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The authors declare they have no conflicts of interest.

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The influence of adenylate cycling on mitochondrial calcium-induced permeability transition pore in permeabilized skeletal muscle fibres

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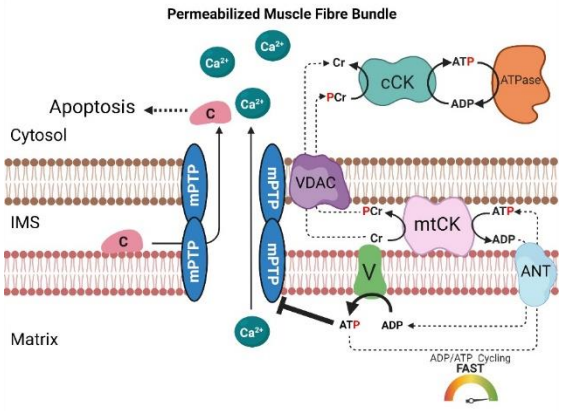
Summary



In isolated mitochondria, calcium-induced mitochondrial permeability transition pore (mPTP) opening is thought to be regulated by adenylates but the relative effects of adenylate cycling in permeabilized tissues is less explored. To determine the effect of adenylates on calcium retention capacity (CRC) as an index of mPTP in permeabilized muscle fibres, separate in vitro assessments of CRC were compared in media that did not contain ADP or received exogenous ADP that naturally equilibrated with ATP through endogenous ATP-dependent pathways (presence of both ADP and

ATP). Comparisons were made to a hexokinase 2-deoxyglucose system that recycles ATP to ADP (depletion of ATP). In permeabilized mouse quadriceps fibres, we found that CRC was increased by ADP suggesting endogenous ADP-ATP equilibria attenuate mPTP. Supplementing ADP with the hexokinase ATP recycling clamp lowered CRC relative to ADP alone but had no effect relative to the absence of adenylates. This finding suggests ADP does not alter calcium-induced mPTP and that its regulation by adenylates is specific to ATP in permeabilized mouse quadriceps fibres. Accelerating matrix ADP/ATP cycling with creatine had no effect on CRC when combined with 5mM ADP but more than doubled CRC (desensitized mPTP) when the hexokinase clamp was included. These results demonstrate the importance of considering adenylate equilibria during the design of in vitro assessments of calcium-induced mPTP in permeabilized muscle fibres.

1. Introduction

Evidence suggests that mitochondria can trigger apoptosis and necrosis through formation of the permeability transition pore mPTP (Rasola and Bernardi, 2011). This event is increasingly linked to numerous cellular processes and pathologies (Bernardi et al, 2021, Rasola and Bernardi, 2011, Rasola and Bernardi, 2007). mPTP is often assessed by *in vitro* fluorescent-based detection of mitochondrial calcium uptake in response to calcium titrations followed by eventual release through the mPTP once calcium retention capacity (CRC) is exceeded. As calcium-induced mPTP is influenced by adenylates (Beutner et al, 1997, Dolder et al, 2003, Duchen et al, 1993, Gizatullina et al, 2005, Sokolova et al, 2013) controlling [ADP], [ATP] and their equilibrium during *in vitro* assessments may influence mPTP measurements. Thus, comparing mPTP assessments with and without adenylates may reveal how the regulation of mPTP by ADP and/or ATP is altered in a given investigation and highlights the importance of careful consideration of *in vitro* assay conditions when assessing mPTP.

In isolated heart mitochondria, micromolar calcium triggers mPTP but this event is partially attenuated by ADP and strongly attenuated by ATP (Duchen et al, 1993). In these experiments, the addition of ADP was supplemented with hexokinase and glucose to recycle mitochondrially-derived ATP to maintain a fixed [ADP] and prevent its depletion. However, in permeabilized tissues or cells, exogenously added ADP creates an equilibrium with ATP because mitochondrially-derived ATP is hydrolyzed by extra-mitochondrial ATPases and other ATP-dependent proteins which does not exist in isolated mitochondria (Saks et al, 1995). Thus, the addition of a fixed concentration of ADP to permeabilized samples results in the presence of both adenylates albeit in an unknown ADP:ATP equilibrium. Given calcium-induced mPTP is robustly attenuated by ATP and partially attenuated by ADP in isolated heart mitochondria (Duchen et al, 1993),

permitting a natural equilibria between both adenylates may offer the advantage of retaining the distinct effects of each albeit without control of the actual concentrations.

As ATP was shown to be a more potent suppressor of calcium-induced mPTP in isolated mitochondria (Duchen et al, 1993), it is possible that combining ADP with a hexokinase system that recycles ATP to ADP would result in a greater mitochondrial sensitivity to calcium-induced mPTP due to depletion of ATP. In contrast, including ADP without a hexokinase clamp could lower the probability of mPTP in response to calcium due to natural equilibration with ATP in permeabilized muscle fibres. To our knowledge, these comparisons in permeabilized muscle fibres have not been performed in the literature.

Mitochondrial adenylate cycling is also sensitive to creatine (Wallimann et al, 2011). By including creatine in assay media, mitochondrial creatine kinase-dependent phosphate shuttling becomes activated which ultimately accelerates matrix cycling of ADP/ATP (Figure 2A). Prior literature has shown that creatine combined with ADP can desensitize mitochondria to calcium-induced mPTP (Dolder et al, 2003). Other literature also supports a role for mitochondrial creatine kinase in regulating mPTP (Datler et al, 2014). Thus, comparing the relative impact of creatine-independent and -dependent control of adenylate cycling could also provide additional insight into how mPTP is altered. The implications of *in vitro* assay design could be considerable given mPTP is altered in a growing list of diseases, stressors and biological contexts.

As the role of mitochondrial stress in contributing to muscle disorders is becoming increasingly popular, there is uncertainty over whether the influence of adenylates and creatine on mPTP is consistent across muscle types, particularly when using permeabilized muscle fibres. Furthermore, it is also unknown if altering adenylates and creatine will lead to unique conclusions of how mPTP responds in models of muscle dysfunction.

The first purpose of this investigation was to compare approaches that modulate ADP and creatine when assessing calcium-induced mPTP. Three major conditions were considered and tested in wildtype mouse muscle: (i) the absence of ADP, (ii) the inclusion of ADP (triggering equilibration with ATP), and (iii) an ATP recycling clamp using hexokinase and 2-deoxyglucose to maintain exogenously added ADP concentrations. All conditions were compared with and without creatine to accelerate matrix ADP/ATP cycling and shift the equilibrium towards ATP. The 2nd purpose of the investigation was to determine whether including ADP and creatine as physiologically relevant regulators altered the interpretation of calcium-induced mPTP events in an example biological context by comparing the D2.*mdx* mouse model of Duchenne muscular dystrophy (Bellissimo et al, 2022) to wildtype mice.

2. Materials and Methods

2.1. Animal Care

Male 4-8 week old CD1 mice were utilized from an in-house colony established at York University (Toronto, Ontario) for the first purpose. For the second purpose, 3 week-old DBA/2J WT mice were ordered directly from Jackson Laboratories (Bar Harbor, USA) due to breeding challenges (McGreevy et al, 2015) and allowed to acclimate for 7 days. These mice were compared to male 4 week-old dystrophin-deficient D2.*mdx* mice were

utilized from an in-house colony established at York University (Toronto, Ontario) and sourced from Jackson Laboratories. All animals were maintained on a 12:12-h light-dark cycle while being provided access to standard chow and water *ad libitum*. All experiments and procedures were approved by the Animal Care Committee at York University (AUP Approval Number 2016-18) in accordance with the Canadian Council on Animal Care.

2.2. Preparation of permeabilized muscle fibre bundles

This technique was adapted from previous methods described elsewhere (Hughes et al, 2019). Muscles were excised carefully from mice while under heavy sedation with 5% isoflurane mixed with medical air at 2.0 l/min flow followed by removal of the heart. Muscles were immediately placed into ice-cold BIOPS containing (in mM) 50 MES Hydrate, 7.23 K_2EGTA, 2.77 CaK_2EGTA, 20 imidazole, 0.5 dithiothreitol, 20 taurine, 5.77 ATP, 15 PCr, and 6.56 $MgCl_2$·6 H_2O (pH 7.1). Muscles were trimmed of connective tissue and fat and separated into small bundle sizes approximately 1.0-2.5mg wet weight in size. Bundles were separated along the longitudinal axis to avoid membrane damage. Once separated and weighed, bundles were permeabilized with 40µg/uL saponin (Sigma Aldrich; Mississauga, ON) in BIOPS on a platform rotor for 30 minutes at 4°C. After permeabilization, bundles were placed in Buffer Y containing (in mM) 250 sucrose, 10 tris–HCl, 20 tris-base, 10 KH_2PO4, and 0.5 mg/mL BSA, supplemented with 4mM EGTA and washed on a rotor at 4°C for 10 min. Fibres were then placed in a second wash of Buffer Y with 10 µM blebbistatin (Cayman Chemicals) to prevent rigor (Perry et al, 2011) until substrate titrations were initiated.

2.3. Mitochondrial calcium retention capacity

Mitochondrial calcium retention capacity was performed according to protocols described previously (Fisher-Wellman et al, 2013, Hughes et al, 2019) with modifications outlined in section *3 Results and Discussion*. Briefly, membrane impermeable Calcium Green-5N (Invitrogen) fluorescence was measured spectrofluorometrically (QuantaMaster 80, HORIBA Scientific) in a cuvette with 300 µL assay buffer containing 1 µM Calcium Green-5N (Invitrogen), 2 µM thapsigargin, 5 µM blebbistatin, and 40 µM EGTA while maintained at 37°C with continuous stirring. Prior to the start of each experiment, the cuvette was placed on a stir plate with 500 µL of water with 10 mM EGTA for a minimum of 10 minutes. The water was then aspirated from the cuvette and replaced with assay buffer, chelating any residual Ca^{2+}. 5 mM glutamate, 2 mM malate and varying concentrations of ADP outlined in section *3* were added to the assay buffer and minimum florescence was recorded. Calcium uptake by the mitochondria was initiated by a pulse of 8 nmol $CaCl_2$ to overcome EGTA. Additional pulses of 4 nmol of $CaCl_2$ are added until the mitochondrial permeability transition pore (mPTP) opening was observed as a spontaneous increase in fluorescence. Two pulses of 0.5 mM $CaCl_2$ were then added to saturate the fluorophore, establishing maximum saturation of the probe (F_{max}). Changes in free Ca^{2+} during mitochondrial Ca^{2+} uptake was then calculated using the known K_d for Calcium Green-5N and equations established for calculating free ion concentration (Tsien, 1989). Fibres are then lyophilized in a freeze-dryer (Labconco, Kansas City, MO, USA) for ≥4 h and weighed on a microbalance (Sartorius Cubis Microbalance, Gottingen, Germany). Calcium retention was then normalized to fibre dry weight in mg.

2.4. Statistics

Results are expressed as means ± SD with the level of significance established as $P < 0.05$ for all statistics. Prior to statistical analyses, outliers were omitted in accordance with ROUT testing (Q=0.5%) and then tested for normality using a D'Agostino–Pearson omnibus normality test (GraphPad Prism 7 Software, La Jolla, CA, USA). All data was found to be normally distributed. To assess statistical differences on the effect of adenylates, a one-way ANOVA was utilized followed by a two-stage step-up method of Benjamini, Krieger and Yekutieli for controlling False Discovery rate (FDR) for multiple-group comparisons. To assess the effect of creatine on CRC, a two-way ANOVA was used followed by a two-stage step-up method of Benjamini, Krieger and Yekutieli for controlling False Discovery rate (FDR) in multiple-group comparisons. Lastly, unpaired t-tests were used to assess differences between the effect of the combination of ADP and creatine on CRC.

3. Results and Discussion

We first examined the effect of ADP on calcium-induced mPTP. This event was assessed by titrating calcium until mitochondrial calcium retention capacity (CRC) was exceeded and mPTP opening was triggered thereby releasing calcium into the assay buffer (Figure 1A). Using permeabilized muscle fibres from mouse quadriceps, exogenous additions of 5mM ADP increased CRC (Figure 1B) which reflects an attenuation in mPTP. This finding is consistent with a previous report in isolated heart mitochondria (Duchen et al, 1993). Notably, this addition of ADP also increased variability in CRC which may be due to the unknown degree of ADP:ATP equilibration dependent upon endogenous ATP-hydrolyzing proteins in permeabilized muscle fibres (Saks et al, 1995). We then repeated the CRC assay in the presence of hexokinase and the glucose analogue 2-deoxyglucose. In this system, ATP synthesized by mitochondria in response to exogenous additions of ADP are immediately reconverted back to ADP (Figure 1C). As shown in Figure 1B, this hexokinase ATP recycling clamp (causing ATP depletion) led to a robust reduction in CRC compared to ADP alone (ADP:ATP equilibria) which reflects a greater propensity for calcium-induced mPTP. These results in permeabilized muscle fibres are consistent with isolated mitochondria from heart whereby ATP attenuated calcium-induced mPTP albeit using exogenous additions of ATP (Duchen et al, 1993). However, this study demonstrated only a small attenuation of mPTP when ADP was combined with the hexokinase clamp to deplete ATP suggesting ADP is much less effective than ATP at attenuating mPTP. In Figure 1B, we show no effect of ADP in the absence of ATP (ADP + hexokinase clamp) suggesting only ATP is effective at attenuating mPTP in permeabilized muscle fibres as noted above when ADP and ATP were permitted to equilibrate.

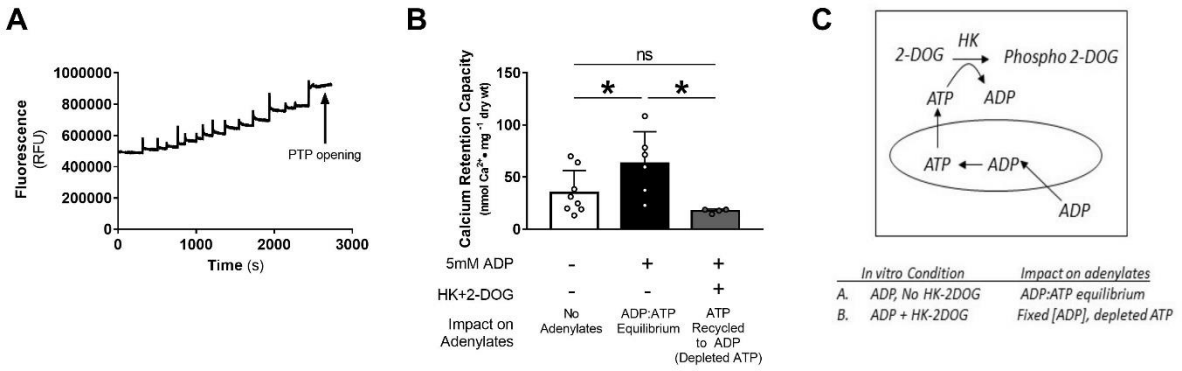


Figure 1. The effect of adenylates on mitochondrial calcium retention capacity in mouse permeabilized quadriceps muscle fibre bundles. (A) Representative trace of calcium titrations prior to induction of mitochondrial permeability transition pore (mPTP) opening. (B) CRC experiments were repeated in separate permeabilized fibre bundles including 5mM ADP with or without hexokinase and 2-deoxyglucose to recycle endogenously synthesized ATP from mitochondria back to ADP. (C) Addition of 2-deoxyglucose (2-DOG) and hexokinase (HK) to the media recycles ATP synthesized and exported by mitochondria (oval) using ADP that was added exogenously. Results represent mean ± SD; * P<0.05; N=4-8.

To further explore the effects of adenylate cycling on CRC, we next compared the effect of creatine on accelerating matrix ADP/ATP. In this system, mitochondrial creatine kinase utilizes mitochondrial ATP synthesized by exogenous additions of ADP, to phosphorylate creatine to phosphocreatine that is then exported for recycling by extramitochondrial creatine kinases (Figure 2A). This creatine-dependent phosphate shuttle shortens the distance required for the slower diffusing adenylates between matrix-inter membrane space domains, thereby accelerating matrix ADP/ATP cycling and shifting the equilibrium towards ATP. Creatine attenuated mPTP (increased CRC) in the presence of 5mM ADP fixed by the hexokinase ATP recycling clamp (depletion of ATP; Figure 2B). This observation is consistent with a prior report showing the combination of creatine and ADP attenuated mPTP albeit in the absence of the hexokinase clamp (Dolder et al, 2003). We then determined whether the presence of the hexokinase clamp altered the effect of creatine. By re-analyzing the data in Figure 1B with a separate experiment of creatine and ADP in the absence of the hexokinase clamp, we no longer observed an effect of creatine on CRC (Figure 2C). Thus, the ability of creatine and ADP to attenuate mPTP was observed only in the presence of the hexokinase ATP recycling clamp. While speculative, this finding suggests that creatine's influence on matrix ADP/ATP cycling is accelerated with exogenous hexokinase which might be facilitated by the higher extramitochondrial [ADP], perhaps by increasing ADP diffusion back to the mitochondria. The precise manner by which adenylate cycling achieves a balance between both kinases is unclear and could be explored in future experiments.

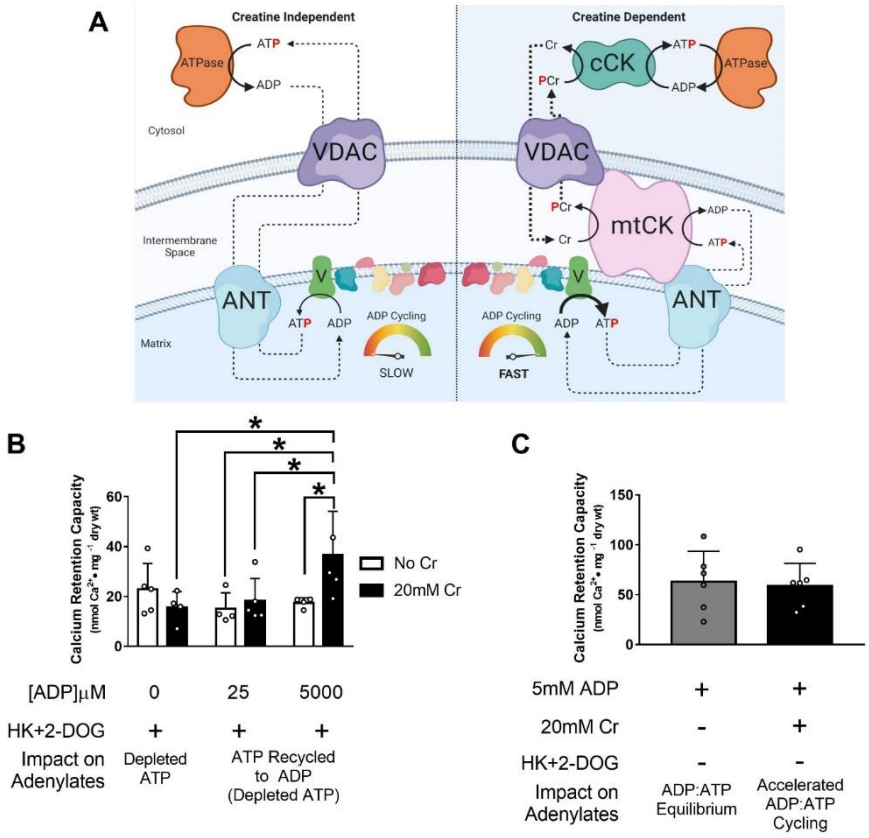


Figure 2. The effect of creatine on mitochondrial calcium retention capacity in mouse permeabilized quadriceps muscle fibre bundles. (A) The regulation of mitochondrial phosphate shuttling. In this system, matrix ATP is exported to the inner membrane space and dephosphorylated by mtCK. The phosphocreatine product is exported to the cytoplasm and recycled by cytosolic creatine kinases back to ATP. As creatine/phosphocreatine diffuses much faster than ADP/ATP, the system minimizes the diffusion distance of the more slowly diffusing adenylates to matrix-inner membrane space compartments and theoretically at local ATP-dependent proteins outside of the mitochondria (ATPases are depicted as only one example). The result is an accelerated turnover of matrix ADP/ATP and a higher rate of oxidative phosphorylation compared to the direct cycling of ADP and ATP between mitochondrial and cytoplasmic compartments as occurs in the absence of creatine in assay media. The experimental evidence for this system is described previously (Aliev et al, 2011, Bessman and Fonyo, 1966, Guzun et al, 2012, Meyer et al, 2006, Meyer et al, 1984, Schlattner et al, 2018, Wallimann et al, 2011). V, Complex V; mtCK, mitochondrial creatine kinase; ANT, adenine nucleotide translocase; VDAC, voltage-dependent anion carrier; cCK, cytosolic creatine kinase. Made with BioRender. (B) Mitochondrial calcium retention capacity was assessed in mouse quadriceps with creatine and ADP in assay media. All data was collected in the presence of the hexokinase 2-deoxyglucose clamp to eliminate ATP and maintain fixed concentrations of ADP. Creatine accelerates mitochondrial ADP/ATP turnover. (C) The effect of creatine on mitochondrial calcium retention capacity in the presence of ADP. Mitochondrial calcium retention capacity in the presence of ADP from Figure 1B was compared to a separate experiment including both ADP and creatine. Results represent mean ± SD; * $P<0.05$; n=4-6.

Thus, while the attenuation of mPTP by creatine in permeabilized fibres is similar to a previous report in isolated mitochondria (Dolder et al, 2003), the requirement of the hexokinase clamp is difficult to explain and the discrepancy between both studies in the absence of the clamp is not apparent. It is unclear if these differences are related to the tissue source of isolated mitochondria wherein expression levels of mitochondrial creatine kinase might be higher in the livers that were genetically modified to express mitochondrial creatine kinase (Dolder et al, 2003) as compared to quadriceps in this study, but such comparisons have not been performed. The assay conditions from this prior study also included 1mM ATP which would result in very low ADP. As creatine increases mitochondrial respiratory sensitivity to sub-maximal ADP concentrations in particular (Schlattner et al, 2018), future experiments could compare the effect of creatine with and without the hexokinase clamp at an even greater range of ADP concentrations used in the present study. Nevertheless, the results from Figure 1B and 1C suggest creatine attenuates CRC in permeabilized muscle fibre bundles from wildtype quadriceps when the hexokinase clamp is present.

One corollary of these findings is that the design of *in vitro* assessments of CRC might reveal distinct aspects of mPTP responses to a given biological stressor or context. For example, repeating CRC assessments with or without adenylates and/or creatine could give insight into whether their regulatory influence on mPTP is altered. To test this possibility, we compared CRC in the D2.*mdx* model of Duchenne muscular dystrophy to wildtype mice using permeabilized cardiac left ventricle fibres (Figure 3A-C). Previously, we demonstrated no difference between D2.*mdx* and wildtype CRC in this muscle, but the assay design did not combine ADP and creatine during *in vitro* assessments (Hughes et al, 2020). As this report also showed mitochondrial insensitivity to ADP and creatine during respiration and H_2O_2 emission, an outstanding question from this work is whether such insensitivity also applies to the regulation of mPTP. To test this possibility, we performed new experiments in D2.*mdx* and wildtype mouse permeabilized left ventricle fibres. We excluded the hexokinase clamp to permit a natural equilibration of ADP and ATP given the results from Figure 1B demonstrated that an equilibrium of both adenylates is more effective at regulating CRC vs the presence of ADP alone. As shown in Figure 3, there were no differences between D2.*mdx* and wildtype CRC whether it was assessed with ADP with or without creatine. This suggests that the prior discovery that mitochondrial creatine insensitivity occurs during respiration and H_2O_2 emission (Hughes et al, 2019) does not manifest as a loss of creatine control of mPTP, at least at this young stage of disease (4 weeks of age), in left ventricle, and when creatine is combined with high ADP concentrations (5mM). While we chose a high ADP concentration for the current experiment, we did not rule out whether creatine would have a different effect at low ADP concentrations in D2.*mdx* mice which is plausible given creatine sensitizes mitochondria to sub-maximal ADP concentrations (Schlattner et al, 2018). Nevertheless, the similar results with either *in vitro* protocol strengthens the prior conclusion (Hughes et al, 2020) that calcium-induced mPTP is not altered in the left ventricle at an early stage of myopathy in dystrophin deficient mice. In so doing, there is clear value in comparing multiple assay conditions when assessing CRC. Lastly, while we reached a similar conclusion with either *in vitro* assay design, our choice of model and muscle type is only one of many biological contexts that could be considered. In this light, the clear influence of ADP and creatine on CRC in quadriceps (Figures 1 and 2) demonstrates the potential opportunity of identifying unique conclusions of how CRC is regulated by adenylates and creatine when designing investigations focused on a variety of biological contexts.

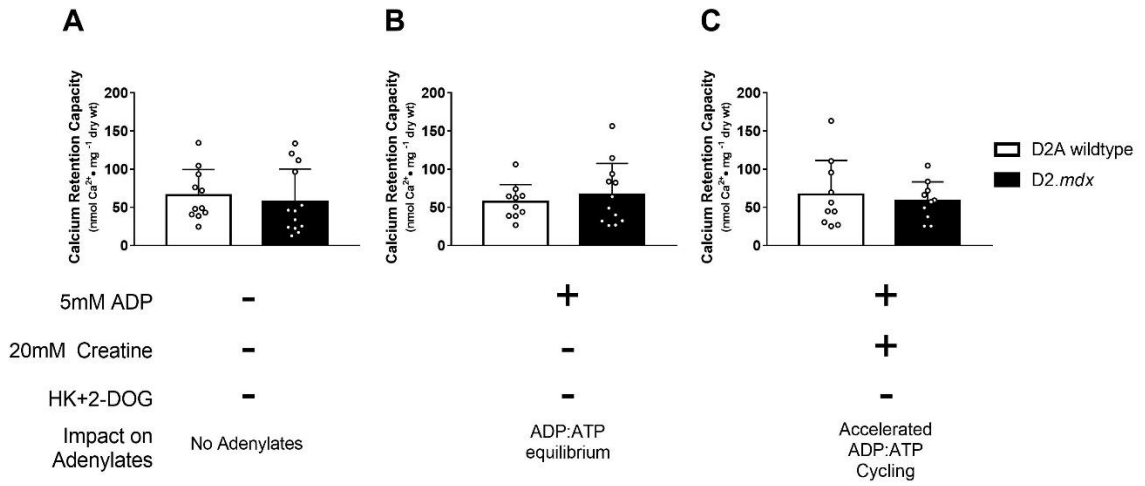


Figure 3. The effects of creatine and ADP on mitochondrial calcium retention capacity in mouse permeabilized left ventricle fibre bundles from D2.mdx mice. Mitochondrial creatine retention capacity was assessed in permeabilized fibres from 4 week old D2A wildtype and D2.*mdx* mice. First, CRC was assessed in the absence of both ADP and creatine (A). Next, CRC was assessed in the presence of 5mM ADP and the absence (B) or presence of creatine (C). The hexokinase 2-deoxyglucose clamp was eliminated to ensure the presence of both ADP and ATP through natural equilibrium pathways endogenous to permeabilized muscle fibres. N=10-13.

4. Perspectives, Limitations and Conclusions

These results demonstrate that *in vitro* assessments of calcium-induced mPTP in permeabilized muscle fibres are influenced by adenylates. Specifically, calcium-induced mPTP is suppressed by exogenous additions of ADP compared to ADP combined with a hexokinase 2-deoxyglucose system of recycling ATP to ADP. As ADP equilibrates with ATP in permeabilized muscle fibres, this finding indicates that the suppression of mPTP by exogenous ADP is due to the presence and cycling of both ADP and ATP. In quadriceps muscle, combining creatine with ADP also suppresses mPTP but only when the hexokinase clamp is present. This finding warrants similar considerations for future experiments in permeabilized systems across cell types and species and using a range of ADP and creatine concentrations. Likewise, consideration of other exogenously added adenylate recycling systems could be considered including the ADP to ATP recycling clamp created by pyruvate kinase and phosphoenol pyruvate (Monge et al, 2008) or clamping ADP and ATP at calculated ratios (Goldberg et al, 2019).

For perspective, while the hexokinase ATP to ADP recycling system would eliminate extra-mitochondrial ATP, it is unlikely to completely eliminate matrix ATP as adenylates will continue to cycle. However, it seems likely that an attenuation of ATP occurs in the matrix which is thought to be the location where adenylates regulate mPTP. However, one study suggested there may also be an adenylate regulatory site of the mPTP outside of the matrix (Gizatullina et al, 2005). Overall, it seems plausible that the hexokinase clamp reduces the net exposure of mPTP to ATP as intended.

Collectively, these results demonstrate the impact of modeling adenylate cycling on calcium retention capacity-based assessments of mPTP in permeabilized muscle fibres.

Careful consideration of the relevance of adenylate cycling to a given experimental question could influence the design of *in vitro* assessments in permeabilized muscle fibres with the aim of gaining greater insight into the regulation of mPTP in various biological contexts.

Abbreviations

CRC	Calcium retention capacity	mPTP	Mitochondrial permeability transition pore
H_2O_2	Hydrogen peroxide		

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