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Triphenyltin as Inductor of Mitochondrial Membrane Permeability Transition

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The effect of triphenyltin on mitochondrial Ca^{2+} content was studied. It was found that this trialkyltin compound induces an increase in membrane permeability that leads to Ca^{2+} release, drop of the transmembrane potential, and efflux of matrix proteins. Interestingly, cyclosporin A was unable to inhibit triphenyltin-induced Ca^{2+} release. Based on these results it is proposed that the hyperpermeable state is produced by modification of 2.25 nmol of membrane thiol groups.

KEY WORDS: Triphenyltin; mitochondrial Ca^{2+}; trialkytin compound.

INTRODUCTION

Early experiments by Aldridge and Cremer (1955) and Rose and Aldridge (1968) showed that trialkyltin compounds inhibited oxidative phosphorylation and induced uncoupling of mitochondrial energy transduction. The effect of trialkyltins on mitochondrial Ca^{2+} transport has also been studied. Stockdale *et al.* (1970) showed that tripropyltin at a concentration of 3 μM induced an increase in calcium uptake. By the same token, Bygrave *et al.* (1978) demonstrated that tributyltin, depending on its concentration, influenced Ca^{2+} movement. For example, at 0.5 μM it increased the initial rate of Ca^{2+} accumulation, while at 2 μM it induces Ca^{2+} release. In addition, trialkyltin reagents promote mitochondrial swelling and exhibit an oligomycin-like action. Such effects are attributed, in general, to a chloride–hydroxide exchange across the inner membrane, followed by leakage of the anion out of the mitochondria (Selwyn *et al.*, 1970a,b). The detergent-like action ascribed to triphenyltin (Stockdale *et al.*, 1970) represents an exception to the proposed mechanism. The present work was aimed at exploring further the mechanism by which triphenyltin increases mitochondrial membrane permeability. The results establish that triphenyltin induces a permeable state, widely known as membrane permeability transition (Gunter and Pfeiffer, 1990), i.e., it promotes Ca^{2+} efflux, membrane deenergization, and release of matrix proteins. It is shown that, conversely to its proposed detergent action, this effect of triphenyltin can be due to modification of critical sulfhydryl groups of membrane proteins. Such a modification might promote the opening of a nonspecfic pore.

MATERIALS AND METHODS

Mitochondria were prepared from rat kidneys in 0.25 M sucrose, and 1 mM EDTA[2] as previously reported (Chávez *et al.*, 1985). Calcium movement was followed spectrophotometrically at 675–685 nm by incubating 2 mg of mitochondrial protein in a medium containing 125 mM KCl, 10 mM succinate,

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[2] Abbreviations: TPT, triphenyltin; CSA, cyclosporin A; HEPES, N-2-Hydroxethyl piperazine-*N*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; $\Delta\psi$, transmembrane potential, DTNB, 5,5′-dithiobis-2-nitrobenzoic acid.

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10 mM HEPES, pH 7.3, 5 μg rotenone, 200 μM ADP, 10 mM acetate, 1 mM phosphate, and 50 μM Arsenazo III (Kendrick, 1976). Uptake and release of calcium were also assayed by using $^{45}Ca^{2+}$ (sp. act. 1000 cpm/nmol). Transmembrane potential was estimated by following the changes in the absorbance of safranine at 511–533 nm (Ackerman and Wikstrom, 1976). Release of matrix proteins was achieved by incubating 2 mg of mitochondrial protein in a medium containing 3 μM triphenyltin. After 12 min of incubation the media spun down at 100,000 × *g* during 60 min. The activity of malate dehydrogenase was assayed spectrophotometrically at 340 nm in 2 ml of the supernatant by following the oxidation of 0.2 mM NADH, using 0.5 mM oxalacetate as the substrate (Chávez *et al.*, 1992). In a parallel experiment, the supernatant was precipitated with trichloroacetic acid, 6% final concentration. After resuspension of the pellet in 500 μl of 2% sodium dodecyl sulfate and 5% β-mercaptoethanol, 5 μl were electrophoresed in 10% acrylamide (Schaaegger and von Jagow, 1987). Membrane thiols were determined by using Ellman's reagent, DTNB (Ellman, 1959), as indicated in the legend of the corresponding figure. Protein was determined by the method of Lowry *et al.* (1951).

RESULTS

The influence of triphenyltin on mitochondrial calcium content is shown in Fig. 1. Trace A indicates that triphenyltin (TPT), at a concentration of 3 μM, induced a fast and total release of the accumulated Ca^{2+}. This effect of triphenyltin appears to be similar to that produced by several reagents that activate membrane permeability transition (Gunter and Pfeiffer, 1990). On the other hand, considerable evidence support the role of cyclosporin A (CSA) as inhibitor of mitochondrial nonspecific permeability (Fournier *et al.*, 1987; Broekemeier, *et al.*, 1989; Halestrap and Davidson, 1990; Bernardi *et al.*, 1993). Taking into account the latter, we explored the possibility that CSA could provide protection against the effect of triphenyltin on Ca^{2+} release. However, Fig. 1B shows that CSA (0.5 μM) was unable to avoid Ca^{2+} efflux as induced by 3 μM triphenyltin. Aldridge and Cremer (1955) showed evidence that trialkyltin compounds had affinity for thiol groups. Accordingly, Fig. 1C illustrates that 100 μM dithiothreitol (DTT) successfully inhibited the damaging effect of

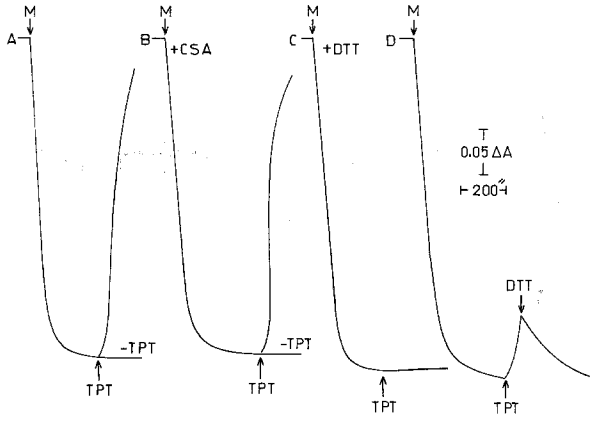

Fig. 1. Effect of triphenyltin on mitochondrial calcium content. Mitochondria (2 mg protein) were added to 3 ml of medium containing 125 mM KCl, 10 mM HEPES, 10 mM succinate, 10 mM acetate, 1 mM phosphate, 50 μM $CaCl_2$, 50 μM Arsenazo III, 200 μM ADP, and 5 μg rotenone. The medium was adjusted to pH 7.3 with KOH. Where indicated, 3 μM triphenyltin (TPT), 0.5 μM cyclosporin A (CSA), and 100 μM dithiothreitol (DTT) were added. Temperature 25°C.

3 μM triphenyltin on membrane permeability. Furthermore, Fig. 1D shows that when DTT was added during the Ca^{2+} efflux phase, the cation was accumulated. The recovery, by mitochondria, of the ability to take up Ca^{2+} eliminates the possibility that the TPT effect could be due to a detergent-like action.

The experiment shown in Fig. 2 was performed to establish a relationship between mitochondrial Ca^{2+} release and modification of membrane thiol groups. Interestingly, a calcium release reaction followed a cooperative-type kinetics, as a function of TPT concentrations. From 0.5 to 2.5 μM triphenyltin induced a very small amount of calcium released, i.e., 3.8 nmol/mg. At such concentrations of TPT the amount of titrated –SH groups ranged from 0.25 to 2.0 nmol per mg protein. However, when TPT concentration was increased to 3 μM, 2.25 nmol/mg of thiol groups was modified and Ca^{2+} efflux reached 50 nmol/mg. A maximal amount of Ca^{2+} release (63 nmol/mg) was obtained after the modification of 3.25 nmol thiol groups per mg protein by 5 μM TPT. From these results, it can be assumed that titration of critical thiol groups is required to activate nonspecific pore.

The inhibitory effect of varying concentrations of DTT on the efflux of Ca^{2+} by 3 μM triphenyltin is presented in Fig. 3. The data indicate that 40 μM DTT diminished Ca^{2+} efflux from 58 to 7.5 nmol/mg. The inset shows that 7.14 μM DTT was required to inhibit 50% of the effect of TPT on Ca^{2+} release.

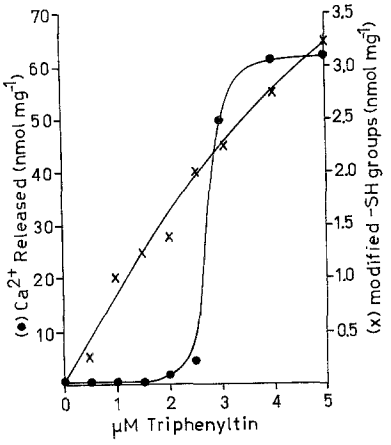

Fig. 2. Effect of increasing concentrations of triphenyltin on mitochondrial calcium content. Mitochondrial protein (2 mg) was incubated in similar conditions as described for Fig. 1, except that the medium contained 50 μM $^{45}CaCl_2$ (sp. act. 1000 cpm/nmol) and the indicated concentrations of triphenyltin. After 5 min of incubation, an aliquot of 0.2 ml was withdrawn to estimate the radionucleotide that was taken. Immediately, the indicated concentrations of triphenyltin were added. After 10 min, aliquots of 0.2 ml were filtered to estimate the $^{45}Ca^{2+}$ contained in mitochondria retained in the filter. The amount of triphenyltin bound to thiol groups was estimated in a parallel experiment. After the incubation time, mitochondria were spun down, the pellet was resuspended, 33.3 μM of 5,5′-dithiobis-2-nitrobenzoic acid was added, and the resulting thionitrobenzoate was estimated at 412 nm. Cysteine was used as standard. The values in the figure represent the difference between total –SH groups (27 nmol/mg) minus the unmodified thiol groups that were titrated by DTNB.

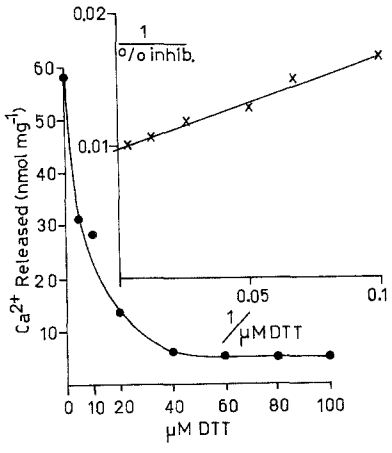

Fig. 3. Protective role of dithiothreitol on the calcium-releasing effect of triphenyltin. Experimental conditions were as in Fig. 2, except for the indicated concentrations of DTT that were added, and 3 μM TPT was used to induce Ca^{2+} release.

An increase in membrane permeability occurs in association with loss of the internal negative membrane potential (Beatrice *et al.*, 1980). In consequence, the effect of triphenyltin on mitochondrial membrane energization was also explored. Figure 4, trace A illustrates the finding that 3 μM TPT, after a lag period, induced collapse of membrane $\Delta\psi$. Trace B shows that 100 μM DTT completely prevented membrane deenergization as induced by triphenyltin. Trace C shows that the addition of 100 μM EDTA similarly restored the membrane potential collapsed by TPT. This effect of EDTA points to the possibility that Ca^{2+} cycling, across the inner membrane (Lotscher *et al.*, 1980; Chávez and Holguín, 1988; Chávez and Bravo, 1988), could be involved in the damaging mechanism of triphenyltin.

In addition to the efflux of Ca^{2+} and other metabolites (Zoccarato *et al.*, 1981; Vercesi, 1985), permeability barrier leakiness in mitochondria is characterized by the release of matrix proteins (Igbavboa *et al.*, 1989; Chávez et al., 1992). Figure 5 shows the electrophoretic pattern of proteins liberated from mitochondria treated with triphenyltin. Line 1 shows the total protein released from mitochondria after freezing and thawing. Line 2 shows the profile protein released from mitochondria under control conditions. Line 3 shows the electrophoretic pattern of proteins released after treatment with 3 μM TPT. Line 4 illustrates the profile of proteins released after the addition of 3 μM TPT and 0.5 μM CSA. The protective effect of DTT on TPT-induced matrix proteins release is illustrated in Line 5. Examination of the electrophoretic pattern shows that the protective effect of DTT corresponds to the closure of nonspecific pore. Assay of mitochondrial malate

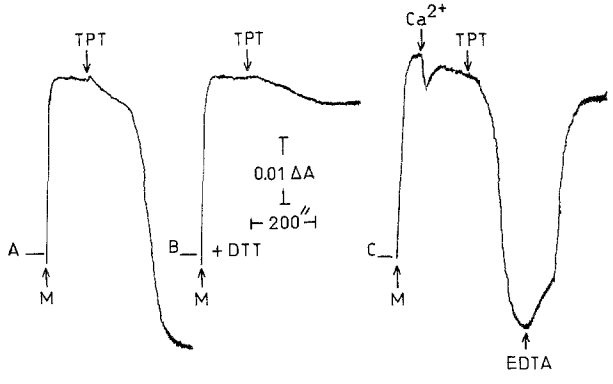

Fig. 4. Effect of triphenyltin on mitochondrial membrane potential. Protein from mitochondria (2 mg) was incubated under the conditions described for Fig. 1, except that 10 μM safranine was added instead of Arsenazo III. Where indicated, 3 μM triphenyltin, 100 μM DTT, 50 μM $CaCl_2$, and 100 μM EDTA were added.

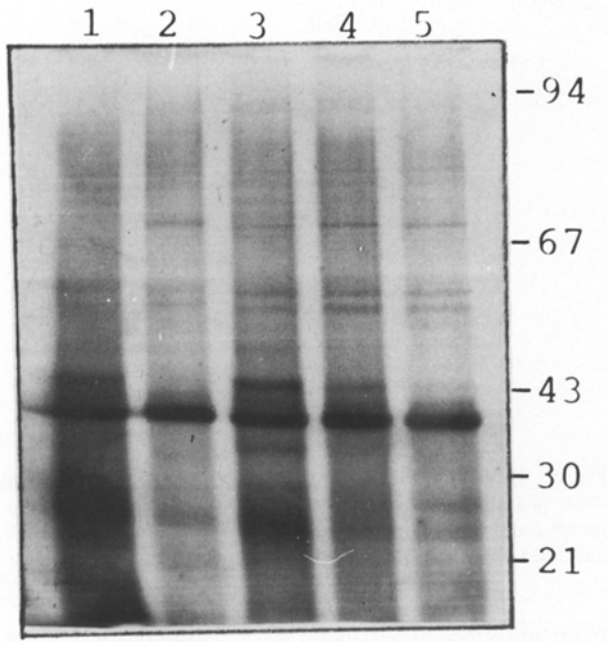

Fig. 5. Electrophoretic profile of matrix proteins released by triphenyltin. Protein from mitochondria (2 mg) was incubated under similar conditions described for Fig. 1. After 12 min of incubation, the media were centrifuged at 100,000 × *g* for 60 min. The supernatants were precipitated with 6% trichloroacetic acid; after centrifugation, the pellets were resuspended in 500 μl of sodium dodecyl sulfate. Silver was used to stain the gel.

dehydrogenase has also been used as an appropriated parameter to monitor activation of membrane permeability transition (Igbavboa *et al.*, 1989; Chávez *et al.*, 1992). Table I shows that, indeed, the transmembrane pathway brought about by triphenyltin allows the outflow of malate dehydrogenase from mitochondria. As observed, addition of 3 μM TPT increased the activity of the enzyme from 32.1 to 62.2 nmol oxidized NADH/10 sec. Such an activity is decreased by the addition of 100 μM DTT, i.e., 24.2 nmol oxidized NADH/10 sec.

Table I. Malate Dehydrogenase Activity Released by Triphenyltin

Additions	nmol NADH oxidized/10 sec
Control	32.15 ± 9.62
+TPT	62.29 ± 1.80
+TPT + DTT	24.20 ± 7.80

Experimental conditions were as indicated under Methods. Where is indicated 3 μM triphenyltin, and 100 μM DTT was added.

DISCUSSION

The data presented in this study show the influence of triphenyltin on mitochondrial membrane permeability. Our results indicate that triphenyltin induces released of Ca^{2+} and matrix proteins, as well as a drop of the transmembrane potential. Thus, TPT appears to be an efficient activator of membrane permeability transition. A previous work (Stockdale *et al.*, 1970) reported that triphenyltin increased mitochondrial permeability by acting as detergent. Certainly, since TPT is an amphipathic molecule, it would satisfy the characteristics of a detergent, i.e., it possesses a hydrophobic region, represented by the triphenyl substituents, and one positive charge located in the tin metal. The possibility that the hyperpermeable state might be caused by a detergent-like action was considered. However, the observation that dithiothreitol reversed the effect of TPT on Ca^{2+} efflux ruled out such a possibility. It is known that simple mitochondrial membrane lysis by detergents is irreversible. Furthermore, EDTA caused a complete recovery of the transmembrane potential, collapsed by the pair TPT–Ca^{2+}. In this regard it can be assumed that EDTA, by removing Ca^{2+}, avoids its cycling across the inner membrane. Such a calcium cycle has been accounted for when trying to explain the membrane damage that leads to a hyperpermeable state (Lotscher *et al.*, 1980; Chávez and Holgúin, 1988; Chávez and Bravo, 1988). However, it is worthwhile to consider the proposal of Valle *et al.* (1993), who postulate that EGTA regenerates membrane potential by impeding the binding of Ca^{2+} to some internal protein responsible for the control of permeability. Similarly, Lenartowicz *et al.* (1991) reported that phenylarsine oxide, a thiol reagent, induced membrane permeability transition even in the presence of EGTA. The authors suggest that Ca^{2+} is necessary in an early step related with the formation of oxygen-derived free radicals.

On the other hand, several reports emphasize that the effects of trialkyltins on mitochondrial functions can be explained through an OH^-/Cl^- exchange reaction (Selwyn *et al.*, 1970a,b). In this respect, Bygrave *et al.* (1978) claimed that tributyltin induces mitochondrial Ca^{2+} uptake as a consequence of the OH^-/Cl^- exchange. Our findings with triphenyltin strongly suggest that Ca^{2+} efflux follows after the binding of TPT to a limited number of specific membrane thiols, i.e., 2.25 nmol/mg. Regard-

ing the latter, there is a large body of evidence in favor of the participation of –SH groups in the control of mitochondrial membrane transport (Pfeiffer *et al.*, 1979; Palmer and Pfeiffer, 1981; Vercesi 1984; Chávez and Holguin, 1988; Chávez *et al.*, 1989). Recently, Lenartowicz *et al.* (1991) have demonstrated that phenylarsine oxide induces permeability transition, through a reaction which is stimulated by Ca^{2+}. Within this context, in a previous work, we reported that Hg^{2+} induces mitochondrial Ca^{2+} release by modification of 1 nmol –SH per mg protein (Chávez *et al.*, 1988). However, the possibility that OH^-/Cl^- exchange and subsequent Ca^{2+} accumulation could result after TPT binding to membrane thiols cannot be discarded at the present.

It is known that, in addition to an inducing agent, a supraphysiological Ca^{2+} load is the first step in the cascade of events that lead to membrane transition (Bellomo *et al.*, 1984; Chávez and Jay, 1987; Crompton *et al.*, 1988). The effect of Ca^{2+} has been associated to either: (a) activation of phospholipase A_2, which leads to the disruption of membrane integrity (Pfeiffer *et al.*, 1979); (b) to its binding to adenine nucleotide translocase, leading to the formation of a nonspecific channel through the transporter of adenine nucleotides, which is inhibited by cyclosporin A (Halestrap and Davidson, 1990); or (c) matrix alkalinization that follows Ca^{2+} accumulation (Petronilli *et al.*, 1993). Such a pore, which is called by the authors a mitochondrial permeability transition pore (MTP), is regulated by the energy state of mitochondria and is also inhibited by CSA. However, our findings indicating that CSA was unable to inhibit TPT-induced membrane permeability in Ca^{2+} loaded mitochondria are noteworthy. The latter is in agreement with the proposed existence of two different pathways for unspecific Ca^{2+} movement, one of them insensitive to cyclosporin (Broekemeier and Pfeiffer, 1989; Davidson and Halestrap, 1990). As shown, the opening of a transmembrane path by triphenyltin allows the efflux of matrix proteins. Based on this finding, it can be assumed that the diameter of the pore must be within a range of 2–7 nm (Igbavboa *et al.*, 1989; Crompton *et al.*, 1992). The question that arises now is whether or not the TPT binds to thiol groups belonging to proteins that are components of the pore. To answer this question, experiments on labeled triphenyltin binding to membrane proteins must be performed.

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