Some Effects of Dibutylchloromethyltin Chloride and Other Reagents on Mitochondrial K$^+$ Flux[1]

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Abstract

Respiration-dependent K$^+$ fluxes across the limiting membranes of isolated rat liver mitochondria, measured by means of ^{42}K, are stimulated by the oxidative phosphorylation inhibitor dibutylchloromethyltin chloride (DBCT). A lack of effect of Cl$^-$ concentration indicates that the stimulation of K$^+$ flux by DBCT is not attributable to Cl$^-$/OH$^-$ exchange activity. The mercurial mersalyl was previously shown to stimulate respiration-dependent K$^+$ influx. The combined presence of mersalyl plus DBCT results in a greater stimulation of K$^+$ influx than is caused by either DBCT or mersalyl alone. The oxidative phosphorylation inhibitor oligomycin, which alone has no effect on respiration-dependent K$^+$ influx, enhances the stimulatory effect of mersalyl on K$^+$ influx. The data are consistent with, although not proof of, a direct interaction of the K$^+$ transport mechanism with the mitochondrial energy transduction apparatus.

Key Words: Mitochondria; DBCT; K$^+$; transport; mersalyl.

Introduction

Unidirectional fluxes of K$^+$ both into and out of rat liver mitochondria depend on respiration (Diwan and Tedeschi, 1975). A direct link between K$^+$ transport and the process of energy transduction has thus been proposed (Diwan and Lehrer, 1978). Alternatively it has been suggested that the respiration dependence of unidirectional K$^+$ fluxes may be explained on the basis of a passive uniport mediating K$^+$ entry and a passive K$^+$/H$^+$ exchanger mediating K$^+$ exit (Chavez *et al.,* 1977). It has been pointed out that the pH dependence of K$^+$ efflux is inconsistent with a passive K$^+$/H$^+$ antiport mechanism (Diwan, 1981).

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[1]Abbreviations used: DCCD, *N,N'*-dicyclohexylcarbodiimide; DBCT, dibutylchloromethyltin chloride.
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The oxidative phosphorylation inhibitor DCCD reacts with a proteo-lipid subunit of the ATP synthase which is involved in H^+ translocation (Fillingame, 1980). The H^+ pump of cytochrome oxidase is also sensitive to inhibition by DCCD (Casey et al., 1980). It has been reported that not only H^+, but also monovalent and/or divalent cations, are transported by isolated, reconstituted preparations of cytochrome oxidase (Saltzgaber-Muller et al., 1980; Fry and Green, 1980; Rosier and Gunter, 1981). Ca^{++} transport activity has been attributed to a protein which copurifies with the cytochrome oxidase (Saltzgaber-Muller et al., 1980).

DCCD increases the apparent K_m for K^+ of the mechanism mediating respiration-dependent K^+ flux into rat liver mitochondria (Gauthier and Diwan, 1979). DCCD also inhibits K^+ flux into beef heart mitochondria (Jung et al., 1980). In contrast, the oxidative phosphorylation inhibitor oligomycin has no apparent effect on respiration-dependent K^+ influx (Gau-thier and Diwan, 1979). The effect of DCCD on K^+ influx is consistent with interaction of the K^+ transport mechanism with the energy transduction apparatus. However, the lack of specificity of DCCD makes such an interpre-tation uncertain.

The site of interaction of DBCT, another covalent inhibitor of the ATP synthase, is not on the DCCD-binding proteolipid (Cain et al., 1977). Yet evidence indicates interaction between binding sites for DBCT and DCCD. Preincubation with DBCT inhibits binding of [^{14}C]DCCD to the proteolipid subunit of the ATP synthase (Kiehl and Hatefi, 1980; Partis et al., 1980). DBCT treatment alters the electron spin resonance spectrum and rate of ascorbate reduction of a nitroxide analog of DCCD bound to beef heart mitochondria (Partis et al., 1980). Stiggall et al. (1979) have suggested that DBCT may react with a dithiol grouping on a soluble protein designated coupling Factor B by Sanadi and co-workers (Shankaran et al., 1975; Joshi et al., 1979). Factor B, which is essential for P_i/ATP exchange, is inactivated by dithiol reagents as well as by mercurial sulfhydryl reagents (Stiggall et al., 1979; Shankaran et al., 1975; Joshi et al., 1979). A recent report suggests that Factor B may also be the site of uncoupler binding to the ATP synthase (Blondin, 1980).

In addition to affecting the ATP synthase, DBCT is reported to inhibit respiration at the level of ubiquinone in plant mitochondria suspended in media of isoosmotic KCl (Moore et al., 1980). While effects of other alkyltin compounds on mitochondrial respiration in high Cl^- media have been attributed to catalysis of Cl^-/OH^- exchange (Aldridge et al., 1977), DBCT was found to cause no alteration of internal pH (Moore et al., 1980).

Mercurial sulfhydryl reagents, such as mersalyl, stimulate mitochon-drial K^+ flux (e.g., Chavez et al., 1977; Jung et al., 1977; Diwan et al., 1977). Mersalyl has been found to increase the V_{max} of unidirectional K^+ influx, while having little effect on the apparent K_m for K^+ of the transport

mechanism (Diwan *et al.,* 1977). Various mechanisms have been proposed for the enhancement of K$^+$ permeability by mercurials, including activation of an endogenous ionophore (Southard *et al.,* 1973), inhibition of P$_i$ exchange (Chavez *et al.,* 1977; Jung *et al.,* 1977), and depletion of membrane-bound Mg^{2+} (Bogucka and Wojtczak, 1979). Evidence against the adequacy of the latter two explanations has been presented (Diwan *et al.,* 1977; Diwan *et al.,* 1980).

The present studies have focused on effects of DBCT, mersalyl, and oligomycin on respiration-dependent fluxes of K$^+$ across the limiting membranes of isolated rat liver mitochondria.

Materials and Methods

Rat liver mitochondria were isolated by standard procedures as in previous studies (e.g., Diwan *et al.,* 1979). The 0.25 M sucrose isolation medium was supplemented with 0.4 mM Tris EDTA or Tris EGTA in the initial stages of preparation. Mitochondria (4–6 mg of Biuret-assayed protein per milliliter) were incubated at 20°C in media containing, unless otherwise indicated, 240 mM sucrose, 8 mM succinate, K$^+$ (added as KOH, see figure or table legends), and trace amounts of ^{42}K, ^{3}H$_2$O, and [^{14}C]sucrose, with the pH adjusted to 7.5 with Tris base. Sampling was by centrifugation through silicone (Harris and VanDam, 1968). Radioisotopes were assayed by liquid scintillation counting. Net K$^+$ levels were determined by atomic absorption spectroscopy. Mitochondrial contents of labeled and total K$^+$ were calculated from the data as in previous studies (e.g., Johnson and Pressman, 1969; Diwan *et al.,* 1979). Values of labeled and total K$^+$ shown in the figures are corrected for contaminating external K$^+$, estimated from the product of the [^{14}C]sucrose distribution space of the sedimented mitochondria and the supernatant K$^+$ concentration. Unidirectional flux rates are calculated from the changes in mitochondrial contents of labeled and total K$^+$ between samples taken after 0.75 and 7 minutes of incubation.

Radioisotopes were obtained from New England Nuclear. Silicone (SF-1154) was a gift of the General Electric Co. DBCT was supplied by K. Cain and D. E. Griffiths. Antimycin A and oligomycin were obtained from Sigma Chemical Co.

Results

Figure 1A shows that 8.4 nmol DBCT per milligram protein stimulates K$^+$ influx, as indicated by the increased slope of the plot of ^{42}K uptake vs. incubation time. At higher DBCT levels there is initially a stimulation of ^{42}K
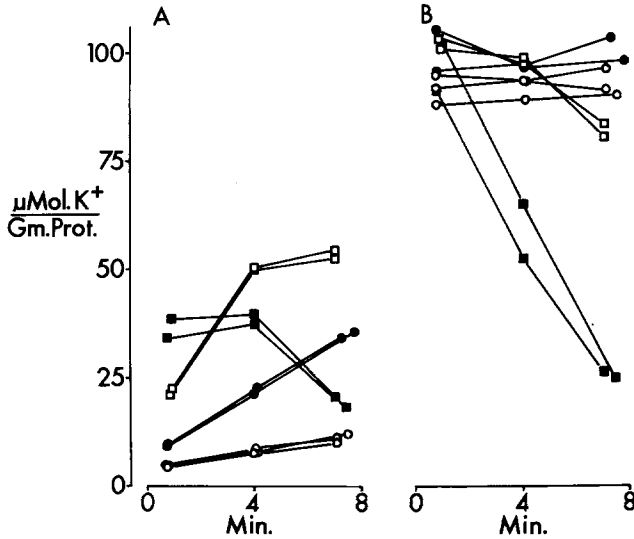
Fig. 1. Effect of DBCT on uptake of labeled K^+, shown in A, and on total mitochondrial K^+, shown in B. The K^+ concentration in the medium was 1.9 mM. DBCT concentrations in the medium, in units of nmol/mg protein: O, zero; ●, 8.4; □ 13.2; ■, 18.5.

uptake, followed by a decline in the influx rate or a loss of the labeled K^+. Figure 1B shows that the higher DBCT levels cause a net loss of endogenous K^+. Unidirectional K^+ flux rates were calculated from data of eight similar experiments and the results summarized in Table I. It should be noted that

Table I. Concentration Dependence of Effects of DBCT on K^+ Flux[a]

nmol/mg protein DBCT	Stimulation of	
	K^+ influx	K^+ efflux
3.7	−	−
4.2	−	−
5.1	−,+	−,−
6.2	+	−
6.7	+	−
7.3	+,+	−,−
7.5	+	−
8.4	+	+
10.2	+	+
11.0	+	+
13.2	+	+
14.5	+	+
15.3	ND	ND
18.5	ND	ND

[a] Individual determinations, each representing at least two measurements, from eight experiments in which the K^+ concentration ranged from 2–3 mM. + = stimulation; − = no stimulation; ND = unidirectional fluxes not measurable due to massive net K^+ loss.

Table II. Respiration Dependence of DBCT-Stimulated K^+ Flux[a]

nmol/mg protein [DBCT]	μmol K^+/g protein (min)			
	K^+ influx		K^+ efflux	
	−Antimycin A	+Antimycin A	−Antimycin A	+Antimycin A
None	2.5		3.0	
	1.7		1.3	
	1.7		0.7	
5	1.7	0.3	1.0	0.2
	2.3	0.3	1.0	0.8
10	8.9	0.7	5.4	3.8
	7.5	0.8	5.9	2.3

[a]The K^+ concentration was 3.2 mM. Antimycin A when present was at 54 ng/mg protein. When included, DBCT and antimycin A were present in the medium prior to the addition of the mitochondria at zero time.

unidirectional flux rates can accurately be determined only under conditions in which isotope uptakes and changes in net K^+ content are sufficiently small that the fluxes may be considered to be initial rates. Thus the flux rates estimated in the presence of some of the higher DBCT levels tested have only qualitative significance. At DBCT concentrations above about 14 nmol/mg protein there is such a massive net loss of K^+ that no estimate of unidirectional flux rates can be made. The results in Table I indicate that a slightly higher concentration of DBCT is required to stimulate K^+ efflux than the level which is effective in stimulating K^+ influx.

K⁺ fluxes stimulated by DBCT remain dependent on respiration, as shown in Table II. Antimycin A inhibits K^+ influx, and to a lesser extent K^+ efflux, in the presence of DBCT. The results are consistent with previously published data showing that, in the absence of DBCT, antimycin A inhibits

Table III. Effects of Mersalyl on K^+ Influx in the Presence of DBCT or Oligomycin[a]

Experiment	Additions	μmol/g protein (min) K^+ influx
A	None	1.22 ± 0.06
	DBCT	1.93 ± 0.30
	Mersalyl	2.32
		2.46
	Mersalyl + DBCT	6.12
		6.74
B	None	0.85 ± 0.10
	Oligomycin	0.87 ± 0.09
	Mersalyl	1.56 ± 0.04
	Mersalyl + Oligomycin	1.89 ± 0.09

[a]In experiment A the K^+ concentration was 2.8 mM. When present DBCT was at 7.5 nmol/mg protein and mersalyl was at 150 μM. In experiment B the K^+ concentration was 1.7 mM. When present oligomycin was at 0.4 μg/mg protein and mersalyl was at 120 μM. Values shown are individual determinations or averages of three determinations $\pm$ standard deviations.

K$^+$ influx to a greater extent than it inhibits K$^+$ efflux (Diwan and Tedeschi, 1975).

The stimulation of K$^+$ influx by the mercurial mersalyl shown in Table III is similar to that reported earlier (e.g., Diwan *et al.,* 1977). In experiment A the K$^+$ influx rate in the presence of DBCT plus mersalyl is shown to be greater than the sum of the rates with the reagents present separately. In experiment B of Table III, addition alone of 0.4 μg oligomycin/mg protein has no apparent effect, consistent with earlier data (Gauthier and Diwan, 1979). However, in the presence of oligomycin the stimulation of K$^+$ influx by mersalyl is enhanced. Thus when perturbed by interaction with mersalyl, the K$^+$ transport mechanism does show some sensitivity to oligomycin.

As mentioned earlier, some effects of other alkyltin compounds on mitochondria suspended in media of high Cl$^-$ content have been attributed to Cl$^-$/OH$^-$ exchange activity (Aldridge *et al.,* 1977; Brierley *et al.,* 1978). In the present experiments, Cl$^-$ was omitted from the reaction media. However, about 1–2 mM Cl$^-$ is present as a contaminant of the ^{42}K stock. Figure 2 depicts the effect of increasing the Cl$^-$ concentration. The slightly lower net
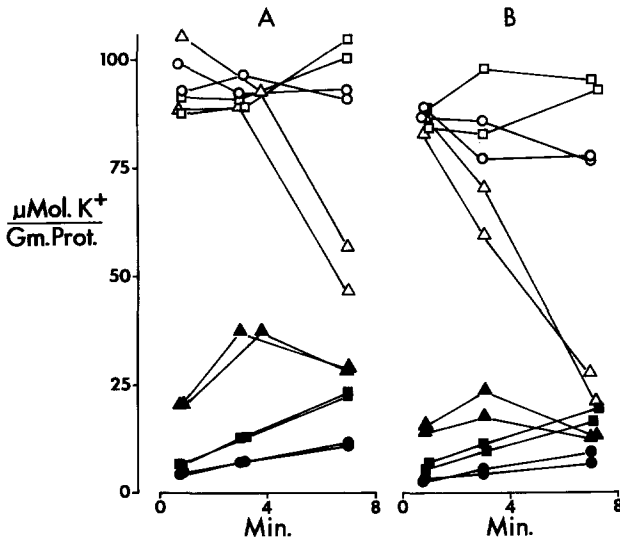

Fig. 2. Dependence on Cl$^-$ of the effect of DBCT. The low Cl$^-$ medium used in A was identical to that described in Methods. The high Cl$^-$ medium used in B was similar to that described in Methods except that 31 mM Tris base was included and the pH was adjusted to 7.5 with HCl. The K$^+$ concentration in the medium was in each case approximately 2.8 mM. The open symbols depict values of total mitochondrial K$^+$, while the solid symbols depict values of labeled K$^+$, both in units of μmol/g protein. DBCT concentrations, in units of nmol/mg protein, were: O or ●, zero; □ or ■, 7.3; △ or ▲, 14.5.

K^+ contents and ^{42}K uptake rates of control samples in the high Cl^- medium may be attributed to the higher level of Tris, which can be taken up by mitochondria (Brierley *et al.*, 1971) and like various inorganic cations probably competes with K^+. Although the flux rates in the two media are thus not precisely comparable, the data appear to rule out any major effect of Cl^-. Only a slight enhancement of the rate of net K^+ loss is observed at the higher DBCT level, as the Cl^- concentration is increased by an order of magnitude.

Discussion

The observations that different concentrations of DBCT are required to stimulate unidirectional K^+ influx and efflux rates may be indicative of distinct mechanisms of transport in the two directions, although the range of concentrations over which DBCT stimulates K^+ influx but not efflux is quite narrow. It should be recalled that the concentration of DBCT binding sites and the concentration of DBCT required for maximal inhibition of ATP-ase activity, estimated by titration of yeast mitochondrial membranes, are both about 10 nmol/mg protein (Cain *et al.*, 1977). This is within the range of concentrations found to stimulate K^+ flux in the present experiments.

Results such as are depicted in Fig. 2 show little evidence of dependence on Cl^- concentration of the effects on K^+ influx of moderate amounts of DBCT (e.g., 5–7 nmol/mg protein). Thus, the stimulation of K^+ influx by DBCT does not appear to relate to any effect on Cl^-/OH^- exchange. This is consistent with the earlier evidence indicating no effect of DBCT on transmembrane pH shifts in plant mitochondria suspended in a high Cl^- medium (Moore *et al.*, 1980).

The finding that the K^+ influx rate in the presence of DBCT plus mersalyl is greater than the sum of the rates induced individually by these reagents suggests separate, although possibly interacting, sites of action. Thus, if the mercurial and/or DBCT are acting via reaction with a dithiol group on Factor B, at least one of these reagents must affect K^+ transport additionally by some other mechanism.

The fact that two more inhibitors of the ATP synthase, DBCT and oligomycin, have been found to alter respiration-dependent K^+ transport, at least under some conditions, is consistent with, though not proof of, a direct relationship between the ATP synthase and the K^+ transport mechanism. It is interesting to note that oligomycin is reported to bind to the same proteolipid subunit of the ATP synthase as that which binds DCCD (Criddle *et al.*, 1979; Fillingame, 1980). As discussed above, DBCT and DCCD bind to separate but interacting sites on the ATP synthase. Thus the ATP synthase binding sites for three reagents which affect K^+ transport, oligomycin, DCCD, and

DBCT, are closely related. Nevertheless, the possibility of secondary effects on K^+ transport of all three reagents, unrelated to their interaction with the energy transduction apparatus, cannot be ruled out.

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