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Communication

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_0-F_1-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)[1] to a stimulus would follow an approximately hyperbolic relation according to a Michaelis-Menten mechanism for the signal transduction (2, 3). With this understanding, the hypothesis that mitochondria detect variations in ATP utilization simply by sensing the variation in cytosolic [ADP] (2, 3) had to be discarded as a general mechanism after studies of the *in situ* dog heart showed 2-fold increases in MOP flux without much change in [ADP] (4). These observations led to consideration of alternative signals but not alternative kinetic functions 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 mitochondria 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 supramaximal percutaneous nerve stimulation (electric pulse duration, 0.2 ms; amplitude, 250–300 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(geno)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, J_p^{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 $\Delta G_p = \Delta G_p^{o'} + RT \ln([ADP][P_i]/[ATP])$, assuming $\Delta G_p^{o'}$ is −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 (Elsevier Biosoft). A modified (sigmoidal) Hill function (10) of the form

$$y = (Max - Min) \cdot (x/x_{0.5})^{n_H}/(1 + (x/x_{0.5})^{n_H}) + Min \qquad \text{(Eq. 1)}$$

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 at half-maximal *y* (the inflection point).

For comparison, the kinetics of [ADP] stimulation of MOP reported for isolated mitochondria at constant, high $[P_i]$ (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 $v'([S]^*)$ (11). Data points were obtained by graphical extraction in all cases except (14) and transformed to $v'([S]^*)$ format, where specific velocity $v' = v/V_{max}$ and specific substrate concentration $[S]^* = [S]/[S]_{0.5}$ 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 inotropic stimulation for *in vivo* canine heart muscle (14) were obtained from Tables 2 and 4 in Ref. 14 and transformed to $v'([S]^*)$ 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).

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[1] The abbreviations used are: MOP, mitochondrial oxidative phosphorylation; PCr, phosphocreatine; ANT, adenine nucleotide translocator.

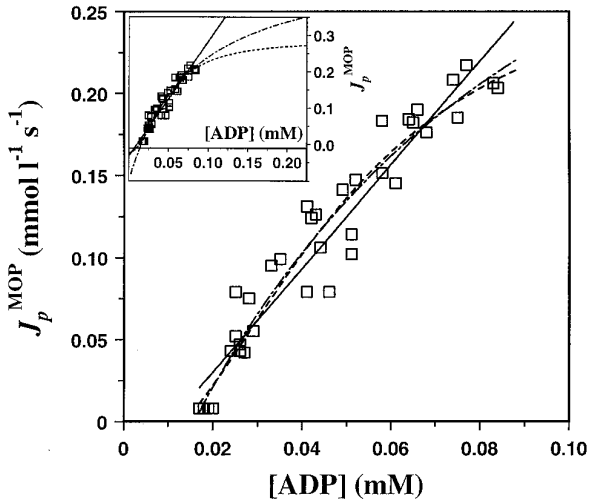

FIG. 1. **Stimulation of mitochondrial ATP synthesis flux, J_p^{MOP} (in mmol ATP liter^{-1} s^{-1}), by increases in the average ADP concentration (in mM) in forearm muscle cells during contraction (pooled data, $n = 6$).** Variables were calculated from ^{31}P NMR spectroscopic data as described elsewhere (8). Over the experimentally achievable range of energy balance states in the muscle, the covariation of [ADP] and J_p^{MOP} was equally well predicted by any of three relations: (i) $y = 3.15 \cdot x - 0.03$ (*solid line*, $r^2 = 0.91$); (ii) $y = 0.51 \cdot (x/0.079)/(1 + (x/0.079)) - 0.10$ (*dashed and dotted line*, $r^2 = 0.93$); (iii) $y = 0.29 \cdot (x/0.048)^2/(1 + (x/0.048)^2) - 0.02$ (*dashed line*, $r^2 = 0.93$). *Inset*, extrapolated covariation of [ADP] and J_p^{MOP} as predicted by each of the three fitted functions over a (nonphysiological) [ADP] range of 0–0.225 mM.

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}, Fig. 1) supplemented by a small glyco(geno)lytic 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 [P_i] in muscle fibers during contraction (for ADP from 0.018 to 0.084 mM (Fig. 1) and for P_i from 3.5 to 21 mM, respectively). The calculated molar cytosolic free energy of ATP hydrolysis, ΔG_p, decreased from approximately −64 to −54 kJ/mol ATP over this range of energy balance states. The calculated MOP flux (J_p^{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 [P_i] in view of the cytosolic concentration ranges of both substrates and the affinity of the ADP and P_i translocators in the inner mitochondrial membrane (the adenine nucleotide translocator (ANT) (17) and the phosphate carrier (18), respectively).

Analysis of Kinetics of [ADP] Stimulation of MOP in Skeletal Muscle—Deduction of the apparent order of the kinetic function for ADP stimulation of MOP from these experimental data requires analysis of the scaled rather than the absolute sensitivity of MOP to cytosolic [ADP] (11), *i.e.* the percentage of change in flux, scaled to the maximal flux, in response to a percentage of change in stimulus. This crucial point is illustrated in Fig. 1, which shows that both first and second order functions statistically fit the covariation ([ADP], J_p^{MOP}) equally well over the experimentally accessible range but extrapolate to widely different flux asymptotes (Fig. 1, *inset*). Thus, analysis of the scaled MOP sensitivity to [ADP] required knowledge of the *in vivo* maximal and minimal MOP fluxes in the muscle cells. These could not be robustly determined experimentally because energy balance steady states outside the sampled physiological range of the ([ADP], J_p^{MOP}) covariation did not exist. One possible approach to estimate the flux asymptotes, curve-fitting of an *ad hoc* kinetic function to the data (3), would bias the analysis. We used an alternative approach to estimate the MOP flux asymptotes in the muscle cells that was not biased toward kinetic mechanism; we analyzed the thermodynamic flow-force relation of MOP in the muscle cells (19, 20). Equation 1 was fitted without any constraints to the covariation (ΔG_p, J_p^{MOP}) (Fig. 2*A*). The fitted maximal and minimal flux were 0.26 ± 0.06 and −0.03 ± 0.02 mmol ATP liter^{-1} s^{-1}, respectively (± S.E. from regression; $r^2 = 0.91$) (Fig. 2*A*, *inset*). This maximum implied that mitochondria in the muscle were stimulated up to 85% of maximal ATP synthesis flux over the full range of sustainable energy balance states (Fig. 2*A*). The inflection point of the sigmoidal function, $(\Delta G_p)_{0.5}$, was −58 ± 1.6 kJ/mol (± S.E. from regression).

The apparent order, n_H, of the kinetic function for cytosolic [ADP] stimulation of MOP could now be determined by curve-fitting of Equation 1 to the ([ADP], J_p^{MOP}) data using these values for the flux asymptotes *Max* and *Min* (Fig. 2*B*). The fitted estimate for n_H was 2.11 ± 0.14 (± S.E. from regression; $r^2 = 0.93$). Clearly, this result was not compatible with the predicted value ($n_H = 1$) in the generally accepted formalism of Chance (1–4). The fitted estimate for $[ADP]_{0.5}$ was 44 ± 1 μM, which was approximately equal to half the full range of steady state cytosolic ADP concentrations in the muscle cells (Figs. 1 and 2*B*).

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. 2*B*, *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 covariation 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 $J_p^{MOP} = f([ADP]^{n_H})$ where $n_H \geq 2$ 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.

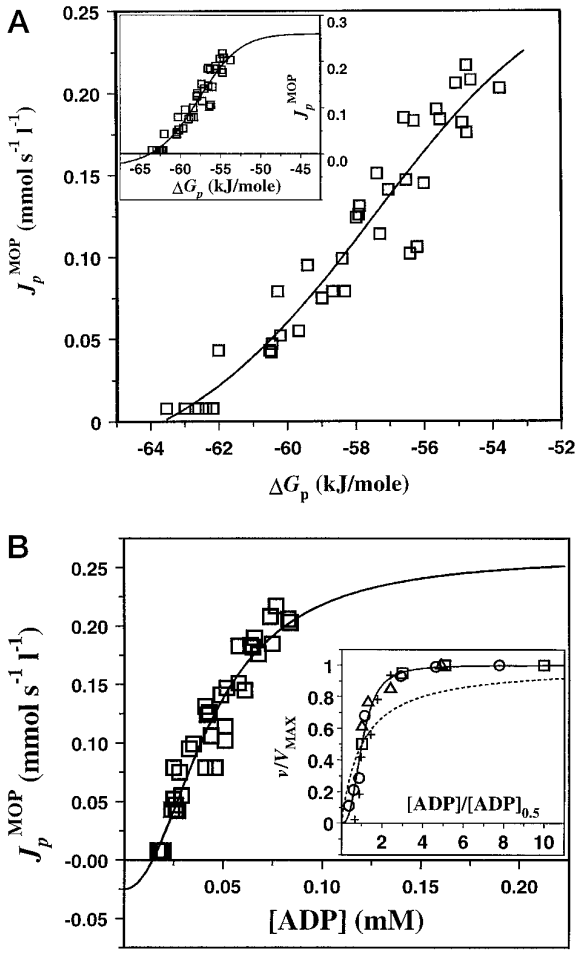

FIG. 2. *A*, flow-force relation of MOP in forearm flexor muscle. The *solid line* represents the fit of Equation 1 to the covariation of the free energy of ATP hydrolysis, ΔG_p (in kJ/mol), and J_p^{MOP} (in mmol ATP liter^{-1} s^{-1}) in contracting muscle (pooled data, $n = 6$). Regression equation: $y = 0.26 \cdot (x/58.1)^{10.3}/(1 + (x/58.1)^{10.3}) - 0.03$ ($r^2 = 0.93$). *Inset*, extrapolation of the flow-force relation over an expanded range of free energy values of −67.5 to −42.5 kJ/mol. *B*, stimulation of mitochondrial ATP synthesis, J_p^{MOP} (in mmol ATP liters^{-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: $y = 0.26 \cdot (x/0.044)^{2.1}/(1 + (x/0.044)^{2.1}) - 0.03$ ($r^2 = 0.93$). *Inset*, dependence of specific velocity v' of MOP (v/V_{max}) on specific ADP concentration [ADP]* ([ADP]/[ADP]$_{0.5}$) in the suspension medium of isolated mitochondria at constant, high [P_i] (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 $V_{max} = [S]_{0.5} = 1$, *Min*/*Max* = 0, to the "classic" Chance and Williams data (12). Regression equation: $y = x^{2.8}/(1 + x^{2.8})$ (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 skeletal muscle (+, present study) was superimposed to illustrate its consistency with isolated mitochondria 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. 2*A*). This is a well established and valid

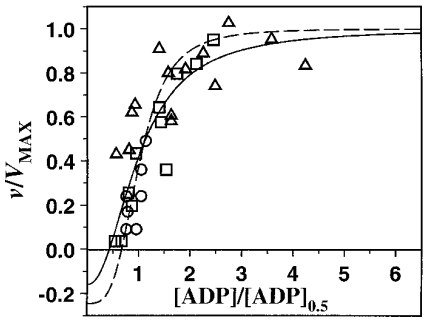

FIG. 3. **Dependence of specific velocity of MOP (v/V_{max}) on specific ADP concentration ([ADP]/[ADP]$_{0.5}$) in different mammalian cell types (□, *in vivo* human skeletal muscle (present study); ○, *in vivo* canine heart muscle (14); △, *ex situ* perfused transgenic mouse liver (15)).** The *solid line* represents the two parameter fit of Equation 1 in reduced form to the pooled data with *Max* = $x_{0.5} = 1$. Regression equation: $y = x^{2.2}/(1 + x^{2.2}) - 0.16$ ($r^2 = 0.73$). The *dashed line* represents an arbitrary Hill relation with $n_H = 3.5$, illustrating that such kinetic order would likewise fit the cardiac muscle data.

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 ΔG 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 kinetic 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 (ΔG_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 μM) 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_i] in muscle corresponding to a ΔG_p range of approximately −64 to −54 kJ/mol. 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

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). Allosteroism of the translocator ANT could be the mechanistic basis for ultrasensitivity of mitochondria 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 that in the latter case cytosolic ADP now had direct access to F_1-ATPase in a major fraction of the preparation. This suggests that the origin of the ultrasensitivity is at the level of the translocation step in MOP, and not the phosphorylation step. Second, Sluse-Goffart *et al.* (27) found second order rate dependence of the ADP-ADP homoexchange on extramitochondrial [ADP] over a wide range of concentrations. Other evidence from studies of the ANT suggested positive cooperativity of adenine nucleotide exchange across the intramitochondrial membrane (25, 26) that would result in allosteroism of the ANT (25, 26) not Michaelis-Menten behavior as was originally proposed (28, 29). There is no such evidence for the P_i carrier (18).

The apparent kinetic order of ADP transduction, n_H, may well be different (but ≥2) for different cell types (*e.g.* skeletal *versus* cardiac muscle cells (Fig. 3)). The mechanistic basis for these possible differences is that specific conditions that affect ANT function such as membrane potential and phospholipid composition (30) may well differ between mitochondria in different cell types. Therefore, although the mechanistic value of n (*i.e.* the number of strongly cooperative binding sites on the enzyme (11)) for ANT in mitochondria in different cell types may be the same, the kinetic, apparent n value, n_H (*i.e.* the actual operation of the translocation) could differ. It is n_H and not n that is measured in kinetic studies.

The new understanding of mitochondrial detection of variations in cytosolic [ADP] proposed in the present study integrates mitochondrial biochemistry into the physiology of mammalian cells. Second or greater instead of first order of the kinetic function for cytosolic [ADP] transduction has broad explanatory power with respect to ATP homeostasis in intact cells (Fig. 3). Energy balance in skeletal and cardiac muscle and liver cells was sufficiently explained by one and the same kinetic function $J_p^{MOP} = f([ADP]^{n_H})$ with $n_H = 2.2$ (Fig. 3). Importantly, explicit consideration of proposed [Ca^{2+}] effects on MOP flux (31) was not required in any of these cell types to explain the energy balance. This implies that [Ca^{2+}] may not be a necessary signal by which cellular ATP utilization flux is transduced to the mitochondria, contrary to what has been proposed (1, 31).[2] This conclusion fits the hypothesis that mitochondria detect variations in cellular ATP utilization during work via reciprocal changes in cytosolic [ADP], a biochemical concept originally proposed by Chance (2). What is a fundamentally novel insight is that the apparent kinetic order of the transduction function of this signal is at least 1 order of magnitude higher than was proposed (2).

Based on current knowledge, it now appears that two amplification mechanisms effectuate ATP homeostasis in the cell: magnitude and sensitivity amplification (24). The first mechanism has been early recognized (1, 31, 33) and involves increases in *absolute* MOP flux (*e.g.* mitochondrial density) to match absolute cellular capacity for ATP utilization flux. The second and newly recognized mechanism is the here described sensitivity amplification by which the *relative* ATP synthesis flux is matched to ATP utilization flux. This mechanism operates independent of mitochondrial density, but the amplification factor may likewise be cell type-specific because of particular conditions that affect the apparent kinetic order of ANT operation. The new challenge in understanding the integration of mitochondrial biochemistry into mammalian cell physiology will be to test this hypothesis.

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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 V_{max} (or "gain" of MOP (32)) and $[ADP]_{0.5}$ (or "operating point" of MOP (32)). Such effects are normalized and thus implicit in the reduced transduction function $v'([S]^*)$. The magnitude of these effects is, however, not constant but subject to specific conditions such as substrate selection (1).