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All-*trans*-Retinoic Acid Binds to and Inhibits Adenine Nucleotide Translocase and induces Mitochondrial Permeability Transition

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ABSTRACT

We investigated the effects of retinoic acids on mitochondrial permeability transition (MPT) measured as changes in rhodamine 123 fluorescence from both isolated heart mitochondria and HeLa cells. We report that all-*trans*-retinoic acid (atRA), 9-*cis*-retinoic acid, and 13-*cis*-retinoic acid induce a drop in mitochondrial membrane potential in isolated mitochondria. The atRA effect was done through the induction of MPT because it was dependent on Ca^{2+}, in a synergic mechanism, and inhibited by cyclosporin A (CsA). Furthermore, atRA also opened MPT in vivo, because treatment of HeLa cells with atRA results in a CsA-sensitive drop of mitochondrial membrane potential. We demonstrated for the first time that retinoic acids inhibit adenine nucleotide translocase (ANT) activity in heart and liver mitochondria. Kinetic studies revealed atRA as an uncompetitive inhibitor of ANT. Photoaffinity labeling of mitochondrial proteins with [^{3}H]atRA demonstrated the binding of a 31-kDa protein to atRA. This protein was identified as ANT because the presence of carboxyatractyloside, a specific ANT inhibitor, prevented labeling. The specific photolabeling of ANT was also prevented in a concentration-dependent manner by nonlabeled atRA, whereas palmitic acid was ineffective. This study indicates that specific interaction between atRA and ANT takes place regulating MPT opening and adenylate transport. These observations establish a novel mechanism for atRA action, which could control both energetic and apoptotic mitochondrial processes in situations such as retinoic acid treatment.

Adenine nucleotide translocase (ANT) is an integral membrane protein of 31 kDa located in mitochondria that catalyzes nucleotide exchange between mitochondria and cytosol. It simultaneously provides ADP for oxidative phosphorylation and ATP to the cytosol for fueling. The existence of two interconvertible conformations of ANT in the mitochondrial membrane has been postulated. The two translocation states of the carrier that correspond to the two conformations are distributed over the cytosol-facing state and matrix-facing state in the presence of ADP or ATP (for reviews, see Klingenberg, 1989; Brandolin et al., 1993).

Three isoforms of ANT have been described in humans (Battini et al., 1987; Cozens et al., 1989; Neckelmann et al., 1989). ANT1 is mainly expressed in heart and skeletal muscle and ANT2 is expressed in tissues able to undergo proliferation, such as liver and kidney, whereas ANT3 is expressed ubiquitously (Stepien et al., 1992; Doerner et al., 1997).

ANT is a major component of the permeability transition pore (PTP) complex, a protein aggregate that resides in contact sites of the inner and outer mitochondrial membrane (Vieira et al., 2000). PTP opening was subsequently implicated in apoptosis induction (Green and Reed, 1998). The core components of the pore complex are ANT and the voltage-dependent anion channel, although other complementary proteins such as cyclophilin D and other molecules also seem to be involved (for review, see Zoratti and Szabo, 1995). Direct interactions of ANT and cyclophilin D (Halestrap and Davidson, 1990) and of ANT and the voltage-dependent anion channel (Crompton et al., 1998) have been shown. In addition, the proapoptotic molecule Bax cooperates with ANT in the PTP complex (Marzo et al., 1998). The opening of the PTP across the inner and outer membranes of mitochondria causes mitochondrial permeability transition (MPT). Its occurrence is associated with depolarization of the mitochon-

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ABBREVIATIONS: ANT, adenine nucleotide translocase; PTP, permeability transition pore; MPT, mitochondrial permeability transition; ATRAC, atractyloside; CATR, carboxyatractyloside; BA, bongkrekic acid; atRa, all-*trans*-retinoic acid; 9cisRA, 9-*cis*-retinoic acid; 13cisRA, 13-*cis*-retinoic acid; CsA, cyclosporin A; PA, palmitic acid; [^{3}H]atRA, [11,12-^{3}H]all-*trans*-retinoic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CCCP, carbonylcyanide *m*-chorophenyl hydrazone; $\Delta\Psi_m$, mitrochondrial membrane potential; CRABP, cellular retinoic acid binding protein; PKC, protein kinase C; UCP, uncoupling protein.

drial membrane potential, loss of the H^+ gradient, uncoupling of oxidative phosphorylation, ATP depletion, and mitochondrial swelling (Kroemer and Reed, 2000; Vieira et al., 2000). A diverse range of stimuli can control the MPT in both isolated mitochondria and intact cells. Among the non-protein effectors, calcium is the most important inducer of MPT (Petronilli et al., 1993). Ganglioside GD3, fatty acids, reactive oxygen species, and nitric oxide can also induce MPT (Green and Reed, 1998). As far as is known, specific ligands of the ANT, atractyloside (ATRAC), carboxyatractyloside (CATR), or bongkrekic acid (BA), as well as ADP and acyl CoA, affect PTP opening. ANT ligands, ATRAC, CATR, and palmitoyl CoA, which stabilize the c conformation of the ANT, act as PTP inducers. Ligands such as BA, matrix ADP, and matrix acyl CoA, which stabilize the m conformation of the ANT, close PTP (Le Quoc and Le Quoc, 1988). Consequently, changes in the conformation of the ANT can regulate PTP opening and trigger cell death. Along these lines is the description that viral protein R, an apoptogenic protein encoded by the human immunodeficiency virus, also induces mitochondrial membrane permeability through direct interaction with ANT (Jacocot et al., 2001). Genetic experiments confirm the participation of ANT in the control of cell death. ANT1 has been described as an apoptosis-inducing gene: as such, overexpression of ANT1 (but not ANT2) induces apoptosis in mammalian cells (Bauer et al., 1999).

Retinoic acid, a natural derivative of vitamin A, affects biological processes such as development, cell growth, and differentiation. There is substantial evidence that retinoids, especially all-*trans*-retinoic acid (atRA), can also induce apoptosis in different tumor cell lines and during embryo development (De Luca, 1991).

The molecular mechanism of retinoic acid action mainly involves the binding and activation of specific nuclear receptors, retinoic acid receptor and retinoid X receptor, that modulate gene expression (Chambon, 1996). In addition to the classical function of retinoids in regulation of gene expression, atRA has been recently described as binding and regulating PKC activity (Radominska-Pandya et al., 2000). This indicates that retinoic acids could be involved in the regulation of numerous signaling processes in which PKC participates. Apart from this important function, retinoids could also regulate some mitochondrial processes. In fact, it was observed that natural retinoids are able to induce swelling through the induction of MPT at least in liver mitochondria (Rigobello et al., 1999). Furthermore, retinoids are extremely potent activators of the uncoupling mitochondrial proteins UCP1 and UCP2 (Rial et al., 1999).

Because retinoids are inducers of liver mitochondria swelling, and ANT is crucial in MPT activity, we investigated the role of natural retinoids as putative regulators of MPT and their effects on ANT activity in heart mitochondria.

Our results are the first evidence for a binding of atRA to ANT that leads to a decrease in ANT activity. AtRA acts as an uncompetitive inhibitor of ANT and, moreover, regulates mitochondrial membrane potential through MPT opening. These findings demonstrate a direct action of natural retinoids upon function and activity of mitochondria and suggest that some of the apoptotic effects of retinoids might occur through this action.

Materials and Methods

Materials. All-*trans*-retinoic acid (atRA), 9-*cis*-retinoic acid (9cisRA), 13-*cis*-retinoic acid (13cisRA), ATRAC, cyclosporin A (CsA), and ADP came from Sigma (St. Louis, MO). CATR was supplied by Calbiochem (San Diego, CA). [^{14}C]ATP came from Amersham Biosciences (Piscataway, NJ). [11,12-^{3}H]All-*trans*-retinoic acid ([^{3}H]atRA) was purchased from PerkinElmer Life Sciences (Boston, MA).

Mitochondria Isolation. Subsarcolemmal mitochondria from bovine heart were obtained by differential centrifugation basically as described previously (Smith, 1967). All subsequent procedures were carried out at 4°C and pH was rapidly readjusted to 7.8 with 2 M Tris after every step. Three hundred grams of fresh bovine heart tissue cut into cubes were passed through a meat grinder. The resulting mince was placed in 400 ml of 0.25 M sucrose and 0.01 M Tris-HCl, pH 7.8, and centrifuged at 1,000*g* for 10 min at 4°C. The pellet was resuspended in 600 ml of sucrose solution (0.25 M sucrose, 10 mM Tris-HCl, pH 7.8, 1 mM succinate, and 0.2 mM EDTA), homogenized by Polytron homogenizer (3 × 10 s, power 6–7), and centrifuged at 1,200*g* for 20 min at 4°C. Supernatant was centrifuged at 26,000*g* for 15 min at 4°C. The resulting pellet usually consisted of three distinct layers. The top layer with damaged mitochondria was discarded. The second precipitated layer (heavy beef heart mitochondria) was removed, mixed with 10 ml of sucrose solution, and decanted, leaving behind the bottom pellet. The resuspended mitochondrial pellet was washed twice with the sucrose solution and finally resuspended to 20 to 40 mg of protein/ml.

Mitochondria were isolated from mouse liver by standard differential centrifugation (Yang and Cortopassi, 1998). Livers excised from male Swiss mice were homogenized in 0.5 g/ml ice-cold homogenization medium (213 mM mannitol, 71 mM sucrose, 2 mM EGTA, 0.2% bovine serum albumin, and 5 mM HEPES/Tris, pH 7.8) by three strokes in a manual glass-Teflon homogenizer. The homogenate was centrifuged twice at 1,500*g* for 10 min at 4°C. The supernatant collected was centrifuged at 10,000*g* for 15 min at 4°C. The pellet obtained was washed and resuspended in 0.5 ml of homogenization buffer without EGTA and bovine serum albumin. Mitochondrial protein concentration was measured using the bicinchoninic protein assay reagent from Pierce (Rockford, IL).

MPT Activity Measured as an Increase in Rhodamine 123 Fluorescence from Heart Mitochondria. Bovine mitochondria were resuspended at 1 mg of protein/ml of 0.3 M mannitol, 10 mM MOPS pH 7.7, 10 mM succinate, 3 μM rotenone, 1 mM KH_2PO_4, and 10 μM EGTA. Changes in the mitochondrial membrane potential were monitored continuously by detection of fluorescence quenching of rhodamine 123 (5 μM) (Tamaka et al., 1998) with a spectrofluorometer (RF-5001 PC; Shimadzu, Kyoto, Japan). Fluorescence excited at 503 nm and emitted at 527 nm was measured. When CsA (2 μM) was used, it was added to the mitochondria before addition of the inducing agents.

Determination of ANT Activity. The determination of the ADP/ATP exchange rate was based on the inhibitor stop method combined with the back exchange (Schultheiss and Klingenberg, 1984). In this procedure, the intramitochondrial adenine nucleotides were first labeled with 100 μM [^{14}C]ATP (5.2 mCi/mmol) for 1 h at 4°C in buffer containing 0.25 M sucrose and 10 mM Tris, pH 7.8. The efflux of radioactive label on addition of variable external ADP concentrations was measured after 10 s in heart mitochondria and after 1 min in liver mitochondria. The time course of the exchange was followed at 2°C by discrete sampling and by stopping the exchange by addition of excess ATRAC (300 μM). The adenine nucleotide translocation as 1:1 exchange between intra- and extramitochondrial nucleotides was calculated as nanomoles of ADP transported per milligram of protein. For the kinetic analysis, external ADP concentrations were between 0 and 80 μM and time sampling was between 0 and 30 s.

Photoaffinity Labeling with [^{3}H]atRA. Direct photoaffinity labeling with [^{3}H]atRA was performed according to a method described previously (Bernstein et al., 1995). Under red safe-light illu-

mination, 10 μCi of [^{3}H]atRA (40–60 Ci/mmol) in 10 μl of ethanol (1 μCi/μl) was added to 1.5-ml microcentrifuge tubes. After the ethanol was removed under vacuum, 20 μg of heart mitochondrial protein were added to each tube, and the final volume was adjusted to 10 μl with 20 mM Tris buffer, pH 7.5, for a final concentration of 20 μM retinoic acid. For the studies of concentration dependence, a final concentration of atRA between 3 and 48 μM was used. The samples were incubated at 25°C and shaken for 1 h in the dark. Open tubes were placed on ice and exposed to an intense 366-nm UV light source for 15 min. The protein samples were boiled in SDS-polyacrylamide gel electrophoresis sample buffer containing 2-mercaptoethanol, and then loaded and run with standard SDS-polyacrylamide gel electrophoresis techniques. The gels were stained with Coomassie blue and then soaked in Amplify (Amersham Biosciences). The dried gels were then used for fluorography at −80°C for 10 days.

Mitochondrial Membrane Potential in HeLa Cells. HeLa cells were grown in the presence of 2% fetal bovine serum in Dulbecco's modified Eagle's medium. The cells were exposed to either 5 or 20 μM atRA for 12 h. atRA was dissolved in ethanol; the final ethanol concentration in the medium did not affect the cells. CsA was added to the cells 30 min before atRA treatment at final concentration of 5 μM. After the exposure of cells to atRA, the cell suspension was incubated in Dulbecco's modified Eagle's medium for 30 min at 37°C. Mitochondrial membrane potential was measured as described previously (Vander Heiden et al., 1997) with minor changes. Cells were loaded with 250 ng/ml rhodamine 123 for 20 min. At the end of incubation period, the cells where washed twice in phosphate-buffered saline, suspended in a total volume of 0.5 ml, and the $\Delta\Psi_m$ was analyzed by flow cytometry in a Coulter Epics-XL-MCL (Beckman Coulter, Fullerton, CA). Where indicated, CCCP at 10 μM was added with the rhodamine 123. Results are expressed as a percentage of the maximum effect produced by the uncoupler CCCP.

Statistical Analysis. Effects of retinoids on ANT activity were tested by one-way analysis of variance with Dunnett multiple comparison test.

Results

Heart MPT Activity Is Regulated by Retinoic Acids. We measured MPT activity as a progressive increase in Rh123 fluorescence from the mitochondria. To associate changes in mitochondrial membrane potential with MPT opening, we used Ca^{2+} ions and ATRAC as inducers of MPT opening and CsA, an immunosuppressive agent that blocks MPT. Ca^{2+} and ATRAC were added to mitochondria after being energized with succinate in the presence of 2 μM rotenone. Figure 1A indicates that Ca^{2+} added to isolated mitochondria at 5, 20, and 30 μM causes a dose-dependent MPT opening measured as changes in $\Delta\Psi_m$. Fig. 1B analyzes ATRAC effect on MPT opening. ATRAC caused an important effect on MPT activity measured as a decrease of $\Delta\Psi_m$ in heart mitochondria. CsA, added to the mitochondria before addition of ATRAC, prevented the decrease in $\Delta\Psi_m$ caused by ATRAC. These results indicate that changes in $\Delta\Psi_m$ are caused by MPT opening of heart mitochondria.

To study the effects of retinoic acids on mitochondrial membrane potential and associate these changes with MPT opening, we analyzed the effects of atRA, 9cisRA, 13cisRA, and MPT regulators on $\Delta\Psi_m$ in heart mitochondria. As Fig. 2A shows, all retinoic acids used in this study induced depolarization of the mitochondrial membrane, but the magnitude of the effect with atRA was greater than with other retinoids at the same concentration. Moreover, the addition of 1 μM CsA completely prevented these effects. Figure 2B describes the effects of atRA at different concentrations. At 10 μM atRA, a slight hyperpolarization was observed. When the concentration of atRA added to heart mitochondria was between 20 and 30 μM, we observed a progressive decrease in $\Delta\Psi_m$ associated with MPT opening. Interestingly, when the mitochondria were exposed to 10 μM atRA and 0.5 μM Ca^{2+} together (Fig. 2C), the drop in $\Delta\Psi_m$ was more important than addition of individual doses of atRA and Ca^{2+}. This result indicates that at low doses the addition of one regulator increases the sensibility of another.

To establish the relation between Ca^{2+} and atRA in MPT opening, we analyzed the changes in $\Delta\Psi_m$ in the presence of EGTA at different concentrations together with 30 μM atRA. The addition of high doses (30–100 μM) of EGTA, which reduces free Ca^{2+} in the medium, progressively prevents

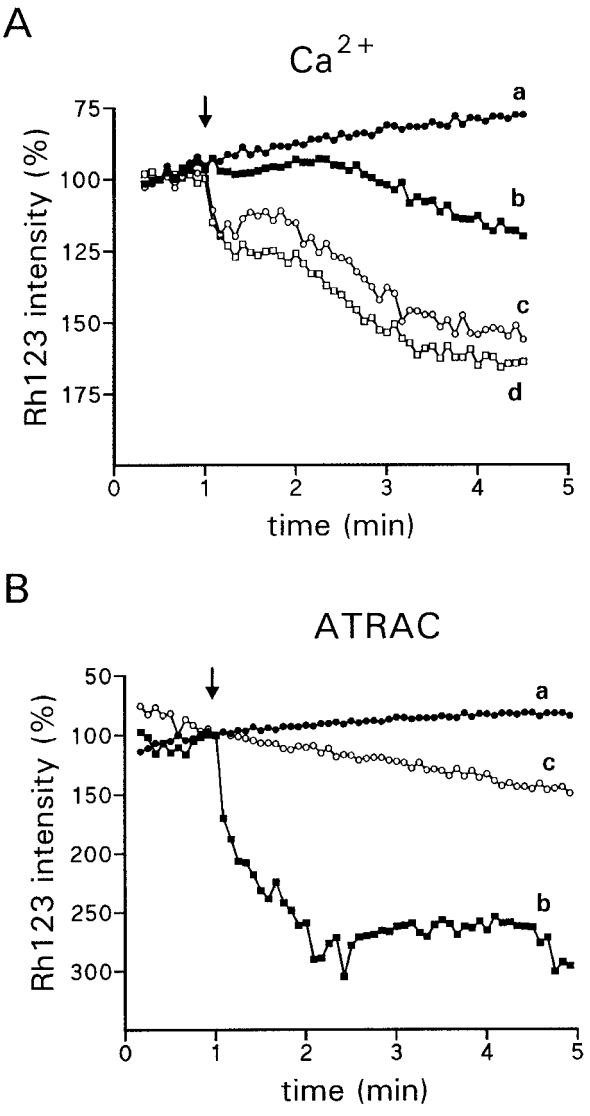

Fig. 1. MPT activity of heart mitochondria. Mitochondria isolated from heart were incubated at 1 mg of protein/ml, as described under *Materials and Methods*. Mitochondria were energized with 10 mM succinate. Rhodamine 123 was added 2 min before adding MPT inducers. A, samples were incubated with increasing micromolar concentrations of Ca^{2+}. a, no additions; b, 5 μM Ca^{2+}; c, 20 μM Ca^{2+}; d, 30 μM Ca^{2+}. B, samples were incubated with 450 μM ATRAC. a, no additions; b, 450 μM ATRAC; c, 450 μM ATRAC + 2 μM CSA. When indicated, mitochondria were preincubated with 2 μM CsA for 2 min before the addition of ATRAC. The data shown here are representative of two independent experiments with similar results.

atRA-mediated MTP induction. This result confirms the synergic mechanism between Ca^{2+} and atRA regulating mitochondrial membrane potential and MPT opening.

Effects of Retinoic Acids on ANT Activity. To find whether ANT is a possible target of the non-nuclear action of retinoic acids, we tested the effects of different doses of retinoic acids, atRA, and 9cisRA in ANT activity in both heart and liver mitochondria. ANT activity was measured as ATP/ADP exchange and expressed as nanomoles per milligram of mitochondrial protein in heart (Fig. 3A) and liver (Fig. 3B). Figure 3A shows that with 5 nmol of atRA/mg of protein, ANT activity decreased to 75% of control values, and with 25 nmol of atRA/mg of protein, it decreased to 30%. Moreover, 12 nmol of 9cisRA/mg of protein inhibits ANT activity at 75% of control, and 25 nmol of 9cisRA/mg of protein inhibits at 50%. As Fig. 3B shows, 10 nmol of atRA/mg of protein inhibits ANT activity in liver to 65% of control and 30 nmol of atRA/mg of protein inhibits ANT activity to 35%. However, when 9cisRA was used, only 15% of inhibition was found at 30 nmol/mg of protein. These results indicate that both atRA and 9cisRA inhibit ANT in isolated mitochondria, but atRA is more efficient than 9cisRA in ANT inhibition in both heart and liver mitochondria.

To establish the type of inhibition by atRA, kinetic measurements were run on heart mitochondria. ATP/ADP exchange was measured in isolated mitochondria by varying external ADP concentration between 5 and 80 μM. The kinetics of this ATP/ADP exchange determined in the presence of 5 and 20 nmol of atRA/mg of protein is shown in Fig. 4. The

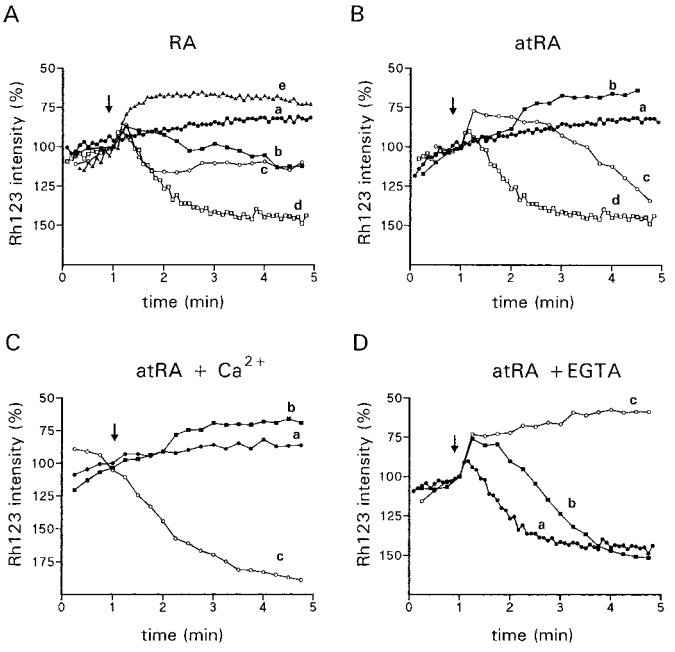

Fig. 2. Effect of retinoic acids on MPT activity in heart mitochondria. Heart mitochondria were incubated at 1 mg of protein/ml, as described under *Materials and Methods*. Mitochondria were energized with 10 mM succinate. Rhodamine 123 was added 2 min before adding MPT inducers. A, effects of 9cisRA (b), 13cisRA (c), and atRA (d) at 30 μM. a, no additions; e, atRA + 2 μM CsA. B, samples were incubated with increasing concentrations of atRA. a, no additions; b, 10 μM atRA; c, 20 μM atRA; d, 30 μM atRA. C, comparison between the effect of atRA and Ca^{2+} individually and in combination. a, 0.5 μM Ca^{2+}; b, 10 μM atRA; c, 0.5 μM Ca^{2+} + 10 μM atRA. D, samples were incubated with atRA at 30 μM and with increasing concentrations of EGTA. a, atRA; b, atRA + 30 μM EGTA; c, atRA + 100 μM EGTA. The data shown here are representative of two independent experiments with similar results.

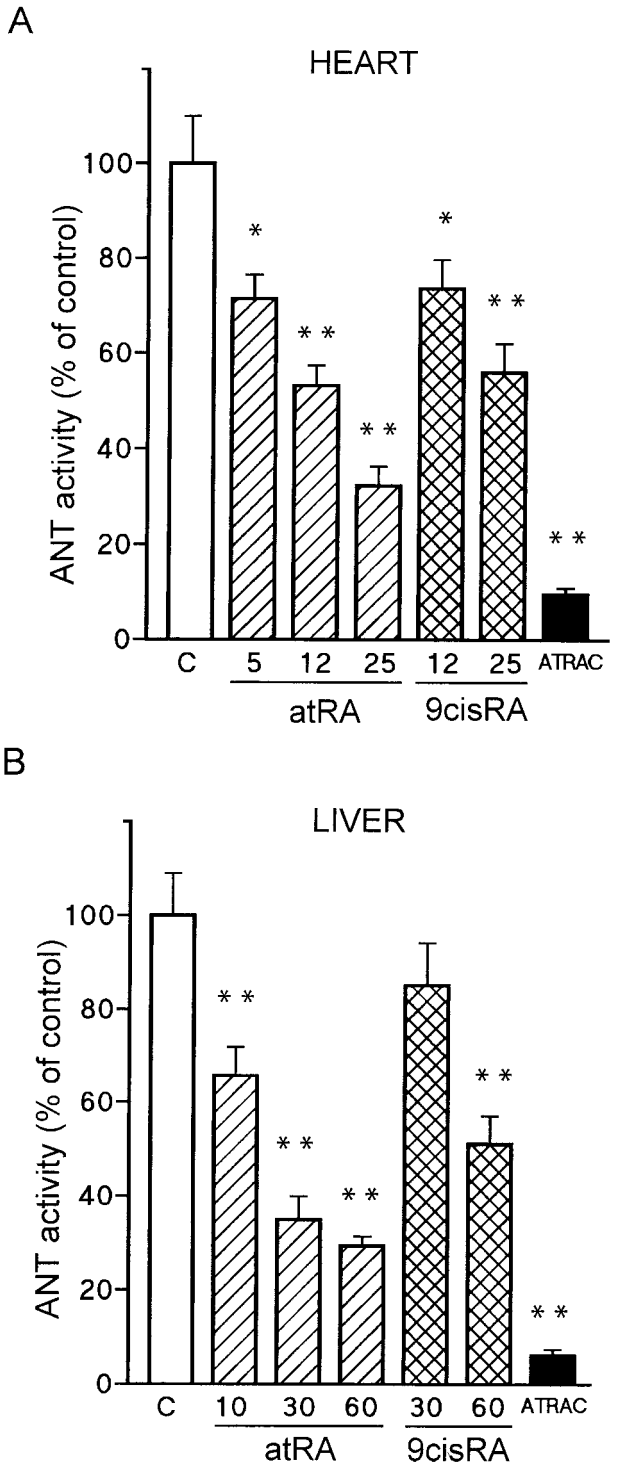

Fig. 3. Effect of retinoic acids on ANT activity of heart and liver mitochondria. Heart and liver mitochondria (40–50 mg/ml) were preloaded with [^{14}C]ATP (100 μM) for 1 h at 4°C. After two successive washings, they were resuspended at 5 mg/ml in fresh buffer and aliquoted (100 μl). The back-exchange was initiated by the addition of unlabeled ADP (200 μM). The exchange was stopped by addition of ATRAC (300 μM) and the samples were processed as described under *Materials and Methods*. When indicated, retinoids or ATRAC were added before the addition of unlabeled ADP. A, heart mitochondria. Concentrations of retinoic acids used were 5, 12, and 25 nmol/mg of protein for atRA (▨) and 12 and 25 nmol/mg of protein for 9cisRA (▩). B, liver mitochondria. Concentrations of retinoic acids used were 10, 30, and 60 nmol/mg of protein of atRA (▨) and 30 and 60 nmol/mg of 9cisRA (▩). Results showed the mean ± S.E.M of three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

results indicate that 5 nmol of atRA/mg of protein caused an important decrease both in V_{max} and K_m in adenylate transport activity. Double reciprocal plot data indicates that V_{max} in control was 1.87 nmol of ADP/min/mg of protein and K_m was 7.04 μM ADP. When the kinetic analysis with 5 nmol of atRA/mg of protein was done, apparent V_{max} was 1.32 nmol of ADP/min/mg of protein and apparent K_m was 4.97 μM ADP. These results indicate that the atRA inhibitor effect is in agreement with an uncompetitive model. Derived K_i from the data measured was 13.7 nmol atRA/mg of protein. However, when the concentration of atRA was 20 nmol/mg of protein, we find a decrease in apparent V_{max} (1.06 nmol of ADP/min/mg of protein) and an increase in the apparent K_m of the transport activity (9.69 μM ADP) (Fig. 4B).

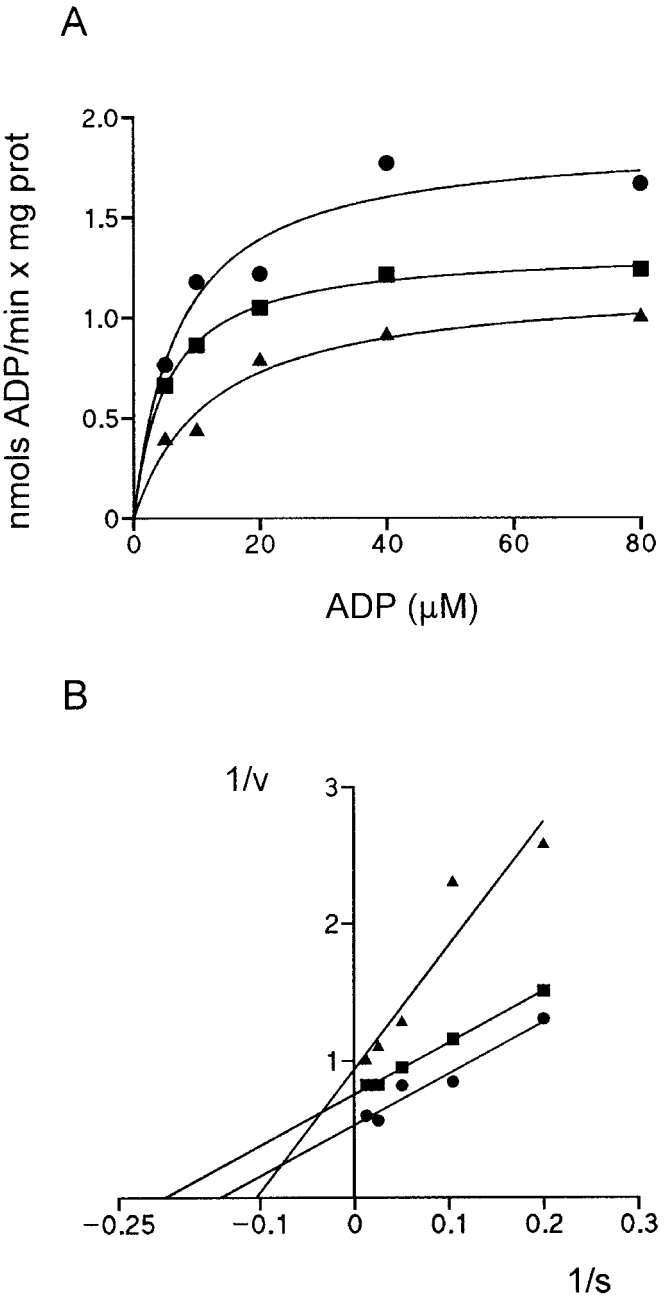

Fig. 4. Inhibition of ANT activity by atRA. Heart mitochondria (40–50 mg/ml) were preloaded with [^{14}C]ATP (100 μM) for 1 h at 4°C; after two successive washings, they were resuspended at 5 mg/ml in fresh buffer and aliquoted (100 μl). The back-exchange was initiated by the addition of various concentrations of unlabeled ADP ranging from 5 to 80 μM. The exchange was stopped after a period of 10 s at 2°C by addition of ATRAC (300 μM) and the samples were processed as described under *Materials and Methods*. A, atRA was added before the addition of unlabeled ADP at concentrations indicated. B, double-reciprocal plot of the velocity of ADP exchange as a function of ADP concentration was depicted. ●, 0 nmol of atRA/mg of protein; ■, 5 nmol of atRA/mg of protein; ▲, 20 nmol of atRA/mg of protein. Values plotted are the average of three independent experiments.

atRA Binds to ANT. To study a direct interaction between atRA and ANT, we used photoaffinity labeling of heart mitochondrial protein with [^{3}H]atRA, which covalently modified proteins within the atRA-binding site and provided direct evidence for atRA binding to proteins. Because atRA is the most effective retinoic acid in MPT induction in heart mitochondria, it was the retinoic acid choice to investigate direct interaction between retinoic acids and ANT. Figure 5 shows mitochondrial proteins labeled with atRA. Surprisingly, only a few proteins bind to [^{3}H]atRA; specifically, a 31-kDa protein was detected. We observed that the photolabeling of the 31-kDa protein was prevented when CATR, a specific ANT inhibitor, was added. This demonstrates that ANT was photolabeled by [^{3}H]atRA. Figure 6A explains the binding of different concentrations of [^{3}H]atRA to purified mitochondria from bovine heart. We observed that the labeling of ANT was a saturable process that increases progressively up to 12 μM [^{3}H]atRA and then reaches a plateau. Figure 6B shows the effect of different atRA concentrations on the binding of ANT with [^{3}H]atRA; the binding was effectively competed with by unlabeled atRA. Palmitic acid (PA) is a fatty acid that has been described as binding to ANT (Schönfeld et al., 1996). To test the specificity of the binding,

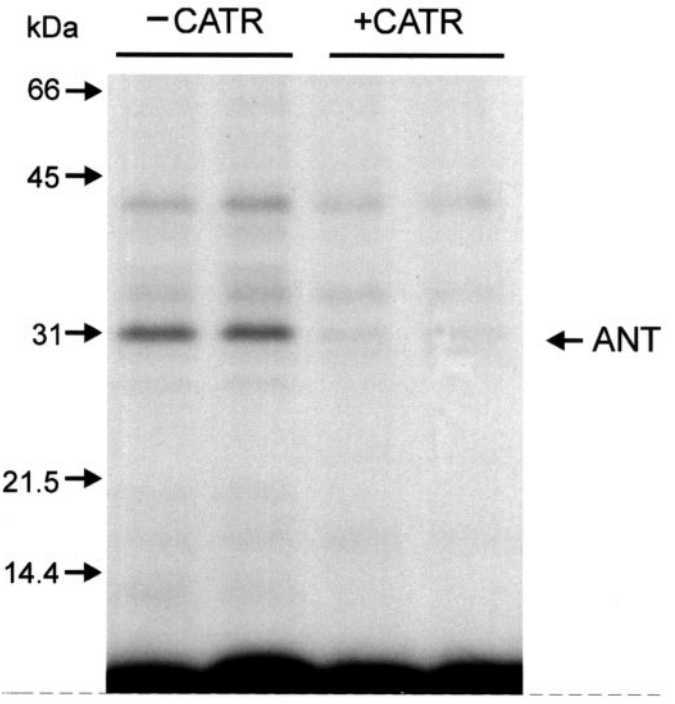

Fig. 5. Photoaffinity labeling of ANT by all-*trans*-[^{3}H]retinoic acid. Heart mitochondrial protein was labeled with [^{3}H]atRA by direct photoaffinity technique described under *Materials and Methods*. The samples were photolyzed for 15 min under UV (366 nm) light source. In fluorography, lanes 1 to 2 correspond to 20 μg of mitochondrial heart protein photolabeled with [^{3}H]atRA. In lanes 3 to 4, 7.5 μM CATR was added to 20 μg of mitochondrial heart protein photolabeled with [^{3}H]atRA.

we made PA compete with atRA to bind ANT. Results showed (Fig. 6B) that atRA and PA did not compete to bind ANT, at least up to 200 μM.

Effects of atRA on Mitochondrial Membrane Potential in Intact Cells. To study the effects of atRA on $\Delta\Psi_m$ and associate the changes with MPT opening in intact cells, HeLA cells were treated with atRA at 5 and 20 μM in the presence or absence of CsA. As can be seen in Fig. 7, 5 μM atRA induces a drop in $\Delta\Psi_m$ that reaches the 50% of the value obtained with 10 μM CCCP, a known mitochondrial uncoupler. The presence of 5 μM CsA prevents completely the atRA effects. The decrease in $\Delta\Psi_m$ observed with 20 μM atRA reaches the same level obtained with 10 μM CCCP, and again CsA decreases this effect. Hence, the effect of atRA on $\Delta\Psi_m$ is done through MPT opening.

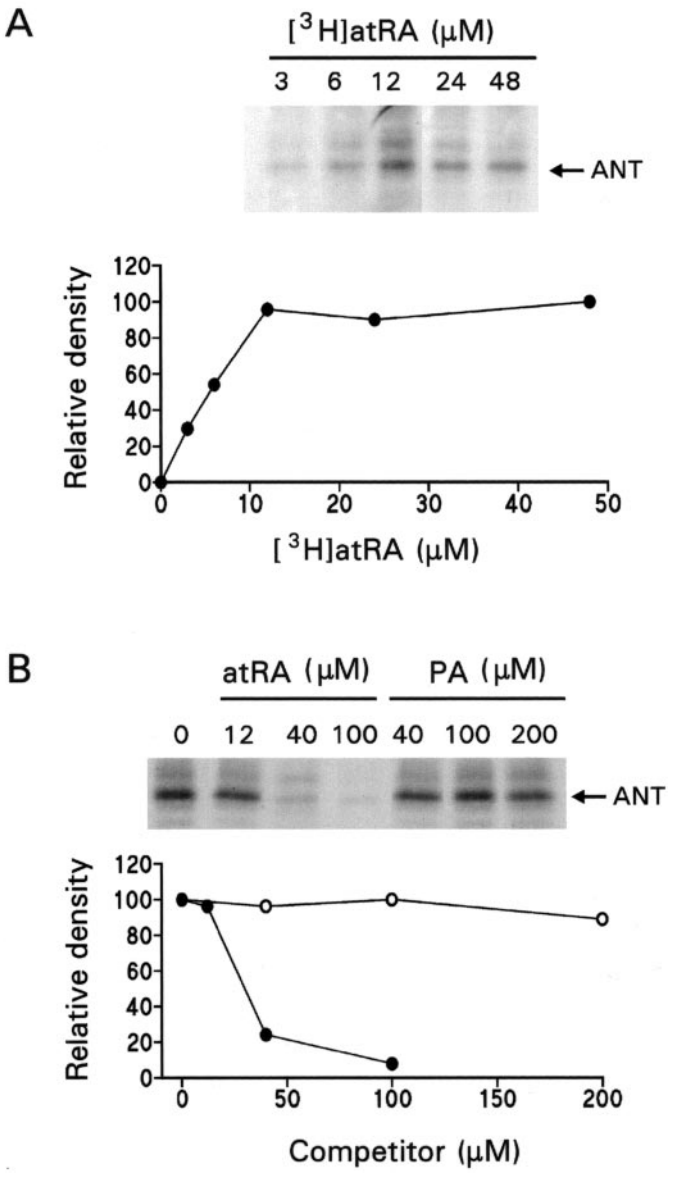

Fig. 6. Photoaffinity labeling of ANT by [^{3}H]atRA and protection with unlabeled atRA and palmitic acid. Direct photoaffinity labeling with [^{3}H] atRA, as described under *Materials and Methods*. A, ANT was photolabeled with increasing concentrations of [^{3}H]atRA (3, 6, 12, 24 and 48 μM). The graph is a plot of relative density versus concentration of [^{3}H]atRA ligand. B, protection of ANT from photolabeling with unlabeled atRA (●) and PA (○). Graph shows the results of the protection by densitometry in the presence of two competitors.

Discussion

In this report, we demonstrate that retinoic acids induce MPT opening in heart mitochondria because their action is dependent on Ca^{2+} and inhibited by EGTA and CsA, a known inhibitor of MPT (Zoratti and Szabo, 1995). Previous research has shown that retinoic acids, especially 13cisRA, could cause mitochondrial swelling and decrease mitochondrial membrane potential in liver mitochondria (Rigobello et al., 1999). However, our results indicate that atRA is more effective in the induction of depolarization of mitochondrial membrane and MPT activity linked in heart. On the other hand, the presence of atRA could decrease the concentration of Ca^{2+} necessary to induce MPT opening (or vice versa). This synergic mechanism between Ca^{2+} and atRA might be important in the induction of MPT opening in biological situations, Thus, high levels of atRA (20 μM) are required to induce MPT; however, when Ca^{2+} are present in the medium (0.5 μM), atRA can activate MPT opening already at 10 μM in heart mitochondria.

Pharmacological and molecular studies have identified ANT as a component of the permeability transition pore (Le

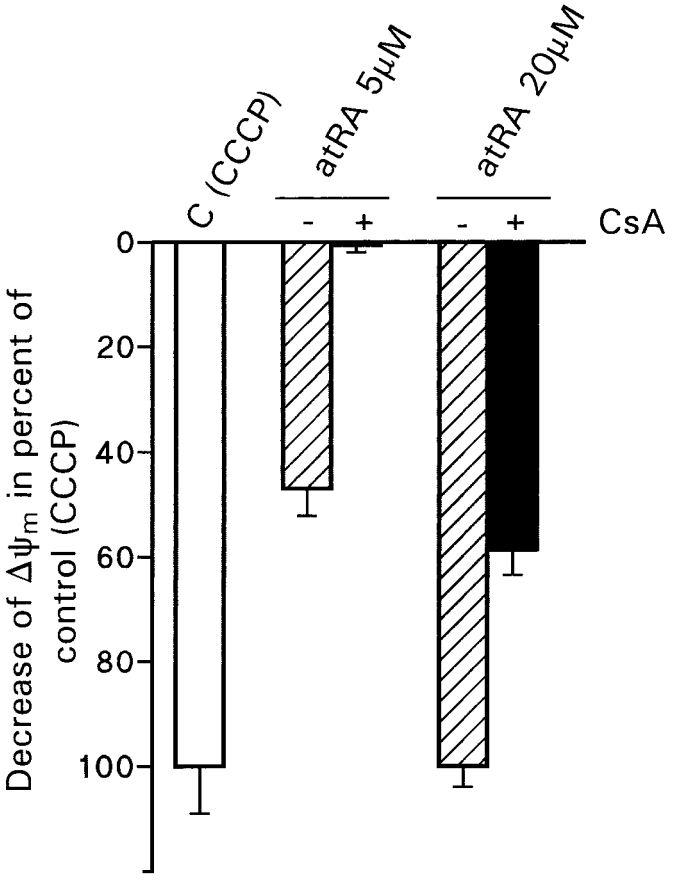

Fig. 7. Effects of atRA on mitochondrial membrane potential in HeLa cells. HeLa cells were recollected 12 h after treatment with 5 and 20 μM atRA and in presence or absence of CsA (5 μM). The cells were loaded with rhodamine 123 and cellular fluorescence was measured as described under *Materials and Methods*. Results are expressed as a decrease of $\Delta\Psi_m$ in percentage of control cells treated with CCCP (10 μM). Results showed the mean ± S.E.M of two independent experiments.

Quoc and Le Quoc, 1988; Marzo et al., 1998). However, the specific mechanism of action of retinoic acids on the induction of permeability transition is not clear. Experiments carried out on yeast demonstrate that mitochondrial proteins that belong to the same protein family of ANT, such as UCP1 and UCP2, are regulated directly by retinoic acids (Rial et al., 1999). Consequently, in this study, we examined whether retinoic acids were putative ANT regulators in heart and liver mitochondria.

Our results are the first demonstration that retinoic acids inhibit ATP/ADP translocation both in heart and liver mitochondria. The inhibition of ANT by atRA is more effective than by 9cisRA in both heart and liver mitochondria. These differences in adenylate transport inhibition could be caused by distinct ANT affinities to retinoic acids. Because ANT1 is the isoform prevailing in adult heart mitochondria (Doerner et al., 1997) and ANT2 is the isoform found in adult liver (Grado et al., 1998), our results suggest that both isoforms can be regulated by retinoids.

Kinetic analysis revealed that atRA inhibits ANT activity uncompetitively at 5 nmol/mg of protein. This kind of inhibition is mediated by the formation of a ternary complex, carrier-ATP/ADP-atRA. However, when isolated mitochondria were incubated with 20 nmol of atRA/mg of protein, the inhibition of ANT activity was changed to mixed-type. The concentration of atRA that inhibits ANT (20 nmol/mg of protein) was of the same order of magnitude as that used for membrane potential experiments in heart mitochondria. In particular it corresponds to 40 μM atRA and the decrease in membrane potential and MPT opening was detected between 20 and 30 μM (Fig. 2B). The transition from uncompetitive to mixed-type inhibition can be attributed to this effect on membrane potential, because similar results were observed when the effects of uncouplers like carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone on adenine nucleotide transport in submitochondrial particles were studied (Lauquin et al., 1977).

atRA and 9cisRA can thus be considered ANT modulators that inhibit the carrier when ATP or ADP are already bound. Previous studies carried out with specific ANT inhibitors showed various kinetic effects: ATRAC acts as a competitive inhibitor, CATR is noncompetitive (Lauquin and Vignais, 1976), and BA is uncompetitive in the presence of ADP (Brandolin et al., 1980). All of these inhibitors were thought to induce conformational changes in ANT and regulate MPT opening. Because atRA and 9cisRA, like ATRAC or CATR, modulate ANT and MPT opening, these retinoic acids could stabilize the carrier in the cytosol-facing state, which is compatible with MPT opening. Further studies will be needed before we know whether atRA and 9cisRA induce changes in ANT conformation and what association they have with MPT opening.

To gain insight into the interaction of atRA and ANT, we photolabeled heart mitochondrial protein with [^{3}H]atRA, because atRA binds covalently to proteins under UV light exposure (Bernstein et al., 1995). The comparison of the ANT amino acid sequence with a specific amino acid motif related to the atRA binding site (Chen and Radominska-Pandya, 2000), does not allow determination of the existence of a putative amino acid sequence related to the atRA binding site. Importantly, autoradiography of the electrophoresed proteins revealed the labeling of only a few mitochondrial proteins. We observed that the atRA binding of a 31-kDa protein was prevented by CATR, a specific ANT ligand, and nonradioactive atRA in a concentration-dependent manner. These results demonstrate the existence of a specific atRA-binding site in ANT in the presence of ADP and ATP. Furthermore, we showed that palmitic acid was unable to compete with atRA, which indicated either that the binding site for retinoic acid is different from the binding site of PA or the affinity for a common binding site is highly different.

Under normal conditions, atRA are present in the plasma at nanomolar concentrations (De Luca et al., 1994), and atRA is considered the most prevalent form of vitamin A in most tissues (Steele et al., 1990) and the main retinoid used in cancer therapy (Benner et al., 1995). Our results show that, in an in vitro system, atRA binds to ANT with high affinity at concentrations above 10 μM (Fig. 6) and inhibits their activity at concentrations above 5 nmol/mg of protein (10 μM atRA). This atRA concentration is higher than normal atRA levels in plasma or animal tissues. However, there are two arguments in favor of biological action of atRA through ANT binding and inhibition and MPT opening.

First, atRA does not exist in the cell in free form but is bound to proteins as cellular retinoic acid binding protein (CRABP) (Ong and Chytil 1978). The existence of a CRABP associated with mitochondria that binds and keeps retinoic acid in the mitochondria has been described previously (Ruff and Ong, 2000). This mitochondrial CRABP could explain how retinoic acids could concentrate and regulate ANT in the mitochondrial compartment in vivo.

Second, the synergic mechanism between Ca^{2+} and atRA, could justify a biological role for atRA. Ca^{2+} is always present in cells, and atRA could regulate ANT and MPT at concentrations near physiological conditions. In this sense, we have demonstrated that atRA induces MPT opening in intact cells, because it produces a CsA-sensitive drop in mitochondrial membrane potential. Interestingly, the concentrations required of atRA for producing the effect in intact cells are below those required with isolated mitochondria, supporting the above-mentioned view that intact cells can be more sensitive to atRA than isolated mitochondria.

Our study, along with others (Rial et al., 1999; Radominska-Pandya et al., 2000), suggests that specific interactions among retinoids and proteins, such as PKC, UCPs, and ANT, which are different from nuclear receptors, take place. Thus, the extra-nuclear action of retinoids seems to be a more general and important phenomenon and to have physiological and pharmacological relevance.

The disruption of mitochondrial ATP/ADP exchange is among the earliest identified events that may initiate apoptosis (Vander Heiden et al., 1999). The drop in cytosolic ATP levels caused by impaired mitochondrial ATP/ADP exchange could stimulate metabolic pathways related to cytosolic acidification in the aerobic cell and initiate apoptosis in these cells (Vander Heiden et al., 1999). The discovery of a decreased mitochondrial adenylate transport caused by retinoic acids could be an important event related to apoptosis in the cell.

In summary, we report that atRA binds to and inhibits ANT protein. Moreover, retinoic acids regulate mitochondrial membrane potential and MPT opening in isolated heart mitochondria and in intact cells. Retinoic acids and synthetic analogs have also been shown to be therapeutically effective

in the treatment of cancer and in apoptosis induction. By using natural retinoids to bind and to alter ANT activity directly, mitochondrial energy processes and apoptosis induction could be controlled.

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