Biochemistry (Moscow), Vol. 70, No. 2, 2005, pp. 187-194. Translated from Biokhimiya, Vol. 70, No. 2, 2005, pp. 231-239.

REVIEW


A Historical Review of Cellular Calcium Handling, with Emphasis on Mitochondria

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Received September 15, 2004

Abstract—Calcium ions are of central importance in cellular physiology, as they carry the signal activating cells to perform their programmed function. Ca^{2+} is particularly suitable for this role because of its chemical properties and because its free concentration gradient between the extra-cellular and the cytosolic concentrations is very high, about four orders of magnitude. The cytosolic concentration of Ca^{2+} is regulated by binding and chelation by various substances and by transport across plasma and intracellular membranes. Various channels, transport ATPases, uniporters, and antiporters in the plasma membrane, endoplasmic and sarcoplasmic reticulum, and mitochondria are responsible for the transport of Ca^{2+}. The regulation of these transport systems is the subject of an increasing number of studies. In this short review, we focus on the mitochondrial transporters, i.e. the calcium uniporter used for Ca^{2+} uptake, and the antiporters used for the efflux, i.e. the Ca^{2+}/Na^{+} antiporter in mitochondria and the plasma membrane of excitable cells, and the Ca^{2+}/nH^{+} antiporter in liver and some other mitochondrial types. Mitochondria are of special interest in that Ca^{2+} stimulates respiration and oxidative phosphorylation to meet the energy needs of activated cells. The studies on Ca^{2+} and mitochondria began in the fifties, but interest in mitochondrial Ca^{2+} handling faded in the late seventies since it had become apparent that mitochondria in resting cells contain very low Ca^{2+}. Interest increased again in the nineties also because it was discovered that mitochondria and Ca^{2+} had a central role in apoptosis and necrosis. This is of special interest in calcium overload and oxidative stress conditions, when the opening of the mitochondrial permeability transition pore is stimulated.

Key words: apoptosis, calcium, channels, endoplasmic reticulum, mitochondria, permeability transition, plasma membrane, sarcoplasmic reticulum

This article is based on a lecture presented at a recent Calcium Symposium [1]. Both authors have had long experience in the field. Saris was introduced to mitochondriology by Britton Chance at the Reeves Johnson Foundation, University of Pennsylvania, Philadelphia, in 1958-59. In the last weeks of his stay Saris discovered that a pH gradient was formed on addition of Ca^{2+} (Ca) to an energized mitochondrial suspension, followed by its equilibration when a certain threshold was exceeded. The formation of the gradient was attributed to the energized uptake of Ca, and the later equilibration to the permeabilization of the inner membrane (now called the mitochondrial permeability transition) ([2, 3], see also review [4]). Now it has become clear that the acidification of the medium on uptake of Ca was due to a chemiosmotic mechanism in which the uptake was driven by the membrane potential, whereby part of the H^{+} pumped out by the respiratory chain remained in the medium [5].

Carafoli was trained in mitochondriology in Giovanni Felice (Licio) Azzone's group at the University of Modena [6] and initiated Ca work on mitochondria in the mid 60s, in the group of Albert Lehninger at the Johns Hopkins School of Medicine in Baltimore, MD (see [4]).

In this review, we'll offer a succinct discussion of the binding of Ca by proteins, and of its transport through biological membranes; we will also discuss briefly its biological functions. Addition of Ca pulses to respiring rat liver mitochondria led to the formation of a pH gradient.

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BINDING AND CHELATION OF CALCIUM IONS

The chemical properties of Ca and their role in the binding of complex molecules have been discussed and compared to those of Mg^{2+} (Mg) and other cations by R. J. P. Williams in several reviews [7-10]. Ca is bound preferentially by multidentate anions and strong acid anions, while Mg has strongest binding to neutral nitrogen groups such as $-NH_2$ and imidazole [7]. Ca is generally bound by proteins more strongly than Mg [8, 9]. Importantly, Ca has far more binding flexibility, i.e., greater variability is tolerated in the number, angle, and distances of its coordination bonds, which makes it easier for dehydrated Ca to permeate membranes and reach binding sites deeper inside proteins [7]. The binding of Ca to individual proteins of biological interest has been discussed in a number of reviews [10, 11]. Some of these are contractile proteins, other are regulatory, or involved in transport processes. An early description of Ca binding to a muscle protein (parvalbumin), as derived from its X-ray structure, has been fundamental [12].

The chelation of Ca and Mg to nucleotides, especially ATP and ADP, is physiologically important. The association constant of Ca to ATP is 4.0, for Mg the affinity is slightly stronger (4.2), while the constants are about two orders of magnitude less for ADP [7]. This means that ATP effectively buffers both Ca and Mg concentrations and that the Mg–ATP chelate is the natural substrate for ATPases and phosphotransferases. Hydrolysis of ATP thus leads to a substantial increase in the free—often called ionized—Ca and Mg.

In most cells the plasma membrane is virtually impermeable for Ca, separating two spaces with a large concentration difference: the free concentration of Ca^{2+} is about 1.2 mM extracellularly and close to 0.1 μM in the cytosol of non-activated cells. This makes Ca an ideal signal substance: even if only traces of it enter the cell through the opening of Ca channels, they still change the free concentration significantly, especially close to the inner channel mouth.

TRANSPORT OF Ca ACROSS PLASMA MEMBRANES

Ca absorption takes place in the first part (abomasum) of the small intestine. A vitamin D-inducible, Ca-binding protein suggested to be involved in the transcellular transport of Ca in the mucosa was described in the mid-60s (see review [13]). The protein was suggested to have a role in the accumulation of Ca close to a plasma membrane Ca transporter.

Plasma membrane channels. The main mechanism for the import of Ca into cells is via opening of Ca channels. The opening is usually of short duration. Many different Ca channels have been described, this being an intensive area of research. A voltage-gated channel was implied by action potentials recordings in crayfish muscle fibers in 1958 [14]. Its conductance is around 20 pS, allowing the passage of about $3 \cdot 10^6$ Ca ions/sec. The channel is present in the heart and in much higher density in the T-tubular membranes of skeletal muscles (see review [11]). Several types of voltage-gated channels have been described that differ in sensitivity to inhibitors (verapamil, diltiazem, and dihydropyridines) and activators (some dihydropyridines and adrenergic neurotransmitters), rate constants, and opening probabilities [11]. The most common channel of this family is the L-type channel with relatively long open times, while the T-type channels open at more negative transmembrane potentials, and the N-type at still more negative potentials.

A channel that has sparked considerable recent interest is the capacitive calcium entry channel, which is activated by the emptying of Ca stores in the sarcoplasmic and endoplasmic reticulum (SR and ER) [15]. It has been found in many cell types (see review [16]). The interaction with the Ca store state is treated in more detail in the SR and ER section below.

The Na^+/Ca^{2+} exchanger was found in the late 1960s to be responsible for most of the efflux of Ca [17-19]. Depending on the concentrations of these cations in the cytosol and external medium and on the direction of the transmembrane potential, Ca can be also taken up by exchange against relatively high internal Na^+ [19]. The exchanger operates with a stoichiometry of 3 Na^+/1 Ca^{2+} [19-21]. Of special interest is the possibility of stimulating the Ca uptake mode of the exchanger in heart ischemia–reperfusion, where Na^+ accumulates in the cytosol during the ischemia, and promotes Ca uptake during the reperfusion phase, causing Ca overload [22, 23].

The Ca-ATPase is necessary to reduce cytosolic [Ca] to the low (100-200 nM) resting levels, which is achieved using the energy of ATP. It was first described in erythrocytes in 1966 by Schatzmann [24]. This pump has high affinity for Ca with a $K_m < 1$ μM, but rather low transport capacity (about 0.5 nmol Ca/mg protein per second [25]). The enzyme is activated by calmodulin [26] (possibly not in liver [27]), but also by acidic phospholipids and unsaturated long-chain fatty acids. Various hormones, i.e., thyroxin, oxytocin, and insulin, have been claimed to inhibit the pump, depending on the tissue and experimental conditions (see reviews [11, 28]).

TRANSPORT OF Ca BY SARCOPLASMIC AND ENDOPLASMIC RETICULUM

Uptake of Ca by microsomes derived from what was later called sarcoplasmic reticulum (SR) of skeletal muscles was described by Ebashi and Lipman [29] and by Hasselbach and Makinose [30, 31] in the beginning of the

1960s. The process required ATP and Mg and was mediated by a **transport Ca-ATPase** of the P-class as that of the plasma membrane [11, 28]. It is now called Ca-ATPase type I and is present in fast-twitch skeletal muscles, while the type II ATPase is found in slow-twitch muscles and a subform of that in other cells [32]. Some types of cells may contain both types of the pump [33]. In the presence of oxalate, large amounts of Ca are taken up, and Ca-oxalate precipitates could be demonstrated in the SR *in vivo* [34]. The role of SR as a Ca store has now been confirmed by numerous studies. The SR is able to reduce the external [Ca] well below 1 μM, which is sufficient to remove Ca from troponin C and cause relaxation of the muscle fibers. The Ca uptake capacity of the ER is increased by various storage proteins that bind Ca with low affinity, i.e. calsequestrins, calreticulins, and endoplasmins.

The ATPase has been purified and reconstituted: it was found that maximally two Ca ions are transported per one ATP molecule hydrolyzed [35]. The ATPase activity can be hormonally regulated via phosphorylation of the proteolipid phospholambdan, present in the SR membrane of slow, heart, and smooth muscles in 1 : 1 stoichiometry with the ATPase [36]. The phosphorylation is carried out by Ca-calmodulin- and cAMP-dependent kinases that phosphorylate different sites [37]. The three-dimensional structure of the pump has recently been solved at atomic resolution [38, 39].

Ca efflux channels. For the release of Ca from the stores on appropriate signals two channels have been described: one is activated by inositol-1,4,5-trisphosphate (IP_3) formed by hydrolysis of phosphatidylinositol-*bis*-phosphate (see reviews [11, 40]), the other is the ryanodine receptor channel, which is activated by increased cytosolic Ca, by caffeine, or by ryanodine (see reviews [11, 41]). The number and kind of channels varies in different cell types. Thus, the Ca stores may differ in localization and mode of excitation–contraction or –mobilization coupling. In muscle, the depolarization of sarcolemma is transmitted to the terminal cisternae of the sarcoplasmic reticulum via invaginations and junctions (T-tubules).

MITOCHONDRIAL CALCIUM HANDLING

Uptake and binding of Ca. In the 1950s-1960s, it was first found that Ca and other bivalent cations were taken up by respiring mitochondria or in the presence of ATP [4]. Even earlier Slater and Cleland [42] found that heart mitochondria (called sarcosomes at that time) took up Ca from the medium during homogenization and isolation and became partly uncoupled. By adding EDTA, this was prevented and well-coupled mitochondria could be prepared. However, Slater and Cleland concluded that the uptake was not active, as it took place at 0°C. Binding of Mn^{2+} and inorganic phosphate (P_i) was found to occur in respiring mitochondria [43] with the formation of a pH gradient [3, 44], and uptake of Sr^{2+} was also observed in the presence of ATP [45]. As mentioned, it was not initially accepted that the uptake was energy-driven, but Britton Chance observed a stoichiometric burst of respiration on addition of small amounts of Ca, which was similar to that seen on addition of ADP [46]. He concluded that it was due to the active transport of Ca. This was confirmed by Saris [2, 3] as noted in the introductory paragraph. Massive accumulation of Ca by respiring liver and kidney mitochondria or in the presence of Mg and ATP was observed in the beginning of the 60s [47, 48] and Rossi and Lehninger found that Ca-phosphate precipitated in the mitochondrial matrix with the stoichiometry of hydroxyapatite [49]. Under these conditions, ADP/ATP uptake was also observed, which Carafoli et al. interpreted as being due to absorption on the hydroxyapatite deposits [50]. Saris also found increased Ca accumulation in the presence of P_i and added ADP [3]. The effect of ADP could at least partly have been due to the increase in negative charges on the matrix side, leading to the decrease in matrix [Ca], and to the dissociation of Ca from the transporter, which would stimulate the rate of uptake and increase the retention of Ca [51]. Saris [3] found a stoichiometry of uptake of 0.7 Ca/ATP hydrolyzed, and Rossi and Lehninger [49] found that the stoichiometry of extra O_2 consumed in the Ca stimulated respiration was on a mole basis half that of ADP. For Sr^{2+}, that is transported with the same mechanism, 1.8-2.0 atoms were transported per electron pair passing each coupling site in the respiratory chain [52].

The Ca uptake mechanism. In the beginning, little information was available on the mechanism of uptake. Saris [3] considered various exchange mechanisms, i.e. $Ca^{2+}/2H^+$, $Ca^{2+}/H^+,K^+$, and exchange of H^+ against Ca^{2+} plus an anion like P_i^-, as well as a pump driven by the hypothetical high energy bonds (~) which in those days were supposed to be formed by energy conservation in the respiratory chain. Chance suggested fast interaction of Ca with a carrier at the expense of these bonds [53]. When the chemiosmotic hypothesis became popular, an uptake mechanism driven by the proton-motive force (a membrane potential) became a natural choice [5]. Gradually, this view became accepted, together with the Mitchellian terms *uniport* for uptake of an ion alone, *antiport* and *symport* for the exchange and cotransport mechanisms. The Ca transporter was then called the *calcium uniporter*.

A uniport mechanism would imply a $2H^+/Ca^{2+}$ stoichiometry [5]. Experimentally, this was not easy to show, since other proton-producing, consuming, and translocating mechanisms had to be excluded. In the end of the 1970s, this stoichiometry was finally established (see review [54]) in the groups of Lehninger [55] and of Saris [56]. When the membrane potential was formed as a diffusion potential for K^+ in the presence of the ionophore

valinomycin, a corresponding stoichiometry of $2K^+/Ca^{2+}$ was found [57, 58].

Purification of the uniporter. The isolation of the Ca uniporter would be essential for the understanding of the mechanism of uptake, i.e., whether it is a carrier or a channel-forming system. Purification of the uniporter has proven difficult and has not yet been accomplished. Early attempts in the beginning of the 1970s in Sottocasa's group produced a Ca-binding glycoprotein from mitochondria suspended in a hypotonic medium in the presence of EDTA. Its Ca-binding was inhibited [59] by the uniporter inhibitor ruthenium red [59]. Antibodies against the protein also inhibited Ca uptake [60]. However, Saris found that the antiserum formed four precipitation bands in Ouchterlony immunodiffusion tests and did not inhibit Sr^{2+} uptake by the uniporter. A protein of 13-14 kD having an isoelectric point of 3.4 was eventually purified from the glycoprotein preparation and called *calvectin* [61]. In 1982, Mironova et al. also isolated a Ca-binding glycoprotein with a molecular mass of 40 kD from beef heart mitochondria and obtained from it a small hydrophobic peptide that formed ruthenium red-sensitive Ca channels when reconstituted in phospholipid black-lipid membranes [62]. Saris tested antibodies against the preparation, obtaining in this case only one precipitation band. The antibodies inhibited the Ca uniporter in rat liver mitochondria deprived of the outer membrane (mitoplasts) and stained mitochondria in fibroblasts [63]. Further purification of the small peptide yielded a product with an apparent molecular mass of only 2 kD [64], which may be a hydrolytic product since inhibitors of proteases were not employed in the purification. The channel formed was Ca-specific, two or more subunits participating in channel formation [65]. Attempts to sequence the peptide and to clone its gene are in process. The absorption of the peptide by Sephadex G-15 indicates affinity for carbohydrates and may explain its association with a glycoprotein.

Recently other groups have also attempted to purify and characterize the Ca uniporter [66, 67]. Zazueta et al. used isoelectric focusing and affinity chromatography with the labeled inhibitor ^{103}Ru360 to specifically locate the Ca uniporter and obtained a 90% purified fraction [67]. An antiserum against a less purified fraction [66] inhibited Ca uptake into phospholipid vesicles reconstituted with the preparation. It labeled 18 and 75 kD fractions. By patch-clamping a Ca-specific channel was characterized in mitoplasts [67]. It conducted Sr^{2+} as well as Ca, but the conductances for Mn^{2+} and Ba^{2+} were much lower. Ruthenium red and Ru360 inhibited, as could be expected for the Ca uniporter.

Other Ca uniport mechanisms. The Ca uptake described above concerns mammalian and plant mitochondria. However, Zvyagilskaya's group has found that fast Ca uptake by mitochondria in the yeast *Endomyces magnusii* [68] is mediated by a uniporter, but is stimulated (!) by ruthenium red [69]. Polyclonal antibodies against the purified beef heart mitochondrial fraction also reacted with a yeast protein fraction. The fast mode of Ca uptake that has been found in heart [70] and liver [71] mitochondria could correspond to it; however, in heart and liver ruthenium red inhibits only at relatively high concentrations.

Regulation of the Ca uniporter. Interest in mitochondrial Ca handling was substantial in the 1960s and 1970s, after it was found that mitochondria acted as Ca sinks, accumulating Ca as hydroxyapatite [4]. However, interest decreased when it was found that mitochondria in resting cells contained little Ca [72] and that the apparent K_m of the mitochondrial system was too high for significant Ca uptake to occur in the cytosol [73]. More recently, it was found that some mitochondria may be located in microdomains close to Ca channels of the SR and ER where cytosolic [Ca] may become high on channel opening [74]. Modulating factors have been found that may activate the uniporter by decreasing its K_m for Ca. Ca itself may be the most important activator, changing the concentration dependence of the uptake shift from sigmoidal to hyperbolic, thereby lowering the K_m [75-77]. Ca also stimulates Sr^{2+} and Mn^{2+} uptake [78]. Mn^{2+} can inhibit or stimulate Ca uptake depending on the experimental conditions, but stimulation has been mostly reported [78-80]. Mg^{2+} and K^+ decrease Ca uptake, presumably by decreasing Ca surface binding and making the kinetics more sigmoidal [81-83]. K^+ acts also under conditions of opening of the ATP-sensitive K^+ channels, thereby decreasing the membrane potential [84]. Polyamines were found to activate the uniporter both in mammalian mitochondria [85, 86] and in those of *Endomyces magnusii* [87].

Efflux of Ca from mitochondria. As expected accumulated Ca is released from mitochondria by protonophorous uncoupler [88], due to reversal of the uniporter when the membrane potential is collapsed. In the presence of ruthenium red to inhibit the uniporter, efflux of Ca from heart mitochondria was found to be induced by Na^+ [89]. Under these conditions, the efflux was stimulated by respiration possibly due to a Ca^{2+}/nNa^+ antiporter with n exceeding 2 [90]. In the absence of ruthenium red, matrix Ca would be influenced by a Ca cycle in which Ca is taken up by the uniporter and released by the Ca^{2+}/nNa^+ antiporter, Na^+ being eventually returned to the cytosol by a Na^+/H^+ antiporter [91]. This cycling implies some energy dissipation, i.e., it stimulates respiration. The Na^+/Ca^{2+} antiporter was found to operate in mitochondria of excitable cells, i.e., muscle and neurons (see review [92]).

In non-excitable cells, the main Ca efflux system is thought instead to be a Ca^{2+}/nH^+ antiporter [92], which cannot be demonstrated in non-energized mitochondria, indicating that it is not electroneutral [93, 94]. A preparation obtained by Ca-affinity chromatography, when

reconstituted into liposomes, had Ca^{2+}/H^+ antiporter activity. Antibodies reacted with bands at 55 and 66 kD [95].

The permeability transition. The permeabilization induced by Ca overloading, originally reported by Saris [2, 3], is now known to be due to opening of a large pore in the inner membrane, which is called the mitochondrial permeability transition (MPT) (see reviews [11, 92, 96]). The opening of the pore will allow the transit of substances with a molecular mass <1.5 kD, i.e. nucleotides are also lost from the matrix so that respiration on NAD-dependent substrates is inhibited. Electron microscopy studies have shown that the mitochondrial population is heterogeneous and the swelling associated with MPT progressed through the population [97]. In addition to the stimulation by Ca and P_i, and to the inhibition by Mg^{2+} and ADP shown by Saris, it is now known [96] that the MPT is promoted by prooxidants and free radicals, by thiol reagents and promoters of S–S bond formation, by decreased membrane potential, by carboxyatractylate, fatty acids and increase in pH, while antioxidants, radical scavengers like butylhydroxytoluene, Mn^{2+}, spermine, lowered pH, and Ca-chelating substances inhibit it. Thus, there seems to be a Me^{2+}-binding regulatory site on the cytosolic side and an opening-inducing Ca-binding site on the matrix side of mitochondria. The Ca sensitivity of the MPT is increased by SH oxidation and cross-linking, with prooxidants modulating also via oxidation of NAD(P)H [98]. The matrix Ca-binding site seems to be cyclophilin D, which becomes bound to the pore, promoting opening [99]. Of great importance for studies on MPT has been the discovery that pore opening is inhibited by cyclosporin A (CsA) [100, 101]. It acts by binding to cyclophilin and by removing it from the pore. There is a complex interaction between Ca, ADP, CsA, and the adenine nucleotide transferase (ANT) in the interaction of cyclophilin with the pore (see review [96]).

The components of the MPT pore are still not known with certainty. ANT is implied by the effect of the ANT inhibitors carboxyatractyloside and bongkrekate, the former promoting the *c*-conformation and pore opening, the latter the *m*-conformation and its closing [102, 103]. Attempts to isolate components of the pore have yielded ANT [104]. Another component is the voltage-dependent anion channel (VDAC) of the mitochondrial outer membrane [105]. The MPT pore is localized to the junctions between the inner and outer membranes; thus, many proteins have been found associated with the ANT. Complex 1 of the respiratory chain has also been recently implicated in the MPT [106]. Recently, it was found that dihydrolipoate—a potent antioxidant (see review [107])—surprisingly stimulated MPT [108]. This was due to the formation of a thiyl radical of this dithiol when it was oxidized in a reaction with free radicals. The latter are formed in one-electron oxidation of other antioxidants that had reacted with reactive oxygen species (ROS) formed in mitochondria [109]. It may stimulate pore opening mainly by reacting with the dithiol of the pore complex, resulting in sulfur bridge formation.

There is a mutual relationship between MPT and ROS formation; ROS stimulate MPT and MPT stimulates mitochondrial ROS formation (see review [110]). This is also promoted by oxidative damage to lysosomes [111]. This is of importance in oxidative stress and Ca overload in ischemia–reperfusion injury [110-112]. The damage does not occur during the ischemic phase, since pH is lowered due to glycolysis, and MPT therefore inhibited, but at reperfusion when oxygen is provided and pH normalized [113].

Cell death in ischemia–reperfusion often results from MPT—a finding that has much increased interest in mitochondrial Ca handling. The MPT increases cell death by releasing cytochrome *c* and apoptosis-inducing factor (AIF) from the inner membrane and the intermembrane spaces [114]. Skulachev has suggested that this mechanism has been developed to dispose of cells producing increased amounts of ROS [115]. There may also be other specific release mechanisms involving Ca. Whether cell death occurs by necrosis or as programmed cell death (apoptosis) depends eventually on the ATP level in the cell (see review [116]). The release of cytochrome *c* can be prevented by Bcl-2 and some related proteins, while other members of the Bcl-2 family promote cell death by forming channels [117].

Increase in permeability by fatty acids. Ca activates phospholipase A_2 (PLA_2) leading to the release of fatty acids. Activation of mitochondrial PLA_2 by Ca causes mitochondrial swelling [118], which is inhibited by PLA_2 inhibitors and related agents [119]. It was also found that PLA_2 activation increases the permeability of liposomes to Ca and H^+ [120] by forming channels [121], which was thought to be the main factor in mitochondrial permeabilization by Ca overload [122]. However, fatty acids also cause uncoupling since they are transported on the ANT as anions, and then diffuse across the membrane as undissociated acids. They also stimulate the MPT [123, 124]. Interestingly, serum fatty acids increase in myocardial infarction [125]. Palmitic acid may activate apoptosis [126, 127], and it has been found that palmitic and stearic acid bind Ca with the highest affinity among mitochondrial lipids [128].

Ca signaling has increased the interest for Ca handling not only in the field of cell death, but also in the area of stimulation of metabolism. This began with finding by McCormack and Denton that Ca stimulated matrix dehydrogenases, which provided a rational for the Ca uniporter (see review [129]). Activated cells would have a greater need for ATP synthesis to meet the increased energy need. Therefore, the respiratory chain and ATP-synthase activities should in principle be increased. That has been difficult to demonstrate. In cancer cells respiration was found to be stimulated by Ca by increased elec-

tron flow through ubiquinone and complex III [130], and Ca was found to activate ATP synthesis more than could be accounted for by the stimulation of dehydrogenases, i.e., by an effect on the ATP-synthase [131]. The stimulation is linked to the phosphorylation of the δ subunit by the platelet-derived growth factor, which operates through tyrosine kinases [132]. Azarashvili et al. found that a 3.5 kD peptide that reacts with antibodies to subunit *c* of the ATP-synthase [133, 134] was phosphorylated in liver mitochondria. The phosphorylation level was modulated by Ca and Mg, which influenced the ATP-synthase and ATPase activities [135, 136].

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