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ACTION OF THE NONSTEROIDAL ANTI-INFLAMMATORY AGENT, FLUFENAMIC ACID, ON CALCIUM MOVEMENTS IN ISOLATED MITOCHONDRIA

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Abstract—The anti-inflammatory agent flufenamic acid was found to inhibit calcium uptake in isolated mitochondria at low concentrations (IC_{50} = 7.2 μM). Similar concentrations were required to promote the release of calcium from mitochondria preloaded with the cation (EC_{50} = 3.5 μM). Identical actions were found with diflunisal, mefenanamic acid and 2,4-dinitrophenol. It was concluded that flufenamic acid was affecting calcium movements across the mitochondrial membrane by virtue of its ability to uncouple oxidative phosphorylation.

The movement of ions across the mitochondrial membrane is important in the control of cellular reactions; for example, mitochondria play a role in the maintenance of calcium levels in the cytosol of a variety of cell types [1]. The uptake of calcium, and its subsequent retention by the mitochondrion, is dependent on the presence of an intact oxidative phosphorylation system [2]. Since we have shown that a number of anti-inflammatory agents are capable of inhibiting mitochondrial ATP synthesis [3], we have examined the effect of flufenamic acid, which was the most potent compound in our study, on the uptake of calcium ions by isolated mitochondria.

MATERIALS AND METHODS

Mitochondria. Tightly-coupled mitochondria were prepared from the livers of rats by the method of Chappell and Hansford [4].

Calcium-stimulated respiration. Calcium-stimulated respiration was measured using a Clark-type oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) [5].

Calcium uptake. Mitochondrial calcium uptake was measured by the method of Reed and Bygrave [6, 7] by following the movement of $^{45}Ca^{2+}$. Mitochondria (2 mg protein) were added to 0.85 ml reaction medium which contained 0.25 M sucrose, 3.4 mM Tris–HCl buffer, pH 7.4, 2 mM phosphate buffer, pH 7.4 and 5 mM sodium succinate. The test compounds were included in the reaction medium at this stage. After 30 sec, the reaction was initiated by the addition of 200 nmole $^{45}Ca^{2+}$ (total activity 2 kBq). The final volume was 1 ml and the temperature was 45°. At 10 sec intervals, samples (0.1 ml) were removed onto 0.45 μm ultrafine Millipore membranes and vacuum dried. Dry membranes were washed once with sucrose–Tris buffer, redried and placed in vials containing 3 ml NE260 scintillation fluid plus 20 mg ascorbic acid. Samples were counted, after 24 hr dark adaptation, in a Beckman LS7500 liquid scintillation counter.

Calcium release. Mitochondria were allowed to accumulate $^{45}Ca^{2+}$ for 5 min, under the conditions described for calcium uptake. Calcium release was initated by the addition of the test compounds, in the presence of ruthenium red (1 μM), to prevent any further calcium uptake, and at 20-sec intervals samples (0.1 ml) were removed for $^{45}Ca^{2+}$ analysis.

Protein. Protein was determined by the method of Gornall *et al.* [8], after solubilisation of the mitochondrial pellet with sodium deoxycholate (0.16% w/v); bovine serum albumin was used as the standard.

Chemicals. Analytical grade laboratory chemicals and biochemicals were purchased from British Drug Houses (Poole, U.K.) and Sigma Chemical Co. (St. Louis, MO, U.S.A.). $^{45}CaCl_2$ was purchased from Amersham International (Amersham, U.K.), scintillation fluid (Micellar Scintillator NE260) from Nuclear Enterprises, (Edinburgh, U.K.) and Millipore filters from Millepor U.K. (Park Royal, London, U.K.). Difluisal was provided by Merck, Sharp and Dohme (Hoddeston, Herts., U.K.), and flufenamic and mefenamic acids by Parke-Davis (Pontepool, U.K.). Insoluble anti-inflammatory agents were added to the reaction media as solutions in dimethylformamide; controls with equivalent amounts of solvent showed that it had no effect on the reactions under consideration.

RESULTS

Calcium-stimulated respiration

When calcium chloride (0.1–335 μM) was added to tightly coupled rat hepatic mitochondria there was a concentration-dependent stimulation of State 4 respiration (ADP absent, substrate and oxygen in excess), increasing the respiratory rate from 26.7 ± 3 ng atoms O_2 min^{-1}mg of $protein^{-1}$ (N = 5) to a maximum value of 234.6 ± 9.3 ng atoms

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Table 1. Effect of flufenamic acid on State 4 and calcium stimulated respirations

Compound	State 4 respiration Succinate EC_{50} (μM)	Calcium stimulated respiration Succinate IC_{50} (μM)	Calcium stimulated respiration Glutamate + malate IC_{50} (μM)
Flufenamic acid	8.4 ± 2.1	13.2 ± 7.0	16.8 ± 4.3
Diflunisal	14.1 ± 3.5	26.6 ± 12.3	37.2 ± 6.2
Mefenamic acid	38.6 ± 4.2	68.4 ± 21.6	172.3 ± 36.2
Ruthenium red	—	4.4 ± 1.0	6.5 ± 1.5
2,4-Dinitrophenol	10.5 ± 1.2	18.6 ± 6.9	19.5 ± 4.2

The oxygen electrode chamber contained, 675 μmol sucrose, 9.2 μmol Tris–HCl buffer, pH 7.4, 10 μmol potassium phosphate buffer, pH 7.4 and 15 μmol sodium succinate (or 10 μmol sodium glutamate plus 10 μmol sodium malate). Rat liver mitochondria (10 mg of protein) were added to the chamber, followed 2 min later by 200 nmol calcium chloride. The temperature was 37° and the final volume was 3 ml. IC_{50} value refers to the concentration required to reduce the maximal effect by 50%. Values are the mean ± SE mean of five different experiments.

$O_2\ min^{-1}mg$ of $protein^{-1}$ ($N = 5$) at a concentration of 335 μM. Higher concentrations of calcium (>335 μM) caused a concentration-dependent inhibition of mitochondrial State 4 respiration and a loss of respiratory control. These experiments confirmed that using "limited-loading" concentrations of calcium (<0.3 mM) respiration returned to the initial State 4 rate without impairment of oxidative phosphorylation, respiratory control or mitochondrial function. Therefore all subsequent experiments were performed using 200 nmol calcium to stimulate State 4 respiration.

Using tightly-coupled mitochondrial preparations, the stimulation respiration which accompanied the uptake of calcium (100 nmol) was inhibited by flufenamic acid (2.5–100 μM). Table 1 shows that the inhibitory effect was independent of the respiratory substrate employed. Similar effects were found with the anti-inflammatory compounds, diflunisal (10–100 μM) and mefenamic acid (10–400 μM), with the calcium uptake inhibitor, ruthenium red (1–20 μM), and with the uncoupling agent (10–50 μM), 2–4-dinitrophenol (Table 1). Comparison of the IC_{50} values for calcium-stimulated respiration and EC_{50} values for the stimulation State 4 respiration indicate the prime action flufenamic acid is on ATP synthesis (Table 1).

Calcium uptake

Under the conditions employed, approximately 30

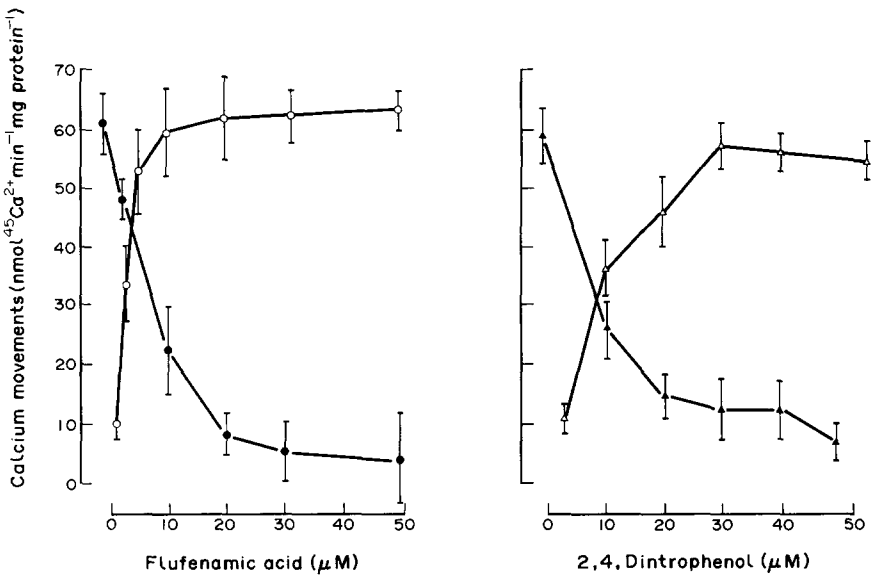

Fig. 1. $^{45}Ca^{2+}$ uptake and release were measured as described in the Materials and Methods section. The incubation mixture contained 250 μmol sucrose, 34 μmol Tris–HCl buffer, pH 7.4, 5 μmol potassium dihydrogen phosphate, 5 μmol sodium succinate and mitochondria (2 mg of protein). The final volume was 1 ml and the temperature 45°. After a 30 sec pre-incubation the reaction was initiated by the addition of 200 nmol $^{45}Ca^{2+}$. For uptake studies 2.5–50 nmol flufenamic acid (○—○) or 10–50 nmol 2,4-dinitrophenol (△—△) were added prior to the addition of $^{45}Ca^{2+}$. While for the release studies either 2.5–50 nmol flufenamic acid (●—●) or 10–50 nmol 2,4-dinitrophenol (▲—▲) were added after the mitochondria had been allowed to accumulate a calcium load. Values are the mean ± SE mean of five different experiments.

Table 2. Effect of flufenamic acid and other compounds on mitochondrial calcium transport

Compound	Uptake IC_{50} (μM)	Release EC_{50} (μM)
Flufenamic acid	7.2 ± 1.2	3.5 ± 1.8
Diflunisal	8.5 ± 1.3	4.2 ± 1.3
Mefenamic acid	68.4 ± 12.2	18.5 ± 4.2
Ruthenium red	0.16 ± 0.02	—
2,4-Dinitrophenol	10.2 ± 1.6	5.8 ± 1.8

$^{45}Ca^{2+}$ uptake and release were measured as described in the Methods section. EC_{50} value refers to the concentration of compound required to stimulate release by 50%. Values are the mean ± SE mean of five different experiments.

per cent of the uptake of calcium could be accounted for by energy-independent binding to the mitochondrial membrane. This binding was insensitive to the action of respiratory chain inhibitors. After determination of the extent of energy-independent binding at the start of each experiment, a correction was made by subtracting the value obtained from the experimentally observed values of total uptake, to give a value for energy-dependent uptake. In control experiments, all the available calcium was taken up by isolated mitochondria within 1–2 min. Flufenamic acid (2.5–50 μM) was found to inhibit energy-dependent calcium uptake in a concentration-dependent manner (Fig. 1), a similar concentration effect was observed when 2,4-dinitrophenol (10–50 μM) replaced flufenamic acid (Fig. 1). Table 2 shows the IC_{50} values for the anti-inflammatory, flufenamic acid, diflunisal and mefenamic acid, the result obtained with ruthenium red is also included for comparison.

Calcium release

Mitochondria which had been allowed to accumulate calcium were found to release the cation into the incubation medium. The release was slow, 11.4 ± 2.1 nmol $^{45}Ca^{2+}$ min^{-1}mg mitochondrial $protein^{-1}$ (N = 5), when compared to the rate of uptake, 65.2 ± 5.1 nmol $^{45}Ca^{2+}$ min^{-1}mg of mitochondrial $protein^{-1}$ (N = 5). Figure 1 shows that in the presence of flufenamic acid (20 μM), the rate of release of calcium was increased to 62.4 ± 5.4 nmol $^{45}Ca^{2+}$ min^{-1}mg of mitochondrial $protein^{-1}$ (N = 5), this concentration-dependent stimulation of release resulted in an EC_{50} value of 3.5 ± 1.8 μM (N = 5) for flufenamic acid. Replacement of the anti-inflammatory agent with 2,4-dinitrophenol (10–50 μM) produced a similar effect (Fig. 1). The EC_{50} values for the effect of the anti-inflammatory agents and the uncoupling agent on calcium release are included in Table 2.

DISCUSSION

Many aspirin-like compounds have been shown to interfere with mitochondrial energy production [9–11]. In particular, we have found that the non-steroidal anti-inflammatory agents, diflunisal, flufenamic acid and mefenamic acid act as uncouplers of oxidative phosphorylation in mitochondria isolated from liver [3]. Compounds which have the ability to uncouple oxidative phosphorylation should also have the ability to modify calcium movements across the mitochondrial membrane [12].

With respect to calcium transport, work in the early 1960s [2] suggested that mitochondria have a large capacity for the cation and thus provide an intracellular sink for calcium when cytosolic levels increase. More recent studies relate the kinetics of calcium transport to mitochondrial energy transduction, with particular reference to chemiosmotic properties [13, 14]. These studies indicate that the mitochondrial inner membrane potential provides the driving force for calcium accumulation, while also revealing the relatively low affinity of the transport system for external calcium.

In terms of calcium distribution across the mitochondrial membrane this is maintained by two distinct transport systems (a) an energy-dependent uniporter for the influx of Ca^{2+} down an electrochemical gradient, and (b) a ruthenium red insensitive electro-neutral carrier, which although slower than the uniporter system is considered essential for the physiological release of Ca^{2+} from the mitochondrion [15, 16].

Evidence also indicates that in undamaged mitochondria the independent efflux pathway operates at a very slow rate, therefore under steady-state conditions efflux occurs through an independent, membrane potential during oxidative phosphorylation being effective in modulating Ca^{2+} distribution by either stimulating, or inhibiting, the efflux pathway. The present studies confirm the ability of flufenamic acid to inhibit ATP synthesis and mitochondrial calcium transport.

Several lines of evidence support the proposal that flufenamic acid interferes with calcium transport by uncoupling oxidative phosphorylation. First, flufenamic acid inhibited the stimulation of respiration elicited by calcium ions in tightly-coupled mitochondria. Second, the energy-dependent uptake of calcium was sensitive to flufenamic acid. Third, the release of calcium from mitochondria preloaded with the cation was stimulated by flufenamic acid. In addition, all three of the above properties were also exhibited by the uncoupling agent, DNP. The fact that the concentrations of flufenamic acid required to modify the above reactions were identical to those required to inhibit mitochondrial ATP synthesis [3] lends further support to the proposal.

In these studies DNP was selected because of its known ability to uncouple oxidation from phosphorylation, and not because of any anti-inflammatory activity. Initial work by Adams and Cobb in 1958 [7] showed DNP to have no anti-inflammatory properties; however, more recent studies have shown its ability to inhibit both ultraviolet erythema in guinea-pigs [18] and dextran/formation oedema in rat [19]. These anti-inflammatory properties are unlikely to be related to its uncoupling activity, as DNP is highly labile in biological systems and is rapidly metabolised in the liver to 2-amino-4-nitrophenol which has no uncoupling activity [20]. A different pattern of activities was found with the specific calcium uptake inhibitor, ruthenium red, thus excluding the possibility that flufenamic acid has a direct action on the calcium

transporters. Experiments on the anti-inflammatory agents, diflunisal and mefenamic acid showed a similar profile of activity to flufenamic acid against both oxidative phosphorylation [3] and mitochondrial calcium movements.

The order of potency with respect to energy metabolism was found to be flufenamic acid > 2,4-dinitrophenol > diflunisal > mefenamic acid, while for calcium transport the order was flufenamic acid > diflunisal > 2,4-dinitrophenol > mefenamic acid. Studies on the physicochemical properties of these compounds by Hansch and Albert [21] confirm them to be weak acids possessing similar pK_a values, flufenamic acid ($pK_a = 3.9$), 2,4-dinitrophenol ($pK_a = 4.07$) and diflunisal ($pK_a = 4.0$). In terms of lipophilicity log P values show the compounds to be lipophilic with a rank order of, flufenamic acid (log $P = 2.74$) > 2,4-dinitrophenol (log $P = 1.50$ > diflunisal (log $P = 0.67$). These physicochemical characteristics are therefore consistent with the ability of the above compounds to act as uncoupling agents and modifiers of mitochondrial calcium transport.

Since the early 1970s evidence has accumulated, suggesting a possible role for calcium ions in acute inflammatory processes [22, 23] and the onset of bronchial smooth muscle hyper-reactivity [24]. Therefore the anti-inflammatory or toxicological properties on non-steroidal anti-inflammatory agents may be due, at least in part, to their ability to alter the distribution of intracellular calcium.

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