The Influence of Adenine Nucleotides and Oxidizable Substrates on Triethyltin-Mediated Chloride Uptake by Rat Liver Mitochondria in Potassium Chloride Media

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In a 100 mM-KCl medium, pH 6.8, containing ATP increasing concentrations of triethyltin cause an uptake of Cl^- into mitochondria with a maximum at $1 \mu\text{M}$. This can be inhibited by atracylate or oligomycin, but is virtually unaffected by the presence of rotenone. When the medium contains substrate (pyruvate, β -hydroxybutyrate or succinate), both in the presence and absence of adenine nucleotides, Cl^- uptake is greater with a maximum at 1 – $10 \mu\text{M}$ -triethyltin. If substrate oxidation is blocked by respiratory-chain inhibitors the Cl^- uptake mediated by triethyltin is inhibited except in the media containing ATP, when the characteristics of Cl^- uptake similar to that found in the medium containing ATP alone are observed. Under all conditions tested Cl^- uptake is decreased by the presence of 2,4-dinitrophenol. It is concluded that energy from either the oxidation of substrate or the hydrolysis of ATP is associated with the generation of sufficient OH^- to enable the triethyltin-mediated Cl^-/OH^- exchange to occur under the metabolic conditions relevant to this action of triethyltin.

When mitochondria are suspended in KCl media in the presence of triethyltin a marked inhibition of oxidative phosphorylation is observed (Rose & Aldridge, 1972) and a chloride-dependent stimulation of both respiration (Aldridge, 1958; Stockdale *et al.*, 1970) and mitochondrial ATPase* activity (Aldridge & Street, 1964) is demonstrable. These effects have been attributed to the consequences of movement of the normally impermeant Cl^- ion (Chappell & Robinson, 1968) into mitochondria mediated by triethyltin (Stockdale *et al.*, 1970; Rose & Aldridge, 1972; Dawson & Selwyn, 1974). Harris *et al.* (1973) were able to obtain direct evidence in a non-phosphorylating medium for a triethyltin-mediated Cl^- entry into mitochondria which could then displace other anions from the matrix and this has been shown to occur in media used in the present studies (Skilleter, 1975). The results support the data of Selwyn *et al.* (1970) which suggested that the primary action of triethyltin was to facilitate a Cl^-/OH^- exchange.

The present study is part of a series attempting to understand the action of triethyltin on mitochondrial functions and examines in detail the triethyltin-mediated Cl^- uptake by rat liver mitochondria in KCl media under conditions when inhibition of oxidative phosphorylation or a stimulation of respiration or mitochondrial ATPase is known to occur. Evidence is presented to support the concept that under these conditions generation of OH^- for

the Cl^- exchange requires energy either as a result of substrate oxidation, or the hydrolysis of ATP. The relevance of this to proton translocation and the binding of triethyltin to mitochondria (Aldridge & Street, 1970, 1971) is also discussed.

Materials and Methods

Materials

Triethyltin sulphate was prepared from triethyltin hydroxide (Aldridge & Cremer, 1955) supplied by the Tin Research Institute, Greenford, Middx., U.K. and dissolved in water. The following were purchased: ATP and ADP were from Boehringer (London) Corp., London W5 2TZ, U.K.; oligomycin, antimycin A, sodium pyruvate, L-succinic acid and DL- β -hydroxybutyrate were from Sigma Chemical Co., St. Louis, Mo., U.S.A.; potassium atracylate was from Calbiochem, Los Angeles, Calif., U.S.A.; rotenone (recrystallized from ethanol by addition of water), 2,4-dinitrophenol, glycylglycine and HClO_4 (60%) were from BDH Chemicals, Poole, Dorset, U.K.; Versilube F.50 silicone oil was from Jacobsen Van Den Berg and Co., London W3 7RN, U.K.; Na^{36}Cl was from The Radiochemical Centre, Amersham, Bucks., U.K.; Instagel liquid scintillator fluid was from Packard Instrument Co., Downers Grove, Ill., U.S.A. All other reagents were A.R. grade.

* Abbreviation: ATPase, adenosine triphosphatase.

Methods

Preparation of mitochondria. Rat liver mitochondria were prepared as described by Aldridge & Street (1971) and suspended in 0.3M-sucrose at a protein concentration of 20–25 mg/ml. Protein was measured by the biuret method of Robinson & Hogden (1940) as modified by Aldridge (1962).

Incubations. Mitochondria (0.15 ml) were added to the incubation medium (2.80 ml) comprising KCl (100 mM), $Mg(NO_3)_2$ (14 mM), EDTA (1 mM), glycyl-glycine (16.7 mM), 50 μ l of $Na^{36}Cl$ (20 μ Ci/ml) and various concentrations of triethyltin at pH 6.8 as indicated in the Results section. Details of further additions to the medium are also given in the Results section and, unless otherwise stated, were present before the addition of the mitochondria. The incubation was carried out at 37°C with shaking in open 25 ml beakers. After 5 min, samples were withdrawn for determination of mitochondrial Cl^- content.

Determination of mitochondrial Cl^- content. Samples (5 $\times$ 0.2 ml) of each incubation mixture were centrifuged through silicone oil into $HClO_4$ (1.5 M) as described by Harris & Van Dam (1968) in 0.4 ml capacity polypropylene tubes in model 3200 Eppendorf centrifuges. Portions (5 $\times$ 20 μ l) of the $HClO_4$ extracts of the incubation mixtures were combined and transferred to 15 ml of Instagel scintillator fluid for determination of ^{36}Cl radioactivity. Counting efficiency was determined by the addition of internal standard. Measurement of mitochondrial Cl^- content based on ^{36}Cl uptake from the incubation media showed close agreement on duplicate determination and over the range 4–10 min did not vary significantly indicating that steady-state concentrations had been reached.

The actual Cl^- content of the mitochondrial matrix in the presence of triethyltin was obtained by subtracting from the measured content the values obtained in the absence of triethyltin. Since Cl^- is a non-permeant anion of mitochondria (Chappell & Robinson, 1968) these values represented the Cl^- present in the intermembrane space and were found to be 290 ($\pm$ 10 S.E.M.) nmol of Cl^- /mg of mitochondrial protein in the KCl medium containing ATP only and 365 ($\pm$ 20 S.E.M.) nmol of Cl^- /mg of mitochondrial protein in the KCl media containing various substrates. These values were in close agreement with those calculated from the value of the sucrose-accessible space as measured by Harris & Van Dam (1968).

Results

Effect of triethyltin on mitochondrial chloride uptake in the KCl medium containing ATP

At pH 6.8 in a KCl medium containing ATP only, triethyltin produces a stimulation of mitochondrial

Fig. 1. Effect of triethyltin on mitochondrial Cl^- uptake in KCl media containing ATP

Mitochondria were incubated in the KCl medium containing ATP (3 mM) (Δ) or ATP (3 mM) plus rotenone (1.1 μ M) ($\blacktriangle$) as described under 'Methods' in the presence of various concentrations of triethyltin.

Table 1. Effect of additions to KCl/ATP medium on triethyltin-mediated Cl^- uptake by mitochondria

Mitochondria were incubated in the KCl media as described under 'Methods' with the indicated additions; ATP (3 mM); 2,4-dinitrophenol (30 μ M); rotenone (1.1 μ M); atractylate (30 μ M); and oligomycin (0.2 μ g/mg of mitochondrial protein: 10 min preincubation at 37°C in the absence of triethyltin).

Additions Triethyltin concentration (μ M) ...	Matrix Cl^- content (nmol/mg of mitochondrial protein)		
	0.1	1.0	10.0
Control*	45	148	90
No ATP	42	59	71
ATP+2,4-dinitrophenol	44	46	41
ATP+2,4-dinitrophenol†	43	154	86
ATP+atractylate	51	73	70
ATP+rotenone	13	19	22
+atractylate			
ATP+oligomycin	49	71	86
ATP+rotenone	28	35	38
+oligomycin			

* Values taken from Fig. 1(a).

† 2,4-Dinitrophenol added after incubation with triethyltin for a further 5 min incubation before determination of Cl^- uptake.

ATPase activity with a maximum at 1 μ M-triethyltin (Aldridge & Street, 1964). In the present study with a similar medium when the triethyltin concentration

was increased from 0 to $10\text{ }\mu\text{M}$ maximum Cl^- content of the mitochondrial matrix was also observed in the presence of $1\text{ }\mu\text{M}$ -triethyltin (Fig. 1). Further increase in the triethyltin concentration from 10 to $80\text{ }\mu\text{M}$ caused a smaller second rise in Cl^- content, but this probably results from an additional non-specific swelling action of triethyltin (W. N. Aldridge, unpublished work), particularly since no corresponding change in mitochondrial ATPase activity can be demonstrated (Aldridge & Street, 1964). The pattern of Cl^- uptake was essentially the same when the mitochondria were incubated simultaneously with rotenone, indicating that inhibition of the NADH dehydrogenase and oxidation of endogenous substrates did not markedly affect the action of triethyltin.

The Cl^- uptake mediated by triethyltin in part appeared to depend on the presence of ATP, since its omission from the medium or inhibition of ATP entry into the mitochondria by atractylate (Klingenberg, 1970) markedly decreased the matrix Cl^- content. Preincubation of the mitochondria with oligomycin to inhibit the mitochondrial ATPase (Lardy *et al.*, 1958) also caused a similar decrease in the Cl^- content as did simultaneous incubation with the uncoupler 2,4-dinitrophenol (Table 1). Addition of 2,4-dinitrophenol after incubation with triethyltin, however, could not reverse Cl^- uptake.

Effect of triethyltin on Cl^- uptake in the KCl medium containing substrate

Pyruvate, β -hydroxybutyrate and succinate are readily oxidized by mitochondria in the KCl media used (Rose & Aldridge, 1972; Skilleter, 1975) and were chosen as examples of substrates with suitably different patterns of oxidation. When these substrates were present in the medium, Cl^- uptake into the mitochondrial matrix mediated by triethyltin was modified from that observed in the medium containing ATP alone. The total matrix Cl^- content was greater at both $1\text{ }\mu\text{M}$ - and $10\text{ }\mu\text{M}$ -triethyltin and a marked fall in Cl^- content was only observed above $10\text{ }\mu\text{M}$ -triethyltin (Fig. 2).

The pattern of Cl^- uptake was similar whether the medium contained substrate only or substrate in the presence of ADP and P_i [conditions under which an inhibition of oxidative phosphorylation can be shown (Skilleter, 1975)] or substrate in the presence of ATP [conditions under which a stimulation of respiration by triethyltin is demonstrable (Aldridge, 1958; Skilleter, 1975)]. This suggests that the Cl^- uptake process under these conditions is largely unaffected by the respiratory state of the mitochondria. In media containing any of the substrates tested the presence of 2,4-dinitrophenol always caused a decrease in the matrix Cl^- content produced by all concentrations of triethyltin (Table 2).

Fig. 2. Effect of triethyltin on mitochondrial Cl^- uptake in KCl media containing different substrates

Mitochondria were incubated in the KCl medium containing substrate only (5 mM) ($\square$), substrate (5 mM) plus rotenone ($1.1\text{ }\mu\text{M}$) ($\blacksquare$), substrate (5 mM) plus ADP (3 mM) and P_i (1.33 mM) with and without rotenone ($1.1\text{ }\mu\text{M}$) ($\circ$), ($\bullet$); and substrate (5 mM) plus ATP (3 mM) with and without rotenone ($1.1\text{ }\mu\text{M}$) ($\blacktriangle$), ($\triangle$) in the presence of various concentrations of triethyltin as described under 'Methods'. The substrates used were (a) pyruvate in the presence of 1 mM-fumarate (Aldridge & Street, 1971), (b) β -hydroxybutyrate and (c) succinate.

When the medium contained pyruvate or β -hydroxybutyrate, either in the presence or absence of ADP and P_i , triethyltin-mediated Cl^- uptake could

Table 2. *Effect of additions to KCl/substrate media on triethyltin-mediated Cl⁻ uptake by mitochondria*

Mitochondria were incubated in the KCl medium as described under 'Methods' with the indicated additions: substrates (5 mM), ATP (3 mM), rotenone (1.1 μ M), 2,4-dinitrophenol (30 μ M), atractylate (30 μ M), antimycin (0.2 μ g/mg of mitochondrial protein) and oligomycin (0.2 μ g/mg of mitochondrial protein: 10 min preincubation at 37°C in the absence of triethyltin).

Substrate medium	Additions Concentration of triethyltin present (μ M) ...	Matrix Cl ⁻ content (nmol/mg of mitochondrial protein)			
		0.1	1.0	10.0	80.0
Pyruvate*	(a) None	25†	200†	209†	65†
	(b) 2,4-Dinitrophenol	24	79	101	83
	(c) Oligomycin	22	192	200	61
Pyruvate*+ADP+P _i	(a) None	20†	165†	225†	110†
	(b) 2,4-Dinitrophenol	26	89	117	107
	(c) Oligomycin	25	162	204	98
Pyruvate*+rotenone+ATP	(a) None	34†	127†	55†	66†
	(b) Atractylate	20	57	54	52
	(c) Oligomycin	21	55	58	50
β -Hydroxybutyrate	(a) None	54*	235†	240†	62†
	(b) 2,4-Dinitrophenol	10	53	72	57
	(c) Oligomycin	48	221	232	55
β -Hydroxybutyrate+ADP+P _i	(a) None	50†	196†	228†	108†
	(b) 2,4-Dinitrophenol	12	56	94	82
	(c) Oligomycin	48	192	223	86
β -Hydroxybutyrate+rotenone+ATP	(a) None	40†	134†	67†	81†
	(b) Atractylate	10	25	21	8
	(c) Oligomycin	9	22	20	26
Succinate	(a) None	41†	248†	241†	83†
	(b) 2,4-Dinitrophenol	27	30	97	77
	(c) Oligomycin	38	237	240	81
Succinate+ADP+P _i	(a) None	24†	202†	266†	98†
	(b) 2,4-Dinitrophenol	20	50	74	70
	(c) Oligomycin	17	205	240	89
	(d) Antimycin	18	207	88	66
	(e) Antimycin+oligomycin	11	41	55	48
Succinate+rotenone+ATP	(a) None	29†	260†	258†	47†
	(b) Antimycin	12	144	88	44
	(c) Antimycin+oligomycin	9	60	75	56
	(d) Antimycin+attractylate	10	63	71	52

* Medium also contained 1 mM-fumarate (Aldridge & Street, 1971).

† Values taken from Fig. 2.

be completely inhibited with rotenone (Figs. 2a and 2b), but was unaffected by oligomycin (Table 2). These results indicate that for pyruvate and β -hydroxybutyrate in the absence of ATP if electron-transfer energy derived from their oxidation is blocked, no triethyltin-mediated Cl⁻ uptake occurs and that under these conditions the activity of the mitochondrial ATPase is not necessary. When electron transfer was inhibited with rotenone, but the medium contained ATP, the characteristics of Cl⁻ uptake observed were similar to those found in the medium containing ATP alone (Figs. 2a and 2b). Further, the mitochondrial Cl⁻ content was decreased by simultaneous incubation with atractylate or by preincubation with oligomycin indicating that in the absence of electron-transfer energy there was a requisite for the mitochondrial ATPase (Table 2).

When succinate was the oxidizable substrate present, Cl⁻ uptake mediated by triethyltin predictably was not inhibited by rotenone (Fig. 2c). In the media containing succinate, ADP and P_i, as with pyruvate and β -hydroxybutyrate, Cl⁻ uptake by the mitochondria was unaffected by oligomycin but the respiratory-chain inhibitor antimycin only decreased the matrix Cl⁻ content in the presence of 10 μ M-triethyltin (Table 2). In view of the site of action of antimycin at cytochrome *b* (Borden & Slater, 1970) it might be expected that this inhibitor would have been more effective at preventing Cl⁻ uptake. However, after preincubation of the mitochondria with both antimycin and oligomycin, Cl⁻ uptake could be inhibited at 1 μ M- and 10 μ M-triethyltin (Table 2). The explanation of these results is not clear, but the data do suggest that impairment of triethyltin-mediated Cl⁻

uptake into mitochondria under these conditions in contrast with that observed with pyruvate and β -hydroxybutyrate requires inhibition of both electron transfer and the mitochondrial ATPase. In the medium containing succinate and ATP in the presence of antimycin the mitochondria behaved as if the medium contained only ATP (Table 2).

Discussion

During respiration and both ATP hydrolysis or synthesis a protonmotive force (Δp) exists across the mitochondrial membrane comprising a potential gradient of protons (ΔE) and a pH differential ($-59\Delta p\text{H}$) (Mitchell, 1968; Mitchell & Moyle, 1969; Nicholls, 1974). The pH of the mitochondrial matrix is decreased by the triethyltin-mediated Cl^-/OH^- exchange (Dawson & Selwyn, 1974) and could thus cause a stimulation of the proton-translocating ATPase or proton-translocating respiration as a means to restore Δp (Nicholls, 1974). The process would continue until steady-state concentrations of Cl^- are achieved in the matrix probably being limited by the electrogenic outward diffusion of Cl^- (Stockdale *et al.*, 1970). The measurements made in the present study represent such steady-state Cl^- concentrations achieved under the conditions relevant to the metabolic actions of triethyltin on mitochondria observed *in vitro*.

When the KCl medium used contained ATP as the sole energy source Cl^- uptake as a function of triethyltin concentration showed a maximum at $1\text{ }\mu\text{M}$ -triethyltin which closely resembled the stimulation of ATP hydrolysis observed under similar conditions (Aldridge & Street, 1964). Further, both activities are virtually unaffected by the presence of rotenone in the medium but are inhibited by oligomycin. The data are a direct demonstration therefore that exchange of OH^- for Cl^- (Selwyn *et al.*, 1970; Harris *et al.*, 1973) can be associated with the hydrolysis of ATP. It is suggested that the OH^- ions may be derived from the ionization of water, the Cl^-/OH^- exchange thus initiating the ATPase with the available protons which are possibly simultaneously translocated by a proton 'channel' in the manner suggested by Mitchell & Moyle (1974). Inhibition of proton translocation would therefore lower the concentration of OH^- ions available for the Cl^-/OH^- exchange.

When the concentration of triethyltin in the ATP medium is raised to $10\text{ }\mu\text{M}$ a decrease in mitochondrial Cl^- content and ATPase activity is demonstrable. Triethyltin has been shown to bind to an insoluble membrane fraction of mitochondria (Aldridge & Street, 1970) probably involving histidine residues (Rose, 1969; Aldridge & Street, 1971). It is attractive to suggest that it may be the hydrophobic F_0 component of the mitochondrial ATPase (Kagawa & Racker, 1971; Capaldi *et al.*, 1973; Beechey, 1974),

the component probably important in the translocation of protons across the mitochondrial membrane (Mitchell & Moyle, 1974), which binds triethyltin under these conditions, particularly since there is some evidence that tributyltin may bind to F_0 (Kagawa & Racker, 1966).

In the studies using media containing different substrates the results indicated that electron-transfer energy resulting from substrate oxidation was essential to support triethyltin-mediated Cl^- uptake, although this need not be accompanied by ATP synthesis from added ADP and P_i as indicated by the ineffectiveness of oligomycin under these conditions to Cl^- uptake. This is in contrast with the inhibitory action of oligomycin when ATP is the effective source of energy present. The apparent redundancy of the mitochondrial ATPase [or ATP synthase, see Racker (1970)] in the Cl^- -uptake process during respiration, compared with its participation when hydrolysing ATP, might, however, explain both the greater Cl^- uptake at $1\text{ }\mu\text{M}$ -triethyltin and the inability of $10\text{ }\mu\text{M}$ -triethyltin to inhibit Cl^- uptake in the presence of substrate, since if triethyltin does indeed bind to the ATPase (F_0) this need not be relevant to the proton-transfer process under these conditions. The inhibitory action of triethyltin above $10\text{ }\mu\text{M}$ on Cl^- uptake is more likely to be due to the loss of functional membrane integrity caused by additional non-specific mitochondrial swelling.

These contentions are consistent with the findings of Pedersen (1975) that the mitochondrial ATPase contains separate sites involved in the hydrolysis of ATP and reversible binding of ADP assumed to participate in ATP synthesis. Further studies on the effects of triethyltin on the isolated ATPase complex may therefore prove useful in establishing whether the binding of triethyltin might be important in the inhibition of proton translocation.

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