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Signaling and cellular mechanisms in cardiac protection by ischemic and pharmacological preconditioning

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Abstract

Ischemic preconditioning (IPC) is a defensive adaptive cellular phenomenon. Brief ischemic stimuli render the heart resistant to subsequent similar stress. Signaling for IPC and pharmacologically induced preconditioning involves several G-protein coupled cell surface receptors, second messengers, specific Ser-Thr-protein kinase-C isoforms, Tyr-kinases, and finally, results in activation of ATP-dependent potassium channels (inward rectifiers) at the sarcolemma and in the mitochondria. In cardiomyocytes these channels regulate cytosolic and mitochondrial Ca^{2+} levels. K^+ influx into mitochondria proves to be a key factor for keeping the mitochondrial permeability transition pore closed. This ensures continuous energy production and prevents cell death by apoptosis or necrosis. Molecular structure, function, and pharmacological properties of the ATP-dependent potassium channels and of the mitochondrial permeability transition pore are discussed. Channel activating agents mimic IPC and also affect reactive oxygen species producing enzymes involved in mitochondrial respiration. Volatile anesthetics, among other drugs, mimic the cardioprotective effects of IPC. Their intracellular signaling and clinical application are briefly discussed.

Introduction: Cytoprotective potential of cardiac preconditioning

Cardiac preconditioning (PC) represents a potent and reproducible method to render the myocardium more resistant against irreversible structural and functional damage induced by a variety of noxious stimuli. The phenomenon of ischemic preconditioning (IPC) was first described in 1986 (Murry *et al*., 1986) in the canine myocardium. By subjecting the heart to four brief ischemic episodes by using ligation of the circumflex coronary artery, interspersed with brief episodes of reperfusion before a prolonged ischemic insult (40 min), the tolerance against cellular injury was increased and the infarct size (necrosis) was significantly reduced from 29 to 7% in the myocardial area at risk. The area at risk encompasses tissue, the blood supply of which comes from the ligated artery and has no compensatory supply by collateral vessels. Without PC, total occlusion of a coronary artery will cause irreversible myocardial damage and cell death within approximately 20 min (Jennings and Reimer, 1991). Interestingly, in most animal studies IPC proved to offer a degree of protection equivalent to prevent cell death by about an additional 20 min (Lawson and Downey, 1993). Several years after the description of acute cardiac protection by IPC, a second delayed window of protection was observed (Marber *et al*., 1993). The first acute or early phase of protection develops within minutes after IPC and lasts for 1–3 h. The second or late phase of protection appears 12–24 h after the first phase has subsided, and lasts for 3–4 days before it fades away (Bolli, 2000). Although the extent of protection in the late phase does not reach the degree of protection during the shorter first phase, it is this longer lasting protective period that may be of particular therapeutic interest for patients with coronary artery disease (CAD).

The endogenous cardioprotective mechanism of IPC could be confirmed in almost all species including mouse, rat, guinea pig, rabbit, dog and pig (Yellon *et al*., 1998). In addition, IPC was also observed in single cells (Marber, 2000), in superfused myocardial specimens (Walker *et al*., 1995), and in isolated hearts *in vitro*. In the past 16 years, the literature exploded with over 4000 published reports on this topic. Under most experimental conditions, IPC restricts infarct size, improves postischemic contractile function and reduces arrhythmias. IPC not only affords protection against loss of function and necrosis but also against the programmed, energy-dependent cell death called apoptosis (Piot *et al*., 1997). All these findings elicited great interest in the preconditioning process for a possible therapeutic use. Indeed, signs of naturally occurring IPC in patients with CAD has been inferred from observations that preinfarction angina reduces myocardial infarct size, improves ventricular function and reduces arrhythmias (Hirai *et al*., 1992; Nakagawa *et al*., 1995;

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Ottani *et al*., 1995). The authors concluded that the anginal episodes (presumably repetitive brief ischemias) preconditioned the heart for the impending coronary occlusion. In addition, repetitive aortic cross-clamping during coronary artery bypass grafting (CABG) as well as coronary artery ballon inflations during percutaneous transluminal coronary angioplasty (PTCA) may serve as preconditioning stimuli (Deutsch *et al*., 1990; Yellon *et al*., 1993). Although prophylactic induction of IPC cannot be applied routinely in patients with CAD to avert impending myocardial infarction (MI), it would be desirable to employ it in the clinical setting, particularly the perioperative period. With increasing life expectancy and improved surgical techniques an ever larger number of elderly patients with cardiovascular disease or risk factors thereof undergo cardiovascular and general surgery. 10–18% of high-risk patients undergoing surgery will suffer from perioperative cardiovascular complications including MI (Raby *et al*., 1989; Mangano *et al*., 1990).

Investigations into the mechanisms of IPC lead to the observation that various pharmacological agents may elicit preconditioning-like effects in experimental animals (Nakano *et al*., 2000). Thus, pharmacological preconditioning (PPC) could provide a safer way than ischemia for inducing cardioprotection in humans.

Results from experimental animal models and similar observations in clinical studies indicate that adenosine receptors, protein kinase-C (PKC), and mitochondrial ATP-sensitive potassium channels (mitoK-ATP channels) may be involved in the pharmacologically induced preconditioning process (reviewed by Nakano *et al*., 2000). Great efforts are ongoing to delineate the intracellular signaling pathways, which relay the surface receptor stimuli to the target sites, and to elucidate the cytoprotective mechanisms. Anesthetics represent one class of drugs with potential cardioprotective properties (Zaugg *et al*., 2003b). Infarct-limiting properties of volatile anesthetics were reported as early as 1983 (Davis *et al*., 1983; Davis and Sidi, 1989). We now know that many anesthetics elicit, enhance, or inhibit the preconditioned state (Zaugg *et al*., 2002a). The use of cardioprotective anesthetics, which can be administered systemically with low toxicity, represents a desirable concept, particularly for high-risk cardiac patients undergoing surgery.

The following aspects of PC will be dealt with in this review: (i) time-relation of early and delayed PC; (ii) PC trigger stimuli such as ischemia-reperfusion, stress-stimuli, and pharmacological agents; and (iii) PC protection against cell death (necrosis and apoptosis), cellular dysfunction (including stunning and hibernation), and arrhythmias. Special focus concerns the signaling and cellular mechanisms for cardioprotection afforded by IPC and PPC with special reference to new insights on anesthetic preconditioning (APC). Due to the wide area covered, reviews containing references to primary literature are often cited. This also helps the newcomer to acquire a balanced overview on this field.

Cell damage by ischemia-reperfusion injury

Limited supply of oxygen and metabolic substrates because of a reduced blood flow to the myocardium, causes metabolic, functional, and morphological changes. This is coupled with a restricted washout and accumulation of ions and metabolites as well as a shift from aerobic to anaerobic metabolism. Reduced energy production results in depletion of the immediate cellular energy stores, ATP and creatine phosphate (CrP). Consequently, contractile function and energy-dependent ion pumps (Ca^{2+}-ATPase of sarcoplasmic reticulum and sarcolemmal Na/K-pump) are depressed (Jennings *et al*., 1991). The resulting increase in cytosolic Na^{+} and Ca^{2+} with a concomitant loss of intracellular K^{+} affects transmembrane ion gradients and the membrane potential. These alterations lead to an accumulation of catabolites and byproducts, to acidosis, increased osmotic load, production of reactive oxygen species (ROS), and activation of various Ca^{2+}-sensitive enzymes. At this point morphological changes are beginning to occur. Activated proteases start degrading myofibrillar and cytoskeletal proteins while lipases may affect phospholipids in the membranes. The ensuing rupture of the cell membrane will ultimately cause cellular death (Sommerschild and Kirkeboen, 2002).

As long as the myocyte has not died during ischemia, one would expect that, upon reperfusion, it is going to recover. Reperfusion, however, adds further strain onto the cell. The return of oxygen and nutrients with reperfusion allows the cardiomyocyte to resume energy production. The cytosolic Ca^{2+}, already high in the ischemic period, may further increase during the early reperfusion phase by entering the cell via the voltage-sensitive Ca-channels (L-type Ca-channel) of the sarcolemma, the Na/Ca-exchanger as well as by Ca^{2+} release from the intracellular sarcoplasmic reticulum (SR) (Du Toit and Opie, 1992; Kusuoka *et al*., 1993). In addition, stimuli triggered by primary messengers such as angiotensin-II, endothelin, catecholamines, cytokines and polysaccharides, which accumulate during the ischemic period, are expected to interfere with the orderly coordination of energy production and its usage by ion pumps and the contractile machinery (Brunner *et al*., 1993; Gao *et al*., 1995; Opie, 1997). Reperfusion arrhythmias due to cytosolic Ca^{2+}-transient oscillations (Du *et al*., 1995) and overstimulation of the tricarboxylic acid cycle (TCAC) because of increased mitochondrial Ca^{2+} (McCormack, 1985), result from a lack of tight control over intracellular signaling coordination. Finally, the Ca^{2+} overload may lead to a particularly cytotoxic burst of ROS production.

Taken together, during ischemia-reperfusion the cardiomyocytes are exposed to a sequence of adaptive and injurious events. In principle, two components can be discerned, one that develops during ischemia (ischemic injury) and a second one, which develops during reperfusion (reperfusion injury). The term 'reperfusion injury' is in fact a misnomer, because it is the severity of

ischemia that sets the stage for the development of reperfusion injury, and the appropriate term should be 'ischemia-reperfusion injury' (Bolli and Marban, 1999). Experimental data indicate that the damage caused by the reperfusion component is always larger than the one caused by ischemia alone. However, the degree of damage caused by the reperfusion component is positively related to the degree of the antecedent ischemic component. Consequently, pharmacological interventions that alleviate the underlying ischemic component of injury (e.g. adenosine, Ca-channel blockers, volatile anesthetics, K-ATP channel openers, abbreviated KCOs) will also indirectly reduce the inevitable damage caused by the reperfusion component. In addition, different areas of the myocardium may be more or less severely strained, depending on the duration and degree of restricted blood supply. Correspondingly, the myocytes may acquire different states of reversible and irreversible damage.

Necrosis and apoptosis, both occurring concurrently?

The predominant cause for early cell death in both ischemia and reperfusion appears to be the mechanical disruption of the sarcolemma. As mentioned above, during ischemia lasting for more than 20 min, the increased cytosolic Ca^{2+} induces enzymatic degradation of structural elements. On the other hand, in the early reperfusion phase mechanical disintegration of the cellular entity may occur for the following two reasons: (i) myocyte hypercontracture induced by the re-energization while cytosolic Ca^{2+} is still high (this phenomenon has been called 'oxygen paradox'), and (ii) swelling of the cell by water uptake when intracellular osmolality is still high, while the extracellular osmolality diminishes fast upon reperfusion (Piper *et al*., 1998). Cell debris in the necrotic area may provoke proliferative inflammation leading to scar development.

Apoptosis, in contrast, is an energy-dependent process occurring after some delay and represents a transcriptionally regulated response to moderate cell injury or to the influence of various cytokines. It is characterised by shrinkage of the cell, condensation of chromatin, fragmentation into membrane-bound apoptotic bodies and rapid phagocytosis by neighbouring cells without induction of inflammatory response (Narula *et al*., 2000; Zhao and Vinten-Johansen, 2002). The apoptotic death pathway becomes irreversible when the endonucleases are activated to degrade the genomic DNA at internucleosomic loci. Although evidence for apoptosis in ischemic-reperfused myocardium and in the border zones of MI has been demonstrated, it is still under debate whether the number of apoptotic cells contributes significantly to the extent of loss of functional tissue after acute ischemia-reperfusion injury. The necrotic process is completed in roughly 24 h and apoptosis within nearly 4 h leaving no traces. Thus, the extent of the contribution of apoptosis to cell death may be largely underestimated (Anversa and Nadal-Ginard, 2002).

While the extent of necrosis is routinely assessed by vital staining with triphenyltetrazolium chloride (TTC), DNA nick end-labeling (TUNEL staining) is taken as an index for apoptosis, which however, requires confirmation by electrophoretic display of the discrete DNA fragments (DNA laddering) and electron microscopic identification of apoptosis-related cell structures. The level of TUNEL-positive myocyte nuclei is very low in healthy hearts (below 0.002% or not detectable at all). Myocyte apoptosis was shown to occur in ischemia-reperfusion damaged myocardium in animal models and in human infarct tissue (for references see Palojoki *et al*., 2001). Apoptosis was also found in viable myocardial areas after MI, in experimental and also in human ischemic heart failure. Under prolonged ischemia induced by coronary artery occlusion in rat and dog models, few TUNEL-positive cells (0.5–1.0%) and no DNA laddering was observed in the infarct area, while in the infarct border zone TUNEL-positive cells increased up to 20–30% after one hour of ischemia followed by 6 h of reperfusion (Zhao and Vinten-Johansen, 2002). Under the latter conditions, the necrotic infarct size peaked at around 24 h and remained constant thereafter, while the number of TUNEL-positive cells in the peri-necrotic zone progressively increased up to 50–60% over the following days and was accompanied by increased DNA laddering. In areas remote from MI the TUNEL-positive myocyte levels were also increased up to 1% as compared to 0.002% in undisturbed hearts. These data suggest that necrosis develops early during reperfusion and is followed by a slower appearance of TUNEL-positive cells in the infarct border zone accounting for a dynamic processing of cell death with concomitant loss of functional myocardium.

Three particular aspects warrant special consideration. First, TUNEL-positive myocytes in biopsies from hearts of patients with dilative cardiomyopathy (DCM) at end-stage failure were shown to exhibit no ultrastructural characteristics of apoptosis (Kanoh *et al*., 1999). Almost all TUNEL-positive nuclei also stained positive for proliferating cell nuclear antigen, suggesting the presence of DNA in these nuclei being processed for either repair or replication. Replication was excluded, however, by negative nuclear staining for the replication-specific antigen Ki-67. In addition, the Taq polymerase-based DNA *in situ* ligation assay, which specifically detects double-stranded DNA breaks with single-base 3′-overhang in cells undergoing apoptosis, was negative in all cases. In conclusion, TUNEL-positive staining may evidence nuclei with active DNA in the process of repair, while nuclear apoptosis still being scarce. Second, the apoptotic process is mediated by activation of caspases (cysteine aspartases) that cleave cytosolic proteins and nuclear targets. The processing of caspases proceeds along two major pathways (Narula *et al*., 2000; Zhao and Vinten-Johansen, 2002). One pathway is initiated by binding of extracellular ligands (Fas ligand, TNF-α) to their cell surface

receptors leading to caspase-8 activation (Borutaite *et al*., 2003). The other pathway is receptor-independent and involves stress signals including ischemia-reperfusion, hypoxia-reoxygenation, calcium overload, and mechanical stretch, all inducing release of cytochrome-c from mitochondria into the cytoplasm. Cytochrome-c is a respiratory chain protein that contributes to oxidative phosphorylation and energy production. It was recently shown that the two pathways may not be mutually exclusive and that cytokines also lead to the release of cytochrome-c, which activates caspase-3 (Luo *et al*., 1998). Caspase-3 degrades cytosolic substrates such as troponin-C (TnC), PKCdelta and probably others, as well as nuclear substrates including poly (ADP-ribose) polymerase (PARP). Caspase-3 is the primary initiator of apoptotic DNA fragmentation by proteolytically inactivating DFF45/ICAD, which releases the active DFF40/CAD endonuclease that preferentially attacks chromatin in the internucleosomal linker DNA (Wolf *et al*., 1999). Release of cytochrome-c from mitochondria thus represents a key step in mediating apoptosis.

The mitochondrial apoptotic signaling pathway operates via the formation of a trimeric complex of liberated cytochrome-c with the apoptotic proteinase activating factor-1 (APAF1) and procaspase-9 (Zhao and Vinten-Johansen, 2002). This complex is instrumental in activating caspase-3, but it is itself under the control of a number of intracellular apoptosis regulating proteins: the Bcl2 protein family (B-cell leukemia/lymphoma2-like proteins). The Bcl2 family is composed of of a group of anti-apoptotic proteins (Bcl2, BclXL, Bclw, Bag1 and BI1) that, when overexpressed, attenuate the expression of a group of pro-apoptotic proteins (Bax, Bak, Bad, Bid and Bim). In addition, the tumour suppressor DNA-binding transcription factor p53 was recently shown to act as an intermediate effector of apoptosis by either activating the transcription of death proteins (Bax) or suppressing the transcription of survival proteins (Bcl2), or both (MacLellan and Schneider, 1997). In isolated beating rat hearts, ischemia without reperfusion did not induce apoptosis or alter the appearance of apoptosis regulating proteins, whereas, a short period of ischemia followed by reperfusion induced a time-dependent reduction in the expression of the Blc2 protein and an increase in the expression of Bax and p53 proteins (Zhao and Vinten-Johansen, 2002).

Third, an additional apoptotic pathway independent of caspases, requires Ca^{2+}-activation of calpain (an unbiquitously expressed cytosolic cysteine-proteinase) with consequential mitochondrial dysfunction (Chen *et al*., 2002). Postischemic reperfusion of isolated hearts results in sodium influx into the myocytes followed by Ca^{2+} accumulation. This leads to calpain activation, which in turn leads to cleavage of Bid. Activated Bid targets the mitochondria, causing dysfunction and release of factors, which commit the cell to death, such as cytochrome-c, Smac/DIABLO, pro-caspases, apoptosis-inducing factor (AIF), and endonuclease-G. The loss of cytochrome-c results in inhibition of oxidative phosphorylation, increased production of ROS and irreversible opening of the mitochondrial permeability transition pore (mitoPTP).

Taken together, the apoptotic process in cardiomyocytes seems to be triggered primarily by ROS originating during reperfusion after ischemia. In the heart, however, the process seems to differ from classic apoptosis in as much as cytosolic structures become degraded while the nuclei are partially preserved retaining their DNA repair capacity. Most cardiomyocytes with ultrastructural evidence of released cytochrome-c and activated caspase-3 exhibited intact nuclei in specimens from failing human hearts. One may speculate that the terminally differentiated cardiomyocytes evolved mechanisms for the nuclei to resist fragmentation despite of the continued cytoplasmic apoptosis (Narula *et al*., 2001). Because apoptosis is an energy requiring process, it is conceivable that a portion of the cells, which entered the apoptotic pathway and have depleted their energy stores during ischemia, may follow the necrotic death pathway. It seems equally plausible, but proves difficult for experimental confirmation, that some of the non-lethally affected myocytes can recover and resume functionality after a steady supply of oxygen and nutrients has been re-established. Finally, it may be concluded that during reperfusion a significant number of myocytes enters the apoptotic pathway, predominantly in the infarct border zone, concurrently with areas of necrosis, whereby their fate remains open for some time before it turns for the better or the worse.

Postischemic contractile dysfunction

The contractile dysfunction of myocytes that have survived ischemia-reperfusion may, even after restoration of the blood supply, persists for hours or days before it recovers completely. This postischemic myocardial dysfunction has been originally observed in dogs after brief coronary artery occlusion (Heyndrickx *et al*., 1975) and was later termed 'myocardial stunning' (Braunwald and Kloner, 1982). The stunning phase may be characterised by the perfusion-function mismatch, i.e., normal blood supply combined with reduced contractility. Myocardial stunning can be elicited in various ways: (i) after a single ischemic episode lasting not longer than about 15 min, (ii) after multiple short ischemic episodes interspersed by reperfusions, (iii) after partial reversible ischemia due to subendocardial infarction, or (iv) after strenuous exercise causing so-called 'high-flow ischemia' (Bolli and Marban, 1999). In the latter condition, the cardiac vascular system cannot provide sufficient oxygen to the myocardium under increased hemodynamic workload. The primary problem is an increase in oxygen demand rather than a decrease in supply.

The underlying pathophysiological etiology for stunning seems to depend on increased ROS and cytosolic Ca^{2+}, both occurring during ischemia and further

augmented during reperfusion. Although the precise mechanism that induces the typical perfusion-function mismatch, has not yet been identified, it is now accepted that the increased Ca^{2+} level is critical for stunning (for more references see Papp *et al*., 2000). The intracellular Ca^{2+}-dependent calpain is believed to be responsible for degradation of myofibrillar and cytoskeletal proteins including troponin-I (TnI), titin, myosin, myosin binding protein-C (MyBPC), microtubules, spectrin, alpha-actinin and desmin. Degradation of most of these proteins as well as contractile deterioration can be prevented in *in vitro* experiments by application of the protease inhibitor calpstatin. In addition, covalent troponin complexes, binding of cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and alphaB-crystalline (a myocardial stress protein) to myofibrils were also blamed for affecting Ca^{2+}-sensitivity and deterioration of contractile parameters in stunned myocytes.

In contrast to stunning, myocardial hibernation describes a state in which the reduced contractile function is accompanied (i.e. 'matched' instead of 'mismatched' in stunning) by a reduced oxygen consumption (Sherman *et al*., 1997). Hibernation may be induced by reduced coronary flow over one to several hours, or alternatively, it may result from repetitive episodes of stunning, which have a cumulative effect and cause protracted postischemic dysfunction (Heusch *et al*., 1997). The term hibernation comes from the zoology vocabulary and describes a state of reduced activity in a situation of reduced energy supply. In the context of CAD, myocardial hibernation was originally interpreted as a chronic, adaptive reduction of contractile function in response to a reduction of myocardial blood supply. In both stunning and hibernation, the myocardium is in a state of preservation with sublethal cell damage, from which it may recover completely, rather than turn into necrosis. Despite their distinct definition, stunning and hibernation may merely represent transitional states in a continuum extending from unimpaired functional tissue to irreversible necrosis. These myocardial tissue areas with recoverable function, would benefit most from therapeutical measures such as interventional reperfusion or surgical revascularisation. As mentioned above, acute IPC may elicit stunning, while PPC usually reduces the extent of stunning (Przyklenk and Kloner, 1998). Some variability in the effects of acute PC may depend on the experimental conditions and may also reflect species differences. In the delayed phase of PC stunning is always reduced irrespective of the type of stimulus.

Preconditioning as adaptation to tissue injury?

A decisive role for the survival of myocardial tissue in the area of an occluded artery also plays the collateral vessel connections to the neighbouring areas (vascular channels that interconnect arteries), the perfusion of which is still sufficient (Schaper *et al*., 1988). Such a collateral circulation is almost absent in the hearts of mice, rats, rabbits and pigs, it is intermediately established in the hearts of dogs and humans, and extensively present in guinea-pig hearts. Accordingly, the infarct size induced under defined experimental conditions is most reproducible in those animals with little collateral circulation, and virtually no infarction develops in guinea-pigs under acute coronary occlusion.

PC may be considered to represent a general adaptive process for cell protection which can be elicited by, beside ischemia, different stress-stimuli such as hypoxia, free radicals, mechanical stretch, thermal stress and endogenous primary messengers (vasoactive peptides, catecholamines and hormones) (Schulz *et al*., 2001; Sommerschild and Kirkeboen, 2002). As long as a perturbation stimulus remains sublethal, the cell will not only recover its original state, but will emerge in a strengthened one to fend off further harm. It seems paradoxical that a sublethal ischemia is able to protect the myocardium from subsequent potentially lethal sustained ischemia. For the individual cell the IPC process means a tightrope walk with an uncertain outcome of survival.

Myocardial preconditioning induced by anoxia has been observed in rainbow trout hearts, which are normally extremely hypoxia intolerant (Gamperl *et al*., 2001). The time course and magnitude of loss of myocardial function during acute anoxia is similar in the trout and the rabbit. These results suggest that PC is a mechanism of tissue protection that preceded the evolution of marine and terrestrial vertebrate life 300–400 million years ago in the mid paleozoic (Ayala *et al*., 1998). In addition, ischemia in organs other than heart can also afford protection of the myocardium (Sommerschild and Kirkeboen, 2002). This effect is called 'remote PC' and is probably caused by neurogenic activation and/or circulating hormones. It was indeed shown that IPC confers protection to both the endothelial and smooth muscle cells of the vascular system and that, consequently, most hypoxia-sensitive organs like the brain, intestines, liver and kidney receive PC trigger stimuli (Rubino and Yellon, 2000; Laude *et al*., 2002; Torras *et al*., 2002). Whether unicellular eukaryotes also possess the capacity for PC is not known. One may speculate that for PC a multicellular organism with a systemic circulation is required, which ensures the transport of primary messengers to the whole body.

In vitro **models for preconditioning**

The isolated beating heart started work in 1895

For the delineation of PC triggers and signaling pathways isolated cardiomyocytes in culture with a defined medium have proven suitable (Jacobson and Piper, 1986; Schaub *et al*., 1997; Marber, 2000). This allows to develop experimental conditions for testing individual steps in a signaling cascade under stimulation with

primary messengers such as catecholamines, hormones, vasoactive peptides or drugs, without signaling interference from other cell types (fibroblasts, vascular endothelial and smooth muscle cells, and neurons). The myocytes comprise around 80% of the heart mass but represent only 35% of the total cell number (Korecky and Rakusan, 1978). A second crucial step consists in establishing the link between a distinct signaling pathway and a specific cellular response. Beside isolated cells, tissue strips and papillary muscles in culture systems, the intact beating heart is particularly suited for measuring functional parameters be it *in situ* or as isolated organ in the Langendorff apparatus. The isolated perfused heart has the advantage that external variables, unlike in the *in situ* situation, may be readily standardised with respect to temperature, pH, ion concentrations, energy substrates and drugs. Since the first description by Langendorff (1895), the isolated perfused heart is still the first choice model for studying the effects of IPC and PPC on contractile function together with ECG, coronary flow, metabolic, biochemical, and immunohistochemical parameters.

Figure 1 shows the contractility recordings of isolated rat hearts in the Langendorff setting comparing the effects on contractile recovery during the reperfusion phase after the test ischemia alone or with prior IPC and APC by isoflurane, a commonly used volatile anesthetic. Both IPC and APC significantly improve the contractile recovery during the reperfusion phase. With APC the original extent of ventricular pressure development is reached after 30 min of reperfusion. Beyond that time point the hearts having received APC perform as well as controls without ischemic challenge. Almost as soon as perfusion is stopped (at the beginning of the test ischemia), cardiac contracture starts. Not only systolic contraction but also diastolic relaxation recovers faster and more completely during reperfusion with prior PC, and particularly so with APC (Figure 1B, C and D). Rapid decline of ATP and cytosolic Ca^{2+} overload contribute to the contracture during zero-flow even when it lasts only for 5 min during IPC. Complete oxidation of 1 mol of glucose yields 36 mol of ATP. In the absence of oxygen supply, only 2 mol of ATP are generated from anaerobic metabolism of 1 mol of glucose. Thus an 18-fold increase in glycolytic flux would be required for compensation of the loss of aerobic metabolism (Jennings and Reimer, 1981). Since the entire ATP content of the heart is turned over within about 5 s, it is not surprising that in the absence of oxidative phosphorylation neither contractility nor ion pump activities can be maintained.

In similar experiments with isolated rat hearts reperfused for 3 h after a 40 min test ischemia the total number of cell nuclei, the nuclei of damaged cells, and the TUNEL-positive nuclei were counted on longitudinal sections with an area of around 80 mm^2. The sections covered almost the entire extension of the left ventricular free wall and were partitioned by a continuous grid with 40–60 squares (1.44 mm^2 each) per

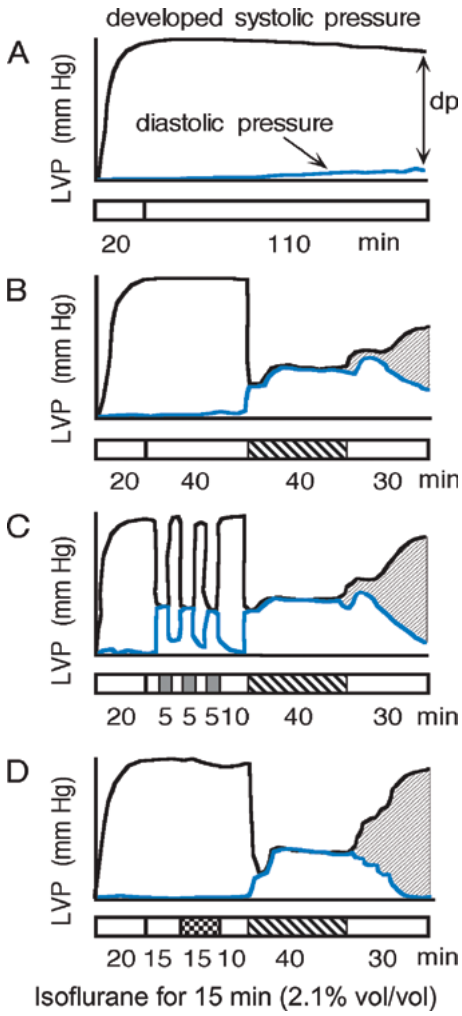

Fig. 1. Contractility recordings of isolated rat hearts during ischemic (IPC) and anesthetic (APC) preconditioning, followed by test ischemia and reperfusion. The hearts (beating at a rate of 280–300 per min) were perfused over 130 min (20 min served as equilibration period) in the Langendorff apparatus at constant pressure (80 mmHg) with Krebs–Henseleit buffer gassed with 95% oxygen and 5% carbon dioxide and glucose. (A) Control heart without treatment; (B) test ischemia of 40 min without prior preconditioning, followed by 30 min of reperfusion; (C) IPC protocol with three episodes of 5 min zero-flow interspersed by short reperfusions, before test ischemia and reperfusion; (D) APC with 15 min 2.1% (vol/vol) isoflurane in the perfusate, followed by test ischemia and reperfusion. LVP, left ventricular pressure; dp, developed pressure; upper black line, systolic developed pressure; lower line, diastolic pressure. The shaded area between the two lines reflects the ongoing recovery process during reperfusion after the test ischemia. IPC significantly improves the contractile recovery compared to the protocol with test ischemia alone. With APC the contractile performance reaches after 30 min of reperfusion almost 100% of the pre-ischemic value. For further explanations see text.

section. An area of 3.9% of each square was counted, hence 3.9% of the entire left ventricular heart sections were counted. Total cell nuclei counted per section ranged from 13,000 up to 26,000. For the assessment of damaged tissue, the hearts were perfused for the last 15 min with the nuclear stain propidium iodide. Propidium iodide stains only nuclei of damaged cells since it does not penetrate the cell membrane of viable cells. After test ischemia without pretreatment, an average of

66% nuclei stained for propidium iodide, and 2.1% nuclei were TUNEL-positive. The value for propidium iodide dropped to 0.2%, while that for TUNEL-positivity remained at 2.1% in hearts with 15 min pretreatment by isoflurane (2.1% vol/vol). The results indicate that isoflurane fully protects the heart tissue from cell death under these conditions but leaves the low percentage of TUNEL-positive nuclei unchanged (unpublished results).

With the isolated heart *in vitro* it is only possible to study the early phase of protection. *In vivo* experiments or cell cultures are required for testing the delayed protection phase. However, preconditioning trigger-dependent transcriptional changes may be detectable following only 2 h of perfusion in isolated hearts and may serve as early markers of a delayed cardioprotective phenotype. Relatively few studies are available that test the delayed phase with chronically instrumented conscious animals (usually rabbit, dog or pig) up to 72 h after PC (Tissier *et al*., 2002). The most often employed protocol, also suitable for small laboratory animals like mouse or rat, applies IPC or PPC on the first day. The animals are then sacrificed 24 h later for testing the isolated working heart for ischemic susceptibility. Under certain conditions, however, 24 h may be too short a period for the development of delayed protection.

Isolated cardiomyocytes in culture

If isolated ventricular myocytes in culture are chosen for unequivocal identification of individual signaling components and PC mechanisms, one has to distinguish between the following preparations: (i) embryonic (usually chick myocytes), (ii) neonatal (usually chick, rat or rabbit), and (iii) adult myocytes (usually, rat, rabbit or pig). For short-term experiments within a period of 12 h, freshly isolated myocytes are seeded and kept in serum-free medium, where they rapidly attach to the substratum, preserve their original elongated shape and remain Ca^{2+}-competent (Jacobson and Piper, 1986). In the presence of serum, myocytes undergo a drastic de- and re-differentiation program with changing surface receptor population and protein isoform composition (Eppenberger *et al*., 1988; Donath *et al*., 1994). Adult cardiomyocytes are terminally differentiated cells that have lost their ability to divide and represent a higher level of differentiaton compared to embryonic, neonatal or atrial myocytes (Marber, 2000). Freshly isolated adult ventricular myocytes are preferred for PC experimentation as they more closely reflect the response capacity of the intact adult myocardium. Their disadvantage is the low transfection rate with calcium phosphate as compared to the high degree of transfection in immature cardiomyocytes. Obtaining consistent results with myocytes from human atrial appendices has proven to be difficult. Some work was also done on atrial myocyte cell lines derived from human atrium (Girardi cell line) (Carroll and Yellon, 2000) and from the AT-1 mouse atrial tumour lineage (HL-1 cell line) (Claycomb *et al*., 1998). However, these cell lines, usually cultured in the presence of serum, exhibit a low differentiation level and a structural phenotype far from that of normal cardiomyocytes.

An interesting observation was recently reported for the isolation and culturing of adult mouse ventricular myocytes up to 72 h with the percentage of viable, rod-shaped cells declining from 100 to 60–70% (the original plating efficiency was around 70% rod-shaped myocytes). Mouse myocytes, unlike rat or rabbit myocytes, are highly susceptible to contracture in culture, which causes rod-shaped myocytes to become rounded and lesioned, often ending in death. The long-term myocyte preservation relies on supplementation of the serum-free medium at pH 7.0 (instead of the usually adopted pH 7.4) with 10 mM of the contractile inhibitor 2,3-butanedione monoxime (BDM) and very low levels of insulin, transferrin and selenium (O'Connell *et al*., 2003). Adenovirus-mediated expression of an exogenous beta-galactosidase reporter gene was maintained by the cultured myocytes for the entire period of 72 h. This opens the avenue for studies on signaling for the delayed PC in cultured myocytes including either vector-based RNA interference (RNAi) or the expression of dominant negative signaling proteins.

After application of exogenous PC triggers (anoxia for 5–20 min in the absence of glucose, or PC inducing substances and drugs) alone or in combination with activators or inhibitors of putative signaling components, ventricular adult rat cardiomyocytes (VARC) in serum-free short-term culture may be challenged by a longer periods (60–180 min) of simulated ischemia (80–99% nitrogen plus carbon dioxide in the absence of glucose). Assessment of the portion of dead and viable cells after the simulated test ischemia serves for end points such as myocyte contractility, release of the cytosolic enzymes creatine kinase (CK) or lactate dehydrogenase (LDH), and nuclear staining with propidium iodide or trypan blue uptake by lesioned cells (Marber, 2000).

A more subtle way to follow the cellular response to PC triggers involves measuring the mitochondrial flavoprotein fluorescence by time-lapse live cell imaging microscopy. This represents a functional characterisation of the PC process and may complement the end point assessment of cell death after test ischemia. Mitochondrial autofluorescence is caused by the reduced pyridine nucleotides, NAD(P)/H, and by the oxidised flavoproteins (succinate dehydrogenase, glycerol-3-phosphate dehydrogenase, acyl-CoA dehydrogenase). These endogenous fluorophores transfer electrons to oxygen in the inner mitochondrial membrane, ultimately leading to formation of water and synthesis of ATP. The excitation of flavoproteins is maximal under full oxidation and minimal under full reduction, whereas the opposite is true for NAD(P)/H. The ratio of the concentration of oxidised and reduced electron carriers or of their fluorescence, respectively, therefore provides a measure of the cellular metabolic

state. On the other hand, Marban and his group showed that the redox state of these endogenous fluorophores directly reflects mitoK-ATP channel activity, and that opening of this channel is closely associated with protection against ischemia (Romashko *et al.*, 1998; Sato *et al.*, 2000).

By the same methodological approach, we recently demonstrated that volatile anesthetics mediate their protection in isolated VARC by priming the mitoK-ATP channels as reflected by changes in flavoprotein fluorescence (Zaugg *et al.*, 2002a, b). Figure 2 shows that the volatile anesthetic sevoflurane (SEVO), which is known to offer cardioprotection, further enhances the diazoxide (DIAZO) induced fluorescence level. As DIAZO is a KCO the results suggest that the cytoprotective property of SEVO may, in part at least, depend on its ability to increase the open probability of the mitoK-ATP channel. In contrast, R-ketamine (R-KET) reduced the activity of this channel, which may indicate that R-KET has an unfavourable effect on the PC process. Repeated time-lapse sampling allows to record the drug-induced time-course and amplitude of fluorescence changes in many individual myocytes for statistical analysis. In the experiments shown, isoflurane with the isolated heart (Figure 1) and SEVO with the isolated VARC (Figure 2) were applied in concentrations as clinically used for anesthesia.

Signaling in the early and delayed cardiac PC

Downey and his group were the first to discover that IPC is receptor-mediated (Liu *et al.*, 1991; Cohen *et al.*, 2000). In rapid succession, a number of surface receptors capable of inducing PC or contributing to PC was identified, all of which belong to the heptahelical transmembrane G-protein coupled receptor type (GPCR). The simplified scheme in Figure 3 summarises the major signaling pathways for the acute early and the second delayed IPC phases. Almost all GPCRs present in cardiomyocytes are capable to induce PC. Beside a systemic surge of catecholamines under stress, all the primary messengers for IPC may originate from the heart tissue and, by local diffusion, transmit the stimulus beyond the ischemic region also to adjacent non-ischemic tissue (Przyklenk *et al.*, 1993). Transient periods of rapid heart rate (pacing) or volume overload (myocyte stretching) can also induce the early phase of

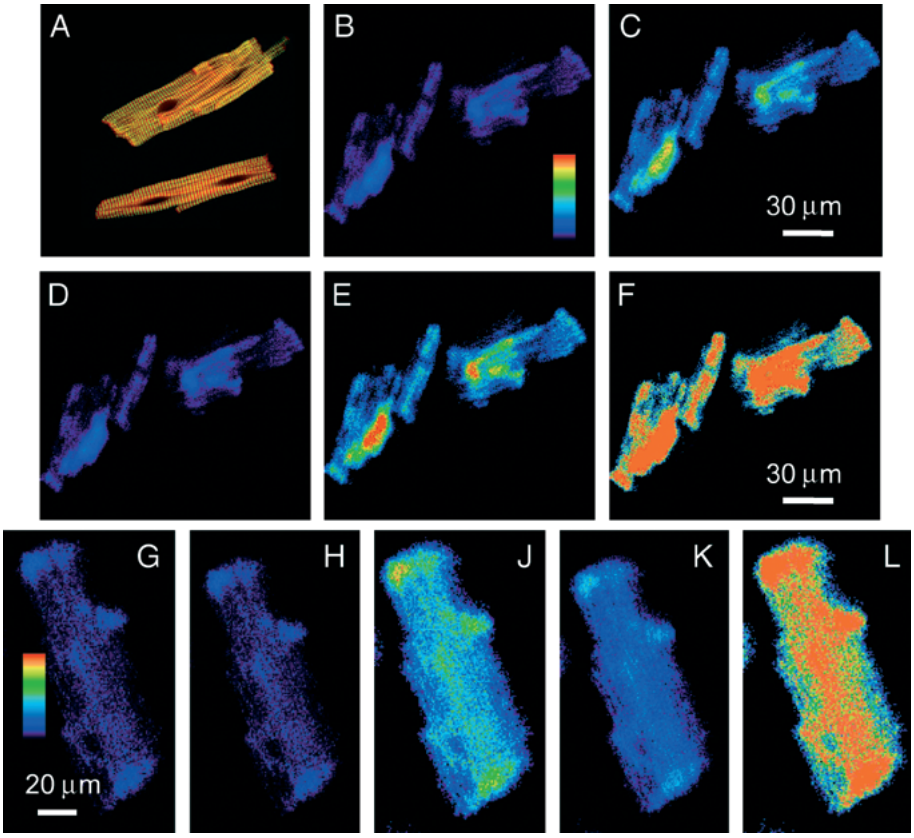

Fig. 2. Effects of the anesthetics SEVO and R-KET on DIAZO-induced flavoprotein oxidation in isolated VARC excited at 480 nm. Emitted fluorescence was recorded at 530 nm (dark blue indicates reduced, and red indicates fully oxidised flavoproteins). (A) Freshly isolated VARC immunostained for the M-line protein myomesin and with phalloidin–rhodamine for actin filaments; (B) flavoprotein fluorescence at baseline; (C) 100 µM DIAZO; (D) SEVO (2.8%, vol/vol); (E) 100 µM DIAZO preceded by SEVO; (F) 100 µM 2,4-dinitrophenol (DNP) for calibration; (G) flavoprotein fluorescence at baseline; (H) 10 µM R-KET alone; (J) 100 µM DIAZO; (K) 100 µM DIAZO plus 10 µM R-KET; (L) 100 µM DNP. Note, SEVO and R-KET do not affect baseline fluorescence but SEVO enhances the DIAZO-induced fluorescence level and R-KET reduces it (results from references Zaugg *et al.*, 2002a, b).

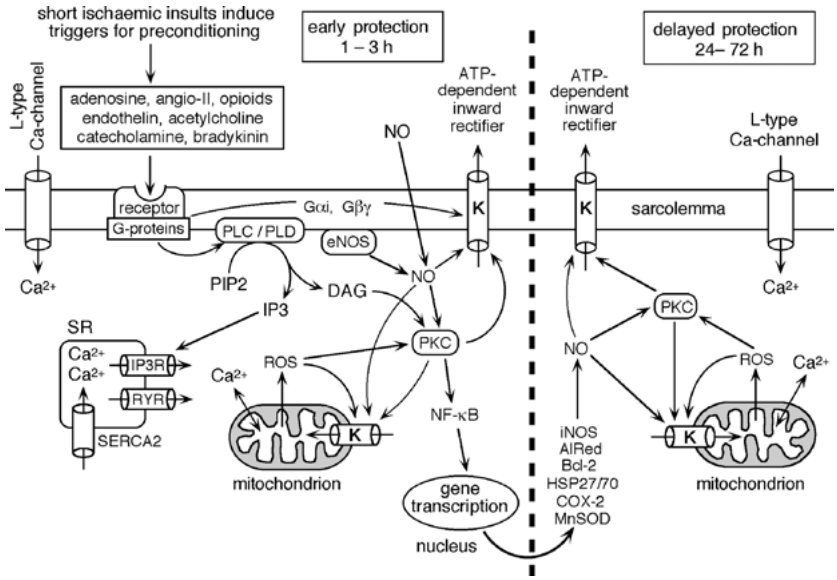

Fig. 3. IPC signaling for the early (on the left of the hatched line) and the delayed (on the right of the hatched line) phase of protection. Several distinct G-protein coupled receptors (GPCR) mediate the primary IPC signals for the early protection by activation of phospholipases (PLC and PLD), production of DAG, which activates different isoforms of PKC. The PKC isoforms further relay the signal to the well-defined ATP-sensitive membrane potassium (sarcoK-ATP) channels, and to the less well-known mitochondrial K-ATP (mitoK-ATP) channels. In addition to direct signaling for the early protection phase, PKC also activates transcription factors (including NF-kB), which lead to the expression of a number of proteins thought to be involved in promoting the delayed protection phase. The delayed phase is characterised by a sustained maintenance of similar signaling pathways and activation of the sarco- and mitoK-ATP channels similar to the early phase. This is achieved by an increased expression of proteins ensuring continuous production of NO, antioxidative, cytoprotective, and anti-apoptotic potential. AlRed, aldose reductase; COX-2, cyclooxygenase type-2; Gαi, inhibitory Galpha protein; Gβγ, heterodimeric G-protein; HSP, heat shock protein; eNOS, intracellular constitutively expressed NO synthase; iNOS, inducible NO synthase; IP3, inositoltrisphosphate; IP3R, IP3 receptor; MnSOD, manganese superoxide dismutase; PIP2, phosphatidylinositol bisphosphate; ROS, reactive oxygen species; RYR, ryanodine Ca^{2+} release channel; SERCA2, SR Ca-pump; SR, sarcoplasmic reticulum. Further explanations in the text (for references see: Rubino and Yellon, 2000; Shinmura *et al.*, 2002; Sommerschild and Kirkeboen, 2002; Zaugg *et al.*, 2003b).

PC, while a heat shock may promote the delayed PC phase (Sommerschild and Kirkeboen, 2002). Finally, short-lasting strenuous bursts of physical exercise with an up to 15-fold increase of plasma catecholamine levels (Paterson, 1996), were shown to increase myocardial ischemic tolerance in experimental animals and in humans.

In view of the number of primary messengers for PC, the rather stringent signaling pathway downstream of the GPCR surprises. It involves activation of phospholipases (PLC, PLD) and liberation of the second messenger diacyglycerol (DAG) for directly targeting the PKC (Bolli, 2000; Schulz *et al.*, 2001, 2002; Zaugg *et al.*, 2003b). PKC represents a key signaling molecule, which, by phosphorylation, may activate both the sarcoK-ATP and the mitoK-ATP channels, transcription factors including the nuclear factor kB (NF-kB), and modify other myofibrillar and cytoskeletal proteins (Schaub *et al.*, 1998; Hahn *et al.*, 2002). Alternatively, PKC and also the K-ATP channels may be activated directly by NO (nitric oxide) from intracellular constitutively active NO synthase (eNOS) or by NO from extracellular sources. PKC can also be activated by ROS arising from mitochondria either during the short ischemic or the following repetitive reperfusion episodes. ROS reacting with NO forms peroxynitrite and other reactive nitrogen oxide species (RNOS), which in turn also may activate PKC and the mitoK-ATP channel. In addition, several types of non-receptor tyrosine kinases (particularly Src and Lck) (not shown in Figure 3) may be interlinked in the signaling cascades either upstream and/or downstream of PKC and their targets, the K-ATP channels. The G-protein subunits Gαi and the Gβγ heterodimer were also shown to activate directly the sarcoK-ATP channels.

In addition to direct signaling for the early protection phase, PKC also activates several transcription factors, which lead to the expression of a number of proteins thought to be involved in promoting the second delayed protection phase. The late phase is characterised by a sustained maintenance over several days of similar primary signals as in the early phase. This is achieved by an increased expression of proteins ensuring continuous production of NO, and of proteins with antioxidative, cytoprotective and anti-apoptotic potential (Figure 3). NF-kB was the first transcription factor that was recognised to be essential for the development of the delayed protection phase. Activated PKC is able to stimulate NF-kB by phosphorylation of a serine-threonine kinase (IKK), which in turn phosphorylates the NF-kB inhibitor (IkB), whereupon the latter dissociates from NF-kB, which can then translocate to the cell nucleus (Li *et al.*, 2000). Also, in this signaling pathway, a non-receptor tyrosine kinase (Lck) may be interlinked

downstream of PKC. NF-kB is directly involved in regulation of iNOS, cyclooxygenase type-2 (COX-2) and aldose reductase (AlRed), which all contribute to delayed cell protection. iNOS-deficient mice have been used to show that NO produced by this enzyme is a key component in the late phase of IPC (Guo *et al*., 1999). Additional transcription factors such as the 'immediate early genes' (c-fos, c-myc, Egr-1 and jun-B) and the AP-1 (heterodimer of c-Fos and c-Jun) become activated and contribute to gene expression after IPC. Expression of some of these transcription factors depends on mitogen activated protein kinase (MAPK) signaling. Finally, heat shock proteins (HSPs), chaperone proteins involved in the folding and function of various cellular proteins, were also shown to be expressed in PC. Although their role in the delayed PC phase is presently unclear (Bolli, 2000; Schulz *et al*., 2001), expression of HSPs in response to temperature stress and other forms of stress occurs in species of all 30 eumetazoan phyla (Feder and Hofmann, 1999).

Similarities in signaling between the early and delayed phases also concern the steps downstream of PKC. In both cases PKC signaling is tied in to a positive feedback loop circling through the mitochondria via the mitoK-ATP channel that directly stimulates mitochondrial ROS production (Figure 3). This PC enforcing circuit is further driven by joining in of NO. In the late PC phase, NO is steadily produced at a high level by the induction of iNOS expression over a longer period of time. As a corollary of the changed gene expression profile in delayed cardioprotection, the early and the late phase are expected to differ from one another not only in time and degree of protection. IPC reduces infarct size more in the early than the late phase. On the other hand, myocardial stunning is more mitigated in the late than the early phase (Bolli, 2000). The duration of the short early phase can be extended neither by continuous infusion of pharmacological triggers nor by repeated brief ischemic episodes, while administration of an adenosine receptor agonist at 48 h intervals resulted in the maintenance of continuous protection against infarction in a rabbit model for at least 10 days without evidence of downregulation of receptor function (Dana *et al*., 1998). However, the cellular mechanisms involved in early and delayed PC are likely to show some variation depending on type and degree of the induction stimulus.

Memory, redundancy, and specificity in PC signaling

In the context of early and delayed PC, four aspects have emerged as particularly interesting in PC research: (i) the memory state of the preconditioned myocyte; (ii) the redundancy in the primary IPC signaling; (iii) signal specificities for PC; and (iv) the role of the mitoK-ATP channel in PC as trigger, mediator, or end effector.

(i) A characteristic feature of PC is the 'memory' phenomenon. The cell 'remembers' that it has been exposed to ischemic stress, hence, the initial stimulus induced alterations in the cell, which can last over a certain time period, either for 1–3 h or from 24 up to 72 h. Different mechanisms must be responsible for the short- and the long-term memory, but in both cases significant changes in the intracellular homeostasis must have occurred. The mechanisms for the long-term memory may be intuitively easier to apprehend. The ischemia-reperfusion stress the myocyte was exposed to, was sufficiently severe so that the increased cytosolic Ca^{2+}, the primary messengers, and ROS were able to alter gene expression (Figure 3). From the rabbit model, where continuous protection resulted from repeated administration of an adenosine agonist (Dana *et al*., 1998), it may be inferred that the release of primary messengers, sufficient for maintaining the PC gene expression program, persists for 3–4 days provided the original IPC stimulus was strong enough.

For the short-term memory over 1–3 h, de novo protein synthesis seems not to be required, although some of the immediate early genes may already be activated during this phase. Most of these early genes are transcription factors, which activate further genes encoding proteins needed later in the process. The short-term memory appears to depend on trigger-induced activation of an ensemble of protein kinases and phosphatases including PKC. Depending on the phosphorylation state, protein kinases may translocate to particular subcellular compartments such as sarcolemma, mitochondria, intercalated discs or nuclei, where they are poised near their targets should the need arise for quick activation (Cohen *et al*., 2000; Newton, 2003). However, the data supporting the translocation hypothesis are controversial. Few studies have shown a correlation of PKC translocation with the presence of a protected state (Kawamura *et al*., 1998).

(ii) The majority of hormones, neurotransmitters, and vasoactive peptides, which are able to induce PC exert their effects through GPCRs (Figure 3). Therefore, considerable redundancy exists among the primary endogenous messengers (Cohen *et al*., 2000; Schulz *et al*., 2001). About as many GPCR subtypes as there are primary messengers, are responsible for transmission of their signal to the cell interior (Figure 4). The redundancy was shown in the rabbit heart model with the main three endogenous PC triggers, adenosine, bradykinin and opioids. In the rabbit, a single brief ischemic event suffices to induce PC. Blocking any of the three respective receptors completely abolished PC, suggesting that the threshold for PC induction was no more reached. However, blocking of the bradykinin B2 receptor with HOE140 did not abrogate PC after four brief repetitive ischemic episodes. Thus a stronger IPC stimulus led to sufficient release of adenosine, opioids and other triggers to overcome the blockade of the B2 receptor. These results indicate that contributions to PC by the individual triggers are additive, but their relative importance may vary according to the type of stress stimulus and species.

From the GPCRs the signals are transferred to four types of G-proteins with different specificities for en-

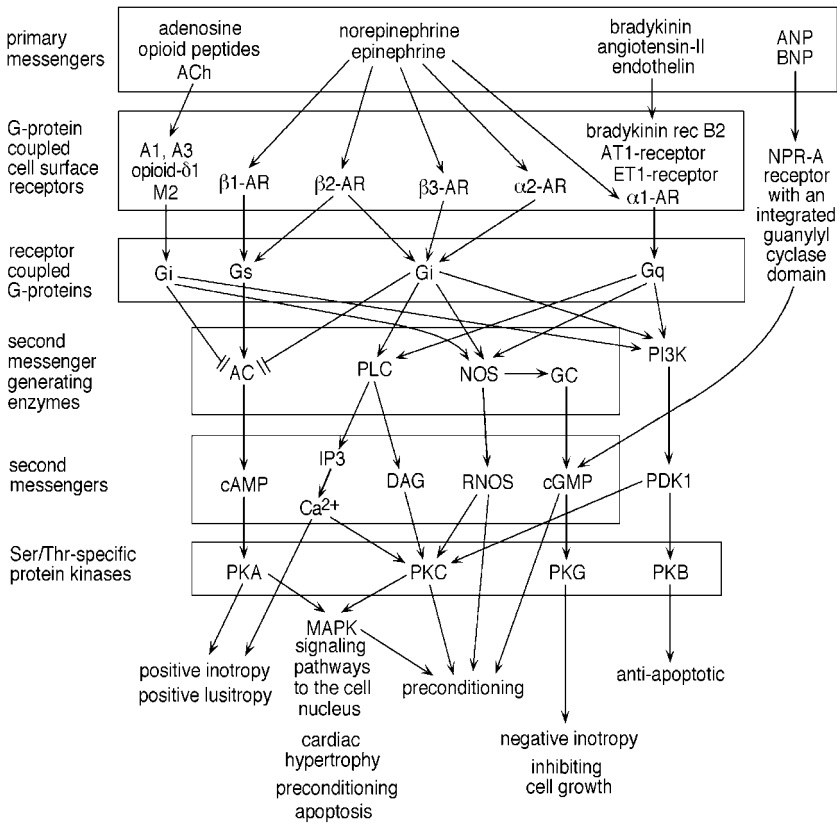

Fig. 4. Surface receptor signaling system for PC. Most PC signals from the extracellular primary messengers are transmitted to the intracellular secondary messengers by the G-protein coupled receptors. Further downstream signaling is mediated by target specific serine-threonine kinases (PKA, PKB, PKC, PKG). Note the intimate cross-talk between the individual signaling pathways. Lines with blunted end (=) indicates inhibition. A1, A3, adenosine receptors; AC, adenylyl cyclase; ACh, acetylcholine; ANP, BNP, atrial and brain natriuretic peptide; AR, adrenergic receptor; AT1, angiotensin-II receptor; DAG, diacylglycerol; ET1, endothelin receptor; GC, cytosolic soluble guanylyl cyclase;GC-A, membrane receptor for ANP with integrated guanylyl cyclase; Gi, Gs, Gq, G-protein subunits; IP3, inositoltrisphosphate; M2, muscarinic acetylcholine receptor; NOS, NO synthase; NPR-A, natriuretic peptide receptor type-A; PDK1, phosphoinositide-dependent kinase-1; PI3K, phosphoinositide-3 kinase; PLC, phospholipase-C; RNOS, reactive nitric oxide species. Further explanations in the text (for references see: Brodde *et al.*, 2001; Rockman *et al.*, 2002; Zaugg *et al.*, 2002c; Neves and Iyengar, 2002; D'Souza *et al.*, 2003; Newton, 2003).

zymes, which in turn, produce the second messengers. These specifically activate distinct serine-threonine protein kinases (PKA, PKC and PKG). At this stage, several signals known to induce PC, are channelled via PKC to their targets at the surface membrane and at the mitochondria (Figure 4). Within the Gi-protein family, the signaling flows equally through both the Gαi and the Gβγ complex, which latter may directly affect the sarcoK-ATP channel in the sarcolemma (Neves *et al.*, 2002; Rockman *et al.*, 2002). It was recently shown that the brain (or ventricular) natriuretic peptide (BNP) is also able to induce PC in isolated rat hearts by elevation of the second messenger cGMP (D'Souza *et al.*, 2003). BNP binds to the natriuretic peptide receptor type-A, a membrane-bound particulate guanylyl cyclase, different from the GPCR family.

In view of the extensive cross-connections between the different signaling pathways, it cannot be expected that the signaling for PC is very specific. The redundancy among the primary messengers continues along the downstream signaling pathways. In fact, the signaling for PC activates at the same time, a multitude of signaling pathways and, consequently, affects also a multitude of cellular properties including contractility, metabolism, and gene expression.

(iii) Nevertheless, some specificity in signaling for the effects characteristic for PC must be secured. It seems that PKC is the key player in the coordinated signal transfer to the appropriate sites of action. The PKC exists in a variety of isoforms with functional specificities. There are 10 mammalian PKC isoforms. The four conventional PKCs (α, β1, and a splice variant β2, and γ) require Ca^{2+} and diacylglycerol (DAG) for activation; the four novel PKCs (δ, ε, η/L and θ) are Ca^{2+}-independent but require DAG for activation; finally, the two atypical PKCs (ζ and λ) lack both the Ca^{2+} and the DAG binding domains (Newton, 2003). In the inactive conformation in the cytosol, PKC binds a pseudosubstrate sequence to the substrate-binding cavity. Generation of DAG (and Ca^{2+}) recruits PKC to the membrane. The membrane binding provides the energy to release the pseudosubstrate sequence from the active site, allowing substrate binding and phosphorylation for full activity. Which PKC isoform may be the major one for inducing PC, or which isoform is targeted to which membrane sites was shown to depend on the animal

model and stress stimuli (Pepe, 2001; Schulz *et al*., 2001). PKCε seems most important for PC in rabbits, PKCδ in rats, and PKCα in dogs. On translocation to membranes, PKCε interacts with an anchoring protein called RACK2 (receptor for activated C-kinase). In fact, each PKC isoform may bind only to its specific RACK protein (Mackay and Mochly-Rosen, 2001). When recruited to the mitochondria, PKCε was shown in mouse myocytes to associate with several other proteins as well, including the voltage-dependent anion channel (VDAC), the adenine nucleotide translocator (ANT) (for characterisation of VDAC and ANT see Section 'what makes up the mitochondrial permeability transition pore?'), hexokinase-II, and with mitogen activated protein kinases (MAPK) (Baines *et al*., 2002, 2003). After IPC and anesthetic PC with isoflurane, we observed in rat hearts that PKC*δ* translocated to mitochondria but not to the sarcolemma, and PKCε translocated to the sarcolemma and intercalated discs but not to mitochondria (Uecker *et al*., 2003). Signal specificity in IPC and PPC is thus achieved by specific interactions of distinct PKC isoforms with other structural or functional components forming a signaling module at the site of action and not by individual signaling pathways attributable to a particular function.

(iv) To address the questions, as to which role the mitoK-ATP channel plays in PC, and whether it acts as trigger, mediator, or end effector, a look into its protein structure and function is required first. Furthermore, the contribution of the mitochondria to the cellular mechanisms of PC and their relation to this channel warrants some detailed consideration. This is done in the Sections 'Key players in ischemic and pharmacological PC' and 'ATP-sensitive potassium channels'. The question about the significance of the mitoK-ATP channel in PC is discussed in Section 'Is the mitochondrial K-ATP channel playing the main role in PC?'.

Signaling amplification cascades

Figure 4 indicates the primary signaling for PC down to the level of the serine-threonine protein kinases. Among the latter, the PKC isoforms are most prominent and provide specificity for distinct subcellular effects involved in PC. This primary signaling needs to be seen in context with the major signaling cascades of the mitogen activated protein kinases (MAPK) and the stress activated JAK-STAT pathway as well (Figure 5). The MAPK cascades represent a characteristic phosphorelay system, in which a series of three protein kinases phosphorylate and activate one another (Johnson and Lapadat, 2002). The extracellular signal regulated kinases (ERK pathway) function in the control of cell division; the c-Jun N-terminal kinases (JNK pathway) are critical regulators of transcription; and the p38 MAPKs (p38 pathway) are activated by inflammatory cytokines and environmental stresses, and may contribute to diseases like asthma and autoimmunity.

The interconnections between the different signaling pathways given in Figure 5 represent only those reported, but probably many more exist in reality (Hefti *et al*., 1997; Bolli *et al*., 1001; Molkentin and Dorn, 2001). Co-activation of different pathways, may thus be expected. In fact, the involvement of all three MAPK cascades as well as the JAK-STAT pathway in PC was reported (Bolli *et al*., 2001; Heidkamp *et al*., 2001; Xuan *et al*., 2001; Baines *et al*., 2002; Steenbergen, 2002; Da Silva *et al*., 2003). The JAK-STAT pathway is now recognised as an important membrane-to-nucleus signaling relay for a variety of stress responses including ischemia and oxidative stress. Angiotensin-II-induced PC involves the JAK-STAT pathway (Hattori *et al*., 2001). Erythropoietin is a paracrine mediator of ischemic tolerance and was recently shown to signal via its cytokine receptor to JAK and the anti-apoptotic signaling pathway involving the phosphoinositide-3 kinase (PI3K), phosphoinositide-dependent kinase-1 (PDK1) and protein kinase-B (PKB) (Ruscher *et al*., 2002; Calvillo *et al*., 2003). Growth factors such as insulin-like growth factor-1 (IGF-I) or epidermal growth factor (EGF) as well as some cytokines stimulate the expression of cytoprotective mediators, which indirectly improve the recovery from oxidative stress. Big MAP kinase-1 (BMK1), also known as ERK5, is a newly identified member of the the MAPK family and is reported to be sensitive to oxidative stress. On PC12 cells (pheochromocytoma cells) it was shown that BMK1 activated by hydrogen peroxide counteracts ischemic cell damage probably by activation of the transcription factor MEF2C (Suzaki *et al*., 2002).

The pattern and degree of activation of distinct signaling pathways seems complex, but it may depend on the type and intensity of the stimulus, time of sampling of the tissue (before, during or after the test ischemia), and on the species examined. It appears that most effects stimulated by the MAPK cascades and the cytokine type of receptors, act via modification of gene expression and may therefore, be more important for late cardioprotection.

Key players in ischemic and pharmacological PC

Mitochondria as target for cytotoxicity and cytoprotection

The myocardium, as a continuously working pump, depends on an efficient and continuous energy production. The heart constitutes less than 0.5% of the body mass, yet it consumes around 11% of the total energy required for living. By complete oxidation of glucose to carbon dioxide and water, the mitochondria are able to extract about 12 times more energy than by anaerobic metabolism in the cytosol. Of the total ATP production, 60–70% is used for the contractile apparatus and 10–25% for ion movements (Ca- and Na/K-pumps). However, a great part of the energy from ATP dissipates

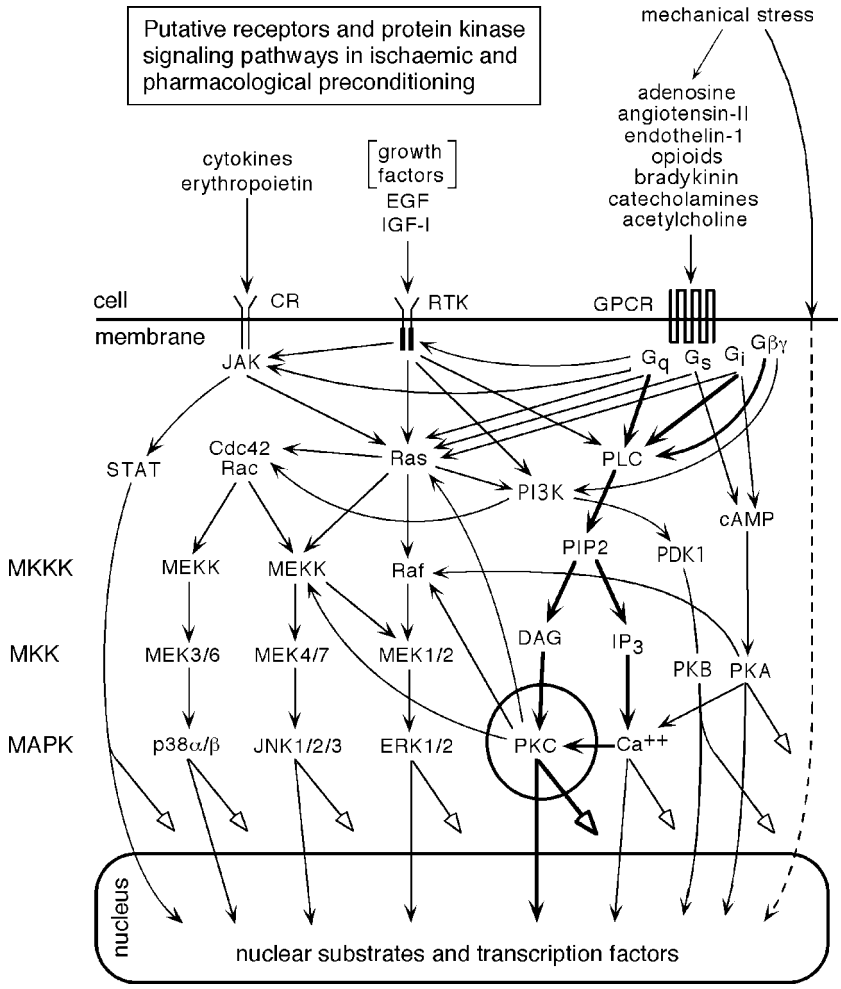

Fig. 5. Relation between PKC signaling for preconditioning (PC) and the major signaling cascades of the MAPK and the JAK-STAT pathways. The heavy arrows follow the main signaling pathways operative in both ischemic (IPC) and pharmacological (PPC) preconditioning. Both IPC and PPC may also activate the MAPK cascades. The MAPKs are part of a phosphorelay system composed of three sequentially activated kinases (MKKK–MKK–MAPK), and, like their substrates, MAPKs are regulated by phosphorylation. Note the intimate cross-connections between the GPCR signaling and the MAPK cascades. Some growth factors and cytokines are also able to induce PC. Open arrows indicate cytosolic effects. Dashed arrow indicates hypothetical direct mechanical signaling to the nucleus. Cdc42, Rac, Ras, small monomeric GTPases; CR, cytokine receptor; DAG, diacylglycerol; EGF, epidermal growth factor; ERK, extracellular signal regulated kinase; Gi, Gs, Gq, Gβγ, different G-protein subunits; IGF-I, insulin-like growth factor-1; IP3, inositol trisphosphate; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; MEK, mitogen activated ERK activating kinase; MEKK, MEK kinase; MKK, MAPK kinase; MKKK, MAPKK kinase; p38, p38 MAP kinase; PDK1, phosphoinositide-dependent kinase-1; PI3K, phosphoinositide-3 kinase; PIP2, phosphatidylinositol bisphosphate; PKA, PKB, PKC, specific serine-threonine kinases; PLC, phospholipase-C; Ras, Rac, Cdc42, monomeric small GTPases; RTK, receptor tyrosine kinase; STAT, signal transducer and activator of transcription. Further explanations in the text (for references see Hefti *et al.*, 1997; Bolli *et al.*, 2001; Michel *et al.*, 2001; Molkentin and Dorn, 2001; Baines *et al.*, 2002; Calvillo *et al.*, 2003).

into heat and only around 20–25% are converted into external mechanical work (Opie, 1997).

Cardiomyocytes have the highest mitochondrial content with a volume fraction inversely proportional to body size varying from 22% in pigs up to 38% in mice (Canale *et al.*, 1986). As the energy consuming mechanical function of the myocyte depends on a continuous energy production by the mitochondria, the two require a tight coupling and coordinated regulation. The myocyte and the mitochondria represent two compartments with different and highly specialised functions like a small cell encased by a bigger one (Figure 6). In fact, this situation reflects its evolutionary history, according to which the eukaryotic cells started without mitochondria. More than a billion years ago, when oxygen in substantial amounts entered the atmosphere, the eukaryotic cells established a stable endosymbiotic relation with a bacterium, whose oxidative phosphorylation system they subverted for their own use (Sogin, 1997). The mutual integration in the eukaryotic cells is secured in two ways: (i) the most prominent second messenger, namely Ca^{2+}, acts as a major communicator between the cytosolic and the mitochondrial matrix (MM) compartments (Carafoli, 2002), and (ii) a continuous metabolic relay system links the cell surface electrical events directly to the energy production centre in the mitochondria (Dzeja and Terzic, 2003). This is achieved through a coupled near-equilibrium enzymatic network involving several shuttle systems. ATP delivery is facilitated by creatine kinase (CK) adenylate kinase

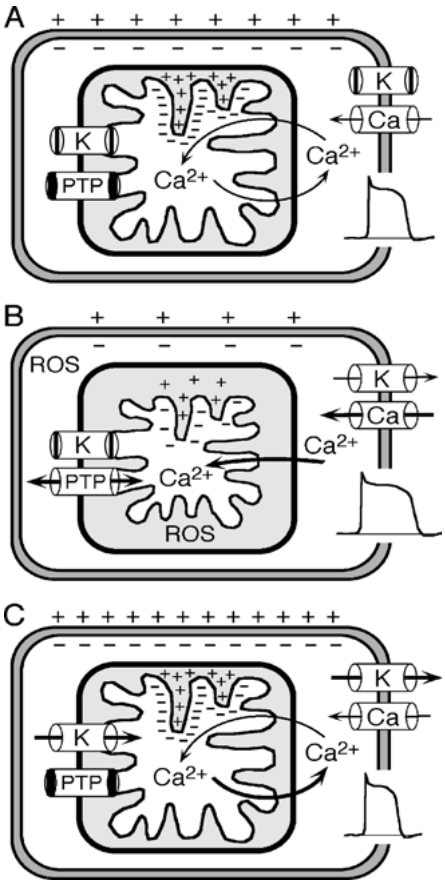

Fig. 6. Hypothetical mechanisms of cardiomyocyte ischemia-reperfusion injury and its potential prevention. Ca, sarcolemmal L-type Ca-channel; K, sarco- and mitoK-ATP channels; PTP, mitochondrial permeability transition pore; ROS, reactive oxygen species. (±) stands for voltage element. (A) Under normal conditions with high ATP/ADP ratio the K-ATP channels are closed and mitochondrial matrix (MM) Ca^{2+} is in equilibrium with cytosolic Ca^{2+}. (B) Lack of oxygen prevents ATP production, causing a decrease in diffusive K^+ influx. The MM volume shrinks on the expense of the intermembrane space, which expands. The low ATP/ADP ratio in the cytosol enhances the open probability of the sarcoK-ATP channel, while the mitoK-ATP channel remains closed in presence of ADP. Consequently, both cytosol and MM become Ca^{2+} overloaded, and as soon as reperfusion starts the PTP opens inducing massive mitochondrial swelling, outer membrane disruption and cell death. (C) Opening of the mitoK-ATP channel by IPC or PPC (e.g. with K-ATP channel openers) regulates volume of MM by moderate swelling, this allows ATP production to restart at reoxygenation, the mitochondrial inner membrane potential can be restored and the PTP remains closed. At the same time, shortening of the action potential duration decreases the cytosolic Ca^{2+} overload. Further explanations in the text (for references see Crompton *et al.*, 2002; Dos Santos *et al.*, 2002; Halestrap *et al.*, 2002; Zaugg *et al.*, 2003b).

(AK), and the glycolytic system comprising hexokinase, pyruvate kinase, and phosphoglycerate kinase (PGK). ADP is removed by the CK, AK, and PGK shuttles. As these shuttle systems work in parallel, a diminished activity of a single enzyme may well be tolerated. Only malfunction of several enzymes together will severely compromise the communication between ATP-generating and ATP-consuming sites. Thus, the mitochondria are at the cell's crossroad of life and death. Below, the contribution of the mitochondria to the cellular mechanisms involved in ischemia-reperfusion injury and in potential protective interventions will be discussed.

Mitochondrial calcium, friend or foe?

The cytosolic Ca^{2+} levels fluctuate between 10^{-8} and 10^{-6} M. The fast Ca^{2+} transients for contraction are mainly governed by Ca^{2+} release from the SR via the ryanodine Ca^{2+} release channel (RYR), and reuptake by the SR Ca-pump (SERCA2) (Figure 3). Ca^{2+} from outside enters the myocyte through the L-type Ca-channel, the so-called dihydropyridine receptor (DHPR), and the sarcolemmal Na/Ca-exchanger extrudes Ca^{2+} from the cell in exchange for Na^+ uptake. During the action potential plateau, Ca^{2+} extrusion is inhibited and the exchanger may uptake Ca^{2+} by working in the reverse mode. Ca^{2+} in the MM tends to follow cytosolic Ca^{2+} levels, being taken up via the Ca-uniporter and released through the mitochondrial Na/Ca-exchanger. The Ca-uniporter is sensitive to ruthenium red and is highly dependent on the mitochondrial inner membrane potential ($\Delta\Psi m$). The Na/Ca-exchanger can be inhibited by diltiazem (a benzothiazepine and Ca-channel blocker) and clonazepam (a benzodiazepine). Both the sarcolemmal and the mitochondrial exchanger move $3Na^+$ ions against $1Ca^{2+}$ ion (Suleiman *et al.*, 2001). Fast Ca^{2+} exchange of the MM with its surroundings is facilitated by the close structural proximity of mitochondria to the SR membranes from where Ca^{2+} may be released (Rizzuto *et al.*, 1998). In the MM Ca^{2+} stimulates the mitochondrial metabolism by regulating three key enzymes (pyruvate-, isocitrate- and oxoglutarate-dehydrogenases) of the TCAC (Jouaville *et al.*, 1999). This is paralleled by a rapid increase in NADH levels, feeding protons into the respiratory chain at the inner mitochondrial membrane (IMM) for ATP production. Any increase in cytosolic Ca^{2+} entails a corresponding Ca^{2+} increase in the MM (Figure 6). As long as these Ca^{2+} fluctuations remain within the physiological limits, it seems reasonable that higher myocyte activity calls for an increase in oxidative energy production by stimulating the TCAC. While the human heart pumps 6–8 l of blood per min at rest, this may increase up to 8-fold during physical exercise. The relation between energy demands and MM Ca^{2+} is given by the scheme:

Increased workload and/or hormonal stimulation
$\longrightarrow$ energy demand $\uparrow \longrightarrow$ cytosolic $Ca^{2+}\uparrow$
$\longrightarrow$ mitochondrial $Ca^{2+}\uparrow \longrightarrow$ dehydrogenase activities $\uparrow$
$\longrightarrow$ NADH $\uparrow \longrightarrow$ ATP $\uparrow \longrightarrow$ energy supply $\uparrow$

Mitochondrial Ca^{2+} overload occurs, however, when mitochondria are exposed to maintained Ca^{2+} levels (not transients) exceeding 1–2 μM. When cardiomyocytes loaded with the fluorescent Ca^{2+} indicator aequo-

rin (a 21,000 Da protein originally extracted from the jellyfish Aequorea forskalea) are subjected to anoxia, a progressive rise in resting cytosolic Ca^{2+} is observed (Crompton *et al*., 2002). This increased cytosolic Ca^{2+} reverses on reoxygenation provided the limit of 1–3 μM Ca^{2+} is not exceeded. If it is exceeded the myocytes undergo irreversible lethal injury. The combination of high MM Ca^{2+} levels, together with high inorganic phosphate (Pi), oxidative stress, and low extramitochondrial ATP unequivocally leads to the so-called mitochondrial permeability transition. This process involves opening of the mitochondrial permeability transition pore (mitoPTP), a non-specific pore, permeant to any molecule smaller than 1500 Da, that opens in the IMM. As a consequence of influx of solutes and water from the cytosol, the open pore induces massive swelling of the mitochondria, which can be accomodated by the extensively folded IMM, but not by the tightly fitting outer mitochondrial membrane (OMM). Consequently, the OMM becomes disrupted, which is followed by release of components from the mitochondrial intermembrane space (MIMS) that trigger apoptosis and/or necrosis. In addition, mitochondria become depolarised, causing inhibition of oxidative phosphorylation and stimulation of ATP hydrolysis, in particular by the different phosphorelay systems working near equilibrium. Even the mitochondrial ATP synthase may hydrolyse ATP by working in the reverse mode under these conditions.

What makes up the mitochondrial permeability transition pore?

The Ca^{2+} induced mitochondrial permeability transition was first described in 1979 (Hunter and Haworth, 1979; Haworth and Hunter, 1979). But the structure of the mitoPTP is still debated. Halestrap and his group present a model, in which the adenine nucleotide translocator (ANT) spans the MIMS from the IMM to the OMM, thus facing on the inner side the MM where under normal conditions ATP is bound, and on the outer side the cytosol, where ADP is bound (Halestrap *et al*., 2002). The nucleotides stabilise its structure. The MgATP complex does not bind to the ANT. Binding of Ca^{2+} to an intramitochondrial loop of the ANT triggers a conformational change required for pore formation, which is, however, antagonised by Mg^{2+} and other divalent cations. The closed pore is called the m-state and the open pore the c-state. The transition from the closed m- to the open c-state is brought about by the mitochondrial cyclophilin-D (CyPD), a peptidyl-prolyl *cis–trans* isomerase in the MM. Cyclosporin-A (CsA) and its analogues inhibit CyPD and thus inhibit pore formation. Under energised conditions (but not de-energised conditions) atractyloside, and more specific carboxyatractyloside (CAT), favours pore formation while bongkrekic acid and trifluoperazine stabilise the closed form. A pH below 7 is a potent inhibitor of pore formation, probably resulting from protons competing with Ca^{2+} for its binding at the trigger site. This model of Halestrap comprises the minimum configuration of the mitoPTP requiring no other outer membrane protein. However, MIMS proteins including Bcl2, Bax, and others may interact with the ANT to regulate the mitoPTP.

Crompton an his coworkers believe the IMM and OMM are held together by junctional complexes of the ANT at the inner and the voltage-dependent anion channel (VDAC or porin) at the outer membrane (Crompton *et al*., 2002). Under normal condition the VDAC is permeable to solutes up to about 5000 Da (Kroemer and Reed, 2000). Thus, whereas the MM contains a highly selective set of small molecules, the MIMS is chemically equivalent to the cytosol with respect to low molecular weight solutes. Three isoforms of both the VDAC and the ANT have been recognised. These junctional complexes may act as dynamic recruitment centres, binding a range of proteins according to functional needs. Some of the additional constituents involve protein kinases, enzymes of lipid transfer, and others. It was also proposed that the peripheral benzodiazepine receptor might be an integral component of the mitoPTP (Zoratti and Szabo, 1994). Physical disruption of the functioning complex facilitates the pore opening, which may be considered to represent a pathological process. The complex of ANT–VDAC together with CyPD reconstitutes Ca^{2+}- and CsA-sensitive mitoPTP activity when incorporated into proteoliposomes (Crompton *et al*., 2002). This tertiary complex is considered to represent a physiological entity in the mitochondria of healthy cardiomyocytes. Two specific functions need mentioning. First, the tertiary complex seems to take part in intermitochondrial junctions, providing ionic continuity between the matrix spaces of the conjugated mitochondria, and allowing the proton electrochemical gradient to be conducted from one mitochondrion to the next. In cardiomyocytes, conjugated mitochondria form intermyofibrillar networks for the efficient distribution of the proton motive force throughout the cell (Skulachev, 2001). Second, the outer protein VDAC is also able to recruit hexokinase and glycerol kinase from the cytosol in order to bring sites of ATP usage in the cytosol close to the sites of mitochondrial ATP export. These observations would point to a functional coordination in nucleotide translocation between the VDAC and the ANT. These two proteins are the most abundant in the mitochondrial membranes.

But yet another protein seems to be involved in mediating translocation of energy phosphoryls between the inner and outer mitochondrial membranes. As ADP is poorly diffusible in the cytoplasm, mitochondria need to work in tandem with a cytosolic shuttle system for efficient distribution of ATP energy throughout the myocyte. Wallimann and his group propose a still more sophisticated mitochondrial contact site between IMM and OMM by placing the mitochondrial creatine kinase isoform (mitoCK) between the ANT and the VDAC (Dolder *et al*., 2001, and references therein). MitoCK

forms an octameric cube structure with a hollow pore in its centre. Physical contact of octameric mitoCK with the VDAC is Ca^{2+}-dependent and can be experimentally demonstrated *in vitro*, while physical contact with the ANT remains elusive (Schlattner *et al*., 2001). However, it was shown that the proper mitoCK octamer in the ternary complex with the ANT and the VDAC, is able to regulate functionally the transition of the ANT between the open c-state and the closed m-state. If CyPD is present as well, the quarternary complex regains its CsA-sensitivity.

Together with the ANT of the IMM and the VDAC of the OMM, mitoCK may form a functional microcompartment in the MIMS. ATP produced by oxidative phosphorylation in the MM is exported by the ANT, and channelled to the active site of mitoCK for transphosphorylation to CrP. CrP that is well diffusible is then funnelled to the VDAC to be expelled to the cytosol, while ADP is channelled back to the mitochondrial MM via the ANT to further support oxidative phosphorylation. In view of the limited permeability of the OMM for adenine nucleotides, this model assures that the ATP and ADP pools in the cytosol and MIMS remain separated and do not intermix. Furthermore, mitoCK in this position exerts a major control over oxidative phosphorylation. Factors impairing the activity and/or destabilising the octameric structure of mitoCK were shown to destabilise the cohesion of the junctional protein complex and to increase the mitoPTP open probability (Dolder *et al*., 2003). Dimeric or monomeric mitoCK does not stabilise the ANT as does the octameric structure. MitoCK is particularly sensitive to reaction with ROS, which modify its active site Cys278. This represents a well defined pathway for ROS cytotoxicity during reperfusion injury. It helps understanding the apparent paradox that the most serious damage does not occur during the anoxic phase, but after reoxygenation.

Does opening of the mitoPTP irrevocably lead to cell death?

The complex of several proteins, each with specific translocator and/or enzymatic properties, is required for the physiologically meaningful translocation of energy phosphoryls from the MM to the cytosol. Physical disruption of the functioning complex facilitates the pore opening, which may be considered as a pathological process.

Whether the open pore is formed by the ANT alone or in conjunction with the VDAC is not clear at present. The pro-apoptotic components Bax, atractyloside, Ca^{2+}, palmitate, and thiol oxidants, induce the open pore state of the ANT. However, *in vitro* experiments have shown that the VDAC can assume an open state large enough for cytochrome-c with a molecular weight of 12,000 Da to pass through. This open state is enhanced by Bax and inhibited by the anti-apoptotic Bcl2. Thus it seems feasible that the VDAC may open and close independently of the ANT, releasing cytochrome-c, apoptosis-inducing factor (AIF), certain pro-caspases, Smac/DIABLO, and endonuclease-G from the MIMS to the cytosol (Kroemer and Reed, 2000). Both Bax and Bcl2 are primarily found in the OMM, however, they are also involved in regulation of the ANT component. Furthermore, Bax can oligomerise and form an autonomous protein translocating channel in the OMM, the activity of which may be enhanced by Bid, independently of the VDAC. Nonetheless, the open mitoPTP controls the permability of both the inner and outer mitochondrial membranes. At the contact sites, changes in the conformation of the ANT, modulated by its interaction with CyPD, may directly impinge on the function of the VDAC, and *vice versa*.

The IMM potential $\Delta\Psi m$ results from the electron transport chain, pumping protons out of the MM, and is indispensable for driving the ATP synthase, which phosphorylates ADP to ATP. ATP generated on the matrix side of the IMM is then exported in exchange for ADP by the ANT. Upon extensive pore opening, the $\Delta\Psi m$ collapses and ATP dissipates, which is accompanied by a rapid loss of cell viability. Rupture of the OMM releases cytochrome-c, and triggers the caspase cascade.

The permeability transition of any individual mitochondrion is thought to be an all or none phenomenon. When widespread irreversible mitoPTP opening occurs throughout a cell's mitochondria, cell death is inevitable. However, within a population, individual mitochondria may possess different sensitivities to openers of the mitoPTP. In fact, by entrapping Ca^{2+} buffers in the MM space in the presence of Ca^{2+} transport inhibitors, it could be shown in isolated mitochondria that the mitoPTP open and close continuously at a frequency that is determined by the matrix Ca^{2+} level. No critical Ca^{2+} threshold was apparent, and the relation between pore opening and matrix Ca^{2+} remains linear up to 20 μM Ca^{2+} (Crompton *et al*., 2002). Thus a limited degree of pore opening may be tolerated by the cell. The energy transduction would be less efficient, but this would not constitute an immediate threat to cellular survival, provided a sufficient minimal ATP level can be maintained. At any matrix Ca^{2+} level, there is a dynamic steady state between mitochondria with open pores and those with closed pores. At low MM Ca^{2+} loads, when only a small fraction of mitochondria have open pores at any given time, the $\Delta\Psi m$ is maintained by the remainder. It was further shown, that transient pore opening is sufficient to release cytochrome-c (Gogvadze *et al*., 2001). Whether transient mitoPTP opening with concomitant cytochrome-c release occurs *in vivo* is not known. What could be the physiological meaning of a low level pore activity with concomitant release of small amounts of cytochrome-c? The pore open probability may be coupled to the IMM $\Delta\Psi m$, and the cytochrome-c may, by activating catabolic enzymes in the cytosol, contribute to the turnover of cell constituents. As the

fluctuating pore activity depends on the Ca^{2+} level, one may speculate that it represents yet another intracellular Ca^{2+} regulated control device.

Cellular cytotoxic mechanisms

While the phosphoryl translocation between MM and cytosol represents the physiological function of the mitoPTP protein complex, its conversion to the pathological open state can lead to cell death. Why is sustained pore opening lethal for the cell? It produces two major detrimental effects: (i) swelling of the MM until physical disruption of the OMM occurs, inducing cell death signaling pathways, and (ii) collapse of the $\Delta\Psi m$, which interrupts energy production. Furthermore, pore opening is mainly occurring upon reoxygenation during reperfusion, when the cytosol and the MM are Ca^{2+} overloaded and cytotoxic concentrations of ROS are generated.

ROS are generated as byproducts of the cellular metabolism, primarily by xanthine oxidase and by the mitochondrial respiratory chain. Thus mitochondria serve both as producers and as targets of ROS. ROS function as subcellular messengers, play a role in gene regulation, and are involved in defensive mechanisms against oxidative stress (for review see Droge, 2002). The superoxide radical (O_2^-) and consequently hydrogen peroxide (H_2O_2) (as a product of the mitochondrial superoxide dismutase, Mn-SOD) originate primarily from complex-I and complex-III of the respiratory chain. Excess and/or sustained ROS production exceeding the antioxidant capacity, leads to damage of membrane lipids, proteins, and DNA. ROS have direct effects on several components of the respiratory chain, the ATP synthase and the ANT, probably by modification of protein thiol groups (Suleiman *et al*., 2001). ROS can also damage sarcolemmal membrane ion pumps and, therefore, exacerbate the effects of ATP deprivation on cell ion dysregulation. NO was also proposed to induce mitochondrial dysfunction. NO can reversibly inhibit cytochrome oxidase and thus affect mitochondrial respiration; but its more permanent damage may occur through NO-enhanced production of mitochondrial ROS upon reperfusion.

Recognising the steps occurring in sequence during reperfusion injury, allows to develop strategies to stop the process at different points. The schematic drawings in Figure 6 visualise the intracellular relations between the activities of the sarco- and mitoK-ATP channels, the sarcolemmal L-type Ca-channel, the mitoPTP, and their effects on the sarcolemmal and mitochondrial membrane potentials and Ca^{2+} homeostasis.

First, under normal conditions, the K-ATP channels and the mitoPTP are virtually closed, and Ca^{2+} equilibrates between the cytosol and the MM (Figure 6A). Second, the scheme in Figure 6B depicts the situation at the end of the ischemic period, at the moment when reperfusion would start. Due to the lack of oxygen during ischemia, the $\Delta\Psi m$ depolarises somewhat, causing a decrease in diffusive K^+ influx. As K^+ in the MM is the main volume regulator, the matrix volume contracts by 10–20% until the K/H-antiporter brings influx and efflux into balance, at which time the matrix volume achieves a new steady state at lower levels (Dos Santos *et al*., 2002; Halestrap *et al*., 2002). The concomitant expansion of the MIMS destabilises the mitoPTP complex, thereby impairing the transfer of cytosolic ADP to the MM in exchange of ATP. The instability of the mitoPTP renders the VDAC permable for the nucleotides, which then equilibrate across the OMM. As a corollary, the ATP synthesis comes to an almost complete halt and ATP is rapidly degraded to ADP and AMP. The deficient energy supply to the cell periphery inhibits the sarcolemmal Na/K-pump, which entails a decrease of intracellular K^+ and an increase of Na^+ and Ca^{2+}. This shift of intracellular ion levels causes a reduction of the resting potential. The action potential duration (APD) increases because of the delayed repolarisation, and due to both, the reduced resting potential and the prolonged APD, the cytosol becomes further overloaded with Ca^{2+} that overflows into the MM (Figure 6B). The increased ADP in the cytosol moderately stimulates the sarcoK-ATP channels, while the mitoK-ATP channels still remain closed by the increased ADP in the MM (Dos Santos *et al*., 2002). However, this limited activation of the sarcoK-ATP channels is able to revert neither the decrease of the resting potential nor the prolongation of the APD. If reperfusion starts after a period of prolonged ischemia at the point shown in Figure 6B, the mitoPTP opens with its fatal consequences for the cell: production of ROS, collapse of the $\Delta\Psi m$, massive mitochondrial swelling and OMM rupture. When the permeability barrier of the IMM is disrupted, the mitochondria become uncoupled, and even glycolytically derived ATP is hydrolysed, as the ATP synthase reverses in the absence of a membrane potential or pH gradient.

The main triggers for opening of the mitoPTP are high Ca^{2+}, ATP depletion, and oxidative stress (ROS). During ischemia, these stresses might not open the pore because the pH is low as a result of lactic acid accumulation (Suleiman *et al*., 2001). pH below 7 strongly inhibits pore opening by the protons competing with Ca^{2+} for the binding sites at the ANT. With the reperfusion, a further influx of Ca^{2+} into the MM occurs, a burst of ROS is produced, and the pH returns to preischemic values as the lactate is washed away. The high Ca^{2+} is the main responsible for opening the pore. The route of Ca^{2+} entry into the mitochondria under conditions of hypoxia or ischemia, surprisingly, is not the Ca-uniporter (ruthenium red does not inhibit the Ca^{2+} accumulation), but the Na/Ca-exchanger operating in the reverse mode (can be inhibited by clonazepam), taking up $1Ca^{2+}$ ion in exchange of $2Na^+$ ions. The Ca-uniporter is possibly inhibited at the lower $\Delta\Psi m$ during ischemia, and the Ca^{2+} entry is likely to shift from electrogenic to electroneutral (Griffiths *et al*., 1998).

Cellular cytoprotective K^+-dependent mechanisms

Cardioprotective interventions aim at preventing irreversible opening of the mitoPTP in order to keep the myocytes alive that have not died during the ischemic phase. Therefore, IPC or PPC must modify three major pathophysiological characteristics, which are responsible for opening of the mitoPTP: high Ca^{2+}, ROS production, and energy depletion. Cell life is only compatible with closed mitoPTP and restoration of the $\Delta\Psi m$ to ensure a sufficient proton gradient across the IMM for energy production.

As mentioned in section 'What makes up the mitochondrial permeability transition pore?', CsA is a potent inhibitor of mitoPTP opening and prevents consequent cell death. FK506, like CsA, inhibits the Ca^{2+}–calmodulin regulated phosphatase calcineurin, does not inhibit mitoPTP opening (Suleiman *et al*., 2001). However, CsA has a narrow concentration range over which it is effective, and has side effects that make it unsuitable for routine therapeutic use. The side effects of CsA may derive from the inhibition of calcineurin via its complex with the cytosolic cyclophilin-A (CyPA). Pyruvate added to the perfusion medium, prior to ischemia and during reperfusion, inhibits opening of the mitoPTP and improves the recovery of heart function. Three mechanisms may be responsible for the protective effect of pyruvate: (i) it increases lactic acid production and, thus, maintains a low pH in the MM; (ii) it acts as a scavenger of ROS; and (iii) it is an excellent respiratory fuel to energise mitochondria and so, enhance ATP production. Lowering the mitochondrial Ca^{2+} under conditions of hypoxia-reoxygenation by affecting selectively the Ca-uniporter with derivatives of ruthenium red, and the Na/Ca-exchanger with clonazepam, were performed on isolated cardiomyocytes, but these drugs are not cardio-specific, and these studies have not been extrapolated to the whole animal.

A recent development involves the ATP-dependent inward rectifying K^+ channels in IPC as well as in PPC. Structure and pharmacological properties of the well-defined sarcoK-ATP channel and the less well-defined mitoK-ATP channel will be described in some detail later. Here, it suffices to mention that both ATP and ADP inhibit the mitoK-ATP channel, while the sarcoK-ATP channel is inhibited by ATP and activated by ADP (Dos Santos *et al*., 2002). The mechanisms for IPC and PPC are still debated, but both sarco- and mitoK-ATP channels are activated by multiple signaling pathways (see Section 'Signaling in the early and delayed cardiac PC'). Export of K^+ ions by activation of the sarcoK-ATP channel hyperpolarises the cell, shortens the APD and, hence, reduces cytosolic Ca^{2+} loading (Figure 6C). The lower cytosolic Ca^{2+} is beneficial for cell survival under stress, since it reduces most cell activities and, thereby, lowers the energy demand; it also lowers the Ca^{2+} uptake into the mitochondria.

At the mitochondrial level, K^+ influx via the activated mitoK-ATP channel has several effects, which are difficult to distinguish form one another and to arrange into a sequential order of appearance. Taken together, these effects are beneficial for cell survival (Hausenloy *et al*., 2002; Oldenburg *et al*., 2002). This can be shown by the use of drugs that activate or inhibit the mito K-ATP channels (Table 1). Activating drugs mimic the IPC effects, while inhibiting drugs abrogate the beneficial effects of both IPC and PPC. But the interpretation of the experimental data is again difficult, because most of the drugs are not highly specific in targeting the mitochondrial channels, but also affect different enzyme

Table 1. Pharmacological properties of native tissue and defined, heterologously expressed K-ATP channels assessed by current measurements or by mitochondrial flavoprotein fluorescence (as indicator for channel activity)

Present in tissues	Molecular channel composition $(Kir6/Sur)_4$	Cell expression system	Diazoxide	Pinacidil	P1075	Glibenclamide	5HD	HMR1098	MCC134
Not identified in tissue	Kir6.1 Sur2A	HEK293	NE	A	A	I	NE	NE	ND
Vascular smooth muscle	Kir6.1 Sur2B	HEK293	A	A	A	I	NE	NE	A
Cardiac sarcolemma	Kir6.2 Sur2A	HEK293; Cos1; Xenopus oocytes	NE	A	A	I	NE	I	A
Non-vascular smooth muscles	Kir6.2 Sur2B	HEK293	NE	A	A	I	NE	NE	A
Pancreatic beta-cells	Kir6.2 Sur1	HEK293; Cos1	A	A	A	I	I	I	I
Amphibian retinal glial (Müller) cells	Kir6.1 Sur1	HEK293	A	A	NE	I	I	NE	ND
Mitochondria	Unknown		A	A	NE	I	I	NE	I

Abbreviations: A, activation; I, inhibition; NE, no effect; ND, not determined.
Data from the literature: Gribble *et al*. (1998); Shindo *et al*. (2000); Liu *et al*. (2001); Marban (2002); Eaton *et al*. (2002); Sasaki et al. (2003); Seino and Miki (2003); Singh *et al*. (2003).

systems including components of the respiratory chain. In addition, it was hypothesised that under ischemic conditions when MM Ca^{2+} increases, the ANT itself may also function as a K^+ ion selective channel (Halestrap *et al*., 2002). Pyrophosphate (PPi) is believed to compete for the nucleotide binding sites at the ANT, and, when bound, induces the K^+ conductivity. PPi accumulates in the MM where Ca^{2+} is increased, because Ca^{2+} is a strong inhibitor of the matrix enzyme pyrophosphatase.

The main effect of K^+ entry, by which ever route, seems to be the volume regulation of the MM (Fryer *et al*., 2000; Dos Santos *et al*., 2002; Halestrap *et al*., 2002). K^+ influx induces a moderate MM swelling by 10–40% due to concomitant osmotic water uptake. Two situations may be distinguished. First, in not severely stressed mitochondria, the matrix swelling is small, but the increased K^+ level prevents, or reduces, subsequent swelling during ischemia. This ensures that the mitoPTP protein complex remains functional for high energy-phosphoryl transfer and is not destabilised by space expansion as would occur when the MM shrinks. A further protective effect consists in the slight, but significant, reduction of the $\Delta\Psi m$ when the MM K^+ is increased. The lower $\Delta\Psi m$ reduces the electrogenic driving force for Ca^{2+} uptake through the uniporter during the ischemic period (Murata *et al*., 2001). Taken together, K^+ influx into the MM prevents the mitochondria from turning into a destabilised state prone to mitoPTP opening. Second, in severely stressed mitochondria with high Ca^{2+} overload, shrunken MM volume and expanded MIMS, the mitoPTP is still closed, but its translocator function may be compromised. The mitoPTP protein complex is physically destabilised by the MIMS expansion, and the pore would open as soon as reperfusion should start (Figure 6B). Under these conditions, K^+ influx may still prevent pore opening and restore its proper translocator function by inducing significant swelling of the MM, reduction of the intermembrane space and concomitant stabilisation of the mitoPTP protein complex. Ca^{2+} influx is inhibited by the lower $\Delta\Psi m$, and excess Ca^{2+} leaves the MM via the Na/Ca-exchanger into the cytosol, where the free Ca^{2+} is low, because of APD shortening and membrane hyperpolarisation due to the activation of the sarcoK-ATP channels (Figure 6C).

If the K^+ influx into the MM, stimulated by IPC or PPC, successfully prevented the opening of the mitoPTP, the $\Delta\Psi m$ is also preserved and ready to maintain oxidative energy production upon reoxygenation. Even after a brief opening of the mitoPTP, K^+ influx may be able to preserve the $\Delta\Psi m$ from total collapse, and restore it sufficiently to allow ATP generation during reoxygenation, which by binding to the ANT induces closure of the open pore. The stabilisation of the $\Delta\Psi m$ by K^+ influx represents an important contribution to the cardioprotective potency of IPC and PPC. Without conservation of the $\Delta\Psi m$, cytotoxic amounts of ROS are produced by the uncoupled respiratory chain during reoxygenation (confer Figure 6B and C). However, K^+ influx into the MM can exert the cardioprotective effects only as long as the OMM remains intact. Loss of cytochrome-c through a disrupted OMM causes irreversible respiratory inhibition. Since the molecular identity of the mitoK-ATP channel is not yet established, the question cannot be resolved at present, whether it is physically linked to the mitoPTP protein complex, or whether it exerts its control over the pore opening only indirectly via changes of K^+ ion fluxes and matrix volume.

Cellular cytoprotective K^+-independent mechanisms

More recently it was recognised that many of the KCO as well as some of the inhibitors, affect the myocytes by channel-independent targets (Hanley *et al*., 2002a, b; Dzeja *et al*., 2003). During the ischemia-reperfusion challenge, the adenine nucleotide homeostasis depends critically on the activity of nucleotide-metabolising enzymes, and the oxidative stress relates to the rate of dehydrogenase-catalysed reactions. Protection of mitochondria energetics, attenuation of oxidative damage, and reduction of infarct size can be induced by KCO such as DIAZO, pinacidil and several volatile anesthetics. Some of these effects were shown to be independent of mitoK-ATP channel activation. In fact, several enzymatic targets in the mitochondrial respiratory chain could be identified for the different drugs, which are summarised in Figure 7.

Inhibition of the respiratory chain at different sites potently reduces ROS production during the reoxygenation phase. Conversely, the mitoK-ATP channel inhibitor 5-hydroxydecanoate (5HD) represents a short-chain fatty acid that may serve as substrate for the acyl-CoA synthase. The acyl-CoA ester 5HD-CoA may represent the active form of 5HD. The further metabolism of 5HD-CoA circumvents the inhibitory effect of DIAZO on the succinate dehydrogenase of complex-II, which links the TCAC to the respiratory chain (Figure 7). The increased supply of electrons via ETF (electron transfer factor) to ubiquinone (Q) at the flavoprotein level of acyl-CoA dehydrogenase, indeed, was shown to reverse the DIAZO-induced attenuation of ROS production. Thus 5HD is able to counteract the cytoprotective effects of DIAZO as far as these effects operate via the respiratory enzyme cascade. The PC induced by pinacidil and volatile anesthetics may, in part, also be operating by inhibition of the respiratory complex-I, which is sensitive to 5HD. On the other hand, the KCO nicorandil (a pyridyl nitrate), which can also produce PPC, is known to liberate NO, a potent inhibitor of the complex-IV. As the complex-IV target lies downstream of the electron acceptor Q, the effects of nicorandil should not be completely reversible by 5HD. Carvedilol used in the treatment of congestive heart failure and MI, is a beta1-, beta2- and alpha1-adrenergic receptor blocker with potent antioxidant properties (Oliveira *et al*., 2001). It protects myocardial function from

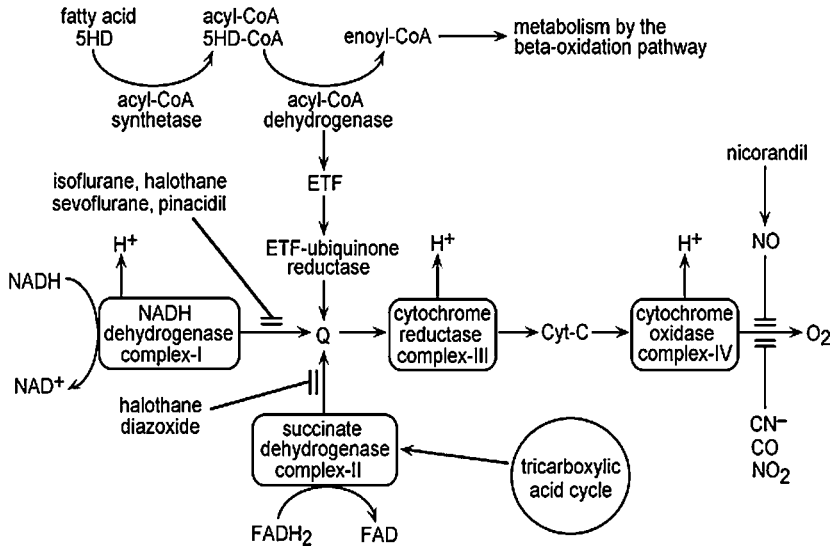

Fig. 7. Scheme with sites of action of drugs and some general inhibitors of the mitochondrial respiratory chain. Lines with blunted ends (⊣) indicate inhibition. Pinacidil and the volatile anesthetics inhibit the NADH:ubiquinone oxidoreductase of complex-I. Halothane also inhibits the succinate dehydrogenase of complex-II. 5HD serves as substrate for the acyl-CoA synthase, and the main product, 5HD-CoA (an acyl-CoA ester) may serve as substrate acyl-CoA dehydrogenase or 5HD-CoA may inhibit this dehydrogenase. Nicorandil inhibits the electron transport via the production of NO at comlex-IV. CN^-, cyanide ion; CO, carbon monoxide; Cyt-C, cytochrome-c; ETF, electron transferring flavoprotein; 5HD, 5-hydroxydecanoate; 5HD-CoA, 5-hydroxydecanoyl-CoA; NO, nitric oxide; NO_2, nitrous oxide; Q, ubiquinone. Discussion in the text (modified from Hanley *et al.*, 2002a, b).

oxidative damage during ischemia-reperfusion by inhibiting the cardiac exogenous NADH dehydrogenase, which is a powerful generator of superoxide radicals located in the IMM. Furthermore, carvedilol also acts as a weak protonophore increasing the permeability of the IMM to protons, but not to K^+, and thus stimulates respiration. Both effects are independent of the mitoK-ATP channel and cannot be blocked by 5HD.

It needs emphasising that these effects on the mitochondrial redox enzyme system occur under therapeutically relevant concentrations. On balance, it seems that PPC depends on both effects on the mitoK-ATP channel (with a distinct molecular identity, or connected to the mitoPTP protein complex) and on channel-independent targets, coupled to the mitochondrial energetics machinery.

ATP-sensitive potassium channels

Subunit organisation of the K-ATP channels

Ligand-gated K-ATP channels belong to the voltage-independent inward rectifying K-channel superfamily with over 60 members (Meneton *et al.*, 1998). K-ATP channels were first discovered in the sarcolemma (sarcoK-ATP channel) of cardiac myocytes (Noma, 1983) and were later found in many other tissues (for reviews see Babenko *et al.*, 1998; Seino, 1999; Seino and Miki, 2003). Beside at the cell surface, K-ATP channels were also found in the mitochondria (Inoue *et al.*, 1991, Paucek *et al.*, 1992; Suzuki *et al.*, 1997). K-ATP channels are thought to be involved in linking the cell metabolic state to the membrane potential, thus regulating a variety of cellular functions including insulin secretion from pancreatic beta-cells, glucose uptake in skeletal muscle, excitability of skeletal and cardiac muscles and neurons, K^+ recycling in renal epithelia and cytoprotection in cardiac and brain ischemia.

The activity of K-ATP channels is mainly regulated by intracellular ATP and ADP, in particular by MgADP and the ATP/ADP ratio (Seino and Miki, 2003). An increase in the ATP/ADP ratio closes, while a decrease of this ratio opens the K-ATP channels. The functional role of K-ATP channels has best been characterised in pancreatic beta-cells (Seino *et al.*, 2000). Increased glucose metabolism in beta-cells induces an increase in cytosolic ATP, which closes the K-ATP channels and depolarises the cell membrane leading to the opening of the voltage-dependent L-type Ca-channels. Consequently, the cytosolic calcium concentration rises and thus triggers exocytosis of the insulin-containing granules.

Beyond the endogenous regulation of the gating mechanism by nucleotides, K-ATP channels can be pharmacologically targeted by both activators and inhibitors. Sulfonylureas such as tolbutamide and glibenclamide, in clinical use for the treatment of non-insulin-dependent diabetes mellitus long before their targets were recognised, stimulate the insulin secretion by closing the K-ATP channels in the pancreatic beta-cells directly. This implies that the sulfonylurea receptor is the K-ATP channel itself, or that it is a regulatory protein associated with the channel. The pancreatic plasma membrane K-ATP channel and its regulatory subunit were first cloned in 1995 (Inagaki *et al.*, 1995; Aguilar-Bryan *et al.*, 1995). This allowed to establish the

K-ATP channel organisation as a 4:4 stoichiometric hetero-octamer comprising two types of subunits, four pore-forming Kir6.0 (either Kir6.1 or Kir6.2) subunits and four regulatory sulfonylurea receptor (Sur) subunits (Sur1 or Sur2), which are structurally not related to one another (Clement *et al*., 1997; Shyng and Nichols, 1997).

The mapping of the Kir6 and Sur genes to human chromosomes showed their pairing, Kir6.2 and Sur1 are both located on the short arm of chromosome-11 (11p15.1) while Kir6.1 and Sur2 are in position 12p11.23 (Inagaki *et al*., 1995a, b). Why these genes are clustered into pairs is unknown, especially since the Kir6.2 subunit appears to pair with both Sur1 or Sur2 subunits expressed in different tissues. It does, however, suggest they may have derived from a fused ancestral gene that has been split and subsequently duplicated (Babenko *et al*., 1998).

The Kir6.0 pore protein belongs to the inward rectifying Kir family with 15 members containing two putative transmembrane domains (TMS) and a conserved H5 region with the sequence Gly-Phe-Gly conferring the specificity for K^+ ion translocation (Meneton *et al*., 1999). In all other pore-forming K-channel subunits the K^+ ion filter depends on the sequence Gly-Tyr-Gly, instead. Four subunits together form a pore. Kir6.0 channels are unique in that they require a structurally unrelated regulatory subunit in order to form a functional channel (Babenko *et al*., 1998). The rat Kir6.1 and Kir6.2 proteins contain 424 and 390 amino acid residues with corresponding molecular weights of 48 and 44 kDa, respectively, and share 71% sequence identity (Seino, 1999). Both N- and C-termini are located at the cytosolic side of the cell membrane. Potential phosphorylation sites for protein kinase-A (PKA) and PKC are located in the intracellular C-terminal region.

Sur1 binds sulfonylurea with high affinity in the nanomolar range while Sur2 exhibits a 500 times lower affinity. The rat Sur1 and Sur2 proteins have 1581 (177 kDa) and 1545 (174 kDa) amino acid residues and share 68% sequence identity (Seino, 1999). Both proteins contain three transmembrane domains, TMD0 with five, TMD1 and TMD2 each with six transmembrane segments. They belong to the ATP cassette protein family with two intracellular nucleotide binding domains (NBD), NFD1 located within the loop between TMD1 and TMD2, and NBD2 located in the C-terminal region. The N-terminus lies extracellularly. The Sur proteins have several extracellular glycosylation sites and several intracellularly located phosphorylation sites for PKA and PKC. Two major splice variants are derived from the Sur2 gene: the originally cloned Sur2, later renamed Sur2A, and Sur2B, which differs by 42 amino acids in the C-terminus from the originally cloned Sur2, later renamed Sur2A. However, Sur2B shares these 42 amino acids with Sur1 (Seino and Miki, 2003). Further minor splice variants of both Sur1 and Sur2 have also been identified. A functional channel may vary in molecular weight between 872 and 900 kDa according to its protein subunit composition, and glycosylation seems to further increase it up to around 950 kDa. However, the intricate interaction between the Kir6.0 channel and the regulatory Sur subunits and their phosphorylation pattern for maximal activity has not yet been unravelled.

Molecular and functional diversity of the K-ATP channels

The functional diversity of the K-ATP channels results from their heteromeric assembly of Kir6.0 and Sur subtypes (Seino and Miki, 2003). The Kir6.1-based channels differ from the Kir6.2 channels mainly by their smaller unitary conductance of around 30 pS as opposed to 70–90 pS. The three main regulatory Sur subunits (Sur1, Sur2A and Sur2B) display in addition to their differing affinity for sulfonylureas (high with Sur1 and low with Sur2A and Sur2B), a rich pharmacological spectrum, which is used for tissue-specific channel characterisation. By heterologous expression of Kir6.0 and Sur subunits in differing combinations, different types of K-ATP channels can be reconstituted with distinct electrophysiological properties and varying nucleotide and pharmacological sensitivities. Table 1 lists the subunit combinations, which were expressed in different cell systems, and their identification by immunohistochemistry or as functional channels in different tissues (literature given in Table 1). Kir6.1/Sur2A was not found in any tissue so far, nor has the molecular identity of the mitoK-ATP channel been established yet. Current activity of surface K-ATP channels can be measured directly by the whole-cell patch-clamp method while the changes of intrinsic flavoprotein (succinate-, glycerol-3-phosphate- and acylCoA-dehydrogenases) fluorescence reflect the redox-state coupled to the mitoK-ATP channel activity (Liu *et al*., 2001).

Table 1 further summarises the pharmacological responses of channel activity assessed in the presence of three channel openers (DIAZO, pinacidil, P1075) and three inhibitors (glibenclamide, 5-hydroxydecanoate (5HD), HMR1098). The inhibitory action of the latter three compounds is usually tested on DIAZO- or pinacidil-induced K-ATP channel activity. Aprikalim (a thioformamide, not listed in the table) is a non-selective KCO. MCC134 derived from aprikalim was first recognised as a vascular relaxing agent (Shindo *et al*., 2000), but was subsequently shown to differently affect channels with either Sur1 or Sur2 subunits. MCC134 activates surface K-ATP channels comprising Sur2A or Sur2B, albeit in combination with either Kir6.1 or Kir6.2, while, at the same time, it inhibits both the pancreatic type channel with Kir6.2/Sur1 as well as the mitoK-ATP of unknown composition (Liu *et al*., 2001; Sasaki *et al*., 2003). The same Sur subunit specificity also applies to 5HD, which inhibits the recombinant Kir6.2/Sur1 channel with similar potency as native mitoK-ATP channels (Liu *et al*., 2001), while leaving the surface channels with either Sur2A or Sur2B

unaffected. 5HD also blocks mitoK-ATP channels reconstituted in liposomes and in isolated mitochondria with similar potency (Jaburek *et al*., 1998). No channel subunit specificity can be attributed to the KCO P1075 since it activates all recombinant combinations but inhibits Kir6.1/Sur1 as well as the native mitoK-ATP channel. The identical pharmacological profile of mitoK-ATP channels tested in isolated cardiomyocytes with various activators and blockers, indicates that the mito K-ATP channel closely resembles the recombinant Kir6.1/Sur1 channel (Table 1).

The Kir6.1 gene is ubiquitously expressed with higher levels in the heart (Inagaki *et al*., 1995a, b) and its protein was localised in skeletal muscle by immunogold staining at the inner mitochondrial membrane (Suzuki *et al*., 1997). Sulfonylureas inhibit non-specifically all types of K-ATP channels by binding to the regulatory Sur subunits. With a fluorescent derivative of glyburide, another sulfonylurea, a 63 kDa protein in brain mitochondria was labeled with high affinity in the nanomolar range, which may represent the mitochondrial Sur subunit (Bajgar *et al*., 2001). Despite its much lower molecular weight than that of the known Sur subunits (around 175 kDa), it seems to contain structural motifs involved in drug and ATP binding similar to those in Sur1. Using a panel of subunit-specific antisera, the channel components Kir6.1 (48 kDa), Kir6.2 (40 kDa) and Sur2A have recently been identified in mitochondria of isolated adult rat ventricular myocytes by confocal microscopy and immunoblotting of subcellular fractions (Singh *et al*., 2003). Kir6.1 seems to be the predominant pore-forming subunit while Kir6.2 is mainly localised in the sarcolemma and only little in the mitochondria. Combinations of these subunits with Sur2A would, however, not explain the reported pharmacology of the mitoK-ATP channel (Liu *et al*., 2001). The low sensitivity to either the activator DIAZO or the inhibitor 5HD conferred on the K-ATP channels by the Sur2A subunit argues against a primary role for the combination Kir6.1/Sur2A in mitochondria. Sur1 with a molecular weight around 175 kDa has not yet been identified in cardiac mitochondria.

The validity of the above considerations rests on the assumption of a one-to-one stoichiometry with one type of Kir6.0 and one type of Sur subunits. However, some reports suggest the hetero-multimerisation of Kir6.1 and Kir6.2 within the same channel complex (Kono *et al*., 2000; Pountney *et al*., 2001; Singh *et al*., 2003) while others do not (Seharaseyon *et al*., 2000). K-ATP channels with distinct isoform compositions thus regulate diverse processes through interaction with the metabolic environment unique for each cell type. Moreover, the co-existence of channels with different subunit composition in one cell (at the surface membrane as opposed to subcellular organelles) would allow for their compartmental distribution with specialised functions. Indeed, K-ATP channels have, beside in mitochondria, also been found associated with the nuclear envelope (Quesada *et al*., 2002).

Ligand binding and regulation of the K-ATP channels

Ligand binding and regulation of the ATP-sensitivity of ther Kir6.0 potassium conductivity has recently been described in detail (for review see Seino and Miki, 2003). While all three Sur subunits bind two nucleotides at their NBD1 and NBD2 sites (Matsuo *et al*., 2000), Kir6.1 and Kir6.2 were also shown to bind one ATP per subunit near its C-terminus causing closure of the pore in the absence of Sur subunits (Tanabe *et al*., 2000; Vanoye *et al*., 2002). Generally, ATP is inhibitory and ADP stimulatory. In the intact hetero-octameric channel the Sur subunits lower the affinity of Kir6.0 to ATP, thus increasing the channel gating activity, when at low ATP/ADP ratio, NBD1 binds ATP and NBD2 binds MgADP. Sur2A has been shown to hydrolyse ATP at NBD2 inducing a conformational transition, which is thought to trigger the activity of the channel (Bienengraeber *et al*., 2000; Zingman *et al*., 2002). Under normal conditions, with cellular ATP far in excess of ADP, MgADP dissociates from NBD2, resulting in release of ATP from NBD1 and closure of the channel (Ueda *et al*., 1999). In this way, K-ATP channels represent enzyme multimers with the regulatory ATPase subunit as intramolecular signaling system for nucleotide-dependent channel gating.

The binding site for sulfonylurea at the Sur subunits is still not yet defined and different Sur subunits confer varying sensitivities to different drugs (Seino and Miki, 2003). For example, Sur1 contains both one sulfonylurea and one benzamido binding site, while Sur2 lacks the sulfonylurea site. On the other hand, glibenclamide comprises both sulfonylurea and benzamido moieties, while tolbutamide has only the sulfonylurea moiety. Nevertheless, Sur2B containing channels are also blocked by tolbutamide indicating that the C-terminal 42 amino acids shared between Sur1 and Sur2B may be involved. These differences could account, in part, for the tissue specificity of the various sulfonylureas. Moreover, the stimulatory effects of the KCO diaxozide and pinacidil on different recombinant channel species vary somewhat in dependence of the cell system used and whether MgADP was present during current measurements. Depletion of cytosolic ATP by addition of dinitrophenol (DNP), which uncouples oxidative phosphorylation in the mitochondria, also enhances the activatory effects of KCO on the surface channels (Sasaki *et al*., 2003). Channel activation is usually more pronounced with MgADP bound to NBD2. The drugs seem to either enhance the ATP hydrolysis at NBD2 or stabilise the MgADP bound state, or both (Moreau *et al*., 2000; Matsushita *et al*., 2002).

In addition to the above mentioned pharmacological profiles of P1075, 5HD, and MCC134 (Table 1), also the agonist diazoxide and the antagonist HMR1098 exhibit specificities useful for channel type characterisation. While DIAZO strongly activates the pancreatic surface channel and the putative mitoK-ATP channel Kir6.1/Sur1, it only weakly stimulates the cardiac sarcolemmal

channel requiring about 2000 times higher concentrations. HMR1098 selectively blocks the cardiac sarcoK-ATP and the pancreatic K-ATP channels. All effects reported in Table 1 are evoked at high potency with dissociation constants between 1 and 50 μM, including the non-specific activation of all channels by pinacidil and non-specific inhibition by glibenclamide.

Is the sarcolemmal K-ATP channel required for PC?

Kir6.2/Sur2A, the cardiac sarcolemmal channel composition (Table 1), regulates the membrane potential according to the cellular metabolic state. It is a weak inward rectifier during diastole and, when open, it stabilises the resting potential and shortens APD. It is expressed at much higher density than other K-channels and it has been suggested to be critically involved in myocardial ischemia and PC (reviewed by Nerbonne *et al*., 2001). Given the high channel density, the APD is expected to shorten by about 50% if sarcoK-ATP channels are activated to only 1% of the maximal conductance. Elevation of the ST-segment of the electrocardiogram (ECG), characteristic of acute ischemia, is presumed to result from dispersion of ventricular repolarisation. In Kir6.2 deficient mice the ST elevation is absent, supporting the hypothesis that it is caused by variable surface K-ATP channel activation and consequent heterogeneities in repolarisation.

The surface channel activity is tightly coupled to cell metabolism via a sequence of key enzyme activities involving creatine kinase (CK), adenylyl kinase (AK) and the muscle form of lactate dehydrogenase (M-LDH) (Carrasco *et al*., 2001; Crawford *et al*., 2002a, b). In fact, M-LDH (but not the cardiac form H-LDH) is physically associated with its N-terminus at the Kir6.2 subunit and with its C-terminus at the Sur2A subunit, while CK primarily interacts with the Sur2A component. Despite high ATP levels in normal cardiomyocytes these two enzymes regulate the sarcoK-ATP channel activity. Under physiological conditions, CK catalyses the production of ATP from creatine phosphate (CP) and ADP, maintaining a high ATP/ADP ratio in the channel microenvironment and keeping the channel closed (ATP on both subunits Kir6.2 and Sur2A). CK promotes Sur-ATPase cycling by removal of ADP from the Sur subunit, thus keeping the channel closed. Under metabolic stress induced by ischemia and/or hypoxia, CP is the first high energy phosphocompound to become lowered. Lowering of the CP/ATP ratio drastically slows the CK phosphoryl transfer activity. These processes promote a drop in ATP synthesis with a concomitant increase in ADP within the channel vicinity resulting in facilitation of channel opening (ADP acting on the Sur2A subunit). AK amplifies the metabolic signal by catalysing the reaction ATP + AMP $\leftrightarrow$ 2ADP. Under stress the mitochondrial ATP synthase consumes cellular ATP and generates ADP, which is delivered to the surface K-ATP channel through a chain of sequential AK-catalysed phosphotransfer reactions. Thus, the complex of the sarcoK-ATP channel with CK, M-LDH, and AK allows for highly regulated transduction of the metabolic status into membrane excitability.

The increased K^+ efflux stabilises the membrane potential at a more negative level and shortens APD resulting in a lower Ca^{2+} influx. This may protect the myocyte against hyperactivity and irreversible cell damage during the ischemia-reperfusion phase. Kir6.2$^{-/-}$ knockout mice display a phenotype of decreased tolerance to stress, reduced exercise capacity, impaired intracellular Ca^{2+} handling, increased susceptibility to fatal arrhythmia under sympathetic stimulation, and failure in developing cardioprotection by preconditioning (Seiko and Miki, 2003; Bienengraeber *et al*., 2003). Kir6.1 deficient mice do not show any of these effects. The myocardial infarct size of wild-type mice with IPC before test ischemia was significantly smaller than in wild-type mice without IPC, while the Kir6.2$^{-/-}$ mice were resistant to the beneficial effects of IPC. Myocytes from animals lacking the surface K-ATP channels have an intact flavoprotein fluorescence response to DIAZO. Treatment with HMR1098, the sarcoK-ATP channel blocker, but not 5HD (specific for blocking the mitoK-ATP channels) produced an impairment of the contractile function (negative inotropy) in wild-type hearts comparable to that of knockout hearts (Suzuki *et al*., 2002). The reduction in contractile function by the KCO pinacidil was equally abrogated in mice lacking Kir6.2.

At face value, these data indicate that the sarcoK-ATP channels are indeed prominent in modulating ischemia-reperfusion injury in the mouse. The rapid heart rate of the mouse (over 600 beats/min) may magnify the relative importance of sarcoK-ATP channels during ischemia, prompting caution in the extrapolation of this conclusion to larger mammals. In larger animals, including humans, with heart beat rates below 200, the sarcoK-ATP channels may not be important for PC. Furthermore, the conclusion hinges on the presumption that ischemic injury is not worse in the knockout than in the wild-type mice. Indeed, this appears not to be the case (Sasaki *et al*., 2003). During ischemia, hearts from the knockouts developed contractures more intensely and more rapidly than those from controls. In addition, functional recovery was much worse in the Kir6.2 knockouts in the absence of any PC stimulus. Thus, knockout of surface K-ATP channels might artefactually enhance ischemic injury and cancel the effect of IPC. This undermines the conclusion that the sarcoK-ATP surface channels are primarily responsible for cardioprotection by IPC. Additionally, it reminds one to exert caution in data interpretation when gene disrupting technology is used for elucidation of functions depending on a complex cellular signaling network. The direct link with metabolism is underscored by the observation that the knockout of M-CK or AK genes (which encode the major cardiac creatine kinase and adenylyl kinase isoforms) impairs proper signal communication to sarcoK-ATP channels, resulting in

abnormal regulation of membrane excitability (Bienengraeber *et al*., 2003). Experimental evidence indicates that activation of the surface K-ATP channels is causally related to ischemia-reperfusion induced arrhythmias and may contribute, among other protective mechanisms, to the delayed phase of cardioprotection.

Is the mitochondrial K-ATP channel playing the main role in PC?

If the mitoK-ATP channel exists as a distinct molecular entity, the answer is yes. The receptor-mediated activation of mitochondrial protective mechanisms via distinct signaling pathways involving mitochondrion-targeted PKC isoforms (section 'Memory, redundancy, and specificity in PC') before the test ischemia, favours the existence of a phosphorylatable channel protein at the mitochondrial membranes. Several KCO similarly affect the sarco- and the mitoK-ATP channels including pinacidil (a cyanoguanidine), cromakalim (a benzopyran) or diethylaminoethylbenzoate. The same holds true for channel inhibitors such as ATP and glibenclamide. Interestingly, some agents display opposite effects, namely activation of the sarcoK- and inactivation of the mitoK-ATP channel, these include ADP, MCC134 and long-chain fatty acid CoA esters (Grover and Garlid, 2000; Liu *et al*., 2001). The pharmacological similarities suggest grossly similar protein structures between the two types of channel. On the other hand, the dissimilarities with certain agents may not be surprising in view of the differences in the Sur subunits, which comprise the pharmacological target sites of the channels; the Sur2A of the sarcoK-ATP channel has a molecular weight of around 175 kDa and the putative Sur subunit of the mitoK-ATP has one of only 63 kDa (see Section 'Subunit organisation of the K-ATP channels').

The different pharmacological properties allowed to differentiate the individual contributions by the sarco- and the mitoK-ATP channels to PC (reviewed by Nakano *et al*., 2000). Using the selective mitoK-ATP channel inhibitor 5HD and activator DIAZO, this channel was shown to be predominantly important for the early phase of PC (Garlid *et al*., 1997; Liu *et al*., 1998). Since inhibition of signaling upstream of the mitoK-ATP channel abolishes subsequent PC, the channel was considered to represent the mitochondrial end effector (Hausenloy *et al*., 2002; Oldenburg *et al*., 2002). However, protection from PC can also be aborted when a mitoK-ATP channel blocker is present only during the lethal ischemic phase, which suggests that the channel can function as mediator, remaining continuously active during ischemia. In addition, activation of mitoK-ATP channels by DIAZO was also shown to induce the liberation of moderate amounts of ROS from the mitochondria. As this effect could be blocked by 5HD, the mitoK-ATP channel may function as signal transducer or as trigger for ROS-induced PC. The ROS production is apparently a direct result of K^+ influx into the MM because the potassium ionophore valinomycin can mimic the DIAZO-induced ROS production. Interestingly, the effect of valinomycin cannot be inhibited by 5HD, which would be expected since valinomycin does not use the mitoK-ATP channel to promote K^+ movement. Thus K^+ influx into MM seems to be the primary mediator for moderate ROS production.

Taken together, the mitoK-ATP channel may serve at the same time as trigger, mediator and end effector, possibly depending on the type and strength of stimulus, and the time point in the PC process. During the test ischemia, a positive feedback loop comprising PKC, mitoK-ATP channel, and ROS seems to be operative (Figure 3). This view does not preclude the possibility that pharmacological KCO may exert additional cytoprotective effects by interference with enzymes of the respiratory chain and other ROS producing enzymes.

Possible clinical relevance of K-ATP channels

An interesting aspect with regard to human infarct susceptibility is the gender-specific difference in expression of the surface Kir6.2/Sur2A channel found in guinea-pigs (Ranki *et al*., 2001). Female hearts comprise at least three times more protein of both channel subunits in Western blots than those of males. The KCO pinacidil evoked an outflow current in isolated myocytes over twice as high in females than in males. Since the single channel properties were almost identical between the two genders, this finding confirms that the higher current density in female myocytes reflects the larger number of functional K-ATP channels in the sarcolemma. Thus IPC, but in particular also pharmacological interventions, which activate the sarcoK-ATP channels, strengthen the resting potential, shorten the APD and consequently prevent the myocyte from Ca^{2+} overloading (see Figure 6B). This ties in with the fact that isolated cardiomyocytes from female hearts are more resistant to ischemia-reperfusion induced Ca^{2+} loading compared with myocytes from male tissue. Bearing in mind the lower plasma levels of estrogen in men of around 4 vs. 8 pg/ml in women, the results from the guinea-pigs would suggest that estrogens afford a cardioprotective effect by induction of a higher sarcoK-ATP channel expression. This concept is supported by the finding that in female guinea-pigs, but not males, ageing is associated with a decrease in number of the surface channels. In humans, a postmenopausal decrease of the sarcoK-ATP channels may be implicated by the observed postmenopausal increase in prevalence of cardiovascular disease in women (Rich-Edwards and Hennekens, 1996; Ranki *et al*., 2002).

In a recent report, adult (aged below 56) and senescent (aged above 64) patients of either gender, undergoing coronary angioplasty were treated with IPC of 120 s coronary occlusion before the surgical intervention (Lee *et al*., 2002). This treatment significantly lowered the subsequent ischemic burden assessed by ST-segment shift, chest pain score, and myocardial lactate extraction

ratios in the adult group, but not in the senescent group. The impaired preconditioning responsiveness in the elderly patients was reversed by nicorandil (KCO) administration at a dose with no nitrate effect, or with an ischemic period lengthened to 180 s. The nicorandil-induced cardioprotection was abolished by co-administration of glibenclamide (unspecific K-ATP channel inhibitor) in both groups. The results suggest that the impaired PC response in the elderly patients was associated with some defects in the signal transduction for activation of the K-ATP channels, either mito- or sarcoK-ATP channels or both.

Anesthetic preconditioning: a paradigm for pharmacological PC

Three groups of anesthetics including volatile anesthetics, opioids, and ethanol-based hypnotics (chloral hydrate, α-chloralose) were found to elicit preconditioning in cardiac tissue (Zaugg *et al*., 2002a, b), and the preconditioning-related characteristics of these agents were recently reviewed (Zaugg *et al*., 2003b). From a clinical point of view, volatile anesthetics and opioids are the most frequently used anesthetics in patients with a great potential to prevent or attenuate perioperative ischemic events in the myocardium (Zaugg *et al*., 2003a). Since volatile anesthetics also precondition endothelial and smooth muscle cells (De Klaver *et al*., 2002), application of these drugs may protect a wide variety of vital organs. This concept has been recently supported by the findings of the first double-blinded, placebo-controlled study on SEVO preconditioning in patients undergoing coronary artery bypass graft surgery (Julier *et al*., 2003). Using biochemical markers, this study demonstrated a significant postoperative myocardial and renal functional improvement in SEVO-preconditioned patients.

APC and IPC share most signaling steps involved in the establishment of the preconditioned state including activation of G-protein coupled receptors (Roscoe *et al*., 2000; Hanouz *et al*., 2002), various types of interacting protein kinases (Toller *et al*., 1999; Uecker *et al*., 2003; Da Silva *et al*., 2003), ROS (Tanaka *et al*., 2003), and the putative end-effectors sarco- and mitoK-ATP channels, the precise sequence of which has not been clarified yet in either type of preconditioning (Figure 8). Nonetheless, recent studies revealed distinct differences in the cardioprotective phenotype between IPC and APC. These comprise a differential translocation and phosphorylation of PKC isoforms (Uecker *et al*., 2003) as well as a distinct activation pattern of mitogen-activated

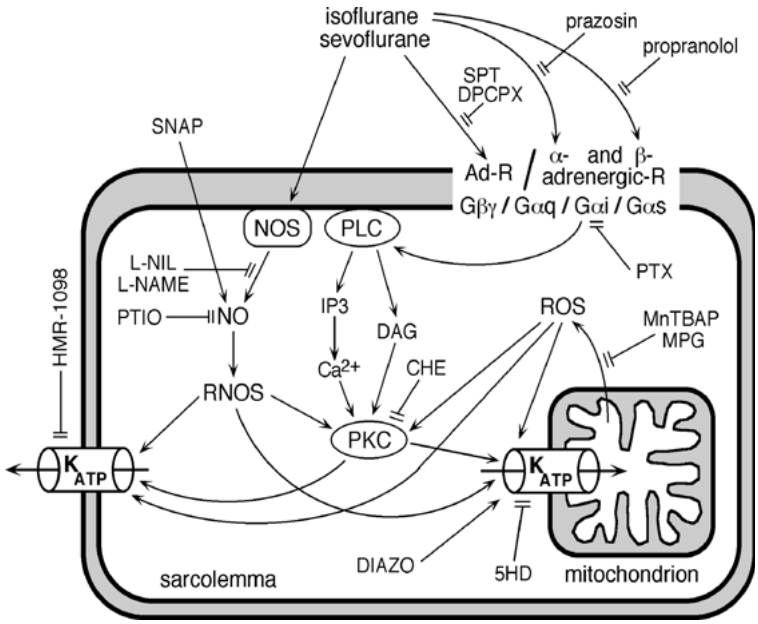

Fig. 8. Simplified scheme of signaling circuits potentially involved in the early phase of preconditioning by activation of the mitoK-ATP channel by volatile anesthetics (VA). Surface G-protein coupled receptors may signal via different classes of G-protein Gαs, Gαi, Gαq, Gβγ. The main routes of activation by VA involve the Gαi-coupled adenosine receptor and the production of nitric oxide (NO), probably by modulation of NO synthase (NOS) activity. These two signaling pathways converge at the level of the protein kinase-C (PKC) although alternative routes for NO via reactive nitrogen oxide species (RNOS) may be operative as well. Lines with blunted ends (⊣) indicate inhibitors used. Most inhibitors partially reduce the myocyte protection by VA. The PKC inhibitor chelerythrine (CHE) and the mitoK-ATP channel blocker 5-hydroxydecanoate (5HD) completely inhibit cardioprotection by VA. The adrenergic receptor blockers prazosin and propranolol, and the sarcoK-ATP channel blocker HMR-1098 have no effect. Diazoxide selectively activates the mitoK-ATP channel and affords cardioprotection. Ad-R, adenosine receptor; DAG, diacylglycerol; DPCPX, specific adenosine-1 receptor blocker; IP3, inositoltrisphosphate; L-NAME, N(G)-nitro-L-arginine methyl ester; L-NIL, L-N6-(1-iminoethyl)lysine; MnTBAP, ROS scavenger; MPG, N-(2-mercaptopropionyl)glycine; PLC, phospholipase-C; PTIO, nitric oxide scavenger; PTX, pertussis toxin; ROS, reactive oxygen species; SNAP, NO donor; SPT, 8-sulfophenyl theophylline. Our results indicate that the adrenergic receptors and the sarcoK-ATP channels may not play a predominant role in VA-induced early PC. Adapted from Zaugg *et al*. (2002b).

protein kinases (Da Silva *et al*., 2003). In addition, trigger-dependent differences in gene expression profiles between APC and IPC were recently detected in our laboratory (unpublished results). As to whether this trigger-dependent transcriptome variability is responsible for the previously reported differences in the memory phase of early and delayed cardioprotection between APC and IPC (Toller *et al*., 1999; Kehl *et al*., 2002; Tonkovic-Capin *et al*., 2002) will need further investigation. Apart from direct preconditioning effects on cardiomyocytes, activation of stretch-dependent gadolinium sensitive ion channels (Piriou *et al*., 2000) as well as inhibition of leukocyte adhesion were further identified as protective mechanisms associated with APC (Laude *et al*., 2002).

Conclusions and outlook

An enormous wealth of facts concerning the cellular mechanisms responsible for IPC and PPC have been published and make it difficult to see the wood for all the trees. We are, however, at an exciting time, which allows some pieces of the puzzle to be put together. IPC can be elicited by so many different primary messengers including adenosine, catecholamines, and peptides and proteins such as BNP and erythropoietin. Corresponding to this variety of primary messengers, the signaling for PC comprises significant redundancy. This redundancy may testify to its very early evolutionary development as a basic adaptive cellular defense against various types of stress. These signals, beside inducing PC, have a multitude of additional effects not only on the heart, but on the whole organism (Figures 4 and 5) including remote PC in other hypoxia-sensitive organs. Specificity in PC signaling is only acquired by recruiting specific PKC isofomrs to structure-related signaling modules at the target sites. The key players in the decision on death or life for the cell, are the mitochondria. All mechanisms for cardioprotection converge on the mitochondrial permeability pore and try to keep this pore closed. Opening of the pore, irreversibly entails cell death. The main component in keeping the pore closed appears to be preservation of the inner mitochondrial membrane potential, and reduction of mitochondrial Ca^{2+}, thus ensuring uninterrupted energy production. This may be achieved by K^+ influx into the mitochondria either through the mitoK-ATP channel or which ever other K^+-selective channel device.

Perhaps the most pressing task is to better characterise the mitoK-ATP channel, to define its molecular identity and its direct physical, or indirect relation to the permeability pore. Furthermore, precise information about protein kinase isoforms and their structural requirements for activation is needed in order to develop new pharmacological strategies to apply PC in patients. A particular rewarding aspect of basic research in the field of PC mechanisms, is its possibility of immediate translation of new discoveries obtained from *in vitro* experiments on isolated cardiomyocytes or on the isolated beating heart, into the clinical setting, particularly the perioperative medicine.

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