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Mitochondrial permeability transition pore and calcium handling

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Abstract

The opening of the a large conductance channel in the inner mitochondrial membrane, known as the mitochondrial permeability transition pore (MPTP) has been shown to be a primary mediator of cell death in heart subjected to ischemia-reperfusion injury. Inhibitors of the MPTP have been shown to reduce cardiac ischemia-reperfusion injury. Furthermore, most cardioprotective strategies appear to reduce ischemic cell death either by reducing the triggers for the opening of the MPTP, such as reducing calcium overload or reactive oxygen species, or by more direction inhibition of the MPTP. This chapter will focus on key issue in the study of the MPTP and provide some methods for measuring MPTP opening in isolated mitochondria.

1. Introduction

The ability of mitochondria to take-up and release calcium is well established. The response of the mitochondria to addition of calcium depends on the amount of calcium added and the level of mitochondrial calcium. Nicholls first reported that mitochondria would buffer extramitochondrial Ca at pCa of ~6.1 until the Ca in the matrix reached a level of ~60nmol Ca/mg protein[1]. He showed that when Ca was added to isolated mitochondria they would accumulate extramitochondrial Ca until they came to this set point and that, when EGTA was added to lower Ca below that set point, mitochondria would release Ca (via a Ca efflux pathway such a Na-Ca or H-Ca exchanger) and return to the same extramitochondrial Ca set point. Ca buffering of the extracellular Ca occurs because at matrix Ca levels above ~4 nmol/mg mitochondrial protein, the Ca efflux pathway is saturated and runs at a constant rate (for rat liver mitochondria this is 5 nmol/min/mg mitochondrial protein) that is independent of matrix or extramitochondrial Ca[1]. When Ca is added outside the mitochondria, the uniporter accumulates Ca until the extramitochondrial Ca falls to a level at which the rate of uptake by the uniporter equals the constant rate of efflux. At this point (which occurs at a pCa of ~6.1 in rat liver mitochondria in the absence of Mg and Na) influx equals efflux and the extramitochondrial Ca is maintained at a constant level. However, this set point only applies when the level of total mitochondrial Ca is above ~4 nmol/mg mitochondrial protein [2]. At levels below this, the efflux pathway is not constant (the Ca efflux pathway is not at Vmax), and a different steady-state extramitochondrial Ca concentration will be achieved. At levels below ~4 nmol/mg protein when Ca is added to mitochondria they begin to take up Ca, the Ca efflux then increases to a new level and eventually a steady-state is achieved where influx and efflux are balanced at a new level and eventually a steady-state is achieved. McCormack et al [3, 4] and others have pointed out that during isolation, mitochondria accumulate Ca and that unless care is taken, the levels of Ca found in isolated mitochondria exceed those that occur in vivo. They further note that at matrix Ca levels above 4 nmol/mg protein, the mitochondrial dehydrogenases are fully activated. Therefore, if the mitochondrial dehydrogenase are regulated by changes in cell and mitochondrial Ca, then it makes sense that the basal, unstimulated matrix Ca level is below a level that fully activates the dehydrogenases.

If sufficient calcium is added to raise matrix calcium out of the physiological range, this leads to a large increase in permeability of the inner mitochondrial membrane which leads to loss of solutes with a molecular weight less than 1.5 kD. This phenomena, termed the mitochondrial permeability transition (MPTP), was described by Haworth and Hunter in 1979 [5–7]. This large conductance was termed the mitochondrial permeability transition pore (MPTP). In an isolated mitochondria preparation, opening of the MPTP leads to swelling of the mitochondria, which is commonly used as a measure of MPTP opening. However, although mitochondrial swelling occurs with isolated mitochondria undergoing MPTP, mitochondrial swelling does not appear to occur in situ because of the high cytosolic level of proteins and solutes (). The MPTP was largely a curiosity only of interest to mitochondrial experts until it was implicated as playing a role in apoptotic and necrotic cell death. Studies in the mid 1980s suggested that inhibition of the MPTP with cyclosporin A would reduce cell dysfunction and death following cardiac ischemia-reperfusion[8]. Indeed, over the ensuing decades, numerous studies have confirmed that inhibition of the MPTP is cardioprotective and the cardioprotective effects of MPTP inhibition have been shown to occur in humans[9].

Although there is considerable interest in the MPTP, the molecular identity of the MPTP remains controversial. Inhibitors of the adenine nucleotide translocator (ANT) can both inhibit and activate the MPTP. Bonkregic acid, which locks ANT in a conformation that faces the matrix (check this), inhibits the MPTP, whereas atractyloside, which inhibits ANT by locking it in a conformation that faces the cytosol, activates the MPTP. Based on these studies and others, ANT was suggested as a possible component of the MPTP. It was speculated that with a high matrix calcium perhaps ANT and the voltage dependent anion channel (VDAC), which come together at contact sites, might adopt an alternative conformation that leads to formation of a large permeability channel (i.e. the MPTP). However, studies in which ANT was genetically ablated showed that MPTP was still present in mitochondria from the liver of these genetically altered mice [10]. Similarly studies in which VDAC was ablated showed that MPTP remained in these genetically altered mice[11]. In contrast, similar studies showed that loss of cyclophilin D resulted in a decrease in sensitivity of the MPTP to activation by calcium or activation during ischemia and reperfusion[12]. These studies strongly supported the concept that cyclophilin D (the binding partner for the MPTP inhibitor, cyclosporin) was an important regulator of the MPTP. It has recently been suggested that the phosphate carrier might be a component of the MPTP, but this will require further validation[13, 14].

In addition to a lack of understanding of the composition of the MPTP, we have little understanding of whether it has any role in cell physiology, or whether it exists only to mediate cell death. The MPTP can function in subconductance states, and it has been suggested that these transient and/or subconductance openings might be important in regulating mitochondrial calcium[15]. The purpose of transient pore opening has not been established, but it has been speculated that it might serve to release matrix Ca [16–18] (Bernardi; Gunter), or perhaps other heavy metal ions. It is generally thought that Ca uptake into the matrix via the Ca uniporter is more rapid than Ca release via the mitochondria NCX. Currently there is controversy as to whether mitochondrial matrix $[Ca^{2+}]$ follows cytosolic Ca^{2+} transients or whether the change in matrix $[Ca^{2+}]$ reflects a more time averaged change in cytosolic $[Ca^{2+}]$. Several studies [19, 20] report that mitochondrial $[Ca^{2+}]$ cycles on a beat to beat basis from ~0.2 μM to 0.9 μM . Others such as Miyata et al [21] suggest that mitochondrial Ca is in the range of 0.1 to 0.2 μM , and increases to a higher steady state as cytosolic $[Ca^{2+}]$ is increased by increasing beating frequency or by addition of isoproterenol. One key issue is whether a mitochondrial release mechanism exists with sufficient time resolution to extrude Ca from the mitochondria on a beat to beat basis. Especially during conditions of elevated cytosolic calcium (such as isoproterenol

stimulation) calcium uptake into the mitochondria might exceed the normal mitochondrial calcium efflux mechanisms. Therefore transient Ca release via MPTP might serve as a Ca release valve[17]. Experimental support for this hypothesis is sparse, likely due in part to the difficulty with measuring small differences in matrix Ca in situ myocytes or cells. Studies by Altschuld et al showed that cyclosporin increased net ^{45}Ca uptake and reduced efflux from myocytes, consistent with inhibition of MPTP being a mechanism for mitochondrial calcium efflux. Ichas et al [18] also reported calcium induced calcium release from mitochondria that was dependent on the MPTP.

A transient opening of the MPTP has been shown to occur in cultured hepatocytes and HMC1 cells[15]. MPTP was measured in these cells by loading mitochondria with calcein-AM, using Co^{2+} to quench cytosolic calcein. This takes advantage of the lack of Co^{2+} entry into mitochondria. Over time the calcein fluorescence in the mitochondria declined and this decline was inhibited by cyclosporin, suggesting that the release of calcein from the mitochondria was by MPTP. Mitochondrial membrane potential was also measured and did not decline over the same time period. These data suggest that the opening of the MPTP and release of calcein was transient and occurred in only a small percentage of mitochondria at any time and therefore did not result in a macroscopic change in mitochondrial membrane potential[15]. These data provide strong evidence that transient MPTP or MPTP flickering occur normally in cells, without apparent detrimental consequences.

During ischemia and reperfusion the MPTP is overwhelmed with high mitochondrial calcium and its normal opening set point may also be altered by ROS generated at the start of reperfusion[22]. In the setting of ischemia and reperfusion, rather than a few mitochondria opening transiently, without detriment to the cell's ability to make ATP, now the majority of the mitochondria in the cell open simultaneously, resulting in a cessation of ATP generation in the cell and increased generation of ROS. This scenario is consistent with the protection afforded by cyclosporin in protocols of ischemia and reperfusion. Low pH inhibits the MPTP and low pH at the start of reperfusion has also been reported to reduce ischemia-reperfusion injury[23]. It has also been suggested that postconditioning protects, at least in part, by maintaining a more acid intracellular pH during early reperfusion[24]. It is also consistent with the reduction in ischemic injury that occurs with interventions that reduce the rise in cell calcium during ischemia and reperfusion. In fact there is considerable data to suggest that preconditioning and many cardioprotective drugs mediate protection at least in part by reducing calcium overload. For example inhibitors of plasma membrane Na-Ca exchange reduce ischemia reperfusion injury and they appear to do so by reducing the rise in cytosolic calcium during ischemia and early reperfusion[25]. Preconditioning has also been shown to reduce the rise in calcium during ischemia[26].

2. Methods for measuring MPTP

2.1 Mitochondrial Swelling Assay

MPTP is commonly measured by following mitochondrial swelling. In the original study by Haworth and Hunter they found that MPTP opening resulted in a conformational change in isolated mitochondria which was accompanied by swelling that can be followed by measuring changes in light scattering. Thus a common and convenient assay for the MPTP is measure changes in light scattering following addition of calcium to mitochondria.

2.2 Calcium uptake

MPTP is also commonly measured by following the ability of mitochondria to take-up and retain calcium. In this assay, isolated mitochondria Ca is added to mitochondria in small

2.2.1 Material—

1. Isolated mitochondria (stock ~ 2 mg/ml; final concentration ~0.25 mg/ml)
2. Calcium uptake assay buffer: 120 mM KCl, 5 mM MOPS at pH 7.4, 5 mM KH₂PO₄, 5 mM glutamate and 5 mM malate.
3. Ca green 5N- tetrapotassium salt
4. Fluorescence spectrometer with stirring capabilities

2.2.2 Method—

1. One ml of calcium assay buffer containing 100 nM final concentration of Ca green 5N is added to the stirred cuvette.
2. Mitochondria at a final concentration of 0.25 mg are added and on addition of the mitochondria the Ca in the buffer will decline because of Ca uptake into the mitochondria. As discussed in the Introduction mitochondrial will reach a set point and the extramitochondrial Ca will stabilize (see figure).
3. Ca is added in successive additions (see figure). The concentration of Ca depends on the source of the mitochondria and it should be adjusted so that multiple Ca additions can be made before the mitochondria release their Ca. Typically additions of 10 μ M, (10 nmol/ml X 0.25 mg/ml = 40 nmol Ca/mg mitochondrial protein) is a good concentration. The concentration of Ca added needs to be adjusted to the mitochondrial concentration. With initial additions of calcium the mitochondria sequester the Ca and return to baseline. As the Ca rises to a level to trigger the MPTP opening, all the mitochondrial Ca is released which leads to saturation of the fluorescent indicator.

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Figure 1.
Successive additions of calcium to isolated mitochondria. See text for details.