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Phosphate-induced efflux of adenine nucleotides from rat-heart mitochondria: evaluation of the roles of the phosphate/hydroxyl exchanger and the dicarboxylate carrier

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Upon the addition of inorganic phosphate, isolated rat-heart mitochondria released endogenous adenine nucleotides. To elucidate the mechanism of this phosphate-induced efflux, we evaluated the relative roles of three inner mitochondrial membrane carriers: the adenine nucleotide translocase, the phosphate/hydroxyl exchanger, and the dicarboxylate carrier. Atractyloside (a specific inhibitor of the adenine nucleotide translocase) prevented this efflux, but did not inhibit mitochondrial swelling. Inhibitors of the phosphate/hydroxyl exchanger (200 μM *n*-ethylmaleimide and 10 μM mersalyl) did not inhibit phosphate-induced efflux. 200 μM mersalyl (which inhibited both the phosphate/hydroxyl exchanger and the dicarboxylate carrier) inhibited the rate of efflux approx. 65% Phenylsuccinate and 2-*n*-butylmalonate (inhibitors of the dicarboxylate carrier) partially inhibited phosphate-induced efflux and adenine nucleotide translocase activity. Mersalyl (200 μM) had no effect on adenine nucleotide translocase activity. Partial inhibition of the adenine nucleotide translocase by phenylsuccinate and butylmalonate could not explain the extent of inhibition of phosphate-efflux by these agents. Moreover, the rates of adenine nucleotide efflux in the presence of phenylsuccinate, butylmalonate, or mersalyl correlated well with the ability of these agents to inhibit succinate-supported respiration. We conclude that phosphate-induced efflux of adenine nucleotides from rat heart mitochondria occurs over the adenine nucleotide translocase, and that the site of action of the phosphate is not the phosphate/hydroxyl exchanger, but is likely the dicarboxylate carrier.

Introduction

Several investigators have shown that during myocardial ischemia there is a loss of adenine nucleotides from heart mitochondria [1–3]; this loss may impair ATP synthesis and could conceivably lead to dysfunction of the post-ischemic heart [4,5]. During ischemia in the heart, the adenine nucleotide content decreases as a result of enzymatic degradation in the absence of ATP

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Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; NEM, *N*-ethylmaleimide; P_i, inorganic phosphate.

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synthesis. ATP is serially hydrolyzed to AMP which is then converted to an adenosine by 5′-nucleotidase in the cell membrane [6]. The adenosine diffuses into the extracellular space and is deaminated by adenosine deaminase to form inosine which is further degraded to the purine bases hypoxanthine and xanthine [6,7]. Consequently, degradation products (adenosine, hypoxanthine, inosine, phosphate, etc.) accumulate in the cell. The adenine nucleotide decrease in the mitochondrial fraction probably results from a direct loss of nucleotides through the mitochondrial membrane, since the enzymes responsible for nucleotide degradation are not located in the mitochondrial matrix. LaNoue et al. [2] reported that during ischemia, the decrease of mitochondrial adenine nucleotides is initially slower than the decrease of whole-tissue adenine nucleotides (ATP + ADP + AMP). This may be indicative of some time-dependent change that eventually triggers the release of adenine nucleotides from the mitochondria. However, since the decrease in tissue adenine nucleotides coincides with many metabolic changes, the causative agent(s) remain unknown. A possible cause of the adenine nucleotide loss from the mitochondria is the accumulation of inorganic phosphate in the cell during the ischemic period [8,9]. Isolated heart [2,4,10] and liver mitochondria [11,12] have been shown to lose endogenous nucleotides in the presence of inorganic phosphate. Previous studies from this laboratory [4] have confirmed that under conditions found in the ischemic heart cell, phosphate can cause the loss of adenine nucleotides from the mitochondrial matrix.

In heart mitochondria, phosphate causes the loss of adenine nucleotides via the adenine nucleotide translocase, since the efflux is prevented by atractyloside [4], a specific inhibitor of the adenine translocase. However, the role of phosphate is unclear. There are two carriers in the mitochondrial inner membrane that are known to transport inorganic phosphate, the phosphate/hydroxyl exchanger and the dicarboxylate carrier. Preliminary studies from this laboratory showed that phosphate-induced efflux of adenine nucleotides from heart mitochondria was not inhibited by mersalyl (10 nmol/mg) but was inhibited by 20 mM phenylsuccinate [4]. These results suggested the lack of a role of the phosphate/hydroxyl exchanger and a possible involvement of the dicarboxylate carrier. The purpose of the present study was to test the hypothesis that phosphate-induced efflux of adenine nucleotides from rat heart mitochondria is mediated by the dicarboxylate carrier and not the phosphate/hydroxyl exchanger.

Materials and Methods

Materials. [^{14}C]Adenosine triphosphate, tetrasodium salt, was purchased from New England Nuclear, Boston. Phenylsuccinic acid and *n*-butylmalonic acid were purchased from Aldrich Chemical Company, Milwaukee. All other chemicals were purchased from Sigma Chemical Company, St. Louis.

Mitochondrial preparation. Male Sprague-Dawley rats (200–250 g) were decapitated and the hearts excised and dropped into a cold (4° C) isolation medium of 120 mM KCl, 1 mM ethylene glycol bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA) and 10 mM Tris (pH 7.4). The fatty and connective tissues were trimmed away and the hearts weighed. The hearts were then sliced into strips which were placed into a centrifuge tube, covered with 10 ml of the isolation meidum, and finely minced with scissors. During the mincing process the overlying solution was removed and new isolation medium (10 ml) replaced twice to remove the excess blood. The tissue (in the final 10 ml of isolation medium) was further disrupted with a Polytron tissue processor for 5 s at a rheostat setting of 4. For each gram of tissue, 1 mg nagarse proteinase (Nagase, Enzyme Development, New York) was added. Then, the suspension was vortexed and allowed to incubate for 2 min at 4° C. Following this incubation, another 10 ml isolation medium was added and the mixture centrifuged for 10 min at 14000 × *g* in a refrigerated centrifuge. The supernatant was discarded and the pellet resuspended in 20 ml of a buffer consisting of 120 mM KCl and 10 mM Tris (pH 7.4) (buffer A) using a glass homogenizer and a motor-driven Teflon pestle. The resulting suspension was centrifuged for 5 min at 500 × *g*, the pellet discarded and the supernatant centrifuged for 10 min at 8000 × *g*. The resulting mitochondrial

pellet was then broken-up and resuspended in 2 ml of the cold buffer A using hand homogenization. To label the endogenous adenine nucleotide pool, the suspension was incubated for 1 h at 4°C in the presence of 2.5 μCi of [^{14}C]ATP (specific activity 50 mCi/mmol). 18 ml of buffer A was then added and the mixture vortexed and centrifuged at 8000 × g for 10 min. The pellet was washed by resuspension in 20 ml of buffer A (using hand homogenization) followed by 10 min of centrifugation at 8000 × g. The washing process was repeated, and the final pellet was suspended in about 2 ml of buffer A.

We did not label the endogenous adenine nucleotide pool for respiration and swelling studies. In these situations, the initial mitochondrial pellet was resuspended in 10 ml of cold buffer A (using hand homogenization), centrifuged for 10 min at 8000 × g, and finally suspended in about 2 ml of buffer A.

All of the procedures were performed at 4°C and the final mitochondrial preparations were kept at 4°C until used. Protein concentration of the final mitochondrial suspension was determined using a Biuret method [13].

Adenine nucleotide efflux. Mitochondria were incubated at a final concentration of 1 mg/ml in buffer A at 30°C. Depending on the experiment, various agents were also added to the buffer (see text and legends for details). Time zero was considered to be 30 s after the addition of mitochondria to the buffer. At time zero, a volume of 60 mM K_2HPO_4 (pH 7.4) was added to give a concentration of phosphate equal to 10 mM. Background efflux was determined by not adding phosphate at time zero. At the appropriate times, extramitochondrial radioactivity was determined as follows: aliquots (0.2 ml) were removed and added to chilled centrifuge tubes containing 0.2 ml of buffer A plus 100 μM atractyloside. The tubes were capped, vortexed, and centrifuged for 30 s at 13000 × g. The supernatants (0.2 ml) were then added to scintillation vials containing 3 ml of Ultrafluor (National Diagnostics) scintillation cocktail. Radioactivity was monitored with a Beckman LS-3155T scintillation counter.

The initial intramitochondrial radioactivity was determined by subtracting the extramitochondrial cpm at time zero (background) from the 'total' cpm of the suspension. The 'total' amount of radioactivity in each mitochondrial suspension was determined by removing 0.2 ml aliquots and adding them to the chilled tubes containing the atractyloside-solution as before; in this case the tubes were only vortexed (not centrifuged) then counted for radioactivity. Adenine nucleotide efflux for a particular time-point was determined by subtracting the extramitochondrial counts at time zero from the extramitochondrial cpm at that particular time. The results are expressed as a percentage of the initial intramitochondrial radioactivity. The total adenine nucleotide content (ATP + ADP + AMP) of the freshly prepared mitochondria was 10.9 ± 0.8 nmol/mg protein (n = 11) determined by HPLC as prevously described [4]. The distribution of the individual nucleotide (ATP/ADP/AMP) was 1.6 : 5.8 : 3.4; the radioactivity was proportionally distributed among the three nucleotides.

Adenine nucleotide translocase. The assay conditions were the same as described above (adenine nucleotide efflux) with the following exceptions: the assay temperature was 15°C; at time zero, inorganic pyrophosphate (5 mM final concentration) was added; and the buffer contained 10 mM NaF. The activity was determined by the percentage of intramitochondrial radioactivity appearing in the extramitochondrial space after 1 min.

Respiratory activity. Oxygen consumption was determined using a Clark electrode at 30°C. Mitochondria (1.8 mg) were suspended in a buffer consisting of 225 mM sucrose, 10 mM Tris, 15 mM KCl, 1 mM EDTA, rotenone (1 μg/mg protein) and 10 mM KH_2PO_4 at pH 7.4 (final volume, 1.8 ml). After 1 min, approx. 400 nmol ADP were added to initiate State 3 respiration. Respiration was allowed to go into state 4 (resting rate). Approx. 1 min into State 4, FCCP was added (final concentration, 0.3 μM) to induce uncoupled respiration. In the presence of some agents, ADP did not stimulate respiration; in this case State 3 (respiration in the presence of ADP) and the resting rate were considered to be equal. Rates of respiration are expressed as a percentage of the State 3 rate of the control condition (no inhibitor added).

Swelling studies. Mitochondria were incubated

at a concentration of 1 mg/ml at room temperature in buffer A. The absorbance was monitored at 520 nm with a Uvikon 860 spectrophotometer (Kontron Instruments). 10 mM phosphate was added 30 s after the mitochondria, and the absorbance was monitored over a period of 6 min. Swelling was indicated by a decrease in absorbance.

Statistical analysis. Significant differences were determined at 95% confidence intervals ($P = 0.05$) using Student's *t*-test.

Results

Phosphate-induced efflux of adenine nucleotides from heart mitochondria

Fig. 1 shows the efflux of adenine nucleotides from isolated rat heart mitochondria. In the absence of phosphate, there was a slow release of endogenous adenine nucleotides. When 10 mM phosphate was added, a rapid efflux occurred; approx. 80% of the endogenous labeled nucleotides were released within 3 min. In the presence of 50 μM atractyloside, phosphate did not stimulate efflux above the slow background rate (absence of phosphate). Phenylsuccinate also inhibited phosphate-induced efflux. Similar results from this laboratory have been reported [4].

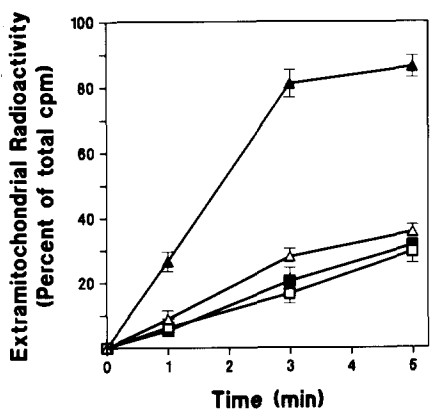

Fig. 1. Efflux of adenine nucleotides from heart mitochondria. Closed triangles (▲), open squares (□) and open triangles (△) indicate the addition of inorganic phosphate (10 mM final concentration) at time zero. Closed squares (■) indicate background efflux (no phosphate or inhibitor added). Open squares (□) indicate the presence of 50 μM atractyloside. Open triangles (△) indicate the presence of 20 mM phenylsuccinate. See Materials and Methods for experimental procedures. Values are means ± S.E.M. of six determinations from two different mitochondrial preparations, each preparation assayed in triplicate.

The ability of atractyloside to prevent phosphate-induced efflux strongly suggests that the adenine nucleotide translocase plays a significant role in this process. One possibility is that, in the presence of phosphate, the translocase facilitates a unidirectional efflux of adenine nucleotides across the membrane. However, the role of phosphate is unclear. Phosphate transport in mitochondria is primarily facilitated by the phosphate/hydroxyl exchanger; however, preliminary results [4] indicated that this carrier was not involved in phosphate-induced efflux, since a low concentration of mersalyl (10 μM) did not inhibit efflux. Phosphate transport facilitated by the dicarboxylate carrier is inhibited by phenylsuccinate. Therefore, the ability of phenylsuccinate to inhibit phosphate-induced efflux suggests a mechanism involving the dicarboxylate carrier. The following studies were done to further explore the possible roles of the dicarboxylate and the phosphate/hydroxyl exchanger in phosphate-induced efflux of adenine nucleotides.

Effect of N-ethylmaleimide and 10 μM mersalyl on adenine nucleotide efflux and succinate-supported respiration

NEM or mersalyl (10 μM) did not significantly affect the rate of efflux of adenine nucleotides in the presence or absence of phosphate (Table I). Since, at the concentrations used, these two inhibitors should completely inhibit the phosphate/hydroxyl exchanger [14,15] without inhibiting the dicarboxylate carrier [16–20], the results suggest that the phosphate carrier is not involved in adenine nucleotide efflux. To verify the effectiveness of these inhibitors under our conditions, we determined their effects on succinate-supported respiration (Table II). In the presence of 10 μM mersalyl, ADP-stimulated respiration was inhibited 100%; however, the resting and uncoupled rates of respirations were inhibited only 27% and 24%, respectively. Similar results were seen with NEM; ADP did not stimulate respiration, but the uncoupler did. We found that preincubation of the mitochondria with NEM for 30 s was not

TABLE I

EFFECT OF MERSALYL AND *N*-ETHYLMALEIMIDE ON EFFLUX OF ADENINE NUCLEOTIDES FROM RAT HEART MITOCHONDRIA

The buffer contained either no inhibitor (control), 10 μM mersalyl or 200 μM NEM. The mitochondria were preincubated for 30 s before the addition of phosphate, except in the case of NEM, where the preincubation time was extended to 3 min to ensure complete inhibition of the phosphate/hydroxyl exchanger. See Materials and Methods for experimental procedures. Values are means ± S.E.M. of six determinations from two different mitochondrial preparations, each preparation assayed in triplicates.

Condition	Percent efflux per 3 min per mg protein
Control (10 mM P_i)	76.88 ± 2.09
Control background (no P_i)	16.33 ± 2.42
10 μM mersalyl (10 mM P_i)	75.63 ± 3.76
10 μM mersalyl background (no P_i)	20.38 ± 2.13
200 μM NEM (10 mM P_i)	91.18 ± 3.10
200 μM NEM background (no P_i)	17.91 ± 2.15

sufficient to inhibit State 3 respiration. Therefore, we used a longer preincubation time with NEM than with mersalyl. The data shown in Table II are consistent with the inhibition of phosphate transport without significant inhibition of succinate transport, which requires either the dicarboxylate carrier or the α-ketoglutarate carrier in rat-heart mitochondria. The results indicate that inhibition of the phosphate/hydroxyl exchanger does not affect phosphate-induced efflux of adenine nucleotides. However, since NEM and mersalyl (10 μM) were apparently unable to inhibit succinate transport completely (as determined by the ability of FCCP to stimulate respiration above the resting rate), the involvement of the dicarboxylate carrier in the mechanism of phosphate-induced efflux cannot be ruled out.

TABLE II

EFFECT OF 10 μM MERSALYL AND 200 μM *N*-ETHYLMALEIMIDE ON SUCCINATE-SUPPORTED RESPIRATION

See Materials and Methods for experimental procedures. Values are means ± S.E.M. of three determinations from one mitochondrial preparation.

	State 4	State 3	Uncoupled
No inhibitor	39.4 ± 1.5	100.0 ± 2.2	131.1 ± 9.3
Mersalyl (10 μM)	23.1 ± 0.7	23.1 ± 0.7	108.2 ± 3.3
NEM (200 μM)	36.3 ± 0.2	36.3 ± 0.2	91.8 ± 0.8

Effects of phenylsuccinate, butylmalonate and mersalyl on phosphate-induced efflux and succinate-supported respiration

If phosphate-induced efflux requires phosphate transport via the dicarboxylate carrier, then inhibitors of this carrier should inhibit adenine nucleotide release in the presence of phosphate. We tested three inhibitors of the dicarboxylate carrier: phenylsuccinate, butylmalonate and mersalyl (see Refs. 14 and 15 for reviews). As shown in Fig. 1, 20 mM phenylsuccinate inhibited phosphate-induced efflux almost as well as atractyloside. Fig. 2 shows the effect of increasing concentrations of phenylsuccinate on the rate of adenine nucleotide efflux. With 10 mM phosphate added, the rate of efflux decreased almost linearly from approx. 25% per min per mg protein in the absence of inhibitor (control) to a rate of approx. 10% per min per mg protein in the presence of 20

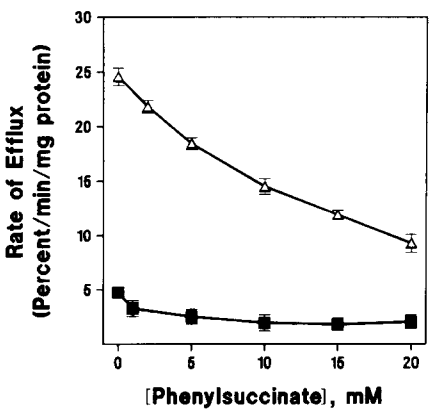

Fig. 2. Effect of phenylsuccinate on the rate of efflux of adenine nucleotides from heart mitochondria. Open triangles (△) indicate the presence of 10 mM inorganic phosphate. Closed squares (■) indicate the absence of inorganic phosphate. For each point, efflux was determined after 3 min. The rate of efflux was found by dividing this value by 3. See Materials and Methods for experimental procedures. Values are means ± S.E.M. of six determinations from two different mitochondrial preparations, each preparation assayed in triplicate.

mM phenylsuccinate. The background rate (in the absence of phosphate) showed a slight decrease with increasing concentration of phenylsuccinate.

The effect of butylmalonate on adenine nucleotide efflux in the presence of phosphate (Fig. 3) was similar to that of phenylsuccinate (Fig. 2). The efflux decreased from 27 to 15% per min per mg protein with 20 mM butylmalonate. Unlike phenylsuccinate, the background rate increased with increasing concentrations of butylmalonate; at 20 mM butylmalonate, the background rate was approximately twice that of the background rate in the absence of inhibitor (Fig. 3).

The results shown in Figs. 2 and 3 indicate that adenine nucleotide efflux in the presence of phosphate was slightly more sensitive to phenylsuccinate than butylmalonate. However, we determined the rate of phosphate-induced efflux by substrating the background rate from the rate in the presence of phosphate. As shown in Fig. 4, phosphate-induced efflux was equally sensitive to phenylsuccinate and butylmalonate. As the inhibitor concentration was increased to 20 mM, the phosphate-induced efflux rate decreased linearly from approx. 20 to 7% per min per mg protein. Thus, at 20 mM, phenylsuccinate and butylmalonate inhibited phosphate-induced efflux by approx. 70%.

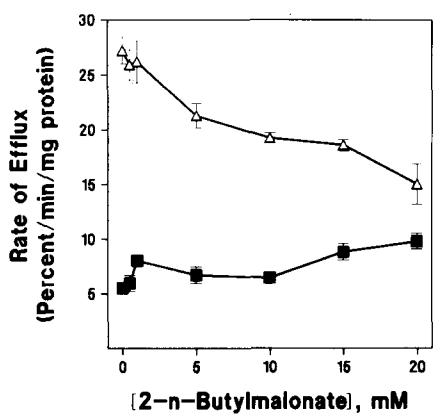

Fig. 3. Effect of 2-*n*-butylmalonate on the rate of efflux of adenine nucleotides from heart mitochondria. Open triangles (△) indicate the presence of 10 mM inorganic phosphate. Closed squares (■) indicate the absence of inorganic phosphate. For each point, efflux was determined after 3 min. The rate of efflux was found by dividing this value by three. See Materials and Methods for experimental procedures. Values are means ± S.E.M. of six determinations from two different mitochondrial preparations, each preparation assayed in triplicate.

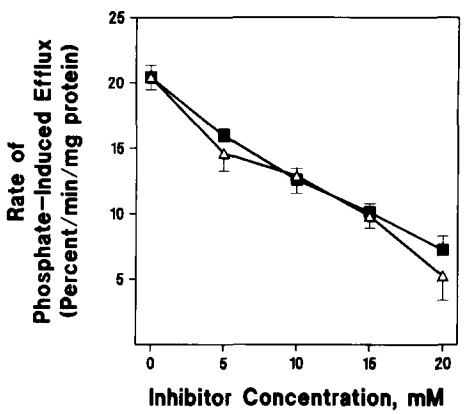

Fig. 4. Effect of phenylsuccinate and butylmalonate on phosphate-induced efflux of adenine nucleotides from heart mitochondria. Closed squares (■) indicate the presence of phenylsuccinate at the concentrations indicated. Open triangles (△) indicate the presence of butylmalonate. The rates reflect the difference between the control and background (no phosphate) values of Figs. 2 and 3. See Materials and Methods for experimental procedures.

The dicarboxylate carrier is inhibited by mersalyl. However, the concentration of mersalyl required for inhibition of this carrier is greater than that required for inhibition of the phosphate/hydroxyl exchanger [15]. Therefore, we determined the effect of high concentrations of mersalyl on adenine nucleotide release. The rates of phosphate-induced efflux in the presence of 0, 50, 100 and 200 μM mersalyl were 20.6 ± 0.4, 12.7 ± 0.6, 9.1 ± 1.1 and 9.0 ± 1.2% per min per mg protein, respectively. Thus, 200 μM mersalyl inhibited efflux by approx. 56%.

As shown in Table III, inhibition of the dicarboxylate carrier by these agents is indicated by inhibition of the uncoupled rate of succinate-supported respiration, due to a limitation of succinate influx over this carrier. Inhibition of respiration was proportional to the concentration of inhibitor used. At the highest concentration of the inhibitors, the slow rates of respiration may be due to residual succinate transport, or to oxidation of endogenous substrates.

TABLE III

EFFECT OF INHIBITORS ON SUCCINATE-SUPPORTED RESPIRATION

See Materials and Methods for experimental procedures. Values are means ± S.E.M. of three determinations from one mitochondrial preparation.

Inhibitor	State 4	State 3	Uncoupled
No inhibitor	43.3 ± 2.8	100.0 ± 3.6	106.0 ± 2.3
50 μM mersalyl	19.9 ± 0.4	19.9 ± 0.4	41.3 ± 4.2
100 μM mersalyl	17.9 ± 1.0	17.9 ± 1.0	24.8 ± 5.1
200 μM mersalyl	16.9 ± 1.4	16.9 ± 1.4	15.8 ± 2.1
5 mM phenylsuccinate	29.8 ± 0.6	53.9 ± 3.6	54.0 ± 0.7
10 mM phenylsuccinate	28.9 ± 0.6	28.9 ± 0.6	28.2 ± 0.2
20 mM phenylsuccinate	14.4 ± 1.8	14.4 ± 1.8	12.6 ± 0.3
5 mM butylmalonate	35.6 ± 3.8	35.6 ± 3.8	27.0 ± 0.7
10 mM butylmalonate	24.1 ± 3.5	24.1 ± 3.5	20.4 ± 0.9
20 mM butylmalonate	14.2 ± 0.2	14.2 ± 0.2	13.3 ± 0.3

The effect of mersalyl, phenylsuccinate and butylmalonate on the adenine nucleotide translocase

Although the inhibition of phosphate-induced efflux by these agents correlates well with their abilities to inhibit the dicarboxylate carrier, the inhibition may be due to an effect on the adenine nucleotide translocase. Therefore, we tested the effects of phenylsuccinate, butylmalonate, and mersalyl on adenine nucleotide translocase activity. The adenine nucleotide translocase of heart mitochondria is very active, and it is difficult to obtain an accurate measurement of exchange rates of adenine nucleotides, even at 4°C [21]. Inorganic pyrophosphate can exchange with intramitochondrial adenine nucleotides via the adenine nucleotide translocase [22–24]; this rate of exchange is slower than with extramitochondrial ADP or ATP [25]. Therefore, we used the rate of exchange of intramitochondrial, labeled nucleotides for extramitochondrial pyrophosphate as an estimate of translocase activity. (It should be noted that 10 mM NaF was added to prevent hydrolysis of the pyrophosphate). As shown in Fig. 5, approx. 60% of the endogenous pool was found to exchange with the pyrophosphate after 1 min. After 5 min, approx. 95% of the label had exchanged with the pyrophosphate. Atractyloside completely inhibited the translocase activity. Moreover, there was a partial inhibition by 20 mM phenylsuccinate. Table IV shows the effects of phenylsuccinate, butylmalonate, and mersalyl on the translocase activity. Mersalyl had little or

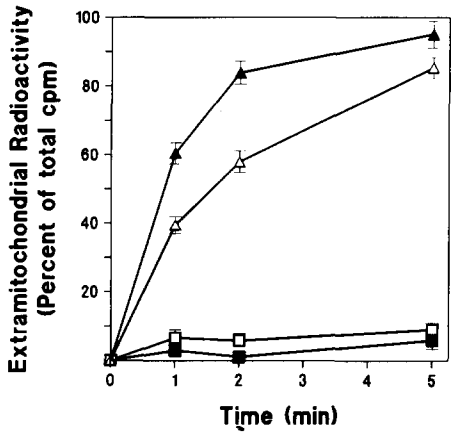

Fig. 5. Adenine Nucleotide Translocase Activity. Closed triangles (▲), open squares (□) and open triangles (△) indicate the addition of inorgnaic pyrophosphate (5 mM final concentration) at time zero. Closed squares (■) indicate background efflux (no pyrophosphate or inhibitor added). Open squares (□) indicate the presence of 50 μM atractyloside. Open triangles (△) indicate the presence of 20 mM phenylsuccinate. See Materials and Methods for experimental procedures. Values are means ± S.E.M. of nine determinations from three different mitochondrial preparations, each preparation assayed in triplicate.

TABLE IV

EFFECT OF INHIBITORS ON INITIAL RATES OF TRANSLOCASE ACTIVITY

See Materials and Methods for experimental procedures. Values are means ± S.E.M. of nine determinations from three different mitochondrial preparations, each preparation assayed in triplicate.

Inhibitor and concentration	Percent exchanged per min per mg protein
No inhibitor	58.42 ± 1.77
50 μM mersalyl	57.51 ± 0.78
100 μM mersalyl	56.79 ± 2.16
200 μM mersalyl	55.36 ± 4.03
5 mM phenylsuccinate	53.98 ± 4.90
10 mM phenylsuccinate	48.32 ± 3.76
20 mM phenylsuccinate	38.36 ± 2.62 [a]
10 mM butylmalonate	50.62 ± 2.23
20 mM butylmalonate	37.34 ± 1.95 [a]

[a] Indicates a significant difference from the control (no inhibitor).

no effect on translocase activity. At 10 mM, phenylsuccinate and butylmalonate inhibited translocase activity by approx. 15%. The inhibition increased to approx. 35% when the concentration of phenylsuccinate or butylmalonate was increased to 20 mM.

Although inhibition of efflux by mersalyl could not be explained by inhibition of the translocase, the results shown in Table IV do not rule out this possibility for phenylsuccinate and butylmalonate. Therefore, we determined the relationship between the rates of phosphate-induced efflux and translocase activity using non-saturating concentrations of atractyloside. Fig. 6 shows the efflux rate plotted as a function of translocase activity. With atractyloside, there was a linear relationship between the translocase activity and phosphate-induced efflux. With phenylsuccinate and butylmalonate, there was a greater inhibition of efflux than could be explained by inhibition of the translocase activity alone. With mersalyl, there was no correlation between translocase activity and efflux rate. Based on the respiratory studies (Table III), we concluded that the dicarboxylate carrier is almost totally inhibited by 200 μM mersalyl, 20 mM phenylsuccinate and 20 mM butylmalonate. These agents inhibited the phosphate-induced efflux by 56%, 65% and 75%, respectively (Figs. 4 and 6). Therefore, while the slightly greater inhibition of efflux by phenylsuccinate and butylmalonate can be explained by an additional affect on the adenine translocase, the majority of the inhibition can be attributed to inhibition of the dicarboxylate carrier.

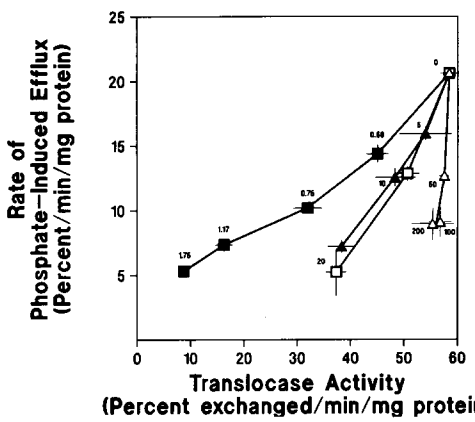

Fig. 6. Rates of phosphate induced efflux plotted as a function of adenine nucleotide translocase activity. Closed squares (■) indicate the presence of atractyloside at the following concentrations: 1.75 μM, 1.17 μM, 0.75 μM and 0.58 μM. Closed triangles (▲) indicate the presence of phenylsuccinate at 5 mM, 10 mM and 20 mM. Open squares (□) indicate the presence of butylmalonate at 10 mM and 20 mM. Open triangles (△) indicate the presence of mersalyl at 50 μM, 100 μM and 200 μM. Control value (no inhibitor) is represented as an open triangle inscribed within an open square (□). Efflux and translocase activities were measured at 30 °C and 15 °C, respectively. See Materials and Methods for experimental details. Values for the translocase coordinate are means ± S.E.M. of nine determinations from three different mitochondrial preparations, each preparation assayed in triplicate. Values for the efflux coordinate are means ± S.E.M. of six determinations from two different mitochondrial preparations, each preparation assayed in triplicate.

Discussion

The phosphate-induced efflux of adenine nucleotides cannot be explained by non-specific leakage from the mitochondria due to swelling. Although phosphate induced swelling, atractyloside enhanced the rate of swelling twofold (data not shown) while inhibiting adenine nucleotide efflux. The results of the present study clearly illustrate the participation of the adenine nucleotide translocase in phosphate-induced efflux of adenine nucleotides from rat heart mitochondria. The adenine nucleotide translocase facilitates the diffusion of ADP and ATP across the mitochondrial inner membrane. The process occurs by a one-for-one exchange of extramitochondrial ADP for intramitochondrial ATP and is sensitive to atractyloside. The total inhibition of phosphate-induced efflux by atractyloside strongly indicates that the efflux pathway occurs over the adenine nucleotide translocase and that either ATP and/or ADP are(is) preferentially exported. Possibly, an external non-nucleotide may exchange for an internal adenine nucleotide. One possibility is that inorganic phosphate exchanges with endogenous adenine nucleotides via the adenine translocase. However, this is not consistent with the observation that 200 μM mersalyl inhibited efflux but did not inhibit the adenine nucleotide translocase. Another possibility is that pyrophosphate may be synthesized by the mitochondria [26,27], and this non-nucleotide may exchange with endogenous

adenine nucleotides [5,22,23,25]. However, inhibition of the electron-transport chain will not inhibit phosphate-induced efflux [4], but has been reported to prevent pyrophosphate synthesis [27]. Another possible mechanism is diffusion of adenine nucleotides through the mitochondrial membrane facilitated by the adenine nucleotide translocase; in this case, no extramitochondrial agent would be required for exchange with endogenous nucleotides. Since phosphate-induced efflux can be inhibited by mersalyl without inhibiting the adenine nucleotide translocase, another component (sensitive to mersalyl) must be involved. This component could be a phosphate-carrier. The phosphate/hydroxyl exchanger does not appear to be required for phosphate-induced efflux, because inhibition of the phosphate/hydroxyl exchanger by *N*-ethylmaleimide and mersalyl (at concentrations that did not inhibit the dicarboxylate carrier) did not inhibit adenine nucleotide efflux. The dicarboxylate carrier is the only other carrier in mitochondria which has been shown to transport inorganic phosphate [14,15,17,19,28–30]. Additional substrates for this carrier are malate, malonate and succinate. The dicarboxylate carrier facilitates a one-for-one exchange of these substrates across the mitochondrial membrane [14]. Inhibition of phosphate-induced efflux by phenylsuccinate, butylmalonate, and mersalyl suggests an involvement of this carrier in phosphate-induced efflux. However, an unexpected finding of our study was the partial inhibition of the adenine nucleotide translocase by phenylsuccinate and butylmalonate. Therefore, it could be argued that inhibition of adenine nucleotide efflux by these agents was due to their action on the adenine nucleotide translocase. A previous report from this laboratory [4] indicated that phenylsuccinate did not affect adenine nucleotide translocase activity of heart mitochondria. There are two possible reasons for this discrepancy. In the previous study, translocase activity was measured by the uptake of [^{14}C]ATP; the rate of uptake may have been too rapid to observe a partial inhibition of activity. The other possible reason is that translocase activity was previously assayed at 4° C, whereas in the present study the assay was done at 15° C; inhibition by phenylsuccinate may be temperature-dependent. We do not know if butylmalonate and phenylsuccinate inhibit the adenine nucleotide translocase directly. Nevertheless, the degree of inhibition of translocase activity by phenylsuccinate and butylmalonate was not sufficient to explain their extensive inhibitory effects upon phosphate-induced efflux. Moreover, mersalyl (50 μM or greater) had no effect on translocase activity, but inhibited phosphate-induced efflux. The results of our study indicate a good correlation of inhibition of efflux with inhibition of succinate transport, as determined by uncoupled respiratory activity.

Phenylsuccinate, butylmalonate and mersalyl do not specifically inhibit the dicarboxylate carrier. The α-ketoglutarate and tricarboxylate (citrate) carriers are also inhibited by these agents [14,15]. The α-ketoglutarate carrier transports α-ketoglutarate, malate, malonate and succinate [14,31], while the tricarboxylate carrier transports citrate, isocitrate, *cis*-aconitate, malate and phospho*enol*-pyruvate [14]. Since the alpha-ketoglutarate carrier is less sensitive than the dicarboxylate carrier to inhibition by phenylsuccinate, butylmalonate, and mersalyl [14,18,31–33], and is not known to transport inorganic phosphate, it is unlikely that this carrier plays a role in the phosphate-induced efflux of adenine nucleotides. While the tricarboxylate carrier in liver mitochondria can be inhibited by phenylsuccinate and butylmalonate [14,15], this carrier is reportedly not as sensitive to mersalyl [14] as are the dicarboxylate and alpha-ketoglutarate carriers. It is not likely that the tricarboxylate carrier is involved in phosphate-induced efflux because it is not known to transport phosphate, and its activity in rat heart mitochondria is minimal at 25° C [34,35]. Furthermore, inorganic phosphate does not induce an atractyloside-sensitive efflux of adenine nucleotides from rat liver mitochondria [12], which have relatively high tricarboxylate carrier activity. Although Sluse et al. [34] observed minimal activity of the dicarboxylate carrier in heart mitochondrial at 4° C, they reported measurable activity at 25° C. Therefore, we conclude that the site of action of phosphate in phosphate-induced efflux of adenine nucleotides from heart mitochondria is the dicarboxylate carrier.

The role of phosphate is unclear. One possibility is an exchange of phosphate, via the di-

carboxylate carrier, for an internal component that can exchange with endogenous adenine nucleotides over the translocase. However, succinate and malate, which exchange with endogenous components via the dicarboxylate carrier, do not induce efflux of adenine nucleotides (data not shown). Therefore, of the reported compounds transported by the dicarboxylate carrier, the induction of nucleotide efflux appears to be specific for phosphate. This specificity may be due to the fact that phosphate has a different binding site on the dicarboxylate carrier than other substrates [14,29]. Although other mechanisms cannot be ruled out, the results of this study suggest that inorganic phosphate can interact with the dicarboxylate carrier of heart mitochondria to trigger a unidirectional efflux of adenine nucleotides over the adenine nucleotide translocase.

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