Stimulus-Secretion Coupling and Mitochondrial Metabolism in Steroid-Secreting Cells

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Ca2+ signal in high-Ca2+ perimitochondrial microdomains is amplified within the mitochondrial matrix and activates Ca2+-dependent dehydrogenases. In steroid-secreting cells, small cytoplasmic Ca2+ signals may also augment mitochondrial Ca2+ concentration. The ensuing formation of NADH and NADPH may have an essential role in supporting the increased steroid secretion.

It was assumed for several decades that the major role of mitochondria in Ca2+ metabolism was Ca2+ sequestration and hence the protection of the cell against Ca2+ intoxication. A previously unknown and perhaps more important role was indicated by the observations of Denton, McCormack, and coworkers (8), who found that the activity of pyruvate, isocitrate, and oxoglutarate dehydrogenases in permeabilized or homogenized mitochondria was enhanced by Ca2+. Two of these enzymes, pyruvate and oxoglutarate dehydrogenase, are half-maximally activated by Ca2+ in the concentration range between 10−7 and 10−6 M. In studies on mitochondrial suspensions, it was also observed that physiological fluctuations of mitochondrial Ca2+ uptake elicited changes in mitochondrial matrix Ca2+ concentration ([Ca2+]m) in that range, which regulates the matrix dehydrogenases (7). Extension of these observations to intact cells was rendered possible by single-cell fluorimetry. It was shown by two groups in 1992 (3, 9) that Ca2+ influx through voltage-gated Ca2+ channels was followed by Ca2+-dependent reduction of mitochondrial pyridine nucleotides [NADH plus NADPH, designated as NAD(P)H]. As reviewed (4), Ca2+ uptake through the mitochondrial membrane potential (about −180 mV inside). Half-maximal transport rate is attained in the range of 10−6–10−5 M Ca2+, and external Ca2+ exerts a positive cooperative effect with a Hill coefficient of 2. The major fraction of Ca2+, taken up into the matrix, is complexed with phosphate and ATP and bound to matrix proteins; therefore, Ca2+ uptake does not proportionally raise the intramitochondrial concentration of ionized Ca2+. Efflux of Ca2+ occurs by means of a Na+/Ca2+ and H+/Ca2+ antiporter. Maximal efflux rate in heart and liver mitochondria is at least two orders of magnitude lower than the maximal influx rate. There are no data available on the dependence of the efflux rate on the concentration of ionized Ca2+ in the matrix; it was reported, however, that the efflux mechanism saturates at mitochondrial [Ca2+] levels concurring with cytoplasmic [Ca2+] ([Ca2+]c) of ~0.5 μM. Since at such [Ca2+]c plasma membrane Ca2+ pump also transports at maximal rate, further elevations of [Ca2+]c will result in steeply increasing Ca2+ flux into the mitochondria. These considerations led to the general view that net mitochondrial Ca2+ uptake occurs only when [Ca2+]c exceeds the micromolar level. (In the case of high mitochondrial [Ca2+]c, mitochondria may rapidly lose ions and molecules smaller than 1,500 Da through the permeability transition pore; the activation of this efflux pathway, however, is not the subject of the present review.)

Several groups reported recently (reviewed in Ref. 15) that the global Ca2+ signal measured in the cytoplasm of the cell is the average of highly focused, short-lived elementary changes of [Ca2+]c, spatially limited by the diffusion of Ca2+ in the cytoplasm. Microdomains containing Ca2+ at high concentrations may exist in the vicinity of the sources of cytoplasmic Ca2+, and the uptake of Ca2+ by mitochondria and subsequent activation of metabolism is determined primarily by the distance of mitochondria from these sources. Although subplasmalemal mitochondria, localized near the orifice of Ca2+ channels, may also be exposed to high [Ca2+]c during channel activity, the development of high-Ca2+ microdomains around the Ca2+-storing endoplasmic reticulum may be especially effective.

High-Ca2+ microdomains and mitochondrial Ca2+ transport

As reviewed (4), Ca2+ uptake through the mitochondrial inner membrane takes place via a high-capacity, ruthenium red-sensitive uniporter. The main driving force is the mitochondrial membrane potential (about −180 mV inside). Half-maximal uptake rate is attained in the range of 10−6–10−5 M Ca2+, and external Ca2+ exerts a positive cooperative effect with a Hill coefficient of 2. The major fraction of Ca2+, taken up into the matrix, is complexed with phosphate and ATP bound to matrix proteins; therefore, Ca2+ uptake does not proportionally raise the intramitochondrial concentration of ionized Ca2+. Efflux of Ca2+ occurs by means of a Na+/Ca2+ and H+/Ca2+ antiporter. Maximal efflux rate in heart and liver mitochondria is at least two orders of magnitude lower than the maximal influx rate. There are no data available on the dependence of the efflux rate on the concentration of ionized Ca2+ in the matrix; it was reported, however, that the efflux mechanism saturates at mitochondrial [Ca2+] levels concurring with cytoplasmic [Ca2+] ([Ca2+]c) of ~0.5 μM. Since at such [Ca2+]c plasma membrane Ca2+ pump also transports at maximal rate, further elevations of [Ca2+]c will result in steeply increasing Ca2+ flux into the mitochondria. These considerations led to the general view that net mitochondrial Ca2+ uptake occurs only when [Ca2+]c exceeds the micromolar level. (In the case of high mitochondrial [Ca2+]c, mitochondria may rapidly lose ions and molecules smaller than 1,500 Da through the permeability transition pore; the activation of this efflux pathway, however, is not the subject of the present review.)

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because the vesicles may form close contacts with the interconnected network of mitochondria (12). The demonstration of high-Ca\textsuperscript{2+} microdomains between endoplasmic reticulum and mitochondria was in harmony with previous observations showing that Ca\textsuperscript{2+}, rapidly released from internal stores through the IP\textsubscript{3} receptor, was much more efficient in increasing mitochondrial [Ca\textsuperscript{2+}] (11) and mitochondrial NAD(P)H formation (14) than Ca\textsuperscript{2+} entering from the extracellular fluid. Since [Ca\textsuperscript{2+}] in these microdomains may attain a concentration of several micromoles (12), it certainly can activate mitochondrial Ca\textsuperscript{2+} uniporter despite its low Ca\textsuperscript{2+} affinity. Due to the positive cooperative effect of Ca\textsuperscript{2+} on the mitochondrial Ca\textsuperscript{2+} uniporter, the perimitochondrial Ca\textsuperscript{2+} spikes are transmitted into the mitochondrial matrix in an amplified manner and the ensuing increase in ATP production will subserve the biological response to the external stimulus.

Ca\textsuperscript{2+} signal facilitates cholesterol transport and NADPH formation in steroid-secreting cells

For stimulation-secretion coupling in steroid-producing cells, which are densely packed with mitochondria, it seems to be essential that Ca\textsuperscript{2+} signal activates mitochondrial metabolism. The mitochondrion is the particle at which the side chain of cholesterol is cleaved off to yield 21-carbon pregnenolone and at which all subsequent steroid hydroxylations of corticosteroid biosynthesis (with the exception of 17\alpha-hydroxylation) occur.

The rate-limiting step of steroid biosynthesis is the supply of cholesterol to the side chain cleaving cytochrome P-450 within the mitochondrial inner membrane. Side chain cleavage and the mitochondrial hydroxylation steps require NADPH, formed by the energy-linked nicotinamide nucleotide transhydrogenase from NADH as well as directly by isocitrate dehydrogenase.

At least two carriers may be involved in the transport of cholesterol from the cytoplasm into the mitochondria. One of them is a 37-kDa protein, the steroidogenic acute regulatory protein (StAR). In addition to being activated by cAMP (via protein kinase A), increased [Ca\textsuperscript{2+}], induces the expression and, via calmodulin-dependent kinase II, also the activation of StAR (1). StAR facilitates cholesterol transport by acting on the outer mitochondrial membrane. The activity of the other carrier, the 3.2-kDa steroidogenesis activator polypeptide (SAP), is claimed to facilitate the intermembrane transport of cholesterol. Its activity is enhanced by both Ca\textsuperscript{2+} and GTP. Intramitochondrial Ca\textsuperscript{2+} has also been suggested to facilitate the transport of cholesterol from the outer to the inner mitochondrial membrane (6); however, no data are available supporting a link between this action of intramitochondrial Ca\textsuperscript{2+} and the function of SAP or StAR.

Membrane-permeant cholesterol derivatives increase steroid production even in the absence of any Ca\textsuperscript{2+} signal. Several observations, however, suggest that increased reduction of pyridine nucleotides also enhances steroid hydroxylation. Classic biochemical studies (reviewed in Ref. 9) revealed that Krebs cycle intermediates support the reduction of pyridine nucleotides.

FIGURE 1. Cytoplasmic Ca\textsuperscript{2+} signal and mitochondrial NAD(P)H level in single glomerulosa cells. Effect of raising extracellular K\textsuperscript{+} concentration from 3.6 to 6.6 mM (top) and the effect of 300 pM angiotensin II (AII) and the subsequent chelation of Ca\textsuperscript{2+} with EGTA (bottom) on NAD(P)H signal and cytoplasmic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i) are shown. Both parameters were monitored by means of fluorimetry in cells preloaded with the Ca\textsuperscript{2+}-sensitive fluorescent dye fura 2. For NAD(P)H excitation wavelength was 360 nm, for Ca\textsuperscript{2+} 395 nm was applied, and the emitted light was measured at 470 nm. (The original records of fluorescence at 395-nm excitation wavelength are shown upside-down for convenience.) Reproduced from Ref. 13 with permission.

FIGURE 2. [Ca\textsuperscript{2+}], and NAD(P)H signal of a large luteal cell evoked by the sarco/endoplasmic reticular (SERCA)-type Ca\textsuperscript{2+}-ATPase inhibitor thapsigargin. Top: [Ca\textsuperscript{2+}], changes in fura-PE3-loaded cells during application of thapsigargin in nominally Ca\textsuperscript{2+}-free medium and the effect of readdition of 1.2 mM Ca\textsuperscript{2+} to the extracellular solution. The first Ca\textsuperscript{2+} signal, observed under Ca\textsuperscript{2+}-free conditions, was brought about by Ca\textsuperscript{2+} release from the endoplasmic reticulum, whereas the second signal, induced by the readdition of Ca\textsuperscript{2+}, reflects capacitative Ca\textsuperscript{2+} influx. Bottom: effect of the same protocol of NAD(P)H, monitored by autofluorescence without dye loading. Reproduced from Ref. 15, with permission.
nucleotides and steroid hydroxylation. In ACTH-stimulated adrenocortical cells, the steroid production rate correlates with the activity of the Krebs cycle. Rotenone, a drug that evokes the accumulation of reduced pyridine nucleotides by inhibiting the mitochondrial electron transport chain, enhances the side chain cleavage of cholesterol by isolated bovine adrenocortical mitochondria.

As demonstrated with online fluorimetric measurements in rat adrenal glomerulosa cells (9, 13), the K+-evoked Ca2+ signal is instantly followed by reduction of mitochondrial pyridine nucleotides (Fig. 1). When K+ concentration was raised from the physiological resting level of 3.6 mM to 6.6 mM, half-maximal NAD(P)H fluorescence followed half-maximal Ca2+ signal with a lag of ~2 s. The pattern of [Ca2+]i, oscillation induced by angiotensin II (Fig. 1) or vasopressin corresponds to that of mitochondrial NAD(P)H oscillation (10, 13). A similar relationship between [Ca2+]i and the reduction of pyridine nucleotides has been observed in rat ovarian luteal cells stimulated with prostaglandin F2α (15). (In both cell types, the mitochondrial NAD(P)H signal had a slowly rising phase only (as opposed to the Ca2+ signal) indicates that no high-Ca2+ microdomain was formed around the mitochondria. Capacitative Ca2+ influx significantly accelerates the accumulation of NAD(P)H induced by the subsequent application of rotenone, showing that the observed change in mitochondrial redox state is in fact brought about by enhanced dehydrogenase activity (15).

Low submicromolar Ca2+ signal is also transferred into the mitochondrial matrix

The fact that cytoplasmic Ca2+ in the low submicromolar concentration range can activate mitochondrial dehydrogenases challenges the concept that [Ca2+]i, has to attain micromolar levels to increase mitochondrial [Ca2+]. Although the characteristics of mitochondrial Ca2+ transport mechanisms do not exclude the possibility that smaller Ca2+ signals are also transferred to the mitochondria, attention has hitherto been focused on the effect of supramicromolar Ca2+ signals. Mitochondrial matrix fails to raise global [Ca2+]i above 300 nM, yet NAD(P)H level is increased (14). In luteal cells (15), capacitative Ca2+ influx induces a steep Ca2+ signal, which, however, does not exceed 200 nM. Nevertheless, even this small Ca2+ signal raises mitochondrial NAD(P)H level (Fig. 2). The fact that the ensuing NAD(P)H signal had a slowly rising phase only (as opposed to that of the Ca2+ signal) indicates that no high-Ca2+ microdomain was formed around the mitochondria. Capacitative Ca2+ influx significantly accelerates the accumulation of NAD(P)H induced by the subsequent application of rotenone, showing that the observed change in mitochondrial redox state is in fact brought about by enhanced dehydrogenase activity (15).

![FIGURE 3. Effect of stepwise increases in extramitochondrial [Ca2+] on mitochondrial matrix [Ca2+]i in a single permeabilized rat luteal cell (top) and a glomerulosa cell (bottom) incubated in a cytosolic solution. The ordinate shows the fluorescence intensity of the Ca2+-sensitive dye rhod 2 related to fluorescence at 50 and 100 nM [Ca2+]i, respectively. (Top reproduced from Ref. 15, with permission. Bottom shows unpublished results of J. G. Pitter, G. Szabadkai, and A. Spät.)](http://physiologyonline.physiology.org/)

![FIGURE 4. Major actions of Ca2+ on a steroid-producing cell. Ca2+ signal in the cytoplasm activates steroidogenic acute regulatory protein (StAR) and thus enhances the transfer of cholesterol to side chain-cleaving cytochrome P450 (P450) in the inner mitochondrial membrane. Ca2+ is transported into the mitochondrial matrix by a Ca2+ uniporter (up.), enhancing cholesterol uptake and the formation of NADH and NADPH. NADPH, also formed from NADH by nicotinamide nucleotide transhydrogenase (TH), supports reducing equivalents to P450 via the adrenodoxin reductase/adrenodoxin (AD) system. P450 converts cholesterol to pregnenolone. Mitochondrial NADPH is also utilized for the conversion of deoxycorticosterone to aldosterone and deoxycortisol to cortisol (not shown).](http://physiologyonline.physiology.org/)
Chondrial [Ca\textsuperscript{2+}] was usually monitored with aequorin, genetically targeted into the mitochondria. This photoprotein, because of its low affinity for Ca\textsuperscript{2+}, is not appropriate for measuring [Ca\textsuperscript{2+}] in the resting or moderately increased range. More recently, Colegrove et al. (2) estimated mitochondrial Ca\textsuperscript{2+} uptake and release as a function of [Ca\textsuperscript{2+}], in sympathetic neurons. They measured the rate at which [Ca\textsuperscript{2+}], declines after depolarization-induced high-Ca\textsuperscript{2+} microdomains in the subplasmalemmal region may have already dissipated. Their results indicated that net Ca\textsuperscript{2+} uptake takes place at [Ca\textsuperscript{2+}] < 300 nM, i.e., at levels much lower than previously thought.

Mitochondrial matrix [Ca\textsuperscript{2+}] was directly monitored in our lab with the application of rhod 2, a Ca\textsuperscript{2+}-sensitive fluorescent dye that, because of its positive charges, accumulates in mitochondria. After permeabilizing the plasma membrane, we could remove any residual dye from the cytoplasm and could precisely adjust the extramitochondrial [Ca\textsuperscript{2+}] with Ca\textsuperscript{2+} buffers. The mitochondria of both luteal cells (15) and glomerulosa cells also responded to elevation of extramitochondrial [Ca\textsuperscript{2+}] in the low submicromolar concentration range (Fig. 3) with a sensitivity at least as great as that observed in sympathetic neurons. When extramitochondrial [Ca\textsuperscript{2+}] was raised from 50 to 180 nM in luteal and from 100 to 300 nM in glomerulosa cells, there was a significant increase in mitochondrial Ca\textsuperscript{2+}-rhod fluorescence. This means that, in steroid-producing cells, Ca\textsuperscript{2+} signals just above the resting level are already transmitted into the mitochondrial matrix. This observation, which may also be valid in other, hitherto unstudied cell types, challenges the exclusive role (but obviously not the efficiency) of high-Ca\textsuperscript{2+} microdomains in activating mitochondrial metabolism.

Another property of steroid-producing cells is the formation of an oscillatory as well as a sustained Ca\textsuperscript{2+} signal, depending on the stimulus applied. In glomerulosa cells, oscillating Ca\textsuperscript{2+} and NAD(P)H signals are induced by angiotensin II (Fig. 1) or vasopressin applied at a physiological concentration. Angiotensin II at a pharmacological concentration induces a sustained Ca\textsuperscript{2+} signal, which, in turn, evokes a sustained NAD(P)H signal (13). Prolonged stimulation with K\textsuperscript{+} (>5 min) also elicits sustained Ca\textsuperscript{2+} and NAD(P)H signal, and the latter lasts at least as long as the stimulus (9, 13). Sustained rise in extramitochondrial [Ca\textsuperscript{2+}] induces sustained rise in mitochondrial Ca\textsuperscript{2+} and NAD(P)H level in permeabilized cells as well (13). This response of glomerulosa mitochondria contrasts with the behavior of mitochondria in hepatocytes, in which only oscillatory Ca\textsuperscript{2+} signal but not sustained rise in [Ca\textsuperscript{2+}], can induce sustained mitochondrial Ca\textsuperscript{2+} and NAD(P)H response (5). The different coupling between [Ca\textsuperscript{2+}], and mitochondrial metabolism probably represents an adaptation to physiological demands: in hepatocytes all Ca\textsuperscript{2+}-mobilizing agonists induce an oscillating signal, whereas in glomerulosa cells an important physiological stimulus (extracellular K\textsuperscript{+}) induces a sustained Ca\textsuperscript{2+} signal. The ability of glomerulosa cells to maintain increased NADPH formation may contribute to the hypersecretion of aldosterone during hyperkalemia, a basic mechanism of vertebrate homeostasis.

The major actions of Ca\textsuperscript{2+} in mediating the effect of stimulatory agents on steroid secretion are summarized in Fig. 4. Cytoplasmic Ca\textsuperscript{2+} signal induces and activates StAR, which facilitates the transport of cholesterol into the mitochondria. The cytoplasmic Ca\textsuperscript{2+} signal is efficiently transmitted into the mitochondrial matrix. The ensuing mitochondrial Ca\textsuperscript{2+} response also promotes the transport of cholesterol to the site of side chain cleavage and activates Ca\textsuperscript{2+}-dependent dehydrogenases. The increased formation of NADPH yields more ATP, obviously required for the maintenance of the intracellular ionic composition, whereas NADPH, formed at the expense of NADH, is utilized for the enhanced steroid production. The stimulus-induced Ca\textsuperscript{2+} signal, by virtue of its effect on both the substrate supply of steroid synthesis and the redox state of the mitochondria, will thus enhance steroid secretion by two major mechanisms.

We apologize that several important reports on the present subject could not be cited because of editorial restrictions on the number of references allowed.

The work in our laboratory was supported by the Hungarian National Science Foundation (OTKA), the Hungarian Council for Medical Sciences, and the Hungarian Academy of Sciences (MTA-AKP).

References