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Review

The regulation of OXPHOS by *extra*mitochondrial calcium

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ABSTRACT

Despite extensive research, the regulation of mitochondrial function is still not understood completely. Ample evidence shows that cytosolic Ca^{2+} has a strategic task in co-ordinating the cellular work load and the regeneration of ATP by mitochondria. Currently, the paradigmatic view is that Ca^{2+}_{cyt} taken up by the Ca^{2+} uniporter activates the matrix enzymes pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and isocitrate dehydrogenase. However, we have recently found that Ca^{2+} regulates the glutamate-dependent state 3 respiration by the supply of glutamate to mitochondria via aralar, a mitochondrial glutamate/aspartate carrier. Since this activation is not affected by ruthenium red, glutamate transport into mitochondria is controlled exclusively by *extra*mitochondrial Ca^{2+}. Therefore, this discovery shows that besides *intra*mitochondrial also *extra*mitochondrial Ca^{2+} regulates oxidative phosphorylation. This new mechanism acts as a mitochondrial "gas pedal", supplying the OXPHOS with substrate on demand. These results are in line with recent findings of Satrustegui and Palmieri showing that aralar as part of the malate–aspartate shuttle is involved in the Ca^{2+}-dependent transport of reducing hydrogen equivalents (from NADH) into mitochondria. This review summarises results and evidence as well as hypothetical interpretations of data supporting the view that at the surface of mitochondria different regulatory Ca^{2+}-binding sites exist and can contribute to cellular energy homeostasis. Moreover, on the basis of our own data, we propose that these surface Ca^{2+}-binding sites may act as targets for neurotoxic proteins such as mutated huntingtin and others. The binding of these proteins to Ca^{2+}-binding sites can impair the regulation by Ca^{2+}, causing energetic depression and neurodegeneration.

1. Introduction: the regulation of OXPHOS

Oxidative phosphorylation is the main process responsible for ATP production and NADH reoxidation. The central mechanism for oxidative ATP synthesis has been known since Mitchell published his chemiosmotic theory [1]. The demand for ATP varies with changes in cellular functions, such as protein biosynthesis, ion transport, secretion, proliferation, differentiation, and contractile work. At present, the mechanisms controlling the balance between production and utilisation of ATP are still poorly understood. Chance and Williams assumed on the basis of experiments with isolated mitochondria that ADP formed by ATP-consuming enzymes represents a feedback signal, which regulates OXPHOS and the rate of ATP synthesis [2,3].

This view has been supported by the inverse correlation of mitochondrial activity with cytosolic phosphorylation potential observed in experiments with mitochondria isolated from several sources [4] and predicted by computer simulations that consider the increased ADP and P_i levels to be direct activators of OXPHOS [5].

However, the results of *in vivo* studies based on ^{31}P-NMR spectroscopy do not fit into the concept of regulation of OXPHOS simply by cytosolic fluctuations in ADP and Pi [6,7,9,10]. These studies have shown that, whereas in contracting white skeletal muscle the phosphorylation potential decreases with increasing work load [7–9], the cardiac muscle is characterised by metabolic stability, as cytosolic

Abbreviations: AK, adenylate kinase; AOA, aminooxyacetate; CAG, DNA code for glutamine; CK, creatine kinase; COX, cytochrome-c-oxidase; CsA, cyclosporin A; DHAP, dihydroxyacetone phosphate; EGTA, ethylene glycol tetraacetic acid; FAD-GPDH, FAD-dependent (mitochondrial) glycerol-3-phosphate dehydrogenase; GP, glycerol-3-phosphate; GPS, glycerol-3-phosphate shuttle; HD, Huntington's disease; htt_{expQ}, huntingtin with expanded CAG repeats; ICDH, NADH-isocitrate dehydrogenase; IF_1, inhibitor protein; IMS, intermembrane space; α-KGDH, α-ketoglutarate dehydrogenase; α-KG, α-ketoglutarate; MAS, malate–aspartate shuttle; PT, permeability transition; PTP, permeability transition pore; PDH, pyruvate dehydrogenase; PDHC, pyruvate dehydrogenase complex; State $3_{glu/mal}$, maximum respiration for the substrates glutamate plus malate; VDAC, voltage dependent anion channel; WT, wild type

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concentrations of ADP, ATP, Pi and PCr do not significantly change in the beating heart during periods of elevated work load [6,10]. It is clear from these data that the mechanisms of regulation of OXPHOS vary according to the type of tissue. In contrast to glycolytic muscle, where OXPHOS is controlled by cytosolic alterations in ADP and Pi [7–9], these mechanisms obviously do not exist in cardiac muscle [6,8,10]. To explain the metabolic stability in cardiac muscle, two hypotheses have been proposed. (i) Energy transfer by creatine kinase (CK)- and adenylate kinase (AK)-mediated pathways, with direct channelling of adenine nucleotides [11–19], and (ii) the parallel regulation by Ca^{2+} of OXPHOS and cell work [6,9,10]. Since energy transfer by CK and AK is reviewed by Saks et al. in this issue, we will focus our review on regulation by Ca^{2+}.

2. OXPHOS regulation by *intra*mitochondrial Ca^{2+}

2.1. Regulation of mitochondrial dehydrogenases by Ca^{2+}

To explain the stimulation of respiration in the absence of cytosolic ADP fluctuations, a hypothesis of parallel activation of OXPHOS and ATPases has been put forward [6,9,20–26]. This hypothesis is based upon the findings that not only the *extra*mitochondrial ATPases, but also the mitochondrial dehydrogenases – such as the pyruvate dehydrogenase complex (PDHC), the NADH-isocitrate dehydrogenase (ICDH) and the α-ketoglutarate dehydrogenase (α-KGDH) [25,26] (Fig. 1, Table 1) – can be activated by increased Ca^{2+} concentrations [21–26].

The activity of the PDHC is regulated by increasing the acetylCoA/CoA and NADH/NAD^+ ratios, and, more importantly, by reversible phosphorylation of three sites of the E1 subunit by highly specific kinases and phosphatases found in mitochondria [22,24]. PDHC is phosphorylated and thereby inactivated by PDH kinase, whereas PDH phosphatase (PDP) activates the enzyme [22,24]. The activation of PDHC by Ca^{2+} is realised by the direct effect of that ion upon PDP ($S_{0.5} = 0.77$) [22,27]. The activity of PDHC is independent of changes in ATP/ADP ratios [21,22,24].

In coupled respiring heart mitochondria, *extra*mitochondrial Ca^{2+} stimulated the α-KG oxidation up to fourfold at low concentrations (0.2 mM) of α-KG, but at saturation concentrations (25 mM) no stimulation occurs [25]. This was explained by the finding that Ca^{2+} reduces the K_M for α-KG from 2.1 μM to 0.2 μM but does not change the V_{max} [23,25]. $S_{0.5}$ for this activation is 0.4 μM Ca^{2+} in the presence of ADP and 0.8 μM Ca^{2+} in the presence of ATP, indicating that the sensitivity of α-KGDH to Ca^{2+} decreases as the ATP/ADP ratio diminishes [24]. At the same time, there is no effect of Ca^{2+} on the K_M for the second substrate NAD^+ [23,25].

Compared with α-KGDH and PDHC, much higher Ca^{2+} concentrations are required to activate ICDH. As is the case for α-KGDH, the sensitivity of ICDH increases with decreasing ATP/ADP ratios

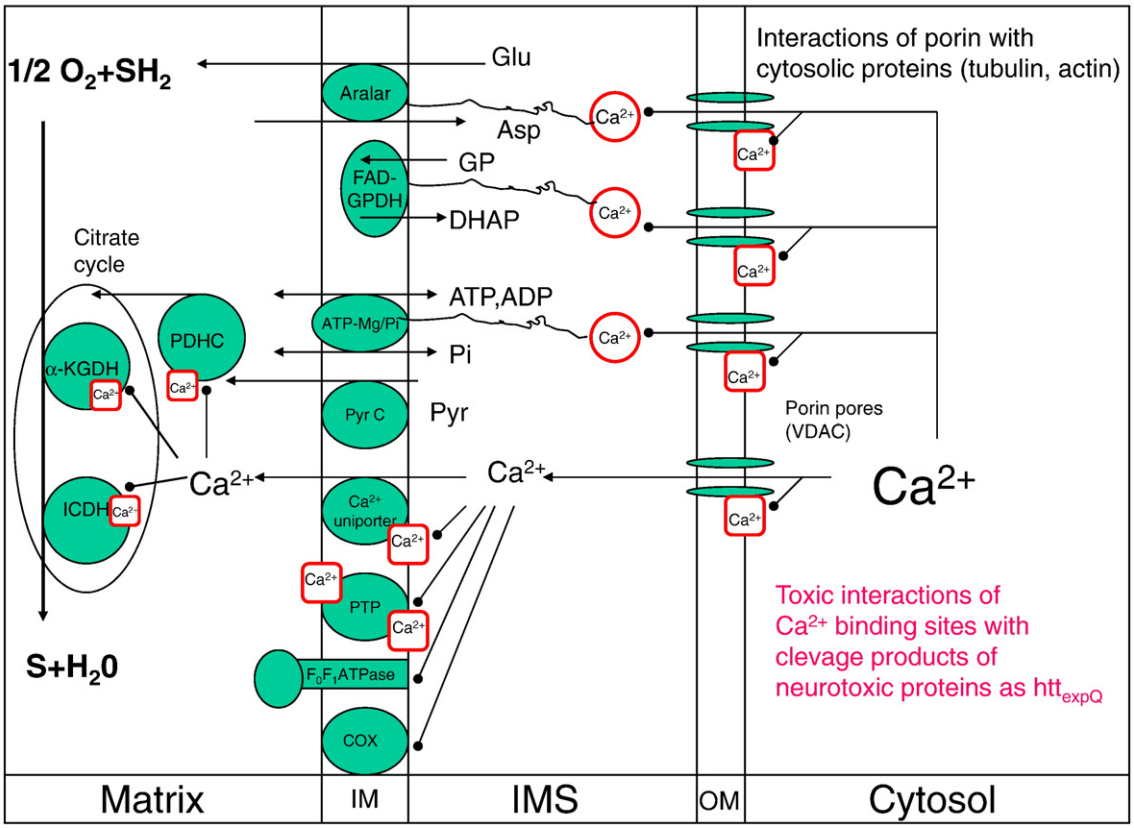

Fig. 1. Accepted and postulated mitochondrial targets for regulation by *extra*- and *intra*mitochondrial Ca^{2+}. There is ample evidence to demonstrate that the matrix enzymes α-ketoglutarate dehydrogenase (α-KGDH), isocitrate dehydrogenase (ICDH) and the pyruvate dehydrogenase complex (PDHC) are activated by rising matrix Ca^{2+} [21–28]. Ca^{2+} accumulation occurs via the Ca^{2+} uniporter [28–35], which has a Ca^{2+}-binding site at the IMS site [30–33]. Ca^{2+} activation of F_0F_1ATPase [20,127–144] is assumed via a release of inhibitor proteins from F_0F_1ATPase [127,131], or by post-translational modifications of the enzyme complex without the necessity of special Ca^{2+} binding sites [20,125]. Subunit I of cytochrome c oxidase can be activated by Ca^{2+}-dependent dephosphorylation occurring *in vitro* at very high Ca^{2+} concentrations [137,138]. A regulatory Ca^{2+}-binding site was assumed to exist on the IMS side of the PTP, where Ca^{2+} supports the closed state of the PTP [141,142]. Increasing intramitochondrial Ca^{2+} induces the opening of PTP by activation of cyclophilin D [129–143]. Recent findings support the possibility that the porin pores have a regulatory Ca^{2+}-binding site that can increase the permeability of porin [120–122]. The following enzymes have EF-hand Ca^{2+}-binding motifs that are localised in the IMS and there sense the cytosolic Ca^{2+} concentration [68]. The MgATP/Pi carrier can increase or decrease the matrix content of adenine nucleotides in a Ca^{2+}-dependent manner [68,82,84–89]. FAD-GPDH oxidises GP to DHAP and supplies the redox equivalents to coenzyme Q [91–99]. In contrast to the other mitochondrial Ca^{2+}-dependent enzymes, Ca^{2+} activation of aralar occurs by increasing V_{max} and not by decreasing K_M. Aralar transports a real substrate (glutamate) for the citrate cycle into the mitochondria and, as part of the malate–aspartate shuttle, it also transports redox equivalents into the mitochondria [68]. Moreover, this substrate supply is electrogenic [69,77,78] and therefore it is a preferred route.

Table 1
Summary of Ca^{2+}-sensitive enzymes of mammalian mitochondria.

Enzyme	RR	$S_{0.5}$ [μM Ca^{2+}]	Kinetic effects of Ca^{2++}	Reference
PDHC-phosphatase	Inhibits	0.77(ADP)	Activation of PDHC	[22]
		0.74 (ATP)	Activation of PDHC	[22]
α-KGDH	Inhibits	0.28 (ADP)	Decrease K_M α-KG	[22,26]
		0.81 (ATP)	Decrease K_M α-KG	[22,26]
ICDH	Inhibits	5.4 (ADP)	Decrease K_M IC	[22,26]
		41 (ATP)	Decrease K_M IC	[22,26]
Uniporter	Inhibits		Increases Ca^{2+} uptake	[30–33]
			K_M>3 μM Ca^{2+}	[31,33]
			Spermine decreases K_M	[34,35]
Aralar	No effect	0.3	Increasing of V_{max} of malate–aspartate shuttle and state $3_{glu/mal}$	[68,70–76] [63,64]
FAD-GPDH	No effect	0.3	Decrease of K_M	[93,95,98]
ATP-Mg/Pi carrier	No effect	1–4	Decrease of K_M	[68,82]
PTP			Shift to closed PTP	[141,142]
Porin		μM range	Increasing permeability	[120,121]
		nM range	Increasing permeability	[122]
	Decreases permeability		Ca^{2+} uptake	[121]
ATPase		n.d.	Increasing activity	[123–136]
COX		>100	Increasing activity	[137,138]

($S_{0.5}$ = 5.4 μM Ca^{2+}at 1.5 mM ADP; $S_{0.5}$ = 41 μM at 1.5 mM ATP) [22,24,26] and Ca^{2+} activation is realised through decreasing K_M for the substrates without changes in V_{max} [22,24,26].

For all three dehydrogenases described it has been shown that their activation by Ca^{2+} is abolished by ruthenium red (RR), an inhibitor of the Ca^{2+} uniporter. Therefore, it was concluded that activation of these dehydrogenases by Ca^{2+} occurs exclusively inside the matrix and thus requires transport of Ca^{2+} into that compartment [21–26].

2.2. Mitochondrial Ca^{2+} accumulation via the Ca^{2+} uniporter

Mitochondria accumulate Ca^{2+}_{cyt} via the Ca^{2+} uniporter, which facilitates the transport of Ca^{2+} down its electrochemical gradient [28–31]. The uniporter can also transport other cations, but these can act as competitive inhibitors of Ca^{2+} accumulation [28]. Kinetic investigation of the accumulation of Ca^{2+} gave sigmoidal plots of transport rate versus [Ca^{2+}] and showed that the mechanism was of second order with respect to [Ca^{2+}], causing a Ca^{2+}-induced reinforcement of mitochondrial Ca^{2+} uptake [29–31]. These results led to the conclusion that the uniporter has both a transport site and an activation site, with different binding affinities [30]. The activation site may interact with nucleotides and divalent cations because both nucleotides and Mg^{2+} can inhibit the uniporter activity [32]. It is also assumed that the Ca^{2+} induced facilitation of Ca^{2+} uptake could be mediated through effects of calmodulin on the uniporter [33,37].

The V_{max} of the uniporter is clearly dependent on the membrane potential [28]. However, its affinity to Ca^{2+} is low, as $K_{0.5}$ ranges from 1 to 189 μM [31,33]. Nevertheless, at lower Ca^{2+} concentrations polyamines – such as spermine – increase significantly the rate of Ca^{2+} influx through the uniporter [34,35]. The amount of polyamines varies somewhat between the cell types, being approximately 1 mM in the cytoplasm of hepatocytes and about 0.3 mM in the cytosol of cardiac myocytes; both of these concentrations are sufficient to activate the uniporter [36].

The Ca^{2+} uniporter plays an important role in connecting cytosolic and *intra*mitochondrial signalling by Ca^{2+} ions. In most cells, the resting free Ca^{2+} concentration is in the range of 100–200 nM in the cytosol [28,38–40]. With physiological stimulation, however, free Ca^{2+} in both cytosol and mitochondria can increase rapidly and transiently by a factor of 10–20, as occurs on a beat-to-beat basis in cardiac myocytes owing to mitochondrial Ca^{2+} uptake [38]. It has been shown that Ca^{2+}_{mit} responds promptly to changes in Ca^{2+}_{cyt}, exactly following the Ca^{2+}_{cyt} oscillations, thus providing a frequency-mediated signal which is specifically decoded by the mitochondria as shown for hepatocytes [39], cardiomyocytes [38] and HeLa cells [41].

Nicholls has shown in extensive studies [42–44] that brain mitochondria start to accumulate Ca^{2+} if the concentration rises above 0.5 μM [42,44]. Similar properties have been found for mitochondria *in situ* within cultured neurons [45–47]. After accumulation of more than 10 nmol Ca^{2+}/mg the parallel accumulation of phosphate causes formation of calcium phosphate in isolated mitochondria [44] but also *in situ* in neurons [45], with a variable calcium-to-phosphate ratio depending on the conditions [48]. This calcium phosphate formation makes it possible that brain mitochondria may act as a "perfect calcium buffer", effectively lowering the Ca^{2+}_{cyt} back to the 0.5 μM. If the cytosolic Ca^{2+} falls below that value the mitochondria release Ca^{2+}_{mit} again by dissolving the calcium phosphate [42]. This means that brain mitochondria act as temporary reservoirs of Ca^{2+}, by taking up Ca^{2+} throughout the stimulation but releasing this ion back into the cytoplasm when the plasma membrane and SR ATPases pump down the Ca^{2+}_{cyt} [42]. As a result, the Ca^{2+} transients in the neurons become smoother and more prolonged [42]. In quiescent neurons, where Ca^{2+}_{cyt} is close to 0.1 μM, the mitochondrial matrix is largely depleted of Ca^{2+} and Ca^{2+}_{mit} varies as a function of Ca^{2+}_{cyt} [42]. Nicholls concluded from these results that the brain mitochondrial Ca^{2+} uptake fulfils two purposes: the activation of mitochondrial dehydrogenases (if Ca^{2+}_{mit} is lower than 10 nmol/mg) and the storage of excess Ca^{2+} (in the range of 10–130 nmol/mg Ca^{2+}) [42].

2.3. Problems with exclusive regulation of OXPHOS by *intra*mitochondrial Ca^{2+}

As noted above, the observation that RR hinders the activation of respiration by the effective inhibition of the Ca^{2+} uniporter has been used as a main argument for the *intra*mitochondrial Ca^{2+} stimulation of mitochondrial dehydrogenases [22,23,49–51]. In some studies with isolated heart mitochondria, only after addition of excessive high (8 μM) RR a reduction in stimulation by Ca^{2+} was observed [51]. Results obtained with such large RR additions must be regarded with caution, because in our hands high RR concentrations caused unspecific inhibitions of mitochondrial functions [Gellerich et al., unpublished]. Although RR carries a strong positive charge, it can enter a number of cell types. Therefore, it has been used to study the effects of *intra*mitochondrial Ca^{2+} on energy metabolism in perfused tissues and cells [50–54]. To overcome the diffusion barrier of cellular membranes in such experiments, higher RR concentrations were used than applied with isolated mitochondria. With this approach it was shown that the activation of PDHC (detected on a basis of altered content of dephosphorylated PDHC in the tissue homogenate) was completely blocked during the perfusion of heart muscle with a medium containing RR; this was taken to indicate complete inhibition of the Ca^{2+}-uniporter [51–54]. However, in these studies the surprising finding was that the work output actually was even increased [52,53,55] despite of the RR inhibition. As expected the decreasing ATP/ADP ratios indicated an impairment of metabolic stability [52,53]. However, the observed small changes did not fit with the assumption that mitochondrial activity is regulated by changes of intramitochondrial Ca^{2+}. Another set of studies performed on MH75 cells has shown that changes in mitochondrial NADH do not correlate with alterations in *intra*mitochondrial Ca^{2+} [55]. In stimulated motor nerve terminals Ca^{2+}_{cyt} – but not Ca^{2+}_{mit} – correlates with the work load adjusted by repetitive stimulations of different frequency and intensity [56–58]. After cessation of stimulations the decrease of Ca^{2+}_{mit} is much slower than that of Ca^{2+}_{cyt} [56–59]. Also, when the stimulation of PDH, ICDH and α-KGDH by *intra*mitochondrial Ca^{2+} was considered in a computer simulation of OXPHOS regulation, the model was not able to predict the data obtained *in vivo* for the beating heart, oxidative skeletal muscle and in hepatocytes stimulated by different hormones

[9,60–62]. Therefore, Korzeniewski postulated the existence of an additional mechanism causing a stimulation of mitochondria by *extra*mitochondrial Ca^{2+} which acts together with the Ca^{2+} activation of the work load (parallel activation) [9,60–62]. However, up to now there was no known biochemical mechanism responsible for that.

Interestingly, the effect of RR on cardiac contractility may be even beneficial, so that RR has been viewed as a cardioprotective drug [54]. The *in vivo* results obtained in the presence of RR do not accord with the understanding that Ca^{2+} ions, after penetrating mitochondrial inner membranes, activate dehydrogenases and thereby exert positive inotropic effects. Obviously, the RR has much less effect on OXPHOS in perfused tissues [50–54] than in isolated mitochondria [23,49]. Such modest RR effects *in vivo* point to a possibility of additional mechanisms of Ca^{2+}-dependent regulation that can operate *in vivo* but not *in vitro*, this difference probably stemming from the substrate-specific and location-specific nature of the regulatory effects of Ca^{2+} (see below). On the other hand, it is possible that ATP formation may be largely preserved in RR-treated hearts by the stimulation of OXPHOS through other intrinsic mechanisms, e.g. through an increase in ADP concentration, which also strongly stimulates the α-KGDH [22,25,26].

Summarising, we can state that there are significant experimental findings *in vivo* which are not in line with a hypothesis of the exclusive regulation of OXPHOS by *intra*mitochondrial Ca^{2+} effects on dehydrogenases. It seems that the importance of these effects has been overestimated in the past. Therefore, an alternative mechanism of OXPHOS regulation by *extra*mitochondrial Ca^{2+} should be considered, as described below (Fig. 1).

3. OXPHOS regulation by *extra*mitochondrial Ca^{2+}

3.1. Stimulation of glutamate-dependent respiration by extramitochondrial Ca^{2+}

We have recently shown that glutamate-dependent respiration in isolated brain mitochondria is reversibly activated by *extra*mitochondrial Ca^{2+} [63,64]. This unexpected discovery was made by using an incubation medium containing 100 μM EGTA, which keeps Ca^{2+}_{free} negligibly low (150 nM Ca^{2+}_{free}). As shown in Fig. 2A we used the multiple substrate inhibitor protocol that was especially designed for quantification of mitochondrial defects in complex-I-related metabolism [65]. With 10 mM glutamate and 2 mM malate as substrates, the addition of 2.5 mM ADP induced an unusually low glutamate-dependent respiration rate (state $3_{glu/mal}$). To clarify whether such a modest respiration rate resulted from an insufficient complex-I-related metabolism, we measured the complex-II-dependent respiration by adding the specific complex I inhibitor rotenone and succinate as a complex-II-specific substrate (state 3_{suc} respiration). State $3_{glu/mal}$ respiration was corresponded to only 69% of state 3_{suc} respiration. However, if the same experiment was performed in the presence of 1.35 μM Ca^{2+}_{free} (Fig. 2B), state $3_{glu/mal}$ respiration was 110% of that of state 3_{suc} (Fig. 2A,B). Thus, the increase in Ca^{2+}_{free} induced a normalisation of the mitochondrial complex-I-dependent respiration in normal brain mitochondria. Ca^{2+} activation was not limited to the mitochondrial capacity of OXPHOS, but rather to its efficacy in metabolising glutamate, as succinate strongly enhanced respiration above state $3_{glu/mal}$. With pyruvate/malate (Fig. 2D), state 3 respiration significantly exceeded that of state $3_{glu/mal}$ (Fig. 2D). However, Ca^{2+} addition did not augment state $3_{pyr/mal}$ respiration (Fig. 2D). Fig. 2E demonstrates that there was also no Ca^{2+} effect on complex-II-dependent state 3 with succinate/rotenone.

In a second approach, state 3 respiration of brain mitochondria was titrated by sequential Ca^{2+} additions (Fig. 2F). Again, state $3_{glu/mal}$ respiration was very low under conditions of low Ca^{2+}_{free}, but increased stepwise until Ca^{2+}_{free} reached 2 μM. Kinetic analyses of Ca^{2+} activation revealed a half-activation constant $S_{0.5}$ of 0.3 μM Ca^{2+}_{free} [63,64]. Further Ca^{2+} additions gave rise to an inhibitory effect. Than the same Ca^{2+} titration was performed but in the additional presence of 250 nM RR. All the data points of state 3 respiration at a Ca^{2+} concentration below 2 μM were identical, irrespective of whether they were measured in the presence or the absence of RR. Differences only occurred at Ca^{2+} concentrations above 2 μM, indicating that at lower Ca^{2+} concentrations under our experimental conditions no Ca^{2+} can be accumulated by the mitochondria since the K_M for this process is >3 μM [Gellerich et al., unpublished]. At higher Ca^{2+} additions respiration becomes increasingly inhibited owing to rising Ca^{2+} overload, while RR prevents this as expected. Ca^{2+} titration with and without RR was also performed under state 4 conditions. A very small but significant increasing of respiration was observed at 1 μM Ca^{2+}_{free}. At higher Ca^{2+} concentrations a further increase of respiration was observed only in the absence of RR followed by an inhibition due to Ca^{2+} overload.

When one compares the metabolic pathways for oxidation of the three substrates glutamate, pyruvate and succinate, it becomes evident that it is not the respiratory chain including F_0F_1ATPase, but rather the substrate supply metabolism with glutamate which should contain the reaction which is exclusively sensitive to changes of *extra*mitochondrial Ca^{2+} under these conditions. A literature search for proteins with an external regulatory Ca^{2+} binding site and, thus, the ability to mediate the *extra*mitochondrial actions of Ca^{2+} pointed to aralar as a potential candidate (Fig. 1).

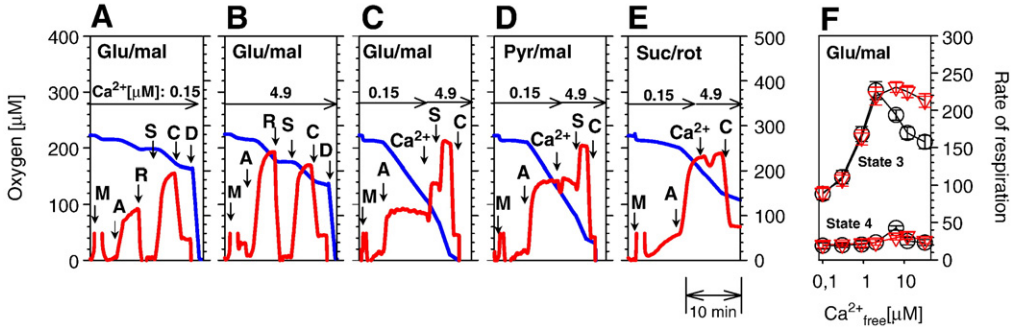

Fig. 2. Exclusive activation of glutamate-dependent state 3 respiration of brain mitochondria by *extra*mitochondrial Ca^{2+} in the nanomolar range. A–E, Respirograms of rat-brain mitochondria obtained by high-resolution respirometry at various concentrations of *extra*mitochondrial Ca^{2+}_{free} as indicated by horizontal arrows. A, Isolated rat-brain mitochondria were incubated with 10 mM glutamate + 2 mM malate as substrates (Ca^{2+}_{free} = 150 nM, adjusted by 0.1 mM EGTA). Additions: M, 0.06 mg/ml brain mitochondria; A, 2.5 mM ADP to activate the phosphorylation-related respiration (state 3); R, 1.5 μM rotenone to inhibit the respiratory chain complex I; S, 10 mM succinate as substrate of respiratory chain complex II; C, 5 μM carboxyatractyloside to block adenine nucleotide carrier; D, a few mg dithionite. Blue lines indicate the oxygen concentration in the oxygraph (left ordinate), whereas red lines represent the rate of respiration (right ordinate). The height of peaks correlates with the rate of respiration (nmol O_2/mg mitochondrial protein). B, as in A but with increased Ca^{2+}_{free} = 4.9 μM. C, as in A but with addition of Ca^{2+} throughout the measurement (Ca^{2+}_{free} = 4.9 μM) and omission of complex I inhibition. D, as in C but with 10 mM pyruvate + 2 mM malate as substrates. E, as in D but with 10 mM succinate + 1.5 μM rotenone as substrates. F, Ca^{2+} titration of glutamate-dependent respiration of brain mitochondria under conditions of state 3 (adjusted by 2 mM ADP) and state 4 (without ADP) without (○) and with (▽) 250 nM RR. *Extra*mitochondrial Ca^{2+} was increased stepwise as indicated.

3.2. Aralar acts as a "gas pedal" of brain mitochondria in response to extramitochondrial Ca^{2+}

Glutamate is taken up by mitochondria either via aralar, leading to its subsequent transamination by aspartate amino transferase (inhibitable by aminooxyacetate AOA, [66,67] and ribulose-5-phosphate [68]), or via the glutamate/OH carrier, followed by its deamination by glutamate dehydrogenase [67,69]. However, the activity of the glutamate/OH carrier is low in most tissues, except liver and kidney [69]. Aralar has regulatory Ca^{2+}-binding sites on its long, hydrophilic amino-terminal extension harbouring EF-hand motifs that face the IMS [68,72,76]. There are two mammalian isoenzymes of the AGC, aralar (occurring in brain and skeletal muscle) and citrin (occurring in liver), while in the heart both isoenzymes are present [68]. As shown by the extensive work of Satrustegui [68,70–75] and Palmieri [76], aralar – as a central enzyme of the malate–aspartate shuttle (MAS) – transports reducing hydrogen equivalents (of cytosolic NADH) into the mitochondria, this reaction being strongly regulated by *extra*-mitochondrial Ca^{2+} with an S_{05} of 300 nM Ca^{2+}_{free} [68,72]; the latter value is in line with the $S_{0,5} = 260$ nM measured for the *extra*mitochondrial Ca^{2+} activation of glutamate-dependent respiration of brain mitochondria [63,64]. It is therefore very likely that activation of state $3_{glu/mal}$ respiration by *extra*mitochondrial Ca^{2+}_{free} is mediated by enhanced transport of glutamate into the mitochondrial matrix by aralar [63,64,71,72,76]. Since the NADH concentration in the matrix is larger than in the cytosol this transport requires energy which is supplied from the membrane potential (Δ_Ψ). The electrogenic nature of the aralar reaction is realized by cotransport of protons in addition to glutamate making this carrier unidirectional [77–79]. The affinity for glutamate ($K_M = 0.2$ mM) is lower than for aspartate ($K_M = 0.5$ mM) and these values do not change with the membrane potential. The transport rate constant increases with membrane potential but does not change the substrate affinities [77–79].

The great importance of the MAS for the supply of substrate to mitochondria is supported by the finding that inhibition of MAS attenuates the respiration of isolated synaptosomes [80] and suppresses the contractile function of the perfused, working heart [81] when glucose or lactate is oxidised. On the other hand, full contractile functionality can be observed if pyruvate is used in the presence of AOA [81]. Satrustegui discovered the stimulation of the MAS by cytosolic Ca^{2+} [68,71–75], but did not take account of the importance of aralar for the regulation of OXPHOS.

3.3. ATP-Mg/Pi carrier

The ATP-Mg/Pi carrier was the first known mitochondrial carrier with an absolute requirement for *extra*mitochondrial Ca^{2+} due to the existence of a EF-hand Ca^{2+} binding motif [68,82]. It transports ATP-Mg in exchange for phosphate and is responsible for the net of adenine nucleotides into or out of the mitochondria. In this way the Ca^{2+}-dependent carrier can change the mitochondrial adenine nucleotide content. Interestingly, there are also calcium-independent mitochondrial ATP-Mg/Pi carriers [83]. Activity of the carrier depends on pH gradient and is suppressed by uncouplers [84]. The fact that the electroneutral net adenine nucleotide transport is not affected by RR demonstrates its strict dependence on *extra*mitochondrial Ca^{2+} [85]. In rat-liver mitochondria, increasing Ca^{2+} from 1 to 4 μM causes a decrease in K_M but does not change the V_{max} of the carrier [85]. In yeast the $S_{0.5}$ is even larger (15 μM Ca^{2+}) [86]. Yeast mitochondria can import cytosolic ATP via the ATP-Mg/Pi carrier for generation of mitochondrial membrane potential [86]. In mammalian cells, such a mechanism could be important under hypoxic conditions [87]. The ATP-Mg/Pi carrier increases the mitochondrial adenine nucleotides in post-natal development [86,87] allowing higher rates of OXPHOS [88–90] because elevated ATP- and ADP-contents can down-modulate the control of the AdN translocator over the OXPHOS [90]. Since this is a long-term process, it remains to be seen whether short-term changes in the concentrations of mitochondrial adenine nucleotides can occur. In summary, owing to the relative insensitivity of the ATP-Mg/Pi carrier to Ca^{2+} it is not probable that this carrier is involved in the short-term adaptation of mitochondria to an increased work load.

3.4. FAD-glycerol-3-phosphate dehydrogenase

The mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase (FAD-GPDH), together with cytoplasmic NAD-linked GPDH, acts as the glycerol-3-phosphate shuttle (GPS) that transports reducing hydrogen equivalents (of NADH) into the mitochondria [91–93]. Since this enzyme reduces ubiquinone without the participation of mitochondrial complex I, the GPS has a reduced energy yield of the transported hydrogen in comparison with the MAS. It has been found that FAD-GPDH is located in the outer site of the mitochondrial inner membrane [94] and is accessible for cytosolic Ca^{2+} [95]. An increased activity of this pathway induced through elevated FAD-GPDH activity was thought to be one possible energy-dissipating mechanism [96]. FAD-GPDH from several mammalian tissues is stimulated by Ca^{2+} [91–93,95–102]. The K_M of FAD-GPDH is high (4–9 mM) but activation by Ca^{2} results in a decrease in the K_M for GP (1 mM), whereas the V_{max} is not changed [91–93,97]. RR does not affect the stimulation by Ca^{2+} of flight-muscle mitochondria, indicating that the Ca^{2+}-binding site is located on the outer side of the mitochondrial inner membrane [97]. The mammalian FAD-GPH contains EF-hand motifs at the carboxy terminus of the enzyme which are responsible for the activation by Ca^{2+} [95]. In mitochondria of pancreatic ß-cells, GP-dependent respiration is strongly activated by Ca^{2+}, with an $S_{0.5}$ between 80 [99] and 300 nM [98]. It has been assumed that the physiological role of the GPS is to keep the cytosolic pyridine nucleotides in the oxidised state, thereby making them effective in sustaining the oxidation of glucose [99]. The activity of FAD-GPDH is increased by T3-induced hyperthyreosis in the heart, liver and kidney, but not in the brain [100]. Brain mitochondria possess a considerable activity of FAD-GPDH, responsible for a Ca^{2+}-dependent activation of ROS formation by reverse electron transport [101]. Since the main substrate of brain cells is glucose, there should be sufficient glycolytic intermediates for transport of reducing hydrogen via the GPS into brain mitochondria. Therefore, this shuttle may contribute to the Ca^{2+}-dependent substrate supply to brain mitochondria.

3.5. Porin pore (VDAC)

Recent results indicate that the porin pore is an important regulator of mitochondrial function [103–105]. All mitochondrial metabolites, e.g. ADP, ATP, Cr, CrP and Ca^{2+} enter the mitochondria by diffusing through the porin pores of outer membranes along their diffusion gradients. There exist evidences that rate-dependent concentration gradients up to 15 μM between the IMS and the cytosol can be formed for adenine nucleotides [11–15,106–109]. These gradients cause compartmentation if the metabolite concentrations are sufficiently small, as is the case for ADP and Ca^{2+} but under normal circumstances not for ATP, Cr or CrP [11,12]. Most data on the permeability of the porin pore have been obtained with isolated mitochondria under isotonic conditions. However, simulating the cellular oncotic pressure with dextrans or albumins resulted in severalfold increased K_M for ADP in regulation of OXPHOS in heart mitochondria [11–15]. Furthermore, the conductivity of the pores inserted into lipid membranes decreases in the presence of added macromolecules [109,110]. It has been shown that also NADH favours closed states of the pore [111]. Very recent data indicate that interaction of porin with the cytoskeletal protein tubulin further increases the diffusion resistance for movement of adenine nucleotides through the pore [112,113]. Likewise, other cytoskeletal proteins – actin [114], and hexokinase-1 [115,116] – interact with porin, decreasing their penetrability. Because tubulin filaments link mitochondria to sarcomeres and

other cellular structures, one can expect mitochondria and ATPases to act as a complex spatial system; hence, muscle contraction should markedly modulate the dynamic compartmentation of ADP [117]. The correctness of this assumption has been recently demonstrated by a series of different experiments [17,19]. In one set of studies, it was shown that endogenous ADP released from the ATPase reaction cannot be fully equilibrated with the ADP pool in the medium surrounding the skinned cardiomyocytes [17,19,118]. Moreover, a powerful ADP-trapping system consisting of pyruvate kinase and phospho*enol*pyruvate was also unable to capture ADP formed by ATPases and stimulating OXPHOS [19,118,119]. On the other hand, it was found that endogenous ADP was much more effective in stimulating OXPHOS than was ADP added to skinned cells [17,19]. Altogether, these results led to conclusion that mitochondria and ATPases indeed form the complexes which at least are partially separated from the cytosol [19,118,119]. Another interesting finding was that strong contractions of skinned cardiomyocytes induced by micromolar concentrations of Ca^{2+} resulted in an increased affinity for ADP of mitochondria within these complexes, revealed as a decreased K_M for ADP in the regulation of respiration [119]. It thus appears that owing to mechanical interactions of mitochondria and sarcomeres, probably mediated by cytoskeletal proteins, the forces transmitted from the latter can dynamically modulate the porin pore's permeability for ADP [119]. In addition to the mechanisms described, the permeability of porin pores for anions and cations can be modulated by the electrical potential [103–105]. Because the porin protein possesses a Ca^{2+}-binding site it is expected that Ca^{2+} ions can also control the status of porin pores [120,121]. Evidences for this assumption have been increased very recently by experiments showing that nanomolar Ca^{2+} concentrations are sufficient to increase the permeability of the MOM towards ADP [122].

3.6. F_0F_1ATPase

First suggestions that mitochondrial F_0F_1ATPase may be regulated directly by matrix Ca^{2+} came from the studies of Harris and Das [123,124]. It is assumed that increasing Ca^{2+} could activate the F_0F_1ATPase, but the mechanism has remained unclear because Ca^{2+} does not activate isolated F_0F_1ATPase [20,126]. It has been suggested that the activity of F_0F_1ATPase is controlled by an inhibitor protein IF_1 which inhibits the F_1ATPase at a molar ratio of 1:1 [126–130] and by a calcium-binding inhibitor protein that is considered to confer Ca^{2+}-dependence upon the F_0F_1ATPase [131,132]. Balaban assumes that *extra*mitochondrial Ca^{2+}, in combination with other proteins, regulates a post-translational process changing the F_0F_1ATPase activity [20,125]. However, the mechanism of this activation remains to be resolved [20]. In a series of studies Territo demonstrated that Ca^{2+} is able to rapidly increase the velocity of ATP production by the F_0F_1 ATPase at a given driving force [20,133–136]. Since all these experiments [133–136] were performed with isolated mitochondria and glutamate/malate as substrates, the conclusions concerning the F_0F_1ATPase may be questioned, especially in view of the fact that these authors did not take into account the possibility that *extra*mitochondrial Ca^{2+} could activate the glutamate entry into the mitochondria which then could cause an activation of OXPHOS by increasing substrate supply [63,64] (see above). Since the *extra*mitochondrial Ca^{2+} activation of isolated heart mitochondria only occurs in the presence of glutamate – but not of pyruvate or succinate – as a substrate [63,64] it seems that the activation observed by Territo et al. is not caused by activation of F_0F_1ATPase but is a consequence of the mitochondrial uptake of glutamate via aralar in dependence on the concentration of *extra*mitochondrial Ca^{2+}[63,64].

3.7. Cytochrome-c-oxidase

Kadenbach demonstrated a cAMP-dependent tyrosine phosphorylation site in liver cytochrome-c-oxidase (COX) subunit I. Its phosphorylation decreases both V_{max} and its affinity for cytochrome c [137,138]. Large concentrations of Ca^{2+} (>100 μM) are required to dephosphorylate COX in intact mitochondria [137,138], indicating that this activation may occur under extreme conditions but cannot contribute to the regulation at normal Ca^{2+} levels [20].

3.8. Permeability transition pore

The Ca^{2+}-linked opening of the permeability transition pore involved in many physiological and pathopysiological processes is described in numerous reviews [eg. 139–141]. Increasing *intra*mitochondrial Ca^{2+} opens the PTP by activating the mitochondrial cyclophilin D [139–143]. In this process Ca^{2+} may have several roles. Increased Ca^{2+} alone and its uptake into mitochondria may cause PT. Further, other stressors may decrease the threshold for Ca^{2+} causing PT even at unchanged Ca^{2+} but at increased other stressors [140]. Bernardi proposed a model were PTP has besides the internal also an external Me^{2+} binding site that can decrease the pore open probability [141,142]. This binding site interacts with *extra*mitochondrial Mg^{2+} and Ca^{2+} [141,142]. Via this mechanism cytosolic Ca^{2+} may act as an inhibitor of PT [141–143].

4. Impaired *extra*mitochondrial Ca^{2+} regulation at transgenic HD models

Huntington's disease (HD) is a progressive neurodegenerative disorder caused by a CAG repeat expansion in the coding region of the huntingtin (htt) gene resulting in an expanded polyglutamine stretch in the htt protein (htt_{expQ}) [144,145]. The CAG repeat length of htt_{expQ} correlates inversely with the time point of disease onset [146]. Unmodified htt, and even htt_{expQ} in HD, are expressed abundantly in most tissues [145], but neither the biological function of htt nor the mechanism of cytotoxic action of htt_{expQ} has been understood [147]. Several lines of evidence suggest that the cell's energy metabolism is impaired in HD [for reviews see 148,149], possibly due to mitochondriotoxic effects of htt_{expQ} [147–154]. Indeed, decreased Ca^{2+} accumulation capacities of mitochondria from brain of YAC_{72Q} mice [150], from skeletal muscle of htt_{140Q} R6/2 mice [151], from liver of htt_{111Q} mice [152], from HD patient's lymphocytes [150], or fibroblasts [157] and from htt_{111Q} striatal progenitor cells [153] have been reported. Furthermore, impaired mitochondrial function and Ca^{2+} dyshomeostasis were detected in PC12 cells after transfection with htt_{expQ} plasmids [154]. In contrast, increased Ca^{2+}-loading capacities were observed in brain mitochondria from several HD mice lines [155,156].

Recently we investigated skeletal muscle mitochondria from 15-week-old R6/2 mice respirometrically [150] by means of the multiple substrate inhibitor titration protocol [65]. At a Ca^{2+} concentration of 0.9 μM, the respirometric properties of HD and WT mitochondria were identical (Fig. 3A,C). In both cases the rates of complex-I-dependent respiration were higher (165%) than those of complex-II-dependent respiration. If, however, the investigation was performed in the additional presence of 10 μM (Fig. 3B,D) the complex-I-dependent respiration was much more inhibited in HD mitochondria than in the controls [151]. On the contrary, the succinate-dependent respiration was nearly unaffected in both HD and WT mitochondria [151]. These data show that htt_{expQ} affects mitochondria not only from brain but also in skeletal muscle and that the impairment can be detectable only at elevated Ca^{2+} concentration.

We therefore developed a protocol for routine investigation of diseased mitochondria by means of Ca^{2+} titrations under state 3 conditions. In an investigation of brain and striatal mitochondria isolated from HD rat, we detected that all rates of state 3 respiration were lower than the corresponding rates in controls [63]. As shown in Fig. 4 inhibition of state 3 respiration of HD mitochondria started at lower Ca^{2+} concentrations than in the controls (Fig. 4). Moreover, we also detected lowered membrane potentials, decreased Ca^{2+} uptake rates and depressed Ca^{2+} threshold values [63]. The impairment of the Ca^{2+} uptake kinetics was abolished by the addition of Cyclosporin

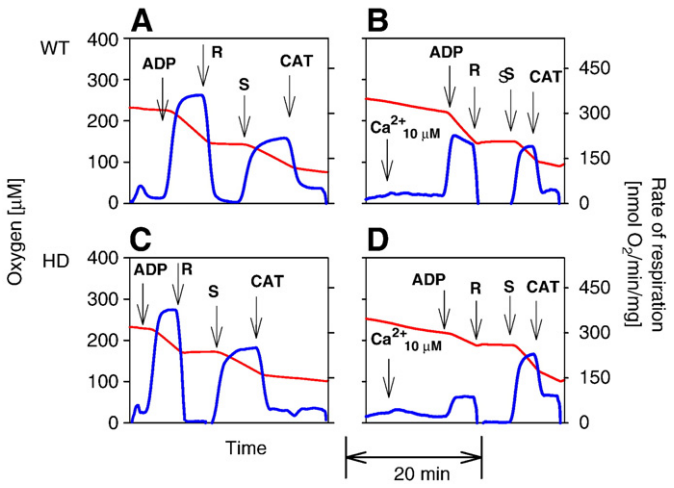

Fig. 3. Ca^{2+}-induced inhibition of complex-I-dependent respiration in isolated muscle mitochondria from wild type (WT) and transgenic R6/2 Huntington mice. Multiple substrate inhibitor titration of respiration of mitochondria isolated from skeletal muscle of WT (Al, B) and HD mice (C, D) at the age of 14 to 16 weeks. Typical protocols of three independent experiments are shown. Isolated mitochondria (0.5 mg/ml) were incubated in medium containing 5 mM $MgCl_2$, 75 mM mannitol, 25 mM sucrose, 100 mM KCl, 10 mM KH_2PO_4, 0.5 mM EDTA, 20 mM Tris, pH 7.4 and 10 mM pyruvate plus 2 mM malate as substrates. Additions: ADP 2 mM adenosine diphosphate, R, 1.5 µM rotenone; S, 10 mM succinate; CAT, 10 µM carboxyatractyloside; $Ca^{2+}_{10\,\mu M}$, 10 µM Ca^{2+}. In the presence of 0.9 µM Ca^{2+} (A, C) respirometric properties of WT and HD mitochondria were not different from each other. State $3_{complex\ I}$ respiration was about 150% of state $3_{complex\ II}$ respiration in both samples but after challenge with 10 µM Ca^{2+} state 3 these values were 100% in WT but 20% in HD mitochondria, indicating a much more pronounced instability of HD mitochondria towards Ca^{2+} stress. For further details see [151].

A [63]. Impaired mitochondrial function was also detected in fibroblasts of an HD patient with htt_{43Q} [157].

Irrespective of the underlying mechanism, htt_{51Q}-dependent changes in mitochondrial regulation may uncover important pathophysiological consequences. For instance, decreased Ca^{2+} thresholds of htt_{expQ} mitochondria for undergoing PT might be responsible for accelerated mitochondrial cell death, in particular under conditions of elevated cytosolic Ca^{2+} concentration. Moreover, we demonstrated that protective CsA effects occur not only under conditions of Ca^{2+} overload, but also within physiological concentration ranges of *extra*mitochondrial Ca^{2+} in htt_{51Q} mitochondria. This may have important, disease-specific consequences *in vivo* if an altered Ca^{2+} homeostasis affects the energy metabolism and *vice versa*.

In summary, our data provide several lines of evidence for htt_{51Q}-induced pathomechanisms underlying the initiation and progression of HD. Ca^{2+}-dependent impairments of mitochondrial oxidative phosphorylation due to a limited substrate supply and/or altered PT were identified as a major cause for the increased vulnerability of HD mitochondria towards Ca^{2+} stress. We assume that the Ca^{2+}-binding sites at the mitochondrial surface regulating the substrate supply for mitochondria can act as targets for mitochondriotoxic proteins (Fig. 1). Regulatory Ca^{2+} binding sites at the surface of mitochondria can be occupied by binding to htt_{expQ} or other neurodegeneratively acting proteins, causing an impaired Ca^{2+} regulation of mitochondria that in turn leads to energetic depression and mitochondrial cell death [149].

5. Conclusions

Since the time when *in vivo* ^{31}P-NMR spectroscopy was first applied to investigate energy metabolism in intact tissues, it has become evident that the original idea of output activation of OXPHOS by ADP could not explain the homeostasis of phosphorylation potentials at increasing work load. In attempts to solve this problem and to explain metabolic stability, two hypotheses have been proposed.

One of them considers that mitochondria and ATPases are linked to each other by specialised systems of energy transfer mediated by CK and AK isoenzymes, which ensure effective transfer of energy-rich phosphate groups from mitochondria to ATPases, in exact response to altered work load, but without fluctuations of adenine nucleotides in the cytoplasm.

The other hypotheses consider that ATPases and OXPHOS are regulated simultaneously by Ca^{2+} ion concentrations. This hypothesis is largely based on the studies of McCormack, Denton and Hansford, who demonstrated in more than 30 papers that PDHC, α-KGDH and ICDH can be regulated by matrix Ca^{2+} after its accumulation via the Ca^{2+}-uniporter. With the development of optical Ca^{2+} sensors it became clear beyond reasonable doubt that matrix Ca^{2+} concentrations change in parallel with cytosolic alterations of Ca^{2+}. This evidence was taken as proof of the mechanism whereby cytosolic Ca^{2+} is taken up by the mitochondria for the regulation of matrix dehydrogenases. In view of current evidence, the *intra*mitochondrial Ca^{2+} activation theory is limited because it ignores the evidence that mitochondrial processes can also be activated by Ca^{2+} ions independently of their accumulation in the matrix. Indeed, there exist at least three mitochondrial processes that can be regulated by *extra*mitochondrial Ca^{2+} ions. Besides the MgATP/Pi carrier there are two enzymes involved in shuttle mechanisms for transporting cytosolic reducing hydrogen (NADH) into the mitochondria: aralar/citrin and FAD-GPDH. The fact that the GPS is activated by *extra*mitochondrial Ca^{2+} was discovered 40 years ago. For several years it has been known that MAS activity is also regulated by Ca^{2+}_{cyt}, owing to the action of aralar. Now, we have observed that glutamate-dependent OXPHOS can be also up-regulated through the activation of aralar by *extra*mitochondrial Ca^{2+} ($S_{0.5} = 0.3$ µM Ca^{2+}). This activation – occurring also in the presence of saturating glutamate concentrations – increases the V_{max} of glutamate-dependent OXPHOS (+300%). In contrast, Ca^{2+} does not increase the V_{max} but rather decreases the K_M of PDH, ICDH, KGDH and FAD-GPDH.

Moreover, interactions with cytosolic Ca^{2+} have been described for the PTP, the Ca^{2+} uniporter and for the porin pores and, in less thoroughly explored state, for the F_0F_1ATPase and the cytochrome c oxidase.

The existence of regulatory Ca^{2+}-binding sites at the surface of mitochondria is important for the glucose-based substrate supply,

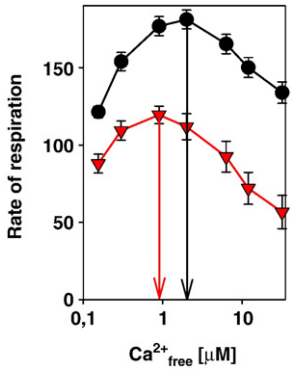

Fig. 4. Impaired mitochondrial function in isolated brain mitochondria of HD rats. The mitochondria were isolated from brains of 24-month-old htt_{51Q} (HD) and 18–27-month-old wild type (WT) rats. Respirometric investigation of WT (●) and HD (▼) mitochondria (0.06 mg mitochondrial protein/ml) were performed in 100 µM EGTA medium with 10 mM glutamate plus 2 mM malate as substrates. After addition of 2.5 mM ADP adjusting state $3_{glu/mal}$ six sequential additions by 20 µM Ca^{2+}_{total} were done increasing the Ca^{2+}_{free} from 150 nM 30 µM. Data are means ± S.E. of 15 (WT) 14 (HD) measurements. Red and black dashed arrows indicate the maximum rates at corresponding Ca^{2+}_{free} concentrations. All respiration rates of htt_{51Q} mitochondria were significantly lower than the corresponding rates of WT mitochondria ($P<0.01$). For further details see [63].

especially in the brain. The brain consumes mainly glucose but it definitely does not use fatty acids or ketone bodies (under normal conditions). Therefore the activity of both shuttles, the MAS and GPS controlled by *extra*mitochondrial Ca^{2+}, can change the hydrogen supply of brain mitochondria. Moreover, aralar can actively pump glutamate, a principal substrate, into brain mitochondria as fuel for Ca^{2+}-dependent dehydrogenases.

Interactions of mitochondriotoxic proteins such as htt_{expQ} with these regulatory Ca^{2+}-binding sites can play a role in the pathophysiology of neurodegenerative diseases by induction of energetic depression and cell death.

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