mediated by some indirect mechanisms and presumably not through a specific PGI_2 receptor.

Preliminarily it is hypothesised that the minor stimulatory action of low concentrations of PGI_2 might be due to some kind of PGE_2 receptor antagonism since PGI_2 is interacting with the PGE_2 receptor in the relevant concentration range (Fig. 1). Maybe low concentrations of exogenous PGI_2, through this receptor antagonism, to some extent could prevent the antilipolytic effect mediated by endogenous PGE_2. This latter suggestion is in agreement with the observations that when the level of endogenous PGs (including PGE_2) was reduced by indomethacin the small stimulatory effect of PGI_2 was partially abolished. The concentrations of PGE_2 and PGI_2 which may be of most "physiological" relevance are probably in the nanomolar range [9]. Accordingly, PGE_2 and PGI_2 could very well have antagonistic actions on the AC complex (and lipolysis) in isolated adipocytes as well as in the adipose tissue *in vivo*. In light of the pronounced production of PGI_2 in adipocytes [7], these latter considerations might possibly explain many of the previous difficulties in determining any action of endogenous PGs in adipocytes by using indomethacin [18, 19] since indomethacin inhibits all PGs including PGE_2 and PGI_2 [7, 17]. Thus, to differentiate between the action of these two PGs highlights the need of specific PGI_2 and PGE_2 receptor antagonists in further studies.

In summary, it is demonstrated that the antilipolytic action of all PGs in adipocytes seems to be mediated by their interaction with the PGE_2 receptor. However, whether the action of low concentrations of PGI_2 also is due to interaction with the PGE_2 receptor is at present an open question.

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Na^+-independent, pyridine nucleotide-linked efflux of Ca^{2+} from preloaded rat heart mitochondria: induction by chlortetracycline

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Chlortetracycline is a polycyclic antibiotic of the naphthacenecarboxamide group which, in a non-aqueous environment, forms highly fluorescent complexes with divalent cations [1]. The initial report [1] describing the properties of CTC* indicated that, at concentrations below 20 μM, CTC did not alter mitochondrial respiration or oxidative phosphorylation. As a result the drug has been widely used as a probe of intracellular Ca^{2+} disposition [2] and mitochondrial Ca^{2+} fluxes [3–6].

Numerous observations suggest, however, that CTC concentrations as low as 5 μM are not entirely without effect on mitochondrial function. Mitochondrial Ca^{2+} uptake measured in the presence of CTC is, under some conditions, transient [5, 6]. In mitochondria from systems as diverse as mammalian liver [3], Jerusalem artichoke tubers [7], and *Tetrahymena pyraformis* [6], CTC uncouples respiration and collapses membrane potential in a Ca^{2+}-dependent fashion. It has therefore been suggested that CTC, like ionophore A23187, mediates the electroneutral release of Ca^{2+} from mitochondria [6].

Agents, including palmitoyl-CoA [8], substrates such as oxaloacetate [9] and *t*-butyl hydroperoxide [10], *N*-ethylmaleimide [11], high concentrations of inorganic phosphate [11, 12], alloxan [13], menadione [14], and divicine [15], induce Ca^{2+} release from preloaded mammalian mitochondria. Release occurs via a process that is Na^+-inde-

* Abbreviations: CTC, chlortetracycline; F-CCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; and MOPS, 3-(*N*-morpholino) propanesulfonic acid.

pendent [16] and appears to be associated with oxidation of mitochondrial pyridine nucleotides [9–11]. The Ca^{2+} release process is characterized by Ca^{2+}-dependent mitochondrial swelling, uncoupling, and membrane potential collapse. A recent, thorough investigation [17] has confirmed earlier reports [18, 19] that such Ca^{2+} efflux is the result of a generalized, reversible increase in the permeability of the inner mitochondrial membrane to substances of molecular weight less than ~1000 daltons. (In contrast, Ca^{2+} efflux mediated by the mitochondrial Na^+/Ca^{2+} exchanger occurs in the absence of mitochondrial swelling or detectable alteration of the membrane potential [20, 21].)

The data presented in this communication demonstrate that CTC, at concentrations far lower than was appreciated previously (0.2 to 10 μM), triggered the release of Ca^{2+} from isolated, preloaded rat heart mitochondria. Ca^{2+} release reflects activation by CTC of Na^+-independent, pyridine nucleotide-linked Ca^{2+} efflux via a generalized increase in inner membrane permeability to small solutes.

Methods

Mitochondria were isolated from the hearts of male Sprague–Dawley rats by a procedure [22] which yields a mixed population of subsarcolemmal and interfibrillar mitochondria. Ca^{2+} fluxes were monitored by means of a Ca^{2+}-selective electrode (Radiometer F2112Ca, K8040 reference) in a chelating-resin-treated buffer (2.0 ml) consisting of sucrose, 100 mM; KCl, 50 mM; MOPS-KOH, pH 7.2, 20 mM; KH_2PO_4, 1.7 mM; to which was added rotenone, 0.8 μM; and 0.4 mg mitochondrial protein. Mitochondria were energized with succinate (1.7 mM); temperature was maintained at 30°. Total Ca^{2+} uptake was determined for each preparation by adding pulses (50 nmole) of Ca^{2+} until spontaneous Ca^{2+} release occurred. Mitochondrial swelling was monitored as a decrease in absorbance at 540 nm in an LKB Ultrospec II spectrophotometer. The redox state of mitochondrial pyridine nucleotides was followed at 340−370 nm in an SLM-Aminco DW-2C dual wavelength spectrophotometer. Membrane potential was measured in the presence of 5 μM safranine at the wavelength pair 511–533 nm [23]. All measurements were made under the conditions used to monitor Ca^{2+} fluxes. Data shown are from representative experiments. Protein was determined according to Lowry *et al.* [24]. Sucrose, rotenone, and MOPS were purchased from Sigma, St. Louis, MO; chlortetracycline was from ICN Biomedicals, Cleveland, OH. All other reagents were of the highest grade available. Aqueous CTC solutions were made fresh immediately prior to use.

Results

Addition of CTC (0.2 to 10 μM) to isolated rat heart mitochondria that had been preloaded with Ca^{2+} induced Ca^{2+} release (Fig. 1, top panel). Release occurred with a lag that decreased with increasing CTC concentration. Release was always complete, regardless of CTC concentration, and occurred in the absence of CTC provided sufficient time was allowed (trace 1).

CTC-triggered Ca^{2+} release was associated with mitochondrial swelling, indicated by a decrease in suspension absorbance at 540 nm (Fig. 1, lower panel). Onset of swelling was a function of CTC concentration and was correlated in time with the onset of Ca^{2+} release. Traces 7 and 8, which were obtained in the presence of 10 μM CTC, illustrate the dependence of swelling on added Ca^{2+}. Ca^{2+} was present for 7, but not for 8. Such swelling reflects the osmotic response of mitochondria suspended in sucrose to a generalized increase in inner membrane permeability to small molecules [18, 19]: sucrose enters, followed by water. It can therefore be proposed that CTC enhances mitochondrial membrane permeability via a Ca^{2+}-dependent process.

Ca^{2+} release triggered by numerous agents is accompanied by net oxidation of pyridine nucleotides [8–10, 14]. CTC likewise induced a Ca^{2+}-dependent oxidation of NAD(P)H, as indicated by a decrease in $A_{340-370}$ (Fig. 2). The reactions mediating this oxidation are unknown. Communication between NADH and the mitochondrial electron transport chain is blocked by rotenone. The oxidation cannot be attributed to uncoupling mediated by either CTC or a CTC–Ca^{2+} complex, as addition of uncoupling amounts of F-CCP was without effect on NAD(P)H redox state (not shown). As observed in other systems [3, 6, 7] CTC collapsed the membrane potential of Ca^{2+}-loaded rat heart mitochondria (data not shown).

These observations are consistent with the hypothesis that CTC triggers Na^+-independent, pyridine nucleotide-associated Ca^{2+} efflux from rat heart mitochondria. The precise mechanism by which CTC acts, however, is unclear. In agreement with other reports [1, 3], at the concentrations employed and in the absence of Ca^{2+}, CTC had no significant effects on respiration or coupling of rat heart mitochondria (data not shown). The data of Figs. 1 and 2 suggest that CTC accelerates the occurrence of a Ca^{2+}-dependent process. The effects of the drug were therefore determined as a function of Ca^{2+} load (Fig. 3). CTC was added prior to Ca^{2+}. CTC was without effect on Ca^{2+} uptake. For a given Ca^{2+} load, the initial rates of Ca^{2+} uptake in the presence (upper panel) and absence (lower panel) of 5 μM CTC were identical. The data indicate, instead, that CTC decreased the amount of Ca^{2+} required to trigger the release process. As the Ca^{2+} load was increased from 72 (trace 2) to 108 nmol (trace 4), the time required for complete Ca^{2+} release from control mitochondria (lower panel) was virtually unaltered. In contrast, in the presence of CTC (upper panel), an increase in Ca^{2+} concentration from 36 (trace 1) to 108 nmol (trace 4) dramatically speeded release. At high Ca^{2+} levels, release was similar in the presence and absence of CTC. CTC decreased the apparent K_m of the release process for Ca^{2+}.

Discussion

The data presented above suggest that CTC at low concentrations (0.2 to 10 μM) promoted the release of Ca^{2+} from isolated, preloaded rat heart mitochondria with attendant mitochondrial swelling and pyridine nucleotide oxidation. All measurements were made in the absence of added Na^+ to preclude operation of the mitochondrial Na^+/Ca^{2+} exchange system [20]. Taken together with earlier reports of Ca^{2+}-dependent uncoupling and membrane potential collapse [3, 6, 7], these findings support the hypothesis that CTC triggers, in the presence of Ca^{2+}, the Na^{2+}-independent, pyridine nucleotide-linked process which results in a generalized increase in the permeability of the inner mitochondrial membrane to small molecules and consequent, electroneutral Ca^{2+} efflux. Specifically, CTC appeared to decrease the matrix Ca^{2+} concentration required for modulation of inner membrane permeability.

These observations have several important logical and operational consequences. First, CTC is, as mentioned previously, in widespread use for measurements of divalent cation fluxes and for cation localization in mitochondrial and cellular systems. The finding that submicromolar CTC concentrations can alter the membrane permeability of isolated mitochondria is immediately relevant to the evaluation and design of investigations using isolated organelles. Applicability to investigations utilizing intact cells will depend on the extent to which CTC effects are modulated by cytosolic compositon. Second, CTC, as a lipid-soluble Ca^{2+} chelator, may be a prototype for a new class of agents capable of triggering Ca^{2+} release and of inducing Ca^{2+}-dependent modulation of mitochondrial membrane permeability. Third, CTC has similar effects on Ca^{2+}-retention and/or membrane potential of mitochondria from mammalian, plant, and protozoan cells. This phylogenetic

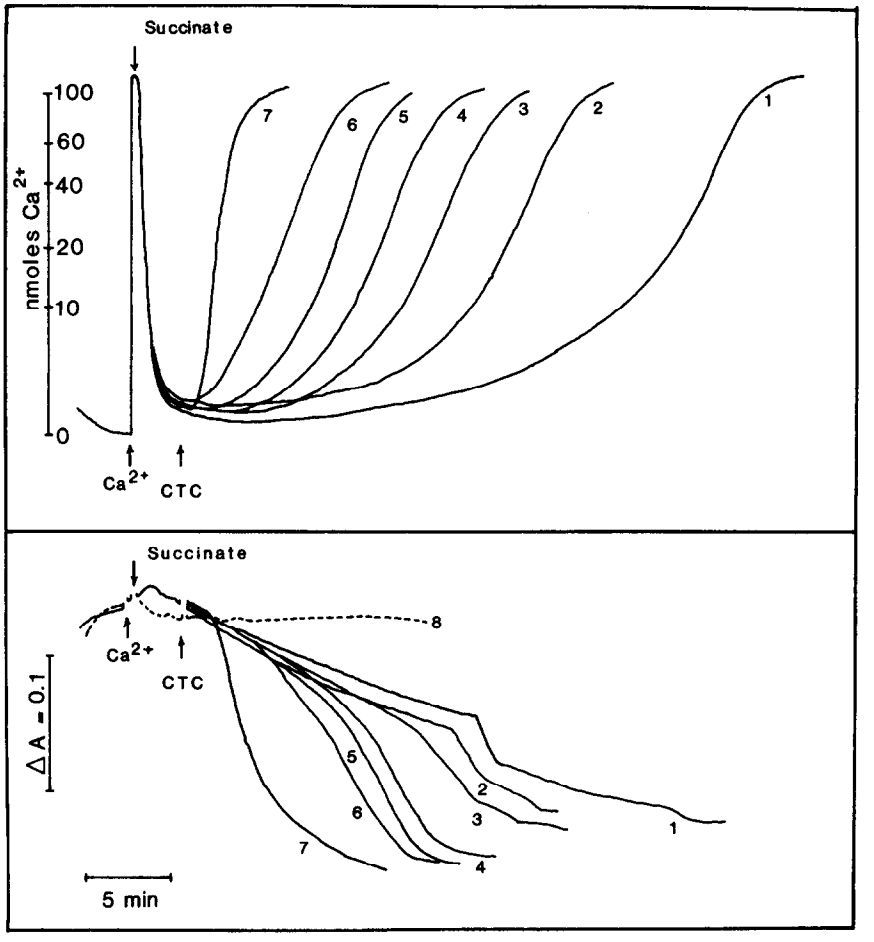

Fig. 1. Effect of chlortetracycline on Ca^{2+} retention by isolated rat heart mitochondria and on mitochondrial swelling. Upper panel: ionized Ca^{2+} concentration. Lower panel: absorbance at 540 nm. Ca^{2+} (100 nmol, 30% of capacity), succinate (1.7 mM), and CTC were added at the points indicated. CTC concentrations were as follows: (1) control, (2) 0.2 μM, (3) 0.5 μM, (4) 1 μM, (5) 2 μM, (6) 5 μM, (7) 10 μM, and (8) 10 μM CTC added in the absence of Ca^{2+}.

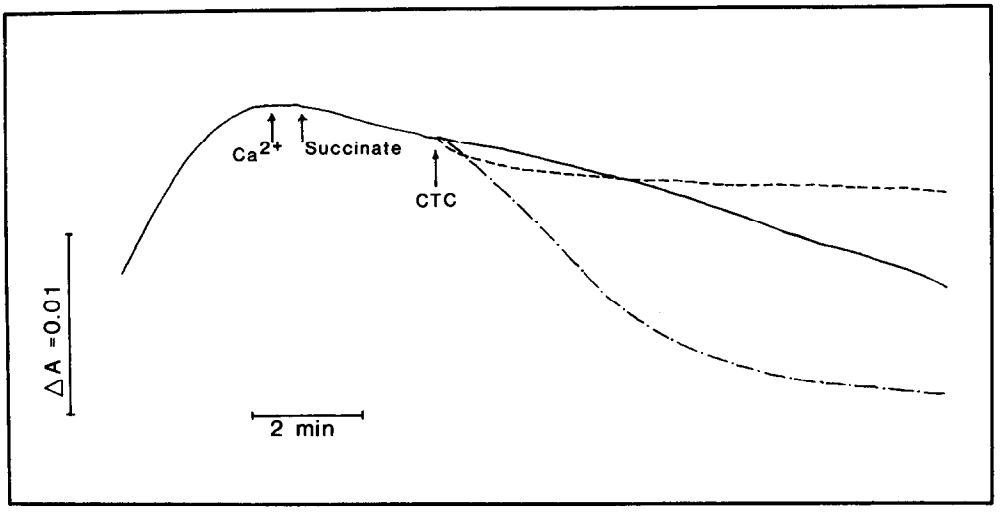

Fig. 2. Effect of chlortetracycline on the redox state of mitochondrial pyridine nucleotides. Ca^{2+} (61 nmol, 25% capacity), succinate and CTC (5 μM) were added at the times indicated. Data shown are for the control (no CTC) (———), and for CTC added in the presence (—·—·—) and absence (– – – – –) of Ca^{2+}. (The abrupt signal decrease due to CTC addition has been eliminated from the drawing).

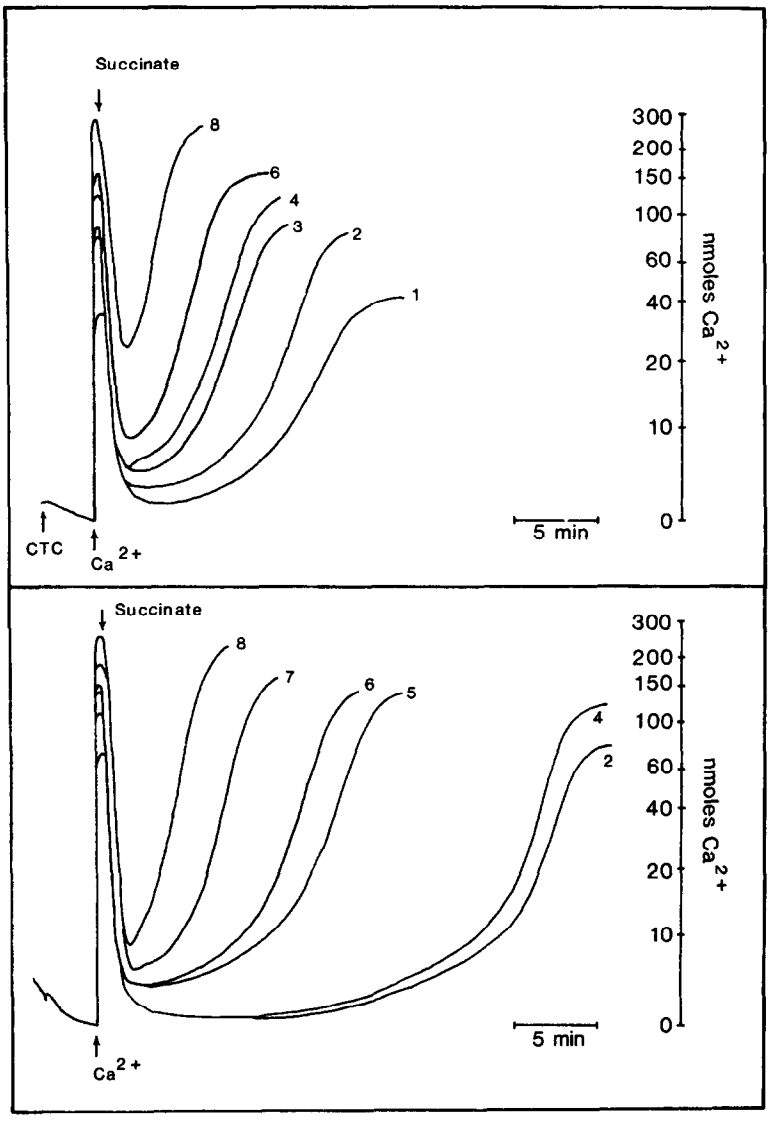

Fig. 3. Dependence of the time course of CTC-induced Ca^{2+} release on mitochondrial Ca^{2+} loading. Mitochondria were incubated for 3 min in the presence (upper panel) or absence (lower panel) of 5 μM CTC. Ca^{2+} and succinate were added as indicated. The amounts of Ca^{2+} added and the resulting percentage of total Ca^{2+} uptake capacity (determined as described under Methods) were (1) 36 nmol, 10%; (2) 72 nmol, 20%; (3) 90 nmol, 25%; (4) 108 nmol, 30%; (5) 126 nmol, 35%; (6) 144 nmol, 40%; (7) 180 nmol, 50%; and (8) 216 nmol, 60%.

persistence implies that the process by which Ca^{2+} modulates mitochondrial membrane permeability is of basic importance to mitochondrial function.

In summary, low concentrations (0.2 to 10 μM) of chlortetracycline triggered the release of Ca^{2+} from preloaded rat heart mitochondria. Ca^{2+} release occurred in the absence of Na^+ and was associated with Ca^{2+}-dependent mitochondrial swelling, membrane potential collapse, and pyridine nucleotide oxidation. These observations suggest that chlortetracycline activates the Na^+-independent, pyridine nucleotide-linked pathway for Ca^{2+} efflux which has been identified in mammalian mitochondria. A review of the literature on chlortetracycline indicates that this transport pathway can also be activated by the antibiotic in mitochondria from sources as phylogenetically divergent as plants and protozoa.

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Biochemical characterization of the antagonist actions of the xanthines, PACPX (1,3-dipropyl-8(2-amino-4-chloro)phenylxanthine) and 8-PT (8-phenyltheophylline) at adenosine A_1 and A_2 receptors in rat brain tissue

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Caffeine, the most widely used psychoactive agent consumed today [1, 2], produces its therapeutic effects by antagonizing the interaction of endogenous adenosine with specific cell surface recognition sites or receptors [1–3]. These may be subdivided into A_1 and A_2 subtypes on the basis of their pharmacological profiles [4] and subserve different functions. Agonists selective for the cardiac A_1 receptor can attenuate arrhythmic abnormalities such as supraventricular tachycardia [5], whereas A_2 selective agonists are potent coronary vasodilators [6]. Antagonists of A_1 and A_2 receptors are also useful as therapeutic agents; theophylline and its ethylenediamine-salt, aminophylline, have been used as anti-asthmatic [7] and cardiotonic agents [8] respectively. However, the use of these agents is limited by their low potency together with unwanted side effects such as phosphodiesterase inhibition [3, 9, 10].

Xanthines substituted in the 1, 3 and 8-positions of the molecule have increased adenosine antagonist activity with a corresponding loss of phosphodiesterase inhibitory activity [9–12]. One of the most potent of these analogs is PACPX (1,3 - dipropyl - 8(2 - amino - 4 - chloro)phenylxanthine), which is the most active A_1 adenosine antagonist known in bovine brain tissue [10]. In contrast to theophylline which has been reported to be a competitive adenosine antagonist, PACPX has been reported to have non-competitive properties [13], a fact that may be attributable to the phenyl substitutent. Examination of the adenosine antagonist activity of PACPX in the driven guinea pig left atria and in the carbachol-contracted guinea pig *Taenia coli* led to the conclusion that PACPX was a non-competitive antagonist at A_1 receptors and a competitive antagonist at A_2 receptors, at which it was less potent than A_1 receptors [13]. In brain tissue, however, the xanthine had been reported to be a competitive A_1 antagonist [10]. The discrepancy between the brain and atrial properties of PACPX was taken as evidence of differences in the A_1 receptors in the two tissues [13].

In the present study, the effects of PACPX and 8-phenyltheophylline (8-PT) on A_1 and A_2 receptor properties were assessed in rat brain tissue using radioligand binding techniques.

Methods

Binding to rat brain A_1 receptors was measured using [^{3}H]cyclohexyladenosine (CHA; sp. act. 25 Ci/mmol) as previously described [14]. For A_2 binding, [^{3}H]5′-*N*-ethylcarboxamidoadenosine (NECA; sp. act. 20 Ci/mmol) was used as a ligand in striatal tissue using 50 nM cyclopentyladenosine (CPA) to block the A_1 component of [^{3}H]NECA binding [15, 16].

For saturation analysis, [^{3}H]CHA was run at ten to twelve concentrations over the range of 0.01 to 20 nM and [^{3}H]NECA over the range 0.1 to 80 nM. Non-specific binding was determined for both ligands in the presence of 10 μM 2-chloroadenosine (2-CADO). Incubations were performed with adenosine deaminase pretreated tissues for 120 or 300 min at 23° as indicated, and receptor–ligand complexes were isolated by vacuum filtration. Data were analyzed using either the Lundon-1 or RS-1 [17] curve fitting programs. Radioligands were obtained from DuPont New England Nuclear, Boston, MA, and 8-PT and PACPX from Research Biochemicals, Natick, MA.

To assess the effects of PACPX on the kinetics of [^{3}H]NECA binding in the presence of CPA, ligand association and dissociation curves were generated in the absence and presence of PACPX. A 5 μM concentration of 2-CADO was used to initiate the dissociation experiments.

In previous experiments [16], IC_{50} values of 10 and 400–