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Ca^{2+}-dependent Depolarization of Energized Mitochondrial Membrane Potential by Chlortetracycline (Aureomycin)*

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The addition of chlortetracycline (CTC) to succinate-energized rat liver mitochondria resulted in depolarization of the membrane potential and decreased respiratory control. CTC inhibited both processes at concentrations that were half maximally effective at approximately 13 and 16 μM, respectively. These inhibitory effects were prevented by either the Ca^{2+} chelator, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, or the inhibitor of mitochondrial Ca^{2+} influx, ruthenium red. These findings are consistent with the formation of a membrane associated calcium-CTC complex and suggest that CTC can alter mitochondrial energy metabolism during transmembrane Ca^{2+} cycling.

Ca^{2+} is now recognized to play an important role in the transduction of diverse metabolic and hormonal signals (1-3). Consequently, the fluorescent Ca^{2+}-chelating antibiotic, chlortetracycline (Aureomycin), has achieved renewed importance as a probe for studying the participation of Ca^{2+} in the regulation of cellular metabolism since the spectral properties of CTC[1] are modulated by Ca^{2+} (4). In this communication, we report a specific Ca^{2+}-related effect of CTC on energized liver mitochondria resulting in depolarization of the membrane potential and inhibition of coupled respiration at concentrations of CTC which have been employed to probe the distribution of intracellular Ca^{2+} in response to physiological and biochemical stimuli. These findings have not been reported previously and although their ramifications may profoundly modify conclusions made by others based on the use of CTC as a Ca^{2+} probe, they also suggest that CTC may serve as an invaluable tool for investigating the role of Ca^{2+} in mitochondrial energetics.

EXPERIMENTAL PROCEDURES

Materials—CTC, EDTA, EGTA, HEPES, rotenone, CCCP, oligomycin, L-ascorbic acid, ADP, succinic acid, arsenazo III, and RR were obtained from Sigma. The RR was further purified by the method of Luft (5). TMPD and DNP were from Eastman and antimycin A from P-L Biochemicals. All other chemicals were of the highest quality available.

Methods—Mitochondria were isolated from livers of male Sprague Dawley rats as described previously (6) using 250 mM sucrose, 2 mM sodium EDTA, and 10 mM HEPES, pH 7.4. The mitochondrial fraction was washed twice with 250 mM sucrose, 10 mM HEPES, pH 7.4, and resuspended in this buffer. Protein was determined by a micro-modification of the Folin method (7).

Membrane potential was monitored at 30 °C in an incubation medium containing: 0.33 mg of mitochondrial protein/ml, 250 mM sucrose, 3 μM rotenone, 1.6 mM sodium phosphate, 3 μM diS-C_3-(5), and 25 mM HEPES, pH 7.4, in a final volume of 3 ml. Membrane potential was estimated using the null point method (8) with the K^+/valinomycin voltage clamp technique (9). Intramitochondrial K^+ concentration was assumed to be 60 to 100 mM, a range which includes reported estimates of this value (10, 11).

Oxygen uptake was measured polarographically at 30 °C using a Clark type oxygen electrode and RCR calculated from the ratio of state 3 to state 4 respiration (12). The incubation medium contained: 1 mg of mitochondrial protein/ml, 75 mM KCl, 20 mM sucrose, 12.5 mM potassium phosphate, 50 mM Tris/Cl, pH 7.4, 5.3 μM rotenone, 16.7 mM succinate, and CTC (as indicated) in a final volume of 2.0 ml. State 3 respiration was initiated by the addition of 200 nmol of ADP.

Ca^{2+} in mitochondria and incubation medium was determined to be >10 nmol/mg of protein using the metallochromic dye arsenazo III.[2] This amount of Ca^{2+} was necessary to observe the inhibitory effects of CTC.

RESULTS AND DISCUSSION

The potentiometric cyanine dye, diS-C_3-(5), was employed to continuously monitor mitochondrial membrane potential. The efficacy and use of diS-C_3-(5) fluorescence as a reliable monitor of membrane potential has been recently reviewed (13). The respective emission and excitation spectra for CTC (400 → 525 nm) and diS-C_3-(5) (622 → 670 nm) are discretely separate and therefore spectral interference was ruled out. Succinate was employed as respiratory substrate since this class of cyanine dyes specifically block NAD-linked (site I) respiration (14). Energization of liver mitochondria with succinate (site II) leads to a decrease in fluorescence intensity, which corresponded to a hyperpolarization of the membrane potential (inside negative) and which was reversed by addition of azide, antimycin A, or CCCP, but not oligomycin. These results are identical to those obtained by Laris *et al.* (15) using the same probe, and are consistent with the chemiosmotic hypothesis of Mitchell (16).

The presence of CTC reduced the degree to which the mitochondria were hyperpolarized by either succinate (Fig. 1) or ascorbate + TMPD (not shown), a site III specific substrate. The energy-dependent membrane potential was diminished by CTC in a concentration-dependent manner. This effect was manifested whether CTC was added before (Fig. 1) or after (Fig. 2) hyperpolarization with succinate. Addition of up to 100 μM CTC to nonenergized mitochondria resulted in no

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[1] The abbreviations used are: CTC, chlortetracycline hydrochloride; diS-C_3-(5), 3,3'-dipropylthiodicarbocyanine iodide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DNP, 2,4-dinitrophenol; K'_{Ca}, apparent association constant for Ca^{2+}; K'_{Mg}, apparent association constant for Mg^{2+}; K_I, apparent concentration for half-maximal inhibition; RCR, respiratory control ratio; RR, ruthenium red; TMB-8, 8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate.

[2] C. R. Fleschner and A. P. Martin, unpublished observations.

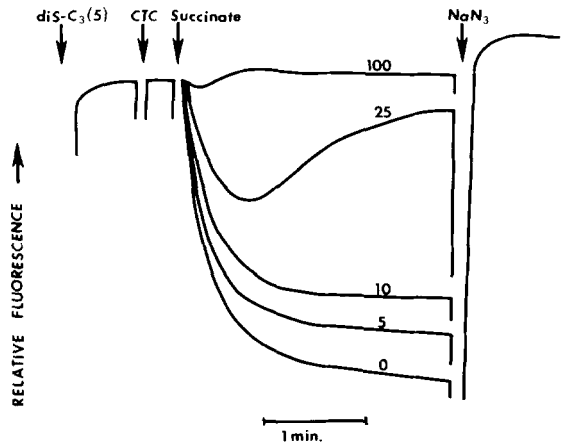

FIG. 1. **Effect of CTC on succinate-dependent hyperpolarization of mitochondrial membrane potential.** *CTC*, sodium succinate (16.7 mM, pH 7.4) and sodium azide (*NaN_3*, 10 mM) were added where indicated. CTC was added to give final micromolar concentrations as indicated by the *numerals* shown. The figure represents reproductions of the actual recordings. *diS-C_3-(5)* (3 μM) was added to the mitochondria suspended in buffer. Rotenone and diS-C_3-(5) were added as suspensions in ethanol whose final concentration in the cuvette never exceeded 0.5%. Identical results were obtained when the respiratory inhibitor antimycin A was substituted for azide. The fluorescent intensity of the dye in the mitochondrial suspension, which was continuously monitored at 622 → 670 nm, achieved a steady level within 15 to 20 s. Other details are described under "Experimental Procedures." The cuvette contents were continuously stirred throughout recordings to ensure adequate oxygenation with air as gas phase. NaN_3 apparently possesses both protonophoretic properties and inhibitory effects on respiration (16). The increased fluorescent intensity above the initial level after NaN_3 addition probably represents uncoupling of a residual H^+ gradient, part of the Donnan equilibrium potential, and is similar to observations made with the protonophore, DNP (15).

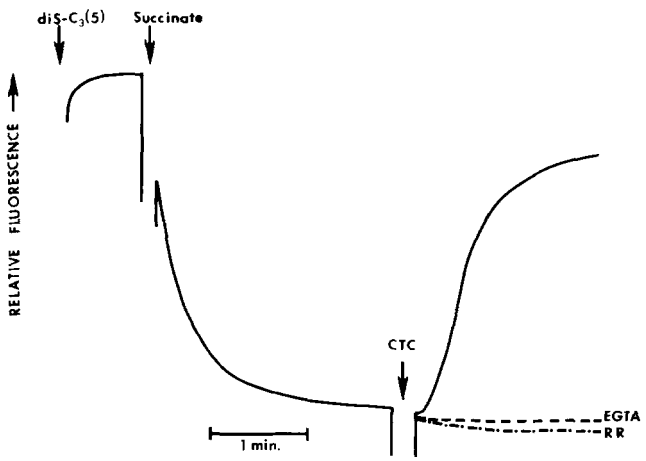

FIG. 2. **Prevention by EGTA and RR of the effect of CTC on succinate-induced hyperpolarization.** The figure represents three separate experimental conditions under which CTC (25 μM) was added to succinate energized mitochondria. Buffer alone (——); with *EGTA* (Tris salt, pH 7.4, 1 mM) (– – –); or with *RR* (1.6 μM) (–·–·–) added to the buffer in the cuvette immediately prior to the addition of mitochondria. *diS-C_3-(5)* and *succinate* were added as shown. All other conditions were the same as described in Fig. 1.

change in fluorescence intensity of diS-C_3-(5) (Fig. 1) suggesting that the depolarizing effect of CTC is specific for the energy-dependent portion of the membrane potential. When energized with succinate (Fig. 1), the membrane potential was estimated to be −145 to −160 mV and is similar to values obtained using this (15, 17) and other (9, 17) methods.

The depolarizing effect of CTC on membrane potential

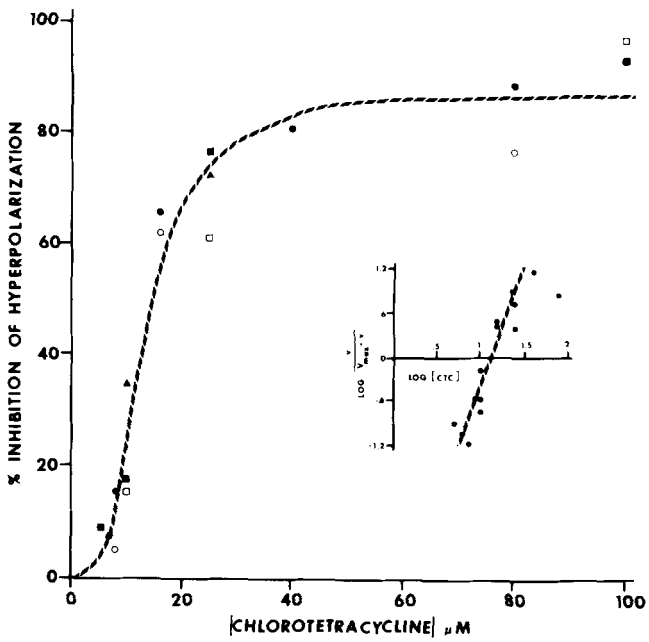

FIG. 3. **Dependence of the degree of succinate-induced hyperpolarization on CTC concentration.** Conditions were the same as those described in the legend to Fig. 1. Data from five separate mitochondrial preparations are shown, *each symbol type* representing a particular preparation. The *curve* shown was obtained by fitting the data to the Hill equation using our adaptation[4] of the iterative computer method of Atkins (19). The *inset* represents the linear Hill plot of the data which had a correlation coefficient of 0.94.

appeared to be dependent on Ca^{2+} and its transport system. When either RR (which specifically inhibits the electrophoretic Ca^{2+} influx uniporter) or EGTA was added first to the incubation suspension, CTC had no effect on the succinate energized hyperpolarization (Fig. 2). EGTA possesses a high affinity for Ca^{2+} and an exceedingly low affinity for Mg^{2+} (18), therefore, the effect does not appear to involve Mg^{2+} directly.[3] Both of the above maneuvers block Ca^{2+} cycling through different mechanisms. The data obtained suggest that for CTC to have its effect on mitochondrial membrane potential, Ca^{2+} must be present (presumably as the Ca^{2+}·CTC complex) and the Ca^{2+} influx uniporter must be operational. Data obtained from five separate mitochondrial preparations (Fig. 3) demonstrated that the depolarizing effect of CTC was most pronounced between 5 and 25 μM, plateauing at approximately 40 μM. A Hill analysis (Fig. 3, *inset*) yielded a K_I for CTC of 13 ± 3 μM and a Hill coefficient between 3 and 4.

CTC also was shown to inhibit coupled respiration as evidenced by successively diminishing RCR with increasing CTC concentration (Fig. 4). The effect also was most pronounced between 5 and 25 μM CTC yielding a K_I for CTC of approximately 16 μM. Addition of EGTA (1 mM) and RR (1.6 μM) prevented the inhibition (not shown). All these characteristics are remarkably similar to those obtained from the membrane potential studies described above. Although Mg^{2+} has been reported to exert a "protective" influence on the inhibitory effect of CTC on O_2 consumption (20), in our hands, $MgCl_2$ (1.6 mM) had no perceptible effect either on the decrease in RCR induced by CTC (16 μM) or on the extent to which energized mitochondria were depolarized.

[3] The K'_{Ca} for CTC and EGTA are $10^{3.36}$ (4) and $10^{7.63}$ M^{-1} (18) at pH 7.4, respectively. Therefore, when both ligands are present in the incubation medium, >99% of the Ca^{2+} is associated with the EGTA. The K'_{Mg} for EGTA is $10^{1.84}$ M^{-1} at pH 7.4 (18).

[4] J. W. Long, Jr., unpublished data.

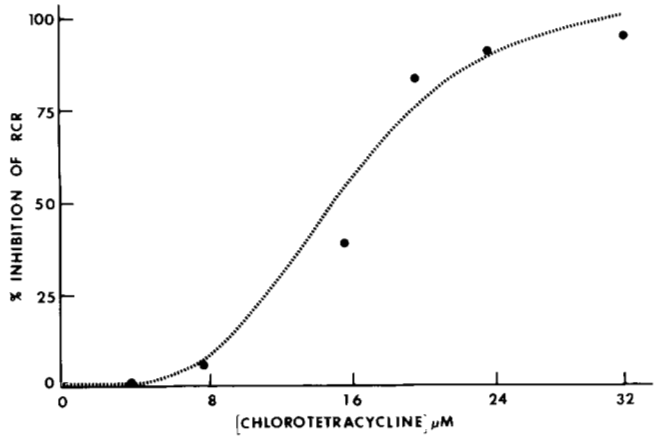

FIG. 4. **Effect of CTC on ADP-stimulated respiration.** Oxygen uptake was measured as described under "Experimental Procedures." Data from two separate mitochondrial preparations are shown. The *curve* shown was obtained by fitting the data to the Hill equation as described in Fig. 3. The *symbols* represent the mean of the observed values. In the absence of CTC, the RCR was 4.4. Per cent inhibition was calculated based on an RCR of 1 as complete inhibition.

Our observations suggest that a Ca^{2+}·CTC association with energized mitochondria can result in depolarization and loss of the energized state. The question arises as to the significance of the Ca·CTC binding sites and their relation to the energy pool. The inhibitory effects of CTC are shown to occur over an exceedingly narrow threshold with a high degree of cooperativity between Ca^{2+}·CTC complexes and specific membrane sites involved in maintaining polarization. The importance of Ca^{2+} in the effects of CTC on oxidative phosphorylation has not been reported previously, although the fact that CTC is capable of disrupting oxidative processes in mitochondria is not a new finding (21). Inhibition of oxidative phosphorylation in rat liver mitochondria by CTC has been attributed to chelation of membrane-bound Mg^{2+} (20, 21) or inactivation of SH-groups (22). Indeed, CTC freely penetrates biological membranes (4) and has been shown to selectively bind to mitochondria in intact cells (23).

CTC gained popularity as a Ca^{2+} chelate probe from the original work of Caswell and Hutchison (24) and others who used it to study membrane-bound Ca^{2+} in liver mitochondria (25, 26). CTC preferentially partitions into hydrophobic regions of biological membranes generating a characteristic fluorescence emission spectrum. In the presence of Ca^{2+}, the intensity of the spectrum is strongly enhanced apparently due to formation of a ternary complex between Ca^{2+}, CTC, and a specific hydrophobic membrane binding site (27). Changes in CTC fluorescence were subsequently employed to record changes in intracellular Ca^{2+} disposition in a variety of stimulated cell types, including pancreatic islet (28) and acinar (29) cells, platelets (30, 31), sperm (32), cardiac muscle (33), endosperm (34), nerve (27, 35), hepatocytes (36), lymphocytes (37), and neutrophils (38–41) of various species. Concentrations of CTC employed ranged from 10 to 100 μM, similar to the range used in our studies. Our observations are important since they imply that the energy state of the cells in the cases cited above may have been severely comprised. To our knowledge, the energy status (for example, estimates of intracellular ATP/ADP ratios) of the cellular systems studied were not assessed. CTC depolarization of the mitochondria could result in a depletion of cellular ATP levels and, since the cell's active ion pumping mechanisms are directly dependent on the presence of a constant supply of ATP, this could result in a severely deranged cellular ionic balance, especially the electrochemical gradients of Ca^{2+}, H^+, Na^+, K^+, Cl^-, and HCO_3^-. This condition could serve as an alternative explanation for observations made by some investigators who have employed CTC to study stimulus-secretion coupling (28, 29). In fact, Täljedal (28), who monitored CTC fluorescence in pancreatic islets, obtained paradoxical findings which lead him to reconsider the potential usefulness of this probe. Furthermore, recent work by Smolen and Weissman (41) and Takeshige *et al.* (39), both using CTC to examine the role of Ca^{2+} in neutrophil stimulus-secretion coupling, resulted in a significant discrepancy with respect to the effects of the intracellular Ca^{2+} antagonist, TMB-8. It is interesting to note that a significant difference in the buffer systems employed by these two groups was the presence of glucose as an extramitochondrial energy source (39). Based on the present work, the possibility of a serious difference in cellular energy status is clearly indicated and should be assessed critically.

Taken together, our findings have these important implications. First, extreme caution should be exercised in interpreting data derived from experiments which employ CTC as a Ca^{2+} probe. Second, the effects of CTC on mitochondrial energy metabolism observed in the present study may serve as an additional mechanism for toxicity in susceptible individuals (42), and may point to a heretofore undescribed mechanism of action of this class of antibiotics on Ca^{2+} homeostasis and energy metabolism in infectious microorganisms. Finally, under carefully defined conditions, CTC may prove to be an invaluable probe for investigation of the involvement of Ca^{2+} in mitochondrial energetics. Recent evidence points to the importance of Ca^{2+} in regulating the mitochondrial ATPase (43, 44) and some mitochondrial dehydrogenases (45). The kinetic parameters describing the inhibitory action of CTC on energy-dependent hyperpolarization and respiratory control, and the Ca^{2+}-related spectral properties of the ligand imply a special usefulness of this probe for investigating the role of Ca^{2+} in mitochondrial bioenergetics.

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