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Pharmacological screening and enzymatic assays for apoptosis

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1. ABSTRACT

Mitochondria play a central role in the intrinsic pathway of apoptosis. In response to many pro-apoptotic stimuli, mitochondria undergo an irreversible process called mitochondrial membrane permeabilization (MMP). The detection of MMP in isolated mitochondria is most often based on assays that monitor either the loss of the inner transmembrane potential ($\Delta\Psi m$; classically with Rhodamine 123), permeability transition (PT, cyclosporin A-sensitive matrix swelling), or the release of critical pro-apoptotic intermembrane space effectors. To gain complementary information on MMP mechanisms, we have systematically used three additional assays optimized for the 96-well microplate format: (1) inner membrane permeability, (2) VDAC-associated NADH reductase activity, and (3) ATP/ADP translocase activity. We report that ad hoc combinations of ANT and VDAC ligands, carbonyl cyanide m-chlorophenylhydrazone (CCCP), mastoparan and Vpr_{52-96} peptide and PT inhibitors, permit to explore relationships between enzymatic functions of sessile mitochondrial proteins (i.e. ANT, VDAC) and MMP. These assays should be useful tools to investigate mitochondrial apoptosis, decipher the implication of inner and outer membrane permeabilization and provide a multi-parametric approach for drug discovery.

2. INTRODUCTION

Apoptosis or programmed cell death has been implicated in metazoan development, as well as during the whole adult life to control cell size population and/or eliminate mutated and surnumerary cells (1). In addition, apoptosis deregulation may lead to diverse severe pathological conditions. For instance, an excess of cell death is observed in neurodegenerative diseases, in ischemia-reperfusion and acute intoxication. In contrast, a defect in apoptosis has been associated with autoimmune diseases and cancer, which are characterized by an uncontrolled cell multiplication (2). In viral infection, host cell apoptosis can be down-regulated in the early phase of infection to promote virus multiplication, whereas apoptosis can be up-regulated at the end of viral cycle to favor virus spreading (3).

Mitochondrial membrane permeabilization (MMP) is a central process in (programmed) cell death pathways and is regulated by Bcl-2 family members via multiple molecular mechanisms (4). Thus, mitochondria collect numerous apoptosis signals, emanating from the extracellular environment (e.g. radiations, growth factors, toxins, death receptors ligands, anoxia) or from the intracellular milieu and other organelles (e.g. calcium

(Ca^{2+}), reactive oxygen species (ROS), Bax, p53, caspases, kinases), integrate them and coordinate the caspase-dependent and caspase-independent degradation steps of apoptosis (5). MMP is currently considered as a point of no return in the apoptosis signaling cascade. As a consequence, MMP modulation has been proposed as a therapeutic goal and the development of robust cellular and subcellular assays to identify biomarkers, therapeutic targets and novel therapeutic drugs is currently a major issue for numerous researchers (2, 6-8).

MMP can be divided into several coordinated steps. These steps can occur concomitantly, sequentially, and depend of the cell type and the apoptosis stimulus. Briefly, MMP can affect only the outer membrane or both mitochondrial membranes, i.e. outer and inner membranes (OM and IM), manisfesting by the so-called mitochondrial permeability transition (PT). In most cases, MMP is accompanied by a loss of transmembrane inner potential ($\Delta\Psi m$ loss) (9), an OPA-1-dependent cristae remodeling (10) and a colloidosmotic matrix swelling due to the massive entry of water and solutes (MM<1.5.kDa) trough the irreversible opening of a megachannel, namely the permeability transition pore complex (PTPC) (11, 12). PTPC is a multi-component pore, whose protein composition appeared to vary with the tissues and the condition (e.g. stress, pathology…) (1). Main components would be the voltage-dependent anion channel (VDAC) in the OM and the adenine nucleotide translocase (ANT), in the IM, and cyclophilin D (CypD), whereas other IM components are not excluded (13). Briefly, ANT and VDAC have been proposed as central mediators and/or regulators in MMP in cooperation with pro-apoptotic members of the Bcl-2 family, such as Bax, Bid and Bak (14-17). The striking consequence of membrane permeabilization is the release into the cytosol of a set of proteins/factors that are normally confined into the intermembrane space (18). This applies to the cytochrome *c* *(*19), the apoptosis-inducing factor (AIF) (20), caspases (21) and caspase activators such Smac/DIABLO (22). It is noteworthy that some genetic studies in rodents and yeast support an implication of ANT, VDAC or CypD in apoptotic and/or necrotic cell death, whereas other studies argue against their requirement (23-27), suggesting that modalities of cell death in specific models must be carefully considered.

Isolated mitochondria have been instrumental to elucidate the molecular events and the regulation of MMP. Detection of MMP in isolated mitochondria, is most often based on the measurement of the loss of the inner transmembrane potential ($\Delta\Psi m$; classically with tetramethylrhodamine ethyl ester (TMRM) or Rhodamine 123), the induction of permeability transition (PT) and of matrix volume changes (cyclosporin A-sensitive matrix swelling by optical density changes), or the release of critical pro-apoptotic intermembrane space effectors into the cytosol (e.g. cytochrome c presence into the mitochondrial supernatant) (28-33). Although there is no doubt on the implication of the OM into MMP, the role of the IM is still debated. Therefore, to gain more insights into MMP mechanisms, and study concomitantly apoptotic and modulation of vital functions of VDAC and ANT during MMP induction, we developed a series of coupled enzymatic assays into the 96-well format. Thus, in addition to swelling and depolarization assays, we measured (1) inner membrane permeability to Acetyl CoA, (2) VDAC-associated NADH reductase activity, and (3) ATP/ADP translocase activity in response to various MMP modulators such as calcium, the prototypic PT inducer, carbonyl cyanide m-chlorophenylhydrazone (CCCP), a protonophore, and two pro-apoptotic peptides known to trigger IM permeabilization, mastoparan (MP), a wasp venom peptide (34), and Vpr $_{52-96}$, the C-terminal moiety of the viral protein R (Vpr) from HIV-1 (35, 36). Here, we show that these various molecules may have different effects, target various mitochondrial membrane proteins and elicit various MMP mechanisms.

3. MATERIAL AND METHODS

When not indicated, chemicals were from Sigma (St Louis, MO). All assays were performed in 96-well plates (200µl) and were performed at 37°C in a spectrofluorimeter (TECAN genios, TECAN, Austria). For absorbance and optical density measurement, transparent microplates were used and for fluorescent measurement, black microplates were used.

3.1. Isolation of mouse liver mitochondria

Mitochondria were isolated from mouse liver (C57BL/6J, female, 6-12 week old, Charles River, France) by differential centrifugation and purified on Percoll gradient according to (37). Protein concentration was determined using the micro-BCA assay (Pierce, Illinois).

3.2. Swelling and depolarization assays

For swelling and depolarization measurements, mitochondria (25µg of proteins/200µl) were diluted in a hypo-osmotic buffer (10mM Tris-Mops, pH 7.4., 5mM succinate, 200mM sucrose, 1mM Pi, 10µM EGTA, 2µM rotenone) containing or not various doses of agents such as Ca^{2+}, CCCP, Vpr_{52-96} and MP. The mitochondrial swelling was immediately measured by the decrease in optical density at 540nm for 1800sec at 37°C. Several pharmacological inhibitors, such as cyclosporin A (CsA), which interferes with the ANT-Cyp D binding (38), bongkrekic acid (BA) and carboxyatractyloside (CAT), which inhibits ANT translocase function (39, 40) and 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid (DIDS), which targets VDAC (41), were added to mitochondrial suspension concomitantly with the inducer or not. Similarly, the depolarization of the mitochondria was measured by the rhodamine 123 (1µM) fluorescence dequenching assay (λexc: 485nm, λem: 535nm, Molecular Probes) (42).

3.3. Inner membrane permeabilization assay

In brief, mitochondria (25µg protein/200µl), were treated or not with the agents for 30min at 37°C to stimulate the swelling as described (43). Thereafter, 100µM 5,5'-Dithio-bis 2-nitrobenzoic acid (DTNB), 300µM acetylCoA and 1mM oxaloacetate were added sequentially

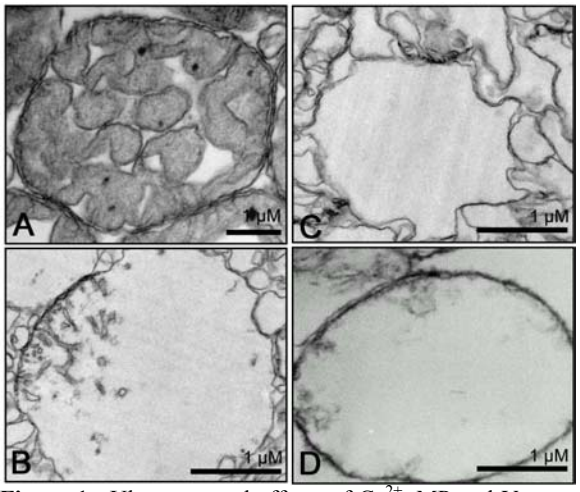

Figure 1. Ultrastructural effects of Ca^{2+}, MP and Vpr_{52-96} on mouse liver isolated mitochondria. Isolated mitochondria were incubated for 15 or 30 min in absence (A) or presence of 25µM Ca^{2+} (B), 2µM MP (C), 3µM Vpr_{52-96} (D)*. Then, their morphology was studied by transmission electronic microscopy (TEM). Magnitude: x 20 000. * Reproduced with permission from (36).

every 10min and the absorbance at 415nm was recorded for 3000sec. Above cited molecules were used.

3.4. NADH ferricyanide assay of VDAC activity

For NADH ferricyanide activity of VDAC, mitochondria (10µg of proteins/200µl) were equilibrated for 10min at 37°C in the same hypo-osmotic buffer in the presence of molecules or not and then, 250µM NADH were added. After 15min of incubation, addition of 300µM ferricyanide was used to start the measurement of the fluorescence (λexc = 360nm; λem = 465nm). The protocol was adapted from (41). Above cited molecules were used.

3.5. ADP/ATP translocase assay

The ADP/ATP exchange rate was then evaluated on 25µg mitochondrial proteins in 200µl of buffer (20mM Hepes, pH 7.2., 5mM succinate, 300mM sucrose, 10mM KCl, 1mM $MgCl_2$, 1mM Pi, 10µM EGTA, 2µM rotenone) per well of 96-multi-well plate. ATP efflux triggered by externally added ADP was monitored in fluorescence (λ_{exc}=360nm, λ_{em}=465nm) by following $NADP^+$ reduction occurring in a solution containing 2.5. mM glucose, 1 E.U. hexokinase (E.C. 2.7.1.1.), 0.5. E.U. glucose-6-phosphate-dehydrogenase (Roche/R-Biopharm, France) and 0.2.mM NADPH as described by (24). The choice of fluorescence instead of absorbance was determined by the higher sensitivity of fluorescence and to allow the combination of multiple mode of measurements. Influence of adenylate kinase-dependent ATP synthesis was evaluated after treatment of isolated mitochondria by 10µM of adenylate kinase specific inhibitor Ap_5A (P_1P_5-diadenosine-5'-pentaphosphate). MT21, and above cited agents were used.

3.6. Statistical analysis

IC_{50} values and standard errors were calculated using Graphpad Prism software.

4. RESULTS AND DISCUSSION

4.1. Ultrastructural effects of Ca^{2+}, MP and Vpr_{52-96} on mouse liver isolated mitochondria

Mouse liver mitochondria were incubated for 15 or 30min in the absence (Figure 1A) or presence of 25µM Ca^{2+} (Figure 1B), 2µM MP (Figure 1C), 3µM Vpr_{52-96} (Figure 1D). Then, their morphology was studied by transmission electronic microscopy. In comparison to control mitochondria, Ca^{2+}, MP and Vpr_{52-96} induced the matrix swelling and the almost complete loss of internal material. The cristae, which are detected in control mitochondria, cannot be distinguished after treatments, indicating that the IM expanded and came in close contact with the OM due to its large surface. Sites of OM discontinuity, while the IM appeared intact, revealed physical rupture of the OM. Of note, Vpr_{52-96} , but not MP, preserved the round shape of mitochondria (Figure 1C, D, (36)) suggesting different modalities of mitochondriotoxicity. Global structural changes correspond to previous observations of Jurkat cells mitochondria following cell treatment with anti-Fas antibody (44). Altogether, these results suggest that, in our conditions, Ca^{2+}, MP and Vpr_{52-96} triggered classical hallmarks of the morphological pro-apoptotic alterations of mitochondria.

4.2. Calcium, MP and Vpr_{52-96}, but not CCCP promote mitochondrial matrix swelling

Mouse liver isolated mitochondria were treated with various doses of Ca^{2+} and changes in optical density of the mitochondria suspension was immediately registered. Thus, Ca^{2+} elicited a dose-dependent matrix swelling, which was reproducibly maximal for 25µM (Figure 2A). This dose was defined as the reference (100%) to estimate the effect of other molecules in percentage for further experiments. Then, we found that MP, and Vpr_{52-96}, but not CCCP, induce a dose-dependent mitochondrial swelling (Figure 2B). The IC_{50} of MP and Vpr_{52-96} were respectively 1.86µM and 0.51µM respectively (Figure 2C). Furthermore, the Ca^{2+} effect was totally inhibited by CsA, and partially by BA and DIDS , as expected from litterature (34, 35, 45). In contrast, MP was only slightly inhibited by DIDS, but was not modulated by CsA and BA, suggesting that MP activity could be mediated via an effect on VDAC, neither ANT nor PTPC. Vpr_{52-96}, also, was not inhibited by CsA, but significantly prevented by BA and DIDS, confirming an effect on both proteins (35, 36). The absence of protective effect of CsA toward both peptides can be explained by differences in mitochondrial origin (rat *vs.* mouse), in purification protocol (mitochondria purification on Percoll gradient or not) and in pre-incubation conditions in previous studies, but not in our conditions (35, 36). Thus, to elucidate the molecular mechanisms of mitochondrial interaction of an unknown molecule, experimental conditions of the assay may be adapted. For instance, the effects of a molecule on mitochondria can be dependent on the order of addition of the various agents e.g. (inducers *vs* inhibitors), the temperature, as well as the presence of salts (e.g. $MgCl_2$) (46).

4.3. Calcium, MP and Vpr_{52-96}, and CCCP induce the mitochondrial depolarization

In parallel with swelling measurement, we

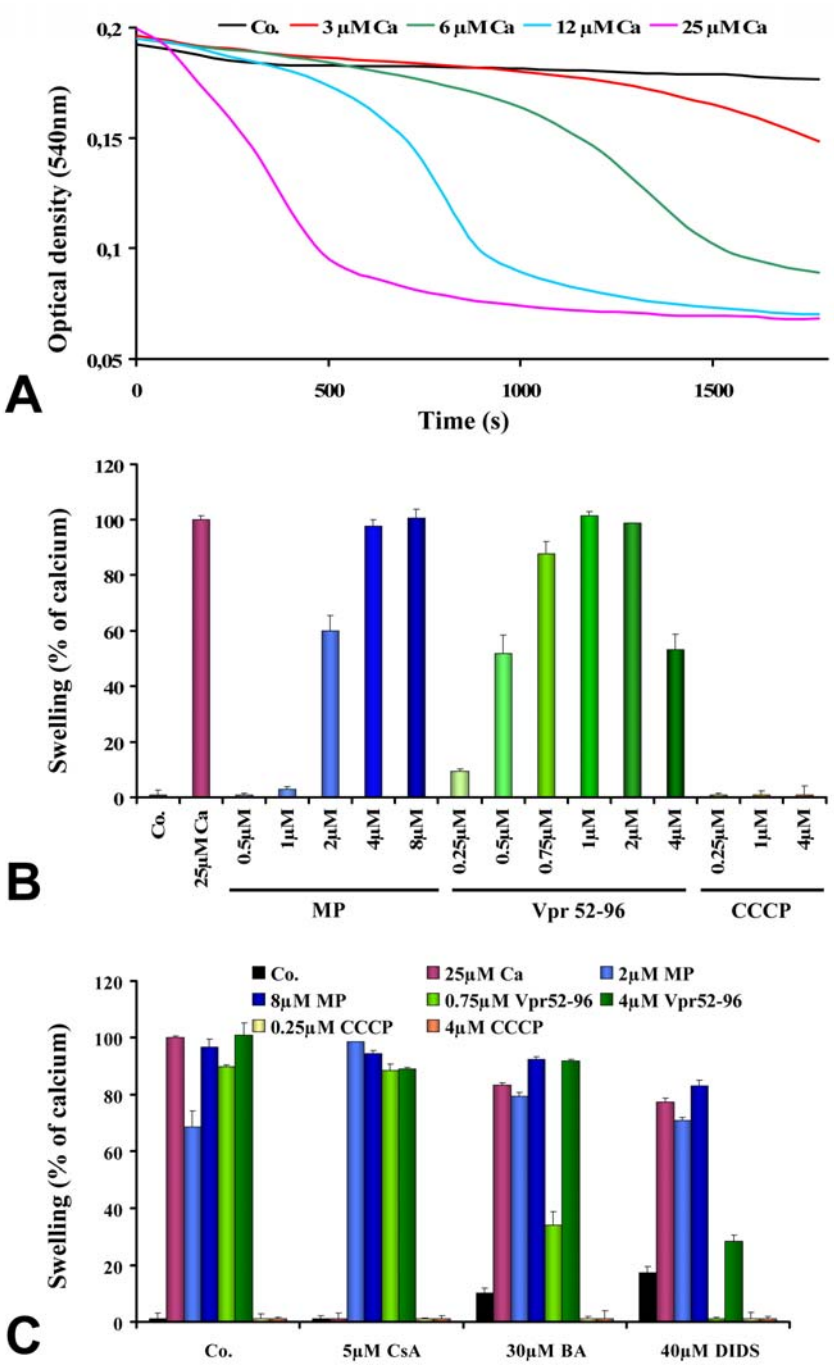

Figure 2. Effects of Ca^{2+}, MP and Vpr_{52-96} on the mitochondrial swelling. Isolated mitochondria (250µg/mL) were incubated at 37°C in a hypo-osmotic buffer. Absorbance was measured at 540nm for 1800sec. (A) Measurements were performed in the absence (Co.) or presence of indicated concentrations of Ca^{2+}. Ca^{2+} is used as a positive control. (B) Measurements were performed in the absence (Co.) or presence of 25µM Ca^{2+}, or various concentrations of MP, Vpr_{52-96} and CCCP. The effect of 25µM Ca^{2+} was normalized to 100%. (C) Measurements were performed in the absence (Co.) or presence of 25µM Ca^{2+}, 2µM and 8µM MP, 0.7.5µM and 4µM Vpr_{52-96} and 0.2.5µM and 4µM CCCP, and/or 5µM CsA, 30µM BA and 40µM DIDS. The effect of 25µM Ca^{2+} was normalized to 100%. Experiments were done in triplicate and repeated three times.

analyzed the transmembrane potential using the methodology based on the dequenching of the fluorescent probe, Rhodamine 123 (42). We found that Ca^{2+} induced a dose-dependent depolarization that is maximal for 25µM (Figure 3A). As expected, CCCP depolarized totally and immediately the mitochondrial membrane (Figure 3B). MP

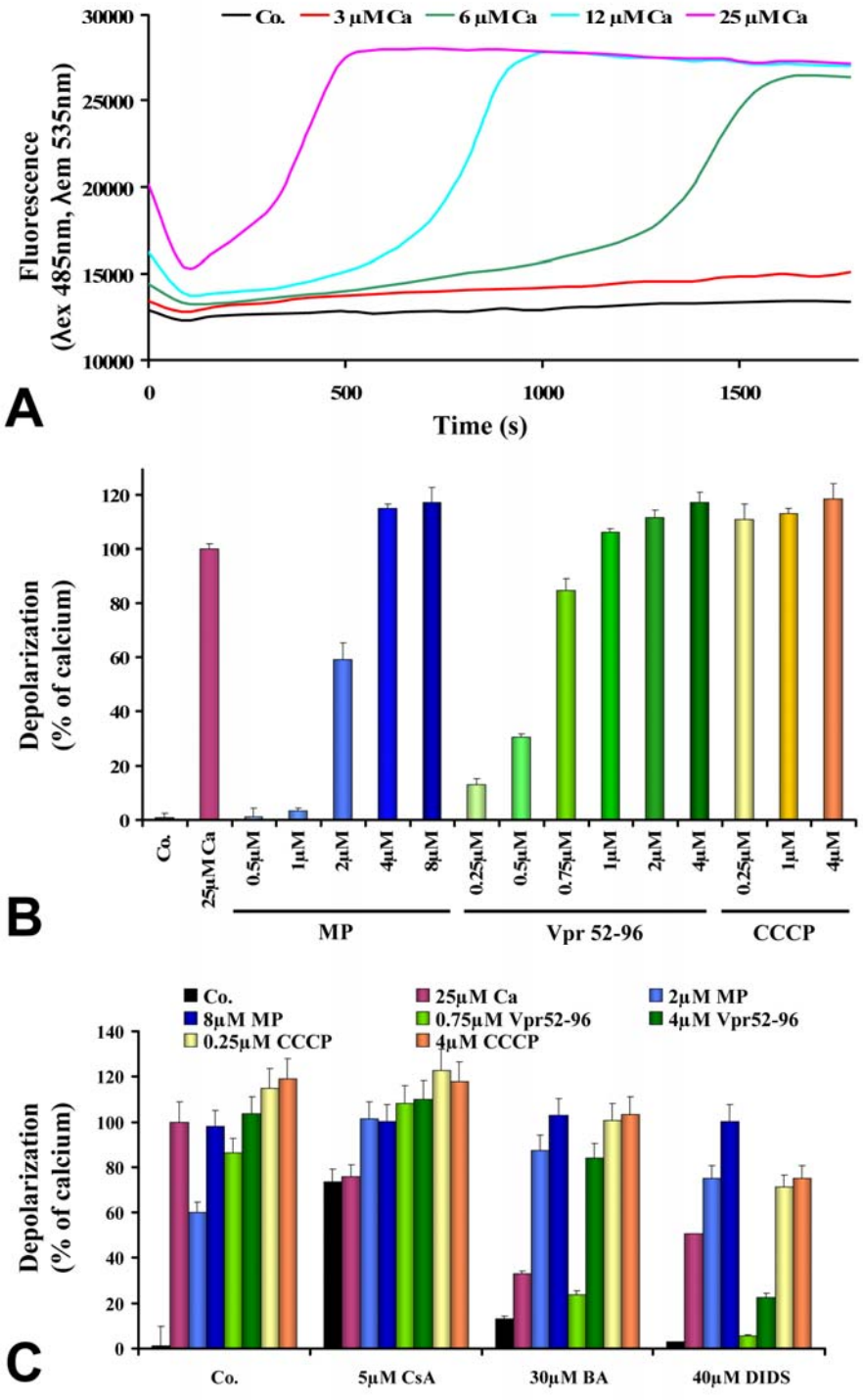

Figure 3. Effects of Ca^{2+}, MP and Vpr_{52-96} on the mitochondrial membrane depolarization. Isolated mitochondria (250μg/mL) were incubated at 37°C in a hypo-osmotic buffer containing 1μM rhodamine 123. Fluorescence change (λex 485nm, λem 535nm) was measured. (A) Measurements were performed in the absence (Co.) or presence of different concentrations of Ca^{2+}used as a positive control. (B) Measurements were performed in the absence (Co.) or presence of 25μM Ca^{2+}, or different concentrations of MP, Vpr_{52-96} and CCCP. The effect of 25μM Ca^{2+} was normalized to 100%. (C) Measurements were performed in the absence (Co.) or presence of 25μM Ca^{2+}, 2μM and 8μM MP, 0.7.5μM and 4μM Vpr_{52-96} and 0.2.5μM and 4μM CCCP, and/or 5μM CsA, 30μM BA and 40μM DIDS. The effect of 25μM Ca^{2+} was normalized to 100%. Experiments were done in triplicate and reproduced three times.

and Vpr $_{52-96}$ depolarized also the IM with IC_{50} of 2μM and 0.4.7μM respectively. According to swelling experiment, CsA, BA and DIDS prevented partially Ca^{2+}-dependent depolarization (Figure 3C). MP-induced depolarization was not prevented by any inhibitor, but was increased by CsA. By contrast and in line with swelling experiment, Vpr_{52-96} effect was decreased by BA and DIDS, but not by CsA. Of note, CCCP-induced depolarization was partially inhibited by DIDS suggesting an (indirect) implication of VDAC in CCCP activity. For charged molecules such as peptides, we cannot exclude a titration effect due the net negative charge of BA and DIDS.

4.4. Calcium, MP and Vpr $_{52-96}$, but not CCCP induce large inner membrane permeability

We used an original enzymatic method to detect changes in IM permeability via the accessibility of a soluble matricial enzyme, citrate synthase (43). This assay can detect IM permeabilization only if exogenous acetyl-coA (MM 809Da) can diffuse into the mitochondrial matrix after opening of a sufficiently large pore, which may be the PTPC, whose exclusion limit is <1.5. kDa (47) (Figure 4A). We decreased the volume of the various constituents of the assay to perform measurement in 96 well-microplate. After the induction of swelling (1800sec), substrates, such as DTNB, acetyl-Coenzyme A and oxaloacetate, were added and the absorbance measured at 415nm. We observed that Ca^{2+}, MP and Vpr52-96 allowed the detection of a citrate synthase activity, suggesting that the molecules stimulated a dose-dependent IM permeabilization (Figure 4B and C). As expected, even at high doses, CCCP did not permeabilize the inner membrane. In line with Korge's conclusion (43), our results support the usefulness of the assay to discriminate compounds acting via the opening of a large pore in the IM or not.

4.5. Vpr $_{52-96}$ but not calcium, MP, CCCP modulates NADH reductase activity of VDAC

VDAC has been demonstrated to be a plasma membrane NADH-oxidoreductase (41, 48, 49). Thus, in the presence of NADH and ferricyanide, VDAC generates ferrocyanide and NAD^+ as schematized in Figure 5A. This activity is inhibited in a dose-dependent manner by DIDS (Figure 5B). When isolated mitochondria have been treated by our three molecules, we showed that MP and CCCP did not affect the ferricyanide reductase activity, that Vpr_{52-96} was able to partially inhibit the VDAC activity with an IC_{50} of 0.86μM (Figure 5C), underscoring again a difference of modality of mitochondriotoxicity of both peptides. Interestingly, a Vpr_{52-96} effect on the VDAC activity is compatible with a direct peptide-protein interaction as previously suggested (35).

4.6. ADP/ATP translocation activity is impaired by Vpr $_{52-96}$, MP, calcium, and CCCP

The physiological function of ANT is to exchange the ADP against ATP in a stoechiometric manner (50, 51, 52). Here, we have developed and used a spectrophotometric assay of ADP/ATP translocation (53, 54) in the microplate format to evaluate the effect of our molecules on the ADP/ATP translocation (Figure 6A). Figure 6B shows a typical analysis based on the use of Ap5A for the inhibition of the adenylate kinase, an enzyme that can interfere with the ADP/ATP translocation assay and of carboxyatractyloside (CAT), as a specific and potent inhibitor of ANT (55). Thus, the specific activity of ANT, after inhibition of adenylate kinase, corresponds to the difference between the Ap5A and Ap5A+CAT curves. In contrast to Passarella *et al.*, ADP/ATP translocation is partially inhibited by Ap5A in our conditions (53). In Figure 6C, we observed that in the presence of CAT, Ca^{2+}, high doses of MP, Vpr_{52-96}, CCCP, and MT21, an ANT inhibitor, described to act independently of permeability transition induction (46, 56), no ADP/ATP translocation activity could be measured. However, when swelling and depolarization have been measured in parallel using the buffer optimized for ATP detection, we noted that for similar concentrations of molecules, (i) CAT did not favored either the swelling and the depolarization (ii) CCCP and MT21 induced the loss of $\Delta\Psi m$ without matrix swelling, and (iii) Ca^{2+}, MP and Vpr_{52-96} triggered both swelling and depolarization (data not shown). However, these discrepancies can be visualized on kinetics curves by the fact that the induction of swelling allows the ATP release from michondria into the medium and modified the level of the CAT curve in presence of the molecule (arrow, Figure 6D). This demonstrates that the enzymatic assay requires the IM integrity. Therefore, this assay is efficient to determine the effect on the ADP/ATP translocase activity of molecules, such as CAT, which preserves IM permeability barrier, but is not suitable to determine the impact of molecules, which can disrupt the membrane potential.

5. CONCLUDING REMARKS

We described the use of three enzymatic assays based on the measurement of a product apparition or a substrate disparition by spectrophoto- and fluorimetry. These assays can be used to characterize the effects of a panel of different molecules on isolated mitochondria as well as to compare mitochondria of various origins. Ca^{2+}, MP, Vpr_{52-96} constituted diverse standards for their differences in their chemical structure and their capacity to promote the pro-apoptotic MMP *in vitro* as well as *in cellulo (*34, 35, 57). CCCP was a negative control, because as a protonophore, it depolarizes mitochondrial IM, but does not trigger a complete process of MMP (57).

The combination of several assays to the classically used swelling and depolarization assays allowed to deepen the study of the mechanisms of mitochondriotoxicity of candidates molecules and thus, to discriminate molecules, which could seem to act similarly to promote MMP. As a result, Ca^{2+}, MP, Vpr_{52-96} activated the swelling, the $\Delta\Psi m$ loss, and IM permeabilization via the opening of the PTPC. Moreover, Vpr_{52-96}, but not MP, affects VDAC activity, in line with previous findings revealing that Vpr_{52-96} requires both ANT and VDAC to kill yeast cells (35). This data was re-enforced by the fact that Vpr_{52-96} mitochondriotoxic effects are prevented by DIDS and BA, but not those of MP. Moreover, on the basis of their IC_{50} in the various assays, Vpr_{52-96} appeared to be more potent than MP. This clearly indicates that the two

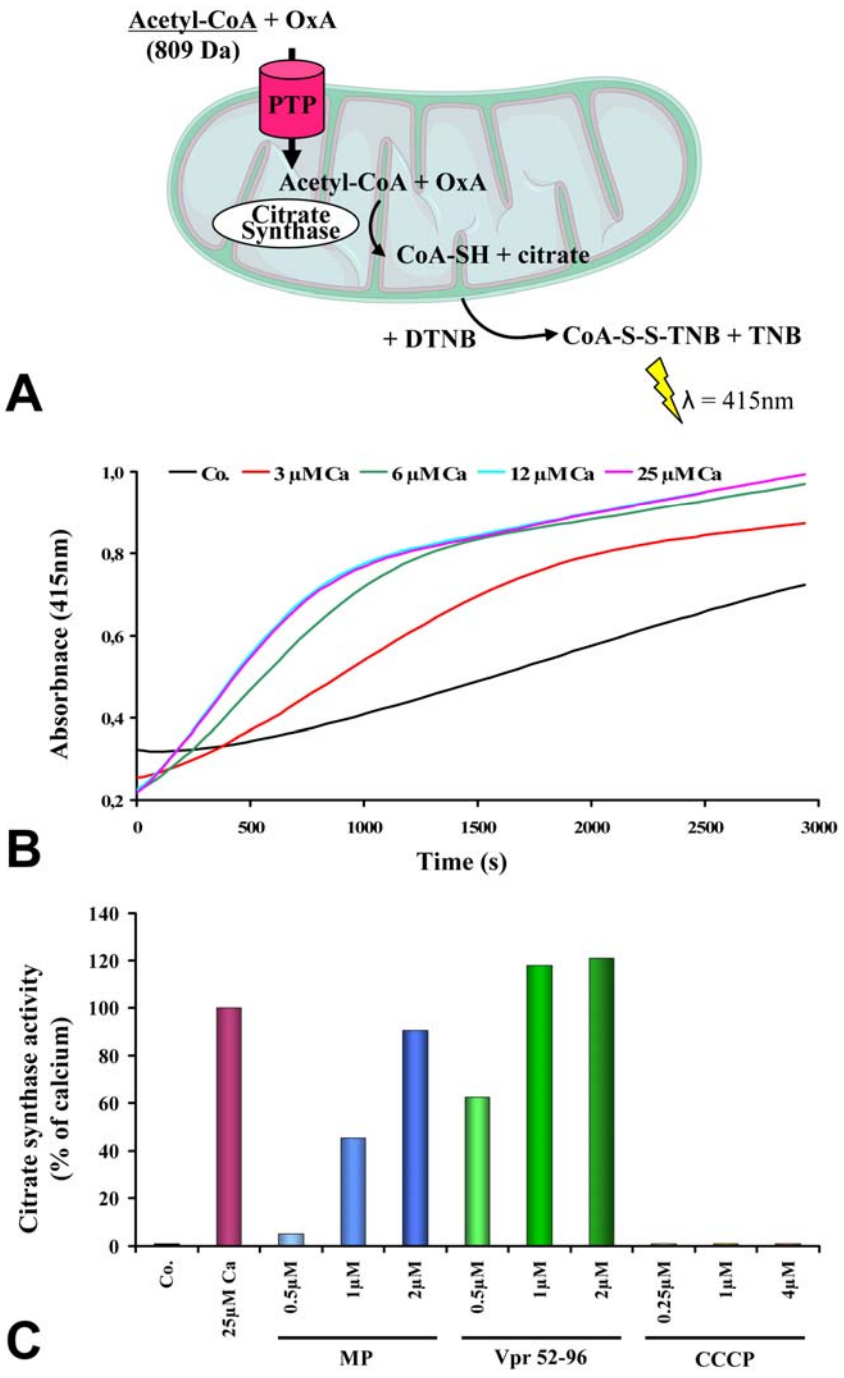

Figure 4. Effects of Ca^{2+}, MP and Vpr_{52-96} on the inner membrane permeabilization. Isolated mitochondria (250µg/mL) were incubated at 37°C in a hypo-osmotic buffer supplemented for citrate synthase activity measurement as described in Material and methods. Following an incubation of 1800 sec, absorbance of CoA-S-S-TNB at 415nm was recorded for 3000 sec. (A) Scheme of the assay. Acetyl-coA (underlined) is the limiting factor of the assay, because it cannot freely diffuse into the matrix. Therefore, if the permeability transition pore (PTP) opens, Acetyl-coA can diffuse into the matrix and be transformed by citrate synthase, a matricial enzyme, in the presence of Oxaloacetate (OxA) into CoA-SH and citrate. Then, CoA-SH and DTNB can be transformed into TNB and CoA-S-S-TNB, which can de detected at 415nm. (B) Measurements were performed in the absence (Co.) or presence of different concentrations of Ca^{2+}. Ca^{2+} induced a concentration-dependent permeabilization of the inner membrane. (C) Measurements were performed in the absence (Co.) or presence of 25µM Ca^{2+}, or different concentrations of MP, Vpr_{52-96} and CCCP. The effect of 25µM Ca^{2+} was normalized to 100%. Experiment was repeated three times.

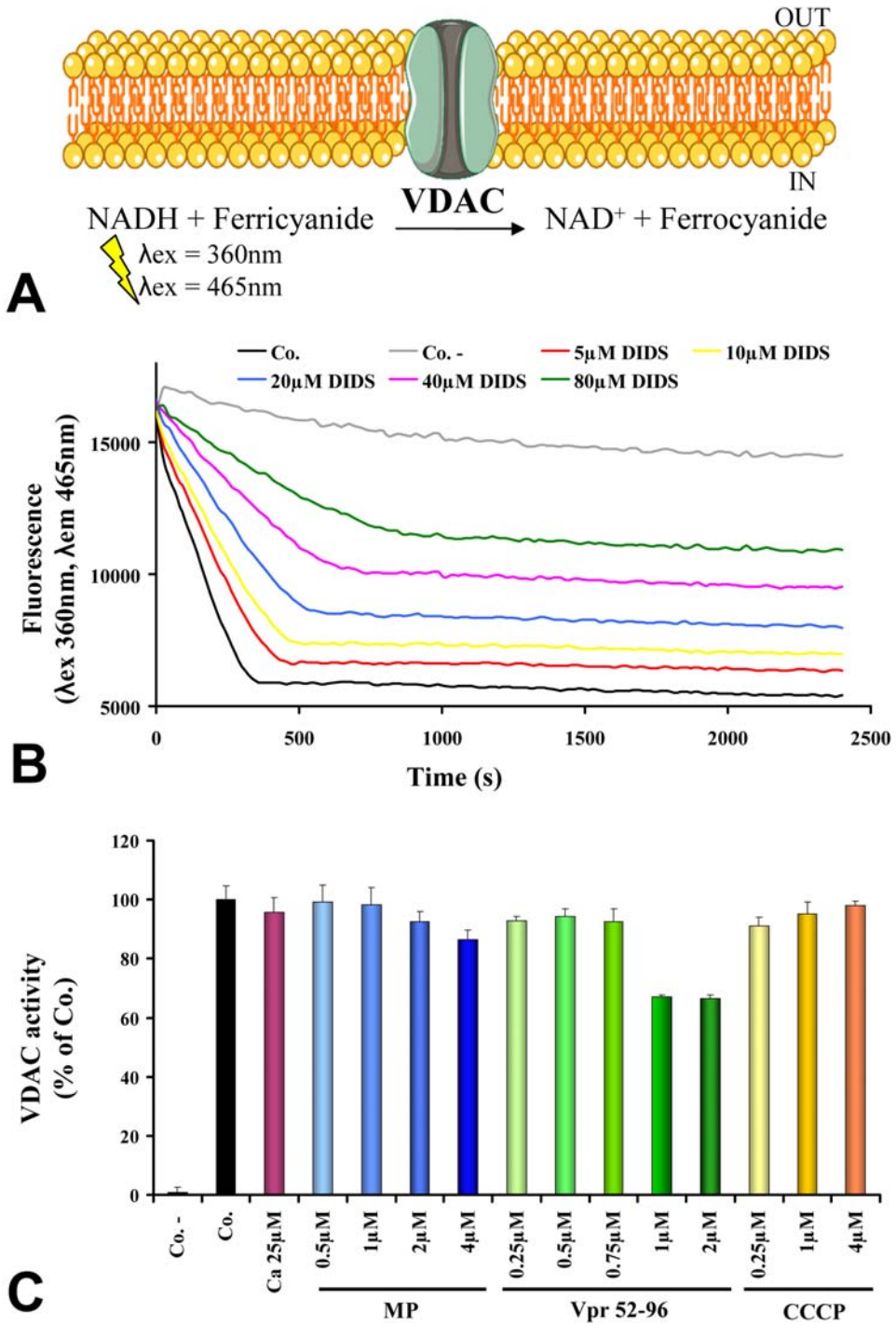

Figure 5. Effects of DIDS, Ca^{2+}, MP and Vpr_{52-96} on the NADH-ferricyanide reductase activity of VDAC. Isolated mitochondria (100μg/mL) were incubated at 37°C in a hypo-osmotic buffer and reagents for reductase activity detection as indicated in Material and Methods. The deacrease of NADH fluorescence change (λex 360nm, λem 465nm) was monitored for 2500 sec. (A) Enzymatic reaction. VDAC, an integral outer membrane protein, catalyzes the oxidation of NADH and the reduction of ferricyanide in ferrocyanide. (B) Measurements were performed in the absence (Co.) or presence of different concentrations of DIDS. Co.- is a negative control without ferricyanide. (C) Measurements were performed in the absence (Co.) or presence of 25μM Ca^{2+}, or different concentrations of MP, Vpr_{52-96} and CCCP. The activity of untreated mitochondria was normalized to 100%. Experiments were repeated three times.

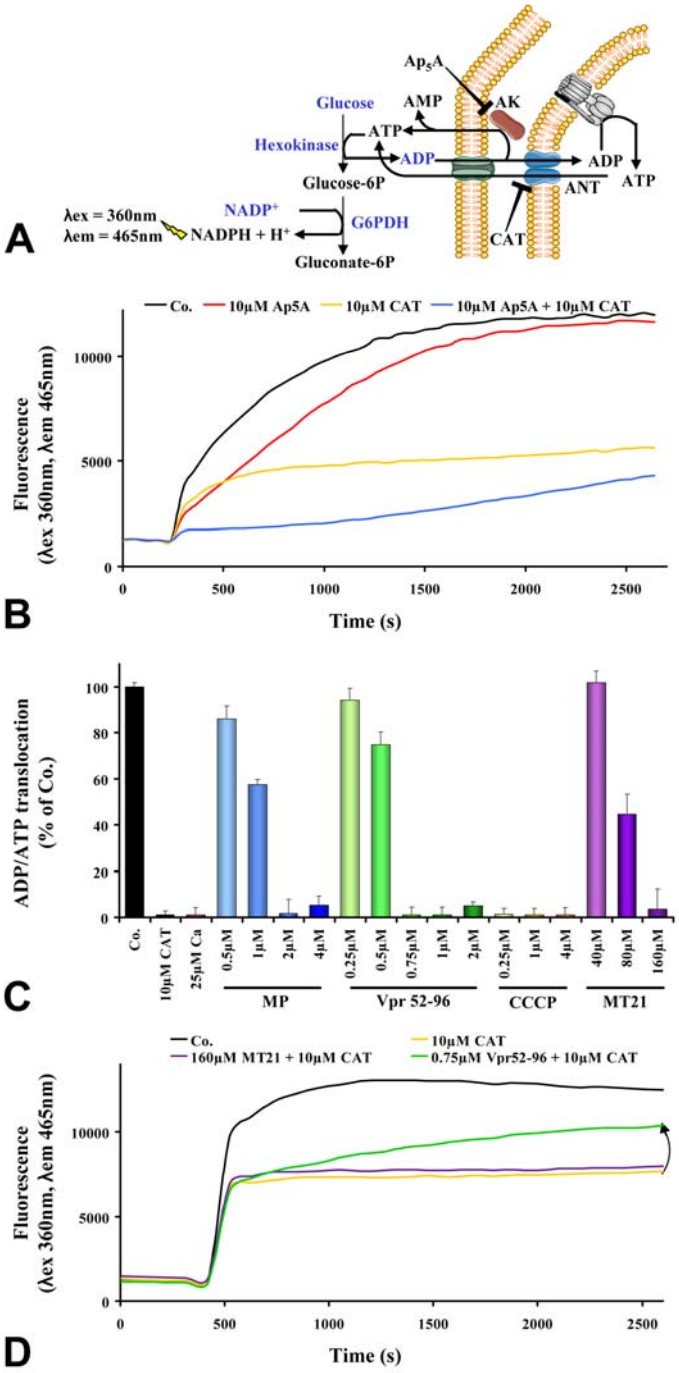

Figure 6. Effects of Ca^{2+}, MP and Vpr_{52-96} on the ADP/ATP translocase activity of ANT. Isolated mitochondria (250µg/mL) were incubated at 37°C in a hypo-osmotic buffer with the ATP detection system (see Material and Methods). Fluorescence of NADPH (λex 360nm, λem 465nm) was recorded for 3000 sec. (A) Scheme of the ADP/ATP translocase assay. ADP is added to mitochondria and diffuse into the intermembrane space through VDAC. Once into the intermembrane space, ADP can be transformed by adenylate kinase (AK) in AMP and ATP or exchange against ATP by the adenine nucleotide translocator (ANT). (B)- Measurements were performed in the absence (Co.) and/or presence of 10µM CAT (ANT inhibitor) and 10µM Ap5A (adenylate kinase inhibitor). (C) All conditions were tested in the presence or absence of 10µM CAT. ANT activity was calculated using the formula: Co. activity – 10µM CAT activity. Measurements were performed in the absence (Co.) or presence of 25µM Ca^{2+}, or different concentrations of MP, Vpr_{52-96} and CCCP. The activity of untreated mitochondria was normalized to 100%. (D) Measurments were performed in the absence (Co.) or presence of 10µM CAT and/or 160µM MT21 and 0.7.5µM Vpr_{52-96}.

peptides exert their effect by different molecular mechanisms and presumably target various constitutive mitochondrial proteins.

These assays constitute additional screening assays for drug candidates. Indeed, during the last decade, the development of high throughput screening has increased the need for robust assays using absorbance, fluorescence as well as luminescence (58). Moreover, since the emergence of a concept of apoptosis-based therapy (6), an ever increasing number of companies are interested in the search of apoptosis regulatory molecules and the identification of their target. Here, we propose three microplate assays that can provide complementary information regarding the permeabilization of the mitochondrial IM and the targeting of the two membrane proteins VDAC and ANT, two main actors of the MMP process.

Thus, the results underscore the usefulness of the combination of multiple assays to decipher fine molecular mechanisms of mitochondriotoxicity of candidate molecules (59) and constitute novel tools, which might contribute to the discovery of novel therapeutic molecules.

6. ACKNOWLEDGMENTS

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Abbreviations: ANT, adenine nucleotide translocase; Ap_5A, P_1P_5-diadenosine-5'-pentaphosphate; BA, bongrekic acid; Ca^{2+}, calcium; CAT, carboxyatractyloside, CCCP, carbonyl cyanide m-chlorophenylhydrazone; Co., control; CsA, cyclosporine A; cyclophilin D, CypD; IM inner membrane; DTNB, 5,5'-Dithio-bis (2-nitrobenzoic acid; DIDS, 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid; $\Delta\Psi m$, transmembrane inner potential; MP, mastoparan; MMP, mitochondrial membrane permeabilisation; OM, outer membrane; PT, permeability transition; PTPC, permeability transition pore complex; VDAC, voltage-dependent anion channel; Vpr, viral protein

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