Topical Review

Pathophysiological and protective roles of mitochondrial ion channels

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Mitochondria possess a highly permeable outer membrane and an inner membrane that was originally thought to be relatively impermeable to ions to prevent dissipation of the electrochemical gradient for protons. Although recent evidence has revealed a rich diversity of ion channels in both membranes, the purpose of these channels remains incompletely determined. Pores in the outer membrane are fundamental participants in apoptotic cell death, and this process may also involve permeability transition pores on the inner membrane. Novel functions are now being assigned to other ion channels of the inner membrane. Examples include protection against ischaemic injury by mitochondrial K_{ATP} channels and the contribution of inner membrane anion channels to spontaneous mitochondrial oscillations in cardiac myocytes. The central role of mitochondria in both the normal function of the cell and in its demise makes these channels prime targets for future research and drug development.

Chemiosmotic hypothesis and energy dissipative ion channels

In 1961, Mitchell proposed the chemiosmotic hypothesis to explain the mechanism of mitochondrial energy transduction (Mitchell, 1961). Paraphrasing the essential postulates of the hypothesis in terms of the current view gives four main features: (1) H^+ translocation down its electrochemical gradient across the mitochondrial inner membrane is reversibly coupled to ATP phosphorylation through the ATP synthase (F_1F_0-ATPase); (2) the flow of reducing equivalents down the electron transport chain (based on the differing redox potentials of substrates in the chain) is coupled to H^+ pumping from the matrix to the intermembrane space, thus establishing the large electrochemical gradient for H^+ (or protonmotive force); (3) exchange–diffusion carrier proteins are present on the inner membrane to transport metabolites and selected inorganic ions into and out of the matrix, and (4) the mitochondrial inner membrane is generally impermeable to ions other than H^+ movement through the ATPase.

As pointed out in the original Mitchell paper (Mitchell, 1961), the last point applies only to mitochondria with tight coupling between oxygen consumption (i.e. electron transport) and phosphorylation of ADP, and the extent of coupling would be expected to vary with the leakiness of the membrane. Since that time, techniques such as mitochondrial swelling assays, patch-clamp recordings, or reconstitution of mitochondrial membrane proteins in lipid bilayers indicate that such 'leak' may be mediated by a variety of inner membrane ion channels with specific ion selectivities, conductances and sensitivities to modulators. Despite the profound effect of opening these energy dissipating ion channels on mitochondrial metabolism, there is remarkably little known about their molecular structure, regulation or physiological role in intact cells. The present article explores the hypothesis that *opening specific mitochondrial ion channels leads to specific functional consequences, either detrimental or beneficial to cell function.* Examples of ongoing studies in our laboratory are given in support of this idea and are complemented by the other articles in this issue.

Channel types in mitochondrial membranes

A wide variety of selective ion transport pathways in mitochondria have been identified (summarized in Table 1), either through swelling or fluorescence assays in isolated mitochondria (Beavis, 1992; Garlid, 1994; Bernardi, 1999), by patch-clamp of isolated mitochondria and mitoplasts (mitochondria with ruptured outer membranes to allow access to the inner membrane) (Sorgato *et al.* 1987; Inoue *et al.* 1991; Moran *et al.* 1992; Sorgato & Moran, 1993), or by

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reconstitution of mitochondrial proteins in bilayers or proteoliposomes (Ballarin & Sorgato, 1995; Brenner *et al.* 2000).

Outer membrane channels

The mitochondrial outer membrane contains one of the most extensively studied mitochondrial channels, the voltage-dependent anion channel (VDAC, or porin) (Colombini *et al.* 1996; Hodge & Colombini, 1997). The high permeability of VDAC in reconstitution experiments has contributed to the impression that the outer membrane plays a rather mundane role in cell physiology. However, resurgent interest in the outer membrane has been spurred by the discovery that a key event of programmed cell death (apoptosis) is the translocation of cytochrome c from the intermembrane space to the cytoplasm. A possible explanation for this is that apoptosis-inducing proteins (e.g. Bax) either form pores across the outer membrane or increase the conductance of endogenous channels like VDAC to allow passage of large molecules. Pore formation by the introduction of some Bcl2-homology proteins into lipid bilayers has been observed (Brenner *et al.* 2000), and there is recent evidence implicating VDAC in the permeability increase (Shimizu *et al.* 2000). It has also been suggested that modulation of outer membrane permeability may control the entry or exit of metabolites, perhaps playing a role in the regulation of mitochondrial energy metabolism (Rostovtseva *et al.* 1997; Vander Heiden *et al.* 2000). In addition, mitochondrial protein import involves pore-forming translocases in the outer (tom) and inner (tim) membranes which can be blocked by protein import targeting peptides (hence the name peptide-sensitive channels (Chich *et al.* 1991; Pelleschi *et al.* 1997). It is interesting to note that the large VDAC conductances seen in bilayers are not generally observed in patch-clamp experiments of intact mitochondria; rather, a variety of conductances spanning the range from 10–307 pS have been reported (Moran *et al.* 1992). At present, functions have not been assigned to these conductances and little is known about their regulation.

Inner membrane channels

The mitochondrial inner membrane was originally assumed to be generally impermeable to ions as a prerequisite for efficient chemiosmotic coupling. It is now clear that a number of ion channels are present and may open under particular circumstances. The major identified channels are listed in Table 1 and discussed briefly below. More detailed reviews are available for the reader interested in mitochondrial ion transport (Antonenko *et al.* 1991; Beavis, 1992; Brierley *et al.* 1994; Zoratti & Szabo, 1994; Garlid, 1996; Szewczyk, 1998; Bernardi, 1999).

Ca^{2+} uniporter. The Ca^{2+} uniporter is believed to play a central role in matching changes in energetic workload to stimulation of mitochondrial metabolism. As part of a complex Ca^{2+} handling cycle, the normal function of the uniporter is to transport Ca^{2+} down its electrochemical gradient into the mitochondrial matrix during periods of elevated cytoplasmic Ca^{2+}. Ca^{2+}-regulated dehydrogenases (pyruvate, isocitrate, and α-ketoglutarate dehydrogenases) are then stimulated to increase the production of reducing equivalents by the Krebs cycle (McCormack *et al.* 1990; Hansford, 1994). The single channel equivalent of the uniporter has not been clearly identified, but the channels are thought to be regulated by divalent ions and nucleotides and are blocked by Ruthenium Red (Gunter & Gunter, 1994; Litsky *et al.* 1997). Ca^{2+} transport also appears to be co-operatively stimulated by high Ca^{2+} (Kroner, 1986; Saris & Kroner, 1990). In addition to stimulating respiration, mitochondrial Ca^{2+} influx can be a significant cellular Ca^{2+} buffer, effectively integrating the response to transient Ca^{2+} stimuli (Hajnóczky *et al.* 1995; Zhou *et al.* 1998; Brandes & Bers, 1999) and loading the mitochondrial Ca^{2+} store. A recent proposal suggests that Ca^{2+} release from this store may be triggered to act as an intracellular signalling mechanism (Ichas *et al.* 1997).

Although not shown in Table 1, a second Ruthenium Red-sensitive rapid Ca^{2+} uptake mode (RaM) has been described (Gunter *et al.* 1998). Since its kinetics and Mg^{2+} sensitivity differ from the classical Ca^{2+} uniporter, it is possible that this pathway involves a new type of Ca^{2+} conductive ion channel.

Permeability transition pore (PTP). The generalized inner membrane permeability increase induced by Ca^{2+} described in isolated mitochondria is thought to be mediated by the opening of a large non-selective ion channel capable of passing solutes up to 1500 MW (Hunter *et al.* 1976; Kroemer *et al.* 1998; Crompton, 1999; Duchen, 1999). An in-depth discussion of this topic is presented by Crompton in this volume (Crompton, 2000). Leading candidates for the single channel underlying this transition are the mitochondrial megachannel (MMC; Szabo & Zoratti, 1992) and the multiconductance channel (MCC; Kinnally *et al.* 1996). Many features of MMC and MCC are similar to those of the permeability transition of intact mitochondria (in lieu of a definitive assignment, they will be referred to collectively as the permeability transition pore (PTP) herein). Common properties include induction of opening under high matrix Ca^{2+} loads, oxidative stress, depolarization, inhibition by Mg^{2+}, ADP, cyclosporin A, and modulation by ligands of the adenine nucleotide translocase (ANT; e.g. bongkrekic acid and atractyloside). PTP openings are characterized by a large number of subconductance states, suggestive of active multimerization of channel proteins in the membrane (Zorov *et al.* 1992). The channels are also modulated by thiol redox state, pH, free radicals, Bax, and in the case of MCC, by mitochondrial signal peptides (Kushnareva *et al.* 1999). Alterations of the properties of MCC by a *tim*23 mutation and block of the channel by a tim23 antibody suggest MCC may be a component of the mitochondrial protein import machinery (Lohret *et al.* 1997).

It is presently unknown whether the PTP plays a role in the normal function of mitochondria, but there is strong evidence that it contributes to cellular injury during

Table 1. Mitochondrial ion channels

Location	Type	Conductance (~150 mM salt)	Modulators/ Inhibitors	Putative Role
Outer membrane	VDAC (porin)	0·5–4 nS	Bax/Bak/Bcl-xL, TOM20, Ca^{2+}, pH, ΔV, NADH, VDAC modulator	Metabolic transport, cytochrome C release/apoptosis, PTP complex
	TOM40 (PSC)	0·5 nS	Signal peptides	Protein transport
	BH proteins	—	Bax/Bid/Bik	Cytochrome C release/apoptosis
	Misc.	10–307 pS	ΔV (for >100 pS)	—
Inner membrane	Ca^{2+} uniporter	—	Divalents, nucleotides	Ca^{2+} uptake
	PTP MCC	0·03–1·5 nS	Ca^{2+}, ΔV, signal peptides, CsA	Protein transport
	PTP MMC	0·3–1·3 nS	CsA, pH, Ca^{2+}, thiols, Bax, ANT inhibition	Necrosis, apoptosis
	UCP	75 pS	Fatty acids	Thermogenesis
	K_{Ca}	295 pS	Ca^{2+}, ΔV, ChTx	Volume regulation
	K_{ATP}	9·7 pS	ATP, GTP, palmitoyl-CoA, Mg^{2+}, Ca^{2+}	Volume regulation, protection, apoptosis
	IMACs	45, 450 pS	ATP	(In yeast) volume regulation
		15 pS (LCC)	Mg^{2+}, pH, P_i, thiols, DIDS,	
		107 pS (centum pS)	Cationic amphiphiles	Volume regulation

A summary of mitochondrial ion channel types identified either in isolated mitochondria, proteolipid bilayers, or in patch-clamp experiments. Detected single channel conductances have been tentatively assigned to a given type, but these assignments have not been unequivocally proven. Abbreviations as follows: VDAC (porin), voltage-dependent anion channel; TOM40 (PSC), TOM20, pore forming translocases in outer membrane; BH proteins, Bcl2 homology proteins; Misc., miscellaneous conductances reported by Moran *et al.* (1992); PTP, permeability transition pore; MCC, multiconductance channel; MMC, mitochondrial megachannel; UCP, uncoupling protein; IMAC inner membrane anion channel; LCC, low conductance channel; Bax, Bak, Bid, Bik, Bcl-xl, apoptosis-related proteins; ΔV, change in membrane voltage; CsA, cyclosporin A; ANT inhibition, adenine nucleotide translocase inhibition; ChTx, charybdotoxin; palmitoyl CoA, palmitoyl coenzyme A; P_i, inorganic phosphate; thiols, redox-sensitive reactive thiols.

ischaemia and reperfusion (Lemasters *et al.* 1999) and may initiate cytochrome c release to trigger apoptosis (for a review of the role of PTP in apoptosis see: Green & Reed, 1998; Crompton, 1999). The link between PTP opening and cytochrome c release is a subject of current controversy (Goldstein *et al.* 2000; Hajnóczky, 2000) and may depend on the type of apoptotic stimulus.

UCP. The uncoupling protein (UCP or thermogenin) of brown fat mitochondria is a prime example of an energy dissipating pathway, as it is responsible for non-shivering thermogenesis. In addition to UCP1, the isoform responsible for heat production in brown adipose tissue, two other widely distributed isoforms have been cloned (UCP2 and UCP3; (Diehl & Hoek, 1999; Ricquier *et al.* 1999). UCP is discussed in detail in the article by Ricquier in this volume (Ricquier & Bouillaud, 2000). Recent evidence suggests that UCP- mediated uncoupling involves fatty acid cycling, with transport of the anionic fatty acid accompanied by protonation/deprotonation and net H^+ transport. While in the case of UCP1, heat production is the primary end, it has been proposed that the presence of UCP2 and UCP3 in other tissues may aid in optimizing the efficiency of energy metabolism by partially uncoupling the mitochondria (Jezek, 1999). This explanation is based on thermodynamic considerations, which suggest that the optimal ATP flow is achieved when the conductance of oxidative phosphorylation is matched to the conductance of the workload (Stucki, 1980). Optimal conductance matching is achieved at coupling ratios less than 1. Additionally, mild uncoupling of mitochondria has previously been suggested to be protective, perhaps by altering the rate of production of free radicals (Starkov, 1997; Korshunov *et al.* 1998). While the carrier-mediated transport of anions by UCP would be too slow to appear in patch-clamp recordings, a 75 pS anion channel attributed to UCP1 was recently reported, leading to speculation that under some conditions the carrier may show channel-like properties (Huang & Klingenberg, 1996).

K_{ATP}. Potassium-selective ion transport is a well-known feature of isolated mitochondria (Garlid, 1996; Bernardi, 1999) and its ATP dependence and sensitivity to K^+ channel openers and sulphonylurea inhibitors has led to the suggestion that a K_{ATP} channel similar to the one present on

the plasmalemma of many cells exists on the inner mitochondrial membrane (Paucek *et al.* 1992; Beavis *et al.* 1993; Szewczyk *et al.* 1993). This was strongly supported by the patch-clamp studies of Inoue *et al.* (1991), who identified a channel in mitoplast membranes whose gating properties, sensitivity to K^+ channel openers, and inhibition by ATP resembled K_{ATP} channels of the surface membrane, albeit with a much smaller single channel conductance (~10 pS in 100 mM KCl). The channel was inhibited by 100 μM ATP and blocked by glibenclamide and 4-aminopyridine.

Work from the laboratory of Garlid showed that diazoxide, an effective opener of pancreatic K_{ATP} channels, was approximately 1000-fold more potent for mitochondrial K_{ATP} channels (mitoK_{ATP}) than the cardiac sarcolemmal K_{ATP} isoform in a reconstituted system (Garlid *et al.* 1997).

While the physiological modulators of mitoK_{ATP} are unknown, reconstituted channels are inhibited by ATP, ADP and palmitoyl- or oleyl-CoA. The inhibited channel can be activated by GTP and GDP (Yarov-Yarovoy *et al.* 1997). Although the patch-clamp studies suggested that the ATP inhibitory site was on the matrix face (Inoue *et al.* 1991) other evidence suggests that the ATP inhibitory site is on the cytoplasmic face (Yarov-Yarovoy *et al.* 1997).

The functional role and pharmacology of mitoK_{ATP} will be discussed further in the section on mitoK_{ATP}.

It is useful to note here that another glibenclamide-sensitive cation conductance that is selective for Na^+ over K^+ has been reported in EDTA-treated mitochondria (Szewczyk *et al.* 1996; not shown in Table 1). The single channel correlate has not been identified, but this conductance is blocked by Ruthenium Red and is modulated by Mg^{2+} (Kapus *et al.* 1990).

K_{Ca}. The most recent addition to the list of inner membrane ion channels is the Ca^{2+}-activated K^+ channel (K_{Ca}) (Siemen *et al.* 1999). The properties of mitochondrial K_{Ca} were similar to those of BK channels in the plasmalemma – they showed sensitivity to charybdotoxin and activation by voltage and Ca^{2+}. The physiological role of mitochondrial K_{Ca} is unknown, but it could potentially be involved in volume regulation during times of increased matrix Ca^{2+} load.

Inner membrane anion channel (IMAC). There is substantial evidence from studies of mitochondrial swelling that a partially anion-selective channel is present in mitochondria (Garlid & Beavis, 1986; Beavis & Garlid, 1987). The channel was activated by matrix Mg^{2+} depletion or alkalinization, and was inhibited by a wide variety of cationic amphiphiles, including Ca^{2+} channel blockers, anti-arrhythmics, β-adrenergic antagonists, local anaesthetics, tricyclic antidepressants, and anticonvulsants.

Two prime candidates for IMAC have been suggested from electrophysiological studies. In the first patch-clamp study of the mitochondrial inner membrane, a 107 pS channel with a permeability ratio of 4·5 for chloride to potassium was detected (Sorgato *et al.* 1987), and this channel comprises the main conductance of the inner membrane. The 107 pS channel, also referred to as the centum pS channel, has an open probability that is steeply dependent on voltage and is predominantly closed at the negative potentials of energized mitochondria ($\Delta\Psi > 150$ mV). In this initial study, since no pH dependence was observed, the authors concluded that the 107 pS was probably not the single channel equivalent of IMAC. However, subsequent investigations have revealed properties of a similar channel in brown fat mitochondria that closely match the IMAC of swelling assays, including inhibition by propranolol, dihydropyridines and the nucleotide analogue Cibacron Blue (Beavis, 1992; Borecky *et al.* 1997).

Another 15 pS anion-selective channel displaying many of the same properties as IMAC has been described by Antonenko *et al.* (1991). This channel was activated by matrix alkalinization and blocked by amiodarone, propranolol and tributyltin.

Several other anion selective conductances have been described, including ATP-sensitive anion channels found in yeast inner membranes (Ballarin *et al.* 1995). At present, nothing is known about their physiological role and distribution in other species.

Since IMAC only appears to conduct ions under alkaline matrix conditions and low divalent concentrations, it is unclear what its physiological role is or if it opens under pathophysiological conditions. It has been suggested to be an important safeguard against mitochondrial swelling as it is poised to counteract the influx of cations (Beavis, 1992). Recent work in our laboratory suggests that IMAC may be activated under metabolic stress in intact cardiomyocytes, as discussed below.

Metabolic oscillations in cardiomyocytes

Spatiotemporal heterogeneity of mitochondrial metabolism

The vectorial orientation of enzyme catalytic sites required for the chemiosmotic model of oxidative phosphorylation is a prime illustration of how structural organization is essential for normal function. Even in the simplest case of aqueous extracts, spatiotemporal patterns of product formation have been observed (Hess & Boiteux, 1971; Rapp, 1979) and the level of complexity increases as structural and kinetic compartmentation is introduced. Examples include the localization of glycolytic enzymes near surface ion channels (Weiss & Lamp, 1987), the association of multiple enzymes at the contact sites between the mitochondrial inner and outer membranes (Marzo *et al.* 1998; Vyssokikh *et al.* 1999) and the compartmentalization of the pathways of oxidative metabolism in the mitochondrial cristae and matrix. This organization is particularly clear in cardiac cells, where

mitochondria take up 30–40 % of the intracellular volume, and are distributed along the myofilaments poised to meet the huge energy demands of the contractile apparatus. While the structure of the mitochondrial network it easily resolved using fluorescent markers, little is known about how it is functionally organized and what factors underly a co-ordinated metabolic response.

Insight into these issues has been gained by studying metabolic oscillation in isolated cardiomyocytes. Originally identified electrophysiologically by the observation of oscillation in sarcolemmal K_{ATP} currents when myocytes were deprived of substrate (O'Rourke *et al.* 1994, 1995), we have subsequently focused on the mechanism of mitochondrial redox oscillation associated with this response (Romashko *et al.* 1998; O'Rourke *et al.* 1999). Changes in mitochondrial redox potential can be monitored using the native fluorescence of endogenous flavoproteins (480 nm excitation/530 nm emission). This fluorescence arises primarily from FAD bound to the mitochondrial dehydrogenase enzymes containing lipoamide dehydrogenase (Fig. 1), and the redox state of the $FAD/FADH_2$ pair is in equilibrium with mitochondrial $NAD^+/NADH$ (flavoprotein oxidation is largely blocked by rotenone, which inhibits NADH dehydrogenase (O'Rourke *et al.* 1999)). Flavoprotein fluorescence increases when the mitochondrial matrix is oxidized (e.g. in the presence of an uncoupler). $\Delta\Psi$ can also be recorded simultaneously using the lipophilic cation tetramethylrhodamine ethyl ester (TMRE; excitation, 547: emission, ~605 nm). In the context of the theme of this article, the opening of an inner membrane ion channel would be expected to cause net oxidation of the matrix (increased flavoprotein fluorescence), as well as a decrease in $\Delta\Psi$ (decreased matrix TMRE fluorescence) if the supply of reducing equivalents is not enough to match the increased flux through the electron transport chain.

Oscillations induced by metabolic stress in cardiomyocytes are characterized by periodic rapid oxidation of mitochondria flavoproteins and the collapse of $\Delta\Psi$. Such oscillations can occur in single chains of mitochondria without affecting neighbouring areas in the same focal plane (Romashko *et al.* 1998). An example of the spatiotemporal pattern of these mitochondrial transitions is shown in Fig. 2, in which a large 'supercluster' of mitochondria repeatedly switches between the polarized and depolarized state. The co-ordinated transition among mitochondria in the cluster without a change in others nearby implicitly suggests that there are physical connections between them, in accord with the idea that subpopulations of mitochondria are connected in units that behave like protonophoric cables (Amchenkova *et al.* 1988). In other cells, we have observed the fast collapse and recovery of $\Delta\Psi$ in 1–2 μm regions of the cell without synchronization of neighbouring mitochondria, demonstrating that the smallest independent oscillator is the individual mitochondrion.

Thus, the cardiac mitochondrial network can be viewed as a network of potential oscillators that are normally synchronized, but can act independently under conditions of metabolic stress. Variable coupling between these oscillators contributes to higher order structures like superclusters or cell-wide reponses. In addition to physical connections between mitochondria, diffusible factors may also influence synchronization. In this regard, we have also observed propagating waves of flavoprotein oxidation that can travel not only within a cell, but also between myocytes connected by an intercalated disc (Romashko *et al.* 1998). Similar heterogeneity of metabolism, presumed to be mediated by the opening of the PTP, has been reported by others in response to various stressors including photo-oxidation (Huser *et al.* 1998) and triggering by spontaneous Ca^{2+} release in local regions of the cell (Ichas *et al.* 1997; Duchen *et al.* 1998; Lemasters *et al.* 1998), leading us to investigate the involvement of inner membrane ion channels.

Effects of mitochondrial ion channel inhibitors

To determine if the properties of the observed mitochondrial transitions induced by substrate deprivation were consistent with those of known mitochondrial ion channels, we tested the effects of a variety of conditions and pharmacological agents. In contradistinction to the properties of the mitochondrial permeability transition observed in isolated mitochondria, metabolic oscillations induced by substrate deprivation were independent of mitochondrial Ca^{2+} overload. Although Ca^{2+} cycling is impaired during the phase of oxidation in stimulated myocytes (O'Rourke *et al.* 1994), depletion of SR Ca^{2+} with EGTA in the intracellular solution does not alter the incidence of oscillation. Ruthenium Red, which should eliminate mitochondrial Ca^{2+} uptake, also does not block the transitions. Furthermore, cyclosporin A, a known inhibitor of the PTP, did not suppress oscillations (O'Rourke *et al.* 1999).

Interestingly, reported inhibitors of IMAC do show significant effects on the oscillations. Figure 3 shows the effect of PK11195, a peripheral benzodiazepine receptor inhibitor that blocks IMAC in isolated mitochondria and also the 107 pS channel seen in patch-clamp recordings. PK11195 (200 μM) suppressed the fast redox transitions in a reversible and reproducible manner. Similar suppression was observed for other inhibitors of IMAC (e.g. amiodarone, amitriptylline, tributyltin, propranolol); but, as for PK11195, concentrations higher than those used in isolated mitochondria or patch-clamp studies (Beavis, 1992) were required (usually >100 μM). This difference in potency in intact cell studies, which is common to most pharmacological attempts to correlate mitochondrial ion channels with a response (see also the section on mitoK_{ATP}), may be due to diffusional barriers present in the intact cell. A note of caution is warranted, however, since non-specific inhibitory effects of amphipathic compounds on metabolism have been reported (Hirsch *et al.* 1989; Fromenty *et al.* 1990).

Nevertheless, the common effect of structurally different IMAC inhibitors, and the lack of influence of agents known to influence PTP, supports the hypothesis that inner membrane anion channels underlie mitochondrial oscillations in substrate-deprived myocytes.

As mentioned above, it has been proposed that anion efflux through IMAC may be a safeguard against excessive matrix swelling. Mitochondrial anion efflux has also recently been implicated in hypoxic preconditioning (Vanden Hoek *et al.* 1998). It remains to be determined if the redox transitions are somehow beneficial to the cell during metabolic stress; however, as we have shown previously (O'Rourke *et al.* 1994), these metabolic oscillations profoundly influence the excitability of the cardiac cell through the activation of sarcolemmal K_{ATP} channels. On the scale of the whole heart, spatiotemporal variation in excitability would have a decidedly detrimental effect by increasing the dispersion of repolarization, thus setting the stage for arrhythmias. Important future goals are to determine whether the oscillations described here are present in the whole heart

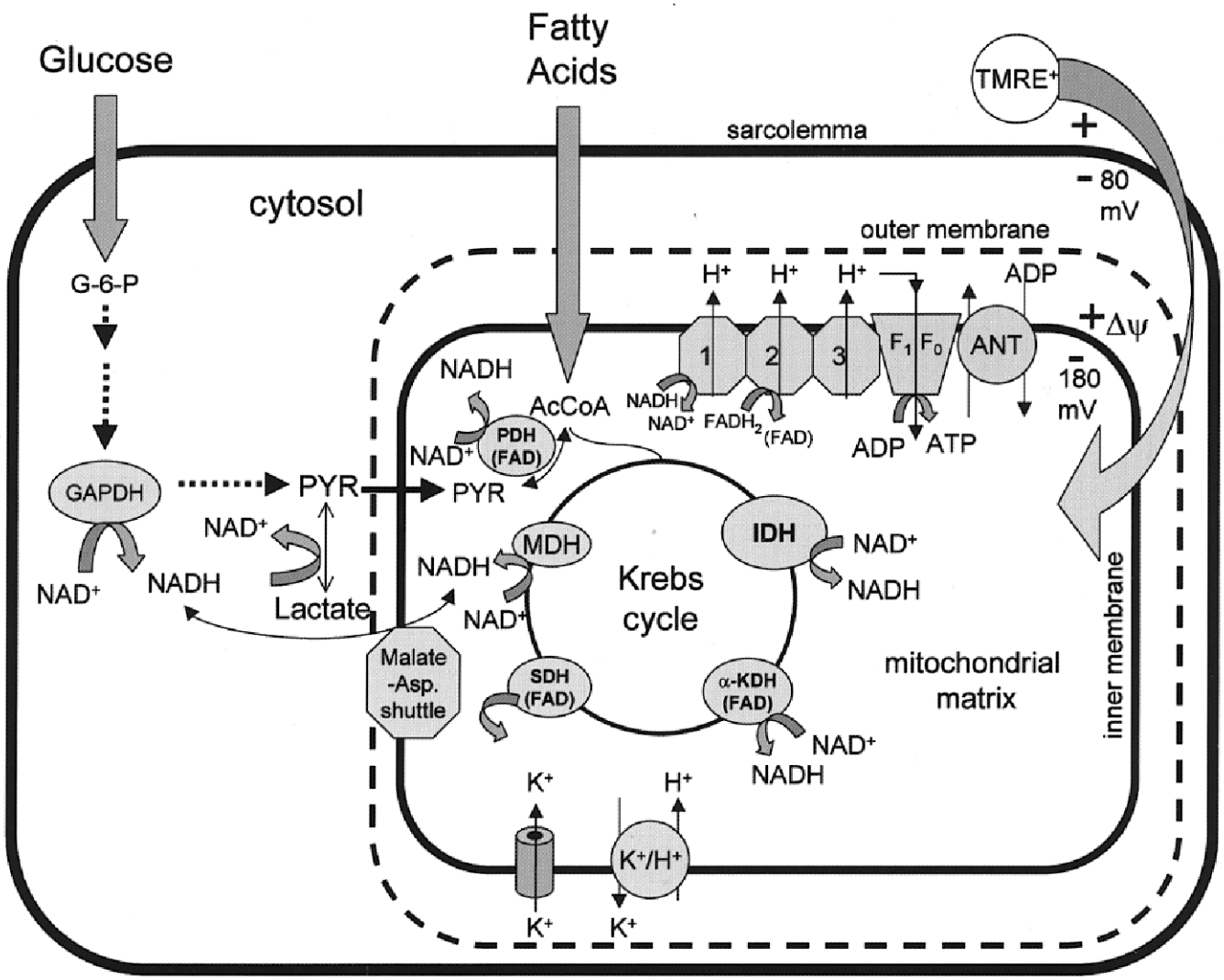

Figure 1. Mitochondrial redox and membrane potentials

The redox state of the cell depends on the rates of production and oxidation of reducing equivalents in the cytoplasm and in the mitochondrial matrix. FAD-linked dehydrogenases in the matrix including pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (α-KGDH) and succinate dehydrogenase (SDH) fluoresce green (excitation 480 nm/emission peak ~520 nm) when oxidized. These flavoproteins are in equilibrium with the mitochondrial $NADH/NAD^+$ redox couple. NADH is also fluorescent, emitting blue light (peak ~450 nm) with ultraviolet excitation (350 nm). Mitochondrial inner membrane potential ($\Delta\Psi$) can be assessed by the distribution of the lipophilic cations like tetramethylrhodamine ethyl ester (TMRE), which emits red fluorescence (excitation 540 nm/emission 605 nm). The opening of an inner membrane ion channel (e.g. mitoK_{ATP}) leads to acceleration of NADH oxidation by the electron transport chain and partial dissipation of the proton gradient through activation of K^+–H^+ exchange. A net change in $\Delta\Psi$ or redox potential occurs if NADH production and proton pumping cannot match the increase in energy dissipation.

during ischaemia/reperfusion and to find out if they can be pharmacologically modulated.

MitoK$_{ATP}$ and protection against ischaemia/reperfusion injury

As described earlier, the existence of a K$_{ATP}$ channel in the mitochondrial inner membrane is supported by studies of isolated mitochondria or reconstituted proteoliposomes (Paucek *et al.* 1992; Beavis *et al.* 1993; Szewczyk *et al.* 1993; Garlid, 1996; Garlid *et al.* 1997) and patch-clamp of mitoplasts (Inoue *et al.* 1991). With the large electrochemical driving force for K^+ entry into the matrix, mitoK$_{ATP}$ opening should lead to net K^+ uptake and subsequent osmotic swelling (assuming that anion transport accompanies the cation). This enhanced K^+ influx is counterbalanced by K^+–H^+ exchange, resulting in concomitant dissipation of the H^+ gradient. Thus, mitoK$_{ATP}$ is part of a volume regulatory K^+ cycle, with energy expenditure as a consequence (Garlid, 1996). While the physiological role of mitochondrial volume regulation has not been fully elucidated, changes in mitochondrial volume influence the rate of oxidative metabolism (Halestrap, 1989) and thus could represent a metabolic control mechanism. By analogy with the proposed role of UCP (Jezek, 1999), optimization of respiration (Stucki, 1980) may also be the result of the partial uncoupling induced by mitoK$_{ATP}$ opening.

In addition to volume homeostasis, recent experiments have revealed a new physiological role for mitoK$_{ATP}$ in protecting cells from ischaemia and reperfusion injury. In particular, ischaemic preconditioning appears to depend critically on the opening of mitoK$_{ATP}$. Ischaemic preconditioning is a self-protective mechanism of the heart in which the injury caused by a long ischaemia is blunted if preceded by short 'preconditioning' ischaemic periods (Murry *et al.* 1986). K$_{ATP}$ channels have been implicated in preconditioning because blockers like glibenclamide attenuate protection and K$_{ATP}$ openers (cromakalin, etc.) mimic the protection (see references within (Gross & Fryer, 1999; Grover & Garlid, 2000). The mechanism of protection was assumed to be through the energy sparing effect of opening sarcolemmal K$_{ATP}$ channels (sarcK$_{ATP}$). sarcK$_{ATP}$ are activated by energy depletion and render the myocytes inexcitable, thus suppressing energy consuming reactions such as Ca^{2+} cycling. However, this explanation was called into question

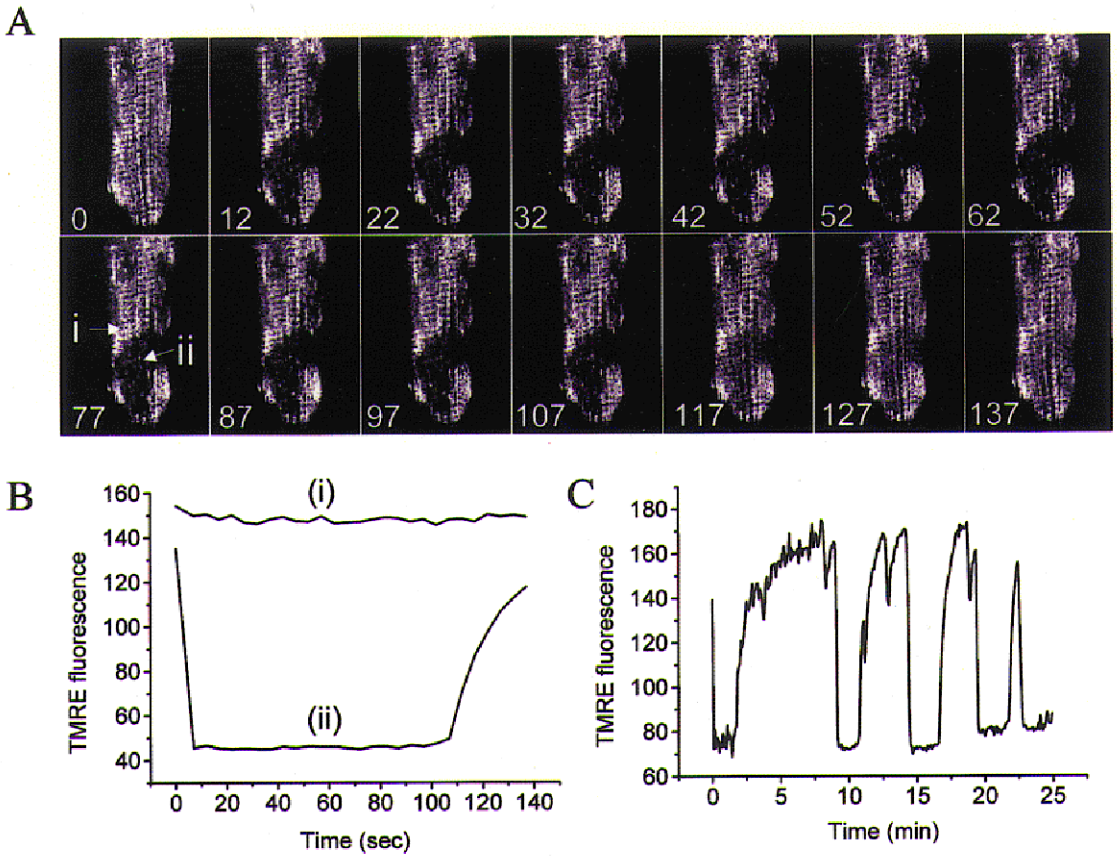

Figure 2. Regional depolarization of mitochondria

A, loss of $\Delta\Psi$ in a substrate-deprived guinea-pig ventricular myocyte can be restricted to 'superclusters' of mitochondria, as shown for this series of images of TMRE fluorescence (100 nM TMRE loading). The timing of the images in seconds is denoted on each frame. *B*, rapid loss and slow recovery of TMRE fluorescence in the cluster (region ii) and unchanging signal in a neighbouring region (i; as shown in *A*) illustrate the independent behaviour of adjacent mitochondria. *C*, the full time course of multiple slow oscillations of $\Delta\Psi$ in the supercluster.

with the finding that low doses of openers that had little effect on action potential shortening still conferred protection (Yao & Gross, 1994; Grover *et al.* 1995) and that artificially preventing ischaemic action potential shortening did not eliminate preconditioning (Grover *et al.* 1996).

To test whether mitoK$_{ATP}$ rather than sarcolemmal K$_{ATP}$ could be an effector of protection, differences in the pharmacological selectivity of the two isoforms were exploited. By comparing cardiac sarcolemmal and mitochondrial K$_{ATP}$ channels reconstituted in proteoliposomes, it was shown that diazoxide was approximately 1000 times more potent in opening the mitochondrial isoform ($K_{1/2}$ 0·8 *vs.* 840 μM for sarcK$_{ATP}$ (Garlid *et al.* 1997)). Furthermore, the effect of diazoxide could be blocked by 5-hydroxydecanoate (5-HD) with a K_i of 45–85 μM. 5-HD is a compound reported to be an ischaemia-selective K$_{ATP}$ inhibitor that can block preconditioning (Auchampach *et al.* 1992). Glibenclamide (K_i 1–6 μM) and 5-HD block mitoK$_{ATP}$ in a state-dependent manner, having no effect on channels activated by removing ATP, but inhibiting channels activated by GTP or K^+ channel openers in the presence of ATP (Jaburek *et al.* 1998).

In parallel with the biochemical studies of mitoK$_{ATP}$, Garlid *et al.* (1997) examined the effects of diazoxide and 5-HD on ischaemic injury in Langendorff-perfused rat hearts. It was found that at concentrations shown to have no effect on sarcK$_{ATP}$ (1–100 μM), diazoxide significantly protected hearts against ischaemic injury. Furthermore, 5-HD (100 μM) eliminated the protective effect of preconditioning.

The opening of mitoK$_{ATP}$ by diazoxide was detected for the first time in intact cardiomyocytes as a reversible increase in the oxidation of flavoproteins, using the same fluorescence method described in the preceding section (Liu *et al.* 1998; Fig. 4). Importantly, at concentrations of up to 100 μM (with a $K_{½}$ of 27 μM), diazoxide had no effect on sarcolemmal K$_{ATP}$ currents in intact cells and 5-HD inhibited the diazoxide effect. The latter finding was an important confirmation, since we have subsequently shown that 5-HD selectively inhibits mitoK$_{ATP}$ (Sato *et al.* 1998). Using the same methodology, we have characterized the pharmacological profile of a number of K$_{ATP}$ openers and inhibitors, and have been able to identify compounds which specifically act on either the mitochondrial or sarcolemmal channels (Fig. 5).

The link between mitoK$_{ATP}$ and protection in cardiomyocytes was demonstrated by examining the extent of cell killing in response to simulated ischaemia using a cell pelleting method (Liu *et al.* 1998). As summarized in Fig. 5, protection was afforded only by compounds capable of activating mitoK$_{ATP}$, and this protection was inhibited only by compounds effective at blocking mitoK$_{ATP}$ (Sato *et al.* 2000). Compounds selective for sarcolemmal channels have no such actions and serve as convincing evidence that sarcolemmal channels play much less of a role in protection.

Studies of intact hearts have confirmed the hypothesis that mitoK$_{ATP}$ is fundamentally involved in cardioprotection. Protection can be mimicked by even a short exposure to diazoxide prior to the long ischaemia and preconditioning

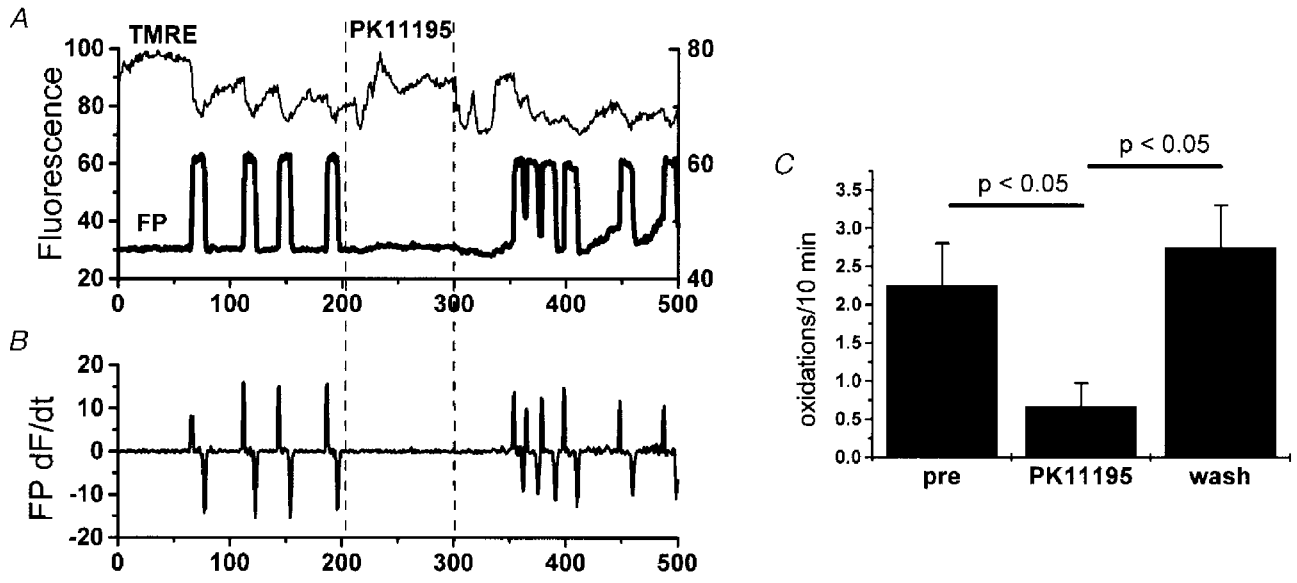

Figure 3. Suppression of oscillations by a benzodiazepine receptor ligand

A, exposure to PK11195 (200 μM applied between dashed lines) reversibly suppressed oscillations in $\Delta\Psi$ (TMRE signal) and flavoprotein redox (FP signal; thick line) as derived from time course image analysis. *B*, taking the first derivative of the redox signal (FP dF/dt) facilitates analysis of the frequency of transitions by eliminating slow baseline changes. Counting fast transitions that exceed a threshold (in this case 20% of peak + dF/dt) permits quantitative determination of drug efficacy *C*, the number of oxidations over a period of 10 min in the presence of PK11195 was significantly suppressed ($n = 12$ cells) compared with before and after application.

induced by brief ischaemia can be blocked by 5-HD or glibenclamide, which have no effects on their own (see references in Gross & Fryer, 1999). The inability of the surface-selective blocker HMR1883 to further eliminate protection supports a mitochondrial *vs.* sarcolemmal site of action (Fryer *et al.* 2000).

The obvious question remains as to how the opening of an energy dissipating ion channel could protect cells against ischaemic injury. Available evidence suggests several possible mechanisms. First, as discussed above, mitochondrial matrix swelling induced by mitoK$_{ATP}$ opening may improve mitochondrial energy production. Recent evidence supports the hypothesis that the pharmacological opening of mitoK$_{ATP}$ improves oxidative phosphorylation during ischaemia and reperfusion and that this effect is inhibited by 5-HD or glibenclamide (Tanonaka *et al.* 1999; Iwai *et al.* 2000; Miura *et al.* 2000).

A second protective influence of mitoK$_{ATP}$ may be an effect on Ca^{2+} handling. Holmuhamedov *et al.* (1999) showed that diazoxide or pinacidil decreased the rate of Ca^{2+} uptake by isolated mitochondria and a similar effect was observed in intact neonatal myocytes. 5-HD inhibited the latter effect. A decrease in the driving force for Ca^{2+} entry due to a 10–15 mV depolarization of $\Delta\Psi$ was thought to be responsible.

A third mechanism is supported by recent work demonstrating that the opening of mitoK$_{ATP}$ channels may alter the rate of mitochondrial reactive oxygen species (ROS) production and contribute to cardioprotection. An early increase in ROS production during hypoxic preconditioning in embryonic myocytes was noted in several studies (Vanden Hoek *et al.* 1998, 2000; Yao *et al.* 1999) and both protection and ROS production were inhibited by 5-HD, the thiol reductant 2-mercaptoproionyl glycine or the mitochondrial site III inhibitor myxothiazol. This suggests that mitochondria were the source of ROS production and that the opening of mitoK$_{ATP}$ could stimulate ROS accumulation. ROS production may in turn activate PKC, which is a known component of cardioprotective pathways, and may also influence mitoK$_{ATP}$ activity (Sato *et al.* 1998; Sasaki *et al.* 2000). Wang & Ashraf (1999) reported that diazoxide induced PKC translocation and protection in Langendorff-perfused rat hearts and these responses could be blocked by PKC inhibitors. In contrast, Miura *et al.* (2000) reported that the PKC inhibitor calphostin C could block adenosine- but not diazoxide-mediated cardioprotection. Thus, the chain

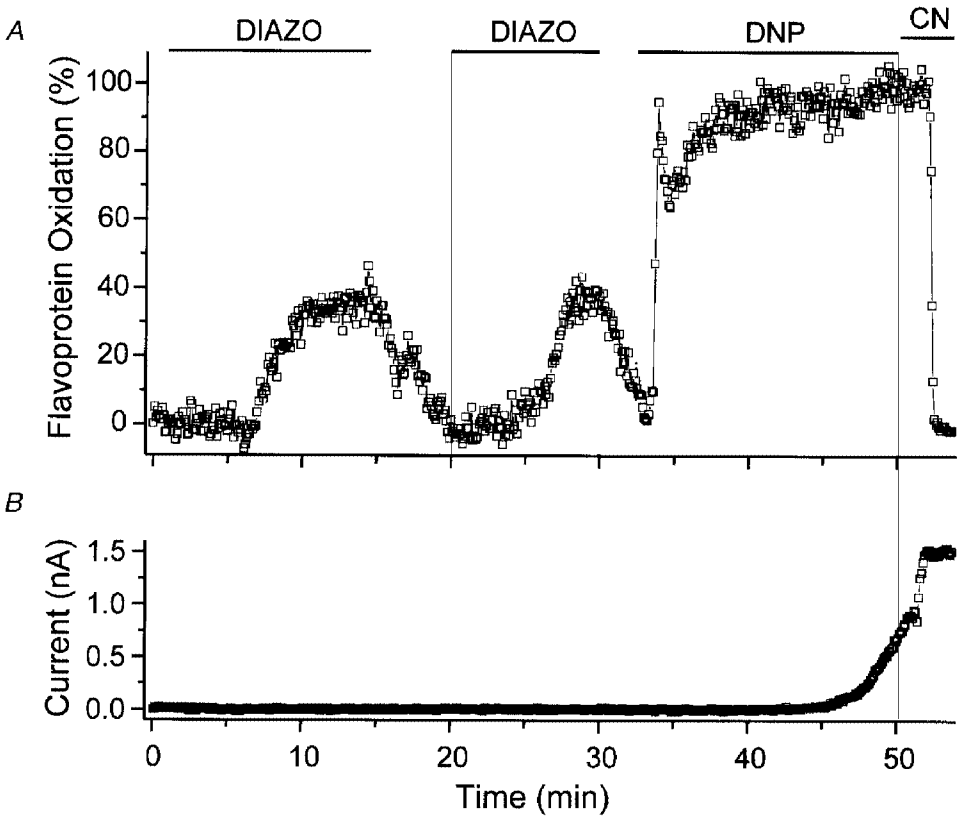

Figure 4. Selective activation of mitoK$_{ATP}$ by diazoxide in cardiomyocytes

A, diazoxide causes reversible and reproducible oxidation of mitochondrial flavoproteins (Diazo; 100 μM). The fluorescence signal can be calibrated by maximally oxidizing (with the uncoupler 2,4-dinitrophenol; DNP) and reducing the mitochondria (with the cytochrome oxidase inhibitor cyanide; CN). *B*, in the same experiment, diazoxide did not activate sarcolemmal K$_{ATP}$ current. Severe metabolic inhibition with DNP or CN activated sarcolemmal K$_{ATP}$, confirming the presence of these channels. From Liu *et al.* (1998).

of events linking mitoK$_{ATP}$ activation, ROS production and PKC activation remains incompletely defined.

Both mechanistic and structure–function studies of mitoK$_{ATP}$ will require a breakthrough in efforts to clone the channel. Thus far, putative channel subunits have been only partially purified, but support the notion that the channel is composed of sulfonylurea receptor (SUR)-like and inward rectifier-like components, although their sizes were smaller than their surface membrane counterparts (Grover & Garlid, 2000). Although it has been reported that antibodies against Kir6.1 immunolocalize to mitochondria (Suzuki *et al.* 1997), a recent effort to knock out Kir6.1 channels by dominant negative suppression in cardiac cells had no effect on the mitochondrial response to diazoxide (Seharaseyon *et al.* 2000), challenging the suggestion that Kir6.1 is a subunit of mitoK$_{ATP}$.

The recent explosion of interest in mitoK$_{ATP}$ as a trigger and/or mediator of cellular protection against ischaemia–reperfusion injury has motivated efforts to develop potent and selective openers of this channel. New compounds with nanomolar affinities for mitoK$_{ATP}$ and little effect on vascular or sarcolemmal K$_{ATP}$ have recently been developed and these agents appear to confer protection without the potentially arrhythmogenic consequences of opening sarcK$_{ATP}$ (Rovnyak *et al.* 1997; Grover, 2000).

SUMMARY

Novel imaging and patch-clamp techniques are now being applied to identify and characterize new mitochondrial ion channel types and the agents that modulate them. These methods complement the myriad studies of isolated mitochondria that have convincingly identified selective conductance pathways in a membrane formerly thought to be largely impermeable to ions. We are just beginning to define the functional roles of these channels and are finding that they are essential for both preserving the function of

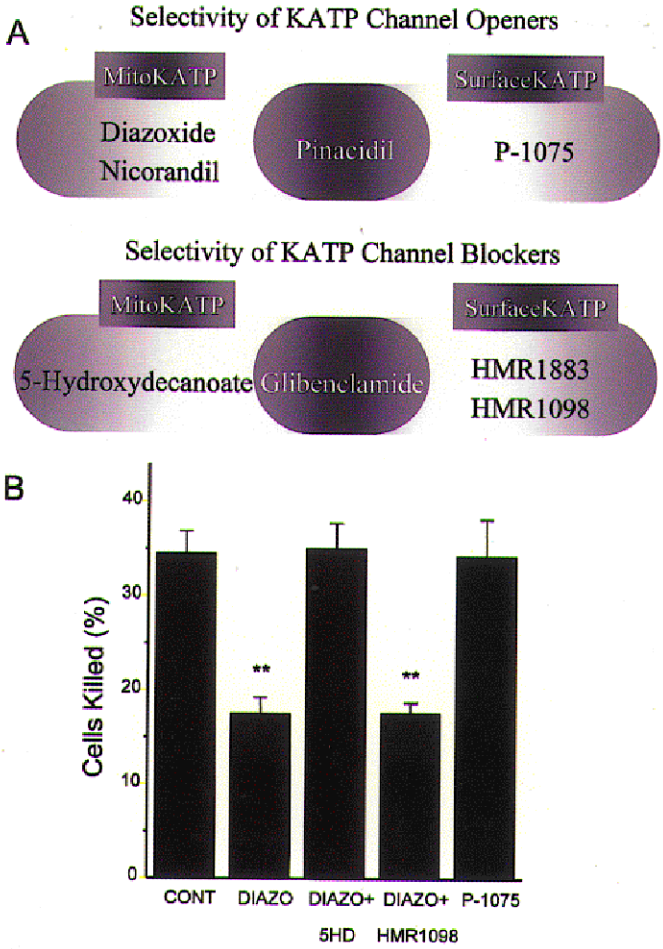

Figure 5. Summary of the selectivity of K$_{ATP}$ channel openers and blockers

A, based on studies of intact myocytes using methods similar to that shown in Fig. 4, the selective mitoK$_{ATP}$ agonists diazoxide and nicorandil have been identified, while pinacidil activates both mitochondrial and surface isoforms. The pinacidil derivative P-1075 selectively activates sarcolemmal K$_{ATP}$ in intact myocytes. *B*, cellular protection against simulated ischaemia is conferred by diazoxide, but not P-1075, and is blocked by 5-HD, but not HMR-1098, thus supporting a mechanism involving mitoK$_{ATP}$ rather than sarcK$_{ATP}$.

the cell and in killing it. In addition to the more widely known Ca^{2+} and PTP channels, we have found that different physiological responses can be initiated by the opening of specific channels. In the case of metabolic oscillation, which could be detrimental in terms of whole heart arrhythmias, inner membrane anion channels may be involved. In contrast, mitoK_{ATP} opening apparently protects both isolated cells and the intact heart against ischaemic injury. Undoubtedly, new types of channels are likely to be identified in mitochondrial membranes and functional assignments will be made for known channels of previously undefined purpose. Progress will be accelerated by future definition of the molecular structure of these channels, which will aid in the development of new drugs to treat or prevent disease.

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