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Mitochondria as a Pharmacological Target

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***Abstract*——Mitochondria play a central role in energy metabolism within the cell. Mitochondrial dysfunctions lead to various neurodegenerative disorders and to the so-called "mitochondrial diseases". A vast amount of evidence points to the implication of mitochondria in such complex processes as apoptosis and cardioprotection. The purpose of this review is to present a recent state of our knowledge and understanding of the action of various therapeutically applied substances on mitochondria. These include antitumor, immunosuppressant, and antiviral drugs, potassium channel openers, sulfonylureas, and anesthetics. Some of these substances are specifically designed to affect mitochondrial functions. In other cases, drugs with primary targets in other cellular locations may modify mitochondrial functions as side effects. In any case, identification of mitochondria as primary or secondary targets of a drug may help us to better understand the drug's mechanism of action and open new perspectives for its application. As far as possible, the molecular mechanisms of the interference of particular drugs in the mitochondrial metabolism will be described. In some cases, metabolic routes in which the drugs interfere will also be briefly outlined.**

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I. Introduction

Mitochondria play a central role in energy-generating processes within the cell. Apart from this important function, mitochondria are involved in such complex processes as apoptosis and cardioprotection. A rapidly expanding body of literature also suggests that mitochondrial dysfunctions play pivotal roles in neurodegenerative disorders ranging from Parkinson's to Huntington's to Alzheimer's diseases. Mitochondrial DNA mutations, whether inherited or acquired, cause impaired respiratory chain functioning. This, in turn, leads to decreased production of ATP, formation of free radicals, and alterations in cellular calcium handling. These events may initiate peroxidation of mitochondrial DNA, proteins, and lipids, and opening of the mitochondrial permeability transition pore, an event linked to apoptotic cell death. Mitochondria are also targets for drugs such as antidiabetic sulfonylureas, immunosupressants, some antilipidemic agents, etc. The aim of this review is to present interactions of various therapeutically applied substances with mitochondria as their primary or secondary targets. To make the mechanisms of these effects comprehensible, we will also briefly describe the metabolic routes in which the drugs in question interfere.

Medically applied substances that interact with mitochondria can be divided into two groups: 1) those that are specifically designed to affect mitochondrial functions, and 2) those for which primary targets are other cellular locations and their interactions with mitochondria are secondary. In any case, identification of mitochondria as primary or secondary targets of a drug may facilitate a better understanding of its mechanism of action and open new perspectives of its application. For example, recent studies on the cardioprotective action of potassium channel openers have revealed that cardiac mitochondria are more important as the primary targets of these drugs than is the plasma membrane. Recognition of drug interaction with mitochondria as secondary targets may help us to understand the mechanisms of side effects and to construct new drugs in which these side effects will be eliminated or minimized.

Mitochondria can also be affected by a number of toxins, especially those that interact with their respiratory and ATP-generating functions. This field is the subject of a recent review by Wallace and Starkov (2000) and will not be dealt with in the present article.

When dealing with particular classes of pharmaceuticals, we will often refer to original experimental work in which the drug is used in concentrations that may exceed those encountered under therapeutic conditions, especially for compounds interacting with mitochondria as their secondary targets. We consider such studies useful because they help us to understand the mechanisms of potential side effects, especially under conditions of chronic administration or overdose. In addition, some drugs may accumulate in particular tissues or organs to attain concentrations higher than those calculated for the whole body. Moreover, mitochondria are unique cellular organelles with alkaline and negatively charged interior, conditions that promote accumulation of some compounds.

II. Mitochondria and the Cell

The number of mitochondria per cell is roughly related to cell energy demands. Somatic tissues contain from a few dozen to several thousand mitochondria per cell. Human spermatozoa contain a fixed number of 16 mitochondria and oocytes up to 100,000. Organs that are very active metabolically, such as muscles, liver, brain, and cardiac and skeletal muscles, contain the largest number of mitochondria and are most susceptible to drugs acting on mitochondria and to mitochondrial pathologies.

In major mammalian tissues, 80 to 90% of ATP is generated by mitochondria in the process of oxidative phosphorylation. The mitochondrial respiratory chain, located in the inner mitochondrial membrane, is composed of enzymes and low molecular weight redox intermediates (coenzymes) that transport "reducing equivalents", in fact hydrogen atoms or just their electrons, from respiratory substrates to molecular oxygen, down the redox potential. This hydrogen/electron current forms three cascades in which the redox energy is high enough to be utilized to extrude protons from the mitochondrial inner compartment, the matrix, to the intermembrane space. The electrochemical proton gradient thus formed, also designated as the protonmotive force Δp, is the driving force for the back flow of protons through the ATP synthase complex (Fig. 1B). This gradient is composed of the electric component ($\Delta\psi$[2]) and the proton concentration gradient. As a result, mitochondria are unique cellular organelles that can build up a transmembrane electric potential of up to 180 mV, negative inside, and whose internal milieu maintains a pH value of about 8 (Nicholls and Ferguson, 1992). As a consequence, they can not only accumulate membrane-permeable compounds of cationic character, but also trap weak acids in their anionic form. Both properties may be of importance in targeting specific drugs into mitochondria (see, for example, *Section III.C.*).

[2] Abbreviations: $\Delta\psi$, mitochondrial transmembrane electric potential; VDAC, voltage-dependent anion channel; ROS, reactive oxygen species; PTP, permeability transition pore; CsA, cyclosporin A; KCOs, potassium channel openers; K_{ATP}, ATP-regulated K^+ channel; mitoK_{ATP}, mitochondrial ATP-regulated potassium channel; SURs, sulfonylurea receptors; Kir, inwardly rectifying K^+ channels; IBP, isoquinoline-binding protein; PBR, peripheral benzodiazepine receptor; NFAT, nuclear factor of activated T cells; TBI, traumatic brain injury; AZT, 3′-azido-3′-deoxythymidine; AIDS, acquired immune deficiency syndrome; HIV, human immunodeficiency virus; mtDNA, mitochondrial DNA; MHC, myosin heavy chain(s); ddC, 2′,3′-dideoxycytidine; HSP, heat shock protein; NSAIDS, nonsteroidal anti-inflammatory drugs; NAEs, *N*-acylethanolamines.

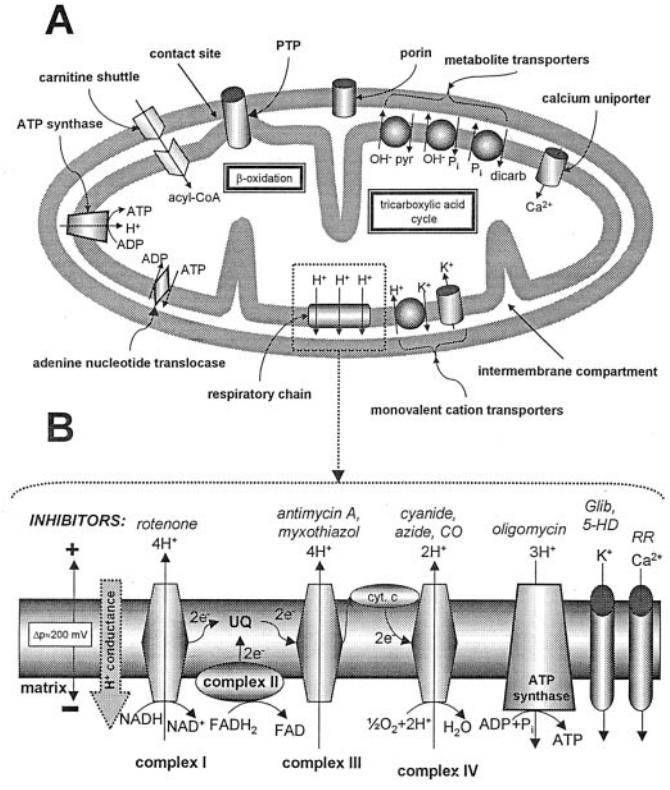

FIG. 1. Schematic representation of the mitochondrion (A) and the respiratory chain (B). pyr, pyruvate; dicarb, dicarboxylate; UQ, ubiquinone (coenzyme Q); cyt. c, cytochrome *c*; Glib, glibenclamide; 5-HD, 5-hydroxydecanoate; RR, ruthenium red.

Because of a vital role of mitochondria in cell metabolism, a constant and intense flux of inorganic ions and metabolites occurs between the cytosol and mitochondria. Due to the presence of a pore protein, termed porin or voltage-dependent anion channel (VDAC) (Mannella et al., 1992), the outer mitochondrial membrane is permeable to polar molecules of up to 5 kDa. In contrast, the inner membrane is freely permeable to just a few compounds such as water, O_2, CO_2, and NH_3. Other hydrophilic metabolites and all inorganic ions of biological importance can cross the membrane due to the presence of specific channels and carrier proteins. Among the latter ones of special importance are carriers for phosphate (P_i), adenine nucleotides ADP and ATP, and the respiratory substrates mono-, di-, and tricarboxylates. In general, these transport proteins operate in the exchange mode, i.e., ADP is exchanged for ATP, P_i for OH^-, dicarboxylic anion for P_i anion, etc. (Palmieri et al., 1992; Kuan and Saier, 1993; Palmieri, 1994; Sluse, 1996).

Channels selective for major inorganic cations K^+, Na^+, Mg^{2+}, and Ca^{2+} have been identified in mitochondria (Bernardi, 1999). Of particular importance are channels for K^+ that will be dealt with later in this review (*Section V.*). Apart from these channels, which must be regulated in a subtle way to prevent collapse of the membrane potential, there are several cation exchangers, e.g., for K^+/H^+, Ca^{2+}/H^+, and Ca^{2+}/Na^+ exchange (Bernardi, 1999). An inner membrane anion channel has also been described (Beavis, 1992), but its molecular identity, characteristics, and control are less well recognized. Recently, a mitochondrial chloride channel has been cloned (Fernández-Salas et al., 1999).

Many lipophilic compounds penetrate the inner mitochondrial membrane freely. Among them, the most important are fatty acids. Undissociated molecules of long-chain fatty acids can easily penetrate the membrane in a "flip-flop" mode (McLaughlin and Dilger, 1980; Gutknecht, 1988). In contrast, a spontaneous crossing of the phospholipid bilayer by fatty acid anions is extremely slow (Kamp and Hamilton, 1992) because of the negatively charged polar carboxylic group. However, in the inner mitochondrial membrane, the transmembrane passage of fatty acid anions is facilitated, probably in an unspecific way, by several mitochondrial anion carrier proteins. This is a process that is essential for the protonophoric action of fatty acids in mitochondria (Wojtczak and Więckowski, 1999). Many lipophilic and amphiphilic drugs are good mitochondrial penetrants.

Although the outer and the inner mitochondrial membranes are well defined structures, each of them possessing different sets of enzymes and fulfilling different functions, intimate contacts between the two membranes have been identified on both morphological and functional grounds (Brdiczka, 1991).

Mitochondria contain circular DNA that encodes about two dozen polypeptide chains participating, as subunits, in mitochondrial respiratory chain complexes and other essential components of the energy-coupling machinery. However, the majority of mitochondrial proteins are encoded by nuclear DNA and synthesized outside the mitochondria. They are imported into these organelles by a complex multistep mechanism (Lill and Neupert, 1996; Schatz, 1996).

Although the end product of the respiratory chain is water that is generated in a four-electron reduction of molecular oxygen by cytochrome oxidase (complex IV), a minor proportion of O_2 can be involved in one-electron reduction processes, generating so-called "reactive oxygen species" (ROS), in particular, superoxide anion radical $O_2^{\cdot-}$, hydrogen peroxide H_2O_2, and the extremely reactive hydroxyl radical $HO^{\cdot}$. Generation of ROS occurs mainly at complex III due to proton cycling between ubiquinone, cytochromes *b* and c_1, and iron-sulfur protein (Sugioka et al., 1988). Some contribution of complex I to this process has also been found. Reactive oxygen species are generally regarded as toxic metabolites and, as such, are decomposed by specialized enzymes: catalase, peroxidases, and superoxide dismutases. Nevertheless, a part of these reactive compounds that has sustained this catalytic removal may have a dramatic effect on mitochondria and the cell as a whole by eliciting a cascade of events leading to programmed cell death, so-called "apoptosis" (see *Section III.*).

This brief description of mitochondrial functions is aimed to point to multiple pathways interconnecting mitochondrial metabolic routes with those of the rest of the cell. Pharmacological agents, which affect either intramitochondrial metabolic processes or transport pathways connecting mitochondria with the cytosol, may therefore have a considerable influence on the total cell metabolism. Figure 1A presents the main mitochondrial processes and their locations within various mitochondrial compartments as well as routes of communication between them and the rest of the cell. It will also help to locate processes and points of pharmacological attack that will be dealt with in subsequent sections.

III. Mitochondria in Chemotherapy-Induced Apoptosis

Programmed cell death, or *apoptosis*, is a common process in multicellular organisms. It enables the elimination of single cells or their assemblies when their natural biological function has come to an end or when the cell has become damaged or mutated to such an extent that its further existence might be dangerous to the whole organism. In particular, apoptosis occurs in embryogenesis, in metamorphosis, and in the growth and maturation of individual organs. Being of such vital importance, apoptosis has attained, during evolution, a high degree of genetic and metabolic control. This broad subject is covered by numerous excellent reviews (e.g., Ellis et al., 1991; Kroemer et al., 1995; Vaux and Strasser, 1996; Hengartner, 2000). Therefore, the present considerations will be limited to the function that is played in apoptosis by mitochondria and to the pharmacological modulations of apoptosis in relation to cancer therapy (for a comprehensive review, see also Deigner and Kinscherf, 1999). It is generally believed that transformations that may lead to malignancy are rather common in cells of highly organized animals, including humans. However, such cells, as a rule, are efficiently eliminated by apoptosis put in operation by mechanisms deeply encoded in their genome. Only cells that have escaped those rescue systems give rise to malignant growth.

A. The Mitochondrial Pathway of Apoptosis

In general, two partly interdependent routes may lead to apoptosis (Hengartner, 2000; Kaufmann and Earnshaw, 2000). One of them is initiated by ligation of the so-called death receptors at the cell surface (Ashkenazi and Dixit, 1999), whereas the other one involves mitochondria (Petit et al., 1997; Green and Reed, 1998; Kroemer et al., 1998; Mignotte and Vayssiere, 1998; Susin et al., 1998; Kroemer, 1999; Desagher and Martinou, 2000; Halestrap et al., 2000). In the latter case, one of the early events leading to apoptosis is the release of cytochrome *c* from mitochondria (Liu et al., 1996; Kluck et al., 1997; Reed, 1997; Yang et al., 1997; Martinou et al., 2000). Along with another mitochondrial protein, the apoptosis-inducing factor (Daugas et al., 2000), it elicits in the cytosol a cascade of events leading to the activation of intracellular proteases of the caspase family (Earnshaw et al., 1999) and, eventually, to a partial self-digestion of the cell (Bossy-Wetzel and Green, 1999) (Fig. 2). The mechanism by which cytochrome *c* is liberated from mitochondria to the cytosol is debatable. According to some authors (e.g., Scarlett and Murphy, 1997; Vander Heiden et al., 1997; Petit et al., 1998), this is preceded by mitochondrial swelling that leads to disruption of the outer membrane.

More recent reports indicate, however, that cytochrome *c* is liberated from mitochondria by special mechanisms under conditions of preserved intactness of the outer membrane (Jürgensmeier et al., 1998; Doran and Halestrap, 2000). A decisive role in this process is played by the mitochondrial permeability transition pore (PTP) and the proapoptotic protein Bax (Marzo et al., 1998; Crompton, 1999). PTP is located in the contact sites between the outer and the inner mitochondrial membranes and, in its open state, enables a free passage

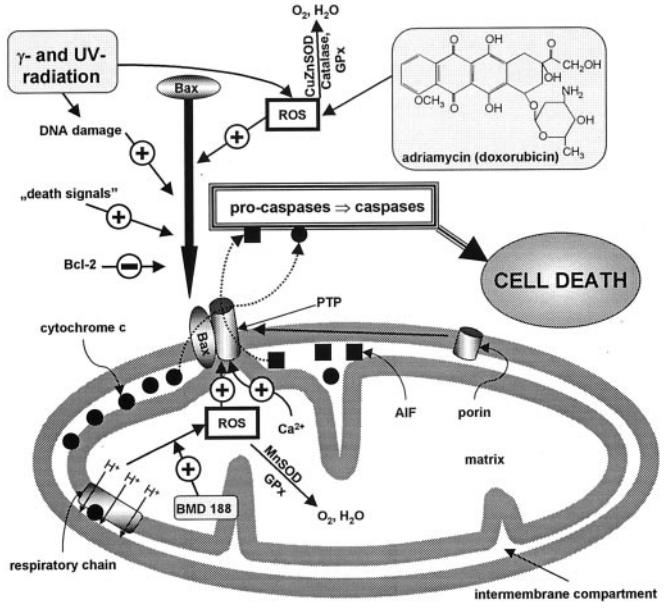

FIG. 2. Simplified scheme of the mitochondrial pathway of apoptosis. The pathway is triggered by various "death signals", such as ROS, DNA damage, etc., that promote binding of the proapoptotic protein Bax with the outer mitochondrial membrane, most likely at the contact sites between the two membranes, and its association with the PTP. This enables the release of cytochrome *c* (●) and the apoptosis-inducing factor (AIF; ■) from the intermembrane compartment to the cytosol. An elevated intramitochondrial Ca^{2+} level and ROS production facilitate this process by promoting PTP opening. Once in the cytosol, cytochrome *c* and AIF, in cooperation with a cytosolic factor, Apaf-1 (not indicated), activate caspase-9 and subsequently other members of the caspase family, thus initiating self-digestion of the cell and nuclear DNA fragmentation, eventually leading to apoptotic cell death. Association of Bax with mitochondria is prevented by the antiapoptotic protein Bcl-2. ROS can be decomposed by Mn-containing (mitochondrial) and Cu, Zn-containing (cytosolic) superoxide dismutases (SOD), catalase, and glutathione peroxidase (GPx). Stimulation of ROS production is exemplified here by UV and ionizing radiation and by two anticancer drugs, Adriamycin and BMD188. Activation is indicated as ⊕ and inhibition as ⊖.

of low molecular weight compounds, up to molecular weight 1500, between the mitochondrial inner compartment (matrix) and the cytosol. It constitutes a complex assembly of porin, adenine nucleotide translocase, and cyclophilin D (Fig. 3). The opening of PTP is favored by factors such as Ca^{2+} accumulation in mitochondria (Hunter et al., 1976), prooxidants (Byrne et al., 1999), and low mitochondrial transmembrane potential (Bernardi, 1992) (for more information see the reviews by Zoratti and Szabó, 1995; Bernardi, 1996; Bernardi et al., 1998; Fontaine and Bernardi, 1999; Halestrap, 1999).

PTP alone is too small to enable the release of cytochrome *c* (13 kDa). However, its association with Bax forms a channel specific for cytochrome *c* and apoptosis-inducing factor (Hirsch et al., 1997; Narita et al., 1998; Jacotot et al., 1999; Murphy et al., 2000). Association of Bax with PTP and its pore-forming activity are prevented by the antiapoptotic protein Bcl-2 (Kluck et al., 1997; Yang et al., 1997; Murphy et al., 2000). Thus, a subtle balance between these two (and other similar) proapoptotic and antiapoptotic proteins and their interaction with PTP are decisive for the survival or apoptotic death of the cell. This balance can be affected by a number of mitochondria-targeted drugs.

B. Mitochondria as Targets in the Control of Apoptosis

Because of the pivotal role that mitochondria play in initiating apoptotic cell decay, they are a vulnerable target for experimental and/or pharmacological intervention. As mentioned above, one of the main physiological factors that regulates the open probability of PTP is calcium ion. Therefore, the role of Ca^{2+} in the regulation of apoptosis has attained much attention, but the picture still remains obscure. Numerous observations have revealed an increased cytosolic Ca^{2+} concentration during apoptosis (McConkey and Orrenius, 1997). Manipulation of Ca^{2+} concentration within the intact cell can be performed, for example, by using thapsigargin, a potent inhibitor of endoplasmic reticulum Ca^{2+}-ATPase (Thastrup et al., 1990), which prevents calcium accumulation in the endoplasmic reticulum. It has been observed (Zhu and Loh, 1995; Waring and Beaver, 1996; Bian et al., 1997) that thapsigargin induces apoptosis in various cell lines. Apoptosis induced in thymocytes by very low concentrations of thapsigargin can be prevented by cyclosporin A (Waring and Beaver, 1996), the well know inhibitor of mitochondrial PTP (Broekemeier et al., 1989), thus pointing to the involvement of mitochondria in this process. On the other hand, it has been observed that thapsigargin also initiates apoptosis after removal of extracellular Ca^{2+} (Bian et al., 1997), i.e., under conditions in which accumulation of calcium ions inside the mitochondria is prevented. Zhu et al. (2000) have recently come to the conclusion that mitochondrial Ca^{2+} depletion promotes apoptosis, whereas mitochondrial Ca^{2+} overload leads to necrosis of cardiac myocytes and neuroblastoma cells. Thus, the role of mitochondria as a target for calcium ions in the regulation of apoptosis still awaits its elucidation.

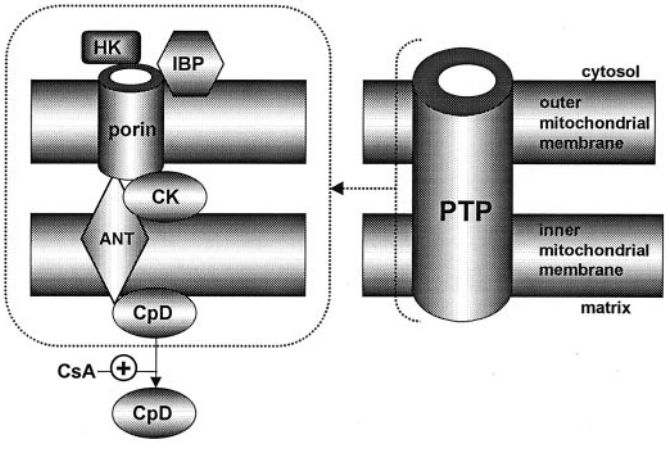

FIG. 3. Molecular composition of the mitochondrial permeability transition pore. The scheme on the right side represents the PTP as situated in the contact site, interconnecting the two mitochondrial membranes. The left-hand diagram shows the subunit composition of the contact site which also comprises the peripheral benzodiazepine receptor. HK, hexokinase; ANT, adenine nucleotide translocase; CK, creatine kinase (in muscle mitochondria); CpD, cyclophilin D. Binding of cyclophilin D by cyclosporin A and its removal from the membrane is indicated as ⊕.

Another apoptosis-promoting factor is ROS (Buttke and Sandstrom, 1994; Slater et al., 1995; Rollet-Labelle et al., 1998; Jabs, 1999; Chandra et al., 2000; Matés and Sánchez-Jiménez, 2000). Excessive production of ROS in the cell can be induced by a number of xenobiotics, transition metal ions, and ultraviolet and ionizing radiations (Halliwell and Gutteridge, 1989, 1990). ROS action on mitochondria results in the opening of PTP (Kowaltowski et al., 1996; Vercesi et al., 1997) and thus triggers the mitochondria-related apoptotic pathway. It is also possible that peroxidative attack may directly damage the outer mitochondrial membrane, resulting in unspecific liberation of intermembrane proteins, including a fraction of cytochrome *c*. Apoptosis induced by ultraviolet radiation can be reduced or completely prevented by glutathione (Slyshenkov et al., 2001) that removes oxygen free radicals. This observation indicates that ultraviolet radiation initiates apoptosis by acting directly on mitochondria rather than on the genomic system. Ionizing radiation (X-ray and γ-radiation), often used in cancer treatment, also acts by inducing apoptosis. Being more energetic than ultraviolet radiation, it also affects DNA and thus puts into operation both the DNA- and the mitochondria-operated apoptosis pathways (Rupnow and Knox, 1999).

Because PTP opens upon collapse of $\Delta\psi$, chemical and physical agents that discharge the mitochondrial membrane potential can be regarded as pro-apoptogenic. However, the picture is complicated by the fact that a complete deenergization of the cell leads to necrosis rather than to apoptosis (Leist and Nicotera, 1997). Therefore, most mitochondrial protonophoric uncouplers do not induce apoptosis. On the other hand, opening of

PTP by other factors results in a $\Delta\psi$ decrease due to an increased proton influx through the pore. Thus, the PTP opening can be either the result or the causative agent of $\Delta\psi$ collapse. Having this in mind, one has to critically evaluate a long list of anticancer agents causing cell death and "disruption" of $\Delta\psi$, as presented by Decaudin et al. (1998).

C. Antitumor Drugs as Apoptosis Promoters

Recent studies have shown that a large number of anticancer drugs exert their therapeutic action by inducing the apoptosis of malignant cells (Debatin, 2000; Preston et al., 2001), mainly due to activation of the cytochrome *c*/caspase-9 pathway (Kaufmann and Earnshaw, 2000). Thus, etoposide, doxorubicin, 1-β-D-arabinofuranosylcytosine (Decaudin et al., 1997), lonidamine (Ravagnan et al., 1999), betulinic acid, arsenite, CD437, and several amphiphilic cationic α-helical peptides (Decaudin et al., 1998; Costantini et al., 2000) induce apoptosis of malignant cells by activating PTP or otherwise affecting the mitochondrial membrane. The proapoptotic action of some of these compounds, e.g., that of lonidamine (Ravagnan et al., 1999), is counteracted by cyclosporin A (CsA) and prevented by overexpression of the antiapoptotic protein Bcl-2. This points to its mechanism of action by the opening of the PTP.

Adriamycin (doxorubicin) is a potent antitumor drug of the anthracycline antibiotic group the proapoptotic action of which is well documented (Müller et al., 1998; Kumar et al., 1999). Its application in chemotherapy is, however, strongly limited by its well known high cytotoxicity (cardio-, nephro-, and neurotoxicity) (Julka et al., 1993; Morgan et al., 1998; Singal et al., 2000). These toxic effects can be alleviated by antioxidants (Singal et al., 1997; DeAtley et al., 1999), thus confirming the notion (Olson et al., 1981; Sokolove, 1994) that Adriamycin and other quinoid anthracyclines are free radical generators (Fig. 2).

A novel anti-prostate cancer hydroxamic acid derivative, *cis*-1-hydroxy-4-(1-naphthyl)-6-octylpiperidine-2-one (designated as BMD188) (Tang et al., 1998; Li et al., 1999), is also an apoptosis-inducing agent. Although its mechanism of action is unclear and, at least in some cell lines, it does not involve cytochrome *c* liberation from mitochondria (Tang et al., 1998), more recent studies have provided evidence that its target is the mitochondrial respiratory chain and that it induces a rapid production of ROS (Joshi et al., 1999). An involvement of PTP seems, therefore, very likely (Fig. 2).

An ingenious way of destroying malignant tissues by synthetic doubly targeted peptides has been proposed (Arap et al., 1998; Ellerby et al., 1999). The authors designed helical amphipathic peptides containing mostly cationic amino acids that preferentially bound to the inner mitochondrial membrane (and prokaryotic cytoplasmic membranes) due to its high transmembrane potential and high content of anionic phospholipids. This binding resulted in the distortion of the lipid core of the membrane, manifested by mitochondrial swelling. These peptides manifested a much lower affinity toward the eukaryotic plasma membrane because of its lower membrane potential and content of mostly zwitterionic phospholipids. For that reason, they affected the integrity of the plasma membrane at concentrations hundreds of times higher than those needed to destroy bacterial or mitochondrial membranes. These peptides were then coupled to short cyclic peptides designed to be targeted toward angiogenic endothelial cells and some malignant cell lines (Pasqualini et al., 1997; Arap et al., 1998). Such hybrid peptides were internalized into the cytosol of angiogenic (but not angiostatic) endothelial cells and cancer cells, and their helical moieties associated with mitochondria, resulting in the swelling of these organelles. This led to classical symptoms of apoptosis. This treatment proved successful not only in killing cells in culture but also in hampering the growth of human breast cancer implanted into mice and in prolonging by several months the survival of such mice (Arap et al., 1998; Ellerby et al., 1999).

IV. Mitochondria and Oxidative Stress, Aging, and Degenerative Diseases

The biological importance of ROS has attracted an enormous interest during recent years due to their major role, both beneficial and noxious, in numerous vital processes. This subject is covered by thousands of original research papers and hundreds of review articles appearing each year. Only some of them can be referred to in this section. We will cite, in particular, the most recent review articles, as they will lead the reader to broader original literature and the most up-to-date information.

Mitochondria are the main source of the superoxide radical and other reactive oxygen species that may generate from them (Chance et al., 1979). The main mechanisms responsible for mitochondrial ROS production are the respiratory chain, in particular its complexes I and III (Beyer, 1992; Cadenas and Davies, 2000; Raha and Robinson, 2000), in the inner mitochondrial membrane, and monoamine oxidase in the outer membrane. Normally, ROS are decomposed or their peroxidation products are neutralized by natural defense systems mainly consisting of mitochondrial (manganese-containing) and cytosolic (containing Cu and Zn) superoxide dismutases (Mn- and CuZn-superooxide dismutase, respectively), glutathione peroxidase, and phospholipid hydroperoxide glutathione peroxidase (Neubert et al., 1962; Fridovich, 1974; Chance et al., 1979; Ursini et al., 1986; Augustin et al., 1997). However, under conditions of increased ROS generation, e.g., in ischemia-reperfusion, action of some xenobiotics, inflammation, aging, and ultraviolet or ionizing irradiation, or conditions of impaired antioxidant defense system, ROS may accumu-

late, exerting a potent damaging effect on the cell and the whole organism (Halliwell and Gutteridge, 1989, 1990; Mizuno et al., 1998; Schapira, 1999; Cadenas and Davies, 2000; Raha and Robinson, 2000). The noxious action of ROS mainly consists of the peroxidation of lipids, in particular phospholipids of biological membranes, and oxidative damage to proteins and DNA (Halliwell and Gutteridge, 1990; Lenaz et al., 1999; Cadenas and Davies, 2000). In particular, the aging of animals and humans is connected with increased mitochondrial production of ROS (Miquel et al., 1980; Lenaz, 1998; Cadenas and Davies, 2000; Lenaz et al., 2000; Sastre et al., 2000; Raha and Robinson, 2000). Mitochondria, being the main site of ROS generation in the cell, are also their primary target. This, in turn, results in damage to the mitochondrial respiratory chain and, as a consequence, a further increase in ROS generation. A *vicious cycle* is thus formed (Lenaz et al., 2000) that may be a causative agent of a number of age-associated dysfunctions of mitochondria and also one of the mechanisms inducing programmed cell death (see *Section III*.).

To protect mitochondria and the cell against such damaging effects, several measures can be applied. One of them is increasing the intracellular glutathione content. This can be done by supplying precursors for glutathione synthesis, e.g., *N*-acetylcysteine, which by itself has antioxidant properties (Benrahmoune et al., 2000). Increasing the cell content of CoA by supplying its precursor pantothenic acid (Slyshenkov et al., 1995) also increases the glutathione level (Slyshenkov et al., 1996), although the mechanism by which an increased content of CoA accelerates glutathione synthesis is not fully understood. Glutathione content and its reduction state can also be increased by incubating the cells with curcumin, the yellow pigment of the Indian spice curry (Jaruga et al., 1998), or with the analgesic drug flupirtine (Perovic et al., 1996); but the mechanisms of these processes are even more obscure. These ways of protecting mitochondria against damaging effects of ROS, although very effective, are not specifically mitochondria-directed because the glutathione content increases in the whole cell and not only in mitochondria.

Other compounds acting as general intracellular antioxidants are ascorbic acid (vitamin C), α-tocopherol (vitamin E), β-carotene (Sies et al., 1992), and α-lipoic acid (Packer et al., 1995). All these compounds are naturally present in the cell, but their contents can be increased when they are additionally administered.

Ubiquinol, the reduced form of coenzyme Q, is a naturally occurring antioxidant which can be targeted more specifically to mitochondria and other cell membranes (Ernster, 1994; Forstmark-Andrée et al., 1995; Andrée et al., 1999; Shi et al., 1999). The redox couple of ubiquinone/ubiquinol plays a key role in the mitochondrial respiratory chain as a link between its complexes I, II, and III (Nicholls and Ferguson, 1992). Much attention has recently been paid to ubiquinone and ubiquinol as potential anti-aging remedies (Lenaz, 1998; Huertas et al., 1999; Lenaz et al., 2000). Some hope has also been expressed as to the beneficial effect of coenzyme Q in retarding and/or reducing symptoms of Alzheimer's, Huntington's and Parkinson's diseases (Beal, 1998; Beal et al., 1998; Cassarino and Bennett, 1999), considered to be caused by a damaging action of ROS in the central nervous system (Mizuno et al., 1998; Schapira, 1999).

The situation with ubiquinol as an antioxidant is, however, complicated by the fact that its operation in the respiratory chain may also be, under specific conditions, a source of free radicals (Beyer, 1992; Nohl et al., 1999; Cadenas and Davies, 2000; Raha et al., 2000). In this respect, ubiquinol resembles some other antioxidants; for example, ascorbic acid and β-carotene, well known to both scavenge and generate free radicals.

An ingenious way of specifically introducing antioxidants to mitochondria within the intact cell has been recently proposed by Murphy and coworkers (Smith et al., 1999; Coulter et al., 2000; Murphy and Smith, 2000). These authors covalently coupled antioxidant moieties with the lipophilic triphenylphosphonium cation. Such compounds, being positively charged, are accumulated within mitochondria up to a thousand-fold, driven by the transmembrane electric potential of about 200 mV (negative inside) in fully energized mitochondria. Such accumulation can be further increased by the electric potential at the plasma membrane of 30 to 60 mV (also negative inside), so that the calculated inside to outside concentration ratio may amount up to 10^4:1. Among the antioxidant moieties coupled to the lipophilic cation were natural products vitamin E and ubiquinol, fullerene derivatives, and a spin trap (Coulter et al., 2000). Tetraphenylphosphonium coupled to vitamin E appeared to exert a potent protective effect against the damage of isolated mitochondria by a hydroxyl radical-generating system (Smith et al., 1999).

Recently, it has been found (Schlüter et al., 2000) that the nonopioid analgesic drug, flupirtine (a fluor-containing triaminopyridine derivative), is an effective antioxidant in mitochondria. Being positively charged, flupirtine most likely accumulates in mitochondria where it efficiently scavenges free radicals. This action of flupirtine explains why this compound prevents apoptosis of cultured cells induced by oxidative stress (Lorenz et al., 1998) and protects rabbit retina against ischemic injury (Osborne et al., 1996).

Finally, it has to be mentioned that an effective way of diminishing mitochondrial production of ROS is a partial uncoupling, i.e., decreasing, of the mitochondrial transmembrane potential ($\Delta\Psi$) and of the redox state of complex I of the respiratory chain (Skulachev, 1996). This can be obtained by increasing the permeability of the inner mitochondrial membrane to K^+ or H^+ by means of the opening of mitochondrial potassium channels (see *Section V*.) or by endogenous uncoupling proteins (see *Section XII*.).

V. Interaction of Potassium Channel Openers with Mitochondria

Potassium channel openers (KCOs) are agents, discovered in the early 1980s, that act by stimulating ion flux through K^+ channels (Edwards and Weston, 1993; Challinor-Rogers and McPherson, 1994; Quast et al., 1994). Many drugs such as cromakalim, nicorandil, and diazoxide have been identified as KCOs (Duty and Weston, 1990; Edwards and Weston, 1995). KCOs act on two types of ion channels: ATP-regulated K^+ channels (K_{ATP} channels) (Ashcroft, 2000) and Ca^{2+}-activated K^+ channels (BK channels) (Lawson, 2000). KCOs were first identified by their antianginal or antihypertensive mode of action (Edwards and Weston, 1993). Now, they are at various stages of development as antiasthmatic (Morley, 1994; Buchheit and Fozard, 1999; Prasad et al., 2000) and cardioprotective agents (Grover and Garlid, 2000). Preclinical and clinical evidence also supports the therapeutic role of KCOs in pulmonary (Wanstall and Jeffery, 1998) and vascular hypertension (Quast, 1992; Atwal, 1994; Quast et al., 1994; Schachter, 1995; Lawson, 1996), and the treatment of overactive bladder (Andersson, 2000).

Until recently, the effects of KCOs were believed to be attributable entirely to the modulation of K^+ channels in cell surface membranes. It is now apparent, however, that new targets for KCOs exist in intracellular membranes including those of sarcoplasmic reticulum (Kourie, 1998), zymogen granules (Thevenod et al., 1992), and mitochondria (Garlid, 1994; 1996; Szewczyk et al., 1996a; Szewczyk, 1997). Mitochondria seem to be particularly important targets for KCOs because the interaction of KCOs with these organelles appears to mediate the cardioprotective action of these compounds (Szewczyk and Marban, 1999; Grover and Garlid, 2000). The protective role of mitochondrial ion channels was recently summarized in an excellent review article (O'Rourke, 2000). Mitochondrial targets for anti-ischemic drugs were recently described (Morin et al., 2001; Suleiman et al., 2001).

A. Potassium Channel Openers and Mitochondrial K^+ Channels

A small-conductance potassium channel, with properties similar to those of the K_{ATP} channel from the plasma membrane, was described in the inner membrane of rat liver and beef heart mitochondria and designated the mitochondrial ATP-regulated potassium channel (mitoK_{ATP} channel) (Inoue et al., 1991; Paucek et al., 1992). The mitoK_{ATP} channel was blocked not only by ATP, but also, similarly to the plasma membrane K_{ATP} channel, by antidiabetic sulfonylureas (Fig. 4) (Inoue et al., 1991; Paucek et al., 1992). These observations raised the question whether the mitoK_{ATP} channel could be activated by KCOs. In fact, an increased influx of K^+ and depolarization of liver mitochondria in the presence

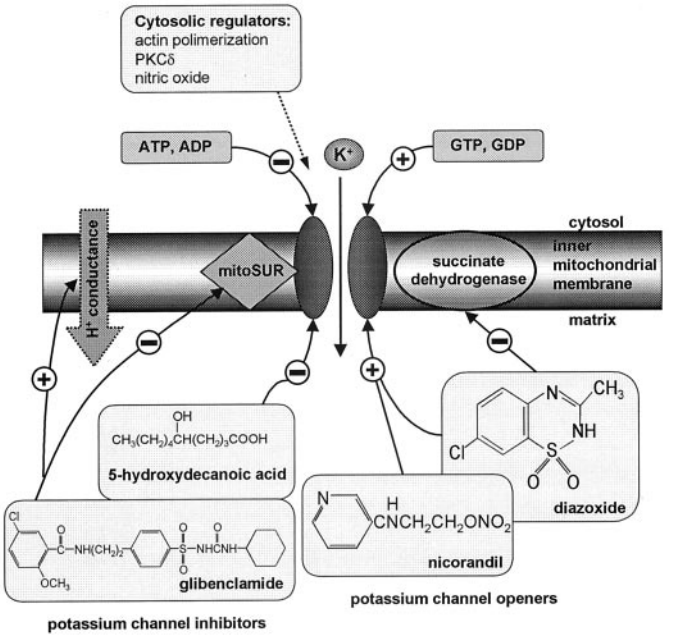

FIG. 4. Interaction of potassium channel openers and inhibitors with mitochondria. Activation is indicated as ⊕ and inhibition as ⊖. PKCδ, protein kinase C-δ; mitoSUR, mitochondrial sulfonylurea receptor.

of KCOs such as RP66471 was observed (Szewczyk et al., 1993, 1995). Also, other KCOs were shown to activate potassium ion transport into mitochondria (Belyaeva et al., 1993; Czyż et al., 1995; Garlid et al., 1996b; Holmuhamedov et al., 1998). Moreover, ATP-inhibited K^+ flux was restored by diazoxide ($K_{1/2}$ of 0.4 μM), cromakalim ($K_{1/2}$ of 1 μM), and two cromakalim analogs, EMD60480 and EMD57970 ($K_{1/2}$ of 6 nM) (Garlid et al., 1996b). KCOs such as pinacidil, cromakalim, and levcromakalim have been shown to depolarize cardiac mitochondria (Holmuhamedov et al., 1998). KCO-induced membrane depolarization was associated with an increase in the rate of mitochondrial respiration and decreased ATP synthesis (Holmuhamedov et al., 1998). Moreover, KCOs released calcium ions and cytochrome *c* from cardiac mitochondria (Holmuhamedov et al., 1998). Despite the effect on K^+ transport, diazoxide also exhibits a direct effect on mitochondrial energy metabolism by inhibition of respiratory chain complex II in liver mitochondria (Grimmsmann and Rustenbeck, 1998). Recently, mitoK_{ATP} channel opener BMS-191095 with no peripheral vasodilator activity was described (Grover et al., 2001).

Using isolated mitochondria or proteoliposomes reconstituted with partly purified mitoK_{ATP} channel and measuring potassium flux, Garlid et al. (1996b) demonstrated that heart and liver mitoK_{ATP} channels share some pharmacological properties with the plasma membrane K_{ATP} channel, i.e., they are both activated by KCOs. The sensitivity of cardiac mitoK_{ATP} channels to diazoxide appeared to be 1000 times higher than that of plasma membrane K_{ATP} channels. This observation es-

tablished that the interaction of KCOs with mitoK$_{ATP}$ channels plays an important role in cardioprotection.

B. Mitochondrial ATP-Regulated Potassium Channel: A Novel Effector of Cardioprotection

KCOs mimic cardiac ischemic preconditioning in the absence of ischemia, whereas K$_{ATP}$ channel blockers, such as glibenclamide and 5-hydroxydecanoic acid, diminish the beneficial effects of short ischemic events on the cardiac tissue. The original hypothesis to explain these observations involved plasma membrane K$_{ATP}$ channels. Recently, it has been proven that the action of KCOs such as diazoxide concerns, in fact, mitochondria and the mitoK$_{ATP}$ channel (Garlid et al., 1997). In a complementary approach, it was shown that diazoxide induced oxidation of mitochondrial flavoproteins, due to the activation of mitoK$_{ATP}$ channel, but did not activate plasma membrane K$_{ATP}$ channels (Liu et al., 1998). The effects of diazoxide were completely and reversibly blocked by 5-hydroxydecanoic acid. Interestingly, exposure to phorbol-12-myristate-13-acetate potentiated and accelerated the effect of diazoxide (Sato et al., 1998). These studies established that the target for the protective effects of diazoxide in cardiac myocytes is the mitoK$_{ATP}$ channel rather than the plasma membrane K$_{ATP}$ channel (Fig. 5). Importantly, evidence for mitoK$_{ATP}$ channels as effectors of myocardial preconditioning has also been demonstrated in human subjects (Ghosh et al., 2000).

The initial observations on the cardioprotective action of KCOs on mitochondria were further confirmed and developed in a series of reports. It has been shown that other KCOs such as pinacidil, cromakalim, and nicorandil modulate mitochondrial membrane potential, respiration, ATP generation, and mitochondrial Ca^{2+} uptake (Holmuhamedov et al., 1998; Sato et al., 2000). Other data suggest that activation and diazoxide-induced translocation of the protein kinase C δ-isoform to mitochondria appears to be important for the protection mediated by the mitoK$_{ATP}$ channel (Wang et al., 1999; Wang and Ashraf, 1999). Recently, it has been shown with the use of diazoxide that ischemic preconditioning depends on the interaction between actin cytoskeleton and mitochondria, and its protective action can be abolished by disruption of the cytoskeleton by cytochalasin D (Baines et al., 1999). Interestingly, diazoxide was also effective in improving the preservation of globally ischemic cold-stored hearts, as it occurs during cardiac transplantation (Kevelaitis et al., 1999, 2000; Ahmet et al., 2000).

The main question remains how the opening of the mitoK$_{ATP}$ channel could protect cells against ischemic injury. First, opening of the mitoK$_{ATP}$ channel followed by mitochondrial swelling could improve mitochondrial ATP production and/or handling (Garlid, 2000). In fact, diazoxide was found to preserve mitochondrial function in ischemic rat heart. It has been shown that hypoxia induces a decrease in the mitochondrial oxygen consumption rate to approximately 40% of the prehypoxic value, and treatment with diazoxide preserves the normal mitochondrial oxygen consumption rate during hypoxia (Iwai et al., 2000). Moreover, ATP concentration was significantly increased in diazoxide-treated hearts (Wang et al., 1999). Second, the protective effect of mitoK$_{ATP}$ activation could be mediated by lowering Ca^{2+} overloading of mitochondria (Holmuhamedov et al., 1998; Crestanello et al., 2000). Third, it has been demonstrated that opening of the mitoK$_{ATP}$ channel may increase ROS generation by mitochondria (Pain et al., 2000). This increase could lead to protein kinase C activation, which is known to be important during cardioprotection. Additionally, the mitoK$_{ATP}$ channel seems to be involved in delayed preconditioning (Carroll and Yellon, 2000), probably due to an altered expression of "protective" proteins. It has been shown that pretreatment of hippocampal neurons with KCOs cromakalim and diazoxide increases the expression level of proteins involved in the control of apoptosis, such as Bcl-2 and Bcl-X$_L$ (Jakob et al., 2000). Moreover, inhibition of apoptosis induced by oxidative stress in cardiac cells was observed (Akao et al., 2001). The presence of a mitochondrial target for diazoxide in hippocampal mitochondria recently has been observed (Dębska et al., 2001).

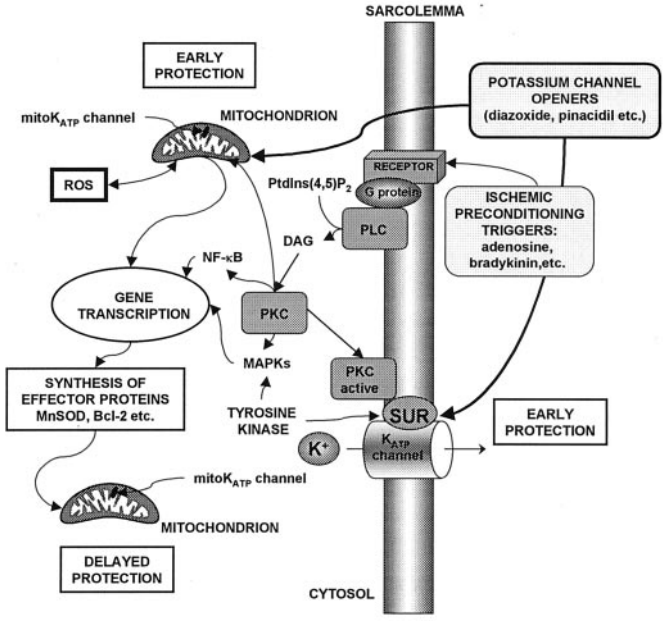

FIG. 5. Mitochondria and intracellular signaling cascade that leads to protection of cardiomyocytes against ischemic injury. The main target points of potassium channel openers are indicated with bold arrows. PKC, protein kinase C; MAPKs, mitogen-activated protein kinases; NF-κB, transcriptional nuclear factor κB; MnSOD, manganese-containing mitochondrial superoxide dismutase; DAG, diacylglycerol; PtdIns(4,5)P$_2$, phosphatidylinositol-4,5-bisphosphate.

VI. Sulfonylureas and Mitochondria

It is well known that antidiabetic sulfonylureas such as glibenclamide (also known as glyburide) or glipizide

bind to high affinity sulfonylurea receptors (SURs) in the plasma membrane of various cell types (Ashcroft and Ashcroft, 1992; Isomoto and Kurachi, 1997). In pancreatic β-cells this causes a closure of the ATP-sensitive K^+ (K_{ATP}) channel (Ashcroft and Ashcroft 1992; Lazdunski, 1994; Ashcroft, 2000) and initiates a chain of events that leads to the exocytotic release of insulin (Miki et al., 1999). The pancreatic β-cell SUR was cloned (Aguilar-Bryan et al., 1995) and identified as an element composing, together with a K^+ pore, the functional K_{ATP} channel (Inagaki et al., 1995). Similar channels are also present in the plasma membrane of smooth, skeletal, and cardiac muscle cells as well as in neurons (DeWeille, 1992). The channels are heterooctamers of four inwardly rectifying K^+ channels (Kir) and four SURs. Two members of the Kir family, Kir6.1 and Kir6.2, appear capable of forming the pores of K_{ATP} channels (Aguilar-Bryan et al., 1998; Babenko et al., 1998; Seino, 1999). These Kir subunits coassemble with SURs encoded by either of two genes, *SUR1* or *SUR2*, to form functional K_{ATP} channels. SURs belong to the superfamily of ATP-binding cassette proteins characterized by the presence of two nucleotide-binding folds within the molecule. Moreover, it is likely that various SUR subunits in combination with Kir6.x subunits contribute to the functional diversity of K_{ATP} channels and determine various pharmacological properties of these channels, including their regulation by KCOs.

A potassium channel, with properties similar to those of the K_{ATP} channel of the plasma membrane, was described in the inner mitochondrial membrane and designated as the mitoK_{ATP} channel (see *Section V.*). This channel is also blocked by antidiabetic sulfonylureas (Inoue et al., 1991; Paucek et al., 1992). The interaction of antitumor sulfonylureas with mitochondria has also been described (Fig. 6) (Howbert et al., 1990).

A. Functional Effects of Antidiabetic Sulfonylureas on Mitochondria

The effects of antidiabetic sulfonylureas on mitochondrial K^+ transport have been observed both in intact mitochondria (Szewczyk et al., 1997) and in proteoliposomes reconstituted with partly purified mitoK_{ATP} channel (Paucek et al., 1992). The mitoK_{ATP} channel became sensitive to glibenclamide only when opened by Mg^{2+}, ATP, and physiological activators such as GTP, or by the KCO diazoxide. In such an induced open state of the mitoK_{ATP} channel, glibenclamide inhibited the channel activity with a $K_{1/2}$ of 1 to 6 μM (Jabůrek et al., 1998).

Equilibrium binding studies performed with [^{3}H]glibenclamide reveal a single class of low-affinity binding sites in intact rat liver mitochondria, with a K_d of 4 μM (Szewczyk et al., 1996b). In beef heart mitochondria the K_d for glibenclamide binding is much lower, 300 nM (Szewczyk et al., 1997). Glibenclamide binding to mitochondria is modulated by SH reagents such as *N*-ethylmaleimide and mersalyl (Szewczyk et al., 1999).

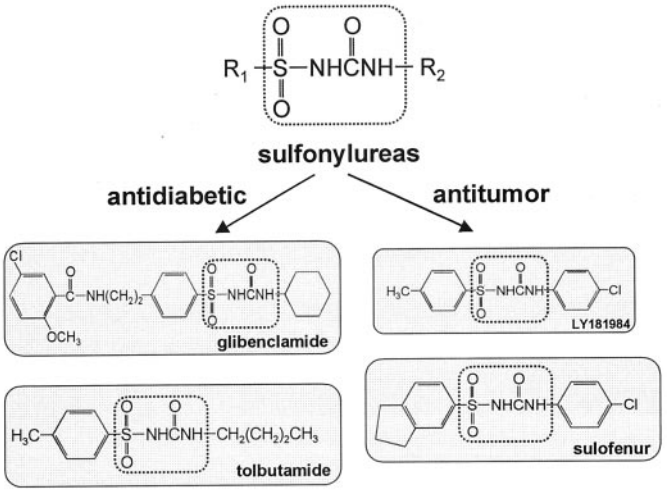

FIG. 6. Chemical structure of antidiabetic and antitumor sulfonylureas.

It is important to mention that, due to the hydrophobicity of its protonated form, glibenclamide is able to increase the proton conductance of the mitochondrial membrane (Szewczyk et al., 1997). Additionally, antidiabetic sulfonylureas such as glibenclamide and tolbutamide affect fatty acid oxidation due to the inhibition of carnitine palmitoyltransferases (Patel, 1986; Cook, 1987) and also block pyruvate carboxylase activity (White et al., 1988).

B. Effect of Antitumor Sulfonylureas on Mitochondria

Diarylsulfonylureas are antitumor agents shown to have therapeutic activity against both rodent solid tumors and xenografts of human tumors in mice (Howbert et al., 1990; Mohamadi et al., 1992; Houghton and Houghton, 1996). Their mechanism of action is unknown but does not appear to be the result of nonselective destruction of actively dividing cell populations. In isolated liver mitochondria, both *N*-(5-indanylsulfonyl)-*N'*-(4-chlorophenyl)urea and its *N*-4-methyl analog uncouple oxidative phosphorylation (Thakar et al., 1991). At concentrations below 50 μM, both compounds exhibited a deleterious effect, causing damage to mitochondrial functions. These data confirm that diarylsulfonylureas may lower cellular ATP by uncoupling mitochondrial oxidative phosphorylation (Thakar et al., 1991).

Diarylsulfonylureas, such as *N*-(4-chlorophenyl)aminocarbonyl-2,3-dihydro-1-indene-5-sulfonamide (sulofenur) and *N*-(4-chlorophenyl)aminocarbonyl-4-methylbenzene sulfonamide (LY181984), have also been shown to be effective antitumor agents (Fig. 6) (Stagg and Diasio, 1990). Mitochondria have been shown to accumulate sulofenur and therefore may be targets of drug action. Many of the diarylsulfonylureas that were effective antitumor agents in animal models were also uncouplers of mitochondrial oxidative phosphorylation (Rush et al., 1992). The mechanism of uncoupling appeared to be related to a dissociable hydrogen ion, inasmuch as these

molecules had pK_a values that ranged from 6.0 to 6.2 and were hydrophobic. However, the mechanism of antitumor activity does not appear to be the result of uncoupling, because no correlation was evident between the inhibition of cell growth and the uncoupling action of a variety of active and inactive antitumor diarylsulfonylureas.

VII. The Mitochondrial Benzodiazepine Receptor

Benzodiazepines are among the most widely prescribed drugs due to their pharmacological actions in relieving anxiety, and as anticonvulsants, muscle relaxants, or sedative hypnotics. These effects are mediated in the central nervous system through postsynaptic plasma membrane $GABA_A$ receptors that are γ-aminobutyric acid-gated chloride channels. In addition to these central-type benzodiazepine receptors, binding sites were also identified in peripheral tissues, and this second class of sites was termed the peripheral benzodiazepine receptor (PBR). The PBR first characterized by Braestrup and coworkers (Braestrup and Squires, 1977; Braestrup et al., 1977) is present in peripheral tissues such as adrenal glands, kidney, and heart, as well as in the brain. The density of the PBR is the highest in endocrine tissues such as adrenal gland, testis, ovary, uterus, and placenta. PBR is also abundant in kidney, heart, and platelets, but densities in these tissues are approximately five times lower than that in adrenal gland. The PBR has been localized in mitochondrial membranes, and nonmitochondrial localizations have been observed in heart, liver, and testis. Additionally, the PBR was found in mature erythrocytes, which lack mitochondria. The properties and role of PBR were described in several review papers (McEnery, 1992; Parola et al., 1993; Gavish et al., 1999).

There is some terminological inconsistency concerning the mitochondrial PBR. Some authors (e.g., Tatton and Olanow, 1999) use this term to describe an 18-kDa polypeptide with high affinity toward benzodiazepine derivatives and isoquinoline carboxamides. This peptide has been isolated from rat adrenal gland and characterized by Antkiewicz-Michaluk et al., (1988a,b). However, other authors (e.g., McEnery et al., 1992; Gavish et al., 1999) consider the isoquinoline-binding protein (IBP) as one of at least three subunits of the mitochondrial PBR. The other two components are the mitochondrial pore protein, porin, also known as voltage-dependent anion channel (VDAC), with a molecular mass of 32,000; and the adenine nucleotide translocase, with a molecular mass of 30,000. The reactivity of the 18-kDa polypetide toward benzodiazepines usually requires the interaction of all three subunits.

IBP is a protein with five transmembrane domains usually associated with the mitochondrial outer membrane. The cDNA for the 850-nucleotide IBP mRNA has been cloned from a number of species. A detailed description of the IBP gene was recently reviewed (Gavish et al., 1999).

The PBR complex is located in the contact sites between the outer and the inner mitochondrial membranes. Its subunit composition roughly coincides with that of the mitochondrial permeability transition pore (Brustovetsky and Klingenberg, 1996; Beutner et al., 1996; Halestrap et al., 1997b) that opens under specific conditions and enables unselective passage of molecules of up to 1.5 kDa between the mitochondrial matrix and the cytoplasm (Bernardi et al., 1994; Zoratti and Szabó, 1995). Recently, much attention has been directed toward this pore, also called the "mitochondrial megachannel", because of its postulated role in events leading to programmed cell death (apoptosis) in multicellular organisms (Kroemer et al., 1997, 1998; Tatton and Olanow, 1999). The role of the 18-kDa IBP in the opening/closing transition of the pore and in its function in eliciting apoptosis is not clear. On the other hand, other components, such as cyclophillin D, have been described as obligatory functional elements of the permeability transition pore (Halestrap et al., 1997a) (for more information see *Section III*. and Fig. 3).

Recently, using a cytoplasmic domain of IBP as a bait in the yeast two-hybrid system, a new protein that specifically interacts with IBP has been cloned (Galičgue et al., 1999). This protein, named PRAX-1 (peripheral benzodiazepine receptor-associated protein 1), exhibits several domains involved in protein-protein interactions such as three proline-rich domains and three leucine-zipper motifs (Galičgue et al., 1999).

In contrast to the central benzodiazepine receptor, PBR exhibits nanomolar affinity to benzodiazepine Ro5-4864 (4′-chlorodiazepam) and the isoquinoline carboxamide derivative PK11195, and low affinity to benzodiazepine clonazepam. Isoquinoline carboxamide derivatives, such as PK11195**,** bind specifically to the 18-kDa IBP subunit, whereas PBR-specific benzodiazepine ligands, such as Ro5-4864, bind to a site consisting of porin, as well as adenine nucleotide translocase and the 18-kDa IBP subunit (Garnier et al., 1994). Other ligands for PBR, such as 2-aryl-3-indoleacetamide (Romeo et al., 1992) and *N*-(2,5-dimethoxybenzyl)-*N*-(5-fluoro-2-phenoxyphenyl)-acetamide (Chaki et al., 1999), were also described.

Benzodiazepine Ro5-4864 and isoquinoline carboxamide PK11195 are the two most widely used PBR ligands. Based on the entropy-driven and enthalpy-driven nature of ligand-receptor interactions, PK11195 has been classified as an antagonist and Ro5-4864 as an agonist (Le Fur et al., 1983). The functional significance of such classification was recently confirmed in studies on the antiapoptotic activities of these PBR ligands (Bono et al., 1999).

Protoporphyrin IX binds to PBR with nanomolar affinity and has been suggested to be an endogenous ligand for PBR (Snyder et al., 1987). During heme biosynthesis, cytosolic coproporphyrinogen III traverses the

mitochondrial outer membrane and is converted via protoporphyrin IX to heme, which is subsequently exported from mitochondria. This suggests an involvement of PBR in mitochondrial heme synthesis (Verma et al., 1987; Woods and Williams, 1996). Another candidate for endogenous ligand for PBR is a 104-amino acid neuropeptide known as diazapam binding inhibitor (Corda et al., 1984). Also, a 16-kDa protein called anthralin inhibits the specific binding of Ro5-4864 to PBR (Mantione et al., 1988).

PBR has been implicated in several mitochondrial functions, but its exact physiological role is still unclear. In vitro studies using isolated cells, mitochondria, and submitochondrial fractions demonstrated that PBR is present in steroid-synthesizing cells and is involved in this kind of cell in the regulation of cholesterol transport from the outer to the inner mitochondrial membrane, known to be the rate-determining step in steroid biosynthesis (Culty et al., 1999). The postulated role of PBR was recently reviewed in detail (Gavish et al., 1999). PBR agonist Ro5-4864 was found to strongly protect against apoptosis induced by tumor necrosis factor-α in human lymphoblastoid cell line U937 (Bono et al., 1999). The potent antiapoptotic effect might represent a major function for this receptor as demonstrated by the lack of antiapoptotic activity of Ro5-4864 in wild-type Jurkat cells (lacking the PBR receptor) and the reappearance of this effect in PBR-transfected cells (Bono et al., 1999). Additionally, the blockade of the antiapoptotic effect of PBR agonist by selective PBR antagonist PK11195 was observed (Bono et al., 1999).

VIII. Immunosuppressant Drugs and Mitochondria

Cyclosporin A has potent immunosuppressive properties due to its ability to block the transcription of cytokine genes in activated T cells (Rovira et al., 2000). It is well established that CsA forms a complex with cyclophylin D, inhibiting the peptidyl-prolyl *cis-trans* isomerase activity of this protein. Additionally, the CsA-cyclophilin complex inhibits the activity of calcineurin (Rusnak and Mertz, 2000). Calcineurin is a Ca^{2+}- and calmodulin-dependent serine/threonine phosphatase (protein phosphatase 2B) that regulates nuclear translocation and subsequent activation of the nuclear factor of activated T cells known as NFAT transcription factor. Prevention of NFAT dephosphorylation, which is an important step for its translocation to the nucleus, blocks cytokine production. In addition to the calcineurin/NFAT pathway, recent studies indicate that CsA also blocks the activation of stress-activated protein kinase JNK (c-jun NH_2-terminal kinase) and the mitogen-activated protein kinase p38 signaling pathway triggered by antigen recognition (for review see Matsuda and Koyasu, 2000). Despite this beneficial role of CsA in organ transplantation, CsA has significant side effects such as hypertension, and renal and muscle toxicity. Probably, these effects of CsA are related to ROS generation (Buetler et al., 2000).

CsA blocks the opening of the PTP (see *Section III.A.*) because of its high affinity to cyclophilin D. This effect is independent of the inhibition of calcineurin and the immunosuppressive action of CsA, because the CsA analog *N*-methyl-Val-4-cyclosporin lacks immunosuppressive properties and is still able to block PTP (Zamzami et al., 1996; Hortelano et al., 1997; Matsumoto et al., 1999; Vergun et al., 1999). The blocking of PTP opening is most likely the mechanism underlying the protective action of CsA against ischemic and ischemic/reperfusion injuries. Such beneficial effects have recently been described in ischemic and traumatic brain injury (TBI) in experimental animals (Scheff and Sullivan, 1999; Sullivan et al., 1999, 2000; Albensi et al., 2000; Li et al., 2000). For example, animals subjected to forebrain ischemia for 30 min exhibited extensive neuronal necrosis and failed to survive, whereas injection of CsA (in combination with an intracerebral lesion to open the blood-brain barrier) prolonged their survival time, ameliorating brain damage and preventing secondary mitochondrial dysfunction (Li et al., 2000).

Although TBI often results in impaired learning and memory functions, the underlying mechanisms are unknown, and there are currently no treatments that can preserve such functions. Recently, the plasticity at CA3-CA1 synapses in hippocampal slices from rats subjected to controlled cortical impact TBI was studied (Albensi et al., 2000). Long-term potentiation of synaptic transmission was markedly impaired after TBI. Post-TBI administration of CsA resulted in a highly significant amelioration of the impairment of long-term potentiation. These data suggest that alterations in hippocampal synaptic plasticity may be responsible for learning and memory deficits resulting from TBI and that agents such as CsA, which improve mitochondrial function, may be effective in the treatment of TBI.

CsA is also effective in protecting against reoxygenation injury in cardiomyocytes (Griffiths et al., 2000). Recently, it has been shown that palmitate-induced apoptosis in cardiomyocytes is prevented by CsA (Kong and Rabkin, 2000). Probably, due to its inhibitory action on PTP, CsA prevents necrotic cell death from oxidative stress, Ca^{2+} ionophore toxicity, Reye's-related drug toxicity, pH-dependent ischemia/reperfusion injury, and other kinds of cell injury (for review see Halestrap et al., 1997a; Lemasters et al., 1999). The protective effect of CsA due to its interaction with PTP could also be of importance in normothermic reperfusion during organ transplantation (Leducq et al., 2000).

FK506 (tacrolimus), another potent immunosuppressant, very often acts via the same signaling pathway as CsA but sometimes is unable to mimic its effect. For example, CsA but not FK506 inhibits creatine uptake by altering surface expression of the creatine transporter

(Tran et al., 2000). Additionally, FK506 has no effect on PTP, induced in isolated mitochondria, that is sensitive to CsA. Swelling kinetics of isolated mitochondria from the hippocampus showed that CsA, but not FK506, inhibits calcium ion-induced PTP opening (Friberg et al., 1998). Lead and calcium produce rod photoreceptor cell apoptosis by opening mitochondrial PTP sensitive to CsA but not to FK506 (He et al., 2000). Interestingly, thymocytes were rescued from apoptosis caused by thapsigargin by incubation with FK506 (Waring and Beaver, 1996).

IX. Disruption of Mitochondrial Functions by Antiviral Drugs

Mitochondria were identified as potential target organelles in toxicity induced by nucleoside analogs used as antiviral drugs (Lewis and Dalakas, 1995; Styrt et al., 1996; for review see Barile et al., 1998). Most of these studies focused on 3′-azido-3′-deoxythymidine (AZT, zidovudine), used for treatment of the acquired immune deficiency syndrome (AIDS). AZT inhibits human immunodeficiency virus (HIV) replication and delays the progression of AIDS. The toxicity of AZT was observed in various tissues such as liver and skeletal muscle. AZT also affects mitochondrial metabolism in heart, leading to a decrease of the ATP/ADP ratio and cardiomyopathy.

AZT causes mitochondrial damage through two mechanisms: a short-term mechanism that directly affects the activity of mitochondrial enzymes, e.g., respiratory chain proteins, and a long-term mechanism that alters the mitochondrial DNA (mtDNA), thus impairing mitochondrial protein synthesis.

The short-term cardiac side effects of AZT were studied in rats to understand the development of AZT-induced cardiomyopathy (Szabados et al., 1999). AZT treatment provoked a surprisingly fast appearance of cardiac malfunctions in developing animals. Electron microscopy showed abnormal mitochondrial structure but normal myofibers. AZT treatment of rats significantly increased ROS and peroxynitrite formation in heart tissues and induced single-strand DNA breaks. Lipid peroxidation and oxidation of cellular proteins, determined from protein carbonyl content, increased as a consequence of AZT treatment. Additionally, a moderate decrease in the activity of respiratory complexes was detected in hearts of AZT-treated animals indicating a damaged mitochondrial energy production. The calculated free ATP/ADP ratio decreased from 340 to 94 in the hearts of AZT-treated rats as a consequence of increased free ADP concentration. These data show that ROS-mediated oxidative damage may play an important role in the development of AZT-induced cardiomyopathy in AZT-treated AIDS patients (Szabados et al., 1999). Recently, AZT has been found to inhibit the ADP/ATP antiport in a competitive manner (K_i value of about 7 μM) in mitochondria isolated from rat heart (Valenti et al., 2000). In contrast, the rate of transport via the dicarboxylate carrier, the oxodicarboxylate carrier, and the tricarboxylate carrier was unchanged in the presence of AZT (Valenti et al., 2000).

The effects of AZT on the mitochondrial energy-generating mechanism were investigated in isolated skeletal muscle mitochondria (Masini et al., 1999). Membrane potential abnormalities, due to a partial impairment of the respiratory chain capability observed in skeletal muscle mitochondria from AZT-treated rats, closely resemble those of control mitochondria in the presence of externally added AZT (Freyssenet et al., 1999; Masini et al., 1999). It was also shown (Valenti et al., 1999) that AZT inhibits mitochondrial nucleoside diphosphate kinase in a competitive manner, with a K_i value of about 10 μM as measured for all tested nucleoside diphosphates (TDP, UDP, CDP or GDP). It has also been shown that high concentrations of GDP prevent AZT inhibition of nucleoside diphosphate kinase (Valenti et al., 1999).

AZT causes oxidative damage to mtDNA both in muscle (De la Asuncion et al., 1998) and liver (De la Asuncion et al., 1999). Liver mtDNA of mice treated with AZT had 40% more oxidized mutagenic nucleoside, 8-oxo-7,8-dihydroxy-2′-deoxyguanosine, than untreated controls. This oxidative damage to mtDNA is caused by a significant increase in peroxide production by liver mitochondria from AZT-treated mice (De la Asuncion et al., 1999). mtDNA deletion analysis by polymerase chain reaction amplification and Southern blot analysis did not show any relevant deletion, whereas mtDNA depletion analysis demonstrated a significant decrease in skeletal muscle mtDNA in AZT-treated rats (Masini et al., 1999). AZT inhibits the polymerase responsible for mtDNA replication. Myocardial alterations caused by this action have also been assessed (McCurdy and Kennedy, 1998). Ventricular muscles from rats treated with AZT were analyzed for cytochrome oxidase activity and for the content of mRNA for the nuclear- and mitochondrial-encoded subunits of this enzyme. In addition, expression of contractile proteins was assessed by examining mRNA levels for α- and β-myosin heavy chains (MHC). The results showed that AZT caused a reduction in cytochrome oxidase activity, in the content of its subunit III mRNA, and in mtDNA levels. There was no decrease in the cytochrome oxidase nuclear-encoded subunit mRNA. MHC expression was altered so that the relative contents of β-MHC protein and mRNA were increased. These data demonstrate that AZT induces a reorganization of cardiac gene expression indicative of changes in cardiac contractile properties. The observed decreases in mtDNA levels along with mRNA for a mitochondrial-encoded protein and cytochrome *c* oxidase activity are consistent with the postulated mechanism whereby AZT induces myopathy by diminishing mtDNA replication (McCurdy and Kennedy, 1998).

Recently, the effects of AZT and other anti-HIV nucleoside analogs, as well as the metabolite of AZT, 3′-amino-3′-deoxythymidine, on mitochondrial function in a human hepatoma cell line were studied (Pan-Zhou et al., 2000). Evidence for a number of mitochondrial defects produced by AZT and it derivatives was found, but only AZT induced a marked rise in lactic acid level. Only in mitochondria isolated from AZT-treated cells were cytochrome oxidase and citrate synthase significantly inhibited. It was hypothesized that liver mitochondria possess an excess of the respiratory capacity so that the inhibition of respiratory enzymes is unlikely to become critical. In contrast, the combined inhibition of the citric acid cycle and electron transport greatly enhances the dependence of the cell on glycolysis and may explain why apparent mitochondrial dysfunction is more prevalent with AZT treatment (Pan-Zhou et al., 2000).

Short-term effects of the anti-retroviral drug 2′,3′-dideoxycytidine (ddC, zalcitabine) on mitochondria were also studied (Rossi et al., 1999; Skuta et al., 1999). It was found that in developing animals, ddC treatment provoked a surprisingly rapid appearance of cardiac malfunctions. ddC treatment of rats significantly increased the formation of ROS in heart and skeletal muscle. A decrease in the quantity of heat shock protein (HSP) 70 was also detected, whereas the level of HSP25 and HSP60 remained unchanged. These data show that the short-term cardiotoxicity of ddC is partly based on ROS-mediated signaling reactions, and the depression of HSP70 levels represents a new mtDNA-independent mechanism for ddC-induced cell damage (Skuta et al., 1999).

X. Nonsteroidal Anti-Inflammatory Drugs and Mitochondria

The nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen and others are among the most frequently prescribed drugs. In particular, NSAIDs have proven to be highly effective in relieving inflammation and pain caused by arthritis (Steinmeyer, 2000). NSAIDs act through the inhibition of cyclooxygenases and therefore diminish prostaglandin production (Kam and See, 2000). Increasing evidence suggests that aspirin and other NSAIDs additionally reduce the risk of colorectal cancer (Arber and DuBois, 1999; Stockbrugger, 1999; Baron and Sandler, 2000). The molecular mechanism responsible for this action is unclear. It may concern several pathways, including cell cycle arrest or induction of apoptosis (Porter et al., 2000). Surprisingly, NSAIDs are also responsible for complications associated with ulcers, such as perforation and bleeding. This is probably due to a multistage process involving mitochondrial damage and cyclooxygenase inhibition, followed by inflammatory tissue reaction (Somasundaram et al., 1997, 2000; Davies et al., 2000).

It has been suggested that gastrointestinal damage may be initiated by the action of NSAIDs on mitochondria. Therefore, the action of various NSAIDs on these organelles of various tissues has been intensely investigated. NSAIDs were found to uncouple oxidative phosphorylation, increase resting state respiration, decrease ATP synthesis, inhibit the adenine nucleotide translocase, and dissipate the mitochondrial transmembrane potential (Mahmud et al., 1996;. Mingatto et al., 1996; Petrescu and Tarba, 1997; Tomoda et al., 1998; Masubuchi et al., 1999; Moreno-Sanchez et al., 1999; Mingatto et al., 2000). In general, the drug concentrations affecting mitochondrial respiration and energy-coupling processes appeared to be in the low micromolar range for diclofenac, diflunisal, mefenamic acid, tolfenamic acid, fulfenamic acid, and piroxicam, and in the submillimolar and low millimolar range for salicylic acid, acetylsalicylic acid, and dipyrone (Mingatto et al., 1996; Petrescu and Tarba, 1997; Masubuchi et al., 1999). Although under treatment with mild doses of these compounds their concentrations within the tissues may not attain such values, they do so under therapy with high doses. For example, in the treatment of inflammation using massive doses of acetylsalicylic acid (aspirin), its peak concentration in blood plasma may reach the value of 2 mM and in tissues as high as 12 mM (Baggott et al., 1992). Corresponding values for salicylic acid are 2 mM and >4 mM, respectively; and, for plasma concentrations of ibuprofen and piroxicam, 400 μM and 20 μM, respectively (Baggott et al., 1992). Therefore, side effects in the form of nephro-, hepato-, and even cardiotoxicity, based on mitochondrial damage, may occur.

The uncoupling effect of NSAIDs can be due, at least partly, to the induction of the mitochondrial PTP (see *Section III.A.*). Yoshida et al. (1992) and Tomoda et al. (1994) were probably the first to link the adverse effects of salicylic acid and its acetyl ester (aspirin) on mitochondria with calcium ions, whereas Biban et al. (1995) found the protection by Mg^{2+} and CsA, the well known inhibitor of PTP (Broekemeier et al., 1989). The loss of mitochondrial membrane potential by salicylates appeared to require the presence of Ca^{2+} and, on the other hand, to induce the release of accumulated Ca^{2+}, these effects being inhibited by cyclosporin A (Al-Nasser, 1999). Interestingly, acetylsalicylic acid was much less effective as a PTP inducer than was free salicylic acid (Al-Nasser, 1999). Nevertheless, oral administration of acetylsalicylic acid at high therapeutic doses may induce mitochondrial dysfunction in vivo (Tomoda et al., 1994) due to the fact that it is hydrolyzed in the organism to its active metabolite, salicylic acid.

Other NSAIDs also appeared to induce PTP opening. For example, incubation of isolated liver mitochondria in the presence of Ca^{2+} and phosphate with ibuprofen induces mitochondrial membrane depolarization and swelling. This process is blocked by CsA, suggesting that, in fact, ibuprofen induces PTP (Al-Nasser, 2000).

Additionally, nimesulide induced mitochondrial Ca^{2+} efflux in a partly ruthenium red-sensitive manner and induced PTP (Mingatto et al., 2000). This was prevented by CsA, ADP, and ATP. The ability of NSAIDs to induce Ca^{2+}-mediated and CsA-sensitive PTP opening was also shown in isolated renal cortex mitochondria (Mingatto et al., 1996; Uyemura et al., 1997; Pigoso et al., 1998). It varied from one compound to another. It was low for piroxicam and acetylsalicylic acid but was very high for diclofenac and mefenamic acid, the two latter compounds acting at concentrations of 20 μM and 10 μM, respectively, according to some authors (Pigoso et al., 1998) and at as low as 2 μM according to others (Uyemura et al., 1997). The latter concentration can certainly be attained under therapeutic doses. However, in contrast to liver mitochondria (Al-Nasser, 2000), ibuprofen seemed to be less effective on PTP in renal cortex mitochondria, as it did not induce Ca^{2+} efflux, whereas salicylic acid was a poor inducer (Yoshida et al., 1992; Tomoda et al., 1994; Pigoso et al., 1998). When compared with salicylate, a classical uncoupler (Gutknecht, 1990,1992) and inducer of PTP (Al-Nasser, 1999), the potency of diclofenac and mefenamic acid was about 50-fold greater and was completely prevented by CsA (Uyemura et al., 1997). Based on inhibitor studies, Pigoso et al. (1998) suggest that diclofenac and mefenamic acid induce calcium ion efflux in mitochondria through mechanisms involving both PTP and the mitochondrial Ca^{2+}/nH^{+} antiporter. Characteristically, NSAIDs inhibited mitochondrial oxidation of NAD(P)-linked substrates but exerted much lower effect on succinate oxidation (Tomoda et al., 1994). This difference can be explained by depletion of nicotinamide nucleotides due to PTP opening.

These multiple effects of NSAIDs on mitochondria are summarized in Fig. 7.

NSAIDs cause an additional range of adverse effects associated with perturbances of lipid metabolic pathways (Manjula and Devi, 1993). Inhibition of β-oxidation was observed in the presence of aspirin and ibuprofen (Fromenty and Pessayre, 1995; Pessayre et al., 1999). Interestingly, β-oxidation was inhibited stereoselectively by *R*-ibuprofen. In contrast, mitochondrial respiration was moderately inhibited by both enantiomers of this drug (Browne et al., 1999). Inhibition of β-oxidation by aspirin (Fromenty and Pessayre, 1995; Glasgow et al., 1999) and induction of PTP (Martens and Lee, 1984; Martens et al., 1986; Trost and Lemasters, 1996,1997) were believed to be implicated in Reye's syndrome, a childhood disorder characterized by liver disease and encephalopathy (De Vivo, 1985; Glasgow and Moore, 1993; Larsen, 1997; Ward, 1997). However, recent studies weaken the validity of the link between the use of aspirin and Reye's syndrome (Casteels-Van Daele et al., 2000).

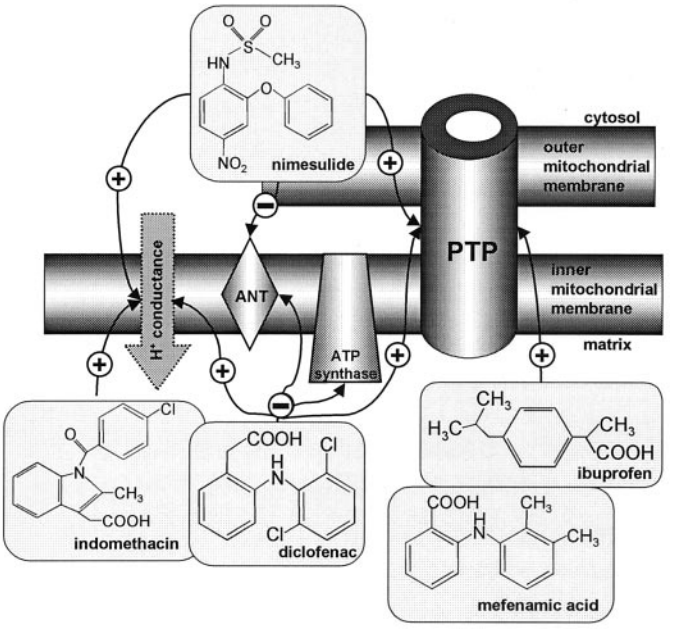

FIG. 7. Main target points of anti-inflammatory drugs within the mitochondrion and examples of active pharmaceuticals. Activation is indicated as ⊕ and inhibition as ⊖. ANT, adenine nucleotide translocase.

XI. Local Anesthetics and Mitochondrial Energy Metabolism

The most relevant physiological effect of local anesthetics is to block the action potential during nerve impulse conduction, thus causing sensory paralysis (Singh and Erwin, 1998; Tetzlaff, 2000). Additionally, local anesthetics can affect a large variety of non-neuronal processes including mitochondrial energy metabolism. They are mostly tertiary amines with pK_a values ranging from 7 to 9, and probably, due to this and their lipophilic properties, they uncouple oxidative phosphorylation (Garlid and Nakashima, 1983; Dabadie et al., 1987; Horakova et al., 1989; Sun and Garlid, 1992). Additionally, it has been shown that they inhibit mitochondrial ATPase (Vanderkooi et al., 1981; Adade et al., 1984, 1987; Dabbeni-Sala et al., 1990; Dabbeni-Sala and Palatini, 1990) and respiratory chain enzymes (Chazotte and Vanderkooi, 1981; Vanderkooi and Chazotte, 1982). Lipid solubility of local anesthetics appears to be the principal physicochemical factor affecting their potency in interfering with mitochondrial bioenergetics (Grouselle et al., 1990). These effects of local anesthetics are secondary to their basic site of action (i.e., the plasma membrane of nerve cells) but are probably responsible for the primary toxic effects observed during analgesia.

The effects of local anesthetics on mitochondrial functions have been observed since the late 1960s. These observations concerned mitochondrial ion transport (Chance et al., 1968; Mela, 1969; Selwyn et al., 1978; Chazotte and Vanderkooi, 1981), including adenine nucleotide translocase (Spencer and Bygrave, 1974), and

metabolic activity (Gotterer, 1969; Haschke and Fink, 1975). Local anesthetics also affect phospholipase activity (Scherphof et al., 1972; Waite and Sisson, 1972). Interestingly, dibucaine and butacaine have opposite effects depending on their concentration. At 10 to 50 μM, dibucaine stimulates, whereas butacaine inhibits, phospholipase A_2 (Waite and Sisson, 1972). At higher concentrations (200–300 μM) dibucaine inhibits, whereas butacaine stimulates, this enzyme; this indicates that local anesthetics might have more than one mechanism of action.

Paradoxically, early reports on the effect of nupercaine, a local anesthetic of the procaine type, also known under the name of dibucaine, pointed to its protective effect on mitochondrial ultrastructure and energy-coupling properties (Scarpa and Lindsey, 1972; Aleksandrowicz et al., 1973). This was, apparently, due to the inhibition of phospholipase A_2, the enzyme mainly responsible for the deterioration of preparations of isolated mitochondria upon prolonged storage. In fact, subsequent investigations have shown that most local anesthetics have uncoupling properties. The local anesthetic bupivacaine was found to uncouple oxidative phosphorylation (Sztark et al., 1997). Its uncoupling effect depends on the respiration state. In state 4 respiration (no ADP phosphorylation), bupivacaine acts as a true protonophoric uncoupler. In contrast, in state 3 respiration (ADP phosphorylation), bupivacaine induces a change in proton pump stoichiometry. Moreover, at high concentration, bupivacaine inhibits the respiratory chain. Both bupivacaine enantiomers tested on rat heart mitochondria appeared to equally inhibit the activity of complex I of the respiratory chain and to uncouple oxidative phosphorylation (Sztark et al., 2000). Recently, it has been shown that bupivacaine inhibits acylcarnitine exchange in cardiac mitochondria (Weinberg et al., 2000).

The cardiac toxicity of bupivacaine stimulated attempts to develop a less toxic local anesthetic. The outcome was ropivacaine. Even though it is structurally very similar to bupivacaine, ropivacaine is less cardiotoxic in both isolated mitochondria and permeabilized heart fibers. A lower lipid solubility of ropivacaine may be responsible for its weaker dose-dependent effects on mitochondrial bioenergetics (Sztark et al., 1998). These effects are strongly enhanced by the lipophilic anion tetraphenylboron (Floridi et al., 1999). Under these conditions and low drug concentrations, state 4 respiration was stimulated and the mitochondrial membrane potential collapsed (Floridi et al., 1999), whereas at higher concentrations state 3 and uncoupled respiration were inhibited by impairment of electron transfer from NAD- and flavine adenine dinucleotide-linked substrates to the respiratory chain. The fact that tetraphenylboron increased the drug effect indicates that stimulation of respiration was due to dissipation of the electrochemical proton gradient caused by its electrophoretic uptake (presumably in the form of an ion complex), although a classical protonophoric uncoupling mechanism could not be excluded. The mechanism for the lower toxicity of ropivacaine in vivo was ascribed to low lipid solubility leading to reduced access to the mitochondrial membrane (Floridi et al., 1999).

Similar effects of ropivacaine and bupivacaine were observed on rat liver mitochondria (Scutari et al., 1998). In these mitochondria, bupivacaine did not alter the ADP-stimulated respiration but strongly affected the resting respiration and decreased the transmembrane electrical potential and the rate of ATP synthesis. Ropivacaine did not alter ADP-stimulated respiration and did not substantially affect the resting respiration. The transmembrane potential was decreased by the anesthetic concentrations higher than 1.2 mM and ATP synthesis was consequently affected. These findings suggest that ropivacaine is also less toxic than bupivacaine in rat liver mitochondria.

Studies on the effects of local anesthetics on the energy metabolism of intact cells are represented by investigations using Ehrlich ascites tumor cells. For example, it was found that the impairment of energy metabolism of these cells by ropivacaine was due to its effect on mitochondrial function. Low concentrations of ropivacaine decreased the rate of oxygen uptake due to inhibition of electron transport in complexes I and II of the respiratory chain. The inhibition of respiration, decrease of ATP content, and depolarization of mitochondrial membrane by this anesthetic were also observed (Floridi et al., 1994; Pulselli et al., 1996; Di Padova et al., 1998). Similar effects were found for bupivacaine (Floridi et al., 1994; Pulselli et al., 1996).

The effects of local anesthetics, which are due to their interaction with mitochondria, are also observed in more complex events such as apoptosis. Mitochondrial swelling and oxidation of membrane protein thiol groups, associated with the activation of PTP, were inhibited by the local anesthetic dibucaine (Kowaltowski et al., 1998). Additionally, dibucaine promoted the inhibition of Ca^{2+}-induced increase in mitochondrial ROS generation (Kowaltowski et al., 1998). It has been concluded that the mechanism by which dibucaine inhibits the mitochondrial permeability transition is related to the decrease in ROS generation induced by Ca^{2+}-promoted alterations of inner mitochondrial membrane properties (Kowaltowski et al., 1998). Recently, it has also been shown that dibucaine inhibits the growth of promyelocytic leukemia cells without inducing arrest of the cell cycle and differentiation to granulocytes (Arita et al., 2000). DNA fragmentation and DNA "ladder" formation, typical for apoptosis, were induced by dibucaine with half-maximal concentration of 100 μM. These effects were completely prevented by the unspecific caspase inhibitor z-Val-Ala-Asp-(OMe)-fluoromethylketone, thereby implicating caspase activation in the process. In fact, dibucaine activated various caspases, such as caspase-3, -6, -8, and -9 (-like) activities, but not caspase-1 (-like) activity, and in-

duced mitochondrial membrane depolarization and the release of cytochrome *c* from mitochondria into the cytosol (Arita et al., 2000). Taken together, these data suggest that dibucaine induced apoptosis of HL-60 cells through activation of the caspase cascade in conjunction with cytochrome *c* release and depolarization of the mitochondrial membrane (Arita et al., 2000).

XII. Mitochondria as a Pharmacological Target of Lipid Metabolism

Many important steps of lipid metabolism are located in mitochondria. Thus, formation of thioesters of CoA with fatty acids, i.e., the so-called "activation" of fatty acids that is the obligatory step in fatty acid metabolism, occurs partly in the endoplasmic reticulum and partly in the outer mitochondrial membrane (for long-chain fatty acids) or in the mitochondrial matrix (for medium-chain fatty acids) (Aas and Bremer, 1968; Aas, 1971). β-Oxidation of fatty acids, the principal route of energy-yielding fatty acid catabolism, proceeds within the matrix compartment (see Eaton et al., 1996 for review). Because the inner mitochondrial membrane is impermeable to acyl-CoA, long-chain fatty acyl-CoA formed in the outer mitochondrial membrane or in the endoplasmic reticulum must be transformed into the acylcarnitine ester. It then crosses the inner membrane and is transformed back to acyl-CoA on the inner side of the inner membrane. These processes are catalyzed by carnitine acyltransferases I and II (mostly represented by carnitine palmitoyltransferases I and II, abbreviated as CPT I and II) located in the outer membrane and the internal side of the inner membrane, respectively (Kerner and Hoppel, 2000) (Fig. 8).

The outer membrane is also the site of the first step in the phospholipid synthesis pathway, i.e., the esterification of α-glycerol phosphate by acyl-CoA to form lysophosphatidic and phosphatidic acids (Zborowski and Wojtczak, 1969; Bremer et al., 1976). Although all nitrogen-containing phospholipids are synthesized outside mitochondria, cardiolipin, the characteristic phospholipid of the inner mitochondrial membrane, is formed within mitochondria (Hostetler and van den Bosch, 1972). The inner mitochondrial membrane is also the unique site of decarboxylation of phosphatidylserine to phopshatidylethanolamine (Dennis and Kennedy, 1972; Zborowski et al., 1983). Thus, mitochondria form an important crossing point for several metabolic pathways in which lipids are involved and therefore may present a sensitive target for pharmacological intervention (Frøland et al., 1997). However, despite this variety of potential points of pharmacological attack, mainly the modulation of β-oxidation and transfer of the fatty acyl moiety across the inner mitochondria are targeted and in use in medical practice.

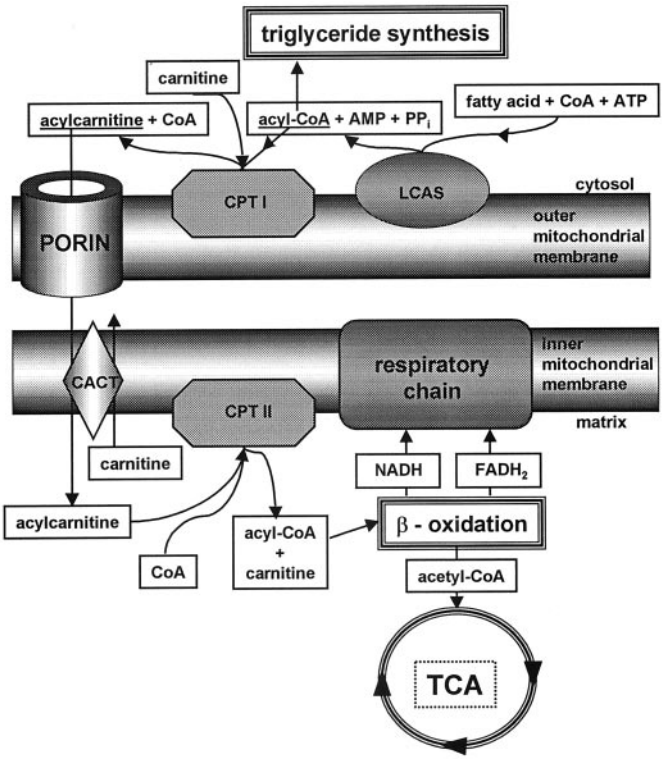

FIG. 8. Main routes of fatty acid metabolism in mitochondria. Long-chain fatty acids react with coenzyme A (CoA) in an ATP-dependent process catalyzed by long-chain acyl-CoA synthetase (LCAS) located in the outer mitochondrial membrane. Acyl-CoA is transformed into acylcarnitine by carnitine palmitoyltransferase I (CPT I) located in the outer membrane and exposed to its external side. The acylcarnitine thus formed penetrates the outer membrane through porin and the inner membrane, in exchange with carnitine, in a process catalyzed by the carnitine/acylcarnitine translocase (CACT). Acylcarnitine is then transformed within the matrix compartment to acyl-CoA by carnitine palmitoyltransferase II (CPT II), and acyl-CoA enters the multistep process of mitochondrial β-oxidation. The end product of the latter, acetyl-CoA, is metabolized to H_2O and CO_2 in the tricarboxylic acid cycle (TCA), whereas reducing equivalents from β-oxidation, in the form of NADH and $FADH_2$, enter the respiratory chain. Acyl-CoA that is not transformed into acylcarnitine and metabolized in β-oxidation can be utilized outside mitochondria for phospholipid and triglyceride synthesis.

A. Inhibition of the Transfer of "Activated" Fatty Acids into Mitochondria and of Their β-Oxidation

Due to complex metabolic and hormonal interrelations, elevated fatty acid oxidation brings about increased gluconeogenesis in liver. In healthy patients (and animals), this process is controlled by the pancreatic hormones insulin and glucagon. However, in noninsulin-dependent diabetes mellitus, one of the ways of preventing hyperglycemia is to decrease β-oxidation. A number of inhibitors of carnitine acyltransferases have been designed that limit the rate of fatty acid oxidation by inhibiting the transfer of the acyl moiety ("activated" fatty acid) into the mitochondrion (Foley, 1992; Foley et al., 1997). They include oxirane carboxylates, irreversible inhibitors of CPT I (Wolf, 1990), and a novel class of reversible inhibitors that mimic the transition state of the acyl transfer reaction (Anderson et al., 1995). They have fewer side effects than irreversible inhibitors (Anderson, 1998; Deems et al., 1998).

Chronic inhibition of β-oxidation by blocking the transfer of acylcarnitine into mitochondria may, however, lead to increased synthesis of triglycerides and undesirable fat deposition. Such effects have been observed as liver steatosis (triglyceride deposition) for L-aminocarnitine, another inhibitor of CPT I (Nagy et al., 2000), and as an increased incorporation of fatty acids into brain lipids for methyl palmoxirate (Chang et al., 1998). Similar pathological changes can also occur when fatty acid oxidation is blocked by inhibitors of mitochondrial β-oxidation (e.g., tetracyclines) and some anti-inflammatory drugs or by sequestration of CoA, e.g., by aspirin or the antiepileptic drug valproic acid (Fromenty and Pessayre, 1997).

Fatty acid oxidation in liver mitochondria is also inhibited by 4-thia fatty acids, analogs of long-chain fatty acids in which the CH_2 group in the fourth position of the hydrocarbon chain is substituted by a sulfur atom (Hovik et al., 1990). These fatty acids are β-oxidized in mitochondria to an intermediate, alkylthioacryloyl-CoA, which is only slowly metabolized further and therefore forms a trap for CoA. In addition, this metabolite is a strong inhibitor of carnitine palmitoyltransferase II (Skrede et al., 1997). As an effect, feeding 4-thia fatty acids to animals induces fatty liver. In contrast, 3-thia fatty acids, in which the CH_2 in the third position is replaced by S, increase β-oxidation and have a hypolipidemic action (Desager et al., 1986). This is due to activation of carnitine palmitoyltransferase I and induction of carnitine palmitoyltransferase II. 3-Thia fatty acids are not β-oxidized in mitochondria (Skrede et al., 1997).

B. L-Carnitine Supplementation

Transfer of the acyl moiety of fatty acids to the mitochondrion via the carnitine palmitoyltransferase/carnitine translocase shuttle depends on the availability of free L-carnitine. Under normal nutritional conditions and in healthy individuals, carnitine availability is not a limiting step in β-oxidation. However, it may become so under certain physiological states, e.g., extreme muscular activity, malnutrition, and some pathological conditions. In such cases, carnitine supplementation may provide beneficial effects. These have been observed under extreme metabolic demands, in experimental diabetes (Paulson et al., 1984), in patients subjected to dialysis therapy and those with peripheral arterial disease, in anorexia, coronary vascular disease, male infertility, hypoglycemia, and chronic fatigue (Folts et al., 1978; Brass and Hiatt, 1998; Kelly, 1998). When administered orally to humans, L-carnitine enhances the performance efficiency of high-intensity muscular exercise (Siliprandi et al., 1990; Vecchiet et al., 1990). It is therefore used as a "legal dope" in sports. In brain tissue, the carnitine shuttle mediates translocation of the acetyl moiety from mitochondria into the cytosol and thus probably contributes to the synthesis of acetylcholine (Nałęcz and Nałęcz, 1996). Administration of L-carnitine, combined with other treatments, also proved effective in the treatment of childhood cardiomyopathy (Winter and Buist, 2000).

In all these cases, carnitine supplementation enhances not only the import of "activated" fatty acids into the mitochondrion but also the export of short- and medium-chain fatty acids that may accumulate in mitochondria. In addition, by shifting the equilibrium between acyl-CoA and acylcarnitine, it may increase the level of intramitochondrial free CoA and thus accelerate other CoA-dependent reactions. These mechanisms may be especially important for nontumor tissues of tumor-bearing individuals and in patients subjected to chemotherapy (Peluso et al., 2000). In this context, it is worthwhile mentioning observations that L-carnitine (McFalls et al., 1986) or propionylcarnitine (Sayed-Ahmed et al., 2000) alleviates the cardiotoxic effect of Adriamycin. Adriamycin is a potent antitumor drug that is, however, highly toxic to nonmalignant tissues due to the generation of reactive oxygen species (see *Sections V.* and *XII.*). Propionylcarnitine induces reversal of the inhibition of β-oxidation produced by Adriamycin in cardiac myocytes and isolated rat heart (Sayed-Ahmed et al., 2000).

This finding substantiates earlier clinical observations on the therapeutic effects of L-carnitine in cases that are now regarded as resulting from peroxidative injury, like heart ischemia (Folts et al., 1978; Thomsen et al., 1979; Paulson and Shug, 1982; Paulson et al., 1984) and Alzheimer's disease (Spagnoli et al., 1991). Protection against staurosporin-induced apoptosis (see *Section V.*) of lymphoidal T (Jurkat) cells by carnitine may occur due to a similar mechanism (Mutomba et al., 2000).

Because medium- and short-chain fatty acids are esterified to corresponding acyl-CoAs in the mitochondrial matrix (see above), a massive supply of medium- and short-chain fatty acids may produce a decrease of the mitochondrial membrane potential due to utilization of intramitochondrial ATP for acyl-CoA synthesis (Schönfeld et al., 1988). As an effect, the feeding of medium- and short-chain fatty acids to animals and humans results in a higher energy expenditure and may even prevent weight gain (Papamandjaris et al., 1998).

C. Nonesterified Fatty Acids as "Natural" Uncouplers: Role in Thermogenesis and Obesity Control

Apart from the multiple effects of various pharmaceutics on the mitochondrial lipid metabolism, nonesterified fatty acids as such can modify the energy-coupling properties of mitochondria by increasing the proton permeability of the inner mitochondrial membrane (Skulachev, 1991; Wojtczak and Schönfeld, 1993; Wojtczak and Więckowski, 1999). Although under normal conditions the concentration of nonesterified free fatty acids in tissues is too low to significantly affect the efficiency of oxidative phosphorylation, it increases under particular physiological (e.g., fasting, high-fat diet, excessive exer-

cise) or pathological (e.g., diabetes, ischemia) states. A partial uncoupling of oxidative phosphorylation was observed in perfused rat liver if the perfusion medium contained fatty acid-serum albumin complex (Soboll and Stucki, 1985).

The dissipation of energy stored in the form of the mitochondrial electrochemical proton gradient by nonesterified fatty acids is of particular importance in brown adipose tissue, which is the unique thermogenic organ in mammals (Nicholls and Locke, 1984). Brown adipose tissue mitochondria contain a specific protein, the "uncoupling protein" (UCP, also called thermogenin), that mediates fast transfer of the fatty acid anion across the inner mitochondrial membrane and, thus, a rapid dissipation of the electrochemical proton gradient (Skulachev, 1991; Garlid et al., 1996a), or forms fatty acid-"lined" proton channels (Klingenberg, 1999). As a result, most of the energy produced by the mitochondrial respiratory chain is not utilized for ATP synthesis but is dissipated in the form of heat. Therefore, the brown adipose tissue is of vital importance in newborn mammals, in cold-acclimatized animals, and during arousal from hibernation. It is also of particular interest that the brown adipose tissue, due to its ability for high energy expenditure, may protect experimental animals against overfeeding-produced obesity (Brooks et al., 1980; Tulp, 1981; Himms-Hagen, 1984; Rothwell et al., 1985).

During recent years, homologs of the brown fat uncoupling proteins have also been found in mitochondria of skeletal muscle, liver, white adipose tissue, and, possibly, other organs (Fleury and Sanchis, 1999; Boss et al., 2000; Ricquier and Bouillaud, 2000). Their expression is under strict hormonal control. The biochemical role of these proteins is not completely resolved. It is supposed that they may participate in temperature control and expenditure of excess nutritional energy, or, in contrast, adaptation to fasting, regulation of mitochondrial ATP synthesis, and protection against the generation of ROS (Nègre-Salvayre et al., 1997). Although the expression of these novel uncoupling proteins can, potentially, be subject to pharmacological stimulation, e.g., by triiodothyronine, our present knowledge about their physiological function is too poor to allow rational application.

D. N-Acylethanolamines

N-Acylethanolamines (NAEs) are derivatives of fatty acids in which the carboxylic group of the fatty acid is bound with the amino group of ethanolamine by an amide linkage (their proper chemical name should therefore be *fatty acyl ethanolamides*). NAEs are present in various tissues at amounts ranging from about 0.1 to over 20 nmol/g (Hansen et al., 2000b). Their content increases up to 500 nmol/g tissue in canine heart during ischemia (Epps et al., 1979). NAEs and their phospholipid precursor (*N*-acyl-phosphatidylethanolamine) also accumulate in cortical neurons as a result of glutamate-induced neurotoxicity (Hansen et al., 1999b) and damage produced by hydrogen peroxide (Hansen et al., 1999a) and sodium azide (Hansen et al., 2000a). *N*-Arachidonoylethanolamine (20:4-NAE), named *anandamide*, has attracted particular attention because it appeared to be an endogenous ligand of the brain cannabinoid receptor (Devane et al., 1992; Di Marzo et al., 1994). Because NAEs can easily penetrate from injured cells, in which they are presumably formed, to adjacent areas, it has been speculated that they could have signaling or cytoprotective effects.

The effect of NAEs on isolated mitochondria has been studied. At low micromolar concentrations, NAEs appeared to prevent increased permeability of the inner mitochondrial membrane produced by Ca^{2+} overloading (Epps et al., 1982). Such action is now interpreted as resulting from closure, or inhibition of opening, of PTP (*Section III.A.*). At higher concentrations, NAEs inhibited mitochondrial respiration and lowered mitochondrial membrane potential (Epps et al., 1982). Preliminary experiments (M. R. Więckowski and L. Wojtczak, unpublished observations) have shown that closure of PTP could be observed not only in isolated liver and heart mitochondria but also in brain PTP reconstituted into phospholipid vesicles.

Anandamide and other NAEs have been found to promote apoptosis and/or inhibit cell proliferation in various types of cells (Schwarz et al., 1994; De Petrocellis et al., 1998; Bannerman et al., 2000; Maccarrone et al., 2000; Sarker et al., 2000). It is, however, doubtful whether the PTP closing properties of NAEs may be involved in this effect.

XIII. Final Remarks

In this review we have presented classes of pharmaceuticals that specifically interact with mitochondrial enzymes and metabolic pathways. We have also described drugs whose interaction with mitochondria is secondary to their primary target but a consistent property for this particular group of compounds; for example, local anesthetics. Apart form such drugs, there are numerous compounds applied in human and veterinary medicine that may interact with mitochondria in a rather accidental way. Here, we may mention some antiarrhythmic drugs such as amiodarone (Yasuda et al., 1996; Card et al., 1998; Moreau et al., 1999), several antibiotics, such as chloramphenicol, which is known to inhibit not only bacterial but also mitochondrial protein synthesis (Kroon, 1965), β-blockers (Dreisbach et al., 1993), and neuroleptic drugs (Balijepalli et al., 1999).

Of special interest also are peroxisome proliferators, among them widely used hypolipidemic drugs, exemplified by clofibrate. Although their main target is peroxisomes, it has been shown that they may also affect mitochondrial functions. Peroxisome proliferators, like thyroid hormones, were found to activate biosynthesis of several mitochondrial enzymes (Hertz et al., 1991;

Schon et al., 1994; Cai et al., 1996; Casas et al., 2000). On the other hand, at high doses they can uncouple oxidative phosphorylation, dissipate mitochondrial membrane potential, and inhibit mitochondrial respiration (Keller et al., 1992; Qu et al., 1999; Zhou and Wallace, 1999). It has been recently shown that these effects may be mediated by increased production of oxygen free radicals and promotion of PTP opening (Qu et al., 2001).

Finally, we want to briefly outline new perspectives to combat genetic defects in mitochondrial functions. Inborn errors in the expression of mitochondrial proteins lead to malfunctions of the mitochondrial respiratory chain and ATP-synthesizing machinery that form the basis of a heterogeneous class of so-called mitochondrial diseases. They comprise various clinical entities classified as encephalo-, neuro-, and myopathies (Luft, 1994; Howell, 1999; Larsson and Luft, 1999). Although the first case of a myopathy related to impaired mitochondrial oxidative phosphorylation was described 40 years ago (Luft et al., 1962), a rapid progress in the recognition of the molecular basis of mitochondrial diseases is the matter of the last decade. Depending on whether the defective gene is located in the nuclear or the mitochondrial genome, the disease is transmitted by either Mendelian or maternal inheritance. Because the central nervous system and skeletal muscles are most susceptible to mitochondrial dysfunction, the diseases are characterized by severe and mostly progressive symptoms and are, as a rule, fatal, the life span ranging from a few days following birth up to adulthood.

Intense studies have been undertaken to develop proper gene therapies. Although this research is mostly beyond the scope of the present article, a few attempts will be mentioned here, as they might be related to pharmacological intervention. A strategy of choice in repairing defective expression of mitochondrial proteins is the introduction of nuclear gene sequences into the mitochondrial genome and their expression inside the mitochondrion (Collombet and Coutelle, 1998). This could be, theoretically, achieved by covalently coupling DNA sequences to short targeting peptide sequences that can enter mitochondria via the protein import pathway (Seibel et al., 1995). In fact, a successful attempt of introducing DNA moiety into the mitochondrial matrix was performed by attaching it to the signaling vector of ornithine transcarbamylase (Seibel et al., 1999). Another strategy is to express the 13 polypeptides that are normally encoded by mitochondrial DNA from nuclear transgenes (de Grey, 2000; Owen et al., 2000). However, the high hydrophobicity of these polypeptides presents a serious problem in their transport into and proper insertion within the mitochondrion. Finally, treatment of mitochondrial defects by introducing sequence-specific antigenomic peptide nucleic acids to specifically inhibit replication of mutant mitochondrial DNA has also been proposed (Taylor et al., 2000). All these attempts, if proved successful, may form what we can call "mitochondrial pharmacology of the future".

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