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Lipoxygenase Metabolites as Mediators of UTP-Induced Intracellular Acidification in Mouse RAW 264.7 Macrophages

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ABSTRACT

In previous studies, we have shown that mouse RAW 264.7 macrophages possess pyrimidinoceptors, coupled to a phosphoinositide-specific phospholipase C, with a higher specificity for UTP than for ATP. In the current study, we explored the mechanism involved in the UTP-induced intracellular acidification seen in this cell line. UTP (30 μM) caused a reversible pH_i decrease of 0.16 $\pm$ 0.01 unit; this effect was not influenced by the removal of extracellular Cl^- or Na^+ ions or by pretreatment with 5-(*N*-ethyl-*N*-isopropyl)-amiloride (10 μM), 5-nitro-2-(3-phenylpropylamino)benzoic acid (100 μM), staurosporine (1 μM), or Ro 31–8220 (1 μM) but was completely abolished by the removal of extracellular Ca^{2+}. UTP (30 μM), thapsigargin (1 μM), and ionomycin (1 μM) each induced a similar extent of external Ca^{2+}-dependent acidification with a similar time-dependency, but the effects were nonadditive. To further investigate the Ca^{2+}-dependent mechanism, we studied the involvement of arachidonic acid (AA) and eicosanoid metabolites. The addition of AA (10 μM) but not arachidic acid (100 μM) produced a reduction in pH_i. UTP, thapsigargin, and ionomycin induced Ca^{2+}-dependent AA release. Furthermore, 4-bromo-phenacyl bromide [30 μM, a phospholipase A_2 (PLA_2) inhibitor], nordihydroguaiaretic acid (50 μM, a lipoxygenase inhibitor), and MK-886 (10 μM, a 5-lipoxygenase-activating protein inhibitor) abolished the UTP- or ionomycin-induced responses, whereas indomethacin (30 μM, a cyclooxygenase inhibitor) and baicalein (10 μM, a selective 12-lipoxygenase inhibitor) had no effect. MAFP (a $cPLA_2$ inhibitor) and REV 5901 (a 5-lipoxygenase inhibitor as well as a competitive antagonist of peptide leukotrienes), but not RHC 80267 (a diacylglycerol lipase inhibitor), also inhibited the UTP-induced response. In contrast, the pH_i response to AA was unaffected by the presence of 4-bromophenacyl bromide or the removal of extracellular Ca^{2+} ions but abolished by addition of NDGA. Exogenous 5-hydroperoxyeicosatetraenoic acid (2 μM) also produced marked acidification, and UTP and ionomycin both induced peptide leukotriene formation. In conclusion, this is the first report indicating that lipoxygenase metabolites act as mediators of the Ca^{2+}-dependent acidification seen in macrophages in response to UTP or ionomycin via activation of $cPLA_2$ and AA release.

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There seem to be multiple homeostatic mechanisms that strictly regulate the pH_i in most cells. Relatively small changes in the pH_i could have profound effects on a variety of cellular functions. For example, pH_i plays a role in the control of DNA synthesis, cellular proliferation (Winkler *et al.*, 1980; Mix *et al.*, 1984; Gelfand *et al.*, 1987), rate of protein synthesis (Chambard and Pouyssegur, 1986), cell fertilization (Winkler *et al.*, 1980), regulation of cell volume (Grinstein *et al.*, 1985), muscle contractility (Fabiato and Fabiato, 1978), formation of second and third messengers (Stella *et al.*, 1995), activity of certain metabolic enzymes (Trivel and Danforth, 1966; Staub *et al.*, 1994), neuronal activity (Irwin *et al.*, 1994), neurotransmitter reuptake (Billups and Attwell, 1996), and apoptosis (Tsao and Lei, 1996). Information regarding the functional properties of pH_i in phagocytic cells is limited, and only a few studies suggest the possible role of intracellular acidification in phagocytes. For example, the respiratory burst that plays an important role in phagocyte microbicidal and tumoricidal activity is accompanied by a burst of intracellular H^+ production, which is associated with the generation of superoxide radicals and an increase in metabolic acid production (Nanda and Grinstein, 1991). Yuli and Oplatka (1987) also proposed that cytosolic acidification functions as an early induction signal for human neutrophil chemotaxis.

Macrophages play a key role in many aspects of acute and

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W.-W.L. and M.-L.W. contributed equally to this study.

ABBREVIATIONS: pH_i, intracellular pH; AM, acetoxymethyl ester; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AA, arachidonic acid; BPB, 4-bromo-phenacylbromide; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DAG, diacylglycerol; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DMEM, Dulbecco's modified Eagle's medium; EIPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride; MAFP, methyl arachidonyl fluorophosphonate; FLAP, 5-lipoxygenase-activating protein; HPETE, hydroperoxyeicosatetraenoic acid; LT, leukotriene; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NDGA, nordihydroguaiaretic acid; PI, phosphoinositide; PKC, protein kinase C; PLC, phospholipase C; PLA, phospholipase A.

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chronic inflammation. Our previous studies first demonstrated the presence in the murine macrophage RAW 264.7 cell line of pyrimidinoceptors that are more selectively activated by UTP and UDP than by ATP and are coupled to the stimulation of PI breakdown and activation of $cPLA_2$ (Lin and Lee, 1996; Lin, 1997), the key enzyme in the release of AA from phospholipids and in the biosynthesis of eicosanoids via the cyclooxygenase or lipoxygenase pathways (Mayer and Marshall, 1993). These findings have stimulated interest in the physiological and pathological roles of endogenous nucleotides, particularly UTP, in macrophage function.

To date, only a few studies have reported that the endogenous protonophore AA and/or its metabolites can induce a decrease in pH_i in certain cell types (Simonson *et al.*, 1988; Sumimoto *et al.*, 1988; Gukovskaya *et al.*, 1989; Astashkin *et al.*, 1993; Wang *et al.*, 1995). In the current study, we first demonstrate the acidification effects of nucleotide analogues in the mouse macrophage cell line RAW 264.7 and then provide evidence that 5-lipoxygenase metabolites mediate both UTP- and ionomycin-induced intracellular acidification.

Experimental Procedures

Materials. DMEM, fetal bovine serum, penicillin, and streptomycin were purchased from GIBCO BRL (Grand Island, NY). [^{3}H]AA (100 Ci/mmol) was from New England Nuclear (Boston, MA). 2′,7′-Bis(carboxyethyl)-5,6-carboxyfluorescein/AM and Fura-2/AM were from Molecular Probes (Eugene, OR). Ro31–8220 and MK-886 (3-[1-(*p*-chlorobenzyl)-5-(isopropyl)-3-*t*-butylthioindol-2-yl]2,2-dimethylpropanoic acid) were from Calbiochem (La Jolla, CA). Baicalein, 5-(*S*)-HPETE, 12-(*S*)-HPETE, and RHC 80267 from BIOMOL (Plymouth Meeting, PA). EIPA and 5-nitro-2-(3-phenylpropylamino)benzoic acid were from RBI (Natick, MA). MAFP and REV 5901 were from Cayman Chemical (Ann Arbor, MI). The enzyme immunoassays for LTB_4 and peptide LTs (C_4, D_4, and E_4) were purchased from Amersham (Arlington Heights, IL). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Cell culture. RAW 264.7 cells, generously provided by Dr. Yen-Jen Sung (Department of Anatomy, National Yang-Ming University School of Medicine, Taiwan), were grown on coverslips (for pH_i and $[Ca^{2+}]_i$ measurement) and in 24-well plates (for AA release) at 37° in DMEM (supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin) in a humidified atmosphere of 95% air/5% CO_2.

Measurement of pH_i. This method has already been described in detail (Wu *et al.*, 1994). In brief, RAW 264.7 cells, grown on a coverslip, were loaded with 5 μM 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein for 30 min at room temperature in HEPES-buffered (i.e., nominally HCO_3^- free) solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 2.0 mM $CaCl_2$, 1.2 mM KH_2PO_4, 10 mM glucose, and 20 mM HEPES, pH adjusted to 7.4 at 37° with NaOH). The cells then were washed with the same solution and excited alternately by 490- and 440-nm wavelength light using a filter wheel (Cairn Research, Kent, England), rotating at 32 Hz. The overall sampling rate was 0.5 Hz. In some experiments, extracellular Ca^{2+} ions were removed, Na^+ ions were replaced with *N*-methyl-D-glucamine, or Cl^- ions were replaced by gluconate. The 490/440-nm emission ratio was calculated and converted to a linear pH scale using *in situ* calibration data obtained at the end of the experiment according to the nigericin technique (Rink *et al.*, 1982). Between pH_i 6.0 and 8.0, the response is linear and fits the equation: $pH_i = pK + \log[(R_{max} - R)/(R - R_{min})] + \log(F_{440min}/F_{440max})$, where R is the ratio of 530-nm fluorescence (490-nm excitation) to 530-nm fluorescence (440-nm excitation); R_{max} and R_{min} are the maximum and minimum ratio values from the data curve, respectively; and pK is the dissociation constant for the dye, taken as 55 nM, pH 7.26.

Measurement of $[Ca^{2+}]_i$. Cells grown on glass slides were loaded with 3 μM Fura-2/AM and pluronic F-127 (0.02% v/v) in DMEM at 37° for 45 min. The fluorescence was monitored on a PTI M-series spectrofluorometer, using dual-excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. The $[Ca^{2+}]_i$ was calculated from the ratio of the fluorescence at the two excitation wavelengths, as described by Grynkiewicz *et al.* (1985): $[Ca^{2+}]_i = K_d(R - R_{min}/R_{max} - R)(S_{f2}/S_{b2})$, where R is the ratio of 510-nm fluorescence (340-nm excitation) to 510-nm fluorescence (380-nm excitation); R_{max} (2 mM Ca^{2+}) and R_{min} (10 mM EGTA in Ca^{2+}-free medium) are the maximum and minimum ratio values from the data curve, respectively; K_d is the dissociation constant for the dye, taken as 224 nM at 37°; and S_{f2}/S_{b2} is the ratio of the 380-nm signals determined at R_{min} and R_{max}.

AA release. AA release was measured as described previously (Lin and Lee, 1996). In brief, cells were prelabeled with 0.3 μCi/ml [^{3}H]AA in DMEM for 24 hr at 37° and then washed twice with HEPES-buffered solution and incubated in HEPES solution containing 0.5% fatty acid-free bovine serum albumin before stimulation with UTP, thapsigargin, or ionomycin (1 μM) at 37° for 30 min. At the end of the incubation period, the medium was removed and centrifuged at 250 × *g* for 5 min to remove floating cells, and the radioactivity in the supernatant was measured.

LT assay. RAW 264.7 cells, washed with HEPES-buffered solution, were treated with various stimuli for 8 min at 37°C and then the medium was collected and subjected to enzyme immunoassay for LTB_4 and peptide LTs (C_4, D_4, and E_4), according to the manufacturer's manual.

MTT assay. After drug treatment, MTT (0.5 mg/ml) was added to the cultures, and the blue color was allowed to develop for 1 hr. After aspiration of the medium, 100 μl of dimethyl sulfoxide was used to solubilize the blue crystals. Samples were read at a test wavelength of 570 nm and reference wavelength of 630 nm. The net absorbance (absorbance at 570 nm minus absorbance at 630 nm) is an index for cell viability.

Statistical analysis. Each experiment was performed several times. Values are presented as mean ± standard error. The statistical significance of differences between the mean values was evaluated using Student's *t* test, with $p < 0.05$ considered significant.

Results

UTP-induced extracellular Ca^{2+}-dependent acidification. Of the various nucleotide analogues tested, UTP was found to be the most potent in causing cytosolic acidification. Fig. 1 shows individual results for the concentration-dependent effect of UTP and the effects of UDP, UMP, and ATP. The minimal concentrations required to induce acidification were 10 μM for UTP and 100 μM for UDP and ATP. The effects were reversible and occurred rapidly, reaching the maximal response within 3 min, after which pH_i returned to basal levels within 10 min. In a series of experiments, 30 μM UTP lowered the pH_i from its basal level of 7.36 ± 0.02 pH unit (20 experiments) to a minimum value of 7.20 ± 0.01 unit (20 experiments), whereas 100 μM ATP or UDP caused a decrease in pH_i of 0.08 ± 0.01 unit (4 experiments) or 0.10 ± 0.02 unit (4 experiments), respectively. No significant intracellular acidification was seen with UMP at concentrations up to 100 μM. These results suggest that UTP is the most potent nucleotide in inducing intracellular acidification.

At least four major types of acid extrusion mechanisms, the Na^+/H^+ exchanger, Cl^-/HCO_3^- exchanger, Na^+/HCO_3^- cotransporter, and Na^+-dependent Cl^-/HCO_3^- exchanger, can be activated during a pH_i decrease (Wu *et al.*, 1994). The first is EIPA sensitive and the others are DIDS sensitive; we

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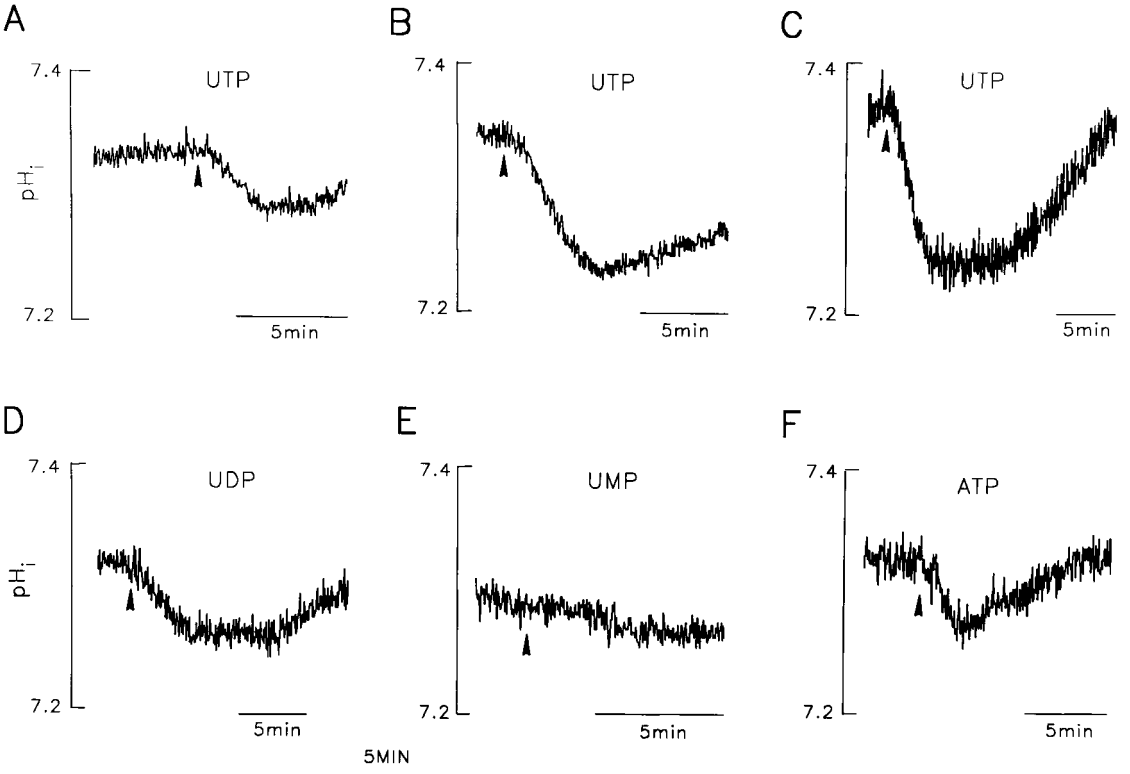

Fig. 1. Effects of nucleotide analogues on pH$_i$. *Arrowheads*, time of nucleotide addition. A, 10 μM UTP. B, 30 μM UTP. C, 100 μM UTP. D, 100 μM UDP. E, 100 μM UMP. F, 100 μM ATP.

therefore tested whether the UTP-induced intracellular acidosis was due to inhibition of these pH$_i$ regulators. The addition of 10 μM EIPA or removal of extracellular Na^+ resulted in an intracellular acidification of 0.06 ± 0.01 (five experiments) or 0.09 ± 0.02 pH unit (five experiments), respectively, probably due to inhibition of the Na^+/H^+ exchanger, resulting in metabolic acid accumulation. UTP stimulation following this acidification, as shown in Table 1, can cause acidification of 0.14 and 0.13 pH unit, respectively, indicating that the Na^+/H^+ exchanger is not involved in the UTP response. 5-Nitro-2-(3-phenylpropylamino)benzoic acid (100 μM), a Cl^- channel blocker, and removal of extracellular Cl^- also had no effect on UTP-induced acidosis, suggesting that HCO_3^- efflux is not the cause of the acidosis. Interestingly, DIDS (500 μM) significantly reduced the UTP-evoked acidification.

TABLE 1
Summary of the pharmacological manipulation on UTP (30 μM)-induced acidification in RAW 264.7 cells

Treatment	Decrease in pH_i (ΔpH_i)	n
None	0.16 ± 0.03	20
Na^+ free (NMG substituted)	0.13 ± 0.02	5
Cl^- free (gluconate substituted)	0.17 ± 0.02	5
Ca^{2+} free (+1 mM EGTA)	0.01 ± 0.01[a]	6
EIPA (10 μM)	0.14 ± 0.02	5
NPPB (100 μM)	0.18 ± 0.03	5
DIDS (500 μM)	0.02 ± 0.01[a]	5
Staurosporine (1 μM)	0.14 ± 0.02	5
Ro 31-8220 (1 μM)	0.15 ± 0.03	5
RHC 80267 (30 μM)	0.16 ± 0.03	5
BPB (30 μM)	0.03 ± 0.01[a]	5
MAFP (50 μM)	0.03 ± 0.01[a]	5
Indomethacin (30 μM)	0.14 ± 0.03	6
NDGA (50 μM)	0.01 ± 0.01[a]	7
MK-886 (10 μM)	0.01 ± 0.01[a]	8
REV 5901 (20 μM)	0.02 ± 0.01[a]	5
Baicalein (10 μM)	0.17 ± 0.02	6

[a] $p < 0.05$ compared with value without drug pretreatment.

Because we previously characterized the UTP-triggered PI signaling cascades in RAW 264.7 cells (Lin and Lee, 1996), we explored the possible involvement of PI/PLC-triggered second messengers in the pH$_i$ decrease. With respect to PKC pathways, we found that 1 μM phorbol-12-myristate-13-acetate, a PKC-activating phorbol ester, had no effect on pH$_i$. (data not shown). When cells were pretreated with either of two PKC inhibitors, staurosporine (1 μM, 20 min) or Ro 31–8220 (1 μM, 20 min), the UTP-induced acidification was unaffected (Table 1). However, the removal of extracellular Ca^{2+} and addition of 1 mM EGTA abolished the UTP response (Table 1 and Fig. 2A). To further confirm the Ca^{2+}-dependency of acidification, we compared the acidosis induced by UTP with that induced by ionomycin (a Ca^{2+} ionophore) or thapsigargin (a molecule known to empty Ca^{2+} stores and elevate $[Ca^{2+}]_i$ in various cell types). Ionomycin (1 μM) caused a pH$_i$ reduction (Fig. 2B), with the effect again abolished by the removal of extracellular Ca^{2+}. The extent and time course of the acidification induced by UTP (30 μM) or ionomycin (1 μM) were similar, but the effects were nonadditive (Fig. 3A). Nonadditivity of the effects of UTP and thapsigargin also was seen (Fig. 3B). This again suggests that a rise in $[Ca^{2+}]_i$ is involved in UTP-induced acidosis.

Involvement of AA and lipoxygenase metabolites in acidification. To further investigate the Ca^{2+}-dependent mechanism, the involvement of AA and eicosanoid metabolites was studied. When applied to RAW 264.7 cells, AA (10

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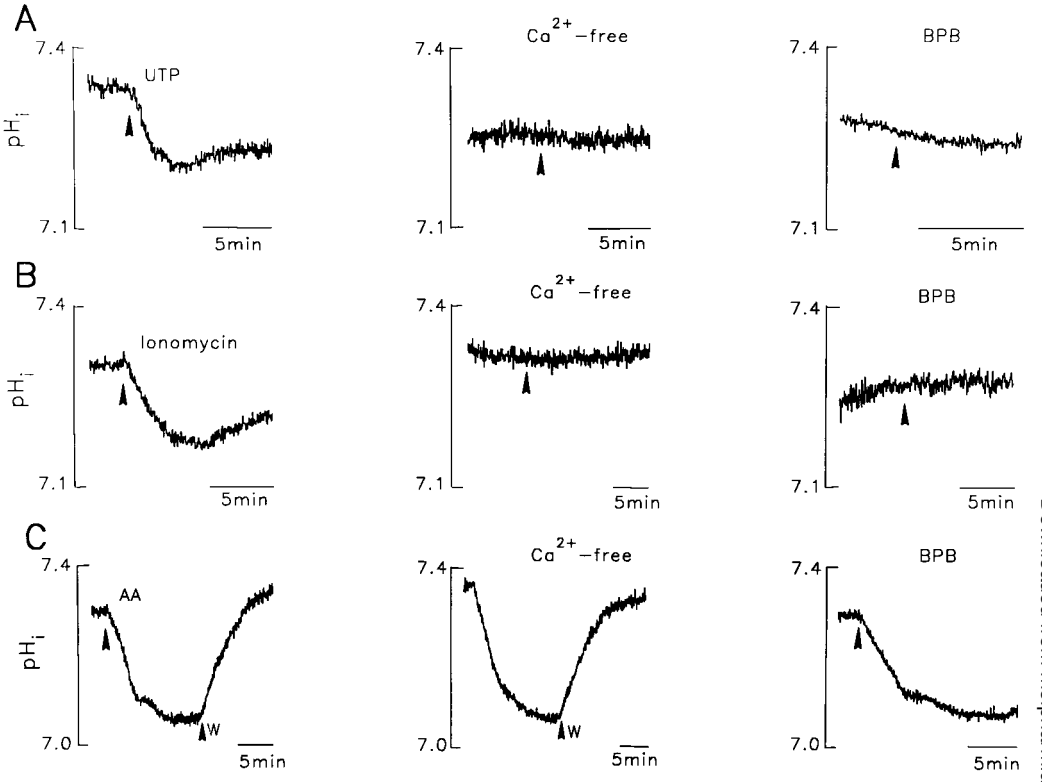

Fig. 2. Effects of Ca^{2+} removal or BPB on UTP-, ionomycin-, and AA-induced acidification. Cells were treated with 30 μM UTP (A), 1 μM ionomycin (B), or 10 μM AA (C) in control HEPES-buffered solution (*left*), Ca^{2+}-free solution (*middle*), or 30 μM BPB-containing solution (*right*). *W*, Washing with solution without AA. *Arrowheads*, time of nucleotide addition.

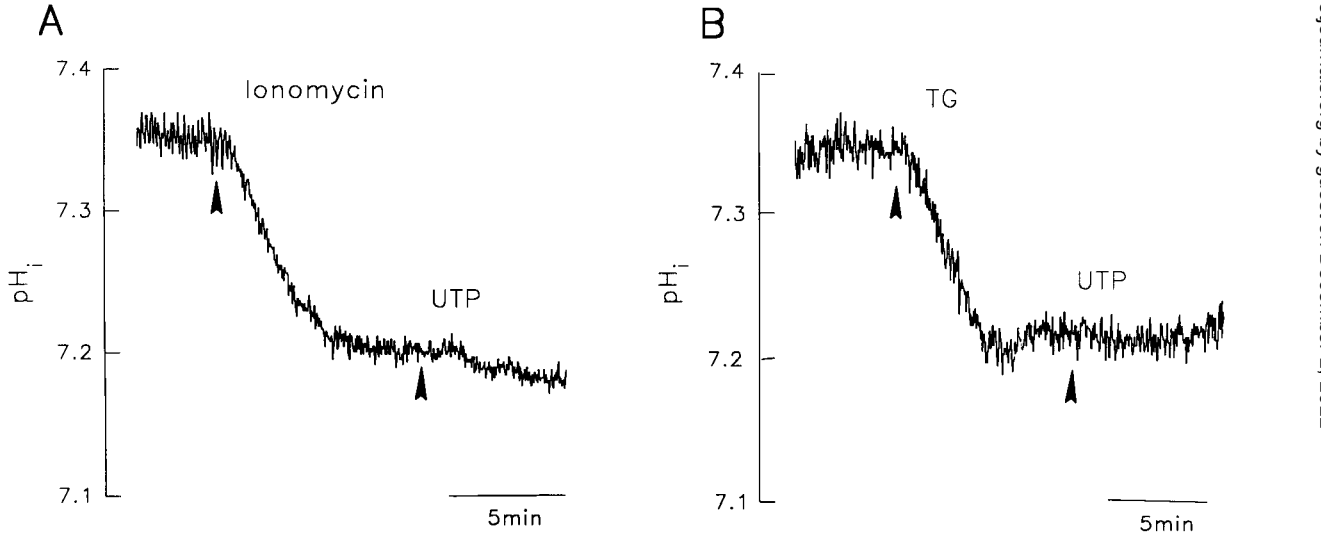

Fig. 3. Nonadditivity of stimulus-induced acidification. *Arrowheads*, time of drug addition. The concentrations used were 30, 1, and 1 μM for UTP, ionomycin, and thapsigargin (*TG*), respectively.

μM) produced a pronounced decrease in pH_i of 0.29 ± 0.03 pH unit (15 experiments), with pH_i remaining at this level for ≥10 min (Fig. 2C). The response was reversible and independent of extracellular Ca^{2+} (Fig. 2C) and decreased the effects of subsequent stimulation with UTP or ionomycin (data not shown). The release of AA is known to occur via two pathways: due to liberation of AA from phospholipids by PLA_2 or to the combined action of PLC (generation of DAG) and DAG lipase (liberation of AA from DAG) (Dieter and Fitzke, 1993). Treatment of cells with BPB (30 μM), a nonselective inhibitor of PLA_2s, abolished the UTP- and ionomycin-induced acidosis but did not affect the response to exogenous AA (Fig. 2 and Table 1). In addition to BPB, we tested MAFP, which is an inhibitor of $cPLA_2$ (Huang *et al.*, 1996). Pretreatment of cells with MAFP (50 μM) significantly inhibited the UTP-induced acidosis by 82% (Table 1). Under the conditions used, neither of the PLA_2 inhibitors had a cytotoxic effect, as determined by MTT assays (data not shown). The DAG lipase inhibitor RHC 80267 was used to investigate the contribution of DAG for AA release (Dieter and Fitzke, 1993). As shown in Table 1, RHC 80267 at a concentration previously shown to inhibit DAG lipase (30 μM) had no effect on the acidification in response to UTP. These results suggest the involvement of $cPLA_2$-, but not DAG lipase-, generated AA pathway in UTP-induced acidosis.

To understand the roles of the downstream part of AA

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pathway, we tested the inhibitors of cyclooxygenase and lipoxygenase. Pretreatment with indomethacin (30 μM), a cyclooxygenase inhibitor, failed to affect the UTP acidification response, whereas NDGA (50 μM), a 5,12-lipoxygenase inhibitor, abolished both the UTP- and AA-induced acidification (Fig. 4 and Table 1). The presence of MK-886 (10 μM), a specific FLAP inhibitor, abolished the UTP- and ionomycin-induced responses and reduced the AA-induced response (Fig. 5), whereas, in contrast, baicalein (10 μM), a specific 12-lipoxygenase inhibitor, had no effect on the UTP or ionomycin responses but attenuated the AA response (Fig. 5). AA-induced intracellular acidosis also was abolished by pretreatment with MK-886 (10 μM) plus baicalein (10 μM) (three experiments, data not shown). REV 5901, a 5-lipoxygenase inhibitor (Musser *et al.*, 1987) as well as a competitive antagonist of peptide LTs (Musser *et al.*, 1987), also markedly attenuated the UTP response (Table 1). All the drugs tested had no cytotoxic effects on RAW 264.7 cells as determined by MTT assays (data not shown).

To further determine whether AA metabolites were involved in acidification and rule out the possibility of the chemical acidity of AA contributing to this event, another AA analogue, arachidic acid, was tested. Arachidic acid at a concentration of 100 μM alone did not induce a pH_i decrease, nor did it alter the effect of AA (Fig. 6A). In addition, 5-(*S*)-HPETE (2–6 μM) produced a concentration-dependent acidification (Fig. 6, B and C). No significant effect on pH_i was seen with 5 μM 12-(S)-HPETE (data not shown), suggesting that 5-(*S*)-HPETE is implicated in UTP-induced intracellular acidosis.

Correlation of the UTP-induced Ca^{2+} increase, AA release, and pH_i decrease. To verify the association of acidification with $cPLA_2$ activation, the AA production induced by UTP, ionomycin, or thapsigargin was studied. Fig. 7 shows that the effects of all three stimuli were inhibited by 30 μM BPB or 500 μM DIDS and completely dependent on extracellular Ca^{2+}, further indicating that the UTP-induced pH_i decrease is mainly due to intracellular AA release.

Using Fura-2 fluorimetry, we then investigated whether UTP-stimulated AA release is dependent on a $[Ca^{2+}]_i$ increase. As shown in Fig. 8A, UTP (30 μM) induced a rapid sustained increase in $[Ca^{2+}]_i$ of 250 $\pm$ 48 nM (eight experiments). BPB (30 μM) did not alter the peak value but attenuated the sustained phase of the UTP-induced $[Ca^{2+}]_i$ increase. In the presence of MK-886 (10 μM), the peak increase in $[Ca^{2+}]_i$ was delayed and the sustained $[Ca^{2+}]$i level was slightly lower. The ionomycin-induced $[Ca^{2+}]_i$ increase was unaffected by either of these treatments (Fig. 8B). In RAW 264.7 cells, neither exogenous AA (30 μM) nor 5-(*S*)-HPETE (5 μM) can induce a $[Ca^{2+}]_i$ increase (data not shown).

UTP increased peptide LT formation. The downstream product of AA metabolism was investigated further in the following experiment. As shown in Table 2, UTP, ionomycin, or thapsigargin can induce the formation of peptide LTC_4, LTD_4, and LTE_4 within 8 min. The increase produced by 1 μM ionomycin was much greater than that induced by either 30 μM UTP or 1 μM thapsigargin. Furthermore, the increase in peptide LT was abolished by pretreatment with MK-886. In contrast, no significant increase in LTB_4 by either UTP or ionomycin was seen within 15 min (data not shown), suggesting the involvement of peptide LTs in UTP-induced intracellular acidosis.

Discussion

The generation of AA and eicosanoids plays a key role in many aspects of acute and chronic inflammation. Many in-

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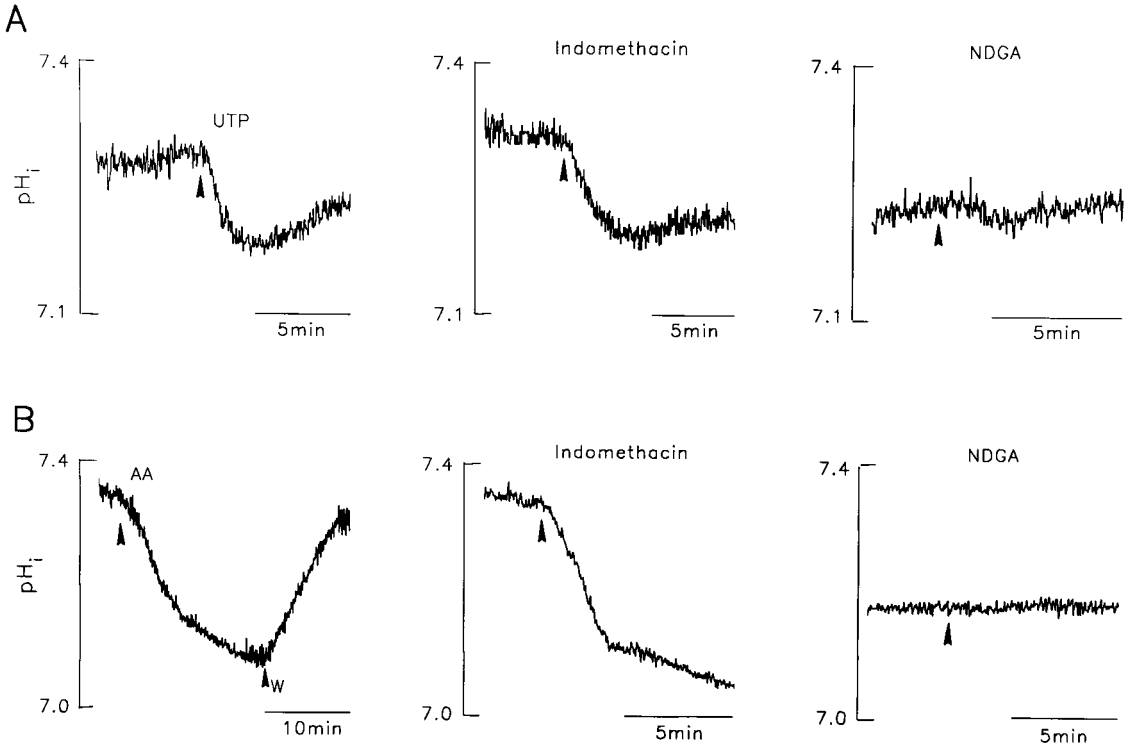

Fig. 4. Involvement of lipoxygenase metabolites in UTP- and AA-induced acidification. A 10-min pretreatment with indomethacin (30 μM, *middle*) or NDGA (50 μM, *right*) was used before 30 μM UTP (A) or 10 μM AA (B) was added (*arrowheads*).

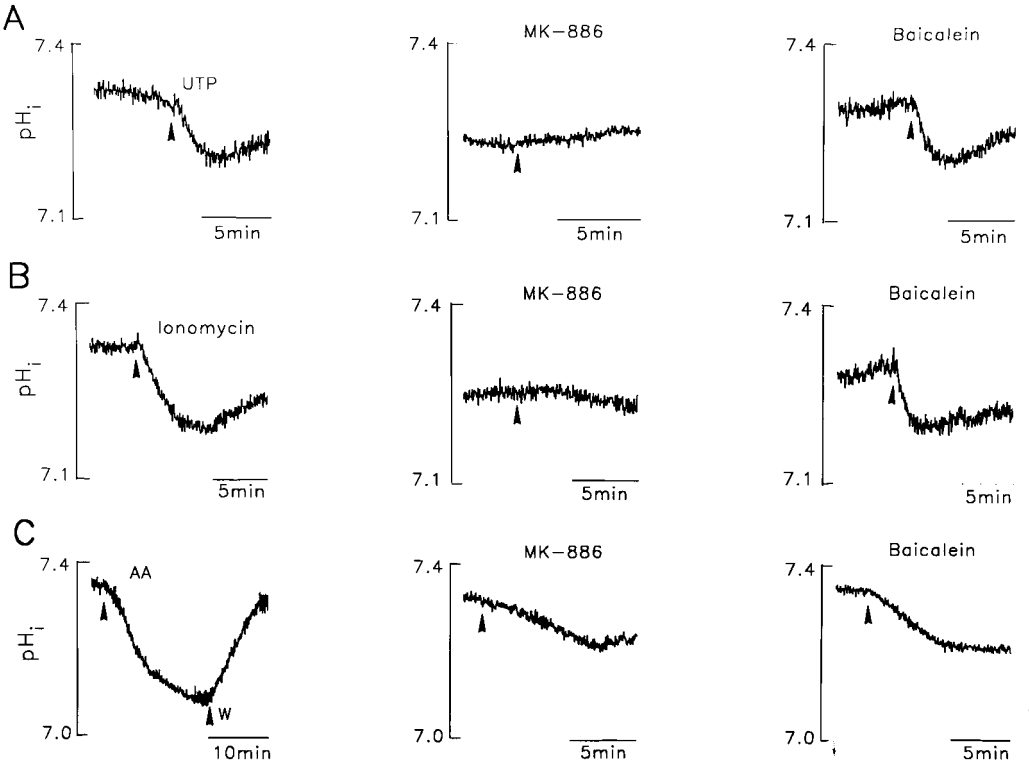

Fig. 5. Effects of MK-886 and baicalein on stimulus-induced acidification. Cells were pretreated for 10 min with 10 μM MK-886 (*middle*) or 10 μM baicalein (*right*) before 30 μM UTP (A), 1 μM ionomycin (B), or 10 μM AA (C) was added (*arrowheads*). *W*, Washing with solution without AA.

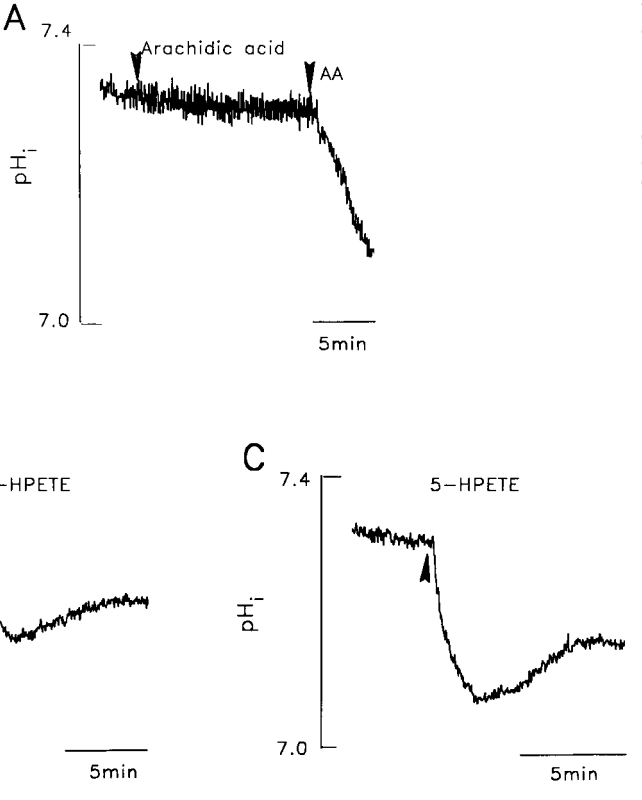

Fig. 6. Effects of arachidic acid and 5-HPETE on the pH$_i$. The concentrations used were 100 and 10 μM for arachidic acid and AA, respectively (A), and 2 (B) and 6 μM (C) for 5-HPETE. *Arrowheads*, time of drug addition.

flammatory mediators (e.g., tumor necrosis factor-α and interleukin-1β) and bacterial endotoxin can stimulate macrophages to release these products (Serhan *et al.*, 1996). The current study is the first to demonstrate that 5-lipoxygenase metabolites act as mediators of the intracellular acidification elicited by UTP, thapsigargin, and ionomycin in macrophages.

After our previous study, which demonstrated the activation of PI/PLC and cPLA$_2$ by pyrimidinoceptors in RAW 264.7 macrophages (Lin and Lee, 1996), we became interested in understanding the cellular signal transduction of UTP and its role in macrophage function. In the current study, we found that cellular acidification can be induced by UTP, thapsigargin, or ionomycin. The dependency on extra-

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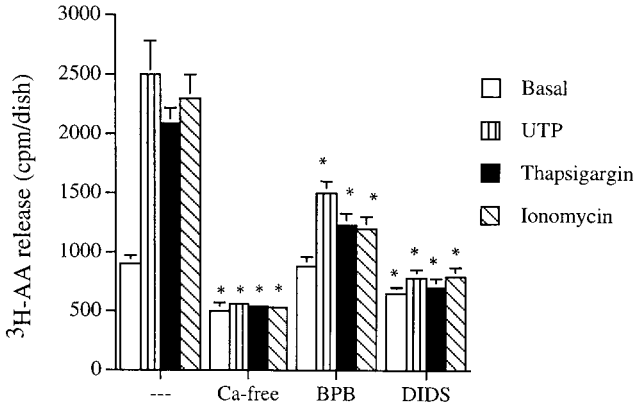

Fig. 7. AA release in RAW 264.7 cells. Cells prelabeled with [^{3}H]AA were stimulated with 30 μM UTP, 1 μM thapsigargin, or 1 μM ionomycin after pretreatment with Ca^{2+}-free physiological salt solution, 30 μM BPB, or 500 μM DIDS for 20 min. Values are mean ± standard error of at least three independent experiments. *, Significant difference ($p < 0.05$) compared with the AA increase without drug pretreatment.

cellular Ca^{2+} and the sensitivity to BPB (an inhibitor of PLA_2-induced AA production) of these three types of stimulus-induced acidification suggested that PLA_2-mediated pathways are involved. The AA-releasing effects of UTP, thapsigargin, and ionomycin and the similar acidification induced by exogenous AA in this cell line strongly supported our conclusion. Although exogenous AA caused a sustained pH_i decrease in RAW 264.7 cells, as seen in thymocytes (Gukovskaya *et al.*, 1989; Astashkin *et al.*, 1993), hippocampal neurons (Wang *et al.*, 1995), and glial cells (Staub *et al.*, 1994), on the basis of the effects of drug inhibitors and 5-(*S*)-HPETE, we found 5-lipoxygenase, but not cyclooxygenase, products to be responsible for the UTP-, ionomycin-, and AA-induced pH_i decreases. Furthermore, the ineffectiveness of arachidic acid (an unsaturated fatty acid that cannot be metabolized by cyclooxygenase or lipoxygenase) on basal pH_i and AA-induced pH_i decrease suggested the AA-induced acidification results from its signaling products and not from the direct effect of exogenous AA on membrane physicochemical properties. In line with this conclusion is our new finding that DIDS (Fig. 7), in addition to its known inhibition of the three HCO_3^--dependent pH_i regulators (Cl^-/HCO_3^- exchanger, Na^+/HCO_3^- cotransporter, and Na^+-dependent Cl^-/HCO_3^- exchanger), can also reduce $cPLA_2$ activation and UTP-induced acidification in RAW 264.7 cells. Similar inhibitory effects on ionomycin- and thapsigargin-induced AA release (Fig. 7) suggest that DIDS acts as an inhibitor of $cPLA_2$ activation. Although the mechanism is still unknown, it seems to be independent of changes in $[Ca^{2+}]_i$, in that the Ca^{2+} ionophore (ionomycin)-induced AA response also was abolished.

The formation of 5-HPETE and LTs from the precursor, AA, is a two-step process consisting of the FLAP-independent/Ca^{2+}-dependent translocation of 5-lipoxygenase to the cell membrane, followed by the FLAP-dependent activation of the enzyme (Rouzer and Kargman, 1988; Woods *et al.*, 1995). FLAP specifically binds to AA and activates 5-lipoxygenase by acting as an AA transfer protein (Abramovitz *et al.*, 1993). MK-886, which inhibits the binding of 5-lipoxygenase to FLAP (Dixon *et al.*, 1990), inhibits both LT synthesis (Table 2) and intracellular acidification (Fig. 5). We therefore concluded that LTs are involved in the Ca^{2+}-dependent acidification in RAW 264.7 macrophages. In this context, the inhibitory effect of REV 5901, a potent inhibitor of 5-lipoxygenase (Musser *et al.*, 1987) as well as a competitive antagonist of peptide LTs (Van Inwegen *et al.*, 1987), on UTP-induced acidification (Table 1) also supports this notion. These findings provide a novel downstream mechanism for AA-induced intracellular acidification. The opposite results, seen in thymocytes, suggest that it is AA, and not its metabolites, that is responsible for the pH_i decrease (Gukovskaya *et al.*, 1989). However, in support of our results, LTD_4 has been shown to elicit cytoplasmic acidification in human mesangial cells (Simonson *et al.*, 1988). Although LTB_4 also is reported to acidify neutrophils (Sumimoto *et al.*, 1988), its involvement in RAW 264.7 macrophages can probably be excluded because we did not detect any significant increase in LTB_4 after UTP or ionomycin treatment.

It has been suggested that cytoplasmic acidification caused by various stimuli, including exogenous AA, may cooperate with calcium mobilization (Naccache *et al.*, 1988; Randriamampita and Trautmann, 1990; Czubayko and Reiser, 1996). In RAW 264.7 macrophages, this cooperativity was seen with pyrimidinoceptor activation, thapsigargin, and ionomycin. Surprisingly, our results show that exogenous AA and 5-(*S*)-HPETE cannot increase $[Ca^{2+}]_i$ (data not shown) and that the AA-induced acidification was extracellular Ca^{2+} independent (Fig. 2), which seems to contradict the Ca^{2+} requirement for 5-lipoxygenase translocation (Rouzer and Kargman, 1988; Woods *et al.*, 1995). There are at least three possible explanations for this observation. First, as shown for alveolar macrophages (Coffey *et al.*, 1992), the 5-lipoxygenase may already be localized in the cell membranes of resting RAW 264.7 cells. Second, as seen in mouse peritoneal macrophages (Randriamampita and Trautmann, 1990), exogenous AA may activate a Ca^{2+} extrusion pathway in an eicosanoid-independent manner, thus compromising the increase in $[Ca^{2+}]_i$. Third, eicosanoids other than 5-lipoxygenase products also may be involved in exogenous AA-induced acidification because both MK-886 (a FLAP inhibitor) and baicalein (a 12-lipoxygenase inhibitor) were required to abolish the response. In line with this evidence, NDGA (a 5- and 12-lipoxygenase inhibitor) abolished the exogenous AA-induced pH_i decrease (Fig. 4). To further investigate the involvement of the 12-lipoxygenase pathway in cellular acidosis, we tested the effect of 12-(*S*)-HPETE. At the maximal concentration (5 μM) at which the solvent (methanol) for commercial 12-(*S*)-HPETE did not alter cell shape, no significant change in pH_i was seen. Although at present we cannot directly demonstrate the profile of eicosanoid products formed by RAW 264.7 cells, as reported in other macrophage types (Laviolette *et al.*, 1988), the lipoxygenase product profile induced by ionophore A23187 or exogenous AA is different.

As demonstrated by the inhibitory effects of BPB on UTP-induced $[Ca^{2+}]_i$ increase, the involvement of eicosanoids in the sustained phase of the UTP-induced $[Ca^{2+}]_i$ increase is suggested. However, the ineffectiveness of exogenous AA and 5-(*S*)-HPETE on the $[Ca^{2+}]_i$ level rules out this possibility and further strengthens our conclusion that the $[Ca^{2+}]_i$ increase is responsible for stimuli-induced $cPLA_2$ activation, which is the upstream signal of intracellular acidification. Whether lysophosphatidylcholine, another metabolite of PLA_2 activation, contributes to the sustained phase of the

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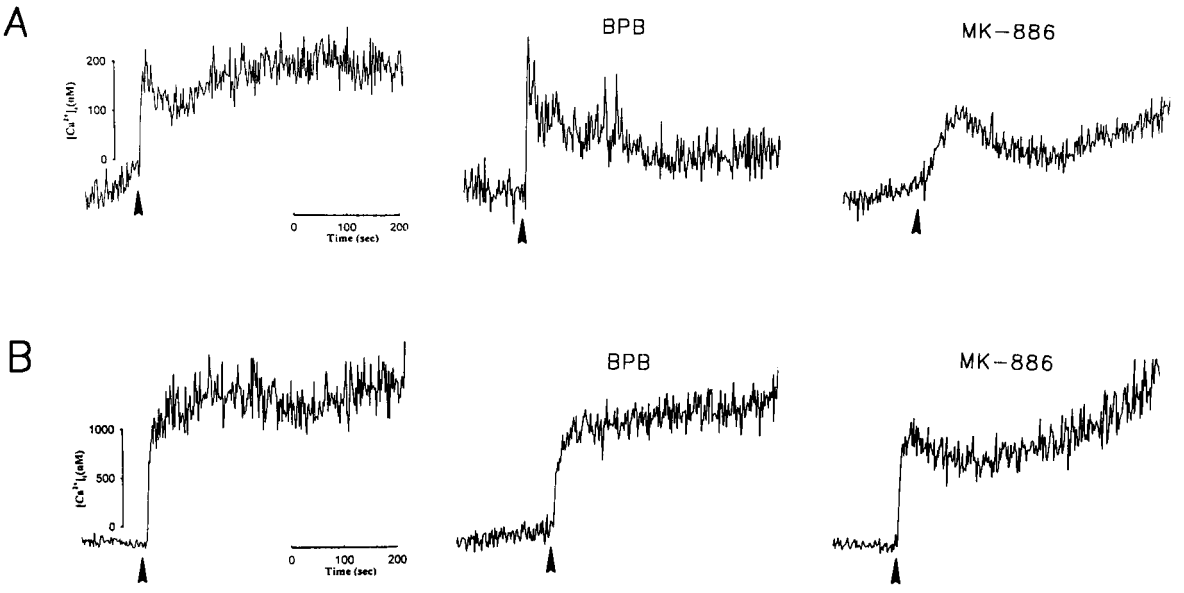

Fig. 8. UTP- and ionomycin-induced $[Ca^{2+}]_i$ increase in RAW 264.7 cells. Cells were pretreated with BPB (30 μM, *middle*) or MK-886 (10 μM, *right*) for 10 min before 30 μM UTP (A) or 1 μM ionomycin (B) was added (*arrowheads*).

TABLE 2
Inhibition effects of MK-886 on stimulus-induced peptide LT formation

Treatment	LTs	
	Control	MK-886 (10 μM)
	pg/well	
Control	55.6 ± 15.1	59.1 ± 8.3
UTP (30 μM)	109.0 ± 15.0[a]	53.1 ± 6.9[b]
Ionomycin (1 μM)	253.5 ± 29.5[a]	52.3 ± 12.2[b]
Thapsigargin (1 μM)	155.4 ± 18.4[a]	Not determined

[a] Significant ($p < 0.05$) increase in peptide LTs.
[b] $p < 0.05$ compared with stimulus-induced peptide LT formation in the absence of MK-886.

UTP-induced $[Ca^{2+}]_i$ increase is under investigation. Although exogenous AA has been shown to inhibit Na^+/H^+ exchange and thus may induce intracellular acidosis in thymocytes (Astashkin *et al.*, 1993), this is not the case in the current study. Neither EIPA nor the use of Na^+-free medium had an effect on the UTP-induced acidification (Table 1), thus excluding involvement of the Na^+/H^+ exchanger in the UTP-induced Ca^{2+}-dependent acidification in macrophages.

Taken together, the $[Ca^{2+}]_i$ increase induced by various stimuli is a crucial step in $cPLA_2$ activation, AA release, and peptide LT formation, which leads to the intracellular acidification of macrophages. However, the physiological role in these cells of the pH_i decrease induced by $cPLA_2$ pathway activation is not yet clear. Recently, it has been established that the inhibitory effect of AA on mitogen-induced lymphocyte proliferation is due primarily to the blockade of transmembrane pH_i signals, associated with a sustained cytosolic acidification (Astashkin *et al.*, 1993). In addition, $cPLA_2$ activity in neurons is stimulated by Ca^{2+} in a pH-dependent manner, with increasing activity as the pH_i is shifted from 7.2 to 7.8 (Stella *et al.*, 1995). In Jurkat T lymphocytes, the intracellular acidification caused by tumor necrosis factor-α and phorbol-12-myristate-13-acetate also potentiates the activation of nuclear factor-κB, a DNA-binding regulatory factor, able to control the transcription of a number of genes (Feuillard *et al.*, 1991). In future studies, we would like to unravel the function of macrophage pyrimidinoceptors associated with cellular acidification and to understand the pH-dependent regulation of $cPLA_2$ signaling efficacy in macrophages.

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