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Inhibition of the Na^+/Ca^{2+} Antiport of Heart Mitochondria by Diethylpyrocarbonate

Michael H. Davis,[1] Dennis W. Jung,[1] and Gerald P. Brierley[1]

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Abstract

Diethylpyrocarbonate inhibits Na^+/Ca^{2+} antiport activity in isolated heart mitochondria. The inhibition is time-dependent with maximum activity developed after 5 min at 25°C. The reaction of diethylpyrocarbonate with the mitochondrial membrane is biphasic with 25–30 nmol mg^{-1} reacting rapidly and an additional 30 nmol mg^{-1} taken up slowly over a 30-min incubation. Inhibition of mitochondrial Na^+/Ca^{2+} antiport by diethylpyrocarbonate decreases the V_{max} of the reaction, and the inhibition cannot be reversed by washing the mitochondria or addition of excess histidine. The inhibition occurs at levels of inhibitor that have little or no effect on Ca^{2+} uptake, Na^+/H^+ antiport, or succinate respiration. A portion of the Na^+-dependent efflux of Ca^{2+} is insensitive to diethylpyrocarbonate and this component is abolished by diltiazem. The mechanism by which diethylpyrocarbonate inactivates Na^+/Ca^{2+} antiport is still uncertain, but may involve the modification of an unprotonated histidine residue in the transporter.

Key Words: Mitochondria; diethylpyrocarbonate; heart; inhibition; sodium; calcium.

Introduction

Heart mitochondria contain a Na^+/Ca^{2+} antiport capable of promoting a Na^+-dependent efflux of Ca^{2+} (Crompton *et al.*, 1976, 1977, 1978; see Brierley and Jung, 1987 for a recent review). This exchanger, in conjunction with the Na^+/H^+ antiport, is thought to promote Ca^{2+} extrusion from the matrix and to balance the influx of Ca^{2+} on the ruthenium red-sensitive uniport (Crompton and Heid, 1978; Nicholls and Akerman, 1982). The Na^+/Ca^{2+} antiport is inhibited by a wide variety of reagents, including diltiazem and other Ca^{2+} antagonists (Vaghy *et al.*, 1982), benzodiazepines (Matlib and

[1]Department of Physiological Chemistry, Ohio State University Medical Center, Columbus, Ohio 43210.

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Schwartz, 1983), amilioride derivatives, trifluorperazine, local anesthetics, spermine, and Mg^{2+} and Ba^{2+} (see Brierley and Jung, 1987). Despite this well-defined inhibitor profile, little is known of the molecular properties of this antiport.

Diethylpyrocarbonate[2] (DEPC) has been used as a modifying agent for proteins and peptides (Melchoir and Fahrney, 1970; Miles, 1977; Lundblod and Noyes, 1984) and in many cases is quite specific for the carbethoxylation of accessible histidine residues. Decarbethoxylation can be accomplished at neutral pH by addition of hydroxylamine. DEPC has been shown to inhibit Na^+/H^+ antiport in bacterial membrane vesicles (Damiano *et al.*, 1985) and in renal microvillus vesicles (Grillo and Aronson, 1986). We have therefore examined the effect of DEPC on Na^+/H^+ and Na^+/Ca^{2+} antiport in heart mitochondria. In the present communication we wish to report that DEPC inhibits the Na^+/Ca^{2+} antiport at levels that do not affect Na^+/H^+ and other mitochondrial transport activities to a significant extent.

Materials and Methods

Beef heart mitochondria were prepared as described by Brierley *et al.* (1984). SMP were prepared by sonication of heart mitochondria in a medium of sucrose (0.25 M), TES (10 mM, pH 7.2), and EDTA (1 mM), essentially as described (Brierley *et al.*, 1984), except that FITC-dextran (2.5 mg/ml) was present to serve as an intravesicular pH indicator (see Rottenberg, 1979).

The amount of added DEPC that reacts with heart mitochondria was estimated by adding excess histidine (8 mM) to DEPC-reacted mitochondria (1 mg/ml) suspended in KCl (100 mM) and TES (5 mM, pH 7.2) at 25°C. The histidine removes all unreacted DEPC, and the mitochondria were then removed by centrifugation in an Eppendorf microfuge. The DEPC-histidine adduct was estimated from the absorbance at 240 nm using an extinction coefficient of $3200\ M^{-1}cm^{-1}$ (Miles, 1977) and compared with the value obtained when histidine is reacted with an identical amount of DEPC added to the supernate after removal of the mitochondria. The difference was taken as the amount of DEPC reacted with the mitochondria.

Na^+-Dependent Release of Ca^{2+}

Heart mitochondria were suspended at 1 mg/ml in a medium of KCl (100 mM), EGTA (10 μM), TES (10 mM, adjusted to pH indicated), succinate

[2] The abbreviations used are as follows: DEPC, diethylpyrocarbonate; SMP, submitochondria particles; FITC-dextran, fluorescein isothiocyanate dextran; TES, *N*-tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid.

(3 mM), rotenone, and antipyrylazo III (10 μM). The temperature was maintained at 25°C and the uptake of Ca^{2+} (65 μM) monitored at 720 minus 790 nm in an SLM Aminco DW-2C spectrophotometer. Ruthenium red (1.0 μM, recrystallized) was added and release of Ca^{2+} initiated with NaCl (20 mM, unless otherwise indicated).

Na^+- or Ca^{2+}-Dependent Release of $^{45}Ca^{2+}$

The uptake and loss of Ca^{2+} was also followed using $^{45}Ca^{2+}$ as described by Crompton *et al.* (1977). $^{45}Ca^{2+}$ (66 nmol mg^{-1} protein) was added to mitochondria suspended in KCl (100 mM), TES (10 mM, pH 6.7), EGTA (10 μM), succinate (3 mM), and rotenone. After 5 min the uptake of $^{45}Ca^{2+}$ was blocked by addition of ruthenium red (1.0 μM) and $^{45}Ca^{2+}$ efflux was initiated by addition of either NaCl (20 mM) or $CaCl_2$ (40 μM). After 2 min the efflux was terminated by addition of La^{3+} (150 nmol mg^{-1} protein) and the mitochondria were removed by centrifugation within 2.5 min. Aqueous samples were added to Beckman Ready-Solv scintillation fluid and radioactivity was determined in a scintillation counter.

Na^+/H^+ Antiport

Na^+/H^+ antiport was followed by the passive swelling of mitochondria in 80 mM Na^+ acetate (see Brierley *et al.*, 1984). Na^+/H^+ exchange in SMP was monitored using an MPF 44 fluorimeter with excitation set at 480 nm and emission at 520 nm (Ohkuma and Poole, 1978). The fluorescence change of FITC-dextran trapped in the SMP lumen reports a ΔpH that depends on succinate respiration and is sensitive to uncouplers. Addition of Na^+ (10–100 mM) causes a change in steady-state ΔpH consistent with Na^+/H^+ antiport activity (see Rosen and Futai, 1980).

Results

The Na^+-dependent release of Ca^{2+} from respiring beef heart mitochondria is strongly inhibited by the addition of DEPC (100 nmol mg^{-1} protein). This inhibition develops rapidly and is readily apparent when DEPC is added at the same time as the Na^+ that initiates the Na^+/Ca^{2+} antiport reaction (Fig. 1). However, the extent of inhibition of Na^+/Ca^{2+} antiport activity increases with increasing time of contact of DEPC with the mitochondrial membrane (Fig. 2A). Half-maximal inhibition is attained after 1 min, and the maximum occurs after about 5-min incubation at 25°C.

Under these conditions the reaction of DEPC with the mitochondrial membrane appears biphasic (Fig. 2B). On the order of 25–30 nmol DEPC per

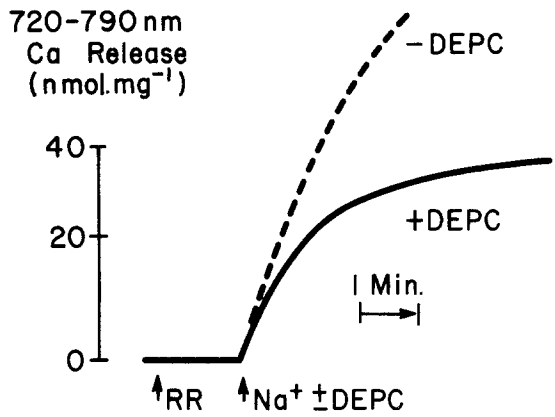

Fig. 1. Inhibition of Na^+-dependent Ca^2 release from heart mitochondria by DEPC. Mitochondria were incubated at 1 mg/ml in a medium of KCl (100 mM), TES (10 mM, pH 7.2), succinate (3 mM), rotenone (2 μg/ml), and antipyrylazo III (10 μM). The mitochondria were allowed to accumulate added Ca^{2+} (60 μM) at 25° C and the release of Ca^{2+} was monitored at 720 minus 790 nm in a DW-2C spectrophotometer. Where indicated, ruthenium red (1 μM), Na^+ (20 mM as the chloride salt), and DEPC (100 nmol mg^{-1} protein) were added.

mg protein is bound rapidly by the mitochondria, and this is followed by the slower uptake of an additional 30 nmol mg^{-1} over a 30-min incubation (Fig. 2B). Inhibition of Ca^{2+} uptake via the ruthenium red-sensitive uniport is seen to commence only after about 10 min of incubation with DEPC, at which point about 40 nmol mg^{-1} of the reagent has reacted (Fig. 2B). Histidine at 10 μmol mg^{-1} protects the mitochondrial Na^+/Ca^{2+} antiport from inhibition by DEPC, but does not reverse inhibition once DEPC has been permitted to react with the membrane.

The inhibition of mitochondria Na^+/Ca^{2+} antiport by DEPC also cannot be reversed by washing the mitochondria. For example, in a typical experiment DEPC inhibited Na^+/Ca^{2+} antiport by 76% when reacted for 7 min under the conditions of Fig 2A. When the mitochondria were reacted under identical conditions, re-isolated by centrifugation and washed in buffered 0.25 M sucrose, the extent of inhibition was 72%. These results are consistent with the expected covalent carbethoxylation of membrane proteins by DEPC (Miles, 1977).

The Na^+/Ca^{2+} antiport of heart mitochondria promotes both a Na^+-dependent efflux of $^{45}Ca^{2+}$ and a $Ca^{2+}/^{45}Ca^{2+}$ exchange (Crompton *et al.*, 1977, 1978). Both of these exchange reactions are inhibited by DEPC, and the titration curves obtained when increasing amounts of DEPC are reacted with the mitochondria for constant times (7 min) are roughly parallel (Fig. 3). Neither Na^+/Ca^{2+} nor Ca^{2+}/Ca^{2+} exchange is completely inhibited by DEPC and both reactions show a maximum inhibition near 100 nmol

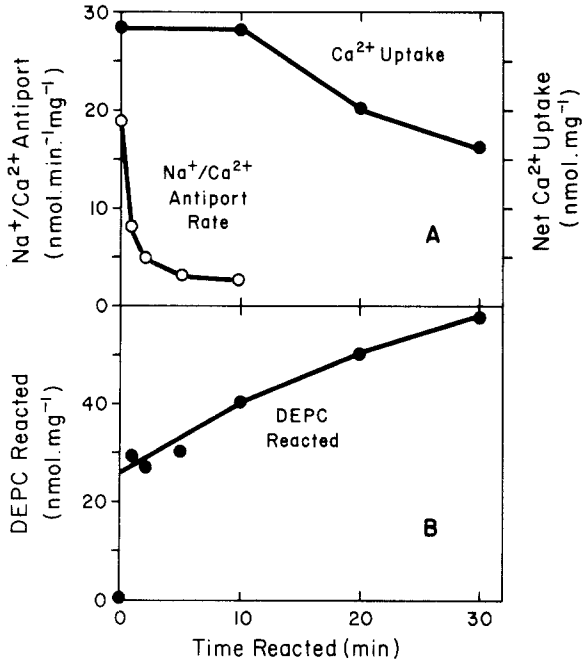

Fig. 2. Inhibition of Na^+/Ca^{2+} antiport and respiration-dependent Ca^{2+} uptake by DEPC as a function of time (A) and of the binding of DEPC (B). Mitochondria were suspended in a medium of KCl (100 mM), TES (10 mM, pH 7.2), succinate (3 mM), rotenone, and EGTA (10 μM) and incubated with DEPC (100 nmol mg^{-1}) for the indicated times at 25°C. The reaction was then terminated by addition of excess histidine and the mitochondria removed by centrifugation. The unreacted DEPC was determined on the supernate as described under Materials and Methods and the rate of Na^+/Ca^{2+} antiport and net Ca^{2+} uptake measured under the conditions of Fig. 1.

DEPC mg^{-1} (Fig. 3). Under these conditions about 40 nmol DEPC mg^{-1} is bound by the mitochondria (Fig. 2B). Half-maximal inhibition is attained when mitochondria are treated with 70 nmol mg^{-1}, and under these conditions about 35 nmol mg^{-1} of the inhibitor is bound.

DEPC inhibits the respiration-dependent uptake of Ca^{2+} only at much higher concentrations of inhibitor (Fig. 3) or when the time of reaction is extended (see Fig. 2A). At DEPC concentrations higher than 300 nmol mg^{-1} the uncoupling of succinate-supported oxidative phosphorylation can be detected and, at even higher levels, inhibition of succinate respiration is seen (data not shown).

DEPC inhibits Na^+/H^+ antiport in other membrane systems, and for this reason we examined the effect of this reagent on the corresponding antiport in mitochondria and SMP. DEPC at high concentrations (up to 5 μmol mg^{-1}) does not affect swelling of heart mitochondria in 80 mM Na^+

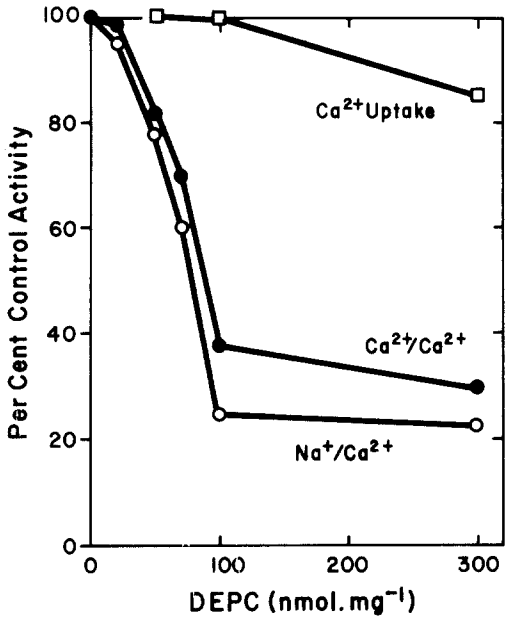

Fig. 3. Effect of increasing amounts of DEPC on $^{45}Ca^{2+}$ uptake, Na^+-dependent release of $^{45}Ca^{2+}$ and Ca^{2+}-dependent release of $^{45}Ca^{2+}$. The reactions were carried out as described in Materials and Methods using a 7-min reaction time and the indicated amount of DEPC.

acetate. This reaction is presently thought to depend on the uptake of Na^+ via Na^+/H^+ antiport (see Brierley and Jung, 1987). SMP containing FITC-dextran, a nonpermeant fluorescent probe sensitive to pH (Rottenberg, 1979; Ohkuma and Poole, 1978), show a ΔpH of about 0.5 pH units when succinate respiration is initiated (records not shown). Addition of Na^+ (20 mM) produces a decrease in internal H^+ consistent with Na^+/H^+ antiport. However, DEPC has essentially no effect on this reaction. It would appear that, in contrast to the situation with microbial vesicles (Damiano *et al.*, 1985), DEPC does not inhibit Na^+/H^+ antiport in heart mitochondria.

The Na^+-dependent release of Ca^{2+} from heart mitochondria shows an optimum at pH 6.6 (Fig. 4). DEPC, like diltiazem, inhibits Na^+/Ca^{2+} antiport activity over the entire pH range examined (Fig. 4). When DEPC is reacted with the mitochondria at different pH values and the mitochondria are re-isolated and Na^+/Ca^+ antiport activity assessed at pH 7.1, there is no change in the percent inhibition as a function of the pH of the initial reaction (pH 6.5–8.2).

Grillo and Aronson (1986) established by kinetic analysis that DEPC inhibits Na^+/H^+ exchange in their vesicle preparation by a reduction in V_{max} with virtually no effect on the K_m for Na^+. An analogous Hanes plot for

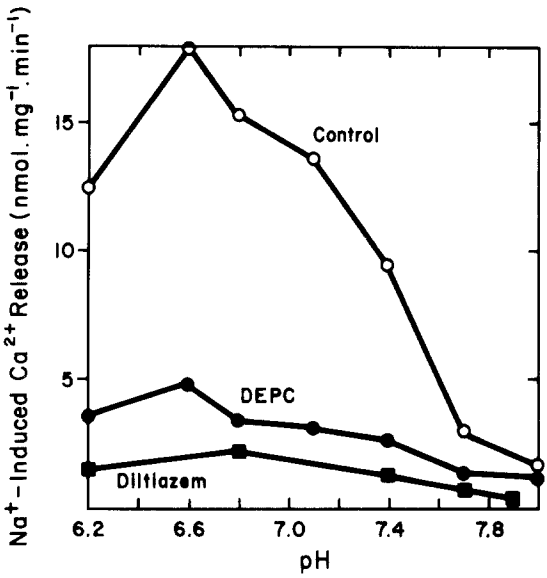

Fig. 4. The pH profile of Na^+-dependent Ca^{2+} release. The rate of Ca^{2+} release was determined at each pH as described in Fig. 1. Where indicated, DEPC (100 nmol mg^{-1}) or diltiazem (0.1 mM) were added 90 sec prior to the addition of Ca^{2+} (60 μM). When Ca^{2+} uptake was complete (4 min after Ca^{2+} addition), ruthenium red (1 μM) was added, followed by 20 mM NaCl to initiate Ca^{2+} release. The values reported are the average of duplicate determinations made with different preparations of mitochondria.

DEPC inhibition of mitochondrial Na^+/Ca^{2+} antiport shows a similar decrease in V_{max} with little effect on K_m (Fig. 5). Attempts to reverse the inhibition of Na^+/Ca^{2+} antiport by DEPC by addition of hydroxylamine (see Miles, 1977; Bindslev and Wright, 1984) have so far given inconclusive results, because of the strong inhibition of this reaction by hydroxylamine itself (not shown).

Discussion

These studies have established that DEPC inhibits the Na^+/Ca^2 antiport of heart mitochondria. The inhibition develops rapidly and is maximal after 5 min incubation at 25°C (Fig. 2A). Inhibition of Na^+/Ca^{2+} antiporter by DEPC is apparent when the antiporter is assayed either as Na^+-dependent Ca^{2+} release or as $Ca^{2+}/^{45}Ca^{2+}$ exchange (Fig. 3) and occurs at DEPC levels that have little or no effect on Ca^{2+} uptake, succinate respiration, or Na^+/H^+ antiport. A portion of the Na^+-dependent Ca^{2+} efflux appears to be insensitive to DEPC (Fig. 3).

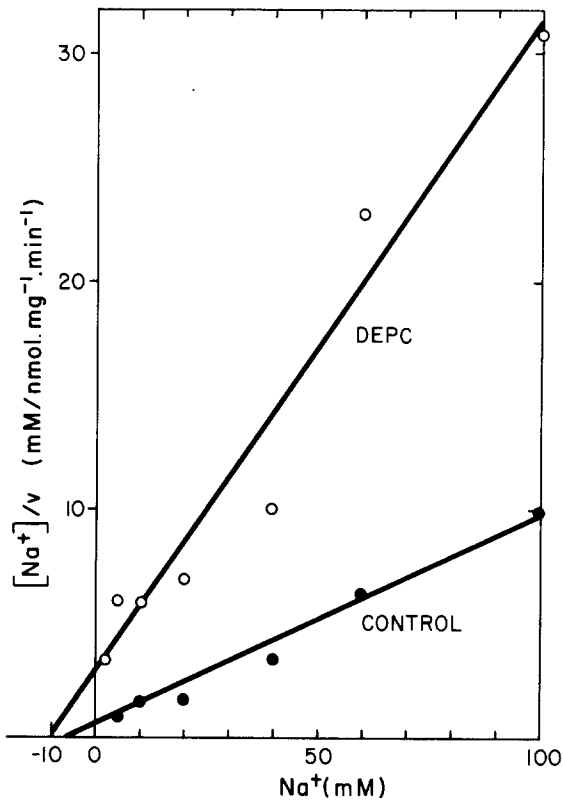

Fig. 5. Kinetics of Na^+-dependent Ca^{2+} efflux in the presence and absence of DEPC. The rates were estimated at pH 6.7 under the conditions of Fig. 1 and reported as an S/V vs S plot (see Grillo and Aronson, 1986). DEPC decreases the V_{max} (calculated from least-square equations) from 11 to 3.5 nmol Ca^{2+} mg^{-1} protein.

The maximum inhibition of Na^+/Ca^{2+} antiport occurs when 30–35 nmol mg^{-1} DEPC has reacted with the mitochondrial membrane (Fig. 2B). Since 25–30 nmol mg^{-1} reacts very rapidly, it appears that the inhibition is associated with the initial stages of the slow second phase of DEPC reaction that proceeds over an extended time of incubation (Fig. 2B). It has been reported that mitochondrial membranes contain a total of about 150 nmol histidine residues per mg protein (Jacobus and Brierley, 1969). The effect of DEPC seems to be on the V_{max} of the antiport reaction rather than on the K_m (Fig. 5). This result is in line with a reduction in the number of available transporters due to DEPC inactivation or to decreased turnover of reacted antiporters.

DEPC can be a reasonably specific carbethoxylation agent for histidine residues in proteins (Miles, 1977; Lundblod and Noyes, 1984). For this reason it is tempting to speculate that the reagent inactivates the Na^+/Ca^{2+} antiporter by interaction with a crucial imidazole group. DEPC inhibition studies have implicated an imidazolium residue in the active site of a Na^+/H^+ antiporter (Grillo and Aronson, 1986) and the Na^+-dependent dicarboxylate transporter of renal brush borders (Bindslev and Wright, 1984), for example. DEPC interacts preferentially with unprotonated imidazole residues (Melchoir and Fahrney, 1970; Miles, 1977). In the present study, DEPC reacted with mitochondria at either pH 6.5, 7.2, or 8.2 produces roughly the same extent of inhibition when the Na^+/Ca^{2+} antiport was subsequently assayed at pH 7.1. This suggests that, if an imidazole group is indeed involved in mitochondrial Na^+/Ca^{2+} antiport, it is largely unprotonated at pH 6.5.

The presence of substrate and Na^+ has been shown to protect the dicarboxylate transporter of renal brush borders from inhibition by DEPC (Bindslev and Wright, 1984). Such protection by the substrate cations Na^+ and Ca^{2+} is not seen with the mitochondrial Na^+/Ca^{2+} antiport, which becomes inhibited by DEPC when it is actively turning over (Fig. 1), as well as when the reagent is reacted with the inactive antiport (Fig. 2, for example). This may be taken as evidence that the putative imidazole group is not at the active site of the antiporter. It should be noted that one of the principal criteria for the reaction of DEPC with histidine, the reversal of inhibition by hydroxylamine, has not been met in the present study. It is therefore possible that the reagent reacts with other groups in the antiporter to produce the observed inactivation.

Further study will be necessary to clarify the nature and role of DEPC-reactive sites on the Na^+/Ca^{2+} antiport. For the present, however, it may be of value to have available a covalent modification reaction that appears relatively specific for this exchange component, especially during isolation and reconstitution protocols.

Acknowledgments

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