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Induction of the Mitochondrial Permeability Transition *in Vitro* by Short-Chain Carboxylic Acids

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We recently reported that acrylic acid (AA) induces the MPT *in vitro,* which we suggested might be a critical event in the acute inflammatory and hyperplastic response of the olfactory epithelium. The purpose of the present investigation was to determine if induction of the MPT is a general response to short-chain carboxylic acids or if there are critical physical chemical parameters for this response. Freshly isolated rat liver mitochondria were incubated in the presence of varying concentrations of selected carboxylic acids. All of the acids that we tested caused a concentration-dependent induction of the MPT, which was blocked by cyclosporine A. Although the C4 carboxylic acids were slightly more potent than the C5 acids, there was no correlation with the degree of saturation, the octanol/water coefficient (log P), or the dissociation constant (pK_a) of the acids that we tested. We conclude that induction of the MPT *in vitro* is a general response to short-chain carboxylic acids having a pK_a of 4 to 5.

Key Words: **liver mitochondria; permeability transition; carboxylic acids; cyclosporine A.**

Short-chain carboxylic acids, which include acrylic acid (AA), propionic acid (PpA), crotonic acid (CA), pentanoic acid (PA), glutaconic acid (GA), glutaric acid (GtA), and pentenoic acid (PeA) have a wide range of commercial application. Acrylic acid is used in the synthesis of esters essential in the production of polymers for assorted paints, adhesives, plastics, and coatings. Propionic acid is a popular antifungal agent in food products. Pentanoic acid is an intermediate in the preparation of perfumes and crotonic acid is extensively used in paper and rubber industry.

Most of these acids produce localized reactions ranging from irritation of the skin and respiratory tract to an overt chemical burn at the site of contact. We recently reported that AA induces the glutathione-independent mitochondrial permeability transition (MPT) *in vitro* (1), which may be responsible for the inflammatory and hyperplastic effects of the chemical. Other acids that have been shown to alter mitochondrial function include clofibrate, 3- nitropropionic acid, fibric acids, salicylic acid, and 5,6-dichloro-4-thia-5-hexenoic acid (2–5).

The observation of induction of MPT by short-chain carboxylic acids is highly significant since MPT has several metabolic consequences, such as uncoupling of oxidative phosphorylation, disturbance of redox regulation, and stimulation of apoptosis (6, 7). The objective of the present investigation was to determine if all short chain carboxylic acids induce MPT and what are the minimum physicochemical requirements. We selected several short chain carboxylic acids with different carbon lengths, degrees of saturation, and number of carboxylic groups to study the structural requirements of short chain carboxylic acids in inducing the MPT. The selected acids include acrylic acid (AA), propionic acid (PpA), crotonic acid (CA), pentanoic acid (PA), glutaconic acid (GA), glutaric acid (GtA), and pentenoic acid (PeA).

MATERIALS AND METHODS

Chemicals. Cyclosporine A was a generous gift from Sandoz Pharmaceutical Corp. (East Hanover, NJ). Acrylic (97% purity) and glutaric acids were purchased from Aldrich Chemical Company Inc. (Milwaukee, WI). Propionic, crotonic, glutaconic, pentanoic, and pentenoic acids were purchased from Fluka (Milwaukee, WI). All other chemicals were purchased at the highest purity available from Sigma Chemical Co. (St. Louis, MO). pKa and logP values for crotonic, acrylic, pentanoic, and propionic acids were obtained from the referred chemical suppliers. The corresponding values for glutaric, glutaconic and pentenoic acids were kindly provided by the U.S. EPA.

Isolation of rat liver mitochondria. Hepatic mitochondria were isolated from adult male Sprague–Dawley rats (200–300*g*) by differential centrifugation (8, 9). Rats were purchased from *Harlan*

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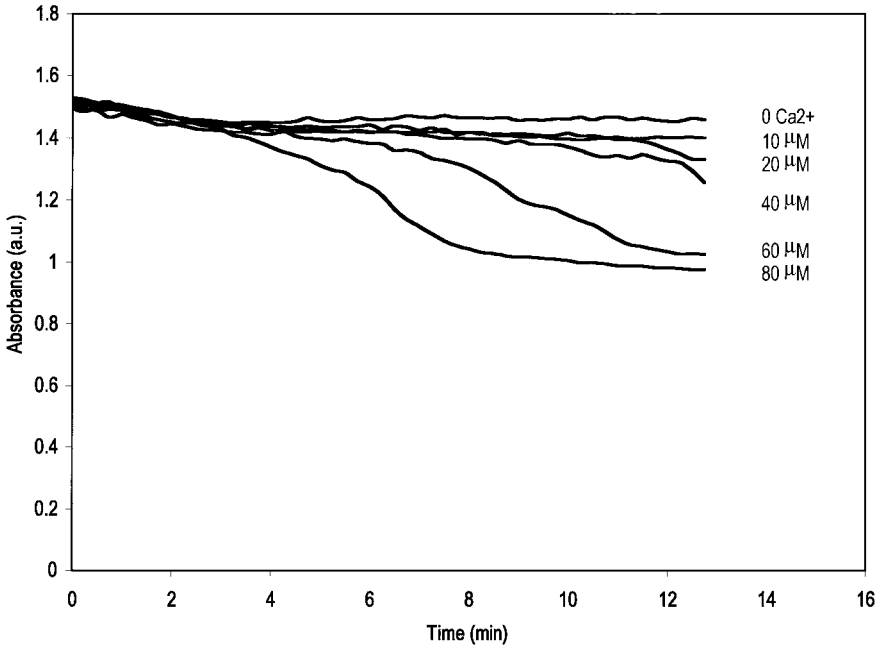

FIG. 1. Calcium-dependent induction of the mitochondrial permeability transition (MPT). Rat liver mitochondria were incubated at 0.5 mg protein/ml in 200 mM sucrose–10 mM Tris–MOPS (pH 7.4)–1 mM KH_2PO_4–10 μM EGTA supplemented with 2 μM rotenone and 1 μg/ml oligomycin. The reaction was stirred continuously and the temperature maintained at 30°C. The mitochondria were energized with 5 mM succinate for 2 min before adding $CaCl_2$ at the indicated concentrations. Light scattering was monitored continuously at 540 nm and served as the basis for assessing changes in mitochondrial volume. The traces depict typical experiments performed on the same day and are representative of three to six repetitions using different preparations.

Sprague-Dawley (Madison, WI) and acclimated in an AAALAC accredited, climate controlled (21°C; 14/10 h light cycle) animal care facility for at least 3 days prior to the experiment. Animals were killed by decapitation and the liver was quickly homogenized (Teflon: glass pestle) in 20 volume (ml/g) of cold 200 mM mannitol–10 mM sucrose–1 mM EGTA–5 mM Hepes (pH 7.4). The homogenate was centrifuged for 10 min at 900*g* and 4°C, and the mitochondria recovered by centrifugation at 10,000*g* for 10 min. The mitochondrial final pellet was resuspended in the same buffer to a concentration of 70–90 mg/ml.

Mitochondrial swelling. The extent of mitochondrial swelling was estimated spectrophotometrically at 540 nm (9, 10). Mitochondria were suspended at 0.5 mg protein/ml in 200 mM sucrose-10 mM Tris MOPS (pH 7.4)-1 mM KH_2PO_4-10 μM EGTA supplemented with 2 μM rotenone and 1 μg/ml oligomycin. The reaction temperature was maintained at 30°C. The mitochondria were energized with 5 mM succinate for 2 min before adding $CaCl_2$ at varying concentrations (for calcium-dependent induction of MPT). Where indicated, the different carboxylic acids were added 2 min after addition of 30 μM $CaCl_2$. None of these reagents interfered with the spectrophotometric analysis.

Data analysis (method of calculation of EC_{50}). The median effective concentrations, EC_{50}s, for the test compounds were calculated graphically from their respective log dose-response curves. The EC_{50} was defined as the concentration which induced swelling in 50% of the mitochondrial population at a specific time. The specific time was arbitrarily defined to be the time when the highest concentration (1 mM) of the test compound produced maximum swelling. The extent of swelling of control mitochondria was subtracted from that obtained from the highest concentration of test compound. The extent of swelling induced by various concentrations of test compounds were expressed as the percentage of maximum swelling. Subsequently, the percent swelling was plotted against the log of the respective dose to get the log dose response curves and the EC_{50}s were calculated graphically.

RESULTS AND DISCUSSION

Mitochondria have a finite capacity to accumulate calcium before undergoing the calcium dependent mitochondrial permeability transition. This is illustrated in Fig. 1 where calcium was added at increasing concentrations to mitochondria that had been energized with succinate in the presence of 2 μM rotenone. Minimum induction of the permeability transition was observed with 10 μM $CaCl_2$ (20 nmol/mg protein). The rate, but not the extent of swelling, was dose dependent up to 80 μM where 100% of the mitochondria were swollen within 8 min. From these results, a calcium concentration of 30 μM (60 nmol/mg protein) was chosen as the standard against which to assess the effect of the different carboxylic acids.

Adding 1 mM of acrylic acid (AA), crotonic acid (CA), pentanoic acid (PA), propionic acid (PpA), glutaconic acid (GA), and glutaric acid (GtA), 2 min after the addition of 30 μM $CaCl_2$, to succinate energized rat liver mitochondria caused an increase in the rate of mitochondrial swelling (Figs. 2 and 3). Ppa, GA, and GtA are less effective in MPT induction since they required 25 min to exert their effects while AA, CA, and PA are effective at 10 min. The rate of swelling was

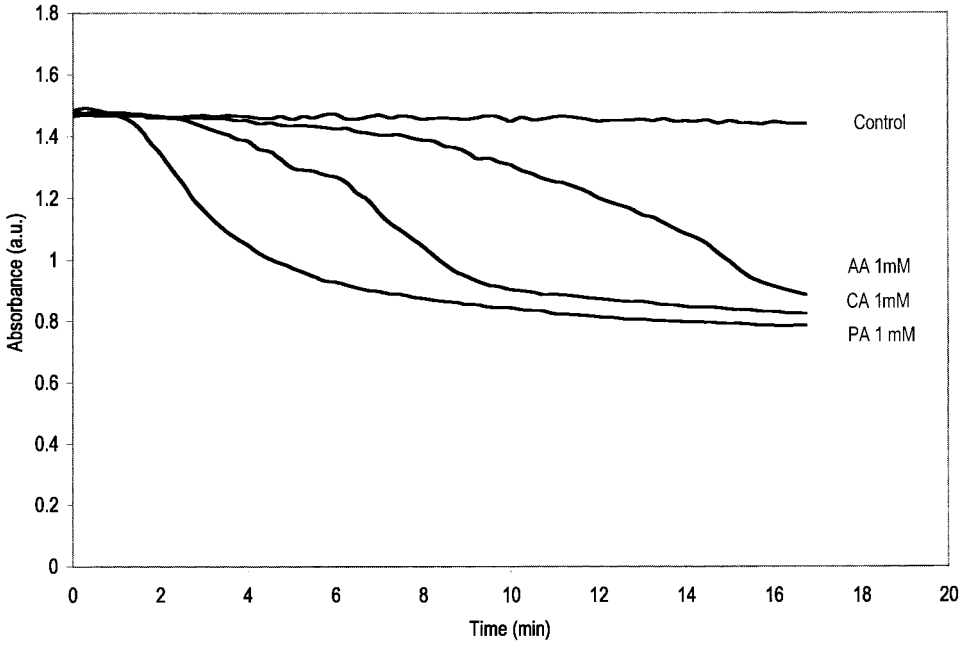

FIG. 2. Induction of the mitochondrial permeability transition (MPT) by acrylic acid (AA), crotonic acid (CA), and pentanoic acid (PA). The reaction conditions were identical to those reported in the legend to Fig. 1, except that the indicated acid was added at 1 mM final concentration 1 min prior to adding 30 μM calcium to initiate the reaction. The traces depict typical experiments performed on the same day and are representative of three to six repetitions using different preparations.

highest with pentanoic acid (PA). Crotonic acid (CA) was almost as effective as the standard pore inducer acrylic acid (AA) in stimulating the rate of swelling. Once initiated, the mitochondria in suspension underwent the same magnitude of volume change, regardless of the nature of test compound. This suggests that the induction of mitochondrial swelling was complete and that the effect of the test compounds was to alter

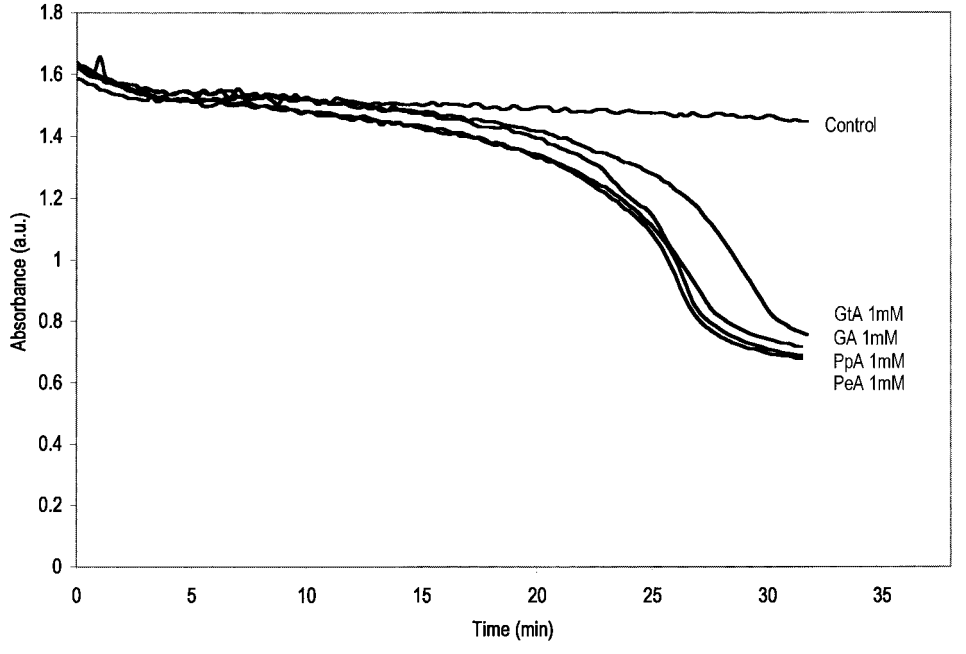

FIG. 3. Induction of the mitochondrial permeability transition (MPT) by glutaric acid (GtA), glutaconic acid (GA), propionic acid (PpA), and pentenoic acid (PeA). The reaction conditions were identical to those reported in the legend to Fig. 2. The traces depict typical experiments performed on the same day and are representative of three to six repetitions using different preparations.

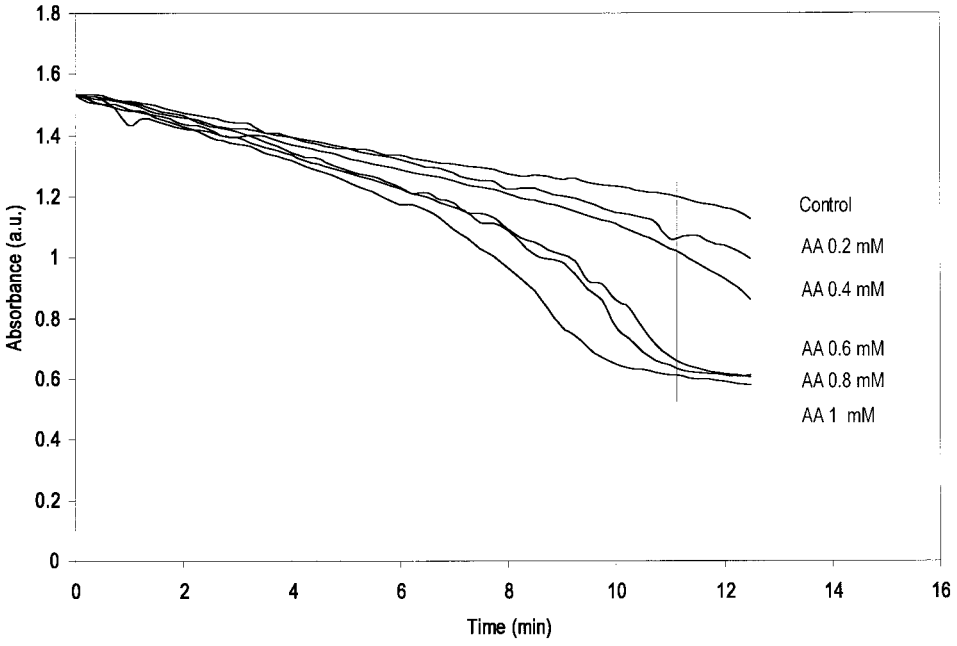

FIG. 4. Dose-dependent induction of the mitochondrial permeability transition (MPT) by acrylic acid, illustrating the method of estimating the EC_{50}. The reaction conditions were identical to those reported in the legends to Figs. 2 and 3. The vertical line indicates the point in time when the fractional swelling was recorded for each concentration of acrylic acid (AA). The traces depict typical experiments performed on the same day and are representative of three repetitions using different preparations.

the time-constant governing activation of the permeability transition pore opening.

The kinetics of induction of swelling by all the carboxylic acids under study was found to be dose dependent (Fig. 4). Addition of 0.85 μM of CyA just prior to the carboxylic acids completely prevented the calcium-induced mitochondrial swelling, while changing the order of addition of test compounds relative to calcium did not (data not shown). These two observations suggest that the change in the volume of mitochondria was due to pore induction and not due to inhibition of respiration.

The EC_{50}s were estimated as described under Materials and Methods section as illustrated in Fig. 4 and summarized in Table 1. The highest estimated EC_{50} value was for crotonic acid (CA), followed respectively by acrylic acid (AA), glutaric acid (GtA), glutaconic acid (GA), pentenoic acid (PeA), pentanoic acid (PA), and propionic acid (PpA).

Although the C4 carboxylic acids were slightly more potent than the C5 acids, there was no apparent correlation between potency and any of several physical chemical parameters examined, including carbon number, degree of saturation, octanol/water partition coefficient (log P), or the dissociation constant (pK_a) of the acids tested. We conclude that induction of the MPT, which may be important in mediating the toxic tissue damage, is a general response to weak carboxylic acids having a pK_a of 4 to 5. Within this series of acids,

TABLE 1

Comparison of Physical, Chemical, and Biological Activities of the Series of Carboxylic Acids

Acid	Formula	EC_{50} (μM)	pK_a[a]	Log P[a]
Crotonic acid	$CH_3CH{=}CH\text{-}COOH$	706.7 ± 70.2	4.61	0.69
Acrylic acid	CH2═CH-COOH	526.7 ± 122.2	4.27	0.16
Glutaric acid	$HOOC\text{-}CH_2CH_2CH_2\text{-}COOH$	500.0 ± 141.4	4.22	−0.63
Glutaconic acid	$HOOC\text{-}CH{=}CHCH_2\text{-}COOH$	470.3 ± 123.5	4.20	−0.77
Pentenoic acid	$CH_3CH{=}CHCH_2\text{-}COOH$	219.5 ± 27.6	4.51	0.81
Pentanoic acid	$CH_3CH_2CH_2CH_2\text{-}COOH$	153.3 ± 15.3	4.82	1.35
Propionic acid	$CH_3CH_2\text{-}COOH$	146.8 ± 25.3	4.74	0.30

Note. The EC_{50} values reflect the means ± SD for 3 separate determinations.

[a] pK_a and log P (octanol:water partition coefficient) values were calculated using the ASTER (Assessment Tools for the Evaluation of Risk) System of the U.S. Environmental Protection Agency.

differences in potency of induction of the MPT cannot be attributed to physical chemical characteristics. Accordingly, induction of the mitochondrial permeability transition *in vitro* by short chain carboxylic acids does not appear to be simply a thermodynamic phenomenon, but rather a carrier or transporter-mediated process.

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