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

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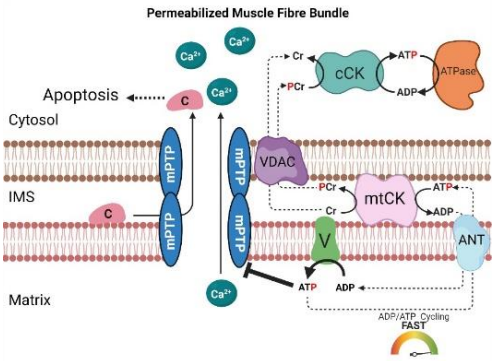
Summary

In isolated mitochondria, calcium-induced mitochondrial permeability transition pore (mtPTP) opening is thought to be regulated by adenylates but the relative effects of inherent adenylate cycling unique to permeabilized tissues is less explored. To determine the effect of adenylate cycling on calcium retention capacity (CRC) as an index of mt-permeability transition (mtPT) in permeabilized muscle fibers, 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 (ADP/ATP cycling). Comparisons were made to ADP combined with a hexokinase 2-deoxyglucose system



recycling ATP back to ADP (ATP depletion). In mouse quadriceps, ADP increased CRC suggesting endogenous ADP-ATP cycling attenuates mtPT. ADP with hexokinase lowered CRC relative to ADP alone but had no effect compared to the absence of ADP (no adenylates). This finding suggests ADP does not alter calcium-induced mtPT and that its regulation by adenylates is specific to ATP in permeabilized mouse quadriceps fibers. Accelerating matrix ADP/ATP cycling with creatine and 5 mM ADP did not affect CRC but more than doubled CRC (desensitized mtPT) when the hexokinase clamp was included. CRC was similar in wildtype and D2.*mdx* mouse cardiac left ventricle fibers whether assessed with ADP and creatine (adenylate cycling) or without ADP, confirming adenylate-dependent and -independent control of mtPT is not altered in this model of myopathy. These results demonstrate the natural cycling of ADP with ATP in permeabilized muscle fibers attenuates mtPT, but not due to ADP itself. Repeating assessments with and without adenylate cycling can add further insight to experimental results.

1. Introduction

Evidence suggests that mitochondria can trigger apoptosis and necrosis through formation of the permeability transition pore (mtPTP) (Rasola, Bernardi 2011). This event is increasingly linked to numerous cellular processes and pathologies (Bernardi et al 2021; Rasola, Bernardi 2007, 2011). Mitochondrial permeability transition (mtPT) is often assessed by *in vitro* fluorescent-based detection of mitochondrial calcium uptake in response to calcium titrations followed by eventual release through the mtPTP once calcium retention capacity (CRC) is exceeded. As calcium-induced mtPT 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 mtPT measurements. Thus, comparing mtPT assessments with and without adenylates may reveal how the regulation of mtPTP 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 mtPT.

In isolated heart mitochondria, micromolar calcium triggers mtPT 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 occur 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 mtPT is robustly attenuated by ATP and partially attenuated by ADP in isolated heart mitochondria (Duchen et al 1993), permitting a natural equilibrium between both adenylates in permeabilized tissues may offer the advantage of retaining the effects of each albeit without control of the actual concentrations. However, to our knowledge, no study has directly determined how calcium-induced mtPT is regulated by the natural equilibration of exogenously added ADP with ATP in permeabilized tissues.

As ATP was shown to be a more potent suppressor of calcium-induced mtPT 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 mtPT due to depletion of ATP. In contrast, including ADP without a hexokinase clamp could lower the probability of mtPT in response to calcium due to natural equilibration with ATP in permeabilized muscle fibers. To our knowledge, these comparisons in permeabilized muscle fibers 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 mtPT (Dolder et al 2003). Other literature supports a role for mitochondrial creatine kinase in regulating the mtPTP (Datler et al 2014). Thus, comparing the relative impact of creatine-independent and -dependent control of adenylate cycling could provide additional insight into how mtPT is altered. The implications of *in vitro* assay design could be considerable given mtPT 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 appreciated, there is uncertainty over whether the influence of adenylates and creatine on mtPT is consistent across muscle types, particularly when using permeabilized muscle fibers. Furthermore, it is unknown if altering adenylates and creatine will lead to unique conclusions of how mtPT 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 mtPT. Three major conditions were considered and tested in wildtype mouse muscle: (*1*) the absence of ADP, (*2*) the inclusion of ADP (triggering equilibration with ATP through endogenous ATP-hydrolyzing proteins in permeabilized muscle fibers (Saks et al 1995)), and (*3*) 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 second purpose of the investigation was to determine whether including ADP and creatine as physiologically relevant regulators altered the interpretation of calcium-induced mtPT 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-to 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 male 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 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 with 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 fiber 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 $MgCl_2 \cdot 6\ H_2O$ (pH 7.1). Muscles were trimmed of connective tissue and fat and separated into small bundle sizes approximately 1.0–2.5 mg wet mass. Bundles were separated along the longitudinal axis to avoid membrane damage. Once separated and weighed, bundles were permeabilized with 40 µg/µL 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_2PO_4, and 0.5 mg/mL BSA, supplemented with 4 mM EGTA and washed on a rotor at 4 °C for 10 min. Fibers 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 measured 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. In experiments that recycled ATP to ADP, 5 mM 2-deoxyglucose and 2 U/mL hexokinase were added. Prior to the start of each experiment, the cuvette was placed on a stir plate with 500 µL 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 mtPTP 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). Fibers 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 fiber dry mass [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 mtPT. This event was assessed by titrating calcium until mitochondrial calcium retention capacity (CRC) was exceeded and mtPTP opening was triggered thereby releasing calcium into the assay buffer (Figure 1A). Using permeabilized muscle fibers from mouse quadriceps, exogenous additions of 5 mM ADP increased CRC (Figure 1B) which reflects an attenuation in mtPT. 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 fibers (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 mtPT. These results in permeabilized muscle fibers are consistent with isolated mitochondria from heart whereby ATP attenuated calcium-induced mtPT albeit using exogenous additions of ATP (Duchen et al 1993). However, this study demonstrated only a small attenuation of mtPT when ADP was combined with the hexokinase clamp to deplete ATP suggesting ADP is much less effective than ATP at attenuating mtPT. In Figure 1B, we show no effect of ADP in the absence of ATP (ADP + hexokinase clamp) suggesting only ATP is effective at attenuating mtPT in permeabilized muscle fibers as noted above when ADP and ATP were permitted to equilibrate.

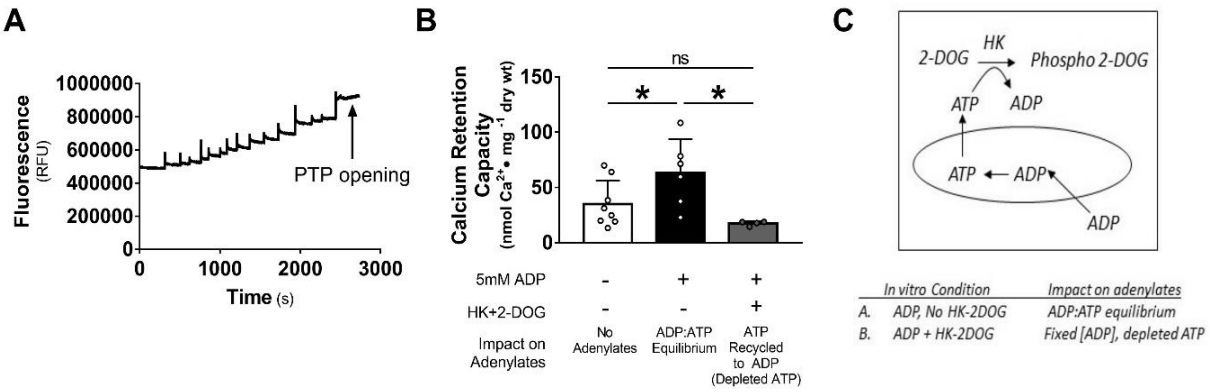


Figure 1. The effect of adenylates on mitochondrial calcium retention capacity in mouse permeabilized quadriceps muscle fiber bundles. (A) Representative trace of calcium titrations prior to induction of mitochondrial permeability transition pore (mtPTP) opening. **(B)** CRC experiments were repeated in separate permeabilized fiber bundles including 5 mM 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 biological replicates.

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 (PCr) 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 mtPT (increased CRC) in the presence of 5 mM 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 mtPT 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 mtPT 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.

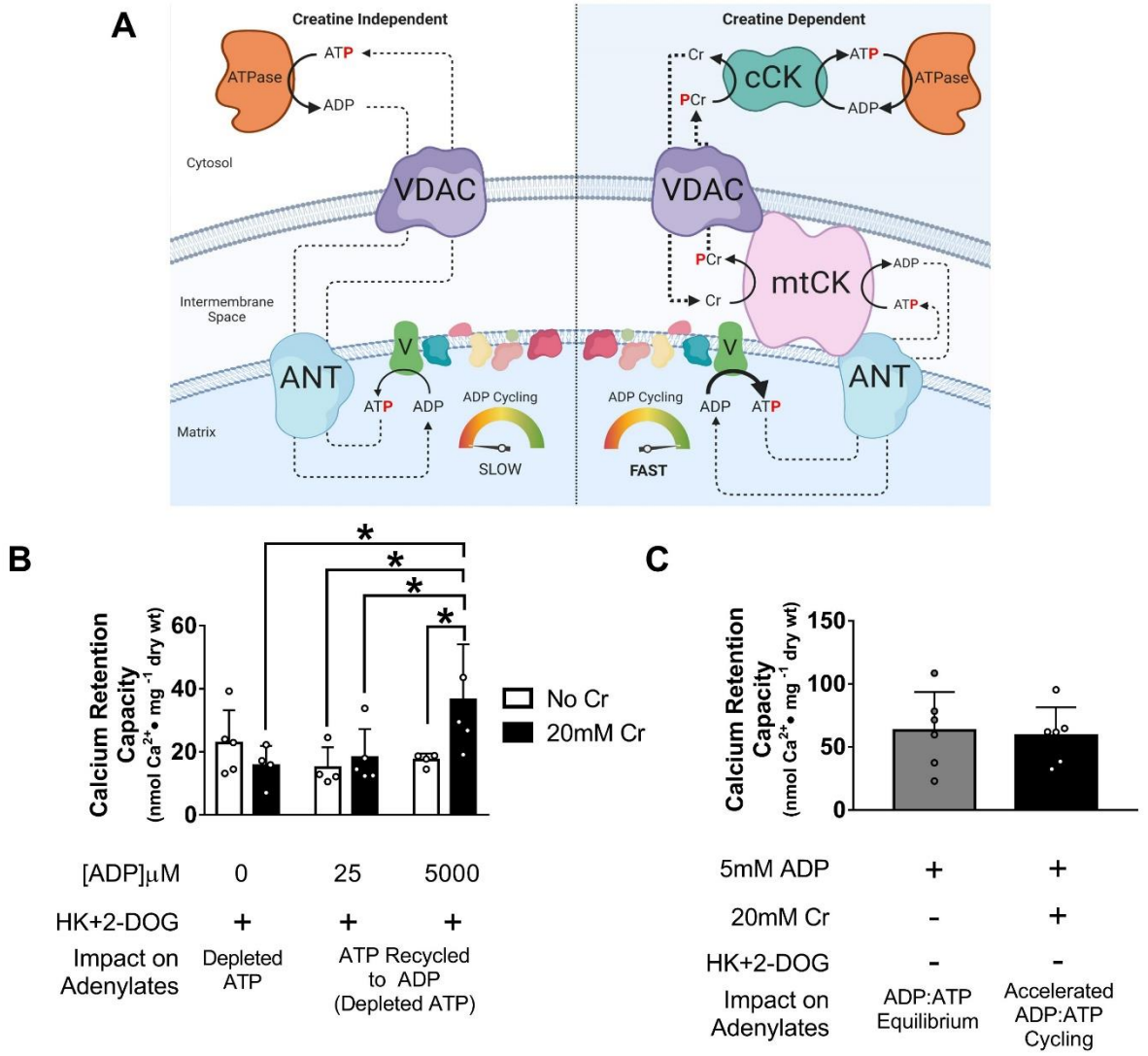


Figure 2. The effect of creatine on mitochondrial calcium retention capacity in mouse permeabilized quadriceps muscle fiber bundles. (A) The regulation of mitochondrial phosphate shuttling. In this system, matrix ATP is exported to the inner membrane space and dephosphorylated by mtCK. The phosphocreatine 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, 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 biological replicates.

Thus, while the attenuation of mtPT by creatine in permeabilized fibers is similar to results in isolated mitochondria reported previously (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 included 1 mM 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 2B and 2C suggest creatine attenuates CRC in permeabilized muscle fiber 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 mtPTP 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 mtPT 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 fibers (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 the mtPTP. To test this possibility, we performed new experiments in D2.*mdx* and wildtype mouse permeabilized left ventricle fibers. 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 the mtPTP, at least at this young stage of disease (4-weeks of age), in left ventricle, and when creatine is combined with high ADP concentrations (5 mM). 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 mtPT 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.

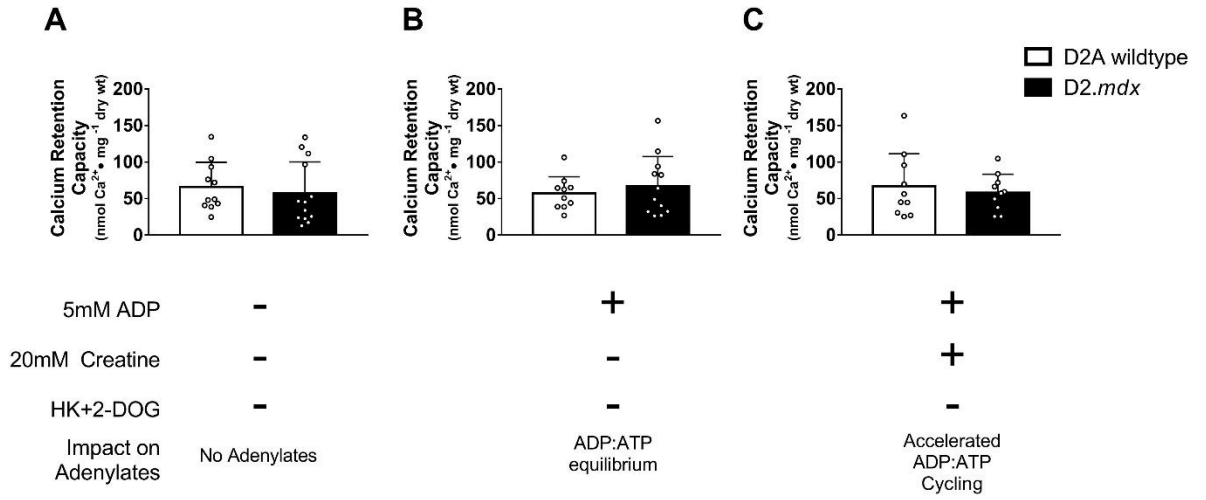


Figure 3. The effects of creatine and ADP on mitochondrial calcium retention capacity in mouse permeabilized left ventricle fiber bundles from D2.mdx mice. Mitochondrial creatine retention capacity was assessed in permeabilized fibers from 4-week-old D2A wildtype and D2.*mdx* mice. First, CRC was assessed in the absence of both ADP and creatine **(A)**. Next, CRC was assessed in the presence of 5 mM 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 fibers. *N*=10-13 biological replicates.

4. Perspectives, limitations and conclusions

Our results demonstrate that *in vitro* assessments of calcium-induced mtPT in permeabilized muscle fibers are influenced by adenylates. Specifically, calcium-induced mtPT 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 fibers, this finding indicates that the suppression of mtPT by exogenous ADP is due to the presence and cycling of both ADP and ATP (Saks et al 1995). In quadriceps muscle, combining creatine with ADP also suppresses mtPT 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 mtPT. However, one study suggested there may also be an adenylate regulatory site of the mtPTP outside of the matrix (Gizatullina et al 2005). Overall, it seems plausible that the hexokinase clamp reduces the net exposure of the mtPTP to ATP as intended.

While we did not measure adenylate concentrations *in vitro*, we nonetheless assumed an equilibrium occurred between ADP and ATP in response to exogenous

additions of ADP based on classic and well-characterized discoveries that endogenous ATP-hydrolyzing proteins in permeabilized muscle fibers remain active (Saks et al 1995). As such, while we do not know the precise equilibrium, we interpret the findings of these experiments in response to exogenous additions of ADP in the context of the 'presence' and cycling of both ADP and ATP, and the absence of ATP when the ATP-recycling hexokinase-based clamp is included as noted above.

Collectively, these results demonstrate the natural ability of permeabilized muscle fibers to cycle exogenous additions of ADP to ATP attenuates calcium-induced mtPT during *in vitro* assessments. Eliminating this ATP with a hexokinase system that recycles ATP back to ADP increases mtPTP sensitivity to calcium. Combining this system with high creatine and ADP concentrations causes a surprising attenuation of mtPT but the kinetics at lower concentrations requires further attention. Careful consideration of the relevance of adenylate cycling to a given experimental question could influence the design of *in vitro* assessments in permeabilized muscle fibers with the aim of gaining greater insight into the regulation of the mtPTP in various biological contexts.

Abbreviations

CRC	calcium retention capacity	mtPT	mt-permeability transition
H_2O_2	hydrogen peroxide	mtPTP	mt-permeability transition pore
mt	mitochondrial	PCr	phosphocreatine

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