Review

Adenosine 5′-triphosphate: an intracellular metabolic messenger

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1. Introduction

It is well established that adenosine 5′-triphosphate (ATP) plays the role of a universal 'high energy' compound. Under normal conditions ATP occurs in cells at a concentration between 2 and 10 mmol/l [1]. The structure of ATP consists of three kinds of building blocks. First, there is a heterocyclic aromatic ring structure, adenine, which is a derivative of 6-aminopurine. Attached to the adenine base through a glycosidic linkage is a molecule of the five-carbon sugar, D-ribose, to which are attached, by ester linkage, phosphate groups at the 5′-position. The ATP molecule as it exists in the intact cell is highly charged; at pH 7.0, each of three phosphate groups is completely ionized. Thus ATP bears four negative charges, which are concentrated around the linear polyphosphate structure. This is an important feature of the ATP molecule with respect to its 'high energy' nature [2]. On the other hand, the structural geometry of the ATP molecule is a matter of functional importance because many enzymes that synthesize and/or utilize ATP have active sites, of tertiary structure implicated by the so-called Walker motifs A and B [3–6], to which the ATP structure must fit exactly (Fig. 1).

The free energy of ATP hydrolysis ($\Delta G°'$ 50 kJ/mol) is significantly higher than that of simple esters,

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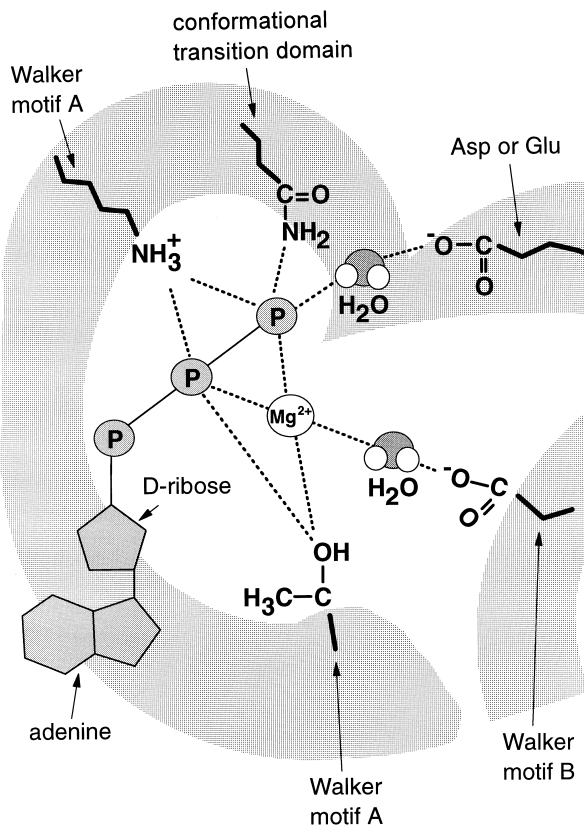E-mail: adam@nencki.gov.pl

Fig. 1. A common nucleotide-binding domain in ATP/GTP-binding proteins of various origin. The Walker A and B consensus motifs are depicted. They consist of the following sequences: the Walker motif A is B-X_4-Gly-X_4-Gly-Lys-Thr-X_5-Leu (or Val), and the Walker motif B is Arg (or Lys)-X_3-Gly-X_3-Leu-H_4-Asp, where B stands for basic, H for hydrophobic, and X for arbitrary (optional?) amino acid residue. Adenosine 5′-triphosphate and its interactions with magnesium ions, water molecules and various amino acid residues are also shown. Other explanations are given in the text.

glycosides, and amides. It is also higher than that of many phosphorylated compounds. The physiological importance of ATP is related, however, to the fact that it occupies an intermediate position on the thermodynamic scale of phosphate compounds [2]. The function of ATP/ADP system is to act as an intermediate 'bridge' or linking system between phosphate compounds having a high phosphate group transfer potential, and other compounds having a low phosphate group transfer potential, thus making possible the transfer of phosphate groups from the former to the latter. ADP serves as a specific enzymatic acceptor of phosphate groups from cellular phosphate compounds of very high potential. The

latter are formed during the energy-yielding oxidation of foodstuffs in the cell, and they conserve much of the energy of the foodstuffs. The ATP so formed can now donate its terminal phosphate group enzymatically to certain specific phosphate acceptor molecules, such as glucose or glycerol (which are low-energy compounds), transforming them to their phosphate derivatives and thus raising their energy content [7].

The second important feature of ATP is not only that is serves as a general energy carrier in all the reactions in the cell which cause phosphorylation of ADP to ATP at the expense of phosphate compounds of very high potential, but also that all the reactions by which the terminal phosphate group is transferred to low-energy phosphate acceptors, are catalyzed by enzymes. Furthermore, nearly all of these enzymes are specific for ATP and ADP, as their active catalytic sites fit only ATP and ADP, and the ATP/ADP system is the primary 'main-line' energy carrier [1,2]. It is worth stressing, however, that ATP-binding proteins comprise macromolecules differing in origin and molecular mechanism of interaction with nucleotides, from ATP hydrolases, through various kinases (including protein kinases), other ATP-utilizing enzymes, such as active transporters of metabolites, endo- and xenobiotics (including multidrug
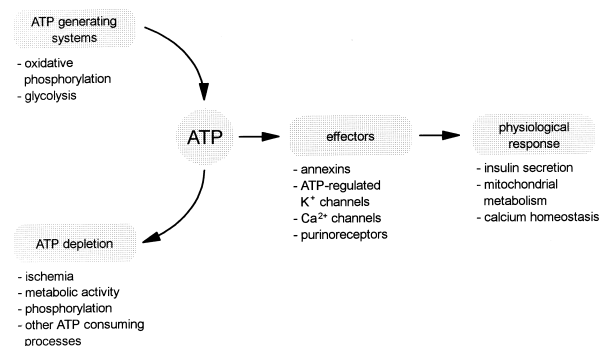

Fig. 2. Schematic illustration of functioning of ATP within the cell, as an intracellular metabolic messenger and important physiological ligand for many transport proteins, enzymes, regulatory and structural proteins. The inter-relationship between ATP-generating systems and ATP-consuming processes, leading to the changes in intracellular ATP concentration (some of them leading to ATP depletion), is shown to underline the importance of adenosine nucleotide homeostasis for cell functioning. The changes in ATP concentration are likely to be propagated by various effector proteins leading to specific functional responses.

resistance or multidrug resistance-associated proteins), to heat shock, contractile and motor proteins [3,4,8].

ATP has a potent physiological impact in different tissues by acting as an extracellular messenger (Fig. 2). Moreover, ATP and related nucleotides were found to be potent Ca^{2+} mobilizing agents and to act intracellularly on both ionotropic (P2X) and metabotropic (P2Y) plasma membrane purinergic receptors for extracellular nucleotides [9–11] that play important physiological and pathophysiological roles in a variety of biological processes, such as neurotransmission, contraction–relaxation cycle, secretion of hormones, immune response, and cell growth. Molecular cloning of purinergic P2 receptors revealed the molecular mechanism underlying the extracellular action of ATP and other nucleotides [12]. P2Y receptors have seven transmembrane spanning regions, are coupled to G-protein, and are involved in inositol triphosphate (IP$_3$) generation and Ca^{2+} release from intracellular stores. P2X receptors are coupled to ion channels related to *Caenorhabditis elegans* degenerins and mammalian amiloride-sensitive Na$^+$ channels [13]. ATP released from presynaptic terminals in a Ca^{2+}-dependent manner may also play a role in long term potentiation (LTP), a process that has been implicated in memory formation [14]. ATP was also proposed as a cytosolic factor involved in pain-sensing of tissue damage [15].

In addition, over the past years, hundreds of observations concerning the direct intracellular effects of ATP on cellular functions have also accumulated. Some of them could be interpreted to support the action of ATP as a metabolic messenger. By the term 'metabolic messenger' we understand a molecule whose cellular concentrations reflect the overall metabolic activity of the cell [16]. The quantitative changes in metabolic messenger concentration are able to trigger various cellular events including insulin secretion [17]. Moreover, it has been found that cell respiration is controlled by ATP via an allosteric inhibition of cytochrome *c* oxidase by the nucleotide which binds to the matrix domain of subunit 1 of this terminal enzyme of the mitochondrial respiratory chain [18]. In addition, the ATP level is proposed to be an important determinant for cell death since maintenance of the mitochondrial membrane potential ($\Delta\Psi$), which is the driving force for mitochondrial ATP synthesis, prevents apoptosis [16]. ATP is able to interact with various enzymatic, regulatory and structural proteins, through its adenine and ribosyl groups. In line with this property, it has been discovered that many proteins are able to bind ATP, but their binding is not accompanied by ATP hydrolysis (e.g. ATP-sensitive K$^+$ channels in plasma [19] or mitochondrial [20,21] membranes or ryanodine receptor/calcium release channel in sarcoplasmic reticulum membranes of muscle cells [22,23]). These observations pointed to the possibility of nucleotides being functional ligands for many effector proteins within a cell (Fig. 2). In the interaction of intracellular ATP with some proteins not involving its hydrolysis, ATP acting as a small ligand or a metabolic messenger, affects directly the activity of enzymes, channels and/or structural proteins [24]. Hence, modulation of the activity of the ATP-binding proteins by the nucleotide depends on two cellular factors.

First are activities of ATP-generating systems in comparison to ATP-depletion processes. A high level of ATP appears within the cell as a result of increased activity of ATP-generating systems: due to glycolytic and oxidative metabolism. It is known that different substrates can contribute to ATP synthesis. Glucose, lactate, free fatty acids and, to a lesser extent, ketone bodies and amino acids, can all be metabolized to produce ATP [1,2]. ATP-depletion within the cell is a result of complex events: of ATP consuming processes such as gluconeogenesis, urea synthesis, protein synthesis, activity of ATPases, and as a result of conditions like ischemia or anoxia followed by mitochondrial dysfunction in ischemia-reperfusion [25]. Different metabolic processes contribute in different ways to the ATP pool present in cells, and it has been proposed that while cytoplasmic concentrations oscillate locally in cells, such oscillations may constitute important cell signals [24]. Hence, one can expect that different ATP-generating processes will regulate to a varying extent the activity of ATP-sensing enzymes. In fact, ATP generated from glycolytic rather than from mitochondrial metabolism is involved in glucose-stimulated insulin secretion [26,27]. There are also observations suggesting that activation of mitochondria directly triggers the exocytosis of insulin in B-cells [28]. Probably in-

sulin secretion is triggered by pleiotropic signaling pathways [17,29–31]. Fatty acids are known to be a primary source of ATP in the heart. There are also lines of evidence that lactate may significantly contribute to the production of ATP [32,33]. Additionally, substrate preferences in the heart are modulated by cardiac ischemia [34].

Second factor is a spatial distribution of ATP-generating systems within the cell, which parallelly with the regulation due to increase or depletion of ATP, maybe important. Glycolysis processes occurring in cytosol and oxidative phosphorylation in mitochondria cause compartmentation of ATP within the cells, affecting in this way the ATP-regulated processes [1,2]. In the heart, glycolytic enzymes may be important in maintaining a high local cytosolic ATP/ADP ratio in the vicinity of the ATP-regulated potassium (K_{ATP}) channels [35,36].

In this review we would like to summarize recent information about the interaction of intracellular ATP with different proteins. Only interactions that do not involve ATP hydrolysis will be taken into account. In other words, the effect of ATP as a ligand, but not as a phosphate group donor, will be described. The article will focus on qualitative changes of cell function due to quantitative changes in cytosolic ATP content.

2. Targets for intracellular ATP

2.1. ATP-regulated ion channels

2.1.1. Plasma membrane K_{ATP}

Plasma membrane K_{ATP} channels, first discovered in cardiac muscle [37], are inhibited by an increase of the intracellular ATP concentration. They play an important role in various cellular responses, such as secretion and muscle excitability, by linking the cell energetic status with the plasma membrane potential [38]. The K_{ATP} channels are present in different cell types such as cardiac, skeletal and smooth muscle cells, pancreatic B-cells or neuronal cells [39]. In pancreatic B-cells the K_{ATP} channels are involved in insulin secretion (Fig. 3). In the brain and in the heart muscle under ischemia the level of ATP is lowered. Decrease of ATP/ADP ratio would cause opening of the K_{ATP} channels, resulting in shortening of the

duration of the action potential, preserving ATP and hence serving a protective function during metabolic stress. In smooth muscle cells the K_{ATP} channels participate in regulation of vascular tone [37–39].

Recently two members of the inward rectifier K^+ channel family were cloned, namely Kir6.1 and Kir6.2 [40–42]. Together with the sulfonylurea receptor (SUR), existing in three isoforms SUR1 and SUR2A/B, they form a functional K_{ATP} channel. Kir6.1 exhibits channel activity without SUR; neither Kir6.2 nor SUR1 exhibit full functional activity. The SUR2A subunit and the Kir6.2 subunit can reconstitute a K_{ATP} channel with properties similar to those found in cardiac and skeletal muscles [43], while the coexpression of Kir6.2 subunit and SUR2B subunit reconstitute potassium channels with similar properties to the smooth muscle cell K_{ATP} channels [44]. Probably various SUR subunits in combination with Kir6.x subunits contribute to functional diversity of K_{ATP} channels [19]. The K_{ATP} channels have weakly inward rectifying properties depending on magnesium or polyamines binding to channel pore [45]. The studies on subunit stoichiometry of the pancreatic B-cell K_{ATP} channels (SUR1 and Kir6.2) indicate that the activity of
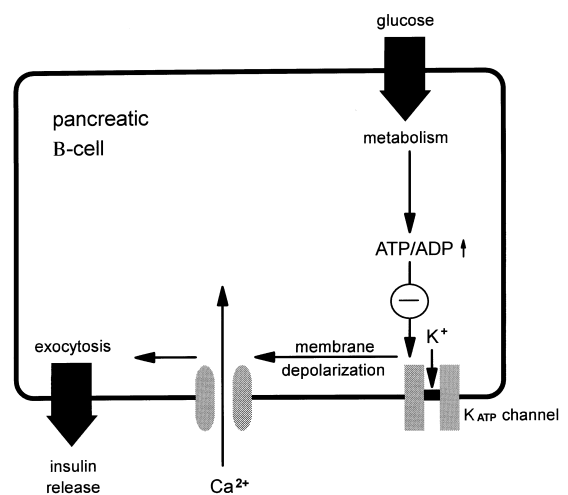

Fig. 3. The K_{ATP} channel functioning in pancreatic B-cells and its connection with the secretion of insulin. The scheme represents a widely accepted mechanism of regulation of the K_{ATP} channel activity, its close association with the overall cellular energetic metabolism, and effect on exocytotic release of the hormone. The details of this mechanism are explained in the text.

K_{ATP} channels is the highest when subunits are coexpressed at a molar ratio of 1:1 [46,47]. Since inward rectifier potassium channels are functioning as tetramers, this suggests that the K_{ATP} channel is active as a multimeric protein, probably as an octamer composed of a tetramer of the Kir6.2 subunit and a tetramer of the SUR1 subunit [47]. Although two different subunits, Kir6.2 and SUR1, are required for formation of a functional K_{ATP} channel, recently it was shown that removal of the last 26 amino acids of Kir6.2 enabled channel activity and ATP inhibition of the channel even in the absence of SUR1 [48]. It seems that the site at which ATP (and ADP) mediate channel inhibition lies on Kir6.2, whereas SUR1 confers sensitivity to sulfonylureas, diazoxide (and probably other potassium channel openers), and potentiation by MgADP [48]. Similarly to ADP, guanine nucleotides have a dual effect on the K_{ATP} channels: at their concentrations exceeding 1 mM the channel is blocked, while low concentrations (100 μM) stimulate its activity [49]. Mutations in the SUR gene appear to be the cause of abnormal insulin secretion in persistent hyperinsulinemic hypoglycemia of infancy (PHHI) [50].

The sulfonylureas such as glibenclamide or tolbutamide are insulin secretagogues widely used as oral hypoglycemic agents in the treatment of diabetes mellitus type II [51,52]. They were shown to inhibit the activity of K_{ATP} channels [53,54]. Molecular cloning of the high affinity SUR [55] has revealed that it is a member of the ATP-binding cassette (ABC) superfamily of proteins [56,57] and has two nucleotide-binding sites that contain Walker A and B consensus motifs [3]. In other ABC transporters these motifs are involved in binding and hydrolysis of ATP. Two residues are of particular importance: an aspartate residue within the Walker B motif coordinates the magnesium ion of MgATP, while a lysine in Walker A motif is critical for ATP hydrolysis. In SUR1 these residues are indispensable for MgADP to be able to activate the K_{ATP} channel activity [58,59]. In addition to ADP, low concentration of guanine nucleotides GTP, GTPγS, GDPγS and GDP activate the K_{ATP} channels in B-cells [49]. As in the case with ADP, this effect of guanine nucleotides requires the presence of magnesium ions. The stimulatory effect of both GTP and GDP was explained by direct interaction of the guanine nucleo-

tides with the K_{ATP} channel and interaction with GTP-binding proteins. Recently it was shown that GTP blocks the K_{ATP} channel by direct interaction with Kir6.2 and the stimulatory effect of GTP is mediated by SUR1 [60]. Moreover, it was shown that potentiating effects of GTP and GDP on the K_{ATP} channel and their ability to enhance diazoxide stimulation are effected via SUR1 [60]. It is believed that Walker motifs play an essential role in K_{ATP} channel activation by MgADP and potassium channel opener diazoxide [61]. The interaction of sulfonylureas with SUR1 abolishes the stimulatory action of MgADP [59].

The K_{ATP} channels have been characterized mostly in pancreatic B-cells where they play the role of a cytosolic ATP sensor triggering the exocytosis of insulin [62,63]. In pancreatic B-cells the K_{ATP} channel regulates insulin secretion in response to elevation in glucose content which is its primary physiological stimulus. The consensus view concerning B-cell stimulus–secretion coupling is that when plasma glucose levels rise, glucose uptake and metabolism by the pancreatic cell is increased. The resulting increase in intracellular ATP, and concomitant lowering of intracellular MgADP result in the closure of the K_{ATP} channels followed by plasma membrane depolarization. This activates voltage-dependent calcium channels, increases calcium influx into the cell and triggers insulin release. Mitochondria are thought to play a central role in the generation of the signal that couples glucose metabolism to insulin secretion in pancreatic B-cells [64,65]. It was shown that probably also factors other than calcium and ATP and generated by mitochondrial metabolism are capable of inducing insulin exocytosis [28].

The K_{ATP} channels also interact with drugs known as potassium channel openers [66]. In contrast to sulfonylureas, the potassium channel openers, such as diazoxide, activate K_{ATP} channels thereby hyperpolarizing the B-cell and inhibiting insulin release. In pancreatic B-cells insulin secretion is an oscillating process, due to metabolic and ATP/ADP level oscillation leading to opening and closing of ATP-sensitive channels [67]. Interestingly, it was also observed that metabolic oscillations in heart cells caused oscillations of the ATP-regulated membrane current [68,69].

2.1.2. K_{ATP} channel in mitochondria

The ion channel highly selective for K^+ present in the inner membrane of rat liver mitochondria [70] (Fig. 4), is blocked by ATP and by the antidiabetic sulfonylurea derivative, glibenclamide [70,71]. Reconstitution of the partially purified mitochondrial K_{ATP} (mitoK_{ATP}) channel into planar phospholipid membranes ('black' lipid membranes) was also performed and enabled to estimate at 30 pS the single channel conductance at saturating K^+ concentration [71]. Recently, results have been shown suggesting that Kir6.1 might be a subunit of the ATP-sensitive potassium channel in the mitochondrion, as well as in the plasma membrane [72].

The mitoK_{ATP} channel is reversibly inactivated by ATP [70], its activity being half inhibited at 0.8 mM ATP and almost completely blocked at > 2 mM. Further studies on inhibition by ATP of the mitoK_{ATP} channel were performed after partial purification and reconstitution of the channel into phospholipid liposomes [71], and a strong inhibitory effect of ATP on K^+ transport, was observed ($K_{1/2}$ 22–30 µM). Mg^{2+} was required to obtain inhibition by ATP, but neither ATP nor Mg^{2+} alone was effective. In contrast, in intact mitochondria potassium transport was inhibited by Mg^{2+} alone. It is not clear why inhibition of the mitoK_{ATP} channel by ADP was observed after its reconstitution into proteoliposomes

[71], but not in patch-clamp studies on mitoplasts [70].

It has been shown that guanine nucleotides reverse the inhibition of mitoK_{ATP} channel by ATP and ADP [73]. GTP and GDP are fully able to activate the channel in the presence of 500 µM ATP with a K_a of 7 and 140 µM, respectively. It has been proposed that the endogenous activators (GTP or GDP) overcome the high affinity binding for ATP and play a role of physiological regulators of the mitoK_{ATP} channel [73]. An important point concerns the orientation of the mitoK_{ATP} channel in the inner mitochondrial membrane. According to Garlid and his co-workers [73,74], the regulatory sites for ATP and GTP face the intramembrane space. There are also some observations suggesting that the ATP binding site faces the matrix compartment [70]. This problem, important for understanding of functioning of mitoK_{ATP} channels within the cells, needs further clarification. The data on unidirectional K^+ transport in mitochondria come from studies on intact (isolated) mitochondria. It is well known that respiring mitochondria exhibit electrophoretic K^+ uptake, the process known as K^+ uniport [75]. In studies on modulation of the activity of this uniport by adenine nucleotides it was demonstrated that K^+ influx was inhibited by adenine nucleotides with IC_{50} values of 0.5 and 2.3 µM for ADP and ATP, respectively [76]. K^+ transport was also inhibited by AMP and by a nucleotide analog belonging to the triazine dye family, Cibacron blue F3GA [76]. It was concluded that the mitoK_{ATP} channel was, at least partially, involved in the K^+ uniport activity [76]. It has been also shown that the K^+ uniport induced by magnesium depletion is inhibited by glibenclamide in a concentration-dependent manner [20,77]. Also other antidiabetic sulfonylureas are able to block the K^+ uniport induced in this way [20,78]. In fact, in intact rat liver mitochondria only a single class of low-affinity binding sites for glibenclamide, with K_d of 4 µM, was found [21]. In beef heart mitochondria K_d for glibenclamide binding is by one order of magnitude lower, e.g. 300 nM, than that in rat liver mitochondria [21].

Similarities in the properties of plasma membrane K_{ATP} channels and mitoK_{ATP} channels concern also their interaction with non-sulfonylurea inhibitors (for review see [20]). It has been shown that a guanidine
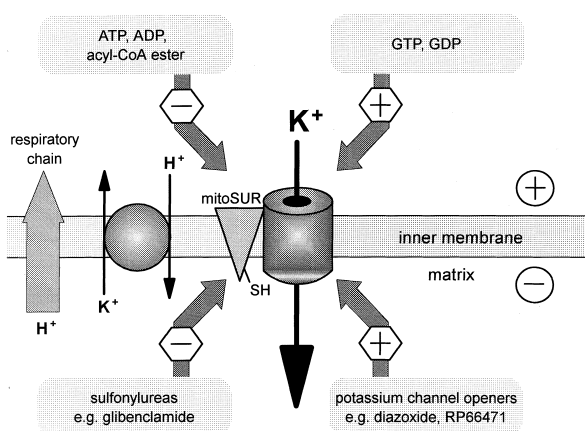

Fig. 4. Interaction of mitochondrial K_{ATP} channel with various effectors, such as ATP/GTP nucleotides, antidiabetic sulfonylureas, and potassium channel openers. This scheme shows a putative role of the mitoK_{ATP} channel and K^+/H^+ exchanger in potassium ions and protons traffic in mitochondria. MitoSUR represents the mitochondrial sulfonylurea receptor.

derivative, U-37883A, acts as a vascular K_{ATP} channel antagonist [79]. This compound was also shown to inhibit the K^+ influx into rat liver mitochondria [80], thus suggesting that it is active against the mitoK_{ATP} channel as well. The mitoK_{ATP} channel is blocked also by quinidine [81].

Plasma membrane K_{ATP} channels are specifically activated by such drugs as diazoxide, pinacidil and minoxidil sulfate, known as potassium channel openers (for review see [82]). It has been found that the potassium channel opener, RP66471, induces a decrease of the mitochondrial membrane potential [83]. Recently, other potassium channel openers were shown to activate potassium transport in mitochondria. ATP-inhibited K^+ flux was restored by diazoxide ($K_{1/2}$ 0.4 μM), cromakalim ($K_{1/2}$ 1 μM) and two cromakalim analogs, EMD60480 and EMD57970 ($K_{1/2}$ 6 nM) [84].

Physiological functions of mitoK_{ATP} channels are not clear because of confusing phenomena involved in the regulation of this channel. In view of the physiological concentration of ATP the mitoK_{ATP} channel should be closed constantly. Only a dramatic lowering of the ATP level should activate the channel and this would lead only to membrane depolarization followed by permanent inhibition of mitochondrial metabolic activity. It may be speculated that, in spite of the fact that ATP inhibits the mitoK_{ATP} channel activity, 'ATP depletion' is not the only physiological process leading to channel activation. Probably other effectors, like GTP and GDP or some proteins, are involved in fine regulation of the mitoK_{ATP} channel activity [73].

Taking into consideration all that is mentioned above, the mitoK_{ATP} channel may have a dual physiological function. Firstly, a concerted action of the electrophoretic K^+ uniport and the electroneutral K^+/H^+ exchange is believed to be the main mechanism responsible for maintaining potassium homeostasis within the mitochondrion, and thus for controlling intramitochondrial osmolality and volume of mitochondria. Regulatory changes of this volume are regarded as one of the important mechanisms of metabolic control at the mitochondrial level (for review see [85]). Observations that glibenclamide and ATP inhibit mitochondrial swelling whereas K_{ATP} openers potentiate the swelling make it likely that this channel, perhaps together with other potassium pathways, is involved in regulatory changes of mitochondrial volume [85].

Secondly, energization of mitochondria is accompanied by a net uptake of K^+, which is simultaneously inhibited by glibenclamide and activated by the well known openers of the plasma membrane potassium channel. This is compatible with the hypothesis that potassium uptake upon energization partly compensates for the electric charge transfer produced by the proton pump and thus enables the formation of ΔpH along with $\Delta\Psi$. This was further substantiated by the observation that the rate of ΔpH formation increases with increasing K^+ concentration in the external medium and thus with increasing rate of K^+ influx [86]. The final steady-state value of ΔpH also increases whereas that of $\Delta\Psi$ decreases at increasing K^+ concentration so that the resulting protonmotive force remains practically unchanged. The assumption that K^+ transport accounts for the formation of ΔpH is also supported by the observation that both the rate of ΔpH formation and its steady-state level in energized mitochondria are increased by the potent opener of K_{ATP} channel, RP66471 [86]. As shown previously [83], this compound decreases $\Delta\Psi$ of energized liver mitochondria by increasing the permeability of the inner mitochondrial membrane to K^+. All this would also suggest possible involvement of the mitochondrial K_{ATP} channel in regulation of processes driven by the mitochondrial transmembrane potential, e.g. adenine nucleotide transport or calcium uptake, and by ΔpH, e.g. phosphate and pyruvate transport.

2.1.3. ATP-regulated potassium channel in zymogen granules

An activity similar to that in the mitoK_{ATP} channel was found in isolated pancreatic zymogen granules in an assay measuring bulk cation influx driven by a proton diffusion potential [87]. Conductance was decreased by monovalent cations in the following order: $Rb^+ > K^+ > Na^+ > Cs^+ > Li^+$. Over 50% of the K^+ conductance was inhibited by millimolar concentrations of ATP or MgATP. The inhibition by MgATP, but not by ATP itself, was reversed by the K^+ channel opener, diazoxide. The inhibitory effect of nucleotides was probably due to non-covalent interactions, as these can be mimicked by non-hydrolyzable analog of ATP and by ADP. The re-

versal of MgATP inhibition by diazoxide may be mediated by phosphorylation as it is blocked by the protein kinase inhibitor, H7. Sensitivity to antidiabetic sulfonylureas, such as glibenclamide and tolbutamide, is also observed. The transport of K^+ by granules was also measured indirectly by ionophore-induced lysis of the isolated secretory granules suspended in a KCl-containing solution [88]. ATP at a physiological level inhibited the K^+ transport in a dose-dependent manner, but ADP did not reverse the inhibition. The antidiabetic sulfonylurea, tolbutamide (0.5 mM), also reduced ionophore-dependent granular lysis by 46%. The ATP sensitivity of K^+ transport was influenced by pH (increased ATP sensitivity with decreasing pH). Preincubation with phospholipase A_2 or lysophospholipids produced a significant decrease, through an unknown mechanism, in the granule K^+ transport. The possible role of granule-associated K^+ transport in pancreatic ascinar cells could be a contribution to granule swelling as a step preceding membrane fusion during exocytosis [88].

2.1.4. Ryanodine receptor

Ryanodine receptors are a family of intracellular Ca^{2+} release channels that were originally identified in the sarcoplasmic reticulum membranes of skeletal muscle cells. In mammalian tissues three members of

this family have been identified so far, the skeletal muscle (RyR1), the cardiac muscle (RyR2), and the brain (RyR3) ryanodine receptors. These proteins are products of different genes and share 66–70% amino acid sequence identity [89–93]. Previously these receptors were thought to be differently expressed, with RyR1 predominantly expressed in skeletal muscle cells, RyR2 mainly expressed in heart, but also in brain, and RyR3 in certain regions of brain, and in smooth muscle cells. Recently, they were found to be widely expressed, most of the tissues expressing more than one RyR isoform [94,95].

RyR1 and RyR2 function as Ca^{2+} release channels from the sarcoplasmic reticulum intracellular calcium store and play a crucial role in the excitation–contraction cycle of skeletal muscles [90,92,96–98]. Both these channels have been purified, reconstituted [99] and characterized. Structurally each is a homotetramer of molecular weight of 4×564 kDa [100,101] with tightly associated four molecules of immunophilin of molecular weight of 12 kDa (immunosuppressant drug FK506-binding protein – FKBP12) with *cis–trans* peptidyl-prolyl isomerase activity [102–105]. Genes encoding both isoforms have been cloned and products of their expression revealed 66% identity [106]. FKBP12, which can be removed from RyR by incubation of skeletal muscle terminal cisternae membranes with an immunosup-
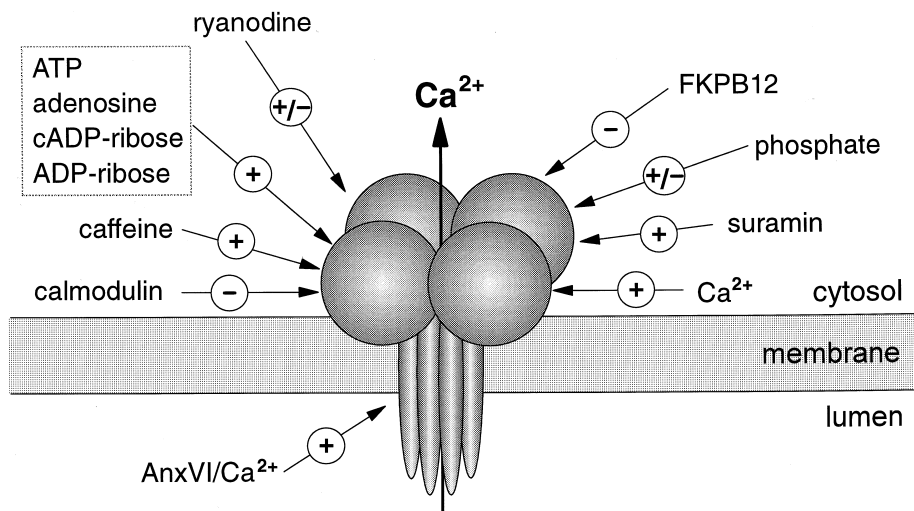

Fig. 5. The interaction of the ryanodine receptor (RyR)/calcium release channel of sarco(endo)plasmic reticulum membranes with calcium ions, various physiological ligands and some drugs. The RyR homoterotetramer is schematically shown; the activation of the channel is marked (+), inhibition with (−), and mixed effects with (+/−). FKBP12 stands for immunorepressant FK506-binding protein of 12 kDa.

pressive drug, rapamycin, seems to coordinate the gating of channel activity in both control and ryanodine-modified RyR [105] and to reduce the frequency of channel subconductance states [103].

RyR isoforms differ, however, from each other in their sensitivity to various effectors and to plant alkaloid, ryanodine [100,107] (Fig. 5). Binding of ryanodine to purified and membrane-bound RyR has been found to be modulated by propranolol, a B-blocker [108]. When incorporated into lipid bilayers purified RyR forms a large conductance channel permeable to monovalent and divalent cations, which can be activated by submicromolar amounts of Ca^{2+}, ATP, and caffeine, and inhibited by millimolar amounts of Ca^{2+}, Mg^{2+}, and Ruthenium red [22,109–111]. RyR isoforms are activated also by ADP-ribose, cADP-ribose, β-NAD [23] and suramin [112]. However, differences in their response to cyclic ADP-ribose [113,114] as well as to inorganic phosphate and perchlorate [115] have been reported. Age-related abnormalities in their regulation has been also observed [116]. Interestingly, nitric oxide, the endothelial-derived relaxing factor, which is known to depress force in smooth and cardiac muscles through the action of guanylyl cyclase and an increase in cGMP, increased the open probability of RyR and inhibited ryanodine binding to RyR. Ca^{2+} release through RyR was induced by nitric oxide free radicals, and was potentiated by cysteine involving formation of nitrosylated cysteine in the presence of O_2, followed by transnitrosation of regulatory thiols on RyR and activation of the channel [117]. Most of the RyR1 and RyR2 ligands interact with the channel domains exposed on the cytoplasmic side of sarcoplasmic reticulum membranes [23,118], as depicted on Fig. 5. There is, however, one exception, that of annexin VI. Annexin VI has been shown, using the patch-clamp technique, to modulate in in vitro experiments RyR1 by increasing 2.7-fold open probability of the channel and by 82-fold the mean open time [119–121], and to interact with the domain of the channel exposed to the lumen of sarcoplasmic reticulum membrane [119]. It should be mentioned, however, that the latter function of annexin, due to its ability to form Ca^{2+} channels in liposomes [122] has been recently questioned [123].

RyR1 and RyR2 bind Ca^{2+} and calmodulin and

become phosphorylated by various protein kinases including Ca^{2+}/calmodulin- and cAMP-dependent kinases [124–126]. Phosphorylation of RyR2 by cAMP-dependent kinase activates the channel [127], while that of RyR1, inhibits Ca^{2+} release [128]. In addition, it has been demonstrated that skeletal muscle RyR is modulated by endogenous phosphorylation of 160/150 kDa proteins of sarcoplasmic reticulum membrane [129], and the kinase involved has been identified [130]. Calmodulin, after binding to RyR1 and RyR2, inhibits transport activity of the channel and decreases the probability of channel opening [23,131]. ATP activates calcium release from sarcoplasmic reticulum cisternae of skeletal and heart muscles; in the presence of physiological concentrations of ATP the probability of channel opening is increased [118,132,133]. RyR2 is also activated by adenosine and its analogs [134]. In RyR1 and RyR2 a domain near Arg^{615} is responsible for the binding of ATP and the same region has been found in the receptor of IP_3 [135].

In the case of RyR3 there is still but a limited number of observations available. Isolation and biochemical characterization of RyR3 have been impeded by its low abundance and its coexpression with other RyR isoforms. Significant progress in understanding the function and regulation of RyR3 has been made due to generation of mice with a null mutation in the RyR1 gene [136]. Recently, the RyR3 cDNA from rabbit uterus was cloned and expressed in HEK293 cells. Recombinant RyR3 was activated by 100 nM Ca^{2+} and inactivated at 10 mM Ca^{2+}. Calmodulin activated the channel at low Ca^{2+}, and inhibited at high Ca^{2+} concentration. The cloned RyR3 was activated by ATP, caffeine, and perchlorate, inhibited by Mg^{2+} and Ruthenium red, and modified by ryanodine; cADP-ribose, however, was without effect [137]. The cloned RyR3 was found to differ from RyR1 in the gating behavior, the extent of maximal activation by Ca^{2+}, and sensitivity to Ca^{2+} inactivation [137].

It has also been found that the activity of a class of intracellular Ca^{2+} release channels, IP_3 receptors, distinct from RyR, is also modulated by ATP. IP_3 receptors are found in all types of cells. They are homotetramers of molecular weight of 4×315 kDa [106,118,138]. Two domains exposed on the cytoplasmic side of endoplasmic reticulum are involved in

binding of second messengers, which is followed by channel opening [138]. IP$_3$ receptors are encoded by four genes, resulting in expression of various IP$_3$ receptor isoforms [138]. These receptors are also regulated by phosphorylation catalyzed by protein kinase C and cAMP- or/and Ca^{2+}/calmodulin-dependent protein kinases [139–141], or, alternatively, undergo autophosphorylation [142]. Calcium ions act on both cytoplasmic and luminal portions of the receptor. ATP has been found to interact with two domains within the receptor molecule, localized on the cytoplasmic side of the membrane, which are responsible for the transmission of conformational changes occurring in the protein molecule upon binding of IP$_3$. Binding of ATP was effective at nucleotide concentrations lower than physiological ones [140,143,144]. This may suggest the involvement of Ca^{2+}-ATPase which may locally reduce ATP concentrations by its hydrolysis [140], ATP was found to act on IP$_3$ receptor biphasically; in the presence of ATP at concentrations below 2 mM the channel is activated, and above 4 mM it becomes inhibited [22]. The inhibition of IP$_3$-induced Ca^{2+} release by caffeine in permeabilized A7r5 cells is prevented by ATP and MgATP (5 mM), suggesting that caffeine may interact with an ATP-binding site on the IP$_3$ receptor or, may be, with other receptor-associated proteins [145].

2.1.5. ATP-regulated cardiac Ca^{2+} channel

It is known that calcium current in cardiac myocytes is dependent on metabolic status of the cells, and is regulated by protein kinase A [146]. Therefore, the effect of ATP on calcium current has been attributed mainly to stimulation of protein phosphorylation. ATP was found to promote channel activity by preventing its Ca^{2+}-mediated inactivation [147,148] or Ca^{2+}-activated proteolysis [148,149]. Interestingly, calcium current was enhanced not only by MgATP, but also by a non-hydrolysable ATP analog [150]. Recently, the possible role of ATP in regulation of L-type channel was investigated in guinea pig ventricular cells [151] and a stimulatory action of MgATP in inside-out patches was observed. Similar effects were also noticed in the presence of 5′-adenylylimidophosphate (AMP-PNP), a non-hydrolysable ATP analog, suggesting that hydrolysis of ATP is not required for the stimulatory effect of the nucleo-

tide on channel activity. In addition, a protein kinase inhibitor H8 did not abolish the effect of MgATP, suggesting that a phosphorylation event is not involved in regulation of channel activity [151].

2.1.6. ATP-dependent anion channels

Transcellular Cl$^-$ fluxes play a pivotal role in the control of salt and fluid secretion, pH balance, osmoregulation, cardiac function, and volume-dependent metabolic effects. The physiological importance of Cl$^-$ transport is reflected by the diversity of Cl$^-$ channels that are expressed in eukaryotic cells, and the numerous intracellular signaling pathways involving Ca^{2+}, cAMP and ATP, that regulate them [152,153].

Recently, an anion channel from rat brain synaptic plasma membrane has been incorporated into planar lipid bilayers and was found to exhibit low anion selectivity to Cl$^-$. The channel was not regulated by changes in membrane potential. Several drugs, efficient inhibitors of anion channels, such as 4-acetoamino-4′-isothiocyanostilbene-2,2′-disulfonic acid, ethacrynic acid, indanyloxyacetic acid, and 5-nitro-2-(3-phenylpropylamino)benzoic acid, inhibited the channel from the cytoplasmic side of plasma membrane. This channel was found to be regulated by intracellular ATP at millimolar concentrations, as well as by other nucleotides, ADP and GTP, which inhibited its activity [154]. In addition, the channel was inhibited by glibenclamide when present at micromolar concentrations, from the extracytoplasmic side of the membrane [154].

Another type of ATP-regulated chloride conductance exists in endoplasmic reticulum-enriched pancreatic microsomes [155]. Chloride conductance in these microsomes was inhibited by stilbene (0.1 mM) and indanyloxyacetic acid (10 μM) derivatives. ATP stimulated the conductance with half-maximal stimulation at 8 μM. Other trinucleotides when used at concentrations not exceeding 100 μM were without effect. The non-hydrolysable ATP analog had the same effect as ATP, while ATPγS, which is a substrate for kinases, was without effect [155]. ATP stimulation was reversed by stilbene derivatives. In addition, evidence has been presented that this channel is not immunologically identical with a cystic fibrosis transmembrane conductance regulator, but rather represents a novel type of chloride channel

which is regulated by ATP. ATP, in this case, does not need to be hydrolyzed and its spatial conformation is important for activating Cl⁻ conductance [155].

2.2. Annexins

2.2.1. Basic characteristics of annexins

Annexins form a family of multifunctional ubiquitous intra- and extracellular calcium- and phospholipid-binding proteins (up to 1–2% of total protein content), that are able to interact with membranes in a calcium-dependent manner. Although their in vivo functions remain unsolved, on the basis of the results of numerous in vitro experiments annexins have been frequently suggested to serve as connecting elements between plasma membrane and intracellular components, such as cytoskeleton (F-actin, spectrin), endosomes, lysosomes, and other vesicular structures [123,156–160]. So far ten different subfamilies in man and many more genus and tissue specific subfamilies and isoforms have been described [123,156–158,161]. In contrast to the superfamily of Ca^{2+}-binding proteins (e.g. calmodulin and troponin C), which are able to mediate various cellular processes in response to changes in Ca^{2+} concentration over the range from 0.1 to 1 μM [162], annexins lack the calcium-binding sites of the 'EF-hand' structure [161–165]. On the other hand, they have specific repeatable motifs, called the 'annexin folds', which represent a second to the C2 domain of protein kinase C, phospholipase C, synaptotagmin, phospholipase A_2, rabphillin, copines, and many other proteins, the major protein motif that regulate calcium-dependent interactions with membrane lipids [166–168]. The annexin fold is similar, with some respect to tertiary structure and mechanism of Ca^{2+} binding, to calcium-binding sites (type II) of cytosolic 85-kDa phospholipase A_2 [168,169]. The analysis of tertiary structure of annexins revealed the existence in these proteins of specialized phospholipid-binding sites exhibiting specificity for certain phospholipid classes [161–165]. Two major regions can be distinguished within an annexin molecule: a conserved core region comprising four (in human annexins I–V, VII, VIII, XI and XIII), or eight (only in annexin VI), repeated amino acid sequences, and a variable, differing in length, regulatory N-terminal region. The core region is the site of a calcium and phospholipid binding to an annexin molecule as evidenced by combinatory mutagenesis [170] or crystal structure analysis [122,171,172], while the terminal N-region contains sequences which are phosphorylated in different annexins by distinct protein kinases [156,157]. Simultaneously these sequences form sites of interaction with various proteins, such as S-100 [123], F-actin [123], profilin [173], tissue plasminogen activator (t-PA) [174,175], apolipoprotein A1 [176], p120^{GAP} (an activator of GTPase p21^{ras}) [177], and ion transporters including RyR [119–121], and the Ca^{2+}-dependent chloride channel from epithelial cells [178,179]. Annexins bind also extracellular matrix proteins, e.g. collagen [180] and tenascin-C [181]. They exhibit lower affinity to Ca^{2+} than the 'EF-hand' calcium-binding proteins and, therefore, seem to be less sensitive to changes in intracellular calcium concentration. In fact, some of the annexin isoforms were found to bind to membranes in a calcium-independent mode [182–186], and in case of annexin II–early endosomes interactions it has been proposed that these interactions could be mediated through specific membrane receptors [187].

On the basis of numerous in vitro observations annexins have been implicated in calcium signal transduction in certain cells and calcium homeostasis. First, the expression of annexins, although consecutive, is frequently tissue-restricted and cell-specific [156]. Genes encoding some annexins are specifically regulated, e.g. by prolactin [156], progesterone [188], glucocorticoids [156], retinoids [189] and thyroid hormones [190], and are often related to the processes of cell differentiation and proliferation. Moreover, a promoter for binding of an early response gene product, AP1, has been identified upstream of the annexin V-encoding gene [191], and annexins V and II are encoded by *fos*-induced genes [192]. Second, an important role in annexin functioning is played by phosphorylation, catalyzed by various tyrosine and serine/threonine protein kinases which participate in transduction of mitogenic signal [193–195]. In turn, annexins were found to be inhibitors of several protein kinases, inhibition of these kinases resulting in changes in permeability of membranes to ions [194] or in modulation of cellular responses to insulin [196]. Third, annexins undergo translocation from cytoplasm to plasma membranes

and phagosomes in response to changes of cytosolic [197–201] or nuclear [202] Ca^{2+} concentrations upon cell stimulation.

Annexins are believed able to perform these functions because almost all them examined to date can form in vitro voltage-dependent ion channels [123,156,157,203,204]. Some of annexins exhibit selectivity towards Ca^{2+}, as shown for example for annexin V. Annexin V which in the pore region of the channel contains Glu^{128}, Glu^{129}, Glu^{130} and Asp^{137} residues, and is acidic, is therefore, expected to be cation-selective [205]. In the case of annexin XII hexamer, the side chains of Lys^{132}, Lys^{128} and Lys^{137} from each of the six monomers provide the region immediately adjacent to the mouth of the pore with a basic environment, expected to reveal selectivity towards anions [206]. In connection with their channel activity, annexins were also implicated in endo- and exocytosis [207], since they are able to modulate membrane aggregation and fusion in a calcium-dependent manner [207–210]. These processes are prerequisite for secretion of hormones and neurotransmitters, and for bile formation [211]. In addition, annexins are involved in regulation, as non-specific inhibitors, of phospholipase A_2 activity [156,158]. Some annexins bind heparin [212,213], signaling molecules and neurotransmitters or their precursors, e.g. choline [176,213].

By the mode of interaction with membranes annexins resemble protein kinase C isoforms [214], as well as 85-kDa cytosolic phospholipase A_2 [215], and even calpains, cytosolic proteases active at the membranes [216]. Like protein kinase C and phospholipase A_2, annexin isoforms alternate within a cell between two major states: soluble, at relatively low Ca^{2+} concentrations in cytosol, and membrane-bound, at higher Ca^{2+} concentrations, mainly associated with the cytoplasmic leaflet of plasma membrane by two distinct mechanisms: one reversible and Ca^{2+}-dependent, and another requiring Ca^{2+} initially but subsequently Ca^{2+}-independent [123]. Annexins were also isolated in association with endoplasmic (sarcoplasmic) reticulum membranes [182,217] and with mitochondria [218,219].

2.2.2. Interaction of some annexin isoforms with nucleotides

In the light of existing binding data and dissocia-

tion constants of annexins for calcium, it is difficult to explain their cellular localization since even in stimulated cells free Ca^{2+} levels are rarely in the micromolar range. Moreover, various annexin isoforms were found to bind to the membranes even when intracellular Ca^{2+} concentrations reached low resting levels. In fact, it has been observed that relocation of annexin V to platelet membranes upon cell stimulation is a phosphorylation-dependent process, as shown by using the protein kinase inhibitor, staurosporine, and immunoprecipitation [220]. In bovine heart, lungs and brain some annexins partitioned in the micellar phase after non-ionic detergent treatment [182,184]. On the other hand, it has been also observed that a Ca^{2+}-dependent binding of annexin VI to membranes is resistant to treatment with detergents and, in addition, annexin VI was found to colocalize with actin binding proteins, α-actinin and fimbrin. This suggests that annexin may interact also with cytoskeleton [221] or other specific membrane receptor [187]. Interestingly, it has been reported that ATP at the physiological concentration range potentiates association of annexin VI with the hepatocyte plasma membrane, especially at submillimolar concentrations of Ca^{2+} [222]. This particular observation may indicate that not only binding of Ca^{2+} to the protein molecule, but also other ligands, nucleotides, may influence the interaction of annexins with membranes.

In fact, some annexins have been shown to interact in vitro with nucleotides [223–225] and biochemical and functional evidence has been provided that they may belong to a superfamily of ATP-binding proteins of various origin. However, one has to remember that structure of annexins does not contain the Walker consensus motifs A and B characteristic of ATP/GTP-binding proteins [3,6]. Specifically, ATP and cAMP were found to bind in vitro to bovine lung annexin I influencing its ability to aggregate liposomes and chromaffin granules, and to form calcium channels [223,226]. Annexin VI was successfully purified by affinity chromatography on ATP-agarose [227]. By comparing primary structures of annexins, cystic fibrosis transmembrane conductance regulator and various cAMP-binding proteins it has been demonstrated that annexins share some homology with cyclic nucleotide-binding proteins [223,228]. The strongest arguments came from in vitro observations

that annexin VI of porcine liver is an ATP-binding protein [224,225], which suggests that a nucleotide-binding domain may exist within the annexin VI molecule exhibiting structural features different from those found in other ATP-binding proteins [225,229]. The nucleotide-binding domain of annexin VI is probably localized in a hydrophobic pocket between two symmetric lobes of the protein molecule, each consisting of four Ca^{2+}- and phospholipid-binding domains [225], in analogy to the hydrophobic pocket within the actin molecule [230], and other related proteins [7,230–233]. The connector between these lobes may participate in creation of an ATP-binding domain of the protein, since in most of annexin VI genus-specific isoforms it contains a unique Trp^{343} residue [122]. These speculations are strengthened by indirect evidence that the binding of ATP to annexin VI implies a rearrangement within the protein molecule tertiary structure leading to change in exposure of the Trp^{343}-containing domain [225] and also by observation made for other ATP-binding proteins revealing the importance of particular tryptophan residue in stabilization of adenine ring of nucleotide while bound to the ATP-binding pocket of protein [234,235].

As it was already mentioned, the large eight-domain annexin VI is able to bind efficiently and specifically ATP with high affinity, $K_d \approx 5$ μM [224,225]. ATP and other adenine nucleotides quench the intrinsic fluorescence of annexin VI which, in the case of trinitrophenyl-ATP, is accompanied by a fluorescence energy-transfer between protein tryptophan residues and the nucleotide [225]. ATP binding to annexin VI changes its in vitro interaction with artificial and biological membranes, and with the cytoskeleton [224], but such an effect of has not been observed in the case of annexin IV [236].

In the next chapter we will focus on two possibilities. First, annexins may react to the local changes in intracellular ATP concentrations which is an additional signal to calcium, resulting in changes of the interaction of annexins with membrane components. As an alternative possibility, annexins bind ATP, this influencing their conformation and function; the nucleotide signal is then stopped by dissociation regulated, e.g. by calcium, after which annexins return to the resting state.

2.2.3. The effect of nucleotide binding on annexin functioning

Annexins, by interacting with membranes and cytoskeleton, may participate in vesicular transport and membrane trafficking; in these processes, in addition to calcium ions, ATP plays a crucial role [123,237]. It has been reported that fusion among endosomes is an important step for transport and sorting of internalized macromolecules and, in the absence of Ca^{2+}, requires ATP and cytosol. Evidence has been presented that annexin II and arachidonic acid are involved in a calcium-dependent fusion event [208], however, no observations have been published yet that annexin II is an ATP-binding protein.

Annexins may act as effectors of the nucleotide signaling pathway within the cell, and changes in nucleotide concentration, especially under metabolic or oxidative stress, may engage annexins as effectors and mediators of various intracellular reactions involved in calcium-mediated signaling and energy transduction. The initial stage in the process of converting a calcium signal into a change in cellular function is binding of calcium to an appropriate calcium-binding protein. Most cells contain many such proteins, among them calmodulin, protein kinase C, calcium-dependent proteases (calpains), and cytoskeleton-associated proteins, particularly those involved in the regulation of actin filaments, one of the best-known examples being gelsolin, an ATP-binding protein exhibiting low affinity for the nucleotide [227,238]. Some of them like synapsins I and II, abundant components of synaptic vesicles, were found to bind ATP with high affinity through a single, evolutionarily conserved glutamate or lysine residues, respectively, and the binding of ATP to synapsins is differentially regulated by Ca^{2+} [239]. In case of some copines, calcium-dependent, phospholipid-binding proteins, conserved from *Paramecium* to humans [167] it has been shown that they bind to an ATP affinity column in a calcium-dependent manner [240]. The function within the cell of many proteins known to interact or bind Ca^{2+} is still not elucidated. Considering the ability of annexins to bind Ca^{2+} and ATP, and form ternary complexes with phospholipid molecules it may be assumed that they play a role of calcium buffering or calcium effectors and serve as transducers of calcium signal.

Recently, the hydrolytic activity of annexin VII

(synexin) towards GTP [241], and phosphodiesterase activity inhibited by phospholipid binding, and myosin-like hydrolytic activity towards ATP of some plant annexins [242,243] have been described. Since GTP and its non-hydrolysable analog GTPγS are known to promote the Ca^{2+}-dependent exocytosis in many cell types by a mechanism thought to involve as yet unknown proteins, Pollard and his co-workers hypothesized that annexin VII may play the role of one of such proteins [241]. They have used streptolysin O-permeabilized cells and have found that the initial rate of annexin VII-driven Ca^{2+}-dependent aggregation of chromaffin granules and phosphatidylserine liposomes is increased by GTPγS > GTP, while 1 mM ATP, CTP, UTP, GDP or GMP were without effect [241]. GTP influenced also liposome fusion driven by annexin VII without changing the apparent $K_{1/2}$ for calcium. These effects were accompanied by specific binding of GTP to annexin VII, which was dependent on Ca^{2+} with two apparent $K_{1/2}$ values for calcium (0.25 and 2.5 mM). Moreover, annexin VII was found to be able both in vitro and in vivo to hydrolyze GTP in a manner dependent on calcium ($K_{1/2}$ 50 μM) which could be substituted by Mg^{2+} ($K_{1/2}$ 400 μM) or another divalent cation. Pollard and his colleagues applied a G-protein-like molecular switch model in which the annexin VII-GTP form was active and revealed fusogenic activity, while upon MgGTP hydrolysis the protein became inactivated; however, it could be reactivated upon elevation of intracellular Ca^{2+} concentration to 50–200 μM [241]. In the opinion of many investigators, annexin VI is not an ATPase nor does its amino acid sequence contain any known consensus sites for ATP/GTP binding [244]. Annexin VII might, therefore, resemble calexcitin, a signaling protein that binds GTP, is activated by calcium, translocates to the cell membrane after phosphorylation, where inhibits potassium channels and increases cellular excitability [245].

Co-distribution of annexin VI and actin in secretory ameloblasts and odontoblasts of rat incisor [246] may suggest a close relationship between nucleotide-binding proteins within a cell. The interaction of annexin VI with F-actin and β-subunit of brain spectrin in neurons [247], where ATP plays the role of an important neurotransmitter, favors this interpreta-

tion [248]. In line with the described phenomena is the observation that translocation of annexin VI from plasma membrane to cytosol in alveolar macrophages under oxidative stress matches elevation of intracellular Ca^{2+} [249]. The rise in cytosol Ca^{2+} concentration has been attributed to inhibition of plasma membrane Ca^{2+}-ATPase by products of lipid peroxidation, to mitochondrial dysfunction and also to annexin VI. Changes in membrane structure together with the ATP depletion and the ability of elevated level of oxidized glutathione to react with protein cysteine residues to form mixed disulfides, may result in detachment of annexin from the membrane and release of a substantial amount of calcium ions [249]. Therefore, the oxidative stress-induced changes in Ca^{2+} concentration [250,251] can modulate the signal transduction for the respiratory burst and many calcium-dependent processes (Fig. 6).

It is very likely that the interaction of annexin VI with ATP play an important role in chondrocytes. These cells take up calcium ions and accumulate them in secretory vesicles, which are primary initiators of extracellular mineral deposition in endochondrial calcification [252]. These secretory vesicles bind collagens II and X which stimulate accumulation of Ca^{2+} in these vesicles. Binding of collagens is modulated by annexins II, V, and VI, expressed at a high level in chondrocytes [253,254]. In addition, chondrocytes excrete ATP which, in turn, is involved in regulation of cell maturation and determines the amount and type of mineral compounds produced by these cells [255]. Moreover, matrix vesicles that accumulate Ca^{2+} during the initiation of mineralization of growing bone, are rich in annexin V, and evidence was provided that annexin V forms a multiconductance Ca^{2+} channel in membranes of these vesicles. Moreover, ATP and GTP were found to differentially modulate the activity of this channel: ATP increased the amplitude of the current and the number of conductance states, while GTP reduced the number of events and of conductance states [226].

The discovery of some annexin isoforms being in vitro intracellular targets for ATP/GTP and other nucleotides, such as cAMP, shed a new light on some general functions of these isoforms in vivo. Thus, annexins would represent an example among the growing in number group of proteins for which
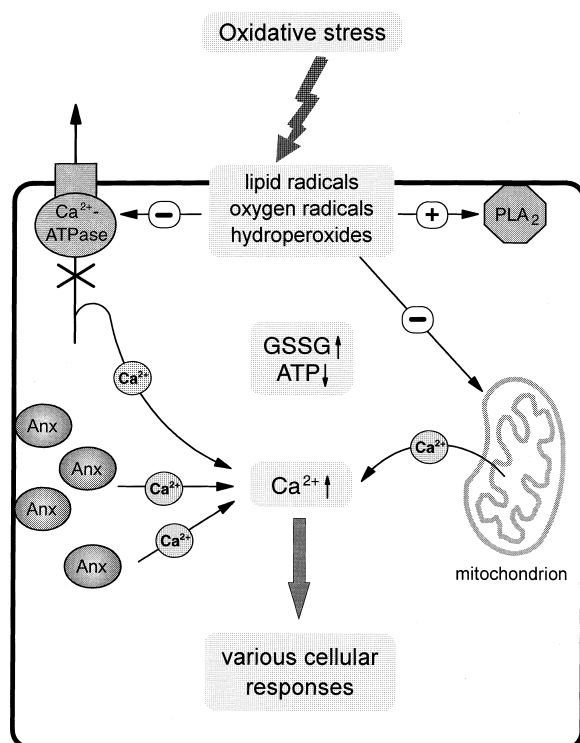
Fig. 6. The effect of oxidative stress on intracellular localization of the annexin molecules (Anx). Lipid peroxidation creates lipid hydroperoxides and free radicals which affect both membrane permeability to calcium ions and calcium-dependent binding of annexins to membranes, while oxygen radicals are deleterious to various membrane and cytosolic proteins and also to nucleic acids. Oxidative stress is accompanied by depletion in ATP and reduced glutathione contents, inhibition (−) of plasma membrane Ca^{2+}-ATPase, disturbances in mitochondrial Ca^{2+} homeostasis, and increase of intracellular content of oxidized glutathione (GSSG). These changes lead to the detachment of annexins from the membrane, and the overall increase of cytosolic calcium concentration. Annexins in cytosol are no longer inhibitory to phospholipase A_2 (PLA_2) which, being activated (+), causes further damage to the membrane lipid barrier. The described changes lead to various cellular responses mediated by calcium-dependent enzymes and proteins. Other explanations are given in the text.

nucleotides would play a role of important ligands and not only be a source of metabolic energy. In fact, even within the known families of proteins sharing a common topology of macromolecules catalyzing ATP-triggered reactions [6], there is a vast number of examples of ATP and other nucleotides playing important structural roles. For example, the cystic fibrosis transmembrane conductance regulator nucleotide binding folds may constitute a portion of

the ion-conducting channel [256]. The major goals of further research should be to identify the region within an annexin molecule responsible for nucleotide binding [229] and find an answer to the question whether binding of ATP/other nucleotides is responsible for any conformational rearrangement within the molecule which might have functional implications.

3. Concluding remarks

In addition to its well-established role in intracellular metabolism [257], ATP has also an important extracellular role propagated via purinergic P2 receptors [9,11]. ATP serves as a fast neurotransmitter in a variety of tissues and its postjunctional actions are curtailed by dephosphorylation to adenosine by membrane and soluble nucleotidases [258]. However, a wealth of data and, particularly, recent observations on various ATP-dependent ion channels and calcium- and phospholipid-binding proteins (such as annexins), being ATP-binding molecules, have unambiguously demonstrated that ATP and probably other adenine nucleotides are ubiquitous intracellular mediators involved in many essential biological processes. In fact, intracellular ATP levels are supposed to be determinants for cell death modes, i.e. apoptosis or necrosis [259], and profound alterations in ATP homeostasis have been reported in hypoxia [260], under oxidative stress [261], and in other pathological states, such as diabetes mellitus [262]. Changes in concentrations of free and bound ATP have been also measured during intracellular Ca^{2+} signaling [263], and, simultaneously, calcium concentrations were affected by the level of ATP, as observed in the case of proximal tubules where ATP depletion caused an increase in cytosol Ca^{2+} concentration above 0.1 mM [264]. Changes in the concentration of ATP seems to be characterized by slow kinetics. ATP levels during cardiac ischemia drop to 20% of their initial value after approximately 15 min [265]. A similar time scale for the lowering of the intracellular ATP level was observed during cardiac hypoxia and inhibition of glycolysis [266]. Spontaneous oscillations of glycolysis lead to oscillations of the ATP/ADP ratio within minutes, resulting amongst other effects, in the opening and closing of

the K_{ATP} channel [267]. Fast changes in free ATP concentrations, in the millisecond range, take place during intracellular calcium signaling and may have some protective role for cells against excitatory calcium signals and the unwanted energy consumption required to maintain calcium movements and calcium-dependent processes [263,268]. However, even long-time scale changes in cytosolic ATP concentrations do not rule out ATP being an important and efficient metabolic messenger. Two factors have to be taken into consideration in further studies of a messenger role for ATP. First, its spatial distribution within cells, and second, the kinetics of local changes of ATP level.

The role of ATP as a ligand and not only as a source of energy has been recently substantiated by many observations coming from various laboratories, concerning even the proteins well known to hydrolyze ATP. It has been also established that in the case of actin, the binding of nucleotide regulates its interaction with myosin [7,230] and protects F-actin against heat and pressure denaturation [231]. In the case of myosin, binding of nucleotide induces closure of the ATP-binding pocket [233], and, thus plays an important role in the catalytic cycle of the protein [269]. Furthermore, ATP takes part in formation of the higher affinity binding site for caldesmon on smooth muscle myosin molecules [270], and affects binding of actin to its accessory proteins such as gelsolin [238]. On the other hand, ATP mediates dissociation of the mitochondrial chaperone Hsp70 system, affecting its activity [271]. In other heat shock proteins (Hsp90), the amino-terminal domain of the protein has been suggested to play a role of an ATP/ADP switch domain that regulates Hsp90 conformation [272]. Another function was described for the nucleotide binding folds of cystic fibrosis transmembrane conductance regulator, which form a portion of the protein ion-conducting channel [256,273].

Moreover, many other proteins, in addition to ion channels and annexins described in detail in the present review, have been shown to interact with ATP via the mechanism not involving ATP hydrolysis, an outstanding example being mitochondrial cytochrome c oxidase [18,274] and synapsins I and II [239]. In the case of acetylcholine receptors, exogenous ATP stabilizes the degradation half-life time of the receptor [275]. Recently, an adenine nucleotide binding-site has been discovered in interferon-α_2, member of a group of related polypeptides that play a major role in mediating the antiviral state, the immune response, cell growth and differentiation [276].

Elucidation of the contribution of different processes to ATP regulation is a difficult task, mainly because of complex regulation of metabolic events and 'cross-talk' between different processes. Studies on ATP-binding proteins have largely contributed to our knowledge of basic, vital cellular functions. Analysis of protein structure resulted in discovery of new chain folds in proteins that bind nucleotides [4,5], and helped, by using these nucleotide-binding folds as particular protein 'finger prints', to establish the real function of many protein families [6,8,277,278]. Better understanding of intracellular ATP interaction with proteins could not only significantly deepen our knowledge of cellular physiology, but also could be taken advantage of in treatment of many diseases or pathologies, such as diabetes mellitus or cardiovascular diseases.

Acknowledgements

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