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MITOCHONDRIAL PERMEABILITY TRANSITION: A LOOK FROM A DIFFERENT ANGLE

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1.1 REGULATION OF INTRACELLULAR CALCIUM IN NEURONS

Calcium ions occupy an exceptional niche in cell physiology and biochemistry because of their chemical properties and unique distribution across the plasma membrane. Ca²⁺ participates in diverse signaling pathways and regulates activity of various intracellular enzymes. Consequently, Ca2+ plays an important signaling and regulatory role in many different cell types. In neurons, fluctuations in cytosolic Ca²⁺ are involved in a variety of processes, including regulation of mitochondrial bioenergetics (Cardenas et al., 2010; Hansford, 1991). Under pathological conditions (e.g., prolonged activation of glutamate receptors), neurons can experience sustained elevation in cytosolic Ca²⁺, or Ca²⁺ dysregulation, which is dangerous for cells and can lead to neuronal injury (Choi, 1988; Manev et al., 1989). The neuronal injury can occur as a result of activation of degradation enzymes such as calpains, which are Ca²⁺-dependent proteases (Brustovetsky, Bolshakov, and Brustovetsky, 2010; Gerencser et al., 2009; Minger et al., 1998; Siman, Noszek, and Kegerise, 1989), and phospholipases A, (Bonventre, 1997; Kim et al., 1995). Ultimately, sustained elevation in cytosolic Ca²⁺ may lead to neuronal death (Tymianski et al., 1993a, 1993b). Therefore, precise regulation of cytosolic Ca²⁺ is critical for neuronal survival.

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Ca²⁺ can enter neurons via different mechanisms such as transient receptor potential channels, Ca²⁺-permeable AMPA/kainate receptors, and acid-sensing ion channels that may, to some extent, contribute to Ca²⁺ dysregulation and glutamate excitotoxicity (Aarts et al., 2003; Gao et al., 2005; Turetsky et al., 1994). However, there is substantial evidence that the N-methyl-D-aspartate (NMDA)-subtype of ionotropic glutamate receptors (NMDAR) plays a major role in massive Ca²⁺ influx into the neuron exposed to glutamate, and thus, is critical for glutamate-induced Ca²⁺ dysregulation (Tymianski et al., 1993b). In addition, the Na⁺/Ca²⁺ exchanger (NCX), operating in the reverse mode and bringing Ca²⁺ into the cell in exchange for cytosolic Na⁺, also significantly contributes to glutamate-induced Ca²⁺ dysregulation and excitotoxic neuronal death (Brittain et al., 2012; Czyz, Baranauskas, and Kiedrowski, 2002; Dietz, Kiedrowski, and Shuttleworth, 2007; Kiedrowski et al., 1994).

Fortunately, eukaryotic cells, including neurons, have elaborate defense mechanisms against unwanted elevations in cytosolic Ca^{2+} (Guerini, Coletto, and Carafoli, 2005). In neurons, NCX operating in the forward mode (extruding Ca^{2+} in an exchange with external Na⁺) provides the highest transport capacity mechanism for extrusion of excessive Ca^{2+} at the expense of the Na⁺ gradient across the plasma membrane (Annunziato, Pignataro, and Di Renzo, 2004). Consistent with the beneficial role of NCX in maintaining low cytosolic Ca^{2+} , down-regulation of different NCX isoforms (particularly, NCX2, NCX3, and NCKX2) exacerbates ischemic brain damage (Cuomo et al., 2008; Jeffs et al., 2008; Jeon et al., 2007), whereas BHK cells transfected with NCX3 become more resistant to hypoxia-reoxygenation injury (Secondo et al., 2007).

Mitochondrial Ca^{2+} uptake is another major mechanism aimed at lowering cytosolic Ca^{2+} under extreme conditions of Ca^{2+} overflow. Mitochondria possess Ca^{2+} channels in the inner membrane (historically called the "Ca²⁺ uniporter") that mediate Ca^{2+} influx into the organelles driven by high membrane potential across the inner mitochondrial membrane (Baughman et al., 2011; De et al., 2011; Kirichok, Krapivinsky, and Clapham, 2004; Perocchi et al., 2010). This allows mitochondria to accumulate significant amounts of Ca^{2+} and hence defer Ca^{2+} dysregulation in neurons induced by prolonged exposure to glutamate. This large Ca^{2+} uptake capacity of mitochondria stems from formation of biologically inactive Ca-phosphate precipitates in the mitochondrial matrix that was convincingly demonstrated by Brian Andrews' group (Pivovarova et al., 2004).

1.2 CALCIUM OVERLOAD AND MITOCHONDRIAL PERMEABILITY TRANSITION

Although the ability of mitochondria to accumulate Ca^{2+} is truly remarkable, mitochondrial Ca^{2+} uptake capacity is finite and mitochondrial Ca^{2+} overload comes with a hefty price. Under certain circumstances (ischemia-reperfusion, prolonged exposure to glutamate, and age-related neurodegenerative disorders such as Parkinson's, Alzheimer's, and Huntington's diseases), excessive Ca^{2+} accumulation



FIGURE 1.1 Glutamate-induced changes in cytosolic Ca^{2+} ($[Ca^{2+}]_c$) and mitochondrial membrane potential in rat cultured hippocampal neurons. Protection with NIM811. Neurons (12–14 days in vitro [12–14 DIV]) were coloaded with 2.6 µM Fura-2FF-AM and 1.7 µM Rhodamine 123 (Molecular Probes, Eugene, OR) to monitor changes in cytosolic Ca^{2+} and mitochondrial membrane potential, respectively. In **a** and **c**, the individual (*thin grey traces obtained from individual neurons*) and average Fura-2FF ($F_{340}F_{380}$, *thick black traces*) fluorescence signals are shown. In **b** and **d**, the individual (*thin grey traces obtained from individual neurons*) and average Rhodamine 123 (Rh123, F/F₀, *thick black traces*) fluorescence signals are shown. Where indicated, neurons were treated with 25 µM glutamate (Glu, plus 10 µM glycine) for 30 seconds. In **c** and **d**, neurons were preincubated for 45 minutes with 3 µM NIM811. NIM811 (3 µM) was also present in the bath solution during the experiment.

in mitochondria can cause mitochondrial damage manifested in the activation of the mitochondrial permeability transition pore (PTP). The role of mitochondrial damage and permeability transition (PT) induction in disrupting Ca^{2+} homeostasis has been illustrated in our experiments with cultured neurons exposed to excitotoxic glutamate (Fig. 1.1). Multiple short exposures to glutamate induce transient increases in cytosolic Ca^{2+} accompanied by transient mitochondrial depolarizations. With increasing numbers of glutamate exposures, neuronal ability to lower cytosolic Ca^{2+} and restore mitochondrial membrane potential gradually vanished. Inhibition of the PTP with NIM811, a nonimmunosuppressive derivative of cyclosporin A (CsA) (Waldmeier et al., 2002), significantly improved recovery of both cytosolic Ca^{2+} and mitochondrial membrane potential, suggesting an important role for PTP induction in glutamate-induced Ca^{2+} dysregulation and mitochondrial depolarization. Inhibition of PTP



FIGURE 1.2 ⁴⁵Ca accumulation in rat cultured hippocampal neurons exposed to glutamate. NIM811 increases ⁴⁵Ca accumulation in mitochondria in situ. Neurons (12-14 days in vitro [DIV]) were incubated in the standard bath solution supplemented with 5 μ Ci of ⁴⁵Ca. ⁴⁵Ca accumulation was measured at 5 minutes after application of 25 μ M glutamate (Glu) plus 10 μ M glycine, as indicated. Nonspecific ⁴⁵Ca binding was measured in the presence of 10 μ M MK801 (an inhibitor of NMDA receptor) and 10 μ M CNQX (an inhibitor of AMPA/kainate receptors). Mitochondrial ⁴⁵Ca accumulation was evaluated by subtracting the amount of ⁴⁵Ca accumulated in neurons in the presence of 1 μ M FCCP and 1 μ M rotenone (Rot) from the amount of ⁴⁵Ca accumulated for 45 minutes with 3 μ M NIM811 before glutamate exposure. NIM811 (3 μ M) was also present in the bath solution during the experiment. **p*<0.05 comparing neurons with and without NIM811 treatment; ***p*<0.01 comparing neurons with and antagonists of glutamate receptors. Data are mean±SEM, N=3.

and protection of mitochondria in neurons resulted in an increase in mitochondrial Ca^{2+} uptake illustrated in the experiments with the ⁴⁵Ca isotope (Fig. 1.2). Neurons exposed for 5 minutes to 25 μ M glutamate (plus 10 μ M glycine) accumulated significant amounts of ⁴⁵Ca. Mitochondrial depolarization and inhibition of mitochondrial Ca^{2+} uptake with a combination of p-trifluoromethoxyphenylhydrazone (FCCP) and rotenone significantly decreased the amount of ⁴⁵Ca accumulated by neurons. This indicates that mitochondria store about 75% of all Ca^{2+} entering the neuron exposed to glutamate and thus play a pivotal role in regulating cytosolic Ca^{2+}

under stressful conditions. Inhibition of the PTP with NIM811 significantly increased the amount of ${}^{45}Ca^{2+}$ accumulated by mitochondria in neurons exposed to glutamate. Thus, inhibition of the PTP augments Ca²⁺ accumulation in mitochondria, stabilizes mitochondrial membrane potential, and defers Ca²⁺ dysregulation. Induction of the PTP, on the other hand, leads to mitochondrial depolarization, inhibition of ATP synthesis and Ca²⁺ uptake, mitochondrial swelling, and the rupture of the outer mitochondrial membrane. The latter may result in the release of mitochondrial apoptogenic proteins such as cytochrome *c*, Smac/DIABLO, endonuclease G, and AIF. It is not surprising, therefore, that studies of the PTP attract significant attention. Recently, several excellent review papers have been published summarizing current views on PTP structure and function (Bernardi, 2013; Elrod and Molkentin, 2013; Javadov and Kuznetsov, 2013; Siemen and Ziemer, 2013; Vianello et al., 2012). In this chapter, I will discuss only a few selected questions concerning PTP function and structure.

Early observations of PT manifestations were made in the 1950s and 1960s (for review see Bernardi, 2013). However, the concept of mitochondrial permeability transition (mPT) was formulated and introduced by Hunter and Haworth only in the late 1970s (Haworth and Hunter, 1979; Hunter and Haworth, 1979a, 1979b; Hunter, Haworth, and Southard, 1976.). In these papers, Hunter and Haworth used the term Ca^{2+} -induced membrane transition in mitochondria to signify the large increase in inner membrane permeability and the key role of Ca²⁺ in triggering this phenomenon (Hunter and Haworth, 1979a). In their seminal work, Hunter and Haworth demonstrated that the Ca²⁺-induced membrane transition in mitochondria leads to mitochondrial uncoupling, release of previously accumulated Ca²⁺, and mitochondrial swelling. The authors described major fundamental properties of mPT such as the requirement for Ca²⁺ entry into mitochondrial energization (membrane polarization), facilitation by membrane depolarization, and inhibition by H⁺, Mg²⁺, and other cations (Haworth and Hunter, 1979; Hunter and Haworth, 1979a).

An early hypothesis concerning the mechanism of the Ca2+-induced mPT postulated that the increase in inner membrane permeability occurs as a result of activation of mitochondrial Ca2+-dependent phospholipase A, and accumulation of free fatty acids and lysophospholipids that disturb the phospholipid bilayer (Beatrice, Palmer, and Pfeiffer, 1980; Pfeiffer et al., 1979). However, the discovery that CsA inhibits mPT (Crompton, Ellinger, and Costi, 1988; Fournier, Ducet, and Crevat, 1987) but does not inhibit mitochondrial phospholipase A, (Broekemeier, Dempsey, and Pfeiffer, 1989) suggested that induction/activation of a proteinaceous pore in the inner mitochondrial membrane is most likely responsible for mPT. Since then, this hypothesis has dominated the field. Nevertheless, under conditions of massive Ca²⁺ accumulation in mitochondria, CsA inhibits mPT only transiently followed by CsA-insensitive PT, in which activated phospholipase A, may play an important role (Broekemeier and Pfeiffer, 1989). Moreover, palmitate, a free fatty acid that is predominately generated because of phospholipase A, activity, can induce a CsA-insensitive pore in the inner mitochondrial membrane (Sultan and Sokolove, 2001) and thus contribute to Ca2+-induced mitochondrial damage beyond the classic CsA-sensitive mPTP.

1.3 THE MITOCHONDRIAL TRANSITION PORE

1.3.1 Evidence for ANT and VDAC as Components of the PTP

In early studies, Hunter and Haworth (1979–1980) described the inhibitory effect of adenosine diphosphate (ADP) acting on PT at the internal matrix side and opposite effects of atractyloside and bongkrekic acid (Haworth and Hunter, 1980; Hunter and Haworth, 1979a), adenine nucleotide translocator (ANT) inhibitors that stabilize ANT in *c*- (nucleotide binding site facing cytosol) and *m*- (nucleotide binding site facing mitochondrial matrix) conformational states, respectively (Buchanan et al., 1976). In ADP-depleted mitochondria, atractyloside stimulated, whereas bongkrekic acid antagonized, PT (Hunter and Haworth, 1979a). This suggested involvement of ANT in PT. Consistent with this, an earlier study by Asimakis and Sordahl (1977) showed that atractyloside and an endogenous ANT inhibitor, palmitoyl-coenzyme A, induced the release of previously accumulated Ca²⁺ from isolated cardiac mitochondria (Asimakis and Sordahl, 1977), apparently as a result of induction of mPT.

Shortly thereafter, Panov, Filippova, and Lyakhovich (1980) demonstrated that ADP, in a carboxyatractyloside-sensitive manner, inhibited H⁺ and K⁺ permeability in isolated liver mitochondria (Panov et al., 1980). This was interpreted as evidence for ANT being a pathway for H⁺ and K⁺ passive transport across the inner mitochondrial membrane. It has also been proposed that ANT operates as a gated pore (Panov et al., 1980). This is in line with the structural model of the ANT that was proposed soon after where ANT was suggested to operate as a gated pore as well (Klingenberg, 1981). Toninello, Siliprandi, and Siliprandi (1983) showed that ADP and ATP protect mitochondria from Ca²⁺-induced membrane depolarization (Toninello et al., 1983). Bongkrekic acid also protected against depolarization, whereas atractyloside accelerated it. Interestingly, bongkrekic acid facilitated the protective effect of adenine nucleotides while atractyloside abrogated this protection. The authors agreed with the contention of Panov et al. (1980) that ANT, operating as a gating pore, might be responsible for the increase in membrane permeability (Toninello et al., 1983). Le Quoc and Le Quoc (1988) showed that carboxyatractyloside, palmitoyl coenzyme A, and pyridoxal phosphate, membrane-impermeant inhibitors of ANT, stimulated mPT, whereas bongkrekic acid and ADP impeded mPT (Le Quoc and Le Quoc, 1988). These authors also provided evidence suggesting that oxidation of sulfhydryl groups linked to mitochondrial pyridine nucleotide oxidation influenced the conformation of ANT, stabilizing it in its c-conformation (Le Quoc and Le Quoc, 1989). The latter was correlated with increased probability of mPT.

In the late 1980s, it was found that CsA binds to cyclophilin, a peptidyl-prolyl cis-trans isomerase involved in folding of different proteins (Fischer et al., 1989; Takahashi, Hayano, and Suzuki, 1989). Later, it was shown that mammalian mitochondria possess a distinct cyclophilin isoform, cyclophilin D (Connern and Halestrap, 1992). Importantly, oxidative stress facilitates recruitment of mitochondrial cyclophilin D to the inner membrane and promotes mPT (Connern and Halestrap, 1994). Consequently, the effect of CsA on mPT was shown to be mediated by CsA interaction with cyclophilin D (Halestrap and Davidson, 1990; Nicolli et al., 1996).

At the same time, studies of the ANT involvement in induction of mPT continued. In 1990, Halestrap and Davidson showed that Ca^{2+} -induced swelling of heart mitochondria was inhibited by ADP and bongkrekic acid, and the effect of ADP was reversed by carboxyatractyloside, a more potent derivative of atractyloside (Halestrap and Davidson, 1990). The authors suggested that Ca^{2+} interacts with ANT when it is in the *c*-conformation and proposed the model in which mitochondrial cyclophilin interacts with ANT in the presence of Ca^{2+} , leading to mPT. Later, Andrew Halestrap's group (Woodfield et al., 1998) experimentally proved this hypothesis by demonstrating mitochondrial cyclophilin D binding to ANT in the inner mitochondrial membrane as well as to purified ANT. However, these authors failed to find evidence for cyclophilin D binding to mitochondrial porin. Later, James Lechleiter's group (Lin and Lechleiter, 2002) also demonstrated binding of cyclophilin D to ANT.

In contrast to Halestrap's findings, Martin Crompton's group (Crompton, Virji, and Ward, 1998) reported that in their experiments, cyclophilin D binds to a complex of mitochondrial porin (voltage-dependent anion channel [VDAC]) and ANT to form mPTP (Crompton et al., 1998). The authors purified and reconstituted porin, ANT, and the glutathione S-transferase/cyclophilin D fusion protein into phophatidylcholine liposomes loaded with fluorescein. These proteoliposomes were permeabilized in the presence of Ca^{2+} plus P_i , and this permeabilization was inhibited by CsA. Thus, the authors concluded that the mPTP includes porin, ANT, and cyclophilin D, and that it is possibly formed at the contact sites between the outer and the inner mitochondrial membranes.

Currently, cyclophilin D is the only regulatory component of the permeability transition pore that is considered to be proven. In our early study, we found a correlation between augmented expression of cyclophilin D and increased sensitivity to the deleterious action of Ca2+ on mitochondria from the striatum compared to mitochondria isolated from the cortex (Brustovetsky et al., 2003). This suggested an increased propensity to mPTP activation that might underlie increased vulnerability of striatal cells in different neurodegenerative disorders. James Geddes' group (Brown, Sullivan, and Geddes, 2006) reported that synaptic mitochondria have higher cyclophilin D levels, resulting in increased susceptibility to induction of mPT (Naga, Sullivan, and Geddes, 2007). Consistent with this, the decreased sensitivity of brain mitochondria to Ca²⁺-induced mPT compared to liver and heart mitochondria was explained by decreased expression of cyclophilin D (Eliseev et al., 2007). Experiments with cyclophilin D-knockout mice showed that genetic ablation of cyclophilin D suppresses mPT induction and increases Ca2+ uptake capacity in mitochondria two- to threefold (Baines et al., 2005; Basso et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005). Based on these studies, it was postulated that cyclophilin D sensitizes the pore to Ca²⁺ (Basso et al., 2005) and mediates neuronal death after focal cerebral ischemia (Schinzel et al., 2005). Shortly after that, we showed that genetic ablation of cyclophilin D improved Ca^{2+} handling in cultured hippocampal neurons exposed to glutamate and increased their survival rate (Li, Brustovetsky, and Brustovetsky, 2009). However, cyclophilin D does not always lead to increased cell vulnerability. In experiments with HEK293 and rat glioma C6 cells, overexpression of cyclophilin D appeared to be protective against oxidative

stress and staurosporine-induced cell death (Lin and Lechleiter, 2002). This protection depended on the peptidyl-prolyl *cis-trans* isomerase (PPIase) activity of cyclophilin D and was independent from binding to ANT. Thus, depending on experimental conditions and applied stimuli, cyclophilin D can be protective because of its PPIase activity or detrimental as a result of sensitization of the mPTP to Ca^{2+} .

The findings that inhibitors and ligands of ANT can influence the mPT suggested ANT involvement in this phenomenon. However, for a long time it was not clear whether ANT could play the role of the pore or if its role was limited to modulatory action. The answer to this question came from experiments with reconstituted systems. Reinhard Krämer's group (Dierks et al., 1990a; Dierks, Salentin, and Kramer, 1990b) demonstrated that the aspartate/glutamate carrier and ANT modified by SH-reagents can be converted from an obligate exchange mechanism to a unidirectional transport mode, suggesting channel-like activity. Using electrophysiological patch-clamp techniques, this group also demonstrated that a P_i carrier from *Saccharomyces cerevisiae* mitochondria can behave as an ion channel, although with relatively low conductance ranging from 25 to 40 pS in the presence of Ca²⁺ and Mg²⁺ (Herick, Kramer, and Luhring, 1997).

In experiments with ANT reconstituted into the planar bilayer phospholipid membrane, Vladimir Skulachev's group (Tikhonova et al., 1994) detected large ion currents following application of the SH-reagent mersalyl. The channel appeared to be nonselective with multiple sublevels of conductance ranging from 200 pS up to 1.5 nS. This study demonstrated the long-suspected ability of modified ANT to operate as a pore. In studies with purified bovine heart ANT reconstituted into giant proteoliposomes, we found a large nonselective channel with multiple sublevels of conductance ranging from 300 to 600 pS in symmetrical 100 mM KCl (Brustovetsky and Klingenberg, 1996). Based on its behavior, the ANT-associated channel could be easily distinguished from the porin channel (VDAC). The ANT channel opening required high Ca2+ that we proposed to bind to cardiolipin molecules, which are tightly associated with the ANT (Beyer and Klingenberg, 1985). The ANT channel depended on pH and closed at pH 5.2. In addition, the ANT channel could be partly inhibited by bongkrekic acid and completely inhibited by a combination of bongkrekic acid and ADP. Carboxyatractyloside as well as CsA were without effect. The latter is not surprising because cyclophilin D was not present in the proteoliposomes. These experiments were based on an early assumption that ANT operates as a gated channel (Klingenberg, 1981) and showed that ANT indeed could be converted into a large nonselective channel by exposure to high Ca2+. In the next study, we reconstituted recombinant ANT from Neurospora crassa into giant proteoliposomes and found a large nonselective channel with multiple conductance sublevels and an open state conductance of 500 to 700 pS in symmetrical 100 mM KCl (Brustovetsky et al., 2002b). The ANT channel required Ca2+ for activation and could be inhibited by ADP and bongkrekic acid. When purified cyclophilin from Neurospora crassa (a single form of cyclophilin is present in both cytosol and mitochondria [Tropschug et al., 1988]) was present, it stabilized the ANT channel in the open state. CsA antagonized the effect of cyclophilin. Thus, these experiments filled an important gap in our

previous study by demonstrating the stabilizing effect of cyclophilin on the ANT channel open state and CsA-induced inhibition. Overall, the properties of the ANT channel strongly resembled the mPTP. This and other observations concerning ANT involvement in the mPT suggested that the ANT could be a key conducting component of the mPTP. This notion is supported by the crystal structure of ANT (Pebay-Peyroula et al., 2003) that revealed a 30Å deep and 20Å wide depression in the carrier molecule. When the ANT is stabilized in the *c*-conformation by carboxy-atractyloside (CAT), this depression appeared to be open to the cytosolic side, providing the structural basis for pore formation.

Dieter Brdiczka's group (Beutner et al., 1996) reconstituted an ANT-porinhexokinase (or creatine kinase) complex in the planar bilayer phospholipid membrane and detected a large channel with a conductance of 6 nS. The ANT-porin-kinase complex was also reconstituted into phospholipid vesicles loaded with malate or ATP. Ca2+ induced the release of malate and ATP, whereas N-methylVal-4-cyclosporin, a derivative of CsA (Nicolli et al., 1996), inhibited the release of solutes. Based on these observations, the authors concluded that the ANT-porin-kinase complex constitutes the mPTP (Beutner et al., 1996). Although the results of this study to some extent resembled our findings (Brustovetsky and Klingenberg, 1996), there was one significant distinction. The ANT-porin-kinase pore detected in electrophysiological experiments did not require Ca²⁺ to operate as a pore, suggesting that porin interaction with ANT alters ANT channel behavior (although in metabolite efflux experiments, Ca²⁺ was necessary to stimulate malate and ATP leakage) (Beutner et al., 1996). In the subsequent study, Brdiczka's group (Beutner et al., 1998) showed that only ANT-porin-hexokinase but not ANT-porin-creatine kinase complex could be stimulated by Ca2+ to release malate and ATP from proteoliposomes in an N-methylVal-4-cyclosporin-sensitive manner. The authors also found mitochondrial cyclophilin D in the ANT-porin-hexokinase complex that could explain sensitivity to N-methylVal-4-cyclosporin. In the following study, Brdiczka's group (Beutner et al., 1998) showed that ANT purified from rat heart mitochondria and reconstituted into asolectin/cardiolipin vesicles loaded with ATP and malate allowed a release of the trapped compounds in response to increasing concentrations of Ca2+ (Ruck et al., 1998). This Ca2+-induced solute leakage was not sensitive to N-methylVal-4-cyclosporin, but ADP inhibited the leakage. On the other hand, atractyloside and HgCl₂ stimulated release of solutes from ANT-reconstituted liposomes. These results strongly suggested that ANT itself is capable of adopting a porelike conformation under conditions known to induce mPT in mitochondria (Ruck et al., 1998).

Soon after these studies, Guido Kroemer's group (Brenner et al., 2000) reported that, consistent with our observations (Brustovetsky and Klingenberg, 1996), ANT reconstituted in the planar bilayer lipid membrane did not mediate ion currents unless Ca^{2+} or atractyloside are applied. Both Ca^{2+} and atractyloside stimulated ANT-associated channel activity, reaching in symmetrical 100 mM KCl a conductance of 250 and 30 pS, respectively. Thus, numerous experimental findings obtained in different laboratories with isolated mitochondria and reconstituted systems converged at the conclusion that ANT can be converted into a pore by Ca^{2+} or other factors promoting mPT, and therefore, modified ANT could be a conducting component of mPTP.

Although the ANT can be converted into a porelike structure without involvement of other proteins (Brenner et al., 2000; Brustovetsky and Klingenberg, 1996; Ruck et al., 1998), the observations that ANT can bind porin (Beutner et al., 1996) and that the ANT-porin complex binds mitochondrial cyclophilin D (Crompton et al., 1998), suggested a role for porin (VDAC) in the mPTP. In fact, for the first time the idea of VDAC involvement in mPT was introduced in the late 1980s and early 1990s by investigators performing electrophysiological experiments with mitoplasts, which are mitochondria with the outer membrane removed. In 1989, a group led by Mario Zoratti (Petronilli, Szabo, and Zoratti, 1989), using excised patches of the inner membrane, detected a large channel with multiple sublevels of conductance ranging from 30 pS up to 1.3 nS in symmetrical 150 mM KCl. These experiments were performed in the presence of 0.1 mM CaCl₂. At the same time, a similar channel in the inner membrane was found by Kinnally, Campo, and Tedeschi (1989). Interestingly, Ca²⁺ was not present in the solution in this study. Initially, the authors of both papers failed to make a connection between the large channels they observed and the mPTP. However, Szabo and Zoratti (1991) reported that CsA applied from the matrix side could inhibit the large channel detected in the inner mitochondrial membrane. Because it was reminiscent of the Ca2+-dependent CsA-sensitive mPTP, the authors concluded that the channel detected in their study was the mPTP. The molecular identity of this channel remained unknown. At the same time, Kinnally's group (1991) found that Ca²⁺ significantly increases the probability of detection of the large channel in patches of the inner membrane excised from mitoplasts (the authors called it the "multiconductance channel" [MCC]), and although they did not demonstrate CsA sensitivity, the authors discussed this channel in the context of the mPTP. This study showed that MCC activity could be induced by Ca²⁺ interacting with the outer side of the inner membrane, and that application of voltage of both signs (>±60 mV) stimulated channel activity. Both observations appeared to be incompatible with the already known regulation of the mPTP: Ca2+ activates the pore from inside of mitochondria and membrane polarization inhibits the pore (Hunter and Haworth, 1979a). In the next study, Szabo, Bernardi, and Zoratti (1992) showed that the large channel (the authors now called it "megachannel") can be activated by Ca²⁺ and inhibited by other divalent cations. In addition, the authors found that low pH favors the closed state of the megachannel. These observations echoed findings made by Haworth and Hunter (1979) and Bernardi's group (1992) that revealed regulation of the mPTP by H⁺ and divalent cations. In the following paper, Szabo and Zoratti (1992) continued to characterize the megachannel. Here they showed that the nonselective megachannel is activated by Ca²⁺ and inhibited by Mg²⁺, CsA, and ADP. Based on these observations, the authors proposed that the megachannel is indeed the mPTP. Simultaneously, Kinnally's group (Zorov, Kinnally, and Tedeschi, 1992) made significant advances in their studies and reported that the MCC in excised patches from heart inner mitochondrial membrane is also inhibited by CsA. In this study, the authors again reported activation of the MCC by voltage that contrasted with mPTP regulation in mitochondria, where the pore is inhibited by voltage and activated by membrane depolarization (Bernardi, 1992; Hunter and Haworth, 1979a). Zoratti and co-workers (Szabo and Zoratti, 1993) reinvestigated voltage dependence

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of the megachannel and again concluded that the megachannel corresponds to the mPTP, but molecular identity of the pore remained unknown. Based on a comparison of voltage dependence of the megachannel and VDAC, the authors concluded that "a VDAC dimer may be the channel-forming component of the mitochondrial permeability transition pore." In the companion paper, the authors further analyzed electrophysiological properties of VDAC and came to the conclusion that "the PTP may consist of two cooperating VDAC channels, plus presumably an adenine nucleotide carrier dimer and a third component known to be a part of the mitochondrial benzodiazepine receptor" (Szabo, De, and Zoratti, 1993). The latter conclusion was based on the effect of Alpidem, a ligand for the mitochondrial benzodiazepine receptor that elicited ion currents from silent mitoplast patches. Because VDAC is the channel of the outer membrane and the outer membrane with VDAC was found to be present on mitoplasts (Schwaiger Herzog, and Neupert, 1987), the authors hypothesized that the mPTP might be preferentially localized at the contact sites between the outer and the inner membranes (Szabo et al., 1993). However, at that time it was already evident that the mPT is an inner membrane event. Correspondingly, the authors hypothesized that, at the contact sites, some VDAC molecules could be translocated from the outer to the inner membrane (Szabo et al., 1993). In the subsequent study the authors tested this hypothesis by trying to insert VDAC into mitoplast membrane, but they failed to obtain conclusive evidence supporting VDAC insertion (Zoratti, Szabo, and De Pinto,, 1994). Later, Bernardi's group (Krauskopf et al., 2006) showed that in VDAC1 knockout mice (VDAC1^{-/-} mice) the permeability transition in liver mitochondria has the same properties as in mitochondria from wild-type animals. However, mitochondria from VDAC1^{-/-} mice contained VDAC2 and VDAC3 isoforms, and therefore, the authors concluded that this study cannot rule out involvement of VDAC in the permeability transition. Jeffrey Molkentin's group (Baines et al., 2007) showed that mitochondria from VDAC1-, VDAC3-, and VDAC1- to VDAC3-null mice have a Ca2+ and oxidative stress-induced mPT that is indistinguishable from wild-type mitochondria. Moreover, the authors found that the mPT in fibroblast mitochondria lacking all three VDAC isoforms was identical to mPT in wild-type mitochondria. The authors concluded that VDAC is dispensable for mPT (Baines et al., 2007). Consistent with this, in our study with isolated rat brain mitochondria, inhibition of VDAC by König's polyanion (Konig et al., 1982) exacerbated mPT rather than inhibited it, arguing against VDAC involvement (Brustovetsky et al., 2002a).

The stimulatory effect of König's polyanion on mPT (Brustovetsky et al., 2002a) may also illustrate a possible role of membrane surface potential in regulating the inner membrane permeability. It was postulated that making membrane surface potential more negative is associated with facilitated pore opening, whereas making surface potential more positive hinders pore opening (Bernardi, Broekemeier, and Pfeiffer, 1994). Free fatty acid- or König's polyanion-induced stimulation of mPT (Bernardi, et al., 1994; Brustovetsky et al., 2002a; Schonfeld and Bohnensack, 1997; Wieckowski and Wojtczak, 1998; Wieckowski, Brdiczka, and Wojtczak, 2000) illustrates the former case, whereas inhibition with spermine and other polycations (Bernardi et al., 1994; Lapidus and Sokolove, 1992, 1993; Rigobello et al., 1995)

exemplifies the latter case. However, there is an exclusion from this rule that cast some doubt on the surface potential hypothesis: a positively charged ceramide appeared to be a potent inducer of mPT in liver mitochondria and mitochondria from HepG2 cells (Novgorodov et al., 2005). Nevertheless, an interesting assumption was drawn from the surface potential hypothesis: the effects of ANT inhibitors and ligands on mPT might result from changes in surface membrane potential associated with stabilizing ANT in different conformational states rather than from changes in conductance of ANT itself (Bernardi et al., 1994).

Indeed, stabilization of the ANT in c- or m-conformation with atractyloside or bongkrekic acid and ADP, respectively, significantly influenced morphology of isolated bovine heart mitochondria, and it was speculated that the changes in membrane surface potential could be one of the causes of these morphological transformations (Klingenberg, 1985; Lee, Xu, and Colombini, 1996). However, measurements of membrane surface potential were not performed in these studies. This was done by Hagai Rotenberg and Miriam Marbach (1989, 1990). In these studies, the authors found a significant effect of ANT conformation on the apparent surface potential of the matrix side of the inner membrane: locking the ANT in c-conformation with atractyloside increased the positive surface charge density, whereas locking the ANT in the *m*-conformation with ADP and bongkrekic acid greatly increased the negative surface potential (Rottenberg and Marbach, 1990). From this study, however, it remained unclear how changes in ANT conformation could affect surface potential on the outer side of the inner membrane, which is obviously the most susceptible for membrane surface potential modifications by exogenous modulators. Schonfeld and Bohnensack (1997) investigated the mechanism of free fatty acid-mediated stimulation of mPT and concluded that an increase in the negative surface charge by free fatty acids seems to be of minor importance for their ability to promote mPT (Schonfeld and Bohnensack, 1997). Nevertheless, a possible regulation of mPTP by membrane surface potential cannot be ruled out. On the other hand, the observations that membrane surface potential changes occur following locking the ANT in m- or *c*-conformation cannot rule out a scenario in which the ANT can be converted into a large channel and operate as a conducting pathway in the mPTP.

Although over the years the ANT hypothesis received significant experimental support from many independent investigators, some studies produced results that seem difficult to reconcile with the key role of ANT as the mPTP. In early studies, Hunter and Haworth (1979a) found the ADP inhibitory effect on the mPTP is mediated by ADP acting at two different binding sites. One of these sites appeared to be high affinity and atractyloside-sensitive, belonging to the ANT, whereas the other is a low affinity and carboxyatractyloside-insensitive site of unknown molecular identity. Novgorodov et al. (1991, 1992) showed that in liver and heart mitochondria the inhibitory effect of CsA was augmented by low concentrations of ADP in a carboxyatractyloside-sensitive manner. In these studies, however, higher concentrations of ADP in combination with CsA inhibited the pore even in the presence of CAT. Based on these findings, the authors proposed that the pore is probably formed not directly by the ANT, and that another ADP-binding protein with low affinity to ADP

could be involved in the pore induction/activation. Gizatullina et al. (2005) also reported that high ADP (2 mM) could inhibit the pore in the presence of CAT (Gizatullina et al., 2005). In this study, the authors provided some evidence that the low affinity regulatory ADP binding site is located on the outer side of the inner membrane. Halestrap's group (Halestrap, Woodfield, and Connern, 1997) reported that in liver mitochondria, K_i for the inhibitory effect of ADP in the absence and presence of CAT is 1.9 ± 0.4 µM and 27.2 ± 5.2 µM, respectively. The authors speculated that this may reflect the existence of two ADP binding sites of high and low affinity on the ANT, of which the former is sensitive and the latter insensitive to CAT. However, it is possible that CAT-insensitive ADP inhibition of mPTP occurs because of ADP binding to a protein different from the ANT, for example, to mitochondrial Ca²⁺ uniporter that also interacts with ATP and ADP (Litsky and Pfeiffer, 1997) and is required for Ca²⁺ uptake by mitochondria to activate mPTP (Hunter and Haworth, 1979a).

The role of the ANT as the mPTP was also questioned in experiments with mitochondria isolated from embryos of the crustacean Artemia franciscana (Menze et al., 2005). These mitochondria possessed ANT but apparently did not undergo classic large amplitude swelling in response to Ca²⁺, and therefore, appeared to be resistant to classic mPT induction. Later, it was determined that the ANT in Artemia franciscana has a stretch of amino acids in the 198 to 225 region with a significant difference compared to ANT from other species (only 48-56% similarity and deletion of three amino acids at position 211, 212, and 219) (Konrad et al., 2011). This correlated with the unusual effects of ANT ligands: adenine nucleotides decreased, whereas CAT increased Ca^{2+} uptake capacity, and bongkrekic acid failed to influence Ca^{2+} uptake capacity and ADP-ATP exchange. In the authors' view, these findings illuminate a correlation between the lack of sensitivity of the ANT to bongkrekic acid and absence of the classic Ca2+- induced mPT (Konrad et al., 2011). In the following study with mitochondria isolated from brown shrimp (Crangpon crangon) and common prawn (Palaemon serratus), the authors found bongkrekic acid-sensitive adenine nucleotide transport without apparent manifestations of the Ca2+-inducible mPT (Konrad et al., 2012). Mitochondria from these species have a large (but finite) Ca^{2+} uptake capacity, which was not sensitive to CsA and bongkrekic acid and did not undergo swelling measured as a change of light scattering at 660 nm. Thus, despite the sensitivity of the ANT to bongkrekic acid, these mitochondria have increased resistance to Ca²⁺ and do not undergo the classic mPT accompanied by large-amplitude mitochondrial swelling. On the other hand, following a larger Ca²⁺ load these mitochondria become damaged and, similarly to mammalian mitochondria, could not further take up Ca2+. This suggests that these mitochondria can undergo some form of mPT that precludes infinite Ca^{2+} uptake, but this mPT is not accompanied by mitochondrial morphological changes and is not sensitive to classic mPTP inhibitors. Interestingly, mitochondria from Artemia franciscana undergo large amplitude swelling in response to HgCl,, which is also an activator of mPT in mammalian mitochondria (Zoratti and Szabo, 1995), but this swelling is insensitive to CsA (Menze et al., 2005). Overall, these findings cannot rule out a key role of the ANT in the mPT in other species, but they suggest existence of multiple mechanisms underlying this phenomenon.

The ultimate test of the ANT hypothesis became possible with the advent of ANTknockout mice. Doug Wallace's group (Kokoszka et al., 2004) generated mice, lacking both ANT1 and ANT2 in liver mitochondria. In a series of elegant experiments, the authors obtained results that argued against an essential role of ANT in mPT. In these experiments the authors, using a light scattering assay, showed that Ca2+-preloaded liver mitochondria from ANT-knockout mice responded to depolarization with FCCP with the same amplitude of light scattering changes as mitochondria from wild-type animals. In experiments with tetraphenyl phosphonium-sensitive electrodes (used to measure changes in mitochondrial membrane potential), the authors found that mitochondria from ANT-knockout mice required a three times higher Ca²⁺ load to completely lose membrane potential, suggesting a threefold larger Ca²⁺ uptake capacity of ANT-deficient mitochondria. In experiments with hepatocytes from ANTknockout mice, the authors found mitochondrial swelling in response to Ca²⁺ influx into the cell mediated by A23187, a Ca2+-ionophore. Overall, these results were interpreted as strong evidence supporting a nonessential role of ANT as the mPTP. However, the data shown by the authors raise some important questions. First, in experiments with light scattering measurements (see Figure 3a,b in Kokoszka et al., 2004) it is not clear why the initial slow decline in light scattering is much steeper with ANT-deficient mitochondria than with mitochondria from wild-type animals. If all conditions were the same, this slope should be the same for both types of mitochondria (for comparison, see Figure 2A in Brustovetsky Shalbuyeva, and Brustovetsky, 2005). Second, it is not clear why the fast drop in light scattering in response to FCCP is much larger with ANT-deficient mitochondria than with mitochondria from wildtype animals (Figure 3a,b in Kokoszka et al., 2004). In fact, this fast light scattering response to FCCP is most likely an artifact produced by addition of clear, ethanolbased FCCP solution to the turbid mitochondrial suspension. A similar initial response to ethanol was shown by other investigators (see Figure 2A in Yamada et al., 2009). If all conditions were the same, the fast response to FCCP should have the same amplitude. Third, the authors claim that A23187 induces mPT in ANT-deficient hepatocyte mitochondria, resulting in mitochondrial swelling. However, on the electron micrograph the ANT-deficient mitochondria look enlarged but not swollen. On electron micrographs, swollen mitochondria are characterized not only by an increase in size, but most importantly by decreased electron density (increased transparency) of the matrix (for comparison, see Guerrieri et al., 2002). This was not the case in the experiment shown by the authors in Figure 4a (Kokoszka et al., 2004). Moreover, on the micrograph that shows ionophore-treated hepatocytes with enlarged ANTdeficient mitochondria, the black inclusions of unidentified nature appeared to be proportionally enlarged compared to similar inclusions on the micrograph with wildtype hepatocytes. Fourth, it is not clear to what extent the conclusions from this study with liver mitochondria are applicable to mitochondria from other tissues. For example, there is a report that skeletal muscle mitochondria, lacking ANT, require eight times larger Ca2+ load to induce mPT (Panov, Andreeva, and Greenamyre, 2004). Such greatly increased resistance to Ca²⁺ hardly can be viewed as nonessential. How much Ca²⁺ would be necessary to induce permeability transition in brain mitochondria lacking ANT remains unknown. However, it is known that ANT1 deficiency in

mitochondria of cortical and hippocampal neurons makes these cells much more resistant to glutamate excitotoxicity (Lee, Schriner, and Wallace, 2009), in which mPT plays an important role (Li et al., 2009; Schinder et al., 1996; White and Reynolds, 1996). Thus, the results reported by Wallace's group (Kokoszka et al., 2004) raise some concerns about the methodologies used and the interpretation of results. Until these concerns are resolved, these results hardly can be considered as unequivocal evidence against an essential role for ANT in forming the mPTP.

1.3.2 Alternative Hypotheses of mPTP Composition

In recent years, new hypotheses regarding composition of the PTP have emerged. Andrew Halestrap (Leung, Varanyuwatana, and Halestrap, 2008) proposed that a P. transporter in the inner mitochondrial membrane could be a conducting pathway that forms mPTP. This is in line with findings made by Reinhard Krämer's group (Schroers, Kramer, and Wohlrab, 1997) that a P₁ transporter can be converted into a channel-like structure following treatment with mercurials. In another recent study, investigators provided evidence suggesting that the c-subunit of F_0 complex in F_0F_1 -ATP synthase could be involved in mPTP functioning (Bonora et al., 2013). Consistent with this, Tamara Azarashvili's group (2002) found decreased phosphorylation of the 3.5 kDa peptide related to subunit c of F_0F_1 -ATP synthase following induction of the permeability transition by Ca²⁺. Incorporation of this peptide into a planar bilayer membrane resulted in increased membrane conductivity as a result of channel activity that was inhibited by antibodies to subunit c. However, in contrast to ANT that under normal conditions translocates bulk ADP³⁻ and ATP⁴⁻ anions with molecular weights of around 500 Da (Klingenberg, 2008), a single subunit c provides only the H⁺ translocating pathway. Therefore, it is not clear whether subunit c of the F_0 component of the F_0F_1 -ATP synthase could be converted into mPTP, permeable for solutes with molecular weights up to 1 to 1.5 kDa (Brustovetsky and Dubinsky, 2000; Haworth and Hunter, 1979). Interestingly, ion currents mediated by subunit c incorporated into a lipid bilayer were recorded in previous studies (McGeoch et al., 2000). However, these currents were found to be activated by cyclic guanosine monophosphate (cGMP) that does not induce the mPTP, and surprisingly, suppressed by Ca²⁺ that is the major activator of the mPTP.

In another recent study, it was shown that mitochondrial cyclophilin D interacts with the lateral stalk subunits of F_0F_1 -ATP synthase, including oligomycin-sensitivity conferring protein (OSCP), subunits *b* and *d* (Giorgio et al., 2009). This binding required P_i and partially inhibited F_0F_1 -ATP synthase activity. CsA disrupted this interaction and increased F_0F_1 -ATP synthase activity (Giorgio et al., 2009). The authors showed that mouse liver mitochondria energized by respiration in the presence of stable levels of ADP maintained by hexokinase plus glucose, an ATPconsuming system, had lower Ca^{2+} uptake capacity than mitochondria energized by ATP hydrolysis in the presence of stable levels of ATP maintained by creatine kinase plus phosphocreatine, an ATP-generating system (Giorgio et al., 2013). Based on these observations, the authors concluded that the catalytic activity of F_0F_1 -ATP synthase (synthesis versus hydrolysis) affects the Ca^{2+} sensitivity of the mPTP. Interestingly, in these experiments lower Ca²⁺ uptake capacity was observed with respiring mitochondria, whereas mitochondria lacking respiratory substrates, and therefore, having negligible respiratory activity, had higher Ca²⁺ uptake capacity. It is also notable that Ca²⁺ uptake capacity in mitochondria energized by ATP hydrolysis was significantly diminished when their respiration was activated by adding respiratory substrates. The reason for that was not provided but a higher level of reactive oxygen species (ROS) production in respiring mitochondria might contribute to this difference.

In the experiments with digitonin-permeabilized HQB17 cells incubated with ATP and an ATP-regenerating system (phosphocreatine and creatine kinase in the absence of respiratory substrates), the investigators found that OSCP knockdown slightly increased mitochondrial Ca^{2+} uptake capacity (by about 30%) (Giorgio et al., 2013). This slight increase in Ca^{2+} uptake capacity was interpreted as evidence for F_0F_1 -ATP synthase involvement in mPTP formation. However, it is also conceivable that OSCP down-regulation could affect F_0F_1 -ATP synthase activity and increase ADP/ATP ratio, resulting in a slight increase in mitochondrial Ca^{2+} uptake capacity. Indeed, in the presence of oligomycin mitochondria accumulated about 25% more Ca^{2+} when incubated with ADP compared to ATP (Giorgio et al., 2013).

Obviously, the most remarkable finding in this study is that dimers of F₀F₁-ATP synthase can form a large channel in the planar lipid bilayer (Giorgio et al., 2013). This channel was Ca²⁺-dependent, could be inhibited by ADP and Mg²⁺, and was insensitive to CsA and ANT inhibitors. To some extent this channel resembled the megachannel described in other studies and attributed to the mPTP (Petronilli et al., 1989; Szabo and Zoratti, 1991; Szabo et al., 1992). Further, it was proposed that this mPTP-like channel is formed at the membrane interface between two adjacent F_o complexes (Bernardi, 2013). Importantly, the channel formed by F₀F₁-ATP synthase dimers required activation by benzodiazepine-423 (Bz-423), whereas the mPTP induction in mitochondria or Ca2+-activated conversion of the reconstituted ANT do not require this agent. This could be explained by the fact that both Bz-423 and CyD bind to OSCP (Giorgio et al., 2009; Johnson et al., 2005), and according to the hypothesis proposed by Giorgi et al. (2013), this binding favors opening of the large channel formed by F₀F₁-ATP synthase dimers. Although the large channel formed by dimers of reconstituted F₀F₁-ATP synthase resembles the megachannel described previously and linked to the PTP (Petronilli et al., 1989; Szabo and Zoratti, 1991; Szabo et al., 1992), the hypothesis that this F₀F₁-ATP synthase conversion into the pore takes place in mitochondria needs additional experimental proof.

In this regard, interesting findings pertinent to the possible role of F_0F_1 -ATP synthase in the PTP were reported by Vladimir Skulachev's group (Shchepina et al., 2002). They found that CsA and oligomycin, but not aurovertin B, suppress TNF α induced cell death in HeLa cells (Shchepina et al., 2002). The inhibitory effect of oligomycin on the mPTP is usually attributed to the change in ADP/ATP ratio. ADP is more effective in inhibition of the mPTP than ATP (Novgorodov et al., 1992). In rat liver mitochondria, oligomycin causes an almost 20-fold increase in ADP/ATP ratio: from 0.15±0.05 to 2.82±0.99, without a change in total concentration of adenine nucleotides (Novgorodov et al., 1994). Both oligomycin and aurovertin B inhibit ATP synthase, but oligomycin interacts with F_0 , whereas aurovertin interacts



FIGURE 1.3 Oligomycin failed to inhibit Ca2+-induced permeability transition in isolated nonsynaptic brain mitochondria. Isolated Percoll gradient-purified rat brain mitochondria were incubated at 37° C under continuous stirring in the incubation medium containing 210 mM mannitol, 50 mM sucrose, 3 mM succinate, 3 mM glutamate, 3 mM KH₂PO₄, 0.5 mM MgCl., 10 mM EGTA, 0.1% BSA (free from fatty acids), 10 mM Hepes, pH 7.4. In a and b, mitochondrial swelling (thick traces) was evaluated by following changes in light scattering of mitochondrial suspension at 525 nm with an incident light beam under 180° C in a 0.3-ml chamber at 37° C and continuous stirring. Simultaneously, $\Delta \psi$ was monitored by following the distribution of tetraphenylphosphonium cation (TPP⁺) between the external medium (initially 1.8 µM TPP⁺-Cl) and the mitochondrial matrix with a TPP⁺-sensitive electrode (Kamo et al., 1979). Where indicated, 2.4 µmol Ca²⁺/mg protein and 1 µM oligomycin were added. In c, recording of mitochondrial oxygen consumption, with oligomycin inhibiting ADP-stimulated respiration, is shown as a positive control. Additions: ADP, 300 µM; oligomycin, 1 µM; 2,4-dinitrophenol (DNP), 60 µM. Mitochondrial respiration was recorded in the 0.3-ml incubation chamber equipped with a Clark-type oxygen electrode and a tightly closed lid. The slope of the O₂ electrode trace corresponds to the respiration rate.



FIGURE 1.4 Ca²⁺ uptake capacity of isolated nonsynaptic brain mitochondria. The effects of oligomycin (Oligo), adenosine diphosphate (ADP), carboxyatractyloside (CAT), bovine

with the F₁ complex of ATP synthase. The mPTP was implicated in cell death induced by TNFa (Shchepina et al., 2002), and it was shown previously that application of oligomycin to isolated rat liver mitochondria inhibits the pore (Novgorodov et al., 1990). Correspondingly, the authors speculated that the protective effect of oligomycin is related to its interaction with the oligomycin-sensitive subunit of F₀ and inhibition of the pore (Shchepina et al., 2002). However, in our experiments with isolated, Percoll gradient-purified brain mitochondria oligomycin failed to inhibit Ca2+induced permeability transition (Fig. 1.3), suggesting that either F₀ does not form a pore in brain mitochondria or oligomycin does not inhibit the pore formed by F₀. In addition, oligomycin failed to increase Ca2+ uptake capacity of brain mitochondria (Fig. 1.4a,b), whereas 100 µM ADP augmented Ca²⁺ accumulation (Fig. 1.4c) and this ADP effect was abolished by 20 µM CAT (Fig. 1.4d). Bovine serum albumin (BSA, 0.1%, free from fatty acids) significantly increased Ca²⁺ uptake capacity of mitochondria incubated with oligomycin and ADP (Fig. 1.4e). CsA further increased Ca^{2+} accumulation in mitochondria (Fig. 1.4f). Thus, the known inhibitors of the mPTP, ADP, BSA, and CsA, increase Ca²⁺ uptake capacity of brain mitochondria, while oligomycin does not.

Taken together, all these observations do not disprove a key role of ANT in the mPT. Consequently, all these data cannot rule out a pore-forming function of ANT in the mPTP. However, the recent data strongly suggest a multiplicity of the mechanisms underlying mPTP. This idea has already been introduced by Zoratti Szabo, and De Marchi (2005) in their excellent review paper on the mPTP and by Martin Klingenberg (2008), a world-renowned expert on ANT, in his brilliant review paper on ANT and adenine nucleotide transport in mitochondria. It seems likely that the multiplicity of mechanisms contributing to mPT is a key to answering many smoldering questions about structure, function, and the pharmacological profile of the mPTP. Consequently, accepting the idea that multiple mechanisms underlie the mPT may help to better understand the complex nature of this phenomenon and resolve many controversial issues concerning molecular identity of the mPTP. Future studies will show whether diversity of the mechanisms underlying the mPTP is a viable idea.

FIGURE 1.4 (*Continued*) serum albumin (BSA), and cyclosporin A (CsA). Mitochondria were incubated at 37° C in 0.3-ml thermostatically regulated chamber under continuous stirring. Ca²⁺ uptake by mitochondria was followed by measuring Ca²⁺ concentration in the incubation medium ($[Ca^{2+}]_{ext}$) with a miniature, custom-made Ca²⁺-selective electrode. The standard incubation medium contained 125 mM KCl, 0.5 mM MgCl₂, 3 mM KH₂PO₄, 10 mM Hepes, pH 7.4, 10 μ M EGTA, 3 mM succinate, and 3 mM glutamate. Where indicated, 10 μ M Ca²⁺ pulses were applied. In **a**, mitochondria were incubated in the standard incubation medium without other additions. In **b**, the incubation medium was supplemented with 1 μ M oligomycin (Oligo); in **c**, with 1 μ M oligomycin and 100 μ M ADP; in **d**, with 1 μ M oligomycin, 100 μ M ADP, and 0.1% BSA (free from fatty acids), in **f**, with 1 μ M oligomycin A (CsA). Time bar in **f** is applicable to all panels.

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