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Phosphate is not an absolute requirement for the inhibitory effects of cyclosporin A or cyclophilin D deletion on mitochondrial permeability transition

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Abstract

CypD (cyclophilin D) has been established as a critical regulator of the MPT (mitochondrial permeability transition) pore, and pharmacological or genetic inhibition of CypD attenuates MPT in numerous systems. However, it has recently been suggested that the inhibitory effects of CypD inhibition only manifest when P_i (inorganic phosphate) is present, and that inhibition is lost when P_i is replaced by As_i (inorganic arsenate) or V_i (inorganic vanadate). To test this, liver mitochondria were isolated from wild-type and CypD-deficient (*Ppif*$^{-/-}$) mice and then incubated in buffer containing P_i, As_i or V_i. MPT was induced under both energized and de-energized conditions by the addition of Ca^{2+}, and the resultant mitochondrial swelling was measured spectrophotometrically. For pharmacological inhibition of CypD, wild-type mitochondria were pre-incubated with CsA (cyclosporin A) before the addition of Ca^{2+}. In energized and de-energized mitochondria, Ca^{2+} induced MPT regardless of the anion present, although the magnitude differed between P_i, As_i and V_i. However, in all cases, pre-treatment with CsA significantly inhibited MPT. Moreover, these effects were independent of mouse strain, organ type and rodent species. Similarly, attenuation of Ca^{2+} -induced MPT in the *Ppif*$^{-/-}$ mitochondria was still observed irrespective of whether P_i, As_i or V_i was present. We conclude that the pharmacological and genetic inhibition of CypD is still able to attenuate MPT even in the absence of P_i.

Keywords

arsenate; cyclophilin D; cyclosporin A; mitochondrial permeability transition (MPT); phosphate; vanadate

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AUTHOR CONTRIBUTION

Christopher Baines conceived and designed the studies. Allison McGee and Christopher Baines performed the experiments and analysed the data. Christopher Baines wrote the paper.

INTRODUCTION

The MPT (mitochondrial permeability transition) pore is a non-selective channel in the inner mitochondrial membrane, the opening of which is a critical event in the progression of cell death and therefore disease (see [1–4] for reviews). Although the specific molecular composition of the MPT pore is still highly debated, pharmacological, biochemical and genetic studies have demonstrated that the matrix peptidylprolyl isomerase CypD (cyclophilin D) is a key regulator of the MPT pore [5–12]. Consequently, pharmacological inhibitors of CypD such as CsA (cyclosporin A), sanglifehrin A and Debio 025 are effective in attenuating the MPT response [5,13,14], as is genetic disruption of the CypD-encoding *Ppif* (peptidylprolyl isomerase F) locus [10–12]. Such chemical/genetic tools have thus been used to establish a critical role for CypD-dependent MPT in the pathogenesis of several diseases, including cardiac [10,12], cerebral [11] and renal [15] ischaemia, muscular dystrophy [14] and neurodegenerative diseases such as Alzheimer's disease [16] and multiple sclerosis [17].

Many factors, including Ca^{2+}, reactive oxygen species, adenine nucleotides, pH, Mg^{2+}, ubiquinones and voltage changes, regulate the MPT pore [1–4]. In particular, elevations in mitochondrial matrix Ca^{2+} are a primary trigger for MPT pore opening, and it has been well established that the presence of P_i (inorganic phosphate) can greatly increase the sensitivity of the MPT pore to the effects of this cation [5,18,19]. In contrast, inhibition of CypD appears to attenuate the MPT response by decreasing the sensitivity of the MPT pore to matrix Ca^{2+} [9–12].

A previous study, however, has suggested that P_i may have a dual effect on the MPT pore such that it can also act as a specific inhibitor of the MPT pore as well as a sensitizer [20]. In that study, it was found that inhibition of CypD, either by gene targeting or with CsA, only attenuated the MPT response when P_i was present, but not when P_i was replaced by other inorganic anions such as arsenate (As_i) or vanadate (V_i). It was consequently proposed that CypD normally masks a P_i-specific inhibitory site on the MPT pore such that blockade/removal of CypD unmasks this site and enables P_i to bind the pore and lower its sensitivity to Ca^{2+} [20]. However, a subsequent study has reported that substitution of As_i for P_i does not affect the ability of CsA to inhibit MPT in both energized and de-energized mitochondria [21].

Consequently, it has yet to be conclusively proven that the effects of CypD inactivation on MPT require an inhibitory action of P_i on the MPT pore. To this end, we wanted to test whether substitution of either As_i or V_i for P_i altered the ability of genetic and/or pharmacological CypD to inhibit MPT. We found that mouse liver mitochondria either treated with CsA or lacking CypD still exhibited an attenuated MPT response when As_i or V_i was used in lieu of P_i. Moreover, these findings were not dependent on the energetic state of the mitochondria, the organ or the strain of mouse from which they were isolated. Thus the ability of pharmacological and genetic inhibition of CypD to attenuate MPT does not appear to be dependent on P_i.

EXPERIMENTAL

Reagents

All chemicals/reagents were from either Fisher Scientific or Sigma–Aldrich.

Mice and rats

The pure strain FVB and C57BL/6 mice used for the CsA studies were purchased from Taconic. The *Ppif*$^{+/+}$ and *Ppif*$^{-/-}$ mice on a mixed C57BL/6-sv129 background have been

described previously [12]. Sprague–Dawley rats were purchased from Taconic. Both the mice and rats were used at ~8–9 weeks of age. All of the experiments involving mice and rats were approved by the University of Missouri Animal Care and Use Committee and conformed to NIH Guidelines for the Care and Use of Animals.

Mitochondrial isolation and swelling

Liver and kidney mitochondria were prepared from the different mice and rats by differential centrifugation in sucrose-based medium, as described previously [12]. The protein concentration was determined using the Bradford assay (Bio-Rad Laboratories), and the mitochondria were resuspended in swelling buffer at 0.25 mg/ml. For energized conditions, the buffer (pH 7.4) consisted of 120 mM KCl, 10 mM Tris/HCl, 5 mM glutamate, 2.5 mM malate and 5 mM KH_2PO_4, KH_2 AsO_4 or Na_3VO_4. The same buffer, but without glutamate and malate, was used for de-energized conditions. When necessary, the MPT inhibitor CsA (1 μM) was included in the swelling buffer. MPT was measured spectrophotometrically as a decrease in light scattering at 520 nm [12] and was induced by the addition of 25 μM (energized) or 250 μM (de-energized) $CaCl_2$.

Statistical analyses

Statistical significance was calculated using Student's *t* test. A P value <0.05 was considered statistically significant.

RESULTS

CsA can still inhibit MPT in the absence of P_i

We first examined the effects of P_i replacement on the ability of CsA to attenuate MPT in energized liver mitochondria from FVB mice. Substitution of either As_i or V_i for P_i did not affect the ability of Ca^{2+} to induce large-amplitude swelling of liver mitochondria (Figure 1). There were subtle differences in the magnitude of the swelling, with the greatest response observed with As_i, followed by P_i and then V_i. However, despite these differences, pre-treatment of the mitochondria with 1 μM CsA was able to significantly inhibit the swelling response irrespective of which anion was present in the buffer (Figure 1). We then repeated these studies in de-energized mitochondria. Interestingly, under these conditions, the largest swelling response was observed with P_i, rather than with As_i, with the least observed in the presence of V_i (Figure 2). Again, however, CsA was still able to significantly attenuate mitochondrial swelling whether the anion was P_i, As_i or V_i (Figure 2).

The P_i-independent effects of CsA are not strain-, tissue- or species-dependent

In the experiments described above, we utilized the FVB inbred mouse strain, as this is a strain commonly used for the generation of transgenic animals. However, the previous study suggesting an inhibitory role for P_i in MPT [20] used C57BL/6 mice. Therefore it was possible that the negative results observed in our FVB mitochondria were simply due to strain differences. To test for this we re-examined the effects of CsA in de-energized liver mitochondria isolated from C57BL/6 mice. The magnitude of the MPT response to Ca^{2+} observed in the presence of P_i, As_i or V_i followed the same pattern in the C57BL/6 liver mitochondria (Figure 3) as it did in the FVB liver mitochondria, i.e. P_i>As_i>V_i. Importantly, 1 μM CsA was still able to attenuate swelling in the C57BL/6 mitochondria independent of the species of anion (Figure 3). We also examined whether tissue differences existed by isolating kidney mitochondria. Interestingly, unlike liver mitochondria, the degree of swelling in response to Ca^{2+} was similar between the three anions in de-energized kidney mitochondria (Figure 4). However, in all three cases, CsA was still able to inhibit MPT

(Figure 4). Finally, we assessed whether mouse liver mitochondria behaved differently from rat liver mitochondria. However, in the rat organelles we obtained results identical with those seen with the mice (Figure 5), both in terms of the $P_i > As_i > V_i$ hierarchy of swelling amplitude and the ability of CsA to attenuate MPT independent of the anion.

Inhibition of MPT by *Ppif* gene targeting is still present in the absence of P_i

In the final set of experiments, we tested the effects of P_i substitution on the inhibitory effect of *Ppif* (the locus encoding CypD) gene ablation on MPT. Ca^{2+} was able to induce robust swelling of energized $Ppif^{+/+}$ liver mitochondria in buffer that contained P_i, As_i or V_i (Figure 6), following the same pattern as that seen in the CsA experiments. In all three cases, the degree of swelling was markedly blunted in the CypD-lacking $Ppif^{-/-}$ mitochondria (Figure 6). This inhibitory effect was even more pronounced in de-energized $Ppif^{-/-}$ mitochondria, and was again independent of the anion species (Figure 7).

DISCUSSION

It has long been known that P_i can sensitize the MPT pore to the effects of matrix Ca^{2+}. Consequently, the majority of studies examining the MPT response in isolated mitochondria, especially with regard to CypD inhibition, include P_i in the assay medium. However, Bernardi's group reported that the ability of CypD inhibition (genetic or pharmacological) to negatively regulate the MPT pore is surprisingly lost when P_i is omitted from the buffer and replaced with other inorganic anions (in order to maintain Ca^{2+} uptake into the mitochondria) [20]. This was consistent with earlier studies suggesting that P_i could strengthen the inhibitory effect of CsA on MPT in isolated mitochondria [22], and that As_i-dependent MPT was less sensitive to CsA [23].

To confirm these findings, we repeated the experiments in mouse liver mitochondria that were either treated with CsA or were completely lacking CypD (i.e. from $Ppif^{-/-}$ mice). However, in direct contrast with the previous study [20], we found that these mitochondria still exhibited a diminished MPT response compared with untreated/wild-type mitochondria regardless of whether it was P_i, As_i or V_i, in the assay buffer. Consequently, our findings are, in fact, more in line with another recent study in rat liver mitochondria where the anti-MPT effects of CsA were apparent in the presence of either P_i or As_i [21]. Brand's group has also reported a maintained ability of CsA to block MPT in the absence of P_i in brown adipose mitochondria [24].

The reason for these discrepancies is not immediately apparent. We used the same species (mouse), organ (liver) and substitute anions (As_i and V_i) as Bernardi's group. One concern was that our initial studies (Figures 1 and 2) were implemented in inbred FVB mice, which we traditionally use for generating transgenic mice, whereas the study by Basso et al. [20] utilized C57BL/6 mice. Our $Ppif^{+/+}$ and $Ppif^{-/-}$ mice are also in a mixed C57BL/6-sv129 background as opposed to pure C57BL/6. Thus the differences in results could purely be due to mouse strain differences. Indeed, profound differences have been reported between FVB, C57BL/6 and/or sv129 strains with regard to their sensitivity to cardiac [25] and cerebral [26] ischaemia/reperfusion injury as well as neuronal excitotoxicity [27], all of which involve opening of the MPT pore.

However, we found that CsA was still able to inhibit MPT in the presence of As_i or V_i in liver mitochondria isolated from C57BL/6 mice. Therefore strain differences do not appear to account for the disparate datasets. Furthermore, this P_i-independent effect of CsA was still observable in mitochondria from another tissue (kidney) and even another species (rat); the latter recapitulating the findings of the earlier rat study [18].

Another difference is that we utilized mitochondrial swelling as our index of MPT, whereas Bernardi's group employed the CRC (calcium-retention capacity) assay [20]. Although unlikely, it is possible that these different assays could yield different results. However, Varanyuwatana and Halestrap [21] simultaneously measured swelling and CRC in energized mitochondria, and found that, in both cases, replacement of P_i did not affect the inhibitory effect of CsA. Also consistent with that study, we found that the anion-independent inhibition of MPT by CsA was observed in both energized and de-energized mitochondria. We observed a similar pattern in the *Ppif*$^{-/-}$ mitochondria too. We did use a higher concentration of anion than Basso et al. [20] and Varanyuwatana and Halestrap [21] (5 mM compared with 1 mM). It is therefore possible that we have bypassed the 'inhibition' aspect of P_i with this higher amount, such that we are only in the 'activation' range. However, this would still fail to account for the fact that, in our study, both CsA and CypD deletion were effective even in the absence of P_i.

Interestingly, in the course of these studies, we observed that, whereas the magnitude of the swelling response to Ca^{2+} followed an As_i> P_i> V_i pattern in energized liver mitochondria, it instead followed a P_i> As_i> V_i pattern under de-energized conditions. Indeed, both of the previous studies reported an As_i> P_i response in energized mitochondria [20,21] and Varanyuwatana and Halestrap [21] also found that this was reversed in de-energized mitochondria. Why the sensitizing effects of P_i and As_i differ depending on energetic state is not clear. Changes in the ability of each anion to bind matrix Ca^{2+} or Mg^{2+} under the different conditions would influence their ability to affect MPT, as has been speculated [21]. Alternatively, under energized conditions, the mitochondrial ATP synthase would use the As_i to create ADP–As adducts instead of ATP [28]. The non-enzymatic hydrolysis of these adducts would create a futile cycle between ADP and ADP–As [29]. This, coupled with a reduction in ATP, would reduce the inhibitory effects of adenine nucleotide levels on the MPT pore, thereby further sensitizing it to Ca^{2+}. It should be noted, however, that the swelling amplitude in the kidney mitochondria was independent of the anion present, i.e. the response was much the same whether it was P_i, As_i or V_i present. The reasons for this are not clear, but may suggest different regulatory factors/proteins in the kidney compared with the liver.

Acknowledgments

FUNDING

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Abbreviations used

As_i	inorganic arsenate
CRC	calcium-retention capacity
CsA	cyclosporin A
CypD	cyclophilin D
MPT	mitochondrial permeability transition
P_i	inorganic phosphate
Ppif	peptidylprolyl isomerase F
V_i	inorganic vanadate

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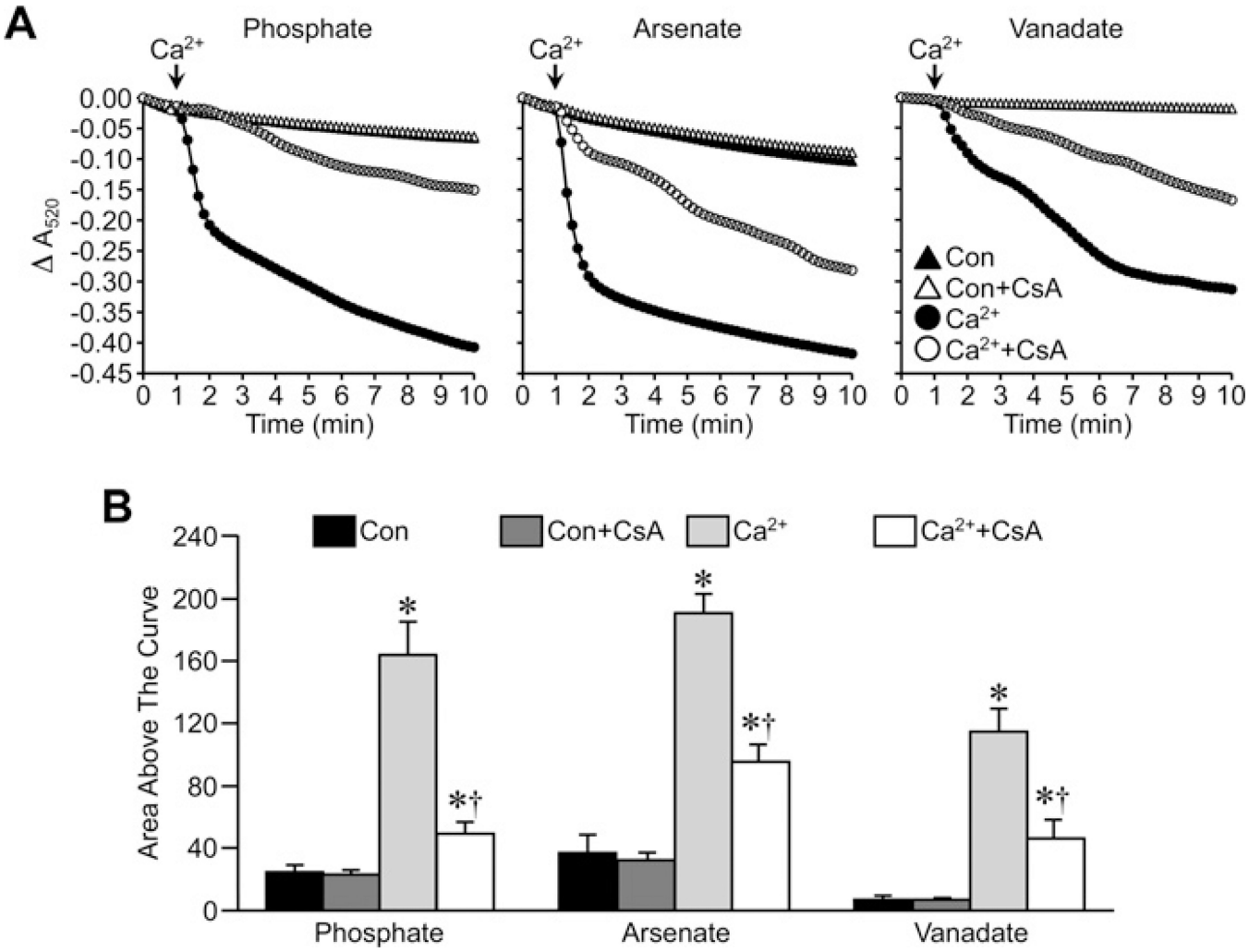

Figure 1. Inhibition of MPT by CsA in energized mitochondria does not require P_i

(**A**) Energized liver mitochondria from FVB mice were incubated in swelling buffer containing 5 mM P_i, As_i or V_i with or without 1 μM CsA. Mitochondrial swelling was then induced by the addition of 25 μM $CaCl_2$ and monitored as a decrease in absorbance (A_{520}). (**B**) Group data, calculated as the area above the curve, for the swelling curves shown in (**A**). All results shown are representative of four independent experiments and are means ± S.E.M. *$P < 0.05$ compared with control (Con) and †$P < 0.05$ compared with Ca^{2+} alone.

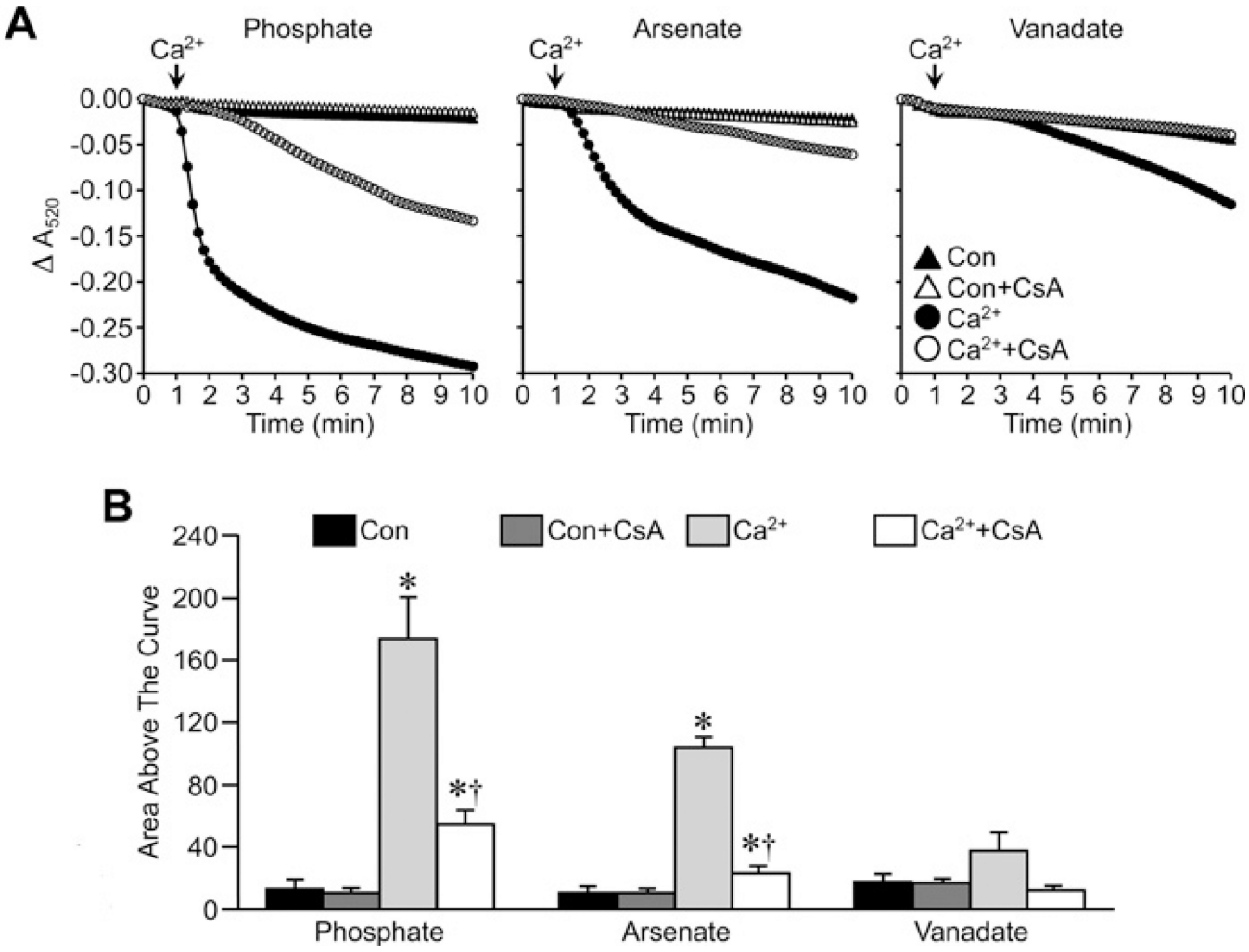

Figure 2. Inhibition of MPT by CsA in de-energized mitochondria does not require P_i

(**A**) De-energized liver mitochondria from FVB mice were incubated in swelling buffer containing 5 mM P_i, As_i or V_i with or without 1 μM CsA. Mitochondrial swelling was then induced by the addition of 250 μM $CaCl_2$ and monitored as a decrease in absorbance (A_{520}). (**B**) Group data, calculated as the area above the curve, for the swelling curves shown in (**A**). All results shown are representative of five independent experiments and are means ± S.E.M. *$P < 0.05$ compared with control (Con) and †$P < 0.05$ compared with Ca^{2+} alone.

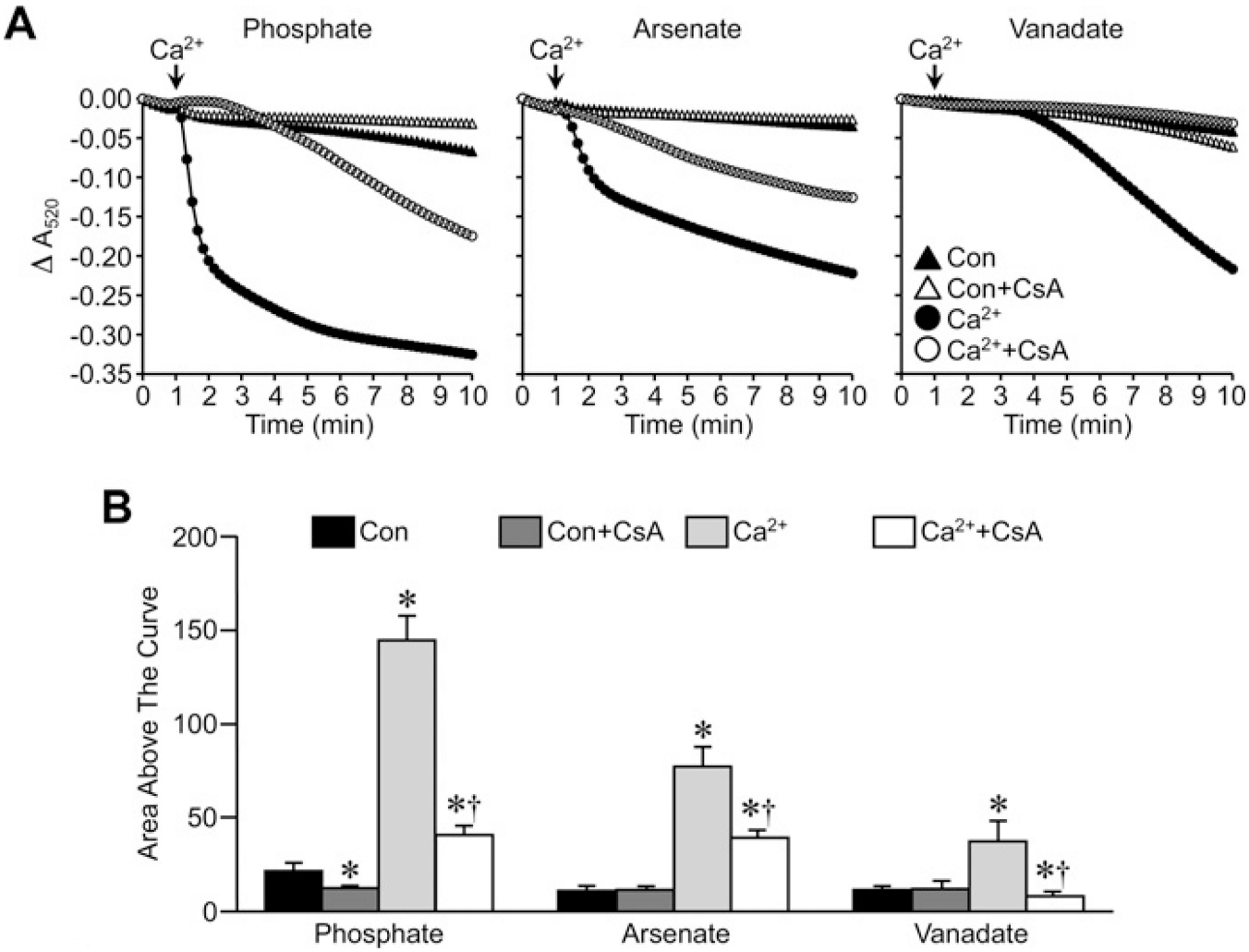

Figure 3. Inhibition of MPT by CsA in mitochondria from C57BL/6 mice does not require P_i

(**A**) De-energized liver mitochondria from C57BL/6 mice were incubated in swelling buffer containing 5 mM P_i, As_i or V_i with or without 1 μM CsA. Mitochondrial swelling was then induced by the addition of 250 μM $CaCl_2$ and monitored as a decrease in absorbance (A_{520}). (**B**) Group data, calculated as the area above the curve, for the swelling curves shown in (**A**). All results shown are representative of five independent experiments and are means ± S.E.M. *$P < 0.05$ compared with control (Con) and †$P < 0.05$ compared with Ca^{2+} alone.

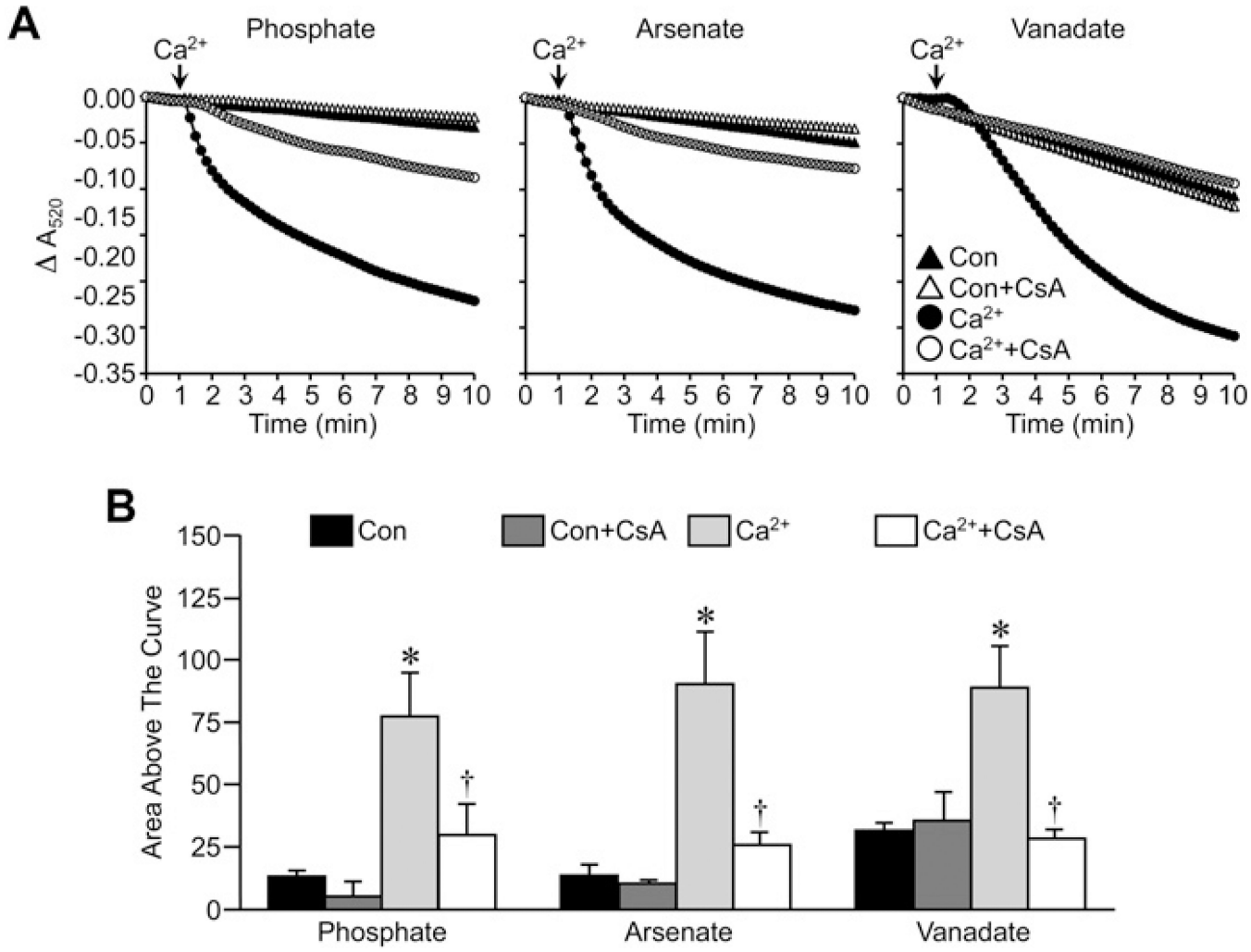

Figure 4. Inhibition of MPT by CsA in mouse kidney mitochondria does not require P_i

(**A**) De-energized kidney mitochondria from FVB mice were incubated in swelling buffer containing 5 mM P_i, As_i or V_i with or without 1 μM CsA. Mitochondrial swelling was then induced by the addition of 250 μM $CaCl_2$ and monitored as a decrease in absorbance (A_{520}). (**B**) Group data, calculated as the area above the curve, for the swelling curves shown in (**A**). All results shown are representative of four independent experiments and are means ± S.E.M. *$P < 0.05$ compared with control (Con) and †$P < 0.05$ compared with Ca^{2+} alone.

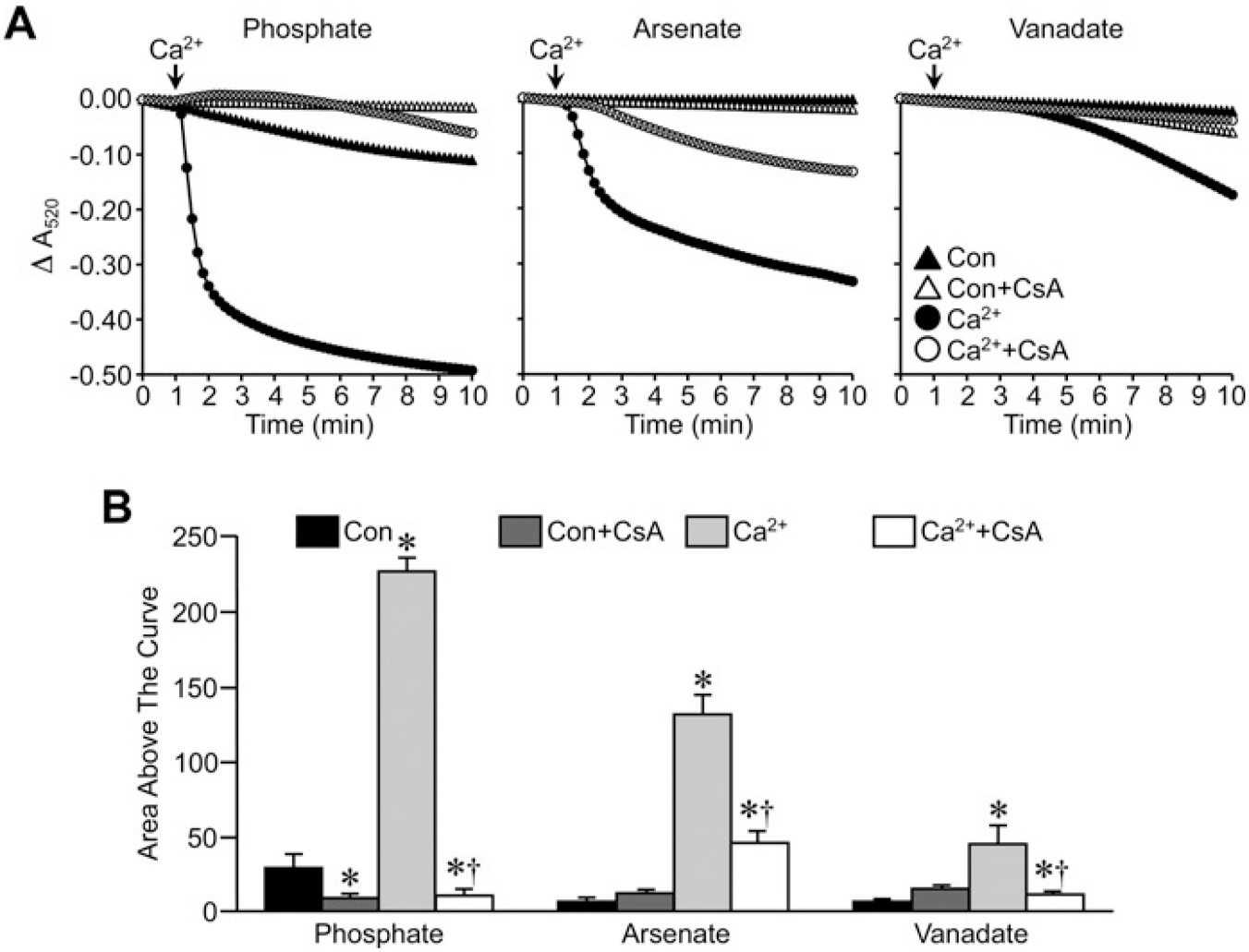

Figure 5. Inhibition of MPT by CsA in rat liver mitochondria does not require P_i

(**A**) De-energized liver mitochondria from Sprague–Dawley rats were incubated in swelling buffer containing 5 mM P_i, As_i or V_i with or without 1 μM CsA. Mitochondrial swelling was then induced by the addition of 250 μM $CaCl_2$ and monitored as a decrease in absorbance (A_{520}). (**B**) Group data, calculated as the area above the curve, for the swelling curves shown in (**A**). All results shown are representative of four independent experiments and are means ± S.E.M. *$P < 0.05$ compared with control (Con) and †$P < 0.05$ compared with Ca^{2+} alone.

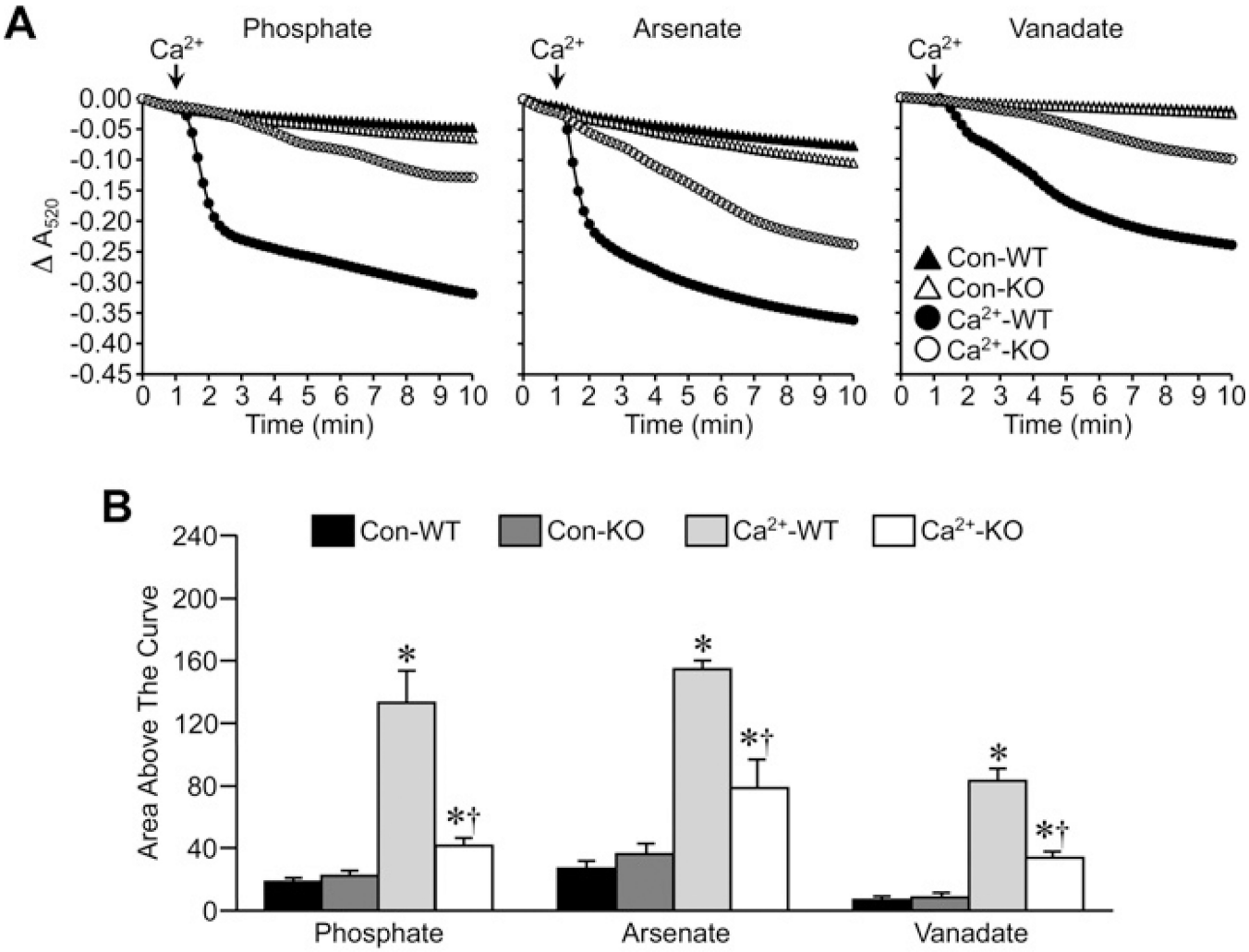

Figure 6. Inhibition of MPT by *Ppif* gene targeting in energized mitochondria does not require P_i

(**A**) Energized liver mitochondria from *Ppif*$^{+/+}$ (WT) and *Ppif*$^{-/-}$ (KO) mice were incubated in swelling buffer containing 5 mM P_i, As_i or V_i. Mitochondrial swelling was then induced by the addition of 25 μM $CaCl_2$ and monitored as a decrease in absorbance (A_{520}). (**B**) Group data, calculated as the area above the curve, for the swelling curves shown in (**A**). All results shown are representative of four independent experiments and are means ± S.E.M. *$P < 0.05$ compared with control (Con-WT) and †$P < 0.05$ compared with Ca^{2+} -WT.

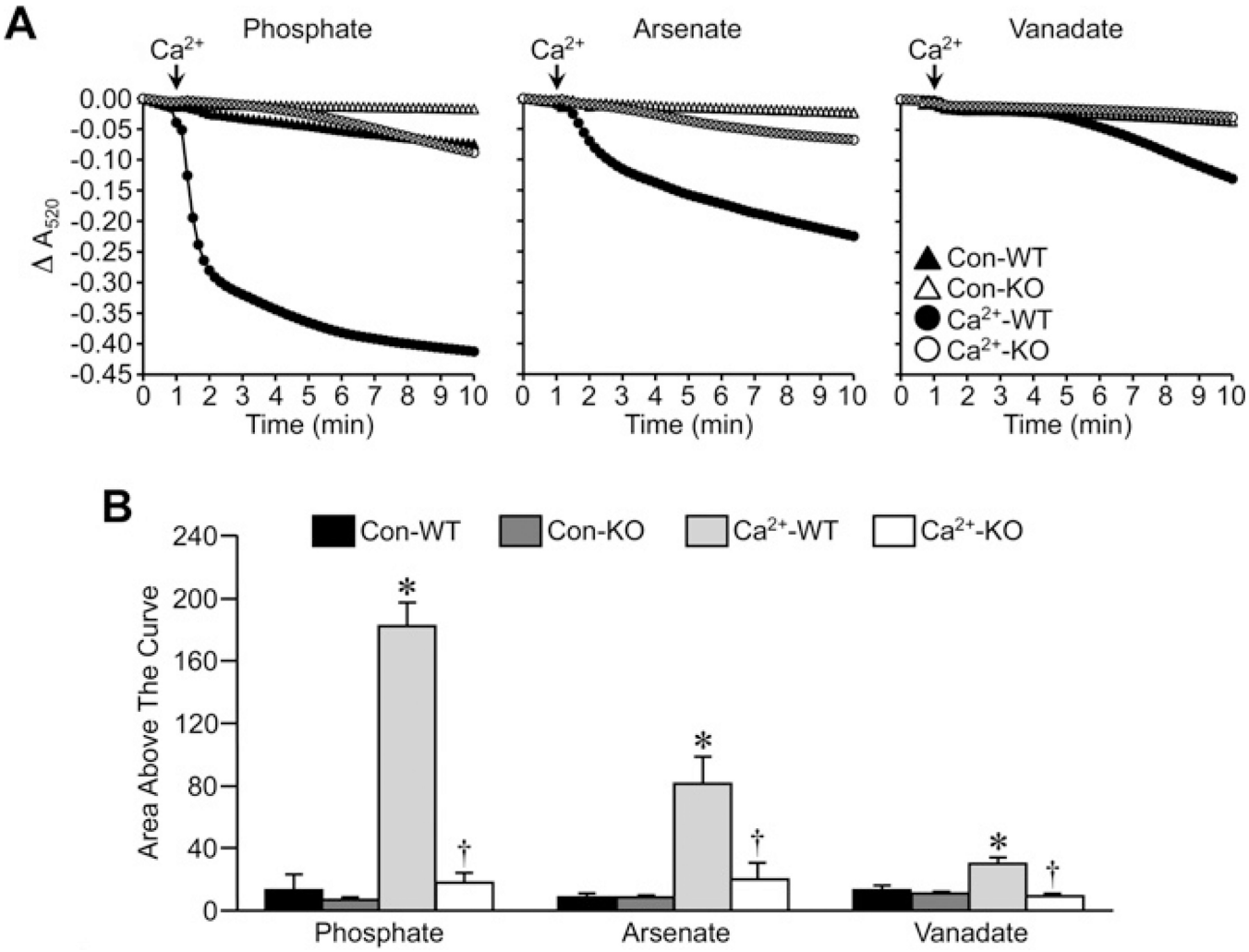

Figure 7. Inhibition of MPT by *Ppif* gene targeting in de-energized mitochondria does not require P_i

(**A**) De-energized liver mitochondria from *Ppif*$^{+/+}$ (WT) and *Ppif*$^{-/-}$ (KO) mice were incubated in swelling buffer containing 5 mM P_i, As_i or V_i. Mitochondrial swelling was then induced by the addition of 250 μM $CaCl_2$ and monitored as a decrease in absorbance (A_{520}). (**B**) Group data, calculated as the area above the curve, for the swelling curves shown in (**A**). All results shown are representative of four independent experiments and are means ± S.E.M. *$P < 0.05$ compared with control (Con-WT) and †$P < 0.05$ compared with Ca^{2+}-WT.