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Inhibitors of the 5-lipoxygenase arachidonic acid pathway induce ATP release and ATP-dependent organic cation transport in macrophages

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ABSTRACT

We have previously described that arachidonic acid (AA)-5-lipoxygenase (5-LO) metabolism inhibitors such as NDGA and MK886, inhibit cell death by apoptosis, but not by necrosis, induced by extracellular ATP (ATPe) binding to P2X7 receptors in macrophages. ATPe binding to P2X7 also induces large cationic and anionic organic molecules uptake in these cells, a process that involves at least two distinct transport mechanisms: one for cations and another for anions. Here we show that inhibitors of the AA-5-LO pathway do not inhibit P2X7 receptors, as judged by the maintenance of the ATPe-induced uptake of fluorescent anionic dyes. In addition, we describe two new transport phenomena induced by these inhibitors in macrophages: a cation-selective uptake of fluorescent dyes and the release of ATP. The cation uptake requires secreted ATPe, but, differently from the P2X7/ATPe-induced phenomena, it is also present in macrophages derived from mice deficient in the P2X7 gene. Inhibitors of phospholipase A2 and of the AA-cyclooxygenase pathway did not induce the cation uptake. The uptake of non-organic cations was investigated by measuring the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by Fura-2 fluorescence. NDGA, but not MK886, induced an increase in $[Ca^{2+}]_i$. Chelating Ca^{2+} ions in the extracellular medium suppressed the intracellular Ca^{2+} signal without interfering in the uptake of cationic dyes. We conclude that inhibitors of the AA-5-LO pathway do not block P2X7 receptors, trigger the release of ATP, and induce an ATP-dependent uptake of organic cations by a Ca^{2+}- and P2X7-independent transport mechanism in macrophages.

1. Introduction

Macrophages are multi-functional and highly plastic cells that display important roles in the immune-inflammatory system and in the homeostasis of mammalian tissue [39,45,50,85]. Macrophage activation is a tightly controlled event, regulated by the production and secretion of pro- and anti-inflammatory mediators and the expression of a diversified panel of receptors and transport systems in their plasma membrane that may change according to their specific differentiation state and microenvironment milieu [25,84]. Lipid metabolism has been a major focus of attention in the study of macrophages due to its involvement in intracellular signaling, production of eicosanoids, atherosclerosis, and obesity, among others. In this regard, inhibitors of lipoxygenase (LO) and cyclooxygenase (Cox) are widely used to study the arachidonic acid (AA) metabolism [20,42,72] although their effect on ion channels and other transport mechanism has not been so well investigated.

Among the several ion channels and other transport mechanisms displayed on macrophage membranes, the ones related to extracellular purine and pyrimidine nucleotides have gained attention in recent years [6,9,53]. Extracellular ATP (ATPe) and other nucleotides can bind one or more of the nucleotide receptors (P2 receptors) expressed in macrophage plasma membrane and other cells of the immune system [3,6,16,22,23,31,40,43,64] as well as inducing the secretion of cytokines [22,27,30,64] and the production of reactive oxygen species (ROS) [16,

Abbreviations: AA, Arachidonic acid; SA, Acetyl salicylic acid; ATPe, Extracellular ATP; BEL, 2-bromo-enol-lactone; $[Ca^{2+}]_i$, Free intracellular Ca^{2+} concentration; CF, carboxyfluorescein; EB, Ethidium bromide; FBS, Fetal bovine serum; PLA_2 α, calcium-dependent PLA_2 α; Cox, Cyclooxygenase; FLAP, 5-lipoxygenase-activating protein; $iPLA_2$, Ca^{2+}-independent PLA_2; LDH, Lactate dehydrogenase; LY, Lucifer yellow; LO, Lipoxygenase; NDGA, Nordihydroguaiaretic acid; NSAD, Non-steroid anti-inflammatory drugs; ROS, Reactive oxygen species; SR-B, Sulforhodamine B; PLA_2, Phospholipase A_2; PI, Propidium iodide

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33,81]. The activation of phospholipases A_2 (PLA_2), the release of AA, and the synthesis and secretion of bioactive eicosanoids have also been reported [4,52,82].

More specifically, the P2X7 receptor is an ATPe-gated cation-selective channel (Na^+, K^+, and Ca^{2+}) that is connected with an intracellular array of signaling pathways [15,21,26,44] and is well known for its involvement in the activation of inflammasome and secretion of IL1-β [24,27,30,32,37,58]. It can also induce macrophage death by necrosis and apoptosis [14,17,35,41,48,49,51]. Its discovery and initial characterization were related to the phenomenon known as ATPe-induced permeabilization of the plasma membrane, characterized by the fast (1–20 min) uptake of fluorescent dyes such as the cation ethidium and the anion Lucifer yellow [18,60,74,77]. The uptake of these large fluorescent organic molecules is the result of a complex transport mechanism coupled to the P2X7 receptor through a poorly understood signaling pathway and involves at least two distinct transport mechanisms, one for cations and another one for anions [11,47,71].

We have previously described the involvement of AA-5-LO metabolism in the ATPe-induced P2X7-dependent apoptosis of macrophages [14]. Mepacrine, a generic inhibitor of PLA_2, 2-bromo-enol-lactone (BEL) – an inhibitor of Ca^{2+}-independent PLA_2 ($iPLA_2$), and the inhibitors of AA-LO pathway nordihydroguaiaretic acid (NDGA) – a general inhibitor of LOs, MK886 – an inhibitor of the 5-lipoxygenase-activating protein (FLAP), and the selective inhibitors of 5-LO Zileuton, and AA-861, but not the inhibitors of Cox, ASA and NS-398, inhibit apoptosis. None of these drugs inhibits necrosis [14]. These results lead us to propose that an $iPLA_2$-5-LO pathway is activated by purinergic P2X7 receptors in macrophages in connection with the induction of apoptotic cell death [14].

During the course of experiments aimed to investigate the involvement of the AA pathways in other P2X7-associated phenomena, we asked whether the inhibitors that blocks ATPe-induced apoptosis could also modulate the ATPe-induced uptake of organic molecules. While performing initial experiments, we observed that some inhibitors of 5-LO induced a fast uptake of the cationic dye ethidium by macrophages in the absence of any exogenous addition of ATP. This unexpected result prompted us to investigate the effects of several anti-inflammatory drugs and other inhibitors of AA pathways on the transmembrane transport of organic molecules on macrophages. Here we describe the induction of ATP release and the characterization of a P2X7-independent transport mechanism selective for organic cations, induced by inhibitors of the AA-5-LO pathway, but not by inhibitors of Cox, in intraperitoneal murine macrophages.

2. Materials and methods

2.1. Materials

DMEM, fetal bovine serum penicillin and streptomycin were obtained from Gibco/BRL (São Paulo, RJ, Brasil); 3-[1-(para-Chlorobenzyl)-5-(isopropyl)-3-t-butylthioindol-2-yl]-2,2-dimethylpropanoic acid (MK886) was from Calbiochem (San Diego, CA, USA); thioglycollate medium was from Difco (Detroit, MI, USA). NaCl, $MgCl_2$, $CaCl_2$, and KCl were from Reagen (Rio de Janeiro, RJ, Brazil); nordihydroguaiaretic acid (NDGA), mepacrine, ATP, 2-bromo-enol-lactone (BEL), EGTA, HEPES, dimethyl sulfoxide (DMSO), ethidium bromide (EB), Lucifer yellow (LY) lithium salt, propidium iodide (PI), carboxyfluorescein (CF), ionomycin, Zileuton, acetylsalicylic acid (ASA), N-[2-(Cyclohexyloxy)-4-nitrophenyl]methanesulfonamide (NS398), 2-(12-Hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-*p*-benzoquinone (AA-861), probenecid, luciferase, CoA, apyrase, brefeldin A, and phosphate buffered saline (PBS), were purchased from Sigma-Aldrich (São Paulo, Brazil); YO-PRO-1, TOPRO-2, TOTO-3, sulforhodamine B (SR-B), Fura-2AM, and D-luciferin were from Life Technologies (São Paulo, Brazil). Most reagents were pre-diluted in a 1000× stock solution either in DMSO (BEL, MK886, Zileuton, Fura-2, NDGA, AA-861, NS398, ionomycin, TOPRO-3, TOTO-3) or in the normal salt solution or Milli-Q® water and kept frozen until use. NDGA was prepared fresh on the day of the experiment. BEL stock solution was prepared in a concentration 100× the highest used dose.

2.2. Animals

Swiss-Webster and C57/Bl6 mice were obtained from the animal facilities of the Instituto de Microbiologia Paulo de Goes and from the Transgenic Animal Facility of the Instituto de Biofísica Carlos Chagas Filho of the Federal University of Rio de Janeiro (LAT-UFRJ). Male mice lacking the 5-LO enzyme gene ($5\text{-LO}^{-/-}$) were obtained from The Jackson Laboratory, and age-matched male wild type (WT) mice (background, strain 129) were used as controls. $P2X7^{-/-}$ were obtained from The Jackson Laboratory and bred at LAT-UFRJ. All animals were eight to twelve weeks old, weighed approximately 16–30 g and were handled according to the guidelines for animal use in scientific experiments of the Instituto de Biofísica Carlos Chagas Filho of the Federal University of Rio de Janeiro and the Animal Care Committee of the Universidade Estadual de Campinas.

2.3. Macrophage isolation and culture

Thioglycollate-elicited macrophages were obtained from the intraperitoneal cavity of mice, collected 4 days after thioglycollate injection, as previously described [18]. In brief, cells were washed in DMEM and kept on ice at a concentration of 10^6 cells/mL until used (freshly isolated macrophages) or plated on 35 mm plastic culture dishes at a concentration of 2×10^5 cells/dish in 2 mL DMEM supplemented with 10% fetal bovine serum, 2 g/L sodium bicarbonate, 0.3 mg/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (complete medium) at 37 °C in a humidified atmosphere containing 5% CO_2. Non-adherent cells were then removed after 1 h and the macrophages were kept for 2–5 days under the same culture conditions. For calcium measurements, cells were plated on glass coverslips at a concentration of 5×10^5 cells/coverslip.

2.4. Dye uptake assay

Unless otherwise specified, cells were kept at 37 °C for 5 min in a salt solution containing, in mM: 145 NaCl, 5 KCl, 1 $MgCl_2$, 1 mM $CaCl_2$, and 10 Na-HEPES, pH 7.4 (normal extracellular solution) and then for 5 min in the presence of 5 mM ATP or an inhibitor of the AA metabolism at the indicated concentrations before the addition of a fluorescent cell-impermeant dye. The dyes were used in the following concentrations for flow cytometry (2.5 μM EB, 2.5 μM YO-PRO-1, 5 μM PI, 1 μM TOPRO-3, 1 μM TOPRO-3, 10 μM SR-B, 2.5 μM CF) or fluorescence microscopy (10 μM EB, 2.5 μM YO-PRO-1, 3 mM SR-B, 5 mM CF, or 3 mM LY). The cells were analyzed either by fluorescent microscopy or by flow cytometry, as described below [14,71].

Fluorescence microscopy was performed using an Axiovert 100 microscope (Karl Zeiss, Oberkochen, Germany) equipped with an HBO lamp, an Olympus digital camera (Olympus American Inc., PA, USA) and Image-pro plus v 6.2 software (Media Cybernetics, Inc. Bethesda, MD, USA). In all fluorescence microscopy observations, cells were also observed using a clear field illumination and at least 50 macrophages were present in each microscope field studied.

Dye uptake was also measured in a flow cytometer (FACSCalibur cytometer, Becton, Dickinson and Co., Franklin Lakes, NJ, USA). In brief, freshly isolated macrophages (10^6/mL) were prepared as described above and the intensity of dye uptake was immediately determined using an excitation wavelength of 488 nm (633 nm for TOTO-3) and an emission wavelength of 590 or 670 nm. At least 5000 data points were collected for each sample. The results were analyzed using the WinMDI program (Multiple Document Interface Flow Cytometry

Application, version 2.8, by Joseph Trotter, The Scripps Research Institute, La Jolla, CA, USA).

2.5. Induction and analysis of cell death

Suspensions of freshly-isolated intraperitoneal cells (2×10^5 cells/200 μL) or adherent macrophages in culture dishes were pre-incubated at 37 °C with/without 5 mM ATP or 50 μM NDGA or vehicle (0.1 or 1% DMSO) for 20 min in DMEM medium containing 2 g/L sodium bicarbonate and buffered with 10 mM Na-HEPES, pH 7.4. After this period, cells were gently washed twice with complete medium to remove the drugs and further incubated for 6 h at 37 °C as previously described [14,47]. The cells were then centrifuged at 5600 ×*g* for 1 min, the supernatant was collected for measurement of LDH content (see below) and the pellet was used for the determination of apoptosis as described [28]. In brief, the cells were gently suspended in 250 μL of a lysing buffer that preserved the nuclei for the determination of DNA content (50 μg/mL ethidium bromide, 0.1% of sodium citrate, 0.1% Triton-X 100). The nuclei were then analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Mountain View, CA, USA) and the degree of apoptosis was quantified by counting the number of hypodiploid events. At least 5000 events were collected per sample and normalized values were calculated by subtracting the spontaneous apoptosis obtained in the controls (absence of any other drugs) from all experimental values and taking the % of the number of hypodiploid events induced by 5 mM ATP as 100%.

Cell lysis (necrosis) was determined by measuring lactate dehydrogenase (LDH) enzymatic activity in the supernatants collected as above, using a commercially available colorimetric assay kit according to the maker's instructions (Doles, Goiania, GO, Brazil) [67]. Control lyses were obtained by using supernatants of untreated cells (0%) and cells treated with 0.1% Triton-X 100 (100%). Each reading was subtracted from the reading obtained in medium containing drugs without cells in order to avoid any interference of the drugs with the reagents in the LDH detection kit.

2.6. Intracellular calcium measurements

Macrophages were plated on glass coverslips for 3–5 days and loaded with 5 μM Fura-2AM at 37 °C in complete culture medium containing 2.5 mM probenecid. The cells were then washed twice and accommodated in a chamber whose base was formed by the coverslip containing the cells. The cells were maintained at 37 °C in the normal salt solution described above or in a Ca^{2+}-free solution in which 1 mM EGTA was substituted by 1 mM $CaCl_2$. This solution was heated to reach 37 °C in the perfusion chamber. Cytoplasmic calcium concentrations of groups of 20–40 cells were monitored continuously with the use of a fluorescence imaging spectrofluorimeter (Easy Ratio Pro equipped with a DeltaRAMX Illuminator, an Olympus IX71 microscope, a QuantEM 5125C camera and the ImagrePro Plus V 6.3 software; PTI Photon Technology International, Princeton, NJ). Fura-2 was excited alternately at 340 and 380 nm, and the emission at 510 nm was measured. The ratio measurement, which is proportional to the cytoplasmic calcium concentration, was determined every 100 ms. ATP application was performed by gently removing half the volume of the incubation chamber (500 μL) and adding back the same volume containing the drug at a 2× concentration with a manual pipette. Maximum and minimum fluorescence values were obtained by adding ionomycin (20 μM) and EGTA (20 mM) sequentially at the end of each experiment.

2.7. Measurements of ATP

The extracellular concentration of ATP from macrophages was measured by luminometry, using firefly luciferin-luciferase, which catalyzes the oxidation of luciferin in the presence of ATP to produce light [76]. For real-time luminometry, glass coverslips containing 2×10^5 macrophages, plated as described above, were mounted in the chamber of a single-channel luminometer as previously described [56]. Before the experiments, cells were bathed in a 300 mosM solution containing (in mM): 137 NaCl, 2.7 KCl, 4.72 Na_2HPO_4, 1.50 KH_2PO_4, 1.32 $CaCl_2$, 1.91 $MgSO_4$, 5 glucose, 0.5% bovine serum albumin, pH 7.4 and kept 20 ºC. After that, 100 μl of medium containing 1% BSA, 195 μM of luciferin, 12 nM of luciferase and 97.5 μg/ml of Co-A were added a few minutes before a stimulus. The final volume was 100 μL. Alternatively, macrophages were plated in 48-well plates and washed twice in the solution described above immediately before the experiments. Luciferin, CoA and luciferase were then added as above in a final volume of 100 μL and kept at room temperature. The stimuli were then added into the bathing solution, gently mixed and luminescence intensity was measured using an Espectra Max model M5e microplate reader (Molecular Devices) registering luminescence intensity 1 min post-stimulus. Cells were stimulated with 50 μM NDGA, MK886 10 μM, equivalent amounts of DMSO (0.1%), or no additions. The concentrations of ATP in the bathing solution were obtained from calibration curves increasing the ATP concentrations from 16 to 150 nM, sequentially added to the assay medium from a stock solution of ATP dissolved in the above mentioned medium. In one set of experiments, the green fluorescence of mepacrine was used as an indicator of intracellular ATP content [10,46]. In these experiments, mepacrine (10 or 100 μM) was added to the bathing solution 20 min before the addition of the stimuli.

2.8. Data analysis

Unless otherwise specified, each experiment was performed at least three times in triplicates and data in bar graphics represent the mean % ± SEM. Data were analyzed using GraphPad InsTat software (GraphPad Software Inc., version 4.0). Values are presented as mean ± s.d. Differences between experimental groups were evaluated by the two-tailed unpaired Student's *t* test. A $^{*}p$ value < 0.05, $^{**}p$ value < 0.01 and $^{***}p$ < 0.001 were considered statistically significant.

3. Results

3.1. NDGA induces the uptake of organic cations by macrophages

ATPe induces apoptosis and the uptake of large organic molecules such as Lucifer yellow and ethidium by macrophages, two phenomena that involve the activation of P2X7 receptors. We have previously shown that inhibitors of AA-5-LO pathway prevent ATPe-induced apoptosis [14], but the effect of these inhibitors on the uptake of organic molecules was not investigated. Therefore, our first experiment was designed to investigate the effects the inhibitors of the AA-5-LO pathway on the uptake of ethidium, a DNA-binding cationic dye that has been widely used to investigate P2X7 activation. To our surprise we observed that treating macrophages with 50 μM NDGA at 37 °C for 10 min, the standard condition used to test the effect of ATPe, induced the uptake of ethidium at similar or higher levels than the uptake obtained by exposing the cells to 5 mM ATP (Fig. 1). The uptake of ethidium was observed in freshly isolated cells (Fig. 1A–B) and in adherent macrophages in the culture dish (Fig. 1C). When compared to dye uptake induced by ATP, the uptake of ethidium induced by NDGA was more intense, as characterized by a more homogeneous distribution in the population and a higher mean fluorescence per cell (Fig. 1). As in the case of ATPe-induced uptake, the localization of the dye in the nuclei is an indicative of a transport mechanism that includes a cytoplasmic soluble phase. The dye uptake is abrogated at low temperatures (Fig. 1C) and has an EC_{50} of approximately 30 μM (Fig. 1D). The fast NDGA-induced ethidium uptake is not an indirect consequence of cell death since, in agreement with our previously published data [14], and contrary to ATPe, it does not induce neither apoptosis nor necrosis in macrophages (Fig. 1E–F). Of note, we also observed that the effect of NDGA and MK886 (see below) is inhibited in the presence of fetal bovine serum

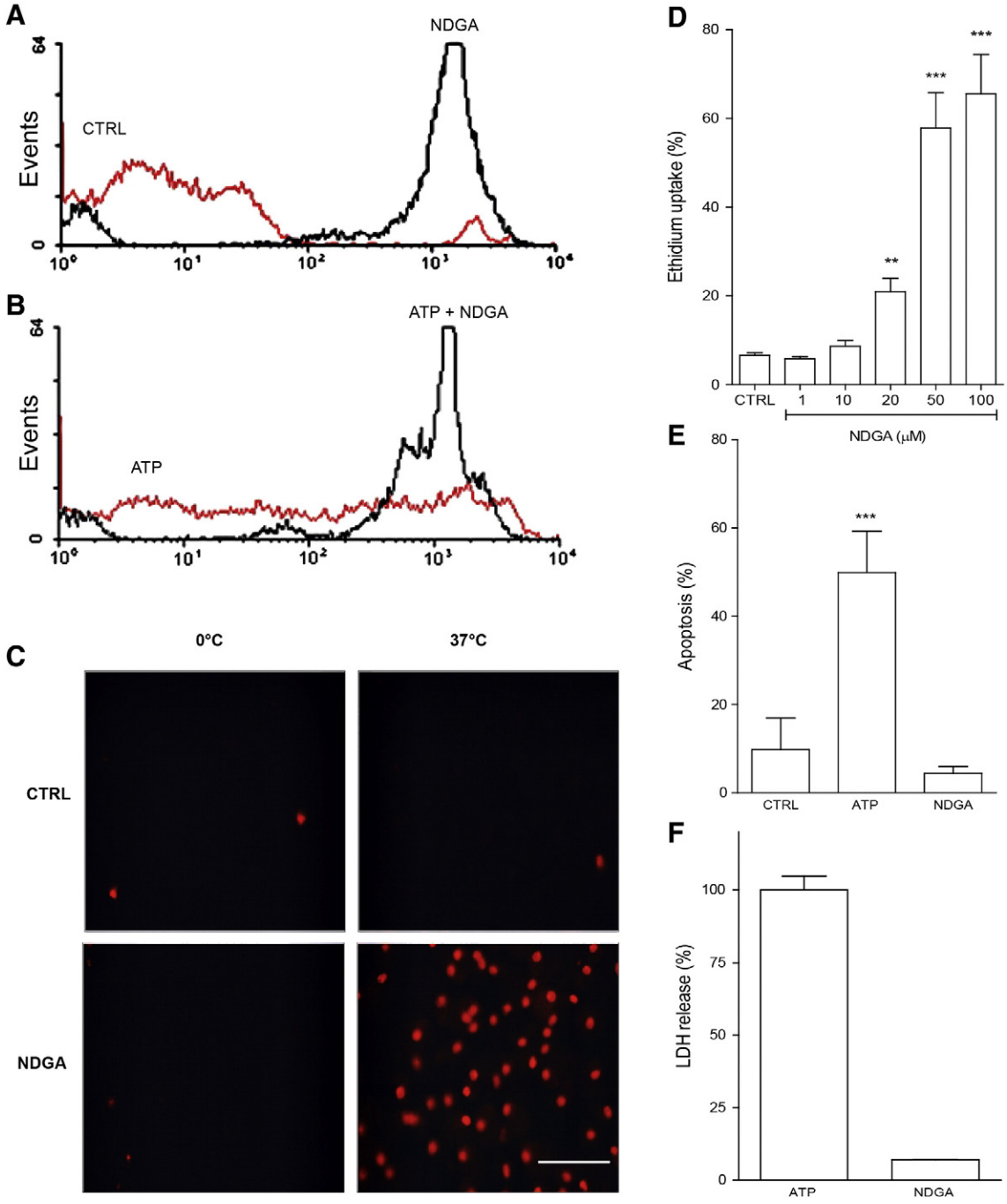

Fig. 1. NDGA induces the uptake of organic cations but not cell death in macrophages. Unless otherwise specified, all experiments were performed using 5 mM ATP and 50 μM NDGA. **Dye uptake**: freshly isolated (A, B, D) and adherent macrophages (C) were incubated for 10 min in normal salt solution containing or not NDGA or ATP and ethidium bromide. Dye uptake was monitored by flow cytometry (A, B, D) or fluorescent microscopy (C). (A) Dye uptake in the absence of drugs (gray line) and in the presence of NDGA (dark line). (B) Dye uptake induced by ATP (red line) and by ATP plus NDGA (dark line). (C) Dye uptake measured by fluorescent microscopy at 0 °C (left panels) and at 37 °C (right panels). (D) Dye uptake obtained by exposing cells to 0–100 μM NDGA. **Cell death**: apoptosis (E) and necrosis (F) were determined in adherent macrophages 6 h after exposure to ATP or NDGA for 20 min in normal salt solution. All experiments (except in the left panels of (C)) were performed at 37 °C. In (F) n = 2. $^{**}p < 0.01$; $^{***}p < 0.001$. Bar in (C): 100 μm.

(Supplementary data). We therefore used only salt solutions to perform our dye-uptake experiments

3.2. Inhibitors of the 5-LO pathway induce the uptake of organic cations by macrophages

We next investigated whether other inhibitors of AA pathways, previously tested for the inhibition of apoptosis [14], also trigger the same ethidium uptake phenomenon. We found that the inhibitors of the 5-LO pathway MK886, Zileuton, and AA-861 that inhibit ATP-induced apoptosis [14] also induce the uptake of ethidium (Fig. 2). However, mepacrine, a generic PLA2 inhibitor, and BEL, an iPLA2 inhibitor, drugs that inhibit some of the enzymes that produce the AA used by the 5-LO and do not inhibit ATP-induced apoptosis [14], as well as the inhibitors of the Cox pathway ASA and NS-398, that also had no effect on ATP-induced apoptosis [14], did not induce any significant dye uptake under our experimental conditions (Fig. 2).

The above results suggested that a selective inhibition of 5-LO enzymes is the triggering step to induce the dye uptake phenomenon. However, transport phenomena induced by NDGA, MK886 and ATP

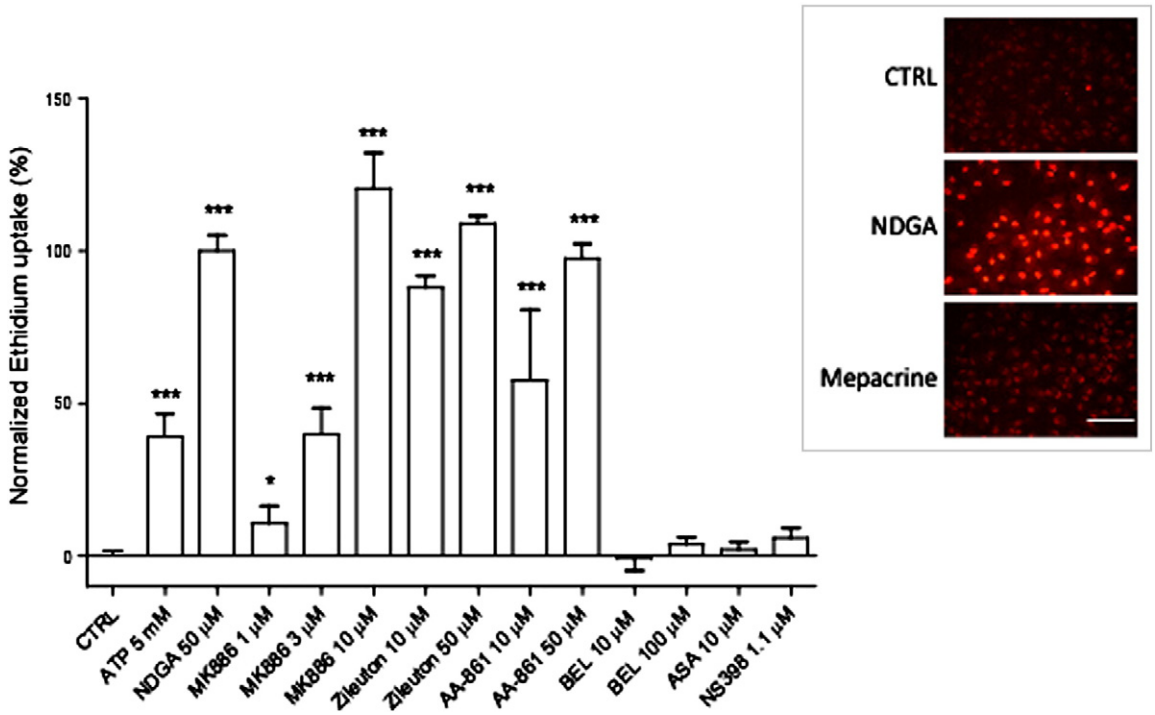

Fig. 2. Inhibitors of the 5-LO pathway induce the uptake or organic cations by macrophages. Freshly isolated macrophages (white bars) and adherent macrophages (insert) were incubated for 10 min at 37 °C in normal salt solution containing or not ATP and several inhibitors of AA metabolism at the indicated concentrations, in the presence of ethidium bromide. $^{**}p < 0.01$ and $^{***}p < 0.001$. Bar in the insert: 100 µm.

are not significantly changed in macrophages derived from 5-LO deficient mice (Fig. 3), suggesting that these inhibitors may have another common target.

3.3. The dye-uptake mechanism is selective to cations and independent of P2X7 receptors

The fast uptake of ethidium by inhibitors of the 5-LO pathway in macrophages resembles the P2X7-associated "membrane permeabilization", a phenomenon characterized by a fast uptake of organic cations and anions limited to a M_r of up to 800–900 Da [75]. We have previously shown this to be a more complex phenomenon involving at least two distinct pathways: one for anions and another for cations [71]. We next performed a series of experiments to compare the two phenomena using a panel of cationic and anionic dyes. We showed that, while ATP can induce the uptake of the anionic dyes (M_r for the free ion in Da) CF (376) and LY (443), the cation-containing dye SR-B (559), the DNA-binding dyes ethidium (314), and TOPRO-3 (417)

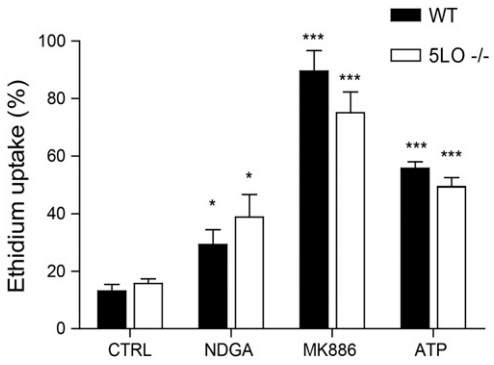

Fig. 3. Dye uptake in macrophages from 5-LO$^{-/-}$ mice. Freshly isolated macrophages were obtained from WT (black bars) and 5-LO deficient (white bars) mice and were incubated for 10 min at 37 °C in normal salt solution containing or not 5 mM ATP, 50 µM NDGA or 10 µM MK886 in the presence of ethidium bromide. $^{*}p < 0.05$ and $^{***}p < 0.001$.

(Fig. 4A–B), propidium (418), and YO-PRO-1 (375) (data not shown), NDGA induce the uptake of all cationic dyes tested, but did not induce the uptake of the anions LY(443) and CF (376) (Fig. 4A–B). In addition, TOTO-3 (847), the DNA-binding cation with the larger M_r tested, was taken up by NDGA-treated cells only (Fig. 4A). Besides the selectivity differences between ATP-induced and LO inhibitor-induced dye uptake mechanisms, we also showed that NDGA can induce ethidium uptake even in macrophages derived from animals deficient for the P2X7 gene (Fig. 4C), while ATP induced no significant effect, as expected [73].

The charge selectivity of this new transport mechanism did not allow us to achieve our initial goal – to investigate the effect the inhibitors AA-5-LO pathway reported to prevent ATPe-induced apoptosis, on the ATPe-induced "permeabilization" – using cationic dyes. We therefore investigated the effect of these inhibitors on ATPe-induced uptake of the anionic dye CF. MK886 (Fig. 4D) and NDGA (data not shown) had no inhibitory effect on ATPe-induced CF uptake, data that suggest that P2X7 receptors are not blocked by inhibitors of AA-5-LO pathway.

3.4. Involvement of ATP release induced inhibitors of the AA-5-LO pathway

The above experiments indicate that the uptake of cationic organic dyes requires neither P2X7 receptors nor active 5-LO enzymes. We then looked for alternative mechanisms involving the 5-LO inhibitors used here. During the course of the experiments aimed to investigate the effect of mepacrine on the uptake of cationic dyes (Fig. 2), one casual observation called our attention to the possibility that 5-LO inhibitors may induce macrophages to release ATP. We noticed that the intensity of its typical green fluorescence, frequently used as an indicator of cellular ATP content [10], displayed a strong decrease after addition of NDGA (Fig. 5A). This observation was consistent with a previous report showing that NDGA could induce the depletion of ATP in a human hepatoma cell line [5]. However, as mepacrine may also concentrate in any acidic organelles [46], we next used the more specific and quantitative luciferin-luciferase luminometry assays and showed that NDGA and MK886 induced a significant increase in extracellular ATP concentration (Fig. 5B and C). The extracellular ATP content showed a rapid increment

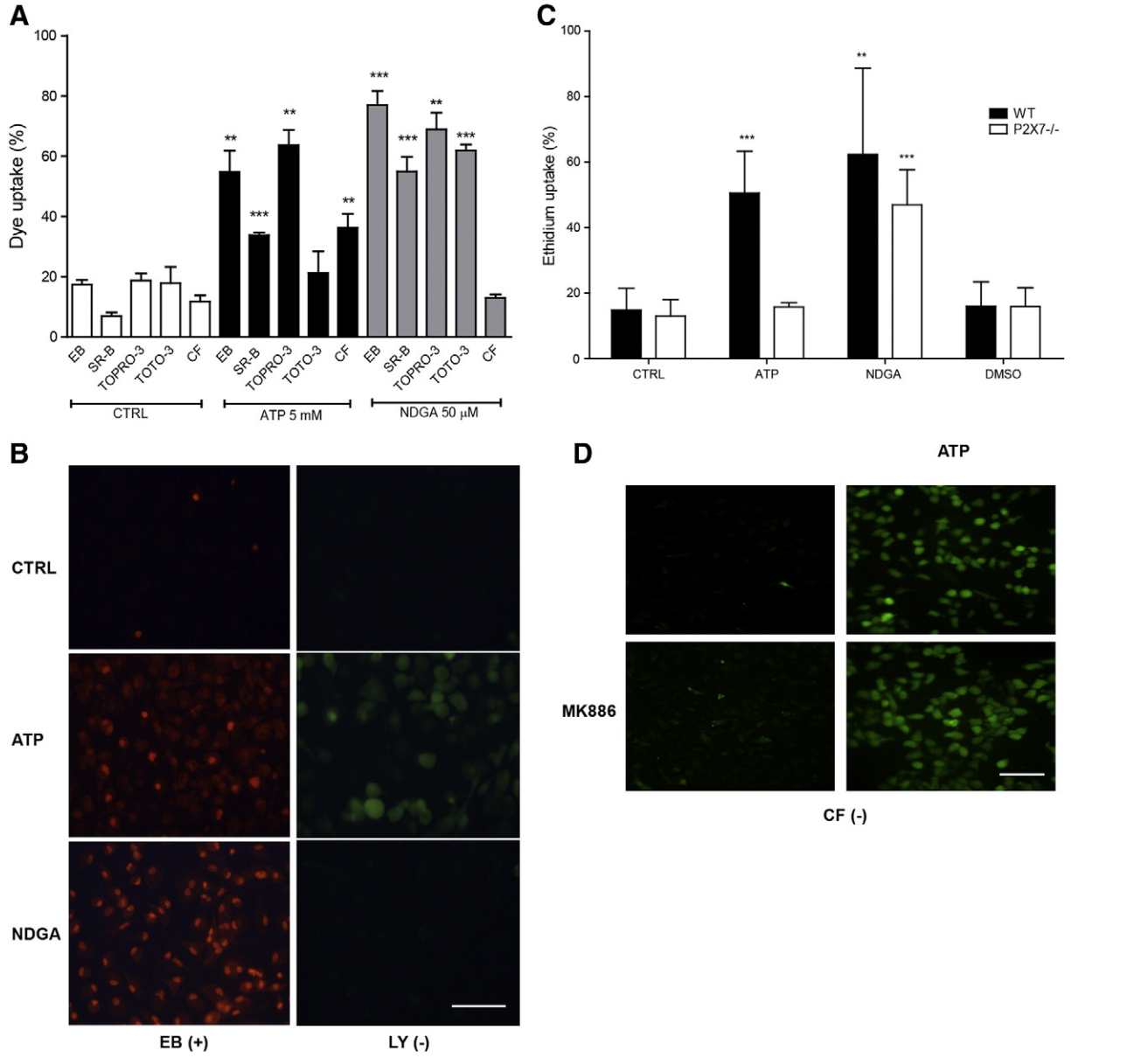

Fig. 4. Dye-uptake mechanism is selective to cations and independent of P2X7. Freshly isolated (A, C) and adherent macrophages (B, D) were incubated for 10 min in normal salt solution containing or not 50 μM NDGA, 10 μM MK886 or 5 mM ATP and different cationic (EB, SR-B, TOPRO-3, TOTO-3) and anionic dyes (CF, LY) as indicated. Dye uptake was monitored by flow cytometry (A, C) or fluorescent microscopy (B, D). (A) Dye uptake in the absence of drugs (white bars), in the presence of ATP (black bars), and NDGA (gray bars). (B) Uptake of ethidium (left panels) and Lucifer yellow (right panels) induced by ATP or NDGA as indicated. (C) Ethidium uptake by freshly isolated macrophages obtained from WT (black bars) and P2X7-deficient (white bars). (D) Uptake of CF induced by ATP (upper right), MK886 (lower felt) or both (lower right). The control, in the absence of any inhibitors is shown in the upper left panel. $^{**}p < 0.01$ and $^{***}p < 0.001$. Bar in (B) and (D): 100 μm.

during the first 1–2 min after the stimulus and its concentration decreased smoothly during the next 20–30 min. The release of ATP was blocked by brefeldin A (Fig. 5C), suggesting the involvement of an exocytic process. We next showed that the addition of apyrase at a concentration that completely abrogates the raise in extracellular ATP concentration (data not show) significantly decreased the dye uptake phenomenon induced by either NDGA or MK886 (Fig. 5D), suggesting the involvement of a paracrine effect ATP in triggering the cation transport.

3.5. Influx of calcium is not involved in dye uptake induced by 5-LO inhibitors

The uptake of cationic dyes and the release of ATP could involve the formation of large non-selective cation channels or the activation of other transport mechanisms. We approached this question by measuring the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and Ca^{2+} influx in Fura-2 loaded macrophages. We showed that the pathway for selective uptake of cationic dyes does not necessarily involve the opening of a non-selective cation channels since NDGA, but not MK886, induced a sustained increase of $[Ca^{2+}]_i$ at the same doses that induced dye uptake (Fig. 6A–D). This increase in $[Ca^{2+}]_i$ is dependent on the influx of Ca^{2+} from the extracellular medium since it is absent when cells are placed in Ca^{2+}-free salt solution (Fig. 6E) and is maintained after addition of thapsigargin (1 μM), an inhibitor of the endoplasmic reticulum Ca^{+2} pump that depletes the intracellular Ca^{2+} store [80] (data not shown). In addition, we also showed that dye uptake does not require the presence of Ca^{2+} ions in the extracellular medium or a rise in $[Ca^{2+}]_i$ since ethidium uptake induced by both NDGA and MK886 is maintained also in Ca^{2+}-free extracellular solution (Fig. 6F).

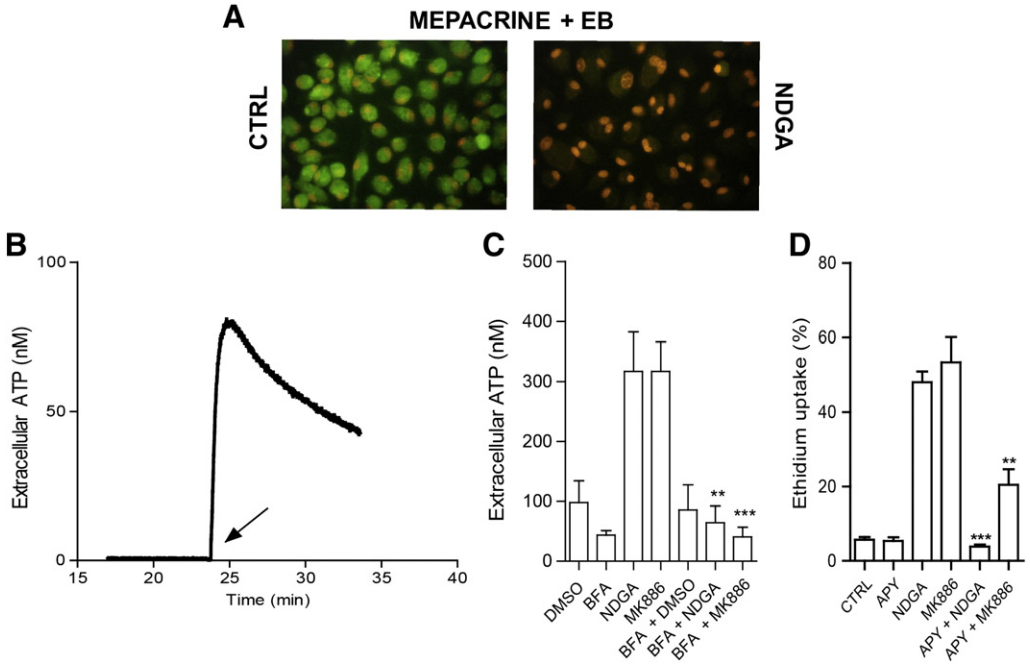

Fig. 5. Inhibitors of the 5-LO pathway induce ATP release and ATP-dependent cation uptake. (A) Mepacrine and ethidium bromide fluorescence in the presence (right panel) or absence (left panel) of NDGA. Adherent macrophages were incubated with normal salt solution for 20 min with 100 μM mepacrine before the addition of 50 μM NDGA for 10 min. Ethidium bromide (10 μM) was then added for 5 min. Cells were kept at 37 °C during the experimental procedure. The emission filter allowed the simultaneous visualization of mepacrine fluorescence (green) and ethidium (orange). (B) Time course of ATP release measured by luciferin-luciferase luminometry. A coverslip with adherent macrophages was placed in the luminometer chamber at 20 °C and ATP release was monitored for 10 min after the addition of NDGA 50 μM (arrow). (C) Extracellular ATP content measured by luciferin-luciferase luminometry. Macrophages were plated in a 48 well plate and ATP concentrations were measured 1 min after the addition of NDGA (50 μM) or MK886 (10 μM) in the presence or not of brefeldin A (BFA 100 nM). Experiments were performed at room temperature. (D) Flow cytometry analysis of ethidium uptake by freshly isolated macrophages in the presence or not of NDGA (50 μM), MK886 (10 μM), or apyrase (2 units). In (C) and (D) DMSO (1%) was used as solvent control. The concentrations of ATP in (B) and (C) were calculated under the same experimental conditions, in parallel experiments using a calibration curve as described in Section 2. At least three independent experiments were performed in each case. $^{**}p < 0.01$ and $^{***}p < 0.001$.

4. Discussion

Many channels and other transport mechanisms are present in macrophage membranes and the study of the effects of anti-inflammatory drugs in these transporters may help to understand the action mechanisms of these drugs and ultimately help to improve their design therapeutic and use. The experiments described in this work were initially planned to investigate the effects of several inhibitors of the AA-pathways on the ATPe-induced, P2X7-mediated dye uptake, usually called "permeabilization", of macrophages [60,74]. The focus on this pathway came from our previous results showing that ATPe-induced apoptosis, but not necrosis, is blocked by the inhibitors of the 5-LO pathway: NDGA, MK886, Zileuton, and AA-861. ATPe-induced apoptosis was also blocked by mepacrine, a general inhibitor of phospholipase A2 (PLA2) and by BEL, a selective inhibitor of calcium-independent PLA2 (iPLA2). The inhibitors of the AA-cyclooxygenase pathway ASA and NS-398 had no effect [14]. It seemed reasonable to ask whether the blockade of apoptosis could be a consequence of blocking the P2X7-associated phenomena.

The surprising observation that inhibitors of the 5-LO pathway activate the uptake of cations interfered with our ability to answer that question but unveiled two yet undescribed transport phenomena present in macrophages – the uptake or organic cations and the release of ATP – and opened new possibilities to understand the action mechanism of some anti-inflammatory drugs. In this work, we describe some of the basic properties of these transport mechanisms.

We initially showed that NDGA, MK886 and other inhibitors of the AA-LO pathway could also induce the uptake of organic dyes. Mepacrine and BEL, which inhibit ATP-induced apoptosis, did not induce significant dye uptake under the same experimental conditions. This set of data suggested that inhibition of P2X7-induced apoptosis and the induction of this new organic cation uptake mechanism are unrelated phenomena.

Our original observation used NDGA, an inhibitor of AA-LO metabolism [1,70] and a potent antioxidant [87]. This drug has been proposed in a large number of medical applications [54] and a number of effects have been reported such as the blockade of protein transport from the endoplasmic reticulum to the Golgi complex [79], the redistribution of Golgi proteins into the endoplasmic reticulum [29], the modulation of free intracellular calcium concentration in different cells types [13,83], the inhibition of cytokine secretion as well as the endocytic uptake of antigens in human dendritic cells [63], the induction of apoptosis [55,78], the regulation of macrophage proliferation [65] and anti-tumor activity in a range of cell lines, including glioma cells, multiple myeloma cells, prostate cancer cells and human breast cells [38,57,66,86].

Then there is a possibility that either a lipoxygenase-related or -unrelated mechanism is involved in the modulation of one or more transmembrane transport mechanisms in macrophages.

In order to better understand the intracellular pathway involved in the NDGA-induced phenomenon, we used a number of more selective inhibitors of the AA metabolisms. We showed that MK886, an inhibitor of 5-lipoxygenase-activating protein (FLAP), considered a more specific inhibitor of the AA-5-LO pathway [69], and the selective inhibitors of 5-LO, Zileuton and AA-861 [36,68] also induced the uptake of organic cations. The inhibitors of the Cox pathway ASA and NS398, and the inhibitors of PLA2 mepacrine and BEL induced no dye uptake. These results strongly indicate that the uptake of cationic dyes here described for macrophages is selectively triggered by inhibitors of 5-LO pathway of the AA metabolism.

In addition, we also notice that the phenomenon described here seems to be unrelated with the anti-apoptotic effect of the inhibitors used since, although the inhibitors of PLA2 mepacrine and BEL inhibit

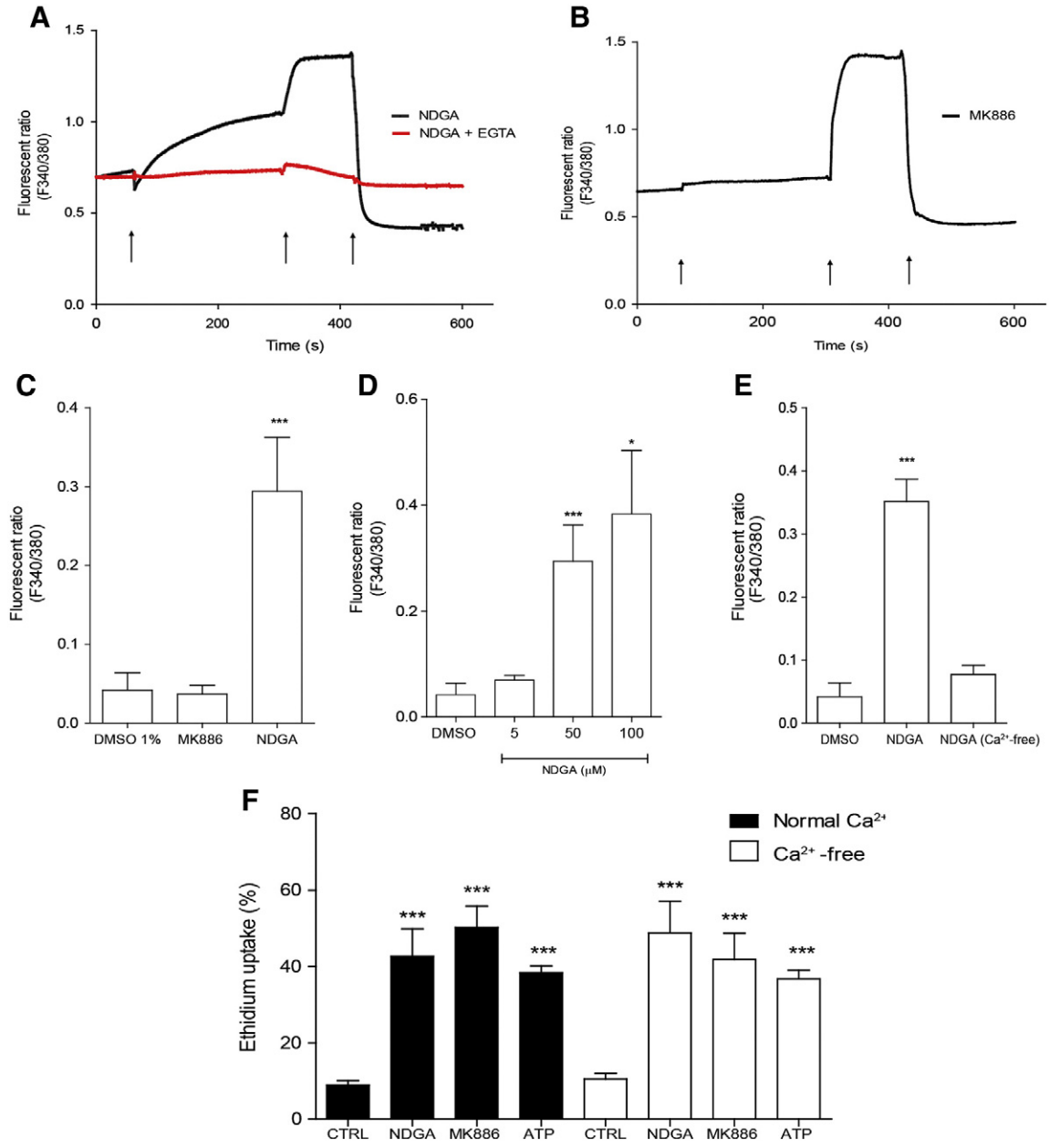

Fig. 6. Influx of Ca^{2+} is not involved in dye uptake induced by 5-LO inhibitors. Free intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ (A-E) and dye uptake (F) of adherent and freshly isolated macrophages respectively. **$[Ca^{2+}]_i$ measurement**: Cells were loaded with Fura-2 as described in Section 2 and $[Ca^{2+}]_i$ was monitored in arbitrary units as F340/380 ratio. Experiments were performed at 37 °C in normal salt solution or in a Ca^{2+}-free solution containing 1 mM EGTA, as indicated. (A) Variations $[Ca^{2+}]_i$ induced by 50 μM NDGA (first arrow), 20 μM ionomycin (second arrow) and 20 mM EGTA (third arrow) in normal salt solution (black line) or in Ca^{2+}-free solution (gray line). (B) Variations $[Ca^{2+}]_i$ induced by 10 μM MK886 (first arrow), 20 μM ionomycin (second arrow) and 20 mM EGTA (third arrow) in normal salt solution. (C-E) Data represent the mean % ± SEM of $[Ca^{2+}]_i$ measured 5 min after addition of: (C) 1% DMSO, 10 μM MK886, or 50 μM NDGA in normal salt solution; (D) 1% DMSO or 5–100 μM NDGA in normal salt solution; (E) 1% DMSO or 50 μM NDGA in normal salt solution or 50 μM NDGA in Ca^{2+}-free salt solution. **Dye uptake**: Macrophages were incubated for 10 min at 37 °C in normal salt solution (black bars) or in Ca^{2+}-free solution (white bars) or not 5 mM ATP, 50 μM NDGA or 10 μM MK886 in the presence of ethidium bromide. Dye uptake was measured by flow cytometry. $^{*}p < 0.05$ and $^{***}p < 0.001$.

ATP-induced apoptosis as do the inhibitors of the 5-LO pathway, only the latter induced the dye uptake phenomenon.

The mechanism of action of the inhibitors of the 5-LO pathway remains unclear. We showed that NDGA- and MK886-induced ethidium uptake is not significantly changed in macrophages derived from 5-LO deficient mice (Fig. 3). These data suggest that other 5-LO-independent mechanism may also participate in the triggering of the cation-uptake mechanism. These inhibitors have different structures and mechanism of action, affecting different targets of the AA metabolism [59,61]. Nonetheless, several reports indicate that some of them may have other common targets. Examples are anti-oxidant activity by NDGA, Zileuton, AA861 and MK886 [19,59], and the 5-LO-independent block of the divalent cation channel TRPM7 by NDGA, AA861, and MK886, but not by Zieluton [12].

Furthermore, it has been described that MK886 and NDGA mediates mitochondrial dysfunction with possible consequences on the intracellular ATP content [5,7,34]. This is especially interesting as we found that NDGA and MK886 induce a fast release of ATP by macrophages. Together, these two ATP-related effects may cause a significant impact on macrophage physiology and response.

Nevertheless, there is a possibility that the phenomena described in our work may result from a double action of the inhibitors: the blockade

of the 5-LO pathway (intrinsically provided when using macrophages derived from 5-LO-deficient mice) and a second 5-LO-independent target. New experiments are required to clarify these possibilities.

After studying several inhibitors of the AA-5-LO pathway, we proceed with the phenomenological characterization of the dye uptake phenomenon induced by MK886 and NDGA.

We first notice that NDGA induces a significant rise in $[Ca^{2+}]i$ in macrophages (Fig. 6). This Ca^{2+} signaling requires the presence of Ca^{2+} in the extracellular medium, and it is not inhibited by thapsigargin, indicating that it is due to the influx of the ion. However, the Ca^{2+} influx seems to be unrelated to the dye-uptake phenomenon described here because it is absent in the MK886-induced phenomenon. This result suggests that we are dealing with a transport mechanism that is selective for organic cations and not with a non-selective large cation channel. Moreover, the phenomenon requires neither the presence of extracellular Ca^{2+} nor its influx, as shown in the dye-uptake experiments performed in Ca^{2+}-free extracellular solution (Fig. 6).

There is a possibility that we are dealing with two separate phenomena, one for NDGA and another for MK886, but more likely, due to the pleiotropic effects of NDGA, the influx of Ca^{2+} induced by this drug results from an unrelated phenomenon. More studies are needed to clarify the mechanism of Ca^{2+} influx.

Regardless of the nature of the organic cation transport mechanism, it is clear that it requires a cytoplasmic soluble stage in order for the DNA-binding dyes to reach the nuclei. The possibility of a large diffusional channel cannot be excluded but it is unlikely due to the absence of Ca^{2+} influx in the MK886-induced phenomenon. Interestingly, NDGA has been reported to disrupt the endocytic pathway in dendritic cells and this effect was inhibited by serum [63]. However neither the 5-lipoxygenase inhibitor Zileuton nor the FLAP inhibitor MK886 had any effect on this putative dye uptake mechanism [63].

Several lines of evidence indicate that the dye uptake induced by inhibitors of the AA-5-LO pathway is not directly related to the ATPe-induced P2X7-associated dye uptake. It is selective for cations and does not necessarily involve Ca^{2+} influx. Moreover, NDGA and MK886, but not ATP, induce dye uptake in animals deficient for the P2X7 gene. Moreover, we also showed that the ATPe-induced uptake of the anionic dye CF, another hallmark of P2X7 activation in macrophages, was not inhibited by inhibitors of the 5-LO pathway (Fig. 4D).

Taken together, our results indicate that inhibitors of 5-LO pathway such as NDGA and MK886 inhibit ATPe-induced apoptosis without blocking P2X7 receptors. The same inhibitors induce the uptake of organic cationic dye that does not require the presence of P2X7 receptors. However, because we and others have shown that the ATPe-induced P2X7-associated transport mechanism can involve different pathways for organic cations and anions [11,47,71], we cannot discard the possibilities that both cation-uptake phenomena are somehow related. In this regard, we should consider a paracrine role of ATP. Our results showing that apyrase inhibits the dye-uptake phenomenon (Fig. 5D) corroborates this possibility. A direct involvement of P2X7 receptors can be discarded by the experiments using P2X7-deficient macrophages. In addition, the lack of a significant Ca^{2+} signal induced by MK886 (Fig. 6) suggests that no Ca^{2+}-mobilizing P2 receptor is being activated in the process. In keeping with this possibility, apyrase added in a concentration that completely abrogates the raise in extracellular ATP concentration did not block the Ca^{2+} signal induced by NDGA (data not shown). Therefore, we need to consider other possibilities. Macrophages may display several P2 and P1 receptors, as well as the ecto-nucleotidases CD39 and CD73 that are potential targets to ATP and its derivatives [2,40,59] and more biophysical and molecular studies are required to clarify these possibilities.

Inhibitors of the AA-5-LO pathway are increasingly being used in allergic, respiratory, cardiovascular, and anti-tumor therapies [8,59,61,62]. We hope that the phenomena of uptake of organic cations and release of ATP described here may help to improve our knowledge of the action mechanisms of these inhibitors.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.04.006.

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