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Short communication

Extracellular nucleotides induce COX-2 up-regulation and prostaglandin E_2 production in human A549 alveolar type II epithelial cells

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Abstract

Extracellular nucleotides regulate ion transport, mucociliary clearance as well as inflammatory properties of the airway epithelium by acting on P2 receptors. Cyclooxygenase-2 (COX-2) is a key enzyme involved in the synthesis of prostaglandins during inflammation. In this study, using calcium imaging, DNA microarray experiments, real-time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and prostaglandin E_2 (PGE_2) measurement, we show for the first time that ATP, UTP or INS365 compound ($P2Y_2$ receptor agonists) up-regulate *COX-2* expression by ~3-fold and enhance the release of PGE_2 in human A549 airway epithelial cells. Our data suggest that P2Y receptors may represent putative targets in airway inflammatory diseases.

Keywords: P2Y receptor; Airway epithelium; COX-2; Prostaglandin E_2; Cystic fibrosis; Extracellular nucleotide

1. Introduction

Nucleotides are released across the membrane of airway epithelial cells into the extracellular compartment in response to different stresses such as mechanical stimulations (Marcet and Boeynaems, 2006). They interact at the cell surface with metabotropic P2Y receptors and thereby regulate the integrated airway epithelial functions like mucociliary clearance, airway defense responses and airway immune function (Marcet and Boeynaems, 2006; Marcet et al., 2007).

Cyclooxygenases (COX-1 and COX-2), enzymes involved in prostaglandin synthesis, are key modulators of airway inflammation (Mitchell and Evans, 1998). The human airway type II alveolar epithelial carcinoma cell line A549 (Lieber et al., 1976), widely used to examine airway inflammation, does not express COX-1 but constitutively expresses COX-2 (Mitchell et al., 1994; Watkins et al., 1999). In addition, nucleotides stimulated the release of arachidonic acid, a rate-limiting step in prostaglandin E_2 (PGE_2) synthesis, and up-regulated inducible nitric oxide synthase expression in A549 cells (Laubinger et al., 2006). Thus, COX-2 and extracellular nucleotides both play a key role in airway inflammation but it remained unknown whether extracellular nucleotides may affect *COX-2* expression. Here, we showed that *COX-2* was a target gene of $P2Y_2$ receptor agonists using microarray technology and real-time RT-PCR and that PGE_2 release was induced by extracellular nucleotides in A549 airway epithelial cells.

2. Materials and methods

2.1. Cell culture

A549 cells, cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum, antibiotics and amphotericin at 37 °C with 5% CO_2/95% air in 6-well plates to 80% confluency, were then stimulated in serum-free medium by nucleotides. ATP, ATPγS, UTP, UDP, NS398, actinomycin D,

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MG132 and PD098059 were obtained from Sigma. INS365 (Up_4U, P^1,P^4-di(uridine 5′-) tetraphosphate) and INS48823 (2′,3′(phenylacetaldehyde acetal)Up_3U) were a gift from Dr B. Yerxa (Inspire, Durham, USA).

2.2. RT-PCR experiments

Total RNA was purified using TRIZOL® reagent (Life Technologies, Netherlands) and Qiagen RNeasy kit (Westburg, Netherlands). PCR experiments were conducted as previously described (Marcet et al., 2007). Briefly, 1 μg of total RNA was reverse transcribed using random hexamers and Superscript-II reverse transcriptase (RT) (Invitrogen, Belgium), then incubated with RNase-free DNase (Invitrogen, Belgium). Negative control experiments were also performed without RT. A set of specific primers for $P2Y_2$, $P2Y_4$ and $P2Y_6$ gene receptors were designed and synthesized (Eurogentec, Belgium): $P2Y_2$ sense, 5′-GCTTCAACGAGGACTTCAAG-3′ and $P2Y_2$ antisense, 5′-CACGCTGATGCAGGTGAGGA-3′; $P2Y_4$ sense, 5′-TGGCATTGTCAGACACCTTGTATGTG-3′ and $P2Y_4$ antisense, 5′-AAGCAGACAGCAAAGACAGTCAGCAC-3′; $P2Y_6$ sense 5′-CCCTGCTGGCCTGCTACTGTCTCCTG-3′ and $P2Y_6$ antisense, 5′-CTAATTCTCCGCATGGTTTGGGG TTGG-3′. PCR experiments were carried out following manufacturer's instructions. The PCR amplification conditions were 94 °C, 5 min, 1 cycle; 94 °C, 1 min, 50 °C, 1 min, 72 °C, 1 min, 35 cycles; 72 °C, 12 min, 1 cycle. A negative PCR control containing water instead of cDNA was performed.

2.3. Microarray experiments

Microarray experiments were performed as previously described (Van Staveren et al., 2006). Briefly, double-stranded cDNA was synthesized from 1 μg of total RNA, followed by production of antisense RNA using an Ampliscribe T7 high-yield transcription kit. After an incubation with 5-(-3-aminoallyl)-dUTP (Sigma-Aldrich) and a labelling with Cy3 and Cy5 (Amersham Pharmacia Biosciences), samples were hybridized onto in-house-manufactured slides containing 23,232 spots with 7541 different identified cDNAs. Microarrays were scanned with a GenePix 4000B scanner (Axon Instruments, Union City, CA). Expression levels were quantified with Genepix Pro 5.0 (Axon Instruments). All hybridizations were replicated with dye swapped. Log_2 ratios averaged over replicates were considered in subsequent analysis.

2.4. Real-time RT-PCR experiments

Real-time RT-PCR was performed as previously described (Marcet et al., 2007). Briefly, diluted cDNA was analyzed using 2X SYBR Green PCR Master Mix (Applied Biosystems, Belgium) by a 7500 Fast Real-Time PCR System and 7500 Fast software (Applied Biosystems) following the manufacturer's protocol. Gene-specific primers were designed according to sequences covering the conserved peptide sequence region and in interexonic gene sequences: human COX-2 forward: GAGAAAACTGCTCAACACCGGA; human COX-2 reverse: CACAACGTTCCAAAATCCCTTG; human IκB-α forward: CTTCAGATGCTGCCAGAGAGT; human IκB-α reverse: GCCTCCAAACACACAGTCATC; human β-actin forward: AGAAAATCTGGCACCACACC; human β-actin reverse: GGGGTGTTGAAGGTCTCAAA. The relative mRNA amount in each sample was calculated based on its threshold cycle in comparison to the threshold cycle of the two most stable housekeeping genes chosen by the software geNorm® from a set of nine tested candidate reference genes. The purity of amplified product was determined as a single peak of dissociation curve. Real-time PCR was conducted in triplicate in three independent experiments, and the mean value was calculated. The results were calculated as follows $(2^{(Ct\ of\ Cox\text{-}2 - Ct\ of\ housekeeping\ gene)})$ ∗(normalization factor) and expressed in arbitrary units. A mean of values obtained in each experiment performed in different experiments were calculated and expressed as a ratio of stimulated values/control values.

2.5. Inositol phosphate measurements

A549 cells were seeded (4.10^5 per well) on 6-well dishes and labelled for 24 h with 10 μCi/ml [*myo*-D-2-^{3}H]inositol in DMEM with 5% fetal calf serum. Cells were incubated for 2 h in DMEM with 10 mM LiCl. The cells were then incubated in the presence of the tested compounds together with LiCl (10 mM) for 20 min (Marcet et al., 2007). The incubation was stopped by the addition of 1 ml of an ice-cold 3% perchloric acid solution. Total inositol phosphates (IP) were extracted and separated on Dowex columns.

2.6. Intracellular Ca^{2+} measurements by confocal microscopy

A549 cells were seeded onto glass coverslips and cultured for 3 days. Cells with 80% confluency were used for single-cell measurement of intracellular calcium concentration. Before experiments, cells were preincubated with 5 μM Fluo-4/acetoxymethylester for 1 h at 37 °C in modified Earle's salt solution containing (in mM): 137 NaCl, 5.36 KCl, 0.8 $MgCl_2$, 1.8 $CaCl_2$, 5.5 glucose, 10 HEPES–NaOH, pH 7.4, then washed twice at room temperature. Cells were then placed into the open-topped circular microperfusion chamber and mounted on a LSM 510 Meta Zeiss fluorescent inverted confocal microscope equipped with a 20× lens (single wavelength excitation, 488 nm and a long band pass filter, 505 nm). For each experiment a new glass coverslip was used and each test solution was freshly prepared. Fluorescence intensity of individual cells was calculated every 2 s and values were plotted versus time. For each experiment fifty cells were arbitrarily selected in the field of view and fluorescence intensity was averaged.

2.7. PGE_2 quantification by ELISA

PGE_2 concentrations were measured by ELISA (R&D Systems) according to manufacturer's instructions. After a brief centrifugation the collected media were stored at −80 °C before ELISA analysis.

3. Results

A549 epithelial cells express $P2Y_2$ and $P2Y_6$ but not $P2Y_1$ or $P2Y_4$ receptors (Communi et al., 1999). However, a recent study indicated the expression of the $P2Y_4$ instead of the $P2Y_2$ subtype in A549 cells (Khine et al., 2006). First, using RT-PCR we showed that, in our culture conditions, A549 cells express both $P2Y_2$ and $P2Y_6$ but not $P2Y_4$ receptors (Fig. 1A), in agreement with a previous study (Communi et al., 1999). Then, we functionally characterized these receptors using inositol phosphates and intracellular Ca^{2+} measurements (Fig. 1B,C). Our data show that both the phosphoinositide pathway and the Ca^{2+} signaling were triggered by $P2Y_2/P2Y_4$ receptor agonists (ATP, UTP, INS365, 100 μM) and a $P2Y_6$ receptor agonist (INS48823, 100 μM) (Pendergast et al., 2001). Altogether, our results indicated that both $P2Y_2$ and $P2Y_6$ receptors are functionally expressed in A549 cells.

DNA microarray experiments were then conducted on total RNA as described in Materials and methods. A549 epithelial cells were stimulated for 1 h with 100 μM ATP, UTP and INS365. DNA microarray analysis revealed that *COX-2* messengers were significantly ($P<0.01$) up-regulated by 100 μM UTP (2.8±0.36, $n=3$), INS365 (3.2±0.06, $n=3$) (Fig. 2A) or ATP (2.4±0.01, $n=3$) (not shown). Fig. 2A shows the time course of *COX-2* transcript expression in response to 100 μM UTP or INS365 from 1 h to 18 h of stimulation. The microarray analysis showed that *COX-2* transcripts were still up-regulated by ~2-fold after 18 h of stimulation by UTP or INS365 (Fig. 2A). Next, we conducted real-time RT-PCR analysis for *COX-2* expression in ATP-, UTP-, INS365- and UDP-treated cells to confirm DNA microarray experiments. As shown in Fig. 2B, after 1 h of stimulation at 100 μM, *COX-2* transcripts were significantly ($P<0.01$) increased by 2.6±0.2-fold in response to ATP ($n=3$), 2.4±0.1-fold for UTP ($n=3$) and 2.7±0.1-fold for INS365 ($n=3$). On the other hand, UDP (100 μM, 1 h) failed to regulate *COX-2* expression. One possible mechanism of *COX-2* transcript regulation involves post-transcriptional mechanisms like mRNA stabilization (Xu et al., 2000). To elucidate whether UTP-induced *COX-2* expression could be due to an increase in stability of *COX-2* mRNA, cultures treated with or without UTP for 1 h were exposed to the RNA synthesis inhibitor, actinomycin D (Fig. 2C). The relative abundance of *COX-2* and *hIκB-α* transcripts after 3 h, was determined from two independent experiments, normalized with the stable housekeeping gene *β-actin* and expressed as percent of the 0 time value. These experiments showed that 3 h following the addition of actinomycin D (5 μg/ml), the level of *COX-2* transcripts in unstimulated conditions was reduced to 83±8%, whereas the level of UTP-induced *COX-2* transcripts was reduced to 59±8%. As control, we showed that the *hIκB-α* transcripts as expected were much less stable than those of *β-actin* or *COX-2* and were dramatically reduced by actinomycin D treatment (Fig. 2C). We observed that following actinomycin D the decrease in *COX-2* mRNA level was faster in UTP conditions than in the control. Our data therefore indicate that UTP-induced *COX-2* mRNA increase was not due to an increased stabilization of *COX-2* mRNA but rather involved transcriptional activation. To elucidate the possible signalling pathway involved in UTP-enhanced *COX-2* expression, inhibitors of nuclear factor-kappaB (NFκB) (MG132) and p42/44 extracellular signal-regulated protein kinase (ERK1/2) (PD098059) were used. MG132 (20 μM, 1 h) failed to significantly ($P>0.05$, $n=3$) prevent the UTP(1 h)-enhanced *COX-2* expression, whereas PD098059 (25 μM, 1 h) inhibited it by 17±2.5% ($P<0.01$, $n=3$) (not shown). Our data suggest that nucleotide-induced *COX-2* expression was independent from NFκB signalling pathway but may rather be dependent on other pathways like ERK1/2.

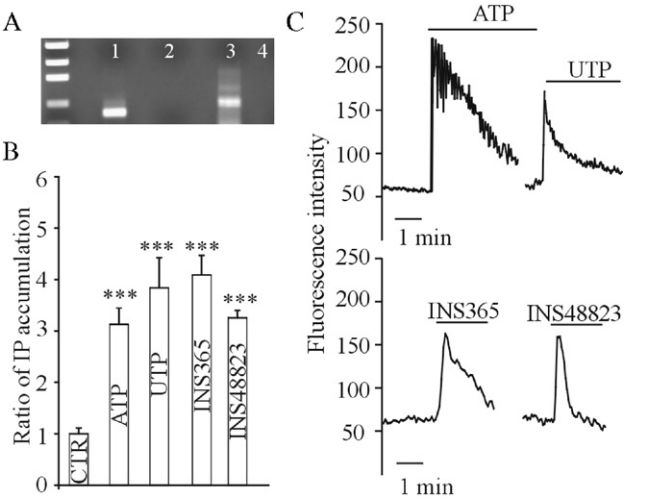

Fig. 1. $P2Y_2$ and $P2Y_6$ receptor expression in A549 cells. (A) $P2Y_2$, $P2Y_4$ and $P2Y_6$ sets of primers were used to detect nucleotide P2Y receptors expressed in A549 cells. Amplification products were obtained with $P2Y_2$ (331-bp, lane 1) and $P2Y_6$ (455-bp, lane 3) but not with $P2Y_4$ sets of primers (lane 2). Lane 4 is the negative RT-PCR control. (B) Effects of ATP, UTP, INS365 and INS48823 (100 μM) on the IP accumulation. The cells were incubated with or without receptor agonists (100 μM) for 20 min. The data represent the mean±S.E.M. ($n=3$). Student's *t*-test was performed using Prism software (GraphPad, CA) (***$P<0.001$). (C) Traces represent typical single-cell Ca^{2+} responses induced either by ATP, UTP, INS365 or INS48823 (100 μM) and expressed in intensity of fluorescence.

Since COX-2 is involved in PGE_2 synthesis, we tested whether nucleotides could modify PGE_2 production. We performed concentration response curves in response to 1 h of stimulation by ATP, ATPγS, UTP, INS365 and UDP in order to measure PGE_2 level in supernatants of A549 cells. As shown in Fig. 2D nucleotide stimulation (1 h) significantly increased the PGE_2 release in a concentration-dependent manner with a rank order of efficacy: UTP>INS365>ATP>ATPγS>UDP ($P<0.01$, $n=3$). Since UDP formation may occur subsequent to UTP hydrolysis by ectonucleotidases, we tested the effect of UDP on PGE_2 release. We found that UDP increased PGE_2 release but to a lesser extent than triphosphate nucleotides, suggesting a minor involvement of the $P2Y_6$ receptors (Fig. 2D). Although it is known that A549 cells express only COX-2 but not COX-1 (Mitchell et al., 1994), we tested the selective COX-2 inhibitor NS398 (5 μM) on the UTP- and ATP-induced PGE_2 production. Fig. 2E shows that NS398 blocked the PGE_2 production induced by UTP or ATP suggesting the involvement of COX-2 in nucleotide-induced PGE_2 release. Furthermore, we

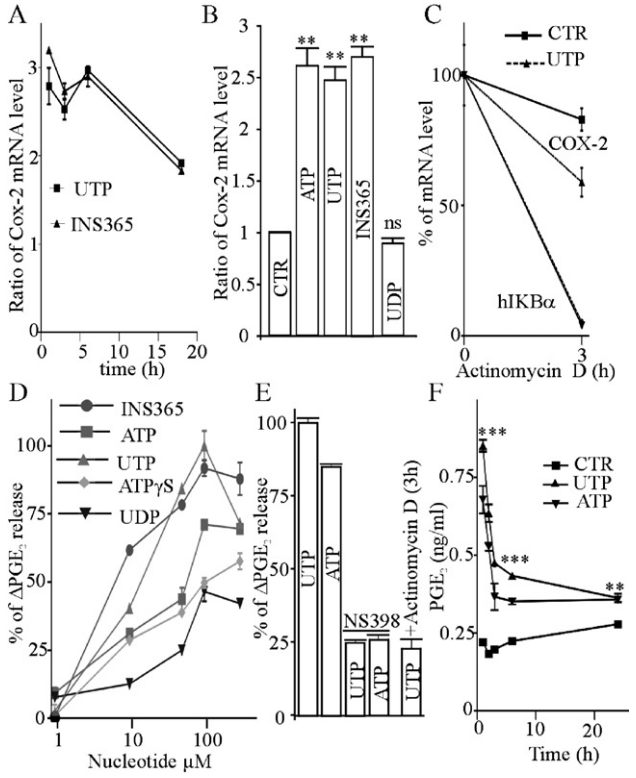

Fig. 2. Effects of ATP, UTP, UDP and INS365 on *COX-2* expression. (A) Time-course experiments of *COX-2* expression in response to UTP or INS365 (100 μM) were performed using DNA microarray experiments as described in Materials and methods. Values are expressed as mean of each ratio±S.E.M. obtained from 3 independent DNA microarray experiments. (B) Cells were treated for 1 h with or without ATP, UTP, UDP (100 μM) and INS365 (100 μM) and real-time RT-PCR analysis was conducted for nine housekeeping genes and *COX-2* transcripts in these RNA samples. Relative level of *COX-2* mRNA after normalization with the two most stable genes in each RNA sample was expressed as ratio between stimulated and unstimulated conditions. Values are mean±S.E.M. of triplicates from 3 independent experiments. One way ANOVA analysis followed by a Dunnett's multiple comparison test was performed using Prism software (GraphPad, CA) (**$P<0.01$). (C) Characterization of *COX-2* mRNA stability after addition of actinomycin D. After 1 h of stimulation with UTP (100 μM), cells were harvested for RNA purification at point 0 h in absence of actinomycin D, and actinomycin D (5 μg/ml) was added to another part of these cultures which were harvested for RNA 3 h after addition of actinomycin D. Real-time RT-PCR analysis was conducted for *β-actin*, *COX-2* and *hIκB-α* in these RNA samples. Relative level of *COX-2* mRNA after normalization with *β-actin* in each RNA sample was converted in ratio between stimulated and unstimulated conditions, and expressed as 100% of starting level (without actinomycin D) for each condition. Values are mean±S.E.M. of duplicates of two independent experiments. (D) Concentration-dependent (1–1000 μM) effects of stimulation by ATP, ATPγS, UTP, UDP and INS365 (1 h) on the enhancement of PGE_2 release. Results were expressed as mean±S.E.M. of duplicate samples ($n=3$ independent experiments), normalized with control and expressed as percent of PGE_2 released. (E) Effect of a selective COX-2 inhibitor NS398 (5 μM for 2 h) and actinomycin D (5 μg/ml, 3 h) treatment on ATP- or UTP-induced PGE_2 release. Results were expressed as mean±S.E.M. of duplicate samples from 3 independent experiments, normalized with control and expressed as percent of PGE_2 released. (F) Time-dependent release of PGE_2 by 100 μM ATP and UTP in A549 cells at 1, 2, 4, 6, and 24 h of stimulation. Each point represents mean±S.EM. of triplicates from 3 independent experiments (**$P<0.01$, ***$P<0.001$, unpaired *t*-test).

observed that actinomycin D treatment (3 h) strongly prevented the UTP-induced PGE_2 release (Fig. 2E), indicating that UTP-enhanced PGE_2 production mainly involved transcriptional mechanisms. Finally, we performed time-course experiments on PGE_2 release in response to 100 μM UTP or ATP. Treatment of A549 cells with UTP or ATP (100 μM) caused a time-dependent accumulation of PGE_2 (Fig. 2F). There was a threefold increase in PGE_2 production, in response to UTP or ATP at 1 h ($P<0.01$). PGE_2 levels declined from 1 h to 24 h but remained slightly but significantly ($P<0.05$) higher in nucleotide-treated cells.

4. Discussion

P2Y receptors play a crucial role in airway epithelium homeostasis, periciliary volume regulation and mucociliary clearance of airway epithelia (Marcet and Boeynaems, 2006). In addition, we have recently shown that nucleotides, by activating P2Y receptor signaling pathways, regulated the expression and the release of CCL20 and IL-8 and the subsequent immune cell recruitment and by this way modulated the immune function of the airway epithelium (Marcet et al., 2007). In A549 cells, ATP or UTP has been shown to promote arachidonic acid release subsequent to a rise in calcium (Laubinger et al., 2006). In the present work, we show that extracellular nucleotides modulate PGE_2 release and *COX-2* expression in the human A549 alveolar type II epithelial cells. To our knowledge, this is the first demonstration that extracellular nucleotides affect the level of expression of the *COX-2* gene in airway epithelium. ATP, ATPγS, UTP and INS365 but not UDP increased the *COX-2* transcript expression, indicating an involvement of the $P2Y_2$ receptor but not of the $P2Y_6$ subtype. *COX-2* transcript level could be affected by transcriptional or post-transcriptional mechanisms like mRNA stabilization (Xu et al., 2000). Our actinomycin D experiments indicated that UTP-induced *COX-2* mRNA increase and UTP-enhanced PGE_2 release were not due to stabilization of *COX-2* mRNA but rather involved transcriptional activation. Our inhibitors' experiments indicated that the nucleotide-enhanced *COX-2* expression is independent from NFκB pathway but partially dependent on ERK1/2 pathway. Such a NFκB-independent but ERK1/2-dependent COX-2 regulation has been previously shown in A549 cells (Lim et al., 2005; Patel et al., 2005). Furthermore, $P2Y_2$ receptor agonists (ATP, ATPγS, UTP and INS365) provoked a stronger increase in PGE_2 production than UDP ($P2Y_6$ receptor agonist), indicating a major contribution of the $P2Y_2$ receptor. Whereas UDP failed to regulate *COX-2* expression, it was capable of inducing a weaker release of PGE_2 than $P2Y_2$ receptor agonists, probably by directly activating free arachidonic release subsequently to a rise in intracellular calcium and via COX-2 constitutively expressed in A549 cells. Our data suggest that UTP stimulation promoted a rapid PGE_2 release due to intracellular calcium mobilization and arachidonic acid release as recently reported (Laubinger et al., 2006), followed by a rapid up-regulation of *COX-2* expression that contributes to maintain a higher level of PGE_2 production. Indeed, the stimulation of PGE_2 production during the first hour following UTP or ATP addition was reduced by both a selective COX-2 inhibitor (NS398) and actinomycin D. Furthermore, the time course of PGE_2 level in the 24 h following UTP or ATP addition

showed a progressive decrease that is likely to be due to the high level of 15-hydroxyprostaglandin dehydrogenase expression in A549 cells (Tong et al., 2006) but the PGE_2 concentration remained significantly higher ($P<0.05$) in the medium of UTP- or ATP-treated cells up to 24 h.

PGE_2 is commonly considered as a potent proinflammatory mediator and is involved in several inflammatory diseases. However, PGE_2 has beneficial actions in airways by limiting the immune-inflammatory response, by preventing bronchoconstriction as well as by controlling tissue repair processes (Vancheri et al., 2004).

In conclusion, our data show that in addition to regulate ion transport and mucociliary clearance in airways, extracellular nucleotides may modulate inflammatory properties of airway epithelium by regulating expression of *COX-2* and release of PGE_2. Given the protective effects of PGE_2 in airways, our results could be of potential interest in further clinical investigations in airway inflammatory diseases like cystic fibrosis for which $P2Y_2$ receptor agonists are currently tested by aerosol therapy to improve mucociliary clearance (Kellerman et al., 2002).

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