Biochemical Pharmacology, Vol. 38, No. 21, pp. 3873–3878, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

EFFECT OF VERAPAMIL ON PHOSPHATE-INDUCED CHANGES IN OXIDATIVE PHOSPHORYLATION AND ATRACTYLOSIDE-SENSITIVE ADENINE NUCLEOTIDE TRANSLOCASE ACTIVITY IN TWO POPULATIONS OF RAT HEART MITOCHONDRIA

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(*Received* 13 *September* 1988; *accepted* 31 *March* 1989)

Abstract—Phosphate (P_i)-induced depression in cardiac mitochondrial function was studied using mitochondria isolated by two different procedures which purportedly yield two distinct populations. Subsarcolemmal mitochondria (SLM) exhibited an enhanced sensitivity to 20 mM P_i with respect to oxidative phosphorylation. Thus, a significant depression in oxidative phosphorylation in this population was seen following only 1-min treatment, whereas interfibrillar mitochondria (IFM) were unaffected. Both populations showed a similar response to 5-min treatment with P_i. The P_i-induced depression in respiration was partially, although significantly, reversed by a 50 μM concentration of the calcium antagonist verapamil, an observation which suggests a contribution of calcium to the P_i-induced defect in respiration. P_i also produced a potent inhibition of ADP uptake in both mitochondrial populations, which was in close agreement to P_i-induced modification of low amplitude shrinkage–swelling responses following ADP addition. Both of these parameters were unaffected by verapamil. Our results show an enhanced sensitivity of SLM to a verapamil-sensitive P_i-induced depression in oxidative phosphorylation. However, the potent, verapamil-insensitive decrease in adenine nucleotide translocase activity by P_i demonstrates that calcium is likely only partially involved in P_i-induced depression in oxidative phosphorylation and that a further partial contribution arises from a decrease in adenine nucleotide translocase activity.

One of the earliest events associated with myocardial ischemia is the accumulation of high concentrations of intracellular phosphate [1, 2], a factor which has been shown to depress cardiac contractility [3–6]. These observations have led to the hypothesis that P_i may account for, or contribute to, the contractile depression associated with early myocardial ischemia [1, 7]. Although interference with cardiac contractile proteins has been suggested as a possible mechanism for P_i-induced contractile depression [6], a precise explanation for the P_i effect remains to be fully resolved. P_i-induced defective mitochondrial function has been well documented [8–11]. The mechanism underlying this effect is uncertain. However, the ability of verapamil to partially reverse P_i-induced mitochondrial swelling and depression in oxidative phosphorylation has led to the suggestion of the involvement of an intramitochondrial calcium pool [10, 11]. Another possible mechanistic basis for P_i-induced mitochondrial injury may be related to the P_i-induced efflux of adenine nucleotides from the mitochondria, thus inhibiting the ADP/ATP translocase activity and resulting in the inhibition of oxidative phosphorylation [8].

Previous reports have demonstrated the existence of two populations of cardiac mitochondria possessing distinct biochemical properties [12, 13]. We have shown previously that subsarcolemmal mitochondria (SLM) demonstrate an enhanced sensitivity to hypoxia, but more so to the injurious influence of P_i, compared to interfibrillar mitochondria (IFM) [14]. In the present report, we examined the interrelationship between oxidative phosphorylation, ADP/ATP translocase activity and low-amplitude swelling following P_i treatment of SLM and IFM. We also examined the ability of verapamil to modify individual parameters under study. We report that verapamil was effective in reversing P_i-induced depression of oxidative phosphorylation, but was without effect on either swelling or translocase activity, suggesting separate mechanisms underlying P_i-induced mitochondrial injury.

MATERIALS AND METHODS

Mitochondrial isolation. Two populations of cardiac mitochondria were isolated from the ventricles of male Sprague–Dawley rats (250–275 g, Canadian Hybrid Farms, Centreville, Nova Scotia), by a modified procedure of Palmer *et al.* [12, 13], as described in detail previously [14]. Both populations of mitochondria were finally suspensed in a buffer containing (in mM) 125 KCl, 5 $MgCl_2$, 2 Tris, pH 7.4, at 25°. All solutions were prepared using distilled and deionized water. Mitochondrial protein concentration was determined by the Lowry method.

Mitochondrial incubation. Mitochondria were incubated in the final suspension medium noted

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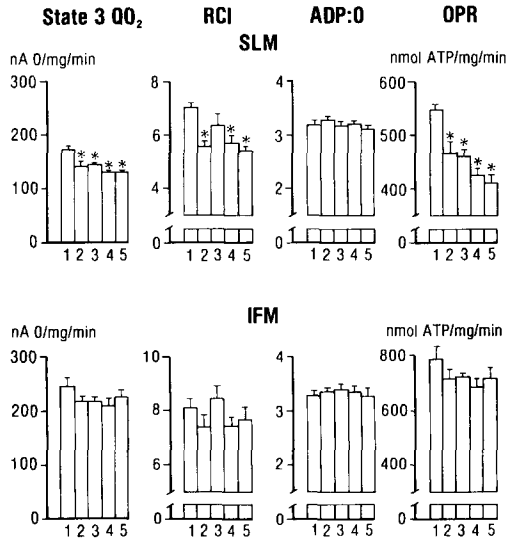

Fig. 1. Oxidative phosphorylation of SLM and IFM after 1-min treatments. Treatment 1 represents oxygenated controls without P_i. Treatment 2 represents oxygenated samples treated with 10 mM P_i, whereas treatments 3, 4 and 5 indicate mitochondria incubated under hypoxic conditions in the presence of 10 mM P_i, 20 mM P_i and 20 mM P_i plus 2 mM succinate respectively. The gassing system consisted of stoppered flasks connected to either a 95% O_2 or 95% N_2 (balance CO_2) gas mixture. Mitochondria were incubated in a shaking water bath at 37°. Each bar is the mean ± SE of six experiments. Asterisks indicate significant differences ($P < 0.05$) from control, as determined by ANOVA followed by SNK multiple comparison tests.

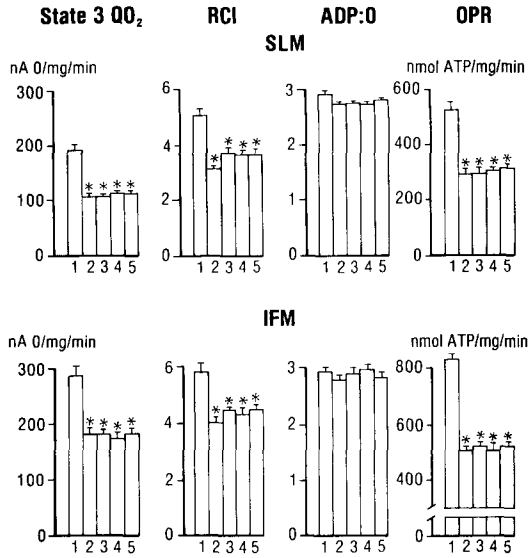

Fig. 2. Oxidative phosphorylation of SLM and IFM after 5-min treatments. See legend of Fig. 1 for experimental details. Each bar is the mean ± SE of six experiments. Asterisks indicate significant differences ($P < 0.05$) from control, as determined by ANOVA followed by SNK multiple comparison tests.

above in a shaker water bath under different conditions as indicated in Results. Incubations were carried out for either 1 or 5 min as noted in Results. For experiments in which the effect of verapamil (50 μM) was examined, the drug or its vehicle was added to the mitochondrial suspension immediately prior to P_i administration. The incubation temperature was kept at 37°.

Oxidative phosphorylation assay. Mitochondrial oxidative phosphorylation was assessed in a Gilson oxygraph as described previously [15]. For each assay, 1 mg of mitochondrial protein was added to a medium (pH 7.4) (SKHEPM) containing 250 mM sucrose, 5 mM KH_2PO_4, 2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM EDTA, 10 mM pyruvate, and 2.5 mM malate. Oxidative phosphorylation was measured at 30° in terms of state 3 QO_2, respiratory control index (RCI), and ADP:O ratio, as described previously [15]. The oxidative phosphorylation rate (OPR) was calculated according to Edoute *et al.* [16] and expressed as nanomoles ATP synthesized per milligram of mitochondrial protein per minute.

Mitochondrial swelling and shrinkage. Mitochondrial swelling and shrinkage was measured spectrophotometrically (Pye-Unicam model SP8-200) by a method modified from Hunter and Smith [17]. Briefly, 1 mg of mitochondrial protein was added to 2.5 ml of SKHEPM buffer. The light absorbance changes at 520 nm were recorded immediately at a sensitivity of 0.1 O.D., using the buffer as reference. Two minutes later, 600 nmol of ADP was added and the solution quickly stirred. The recording of absorbance changes was continued for another 5 min.

ADP/ATP translocase activity. The method for determining translocase activity was modified from Duee and Vignais [18]. The reaction buffer contained 110 mM KCl, 20 mM Tris, 1 mM EDTA, pH 7.4, at 25°. Fifty nanomoles of ADP (with 0.02 μCi [^{14}C]ADP) was added to 150 μl buffer immediately before commencing the assay. Mitochondria (0.5 mg protein) were added to initiate the reaction. The ADP uptake reaction was stopped at different time intervals with 0.1 mM atractyloside as indicated by the reaction time in the figures. Each sample was centrifuged at 25,000 *g* for 5 min. The supernatant fraction was discarded, and the pellet was washed twice with buffer and dissolved in 0.2 ml of 0.1 N NaOH. The mixture was suspended in 2.5 ml of Beckman scintillation solution and radioactivity was counted in a Searle Mark III scintillator counter. Time-dependent ADP uptake per milligram of mitochondrial protein was calculated using [^{14}C]ADP as standard. ADP/ATP translocase activity was determined as atractyloside-sensitive ADP uptake.

RESULTS

Inhibition of mitochondrial oxidative phosphorylation. Figure 1 shows the effects of preincubation under different conditions on mitochondrial respiration. Hypoxia, inclusion of succinate as substrate, or increasing the P_i concentration above 10 mM did not augment the 10 mM P_i-induced mitochondrial depression on state 3 QO_2, RCI and OPR in SLM after 1-min treatment. Other factors tested

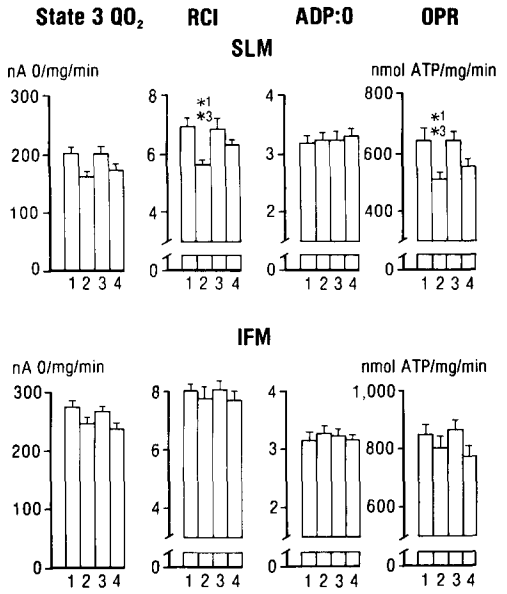

Fig. 3. Effect of verapamil on P_i-induced depression of mitochondrial oxidative phosphorylation after 1-min treatments. Treatment 1 represents control experiments with vehicles of P_i and verapamil only. Treatments 2, 3, and 4 represent samples treated with 20 mM P_i plus verapamil vehicle, P_i vehicle plus 50 μM verapamil, and 20 mM P_i plus 50 μM verapamil respectively. All samples were treated at 37° in the shaking water bath for 1 min under room air. Each bar is the mean ± SE of six experiments. Asterisks indicate significant depression compared with treatments indicated by treatment numbers following the asterisks ($P < 0.05$). The same statistical test was used as for Fig. 1.

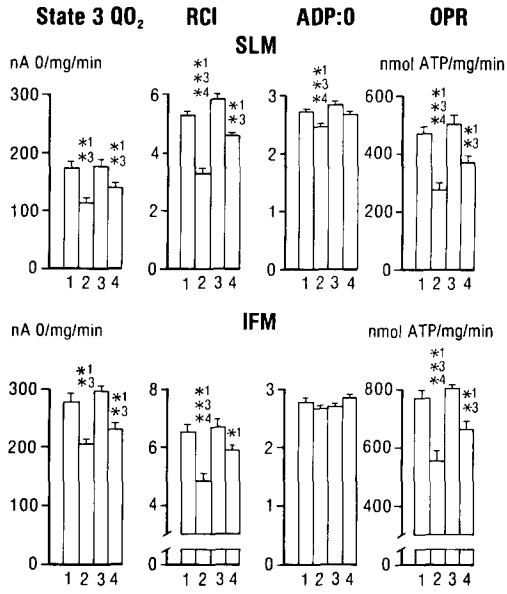

Fig. 4. Effect of verapamil on P_i-induced depression of mitochondrial oxidative phosphorylation inhibition after 5-min treatments. See legend of Fig. 3 for experimental details, except that all samples were treated for 5 min. Each bar is the mean ± SE of six experiments. Asterisks indicate significant depression compared with treatments indicated by treatment numbers following the asterisks ($P < 0.05$). The same statistical test was used as for Fig. 1.

which mimic ischemic conditions including lactate and acidosis produced no direct effect nor did they add to the P_i-induced depression (data not shown). As Fig. 1 shows, IFM were resistant to P_i-induced depression following 1-min incubation. With 5-min incubation, SLM and IFM responded in similar fashion to P_i treatment (Fig. 2).

As shown in Figs 3 and 4, the P_i-induced depression was partially reversed by the addition of verapamil, at a concentration which produced no direct effect on oxidative phosphorylation. Following 1-min treatment (Fig. 3), the significant P_i-induced depression on RCI and OPR in SLM, was partially reversed if verapamil was included. However, 1-min treatment, with or without the presence of verapamil, did not change these functions in IFM significantly.

For the 5-min treated samples (Fig. 4), verapamil also produced a significant partial reversal in RCI, ADP:O and OPR in SLM. In IFM, only RCI and OPR depressions were reversed by verapamil owing to the fact that ADP:O was not depressed significantly by P_i in this population.

Swelling and shrinkage responses. Figure 5 summarizes the swelling–shrinkage responses of SLM and IFM to 5-min preincubation in the presence of different factors. The controls (both populations) showed responses similar to those of untreated samples, i.e. a small initial swelling (indicated by a small decrease in light absorbance) and shrinkage due to ADP addition (indicated by the light absorbance increase). Preincubation for 5 min with 20 mM P_i substantially modified the swelling–shrinkage responses, especially the latter, after ADP addition. Preincubation with verapamil did not alter this response in the presence or absence of P_i. Although less pronounced, P_i effects were observed after 1-min incubation; the overall trend in the swelling–shrinkage response, as well as the failure of verapamil to prevent these changes, remained virtually identical to that of the 5-min incubation study (data not shown).

ADP/ATP translocase activity. Although the atractyloside-nonsensitive portion (zero reaction time) of ADP uptake was not significantly different between untreated SLM and IFM, the atractyloside-sensitive ADP/ATP translocase activity was significantly higher in IFM than in SLM (Fig. 6).

Pretreatment for 1 min with 20 mM P_i significantly depressed ADP/ATP translocase activity in both SLM and IFM. This depression was not affected by verapamil (Fig. 7). With 5-min incubation, ADP/ATP translocase activity was much lower even in the controls, whereas the P_i-treated samples exhibited an almost total loss of atractyloside-sensitive ADP uptake (data not shown). Verapamil had no effect on the P_i-induced depression.

Relationship between mitochondrial ADP/ATP translocase activity and OPR. We initially analyzed the relationship between the atractyloside-sensitive ADP uptake and OPR from six control experiments within each individual time treatment and observed

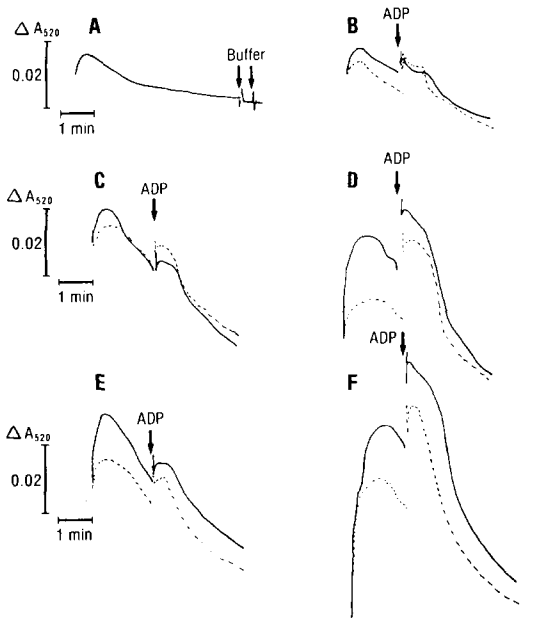

Fig. 5. Typical recording tracings (of four experiments) showing swelling–shrinkage responses of IFM (dashed lines) and SLM (solid lines). Key: (A) control response of untreated IFM without ADP addition; (B) untreated mitochondria; (C) mitochondria pretreated with vehicles of P_i and verapamil; (D) mitochondria pretreated with 20 mM P_i plus verapamil vehicle; (E) mitochondria pretreated with 50 μM verapamil plus P_i vehicle; and (F) mitochondria pretreated with 20 mM P_i plus 50 μM verapamil. All pretreatments used were for 5-min durations at 37°.

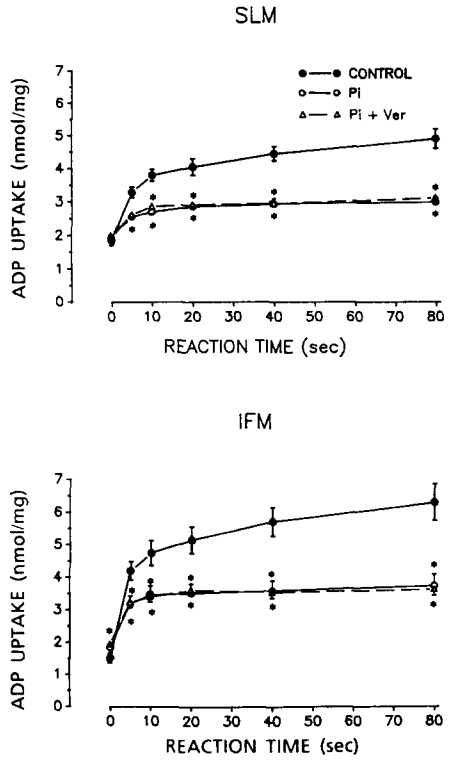

Fig. 7. P_i effect on mitochondrial ADP uptake, with or without the presence of 50 μM verapamil. SLM and IFM were incubated at 37° for 1 min. Each point is the mean ± SE of six experiments. Key: (*) $P < 0.05$ from control (ANOVA).

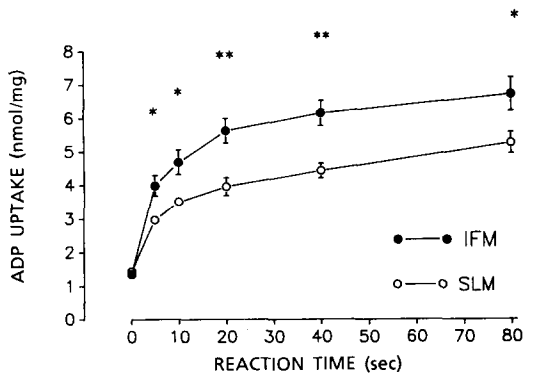

Fig. 6. ADP uptake by untreated mitochondria. Each point is the mean ± SE of six experiments. Key: (*) $P < 0.05$; and (**) $P < 0.01$ between the two populations (two-tailed paired t-test).

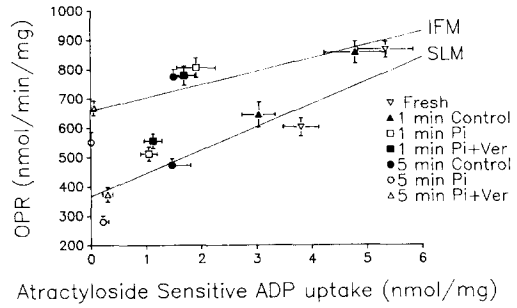

Fig. 8. Relationship between the mean of the maximum atractyloside-sensitive ADP uptake and the mean of mitochondrial OPR. Regression lines are pooled data for SLM and IFM, respectively, after various treatments as indicated by symbols. The slopes for the regression lines for IFM and SLM were $y = 79X + 368$, $r = 0.832$, $P < 0.05$, and $y = 45X + 659$, $r = 0.849$, $P < 0.05$, respectively. Each point is the mean ± SE of six experiments.

no significant correlation between the two parameters. However, when data were pooled for freshly isolated, control incubated or P_i-treated SLM and IFM, a significant correlation between OPR and atractyloside-sensitive ADP uptake was evident for both mitochondrial populations (Fig. 8). However, the slopes of the linear correlations between ADP uptake and OPR were obviously different for the two populations. Although not shown in Fig. 8, the significant correlation was maintained when data were combined for both mitochondrial populations.

DISCUSSION

Although the deleterious effects of P_i on cardiac mitochondrial oxidative phosphorylation have been reported previously [10, 11], our study shows for the

first time a differential sensitivity of two mitochondrial populations to P_i treatment. Moreover, we report a rapid verapamil-insensitive depression in ADP/ATP translocase by P_i. The results reported in the present study may be of importance in the following terms: (1) the rapid mitochondrial dysfunction, especially in SLM, by P_i may suggest a possible mechanism for cardiac contractile failure during early ischemia; and (2) the P_i-induced mitochondrial dysfunction may consist of different components, one of which may be related to an intramitochondrial calcium pool and, therefore, partially reversible by verapamil.

The decrease in cardiac contractility during acute ischemia occurs very rapidly, with a substantial decline after 1 min, and almost total loss within 5–10 min. Considering the importance of an energy source for maintaining normal excitation–contraction coupling, ATP depletion has been suggested as a possible mechanism to explain this rapid contractile failure [19]. One report demonstrated a 25% decrease in ATP and a 50% decrease in creatine phosphate during the first 5 sec of anoxia in the rat heart [19]; however, most others reported only a small reduction in total ATP content of the myocardium accompanying the early ischemia [20, 21]. This dissociation between ATP depletion and contractile depression has led to the speculation that compartmentalization of ATP exists in the myocardium, i.e. a pool sensitive to ischemia-induced depletion [7, 22].

Early myocardial ischemia is accompanied by a rapid rise in intracellular P_i [1, 2]. Although the rapid (within 1 min) accumulation of P_i from 2–5 mM normal physiological levels to 15–20 mM during ischemia or hypoxia is well documented [2, 23], the precise significance of this remains to be firmly established. The present study shows that P_i at physiological concentrations substantially depressed mitochondrial function. These concentrations are compatible with those found to depress contractility of skinned cardiac cells [3, 5]. A particularly relevant observation was the substantial inhibition in SLM oxidative phosphorylation after only 1-min exposure to P_i. The anatomic location of SLM could suggest that these mitochondria play a key role in the maintenance of normal sarcolemmal function. For instance, if SLM provide a critical pool for ATP-dependent sarcolemmal function, then it would be attractive to speculate that a reduction in SLM ATP production to critical levels could ultimately result in defective intracellular homeostasis, which may account for, or contribute to, contractile depression accompanying early myocardial ischemia.

The partial reversal of P_i-induced depression in oxidative phosphorylation by verapamil is in agreement with previous reports from others who used a mixed pool representing both mitochondrial populations [10]. Similarly, ruthenium red, an inhibitor of mitochondrial calcium transport, was also demonstrated to inhibit P_i-induced mitochondrial dysfunction [11]. These authors suggested the possible involvement of intramitochondrial calcium in P_i-induced mitochondrial injury as a basis to explain the verapamil effect [10, 11]. However, our mitochondrial swelling and shrinkage studies failed to demonstrate a similar protective effect of verapamil. Thus, we did not see any substantial modification of the spontaneous mitochondrial swelling due to P_i preincubation by verapamil, even with prolonged monitoring (data not shown). Also, there was a substantial increase of the ADP-induced shrinkage with P_i-pretreated mitochondria, in the presence or absence of verapamil. These findings suggest that (1) the calcium-related hypothesis cannot explain completely the P_i-induced mitochondrial dysfunction, and (2) the P_i-induced low amplitude swelling–shrinkage changes may be related to a remaining distinct component of the mitochondrial functional changes in response to P_i.

The spectrophotometric measurement of mitochondrial "swelling–shrinkage" has been related to the transition of mitochondrial oxidative phosphorylation from the "controlled" to the "active" state following ADP addition and has been generally referred to as "small amplitude shrinkage" [24]. By combining spectrophotometric and electromicroscopic studies, Klingenberg [25] hypothesized that the light absorbance change, i.e. the "swelling–shrinkage" response, represented a reflection of the ADP/ATP carrier location relative to the inner or outer surface of the mitochondrial inner membrane.

Our results clearly demonstrate the increased sensitivity of mitochondria shrinkage responses to ADP after pretreatment with P_i. This observation may reflect a prior P_i-induced efflux of adenine nucleotides [8], which could result in the relocation of the ADP/ATP carrier to the outside of the inner membrane. Exogenous ADP could move more carrier molecules from outside to inside; the greater the difference between the distribution of the carrier concentration across the inner membrane, the greater would be the change in light absorbance. Also, the shorter shrinkage duration following ADP addition is likely a reflection of diminished concentration of carriers on the inner mitochondrial membrane. Thus, the difference in duration and amplitude between IFM and SLM may reflect differences in either the total number of carriers or the arrangement of the carriers in the membrane system, although the responses to P_i preincubation were similar for both mitochondrial populations. This possibility was supported by the ADP/ATP translocase activity assay which demonstrated a similar P_i-induced inhibition for both populations, a phenomenon totally unaffected by pretreatment with verapamil.

In recent years there has been substantial controversy regarding the rate-limiting factor(s) involved in the control of mitochondrial oxidative phosphorylation. It is very likely that numerous factors are involved in this process [see Ref. 26 for review]. In this regard, it is important to note that ADP/ATP translocase activity is an important regulator of mitochondrial oxidative phosphorylation, particularly under pathophysiological conditions. The dissimilar magnitude of depression in oxidative phosphorylation versus ADP/ATP translocase activity suggests that the latter contributes only to a partial extent to the depression in oxidative phosphorylation and that the inhibition in respiration may not be observed until the ADP/ATP translocase system is

inhibited to a critical level. The other component, a verapamil-sensitive P_i-induced inhibition of oxidative phosphorylation, may be related to an intramitochondrial calcium pool; however, because of the inclusion of ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA) in the isolation procedure, as well as the absence of exogenous calcium, the mechanism underlying this phenomenon is difficult to envisage accurately at the present time.

In conclusion, our results show that P_i can produce a rapid depression in cardiac mitochondrial oxidative phosphorylation which is partially reversible by verapamil. SLM exhibited an enhanced sensitivity to P_i-induced depression in respiration. Although the precise mechanisms underlying the P_i-induced decrease in respiration are unknown, the partial reversal by verapamil suggests a calcium-mediated component. In addition, a potent verapamil-insensitive inhibition in adenine nucleotide translocase implicates this as an important contributor towards P_i-induced mitochondrial dysfunctions which could represent the basis for the partial effectiveness of verapamil in reversing P_i effects on oxidative phosphorylation.

Acknowledgements—J. Duan is a recipient of a Canadian Heart Foundation Traineeship Award. The authors thank Sandra Dibb for her excellent technical assistance and Dr Ken W. Renton for providing the use of his Pye-Unicam spectrophotometer.

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