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Inhibition and Uncoupling of Oxidative Phosphorylation by Nonsteroidal Anti-inflammatory Drugs

STUDY IN MITOCHONDRIA, SUBMITOCHONDRIAL PARTICLES, CELLS, AND WHOLE HEART*

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ABSTRACT. The effects of the anti-inflammatory drugs diclofenac, piroxicam, indomethacin, naproxen, nabumetone, nimesulide, and meloxicam on mitochondrial respiration, ATP synthesis, and membrane potential were determined. Except for nabumetone and naproxen, the other drugs stimulated basal and uncoupled respiration, inhibited ATP synthesis, and collapsed membrane potential in mitochondria incubated in the presence of either glutamate + malate or succinate. Plots of membrane potential versus ATP synthesis (or respiration) showed proportional variations in both parameters, induced by different concentrations of nimesulide, meloxicam, piroxicam, or indomethacin, but not by diclofenac. The activity of the adenine nucleotide translocase was blocked by diclofenac and nimesulide; diclofenac also slightly inhibited mitochondrial ATPase activity. Naproxen did not affect any of the mitochondrial parameters measured. Nabumetone inhibited respiration, ATP synthesis, and membrane potential in the presence of glutamate + malate, but not with succinate. NADH oxidation in submitochondrial particles also was inhibited by nabumetone. Nabumetone inhibited O_2 uptake in intact cells and in whole heart, whereas the other five drugs stimulated respiration. These observations revealed that *in situ* mitochondria are an accessible target. Except for diclofenac, a negative inotropic effect on cardiac contractility was induced by the drugs. The data indicated that nimesulide, meloxicam, piroxicam, and indomethacin behaved as mitochondrial uncouplers, whereas nabumetone exerted a specific inhibition of site 1 of the respiratory chain. Diclofenac was an uncoupler too, but it also affected the adenine nucleotide translocase and the H^+-ATPase. BIOCHEM PHARMACOL **57**;7:743–752, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. NSAIDs; uncoupling; oxidative phosphorylation; mitochondria

It is known that, among other alterations, the chronic use of NSAIDs** may induce gastrointestinal bleeding [1, 2]. However, the biochemical mechanisms underlying the action of NSAIDs on gastrointestinal function have not been clearly elucidated. Current thinking suggests that inhibition of COX I is involved directly in the side-effects of NSAIDs, while the therapeutically desirable effects come from inhibition of COX II [3, 4]. COX I synthesizes prostaglandins, which have transient effects such as pain and inflammation, and long-term effects such as protection of the gastrointestinal tract against acidity [4, 5]. Nevertheless, no clear correlation has been found between the inhibition by NSAIDs of COX I activity and damaging effects on gastrointestinal function [6]. In this regard, perturbation of mitochondrial energy metabolism by NSAIDs has been suggested to be involved in the adverse side-effects of NSAIDs [7].

Several reports indicate that many NSAIDs uncouple mitochondrial energy metabolism [8–12], i.e. inhibition of oxidative phosphorylation through the collapse of the H^+ electrochemical gradient with the consequent stimulation of electron transport and O_2 consumption by the respiratory chain [13]. However, in these works [8–12] only the effects of NSAIDs on the respiratory rate, H^+ gradient, or

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** *Abbreviations*: NSAIDs, nonsteroidal anti-inflammatory drugs; COX, cyclooxygenase; TPP^+, tetraphenylphosphonium; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; SMP, submitochondrial particles; and 6MNA, 6-methoxy-2-naphthylacetic acid.

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ATP synthesis were measured. Since only when these parameters are assessed together can an uncoupler be unambiguously distinguished from respiratory inhibitors and activators, it is not yet evident that NSAIDs act as uncouplers. Moreover, there are at least three major types of uncouplers: (a) classic uncouplers such as DNP (2,4-dinitrophenol), FCCP [carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone], CCCP, and valinomycin (in the presence of an excess of K^+); (b) the H^+-leak inducers; and (c) the so-called decouplers, which promote inhibition of oxidative phosphorylation or stimulation of O_2 uptake with no associated collapse of the H^+ gradient [14–16].

Except for one report [11] in which stimulation of O_2 uptake by diclofenac or aspirin in perfused liver was observed, the evaluation of the uncoupling potency of NSAIDs has been carried out solely in isolated mitochondria, and no assays have been made in intact cells or organs. Therefore, to identify the mechanisms of action, a thorough evaluation of the effects of seven NSAIDs on several energy-dependent parameters in mitochondria, submitochondrial particles, cells, and organs was undertaken in this study.

MATERIALS AND METHODS

Tightly coupled rat liver mitochondria [17], tightly coupled rat liver submitochondrial particles [18], and AS-30D hepatoma cells [19] were obtained as previously described. Isolated rat hearts were perfused via the aorta according to the Langendorff method, as described previously [20]. A preparation of hog gastric microsomal fraction enriched in H^+,K^+-ATPase was made according to Rabon *et al.* [21], with the following modifications: the epithelial gastric layers were homogenized in a tissue disintegrator (Polytron), and the density-gradient purification consisted of a centrifugation at 38,000 rpm (247,000 *g*), in a Beckman SW41Ti rotor, of the crude microsomal fraction layered onto a discontinuous sucrose gradient of, from the bottom, 2 mL of 45%, 2 mL of 20%, and 3 mL of 10% sucrose (w/v).

O_2 uptake using a Clark-type O_2 electrode [17], incorporation of $^{32}P_i$ into ATP [17], distribution of [^{3}H]-TPP^+ [22], changes in the absorbance difference of safranine O at 554–520 nm [23], release of P_i from ATP [24], [^{3}H]-ADP uptake [25, 26], and determination of the pH gradient in inside-out vesicles [27] were measured as described elsewhere.

Isolated hearts prepared according to the Langendorff method were perfused with Ringer–Krebs bicarbonate buffer (pH 7.4, 37°) saturated with 95% O_2/5% CO_2 and electrically stimulated at 4.5 Hz. The pressure in the left ventricle was monitored with a latex balloon coupled to a pressure transducer, and O_2 uptake was measured from the difference in O_2 concentration, detected by an O_2 electrode, in the O_2-saturated perfused medium, before and after it had passed through the active heart.

Nimesulide (4-nitro-2-phenoxymethane-sulfoanilide) and nabumetone (4-[6-methoxy-2-naphthalenyl]-2-butanone) were provided by SmithKline Beecham Pharmaceuticals. Meloxicam (4-hydroxy-2-methyl-*N*-[5-methyl-2-thiazolyl]-2*H*-1,2-benzothiazine-3-carboxamide-1,1-dioxide) was obtained from Boehringer Ingelheim; diclofenac (2-[(2,6-dichlorophenyl)amino]-benzeneacetic acid) from Ciba-Geigy; indomethacin (1-[*p*-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid) from Merck Sharp Dohme; and piroxicam (4-hydroxy-2-methyl-3-[pyrid-2-y1-carbamoyl]-2*H*-1,2-benzothiazine-1,1-dioxide) from Pfizer. Naproxen (D-6-methoxy-α-methyl-2-naphthaleneacetic acid) was supplied by Syntex. Stock solutions in DMSO of 2 and 20 mg/mL were prepared and stored at −10°. To avoid precipitation of NSAIDs during their mixing with aqueous solutions, they were always added to media that already contained mitochondria or cells.

RESULTS

Isolated Rat Liver Mitochondria Experiments

With either glutamate + malate (G + M) or succinate (+ rotenone) as substrates, the rate of basal (state 4) respiration of rat liver mitochondria was stimulated by the acidic NSAIDs diclofenac, piroxicam, and indomethacin (Fig. 1). The non-acidic NSAID nabumetone (440 nmol/mg protein) stimulated basal respiration by 69% with succinate as substrate. However, during G + M oxidation, nabumetone (440 nmol/mg protein) induced a 62% inhibition of basal respiration ($N = 3$; $P < 0.01$; see Fig. 1).

Nimesulide and meloxicam* lack carboxylate groups and are apparently more specific COX II inhibitors than diclofenac, piroxicam, indomethacin, and naproxen [28–30]. Surprisingly, these two NSAIDs exhibited a greater potency for respiratory stimulation with either G + M or succinate as oxidizable substrates. Nimesulide induced a 6- to 8-fold stimulation of the respiratory rate at a concentration range of 40–60 nmol/mg protein; meloxicam induced a 4- to 5-fold stimulation at 50–120 nmol/mg protein (Fig. 1). Thus, nimesulide and meloxicam were the most potent NSAIDs for inducing stimulation of the basal respiratory rate.

Concentrations of NSAIDs higher than those shown in Fig. 1 did not increase respiratory rates; instead, these relatively high concentrations decreased O_2 uptake. It is noted that naproxen produced an increased respiratory rate solely at concentrations higher than 500 nmol/mg protein.

The rate of uncoupled respiration (0.3 μM CCCP; 1 mg protein/mL) was also enhanced by the seven NSAIDs with succinate as oxidizable substrate; the order of potency of the NSAIDs was identical to that observed for basal respiration (*cf.* Fig. 1). With G + M, nabumetone inhibited uncoupled respiration (63 ± 4% inhibition with 440 nmol/mg protein; $N = 3$); the other six NSAIDs further increased the uncoupled respiration (data not shown). At higher CCCP

* Engelhardt G, Meloxicam: A potent inhibitor of COX-2. 9th International Conference on Prostaglandins and Related Compounds, Florence, Italy, p. 82, 1995.

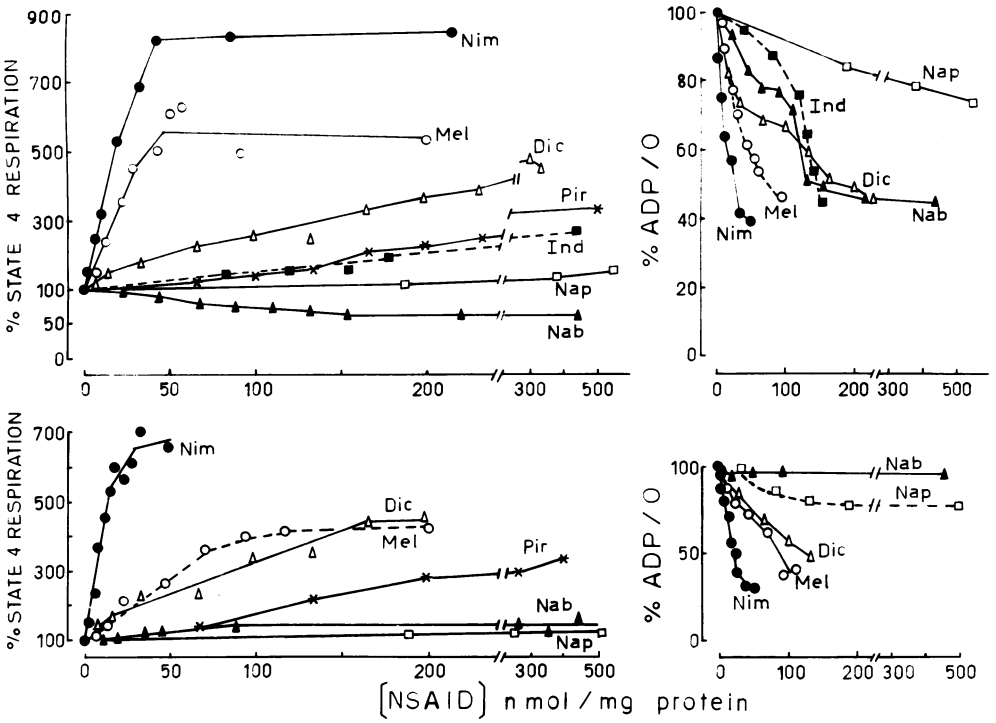

FIG. 1. Effect of several NSAIDs on the rate of basal (state 4) respiration and the ADP/O ratio in rat liver mitochondria. Mitochondria (1 mg protein/mL) were added to 1.9 mL of a medium that contained 120 mM KCl, 20 mM MOPS, 0.5 mM EGTA, 5 mM potassium-phosphate, and 5 mM glutamate + 5 mM malate (upper panels) or 10 mM succinate + 1 μM rotenone (lower panels), of pH 7.2 at 25°. After approximately 2 min, different concentrations of the indicated NSAIDs were added, and the stimulation in the rate of basal respiration was determined. Two minutes later, 600 nmol ADP was added, and the ADP-stimulated (state 3) respiration was obtained. State 3 respiration was allowed to return to basal respiration after exhaustion of added ADP. The ADP/O ratio was estimated from the extra amount of ng atoms of oxygen consumed by the addition of ADP. At 25° and 2240 m altitude, the solubility of O_2 was determined to be 210 nmol (420 ng atoms oxygen/mL). Abbreviations: Nim, nimesulide; Mel, meloxicam; Pir, piroxicam; Dic, diclofenac; Ind, indomethacin; Nap, naproxen; and Nab, nabumetone. The data shown represent the means of at least two different mitochondrial preparations. For clarity, titration curves with either piroxicam or indomethacin were omitted. The values of the rates of basal respiration in the absence of drugs were 10.5 ± 1.6 (12) and 12.4 ± 1.4 (15) ng atoms/(mg protein · min) (mean ± SEM) for glutamate + malate and succinate (+ rotenone), respectively. The control values of the ADP/O ratios were 2.2 ± 0.1 (12) and 1.59 ± 0.06 (15).

(>0.5 μM) concentrations, NSAIDs inhibited respiration. In turn, the rate of NSAID-stimulated respiration was also inhibited by CCCP concentrations higher than 0.5 μM, whereas at low concentrations (<0.5 μM), CCCP induced a further enhancement of the NSAID-stimulated respiration. Moreover, the addition of 0.5 μM CCCP also inhibited the rate of 0.3 μM CCCP-stimulated respiration. Such a dual effect of uncouplers has been documented extensively [see, for instance, Refs. 31 and 32]; the inhibitory effect of high uncoupler concentrations is related to the inhibition of substrate transport by diminution in the pH gradient.

The effect of the NSAIDs on the ADP/O ratio was similar to that observed on state 4 respiration (Fig. 1), i.e. nimesulide and meloxicam were the most potent uncouplers of oxidative phosphorylation, whereas naproxen induced only a slight diminution of the ADP/O ratio. Nabumetone also brought about a sharp diminution of the ADP/O ratio with G + M, but it was ineffective with succinate. Moreover, in agreement with the data in Fig. 1, nabumetone inhibited strongly the rate of ATP synthesis during G + M oxidation, but it only exhibited a slight inhibitory effect during succinate oxidation (Fig. 2). Nimesulide showed again the strongest inhibitory effect on ATP synthesis, while naproxen demonstrated the weakest (Fig. 2). In contrast to the O_2 uptake data, diclofenac exhibited a greater inhibitory potency on ATP synthesis than meloxicam, with either substrate (Fig. 2), suggesting that diclofenac may also inhibit some of the enzymes involved in ATP synthesis (see below).

The stimulation of the basal respiratory rate, diminution of the ADP/O ratio, and inhibition of ATP synthesis by 20 nmol nimesulide or 20 nmol diclofenac (10.5 μM final concentration) diminished as the concentration of mitochondrial protein was increased (data not shown). These results indicated that, due to their hydrophobic nature, the NSAID concentrations must be referred to the mitochondrial protein (which is related to the total amount of phospholipids), rather than to the liquid volume.

For a full characterization of the uncoupling action of NSAIDs, the effect on the membrane potential was determined initially using safranine O. However, a faulty behav-

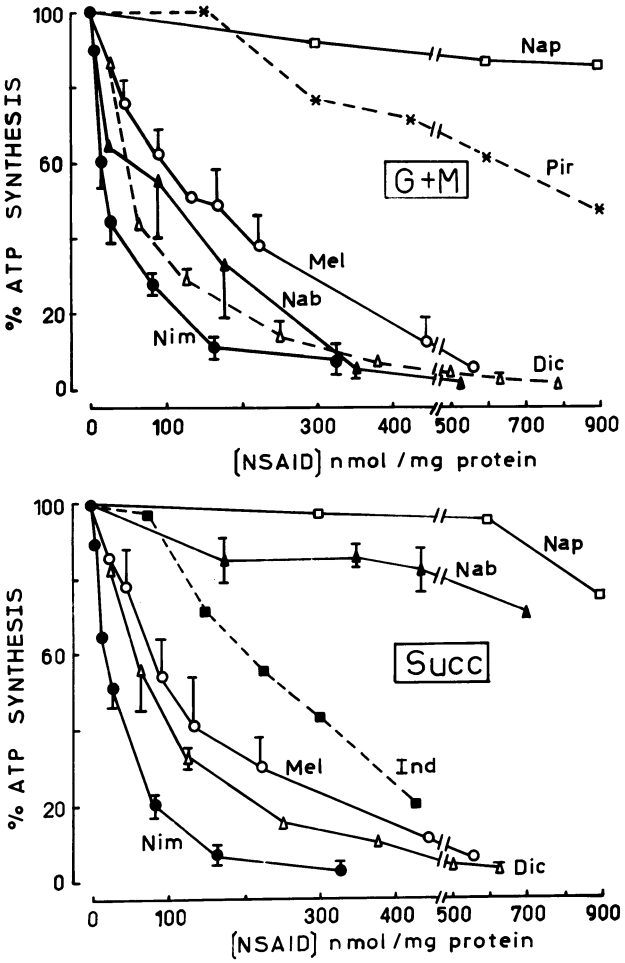

FIG. 2. Effect of several NSAIDs on the rate of ATP synthesis. Oxidative phosphorylation was determined in mitochondria incubated as described in the legend of Fig. 1, with the addition of $^{32}P_i$ (specific activity = 1.6 to 2.5 × 10^5 cpm/μmol, Cerenkov radiation). The incorporation of $^{32}P_i$ into ATP was determined after allowing phosphorylation to proceed for 2 min in the presence of 5 U hexokinase, 10 mM glucose, and 1 mM $MgCl_2$. The control rates of oxidative phosphorylation were 144 ± 11 (15) and 157 ± 10 (14) nmol/(mg protein · min) for G + M and succinate, respectively. The values shown are means ± SEM of 3–4 different preparations assayed.

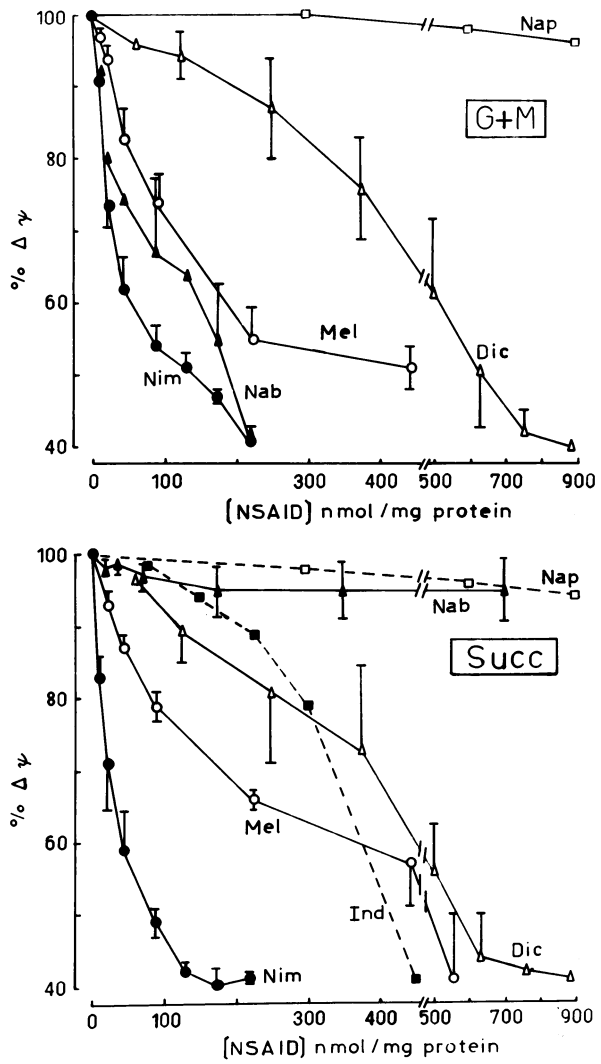

FIG. 3. Effect of several NSAIDs on the magnitude of the membrane potential. Mitochondria (3 mg protein/mL) were incubated, under orbital shaking, in 0.5 mL of the medium described in the legend of Fig. 1 with the addition of 0.8 μM [^{3}H]-TPP^+ (specific activity = 57,000 cpm/nmol). The indicated concentrations of NSAIDs were also added to the incubation medium. After 2–3 min, the mitochondrial suspension was centrifuged at 16,000 *g* for 75 sec at 4°, and aliquots of the supernatant and the pellet were withdrawn for determination of radioactivity; membrane potential was calculated according to Ref. 22. G + M, glutamate + malate; Succ, succinate (+ rotenone); see the legend to Fig. 1 for other abbreviations. The values of the membrane potential in the absence of drugs were 168 ± 3 (16) and 168 ± 2 (17) mV for G + M and succinate, respectively. The values shown are means ± SEM of 3–4 different preparations assayed.

ior of the safranine absorbance changes when monitoring membrane potential has been reported previously [23, 33, 34], in which perturbation of the charge density in the inner phase of the inner mitochondrial membrane by cations can modify the safranine signal without an associated change in membrane potential.

Therefore, membrane potential ($\Delta\psi$) was measured quantitatively by estimating the distribution of [^{3}H]-TPP^+ across the mitochondrial membrane. With either substrate, nimesulide was the most potent NSAID for collapsing membrane potential; nabumetone had no effect with succinate, but it induced a marked diminution of $\Delta\psi$ with G + M (Fig. 3). Diclofenac collapsed $\Delta\psi$ only at concentrations higher (>300 nmol/mg protein) than those that diminished ATP synthesis (*cf.* Figs. 1 and 2). It was not feasible to determine values of membrane potential lower than −60 mV, since the unspecific binding of TPP^+ to the surface of the mitochondrial membranes diminishes the sensitivity of this method when levels fall below −60 mV [22, 32].

Taken together, the data in Figs. 1–3 indicate that nimesulide and meloxicam are true uncouplers of oxidative phosphorylation. In contrast, nabumetone seems to be a respiratory inhibitor of NAD-linked substrates. Diclofenac

TABLE 1. Concentrations of NSAIDs to obtain half-maximal effect (EC_{50}) on several mitochondrial energy-dependent parameters

	EC_{50} (nmol NSAID/mg protein)							
	Basal respiration		ADP/O		ATP synthesis		$\Delta\Psi$	
NSAID (pK_a)	G + M	Succ	G + M	Succ	G + M	Succ	G + M	Succ
Nimesulide (6.5)	17 (2)	11 ± 6 (3)	26 (2)	24.4 ± 3 (3)	21 ± 5 (3)	27 ± 5 (3)	27 ± 4 (3)	21.5 ± 5 (4)
Meloxicam (4.18)	21 ± 6 (3)	48 ± 10 (4)	74 ± 7 (3)	78 ± 15 (4)	142 ± 9 (4)	103 ± 10 (3)	115 ± 4 (4)	180 ± 2 (3)
Diclofenac (4.0)	103 ± 22 (3)	80 (2)	125 ± 54 (3)	102 ± 24 (3)	57 ± 4 (3)	77 ± 11 (3)	415 ± 10 (5)	490 ± 14 (4)
Indomethacin (4.5)	166 (2)	112 (2)	131 (2)	98 (2)	145	260	>500	335
Piroxicam (6.3)	388	>400	>600	>400	835	>900	405	690
Naproxen (4.15)	>550	>800	>1000	>800	No effect	>900	No effect	No effect
Nabumetone	44 (2)	No effect	140 (2)	No effect (3)	142 ± 9 (4)	>700 (3)	68 ± 9 (3)	No effect (3)

Mean ± SEM (N). The pK_a values were taken from the following references: [36] for nimesulide; [37] for meloxicam; and [10] for the other four NSAIDs.

is an uncoupler at high concentrations, but at low concentrations, it may act as a decoupler or as an inhibitor of some of the enzymes involved in oxidative phosphorylation. Naproxen was ineffective at concentrations as high as 500 nmol/mg protein, whereas indomethacin and piroxicam were weak uncouplers.

The concentrations of NSAIDs that induce a half-maximal uncoupling or inhibition (EC_{50}) are shown in Table 1. The EC_{50} values for $\Delta\psi$ were estimated assuming that a membrane potential value of −60 mV was the limit of sensitivity of the method used (0% $\Delta\psi$); −60 mV is also the threshold value for ATP synthesis [22, 32, 35]. With succinate as substrate, the uncoupling potency of NSAIDs was: nimesulide > meloxicam > diclofenac > indomethacin > piroxicam > naproxen, nabumetone. Table 1 also shows the pK_a values of the drugs. The data show that there is no correlation between the uncoupling ability of the NSAIDs and their pK_a values. For instance, nimesulide and piroxicam or meloxicam and naproxen have similar pK_a values, but quite different uncoupling potencies.

The delocalized chemiosmotic hypothesis of energy coupling establishes that a variation in the H^+ gradient should lead to a corresponding variation in the fluxes that depend on the H^+ gradient, such as the rates of respiration and ATP synthesis [13], i.e. a diminution in the H^+ gradient should result in a proportional increase in the respiratory rate and a decrease in the ATP synthesis rate. However, some compounds such as long-chain fatty acids and gramicidins, when added to mitochondria at low concentrations, induce a pronounced inhibition of ATP synthesis and stimulation of the basal respiratory rate, without a concomitant diminution in the H^+ gradient [15]; these compounds are called decouplers.

To determine whether the effects of diclofenac on mitochondrial energy coupling are due to uncoupling or decoupling, its effect on the relationship between membrane potential and the rate of ATP synthesis (or respiration) was determined (Fig. 4). For comparison, the effect of nimesulide was also determined. A proportionality between changes in force (membrane potential) and changes in flux (rate of respiration or ATP synthesis) was observed for nimesulide. Similar flux-force relationships were observed for meloxicam, indomethacin, and piroxicam (data not shown). However, there was a significant variation in flux without variation of force, induced by low concentrations of diclofenac (0–60 nmol/mg protein). Since the H^+

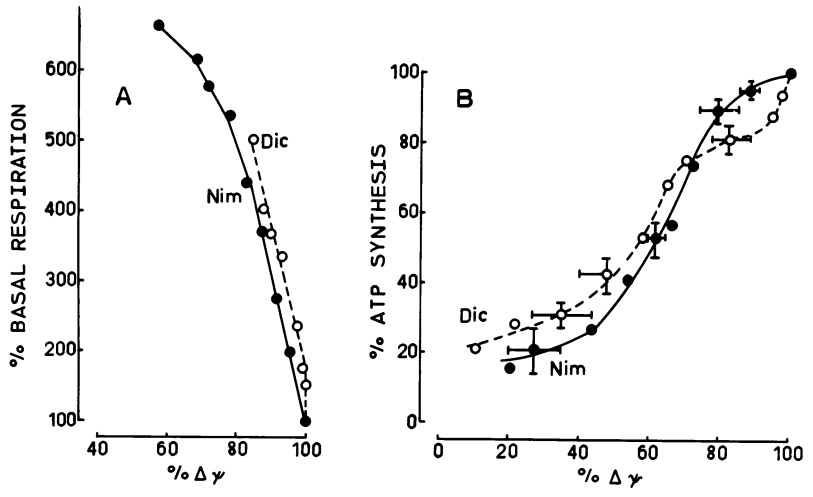

FIG. 4. Relationship between the rate of basal respiration or the rate of ATP synthesis and the magnitude of the membrane potential. The rate of basal (state 4) respiration was determined in the presence of succinate (+ rotenone) (A) as described in the legend of Fig. 1. The rate of ATP synthesis (B) was determined from experiments with mitochondria incubated as described in the legend of Fig. 2 with the addition of $^{32}P_i$ and succinate (+ rotenone) as substrate. Membrane potential was measured as described in the legend of Fig. 3, in the absence (A) or in the presence (B) of 2 mM ADP. The rate of ATP synthesis was 120–160 nmol/mg protein · min (100%). Membrane potential was 130–150 mV (100%) in the presence of ADP (state 3) and with succinate (+ rotenone) as substrate. The data shown are the means obtained from two different preparations (A), or the means ± SD obtained with four different preparations (B), which were assayed simultaneously for O_2 uptake or ATP synthesis and membrane potential.

electrochemical gradient that supports oxidative phosphorylation must be above -60 mV [22, 32, 35], the values of $\Delta\psi$ under phosphorylating conditions (Fig. 4B) were normalized, assuming that 0% $\Delta\psi$ corresponds to −50 mV (because the pH gradient contributed with 10–15 mV, in the presence of 5 mM P_i at an external pH of 7.2). Hence, diclofenac apparently behaves as a decoupler at low concentrations (<60 nmol/mg protein) and as an uncoupler at higher concentrations.

Since diclofenac had a greater efficacy on the diminution of the ADP/O ratios (Fig. 1) and ATP synthesis (Fig. 2), than on the collapse of membrane potential in basal, non-phosphorylating conditions (Fig. 3), this drug could also inhibit some of the key enzymes of oxidative phosphorylation. Thus, its effect on the activity of the adenine nucleotide translocase was examined by measuring [^{3}H]-ADP uptake by mitochondria [25, 26]. Diclofenac (314 nmol/mg protein) diminished the translocase activity by 76 ± 3% (mean ± SEM; N = 3); 0.3 μM CCCP inhibited the activity by 5%. Nimesulide (259 nmol/mg protein) also showed a strong inhibitory effect (60 ± 3% inhibition), whereas meloxicam and nabumetone were ineffective.

SMP Experiments

The use of SMP allows one to test the uncoupling potency of NSAIDs, without the involvement of the ion transport systems. It is also possible to examine the effect of nabumetone on the respiratory chain, without the interference of the NAD-linked dehydrogenases of the Krebs cycle. The energy-dependent parameters measured in SMP were the respiration rate and the formation of a pH gradient with NADH or succinate as oxidizable substrate.

Nabumetone fully inhibited NADH oxidation, but it did not affect succinate oxidation. It also collapsed the pH gradient generated by NADH oxidation, but it did not affect the pH gradient formed by succinate oxidation (Fig. 5). These results indicated that nabumetone was an inhibitor of site 1 of the respiratory chain, and that it did not affect the activity of the other respiratory components. This result is in consonance with the observed inhibition of basal (Fig. 1) and uncoupled respiration, ADP/O ratios (Fig. 1), oxidative phosphorylation (Fig. 2), and collapse of membrane potential (Fig. 3) by nabumetone in mitochondria that oxidized NAD-linked substrates, and its negligible effect on mitochondria that oxidized succinate. The EC_{50} value of nabumetone (55 nmol/mg protein) for the inhibition of NADH oxidation in SMP (Fig. 5) was comparable to the values found in mitochondria (Table 1).

The other NSAIDs brought about a concentration-dependent stimulation of the respiratory rate with succinate in SMP. The order of their potency was: diclofenac (EC_{50} = 100 nmol/mg protein) > nimesulide (EC_{50} = 230 nmol/mg protein) > piroxicam (EC_{50} = 400 nmol/mg protein) > indomethacin (EC_{50} = 600 nmol/mg protein) > naproxen (EC_{50} = 800 nmol/mg protein). Collapse of the pH gradient was observed with diclofenac; it was not possible to assay

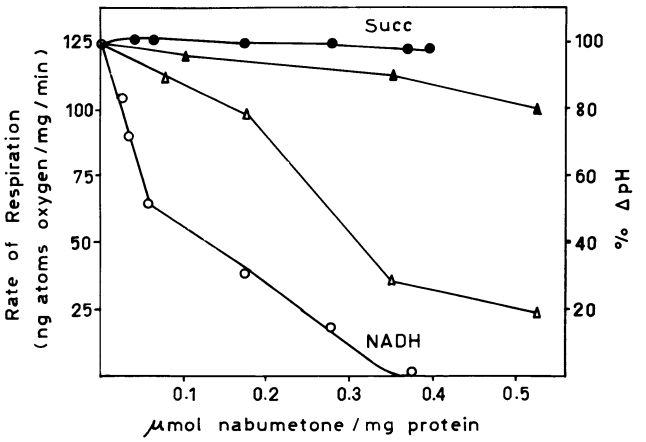

FIG. 5. Effect of nabumetone on respiration and pH gradient in submitochondrial particles. Rat liver submitochondrial particles (150–200 μg protein/mL) were incubated in a medium that contained 250 mM sucrose, 10 mM HEPES, 1 mM EGTA, 1 μM oligomycin, and 10 mM succinate (succ; closed symbols) or 1 mM NADH (open symbols). After 2 min, in the case of NADH, or 6–8 min, in the case of succinate (minimal time required to fully express the activity of succinate dehydrogenase), the indicated concentrations of nabumetone were added and the rate of O_2 uptake was determined (circles). The same protocol was followed to measure ΔpH (triangles) except that the medium also contained 1.5 μM 9-amino-6-chloro-2-methoxyacridine (ACMA) + 3 μM 9-aminoacridine. The percentage of fluorescence quenching induced by addition of succinate or NADH was taken as a measurement of the pH gradient across the submitochondrial particle membranes [27].

nimesulide due to the interference in the fluorescence signal by its strong yellow color.

The effect of NSAIDs on the activity of the ATPase of SMP was also assayed. Diclofenac (418 nmol/mg protein) brought about a 19% inhibition (N = 2), whereas the other six NSAIDs did not affect the ATPase activity. Furthermore, the effect of the NSAIDs was assayed on inside-out vesicles of greater pharmacological relevance such as gastric microsomes, which contain the H^+, K^+-ATPase activity involved in stomach acidification [21]. However, this H^+-ATPase activity, which was 70–80% sensitive to 1 mM vanadate, was not affected by the NSAIDs at the same range of concentrations used in mitochondria and SMP (data not shown).

Intact Cell Experiments

To test whether the uncoupling effect of NSAIDs observed in mitochondria and SMP was also apparent in a more physiological system, i.e. in *in situ* mitochondria, the effect of NSAIDs on the rate of cell respiration was determined in AS-30D hepatoma cells. The rate of respiration in these cells is 88% oligomycin-sensitive [38], which implies that most of the cell O_2 uptake is engaged in oxidative phosphorylation. Despite the presence of an extra permeability barrier (the plasma membrane), the addition of nimesulide (300 nmol/10^7 cells) to cell suspensions induced a 4-fold stimulation of cell respiration, meloxicam (1200 nmol/10^7

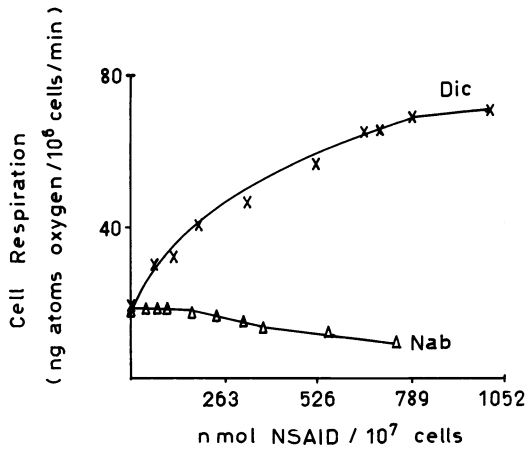

FIG. 6. Effect of nabumetone and diclofenac on O_2 uptake in AS-30D hepatoma cells. AS-30D hepatoma cells (10 million/mL) freshly isolated and kept on ice were added to 2 mL of Ringer–Krebs–HEPES buffer with 5 mM glucose of pH 7.4 at 37°, and the rate of respiration was determined in the presence of the indicated concentrations of NSAIDs. Similar results were obtained with two other cell preparations.

cells) increased it 6-fold, and diclofenac 2.6 times (results for diclofenac are shown in Fig. 6). The EC_{50} values were 120, 700, and 260 nmol/10^7 cells for nimesulide, meloxicam, and diclofenac, respectively. The maximal respiratory stimulation attained by these three NSAIDs in intact cells was consistent with the data obtained in mitochondria and SMP. However, the requirement of a higher concentration of meloxicam to reach half-maximal uncoupling suggests that, in this type of mammalian cells, this drug is less permeable through the plasma membrane and, therefore, has a lower accessibility to mitochondria than nimesulide and diclofenac. Nabumetone diminished the rate of cell respiration (Fig. 6), which was expected since NAD-linked substrates are the physiological substrates of *in situ* mitochondria [39–42].

Isolated Heart Experiments

The use of isolated perfused whole heart preparations allows for a more complete pharmacological examination of the effects of NSAIDs on mitochondrial energy metabolism. The function that can be readily measured, and is exclusively mitochondrial in whole organs, is the rate of O_2 uptake that is sensitive to respiratory and ATP synthase inhibitors. In whole heart [43] and cardiac myocytes [44], the rate of respiration is more than 90% linked to oxidative phosphorylation.

In full agreement with the data obtained in mitochondria and cells, nabumetone inhibited, in a concentration-dependent manner, the rate of O_2 uptake in glucose-supported whole beating heart (Fig. 7, left trace). When this drug was applied as a bolus (870 nmol), a slight decrease of approximately 0.26 μmol O_2/heart in O_2 uptake was observed; this was accompanied by a significant reduction of 12 mm Hg in intraventricular pressure. The addition of an excess of nabumetone (8.7 μmol) produced a fast, transient stimulation of O_2 uptake (which was not due to the added solvent); this was followed by a very pronounced inhibition. After 20 min, the perfused heart was able to recover, although not completely, from the administration of nabumetone. The very severe reduction in intraventricular pressure that accompanied the inhibition of respiration by excess nabumetone was expected, since muscle contraction depends on the ATP supplied by mitochondria.

Nimesulide (Fig. 7, lower right trace), meloxicam (222 nmol), indomethacin (478 nmol), and piroxicam (334 nmol) (not shown) induced a stimulation of respiration together with a reduction in intraventricular pressure in perfused heart (from 46 to 30 mm Hg with 324 nmol nimesulide). This is in agreement with their uncoupling activity. In contrast, 126 nmol diclofenac, although it stimulated O_2 uptake, also induced an enhanced muscle contraction activity resulting in increased intraventricular pressure (Fig. 7, upper right trace).

DISCUSSION

The experiments on the effects of different NSAIDs on the energy-dependent reactions of isolated mitochondria showed that, except for nabumetone and naproxen, the other five NSAIDs stimulated the rate of basal respiration, inhibited the rate of oxidative phosphorylation, and collapsed the H^+ gradient, with either glutamate + malate or succinate as oxidizable substrates. Similar results were reported previously [8–12] for some of the strongly acidic NSAIDs used in this study. However, at variance with the results of Mahmud *et al.* [10], we did not observe a higher potency for respiratory stimulation by indomethacin over diclofenac and piroxicam, using succinate as substrate. The flux-force relationships for nimesulide, meloxicam, indomethacin, and piroxicam showed a proportional variation in both parameters, which indicated that these NSAIDs behave as classic uncouplers. In the case of diclofenac, the flux-force relationship suggested that this drug may behave as a decoupler at low concentrations and as an uncoupler at high concentrations.

On the other hand, nabumetone did not stimulate basal respiration, did not inhibit oxidative phosphorylation, nor did it collapse the membrane potential in mitochondria that oxidized succinate. Mahmud *et al.* [10] also described the lack of respiratory stimulation by nabumetone in mitochondria that oxidized succinate; these authors did not use other substrates. Here, we observed that this drug inhibited basal and uncoupled respiration as well as oxidative phosphorylation with an associated diminution in membrane potential in mitochondria that oxidized glutamate + malate. This indicates that nabumetone is a typical respiratory inhibitor of NAD-linked substrate oxidation. In turn, we found that naproxen did not significantly affect respiration, ATP synthesis, and $\Delta\psi$ at concentrations of pharmacological relevance (<300 nmol/mg protein).

Nabumetone is a non-acidic prodrug, which undergoes

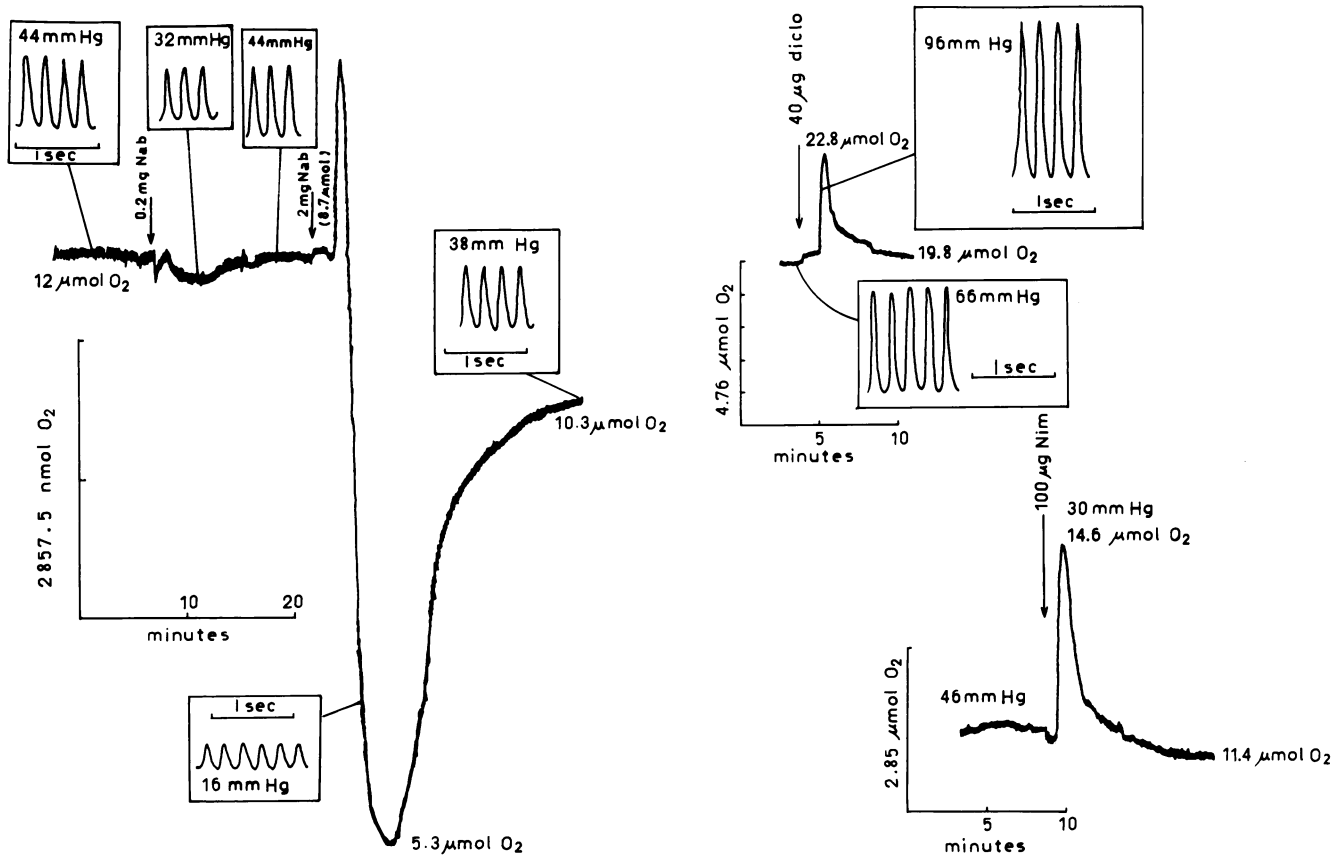

FIG. 7. Effect of several NSAIDs on O_2 uptake and intraventricular pressure of isolated rat heart. Hearts excised from rats of 350–450 g body weight were perfused through the aorta in a retrograde manner with Ringer–Krebs bicarbonate buffer (perfusion flow 10 mL/min) following the Langendorff method. The changes in the concentration of O_2 between the 95% O_2/5% CO_2-saturated medium and the medium that was exposed to the contracting heart were measured with an O_2 electrode. The numbers on the traces establish such changes in O_2 concentration. Since the total volume in the O_2 electrode chamber was 1.8 mL and the rate of medium flow through this chamber (after passing through the contracting heart) was 6 mL/min, the rate of O_2 uptake can be obtained after multiplying the numbers on the traces by 3.77 (6 mL min^{-1}/1.8 mL). At the indicated times, the pressure developed by the left ventricle was registered (inserts); the values shown on the right, lower trace, indicate both the steady-state O_2 concentration (μmol O_2) and the intraventricular pressure (mm Hg).

hepatic metabolism to its active metabolite, 6MNA. More than 90% of nabumetone is metabolized in the liver; of this, 35–38% is transformed to 6MNA and 50% is converted to inactive metabolites [45, 46]. In this context, it is noteworthy that 6MNA uncouples oxidative phosphorylation in a way similar to that of conventional acidic NSAIDs [10]. 6MNA does not undergo significant enterohepatic circulation; thus, the gastrointestinal tract is not exposed to uncoupling concentrations of the drug, which may account for the improved tolerability of nabumetone [10, 47]. Therefore, nabumetone and 6MNA should exert their inhibitory and uncoupling effects mainly on liver metabolism.

The distinct actions of nabumetone and the other NSAIDs on mitochondria were also apparent in submitochondrial particles as well as in intact cells and whole heart. In these systems, nabumetone inhibited O_2 uptake, whereas the other NSAIDs stimulated respiration. These observations indicated that the presence of other permeability barriers, such as plasma and organelle membranes, in cells and organs did not hinder the interaction of NSAIDs with the inner mitochondrial membrane.

It has not been demonstrated that acidic NSAIDs act as H^+ ionophores. It is thus interesting that the non-carboxylated NSAIDs nimesulide and meloxicam behave as classic uncouplers. However, meloxicam (enolic acid and thiazol) and nimesulide (sulfoanilide) have groups with acidic properties [48, 49]. Then, the mechanism of action for nimesulide and meloxicam uncoupling activity could be through the transport of H^+ across the inner mitochondrial membrane; this could be facilitated by the formation of lipophilic ion pairs with appropriate anions, as occurs with amine local anesthetics [14, 16].

The composition of our experimental medium contained Cl^- as the main permeable anion, which is a poor promoter of neutral ion pairs, in particular with hydrophobic amines [14]. To determine whether diclofenac, nimesulide, and meloxicam were protonophores, swelling of non-respiring mitochondria was measured in the presence of potassium-acetate and valinomycin. To induce swelling under these

conditions, a protonophore has to be present [14, 16]. Such a result was achieved with CCCP and, to a lesser extent, with nimesulide > meloxicam > diclofenac (data not shown). The addition of KSCN increased the swelling induced by diclofenac only. Therefore, it appears that nimesulide and meloxicam behave as protonophores, while the uncoupling activity of diclofenac may partially derive from its ability to form lipophilic ion pairs with permeant anions such as SCN^-.

The profile of adverse side-effects for different NSAIDs is very similar, although indomethacin is associated with a greater frequency of both gastrointestinal and renal side-effects [50], whereas a lesser frequency is evident with nimesulide, meloxicam, and nabumetone [28–30, 51, 52]. It has been reported that nabumetone, flurbiprofen derivatives, and the non-acidic highly selective COX-2 inhibitors SC58125 and DuP-697, drugs known to have good gastrointestinal tolerance, apparently do not uncouple oxidative phosphorylation, leading to the suggestion that mitochondrial uncoupling might be related to the side-effects on the gastrointestinal tract [7, 10].

However, our present findings do not support this hypothesis, because the drugs that require the lower doses to achieve an anti-inflammatory response with few side-effects, such as nimesulide (200 mg/day; 649 μmol) and meloxicam (15 mg/day; 44 μmol), were the most potent uncouplers. Moreover, naproxen also produces adverse side-effects [50], but, as shown in this study, it exerts a negligible uncoupling effect. It should also be pointed out that the range of EC_{50} values, obtained for the seven NSAIDs, were approximately three orders of magnitude above the serum *free* concentrations found in humans [46, 47, 50, 53, 54]. This suggests an insignificant effect on mitochondrial metabolism by therapeutic doses of NSAIDs under *in vivo* conditions, particularly for liver and kidney.

However, the cellular uptake of NSAIDs from serum still could enhance the concentrations of the drugs in the cytosol by two to three orders of magnitude, i.e. from nanomolar to micromolar. In addition, oral administration of NSAIDs exposes gastric and duodenal cells to micromolar concentrations, which might prompt uncoupling and inhibition of oxidative phosphorylation and, hence, trigger the severe side-effects on the gastrointestinal tract induced by the oral administration of NSAIDs.

Diclofenac diminished oxidative phosphorylation through a direct inhibitory interaction with the adenine nucleotide translocase and the ATPase, in addition to its uncoupling ability. Moreover, this drug led to stimulation, instead of inhibition, of intraventricular pressure. Diclofenac, indomethacin, and naproxen also stimulated the rates of glycolysis and gluconeogenesis, in parallel with a stimulation of mitochondrial O_2 uptake in liver [11, 55]. Thus, other effects of NSAIDs, in addition to their inhibitory and uncoupling effects on oxidative phosphorylation, might also take place in liver and cardiac cells. This obviously has to be further explored.

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