Stimulus-Secreion Coupling and Mitochondrial Metabolism in Steroid-Secreing Cells

András Spät,1 János G. Pitter,1 Tibor Rohács,2 and György Szabadkai1
1Department of Physiology, Faculty of Medicine, Semmelweis University, H-1444 Budapest, Hungary; and 2Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York, New York 10029

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.

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 transport rate is attained in the range of 10−4–10−6 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+] of ~0.5 μM. Since at such [Ca2+], the plasmalemmal Ca2+ pump also transports at maximal rate, further elevations of [Ca2+] will result in steeply increasing Ca2+ flux into the mitochondria. These considerations led to the general view that net mitochondrial Ca2+ uptake occurs only if [Ca2+] exceeds the micromolar level. (In the case of high mitochondrial [Ca2+], 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+], 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 subplasmalemmal mitochondria, localized near the orifice of Ca2+ channels, may also be exposed to high [Ca2+] during channel activity, the development of high-Ca2+ microdomains around the Ca2+-storing endoplasmic reticulum may be especially effective,
because the vesicles may form close contacts with the interconnected network of mitochondria (12). The demonstration of high-Ca²⁺ microdomains between endoplasmic reticulum and mitochondria was in harmony with previous observations showing that Ca²⁺, rapidly released from internal stores through the IP₃ receptor, was much more efficient in increasing mitochondrial [Ca²⁺] (11) and mitochondrial NAD(P)H formation (14) than Ca²⁺ entering from the extracellular fluid. Since [Ca²⁺] in these microdomains may attain a concentration of several micromoles (12), it certainly can activate mitochondrial Ca²⁺ uniporter despite its low Ca²⁺ affinity. Due to the positive cooperative effect of Ca²⁺ on the mitochondrial Ca²⁺ uniporter, the perimitochondrial Ca²⁺ 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.

Ca²⁺ 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 Ca²⁺ 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 17α-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 steriodogenic acute regulatory protein (STAR). In addition to being activated by cAMP (via protein kinase A), increased [Ca²⁺], 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 Ca²⁺ and GTP. Intramitochondrial Ca²⁺ 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 Ca²⁺ and the function of SAP or STAR.

Membrane-permeant cholesterol derivatives increase steroid production even in the absence of any Ca²⁺ 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.
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 F₂₀ (15). (In both cell types, the mitochondrial origin of the NAD(P)H response has been confirmed by the application of electron transport chain inhibitors.) In K⁺-stimulated rat glomerulosa cells, the pharmacological inhibition of steroid synthesis significantly retards the reoxidation of NAD(P)H after the termination of the Ca²⁺ signal (14). This observation, in accordance with previous data on the enhancement of steroid production by NADPH, suggests that NADPH, formed in excess during Ca²⁺ signaling, is utilized for steroid synthesis.

As measured in bovine glomerulosa cells with aequorin targeted to mitochondria, the [Ca²⁺]ᵢ response to angiotensin II was severalfold amplified within the mitochondria (see Ref. 1). Nevertheless, it should be recalled that the mitochondrial Ca²⁺ and NAD(P)H responses cannot always be attributed to the formation of high-Ca²⁺ microdomains. Although such microdomains may be built up between the IP₃ receptors and the mitochondria during the peak phase of IP₃-induced Ca²⁺ signal, they need not be formed around the mitochondria during Ca²⁺ influx from the extracellular space. In glomerulosa cells, Ca²⁺ influx induced by the store-operated (capacitative) mechanism 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).

**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 $[\text{Ca}^{2+}]$ was directly monitored in our lab with the application of rhod 2, a $\text{Ca}^{2+}$-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 $[\text{Ca}^{2+}]$ with $\text{Ca}^{2+}$ buffers. The mitochondria of both luteal cells (15) and glomerulosa cells also responded to elevation of extramitochondrial $[\text{Ca}^{2+}]$ in the low submicromolar concentration range (Fig. 3) with a sensitivity at least as great as that observed in sympathetic neurons. When extramitochondrial $[\text{Ca}^{2+}]$ 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 $\text{Ca}^{2+}$-rhod fluorescence. This means that, in steroid-producing cells, $\text{Ca}^{2+}$ 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-$\text{Ca}^{2+}$ microdomains in activating mitochondrial metabolism.

Another property of steroid-producing cells is the formation of an oscillatory as well as a sustained $\text{Ca}^{2+}$ signal, depending on the stimulus applied. In glomerulosa cells, oscillating $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$ signal, which, in turn, evokes a sustained NAD(P)H signal (13). Prolonged stimulation with $K^+$ (>5 min) also elicits sustained $\text{Ca}^{2+}$ and NAD(P)H signal, and the latter lasts as long as the stimulus (9, 13). Sustained rise in extramitochondrial $[\text{Ca}^{2+}]$ induces sustained rise in mitochondrial $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$ signal but not sustained rise in $[\text{Ca}^{2+}]$, can induce sustained mitochondrial $\text{Ca}^{2+}$ and NAD(P)H response (5). The different coupling between $[\text{Ca}^{2+}]$ and mitochondrial metabolism probably represents an adaptation to physiological demands: in hepatocytes all $\text{Ca}^{2+}$-mobilizing agonists induce an oscillating signal, whereas in glomerulosa cells an important physiological stimulus (extracellular $K^+$) induces a sustained $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$ in mediating the effect of stimulatory agents on steroid secretion are summarized in Fig. 4. Cytoplasmic $\text{Ca}^{2+}$ signal induces and activates StAR, which facilitates the transport of cholesterol into the mitochondria. The cytoplasmic $\text{Ca}^{2+}$ signal is efficiently transmitted into the mitochondrial matrix. The ensuing mitochondrial $\text{Ca}^{2+}$ response also promotes the transport of cholesterol to the site of side chain cleavage and activates $\text{Ca}^{2+}$-dependent dehydrogenases. The increased formation of NADH 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 $\text{Ca}^{2+}$ 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.

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References