatine of 8.2 and 42.7 mm, respectively, and creatine kinase near equilibrium (8). The molar free energy of cytosolic ATP hydrolysis was calculated according to $\Delta G_{o}$ = $\Delta G_{o}^{\circ}$ + RT $\ln$[ADP]/[ATP], assuming $\Delta G_{o}^{\circ}$ is $\sim$32.8 kJ/mol at 37 °C (9).

Analysis of Kinetics of [ADP] Stimulation of MOP—The kinetics of [ADP] stimulation of MOP in skeletal muscle in situ were analyzed by nonlinear curve fitting using Fig.P software (Elsiever Biosoft). A modified (sigmoidal) Hill function (10) of the form

$$y = \frac{(Max - Min) \cdot (x_{0.5})^{n}}{(1 + (x_{0.5})^{n})} + Min$$

(Eq. 1)

was used to analyze the apparent order of the kinetic function corresponding to the value of the Hill coefficient, $n_{H}$ (11). The parameters Max and Min are the y-asymptotes of the function, and $x_{0.5}$ is the x value corresponding to half-maximal y (the inflection point).

For comparison, the kinetics of [ADP] stimulation of MOP reported for isolated mitochondria at constant, high [P] (5, 6, 12, 13), and in situ canine cardiac muscle (14) and ex situ transgenic mouse liver (15) were analyzed analogously by curve-fitting analysis of the specific velocity function $v^{\prime}(S^{\ast})$ (11). Data points were obtained by graphical extraction in all cases except (14) and transformed to $v^{\prime}(S^{\ast})$ format, where specific velocity $v^{\prime} = v$/$V_{max}$ and specific substrate concentration $S^{\ast} = S$/$[S]_{0.5}$ were obtained from the experimental maximal velocity $V_{max}$ and $[S]_{0.5}$ given in each study. Data on the kinetics of [ADP] stimulation of MOP during pacing and isotropic stimulation for in vivo canine heart muscle (14) were obtained from Tables 2 and 4 in Ref. 14 and transformed to $v^{\prime}(S^{\ast})$ format using a maximal oxygen consumption of in vivo dog heart of 0.45 ml/min/g (16) and 0.074 mm for [ADP]$_{0.5}$ corresponding to the [ADP] in cardiac muscle at half this rate (14).
Results
Dynamic Range of Energy Balance in Skeletal Muscle—The studied range of energy balance states in forearm muscle included the maximal sustainable steady state of energy balance in all subjects as judged from the physiological responses. Typically a maximum occurred at a twitch frequency of 1.6 Hz; higher twitch frequencies resulted in acidosis (8). The calculated rate of contraction coupled ATP hydrolysis in forearm flexor muscle cells at this state was 0.25 ± 0.01 mmol ATP liter$^{-1}$ s$^{-1}$ (mean ± S.E., n = 6). The matching ATP synthesis flux was mostly mitochondrial (maximally 0.22 mmol ATP liter$^{-1}$ s$^{-1}$). Figure 1) supplemented by a small glycolytic ATP synthesis flux. The measured extent of steady state changes in average PCr content and pH$_i$ in muscle fibers over this range of energy balance states corresponded to approximately 4–5-fold increases of the calculated average [ADP] and [Pi] in muscle fibers during contraction (for ADP from 0.018 to 0.084 mM (Fig. 1) and for Pi from 3.5 to 21 mM, respectively). The calculated molar cytosolic free energy of ATP hydrolysis, $\Delta G_p$, decreased from approximately −64 to −54 kJ/mol ATP over this range of energy balance states. The calculated MOP flux ($J_{p\text{MOP}}$) increased approximately 28-fold (from 0.008 to 0.22 mmol ATP liter$^{-1}$ s$^{-1}$) (Fig. 1). This flux was kinetically limited by [ADP] rather than [Pi] in view of the cytosolic concentration ranges of both substrates and the affinity of the ADP and Pi translocators in the inner mitochondrial membrane (the adenine nucleotide translocator (ANT) and the phosphate carrier (18), respectively).

Analysis of Kinetics of [ADP] Stimulation of MOP in Other Systems—To test the generality of this in vivo result, we also analyzed the in vitro kinetics of [ADP] stimulation of MOP reported for isolated mitochondria (5, 6, 12, 13) (Fig. 2B, inset). Likewise, stimulation of MOP by [ADP] reported in these in vitro studies required in each case a Hill coefficient significantly greater than 1 to explain the kinetics. The range of $n_H$ values was 2.1–2.9 (2.6 ± 0.2, mean ± S.E., n = 4) and not significantly different from the value we obtained for mitochondria studied in situ in skeletal muscle. This result was surprising because the description of approximately first order control characteristics of extramitochondrial [ADP] (2, 3) had been formulated based on just these studies (12).

To next test if this apparent kinetic order (i.e., between 2 and 3) for transduction of cytosolic [ADP] to intramitochondrial F$_1$-ATPase explains the coactivation of cytosolic [ADP] and MOP flux also in other mammalian cell types, we analyzed the reported kinetics of [ADP] stimulation of MOP in intact cardiac muscle (14) and liver (15) cells pooled with skeletal muscle data (Fig. 3). The covariation of [ADP] and the rate of MOP in the pooled data from all three cell types was adequately explained by a transduction function for cytosolic [ADP] with $n_H = 2.2 ± 0.4$ (± S.E. of regression, $r^2 = 0.73$).

Discussion
The main result and novel finding of this study is that the kinetic function for [ADP] stimulation of MOP in skeletal muscle is approximately a second order function of the form $p_{\text{MOP}} = f(\text{[ADP]}^{n_H})$ where $n_H = 2$ and not 1 as has hereto been assumed (1–4). This implies that the scaled sensitivity of mitochondria to variations in cytosolic [ADP] is at least 1 order of magnitude greater than has been assumed. This result impacts the understanding of the biochemistry of mitochondria and its integration in the physiology of mammalian cells.
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Fig. 2. A, flow-force relation of MOP in forearm flexor muscle. The solid line represents the fit of Equation 1 to the covariation of the free energy of ATP hydrolysis, \( \Delta G_p \) (in kJ/mole), and \( J_{P}^{MOP} \) (in mmol ATP liter\(^{-1}\) s\(^{-1}\)) in contracting muscle (pooled data, \( n = 6 \)). Regression equation: \( y = 0.26 \times (0.58)^{10} / \left( 1 + (0.58)^{10} \right) - 0.03 \) (\( r^2 = 0.93 \)). Inset, extrapolation of the flow-force relation over an expanded range of free energy values of -6.7 to -42.5 kJ/mole. B, stimulation of mitochondrial ATP synthesis, \( J_{P}^{MOP} \) (in mmol ATP liter\(^{-1}\) s\(^{-1}\)), by cytosolic [ADP] (in mM) in contracting muscle cells. The solid line represents the fit of Equation 1 to the data with the asymptotes constrained to the values obtained from the flow-force relation in A. Regression equation: \( y = 0.26 \times (0.043)^{10} / \left( 1 + (0.043)^{10} \right) - 0.03 \) (\( r^2 = 0.93 \)). Inset, dependence of specific velocity \( v' \) of MOP (in V\(_{MAX}\)) on specific ADP concentration ([ADP]/[ADP]\(_{0.5}\)) in the suspension medium of isolated mitochondria at constant, high [P] (10 mM); ○, rat liver mitochondria (12); □, beef heart mitochondria (13); ■, rat germ cell mitochondria (5). Data from a fourth study (6) were omitted for clarity of presentation. The solid line represents the fit of Equation 1 in reduced form with \( V_{MAX} = [S]_{0.5} = 1, \text{ Min/Max} = 0 \), to the “classic” Chance and Williams data (12). Regression equation: \( y = x^{0.52} / \left( 1 + x^{0.52} \right) \) (\( r^2 = 0.99 \)). The dashed line shows the general inconsistency of the covariation of \( v' \) and [S] predicted by a hyperbolic relation with the experimental data. A formatted data set of specific velocity \( 0.26 \) (solid line) represents the fit of Equation 1 in reduced form to the pooled data with \( \text{Max} = x_{0.5} = 1 \). Regression equation: \( y = x^{2} / \left( 1 + x^{2} \right) - 0.16 \) (\( r^2 = 0.73 \)). The dashed line represents an arbitrary Hill relation with \( n_H = 3.5 \), illustrating that such kinetic order would likewise fit the cardiae muscle data.

**Apparent Kinetic Order of [ADP] Transduction: Analysis**—The crucial piece of information in the analysis of the apparent kinetic order of the transduction function of cytosolic [ADP] to the mitochondrial matrix was knowledge of the maximal and minimal sustainable MOP fluxes in the muscle cells. Dense sampling of the full physiological domain of the ([ADP], \( J_{P}^{MOP} \)) relation in itself did not allow for discriminating between first or second order (or higher order, for that matter) of the transduction function (Fig. 1). We obtained estimates of the flux asymptotes from analysis of the thermodynamic flow-force relation of MOP (Fig. 2A). This is a well established and valid description of the relation between the flux through a reaction and the concentration of its substrates and products (19, 20).

The flow-force relation predicts that under the condition of constant sum of substrate and product concentrations, the flux (or flow) \( J \) through a reaction varies in sigmoidal fashion with the thermodynamic driving force \( \Delta G \) between maximal forward and reverse rates (19, 20). It was previously shown that this description applies to MOP in muscle (21).

Of utmost importance to the analysis, this approach is by definition unbiased toward the specific mechanism of a reaction (20). The only prior knowledge about the nature of the flow-force relation of MOP in muscle that was used in the analysis was that this relation is innately sigmoidal (19, 20). The curve fitting of a four parameter sigmoidal function (Equation 1) to the \( \Delta G_p, J_{P}^{MOP} \) covariation was performed fully unconstrained. The performance of the curve fitting of this function was enhanced by its symmetrical properties.

The fitted estimate of the minimal flux (\(-0.03 \) mmol ATP liter\(^{-1}\) s\(^{-1}\)) predicted that there would be net ATP hydrolysis by the mitochondrial ATPase over a nonphysiological range of [ADP] in muscle (i.e. \( 0 < [ADP] < 13 \) mM) if such conditions were to be achieved experimentally in intact cells. This is not unprecedented. Net ATP hydrolysis has been demonstrated in intact isolated mitochondria (20, 22, 23) and significant ATP hydrolysis flux even at maximal net synthetic flux (22, 23), which is entirely consistent with this relation. The flow-force relation predicted net ATP synthesis by MOP over the entire physiological range of [ADP] and \([P]_p \) in muscle corresponding to a \( \Delta G_p \) range of approximately -64 to -54 kJ/mole. Of course, this was fully expected and consistent with mitochondrial function as the primary source of ATP in the eukaryotic cell. On basis of these considerations, we conclude that the estimates of the flux asymptotes from the analysis of the flow-force relation provided a sound basis for analysis of the apparent kinetic order of cytosolic [ADP] transduction in muscle in situ.

**Higher Order of [ADP] Transduction: Implications**—The result that the apparent kinetic order of cytosolic [ADP] transduction is at least 1 order of magnitude higher than has hereto been assumed is dramatic and impacts both the understanding of the biochemistry of mitochondria and integrative physiology of mitochondrial function in the cell. According to the formalism proposed by Koshland et al. (24), a Hill coefficient greater than 1 implies amplified sensitivity of mitochondria to variations in cytosolic [ADP]. Sensitivity amplification of enzymes and entire metabolic pathways may be achieved by any of a number of kinetic mechanisms but not a Michaelis-Menten
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mechanism (24). Therefore a fundamentally different molecular transduction mechanism for cytosolic ADP must now be considered.

There is considerable in vitro experimental evidence for allosteric instead of Michaelis-Menten kinetics of adenine nucleotide translocation (13, 25–27). Allosterism of the translocator ANT could be the mechanistic basis for ultrasensitivity of mitochondrial to cytosolic [ADP] (24). First of all, our analysis showed that ultrasensitivity of isolated mitochondria to ADP under physiologically comparable conditions of limiting [ADP] and saturating [P$_i$] is abolished upon bypassing the enzyme-catalyzed translocation of ADP, ATP, and P$_i$. We analyzed the published kinetics of [ADP] stimulation of MOP for intact versus digitonin-treated mitochondria (13) and found Hill coefficients of 2.9 ± 0.61, and 1.2 ± 0.05, respectively (± S.E. from regression). MOP flux in each preparation could be 100 and 33% inhibited by ANT inhibition, respectively (13), suggesting regression. MOP flux in each preparation could be 100 and coefficients of 2.9 ± 0.61, and 1.2 ± 0.05, respectively (± S.E. from regression). MOP flux in each preparation could be 100 and 33% inhibited by ANT inhibition, respectively (13), suggesting regression. MOP flux in each preparation could be 100 and

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2 $Ca^{2+}$ can play a role in altering the absolute MOP flux to match ATP utilization flux via “feed forward” (32) modulation of the absolute value of $\nu_T^m$ (or “gain” of MOP (32)) and $[ADP]^n_p$ (or “operating point” of MOP (32)). Such effects are normalized and thus implicit in the reduced transduction function $\nu^+(\theta)$. The magnitude of these effects is, however, not constant but subject to specific conditions such as substrate selection (1).