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Mitochondrial Permeability Transition and Release of Cytochrome *c* Induced by Retinoic Acids

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ABSTRACT. Retinoic acids, structurally related to vitamin A, inhibit the *in vitro* proliferation of different types of normal and neoplastic cells. The effects of all-*trans*, 9-*cis*, and 13-*cis* retinoic acids were tested on mitochondria isolated from rat liver. All the compounds were able to induce the membrane permeability transition observed as swelling and decrease in membrane potential, but 13-*cis* retinoic acid appeared to be the most effective. The latter was also shown to stimulate the release of cytochrome *c* from mitochondria, suggesting a potential target of retinoids in the induction of cell apoptosis. Interestingly, EGTA and cyclosporin A, which strongly inhibit the permeability transition induced by 13-*cis* retinoic acid, were without effect on the release of cytochrome *c* from the mitochondrial intermembrane space. BIOCHEM PHARMACOL **58**;4:665–670, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. cytochrome *c*; mitochondria; permeability transition; retinoic acids

Retinoids are compounds structurally related to vitamin A and largely utilized in clinical practice, particularly in skin disorders and cancer. In fact, they are able to inhibit the *in vitro* proliferation of different types of normal and neoplastic cells [1]. Their action is mediated by two types of receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs) [2], both belonging to the steroid/thyroid hormone receptor superfamily. There is substantial evidence that retinoids exert their effects through the induction of apoptosis in different tumor cell lines [3–6]. In human myeloid leukemia cells (HL-60), retinoids induce differentiation and subsequent apoptosis [5]. Differentiation depends on the activation of the retinoic acid receptors (RARs), while activation of the retinoid X receptors (RXRs) causes the apoptosis of the cell [3–6]. The capacity of the cell to undergo apoptosis is regulated by several factors, including the mitochondrial-associated protein Bcl-2 that increases cell resistance to apoptosis; cells treated with retinoids decrease their expression of Bcl-2 [4–7].

Recent observations indicate that mitochondria play an important role in apoptosis induction [8–10]; in fact, Bcl-2 is localized in the outer mitochondrial membrane [11] and, among other actions, prevents the release of apoptogenic factors such as apoptosis-inducing factor [8] and cytochrome *c* [9]. The latter, after its displacement to cytosol, stimulates the formation of a complex with Apaf-1 and caspase-9 in the presence of dATP. This association leads to caspase-9 activation that, in turn, cleaves and activates caspase-3 and hence DNA fragmentation [12]. Nevertheless, the cytochrome *c* signaling appears to be distinct from that of apoptosis-inducing factor [10, 13]. According to Kroemer *et al.* [8], the release of apoptogenic factors is a consequence of the permeability transition [14, 15] of the inner mitochondrial membrane. However, according to other studies [16–18], the release of cytochrome *c* occurs in the absence of permeability transition and appears to solely involve the outer mitochondrial membrane.

Previously, it was observed that retinol and its derivatives are able to induce swelling [19] and uncoupling of oxidative phosphorylation [20] in isolated mitochondria, these effects being attributed to a general membrane disrupting action [19, 20]. In the present paper, the effects of all-*trans* RA§, 13-*cis* RA, and 9-*cis* RA on mitochondria isolated from rat liver, indicating the occurrence of a membrane permeability transition, are reported. The data are discussed with reference to the possible induction of a mitochondria-mediated cell apoptosis.

MATERIALS AND METHODS

Stock solutions of retinoic acids (10^{-1} M) were stored at −20°; all-*trans* RA was dissolved in absolute ethanol, while 9-*cis* RA and 13-*cis* RA were dissolved in dimethyl sulfoxide. Before use, the retinoids were further diluted in

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§*Abbreviations:* all-*trans* RA, all-*trans* retinoic acid; 9-*cis* RA, 9-*cis* retinoic acid; 13-*cis* RA, 13-*cis* retinoic acid; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; CsA, cyclosporin A; $\Delta\Psi$, transmembrane electrical potential; and TPP^+, tetraphenylphosphonium ion.

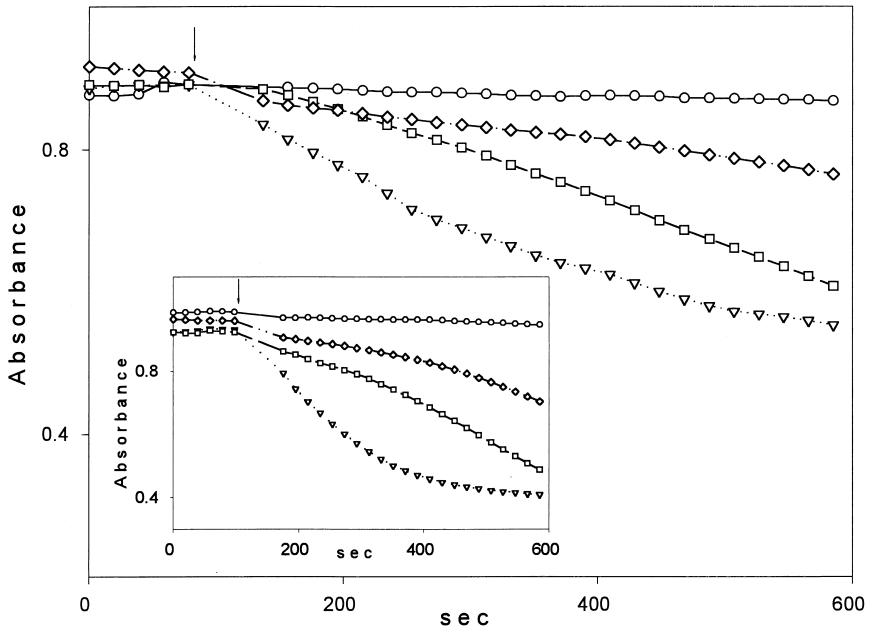

FIG. 1. Effect of retinoic acids on mitochondrial swelling. Rat liver mitochondria (0.25 mg/mL) were incubated at 25° in 213 mM mannitol, 71 mM sucrose, 5 mM HEPES/Tris (pH 7.4), 5 mM succinate, and 3 μg/mL rotenone. Swelling was triggered by 7 μM retinoic acids added at the arrow. Control (○), 9-*cis* RA (◇), all-*trans* RA (□), 13-*cis* RA (▽). The inset reports similar experiments performed in the presence of 6 μM $CaCl_2$, added 1 min before the retinoids.

absolute ethanol. In the control experiments, the same volume of solvent utilized for the samples containing the retinoic acids was added. Rat liver mitochondria were isolated by differential centrifugation essentially as described by Myers and Slater [21], using a medium containing 220 mM mannitol, 70 mM sucrose, 5 mM HEPES (pH 7.0), and 0.5 mg/mL BSA. In the homogenization buffer, 1 mM EGTA was also present. Proteins from the mitochondrial preparation were measured with the biuret test [22], while proteins from the supernatants, obtained after treatment of mitochondria under different conditions, were estimated following the procedure of Lowry *et al.* [23]. Mitochondrial swelling was followed spectrophotometrically by the decrease in absorbance at 540 nm [24].

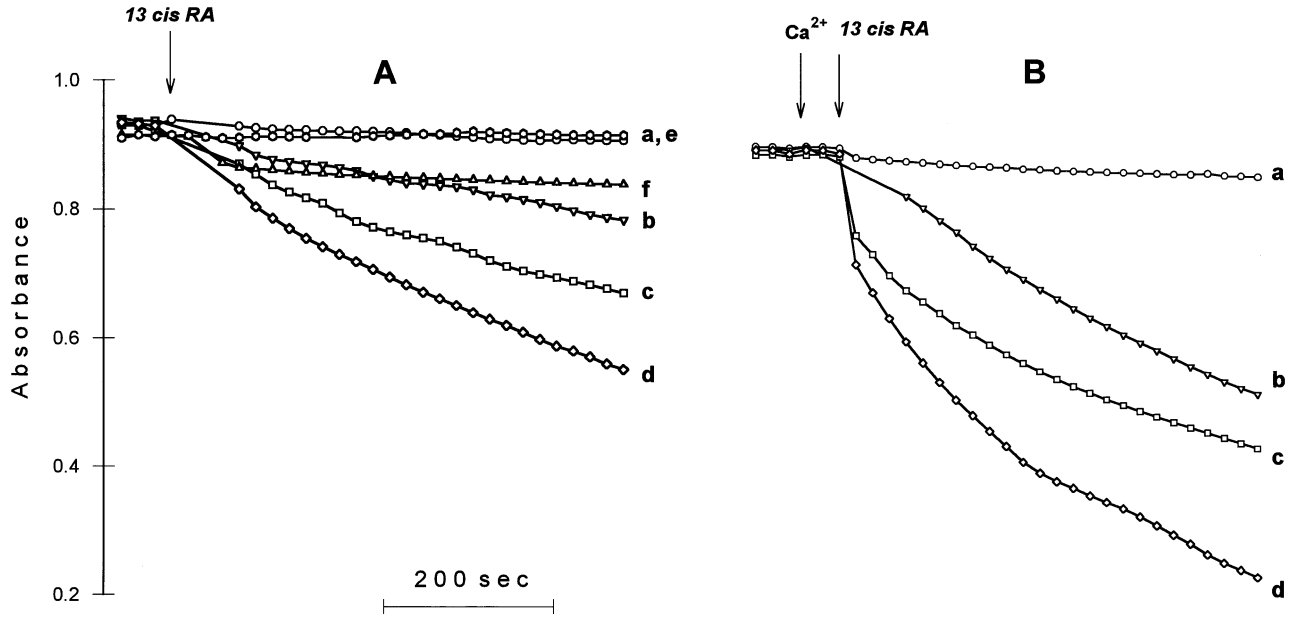

FIG. 2. Effect of increasing concentrations of 13-cis retinoic acid on mitochondrial swelling. Rat liver mitochondria (0.25 mg/mL) were incubated at 25° in 213 mM mannitol, 71 mM sucrose, 5 mM HEPES/Tris (pH 7.4), 5 mM succinate, and 3 μg/mL rotenone. Swelling was triggered by the addition of 13-*cis* retinoic acid at the following concentrations: control (a), 5 μM (b), 10 μM (c), 33 μM (d). Other additions were: 1 mM EGTA + 33 μM 13-*cis* RA (e), 2 μM CsA + 33 μM 13-*cis* RA (f). In B, 6 μM $CaCl_2$ was also present.

The data obtained with 5 sampling points/minute were transferred to a graphics software and utilized for averaging the various curves of swelling, which are the means of 5 to 7 experiments. Membrane potential was assessed by measuring the movements of TPP^+ across the mitochondrial membrane with a TPP^+-selective electrode [25]. The signal output obtained with the TPP^+ electrode was measured by a personal computer [26] with one sampling point/second. The ordinate $\Delta\Psi$ values of the curves were recalculated from the experimental values in order to obtain a linear scale. The reported data are the average of 5 to 6 curves. For the measurements of cytochrome *c* released, aliquots of mitochondrial suspensions were rapidly centrifuged at 13,000 *g* for 1 min and the resulting supernatant was centrifuged again at 24,000 *g* for 15 min. The supernatant obtained was concentrated on Centricon-10 filters (Amicon) at 3400 *g* for 45 min and utilized for protein measurement and spectrophotometric determination of cytochrome *c*. The total amount of cytochrome *c* of untreated mitochondria and of the pellets obtained after cytochrome *c* release was measured after extraction with *n*-butanol according to Richardson and Fowler [27]. Cytochrome *c* concentration was measured spectrophotometrically by reduced minus ferricyanide-oxidized difference spectra; the extinction coefficient was 19.7 $mM^{-1} \times cm^{-1}$ ($\varepsilon_{550} - \varepsilon_{540}$) [28].

Statistical Analysis

All values are the means ± SE of not less than five measurements. Multiple comparisons were made by one-way analysis of variance followed by the Tukey post-test (GraphPad Inc.).

RESULTS

All the retinoic acids (all-*trans* RA, 9-*cis* RA, and 13-*cis* RA) were able to induce swelling (Fig. 1) in rat liver mitochondria. At the concentration used, 13-*cis* RA appeared to be the most efficient, while higher concentrations of all-*trans* RA and 9-*cis* RA were necessary to induce a swelling comparable to that induced by 13-*cis* RA. The presence of calcium ions in the incubation medium stimulated the extent of swelling (inset of Fig. 1). The swelling induced by 13-*cis* RA was concentration-dependent (Fig. 2) and strongly inhibited by the presence of EGTA and CsA (Fig. 2A). The stimulation of swelling by Ca^{2+} ions and the inhibitory effects of EGTA and CsA indicate that retinoids act as inducers of permeability transition of the mitochondrial inner membrane, since Ca^{2+} is a potent stimulator while CsA is considered as the specific inhibitor [14, 15] of the process.

The influence of retinoids on mitochondrial membrane energy levels is apparent in Fig. 3. Rat liver mitochondria, utilizing succinate as respiratory substrate, were able to attain a membrane potential ($\Delta\psi$) of about −180 mV that was stable for several minutes. When retinoic acids were

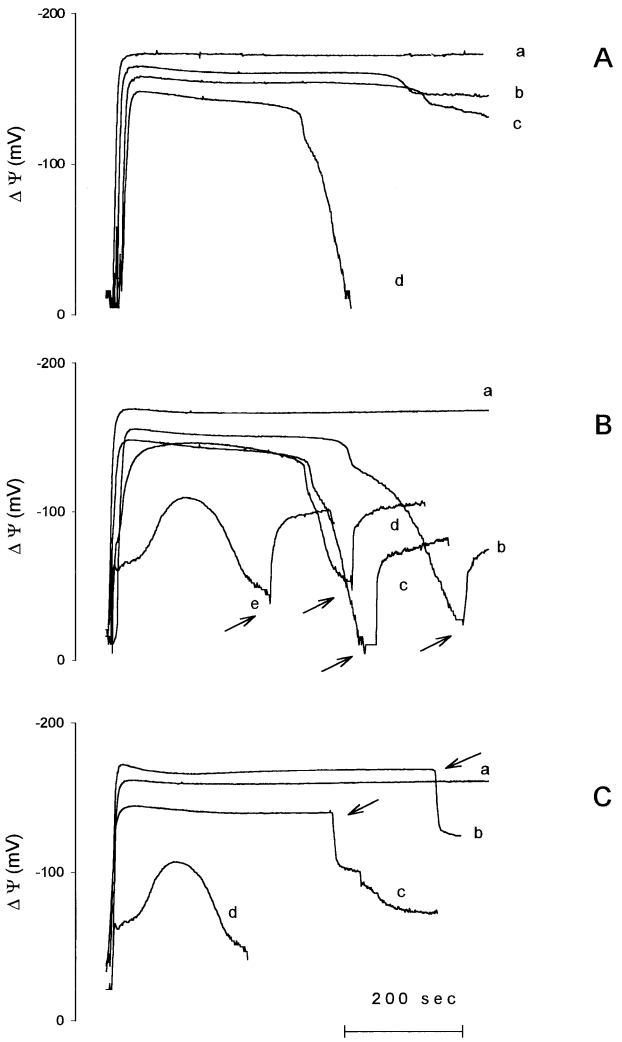

FIG. 3. Effect of retinoic acids on mitochondrial membrane potential. Rat liver mitochondria (1 mg/mL) were incubated at 25° in 106 mM mannitol, 35 mM sucrose, 62 mM KCl, 15 mM HEPES/Tris (pH 7.4), 1.35 mM phosphate, 2 μg/mL rotenone, and 5 μM TPP^+. Energization of mitochondria was obtained by the addition of 4 mM succinate. (A) Effect of different retinoic acids at 16 μM concentration: control without retinoic acids (a), all-*trans* RA (b), 9-*cis* RA (c), 13-*cis* RA (d). (B) Effect of increasing concentrations of 13-*cis* RA: control (a), 8 μM (b), 16 μM (c), 24 μM (d), 33 μM (e). At the arrow, 1 mM EGTA was added. (C) Prevention by EGTA and CsA of membrane potential collapse induced by 33 μM 13-*cis* RA. Control without 13-*cis* RA (a), 13-*cis* RA (d), 13-*cis* RA + 1 mM EGTA (b); 13-*cis* RA + 3 μM CsA (c). At the arrow 0.2 μM CCCP was added.

added at a concentration of 16 μM, it was apparent that, while all-*trans* RA and 9-*cis* RA were scarcely effective, 13-*cis* RA determined a lower extent of membrane potential that was rapidly followed by its total collapse in about ten min (Fig. 3A). At higher concentrations (33 μM), the effect of the various retinoic acids was more pronounced, but, while all-*trans* RA and 9-*cis* RA showed a moderate decrease in the extent of $\Delta\psi$ (not shown), 13-*cis* RA, again, appeared extremely effective. Figure 3B shows the effect of

TABLE 1. Release of cytochrome *c* from mitochondria induced by 13-*cis* retinoic acid or calcium/phosphate

	Cytochrome *c* content (pmol/mg prot)		% cytochrome *c* released	Protein released (mg)
	Pellet	Supernatant		
None (*a*)	109.2 ± 5.5	6.6 ± 1.2	5.7	1.61 ± 0.19
EGTA + CsA (*b*)	104.7 ± 5.4	6.5 ± 0.3	5.8	1.69 ± 0.23
13-*cis* RA (*c*)	100.3 ± 5.1	11.4 ± 1.0†	10.2	1.89 ± 0.26†
EGTA + CsA + 13-*cis* RA (*d*)	104.2 ± 6.3	10.2 ± 0.6§	8.9	1.94 ± 0.22‖
Ca^{2+} + phosphate (*e*)	96.8 ± 4.3*	17.4 ± 0.8‡	15.2	1.81 ± 0.21

Rat liver mitocondria (10 mg/mL) were incubated for 15 min at 25° in 100 mM KCl, 42 mM mannitol, 14 mM sucrose, 17 mM HEPES/Tris (pH 7.4), 0.2 μg/mg protein rotenone and, when indicated, 1 mM EGTA, 5 μM cyclosporin A, 400 μM $CaCl_2$, 12.5 mM phosphate, and 200 μM 13-*cis* retinoic acid. Concentrated supernatants were directly utilized for the estimation of cytochrome *c*, while the total amount of cytochrome *c* of untreated mitochondria and of the pellets obtained after cytochrome *c* release were measured following extraction as indicated in Materials and Methods. Concentration of cytochrome *c* was determined as described in Materials and Methods. Figure 4 reports the spectrophotometric estimation of cytochrome *c* for (*a*), (*b*), (*c*), (*d*), and (*e*). Statistical significance: versus control without additions (*a*): $*P < 0.05$, $†P < 0.01$, $‡P < 0.001$; versus control with EGTA + CsA (*b*): $§P < 0.05$, $‖P < 0.01$.

increasing concentrations (ranging from 8 μM to 33 μM) of 13-*cis* RA on mitochondrial membrane potential. The extent of the latter and the time preceding the total breakdown were inversely related to the concentration of 13-*cis* RA. At 33 μM 13-*cis* RA, a $\Delta\Psi$ of about −100 mV was gradually obtained and was followed by a rapid fall; the addition of EGTA after the collapse of membrane potential was able to restore $\Delta\psi$ only to a very limited extent. However, when EGTA or CsA were present in the incubation medium together with 33 μM 13-*cis* RA, the decrease in membrane potential is largely prevented and could be discharged only by the addition of the uncoupler CCCP (Fig. 3C). Interestingly, if EGTA or CsA was added to the system together with 13-*cis* RA and before the energization of mitochondria, the presence of increasing concentrations of 13-*cis* RA determined a correspondingly lower steady state of $\Delta\psi$, that, on the other hand, was maintained for several minutes and disrupted only by the addition of CCCP (not shown).

The mitochondrial content of cytochrome *c* can be indirectly monitored as oxygen uptake dependent on the electron transport through cytochrome *c* and cytochrome oxidase in the presence of the electron donors ascorbate and tetramethyl-*p*-phenylenediamine. Nevertheless, in our case, this methodology did not provide reliable and quantitative information on the amount of cytochrome *c* released. Therefore, the release of cytochrome *c* in the presence of 13-*cis* RA was directly measured in the supernatants of mitochondria incubated under different conditions (Table 1 and Fig. 4). The spectrophotometric determination of cytochrome *c* released, as differential spectrum in the range 500–600 nm, was reported as the second derivative of the absorption (Fig. 4) in order to increase the sensitivity of the measurement and prevent the interference of the added substances, particularly 13-*cis* retinoic acid. In the presence of the latter, the amount of cytochrome *c* released was almost doubled with respect to the control (Table 1, Fig. 4). The concentration of 13-*cis* RA utilized to induce the release of cytochrome *c* (20 nmol/mg protein) was of the same order of magnitude as that used for swelling and membrane potential experiments (compare Table 1 and Figs. 2 and 3). In particular, it corresponds to the lowest concentration used for swelling experiments (Fig. 2), indicating that relatively low concentrations of 13-*cis* RA are sufficient to induce a significant release of cytochrome *c*. The presence of EGTA + CsA, which are both potent inhibitors of swelling and membrane potential decrease (Figs. 2 and 3), resulted in a negligible protection towards cytochrome *c* release which was instead, strongly stimulated by the presence of Ca^{2+} and phosphate [29–31]. The release of cytochrome *c* under the above conditions was confirmed by polyacrylamide gel electrophoresis of the proteins released from the mitochondria (not shown). It should also be noted that the amount of total protein measured in the supernatants obtained after sedimentation of mitochondria was larger when 13-*cis* retinoic acid was present in the incubation medium, again indicating that a permeabilization of the membrane had occurred.

DISCUSSION

Retinoic acids, in particular 13-*cis* RA, are able to induce mitochondrial swelling and a rapid decrease in mitochondrial membrane potential. Both events are hallmarks of the occurrence of permeability transition that depends on the presence of calcium ions and a large array of inducing agents, chemically unrelated, but all capable of opening a putative "pore" [14, 15] responsible for an increase in membrane permeability. Previous research has shown that retinoic acids act as mitochondrial uncouplers [19] and swelling agents [20] and their effects were attributed essentially to a membranolytic action [19, 20]. Moreover, from our results, it is also apparent that retinoids can be considered as inducers of permeability transition, since their action is stimulated by Ca^{2+} ions and inhibited by EGTA and cyclosporin. At present, the specific mechanism of action of retinoic acids on mitochondrial metabolism and in particular on the induction of permeability transition is not clear. 13-*cis* RA is an inhibitor of the thioredoxin system [32]; the latter is present in mitochondria [33], where it might exert control over the redox balance of

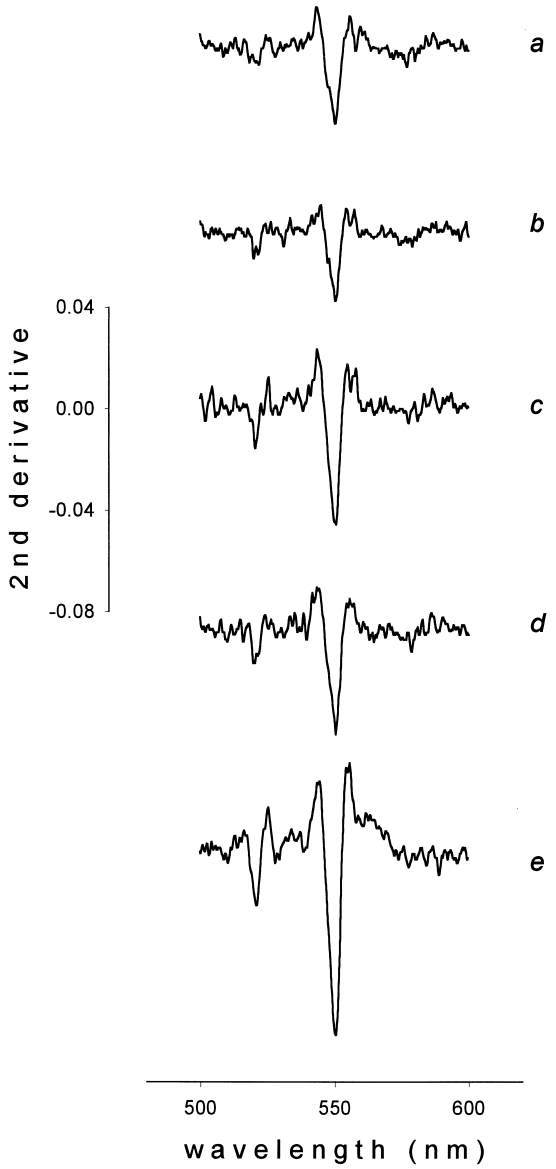

FIG. 4. ***Spectrophotometric determination of cytochrome c.*** **Mitochondria were incubated under the conditions given in Table I. Cytochrome c concentration was measured as indicated under Materials and Methods. In order to enhance minor differences, the absorption spectra are reported as the second derivative ($d^2A/d\lambda^2$). The resulting values were compared to those of a standard curve obtained using commercial cytochrome c; the readings are linear in the range 0.25–2 μM cytochrome c. a: control without additions; b: control with EGTA + CsA; c: 13-*cis* RA; d: EGTA + CsA + 13-*cis* RA; e: Ca^{2+} + phosphate.**

thiols and hence over mitochondrial inner membrane permeability [33].

Cytochrome *c* is present in the mitochondrial intermembrane space loosely bound to the inner membrane and can be released by several conditions, including hypotonic shock in KCl solutions [34], permeability transition [29–31], or an increase in permeability of the outer membrane [16–18]. Table 1 and Fig. 4 show that 13-*cis* RA is able to release cytochrome *c* from the mitochondrial compartment to cytosol; for purposes of comparison, the combination Ca^{2+} + phosphate, which constitutes the classical system utilized for the induction of permeability transition, is also reported. During apoptosis, there is a disruption of the barrier function of the inner and/or outer mitochondrial membrane [13, 18]. However, the extent of involvement of the two membranes and the temporary sequence by which they are affected are not clear [13, 18]. According to some authors [13, 16–18], cytochrome *c* release can occur independently of the permeability transition process. Bossy-Wetzell *et al.* [16] reported that the decrease in the transmembrane electrical potential, possibly mediated by the opening of the mitochondrial permeability transition pore, occurred later than the cytosolic translocation of cytochrome *c* and caspase activation and was not necessary for DNA fragmentation. In addition, Eskes *et al.* [17] observed that Bax, a pore-forming protein associated with mitochondrial membrane, when overexpressed in different cell types or directly added to isolated mitochondria, can trigger the release of cytochrome *c* independently of the activation of membrane permeability transition, suggesting that the permeability transition pore and Bax are two separate entities. Interestingly, in our case, CsA + EGTA, which completely inhibit mitochondrial swelling, were unable to prevent the release of cytochrome *c* induced by 13-*cis* RA (Table 1, Fig. 4). This might indicate that permeability transition and the release of apoptogenic factors are not strictly correlated. Therefore, 13-*cis* retinoic acid might act through two different mechanisms, one involving permeability transition and the other active on the outer membrane and insensitive to cyclosporin and EDTA. The action of 13-*cis* RA on the release of cytochrome *c* requires further study in order to identify the targets responsible for the induction of a specific increase in permeability of the outer membrane.

In the present communication, it has been observed that 13-*cis* retinoic acid is able to induce mitochondrial permeability transition observed as swelling and a decrease in membrane potential in addition to a significant release of cytochrome *c*. The release of the latter also appears to occur independently of permeability transition. The reported results indicate that mitochondria could be, at least in part, a direct target of the antitumor action of 13-*cis* RA.

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