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ORIGINAL ARTICLES

Measuring Ca^{2+} influxes of TRPC1-dependent Ca^{2+} channels in HL-7702 cells with Non-invasive Micro-test Technique

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Abstract

AIM: To explore the possibility of using the Non-invasive Micro-test Technique (NMT) to investigate the role of Transient Receptor Potential Canonical 1 (TRPC1) in regulating Ca^{2+} influxes in HL-7702 cells, a normal human liver cell line.

METHODS: Net Ca^{2+} fluxes were measured with NMT, a technology that can obtain dynamic information of specific/selective ionic/molecular activities on material surfaces, non-invasively. The expression levels of TRPC1 were increased by liposomal transfection, whose effectiveness was evaluated by Western-blotting and single cell reverse transcription-polymerase chain reaction.

RESULTS: Ca^{2+} influxes could be elicited by adding 1 mmol/L $CaCl_2$ to the test solution of HL-7702 cells. They were enhanced by addition of 20 μmol/L noradrenalin and inhibited by 100 μmol/L $LaCl_3$ (a non-selective Ca^{2+} channel blocker); 5 μmol/L nifedipine did not induce any change. Overexpression of TRPC1 caused increased Ca^{2+} influx. Five micromoles per liter nifedipine did not inhibit this elevation, whereas 100 μmol/L $LaCl_3$ did.

CONCLUSION: In HL-7702 cells, there is a type of TRPC1-dependent Ca^{2+} channel, which could be detected *via* NMT and inhibited by La^{3+}.

Key words: Non-invasive Micro-test Technique; Ca^{2+} channels; Transient Receptor Potential Canonical 1; Gene expression; HL-7702 cells

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INTRODUCTION

Changes in the concentration of Ca^{2+} in the cytoplasmic space play a central role in intracellular signaling pathways in liver cells, including glucose, fatty acid, amino acid and xenobiotic metabolism, bile acid secretion, protein synthesis and secretion, the movement of lysosomes and other vesicles, the cell cycle and cell proliferation, and apoptosis and necrosis[1-4]. In earlier reports, it has been shown that ligand-gated, store-operated, receptor-activated, and stretch-activated Ca^{2+}-permeable channels are expressed in hepatocytes and in liver cell lines. No voltage-operated Ca^{2+} channels (VOCCs) have been detected[5-8]. There is increasing evidence that members of the canonical subgroup of Transient Receptor Potential (TRP) proteins constitute tetramers of both receptor-activated and store-operated Ca^{2+} channels (SOCs)[9-11], and Transient Receptor Potential Canonical 1 (TRPC1) is considered as one of the most likely candidates in forming Ca^{2+} channels in mammalian cells[12-15].

The Non-invasive Micro-test Technique (NMT) was developed in the late 20th century, and is a new

technology for obtaining dynamic information on specific ionic/molecular activities on material surfaces, non-invasively. This technique incorporates different temporal and spatial resolution domains from other traditional methods, and its 3-dimensional measurement capability enables us to observe the physiological characteristics of biological phenomena that would be difficult or even impossible with other techniques[16]. To date, Ca^{2+}, H^+, K^+, Cl^-, NO^-, Mg^{2+}, Cd^{2+}, Al^{3+}, and O_2 have be detected as sensors for ionic/molecular species.

In the present study, we used NMT to study the Ca^{2+} influxes elicited by extracellular elevations of Ca^{2+} concentration, and the inhibitory effects of several Ca^{2+} channel blockers, to investigate the role of TRPC1 in regulating Ca^{2+} fluxes in HL-7702 cells.

MATERIALS AND METHODS

Materials

Nifedipine, noradrenalin, protease inhibitor Cocktail, Fast Red TR, Naphthol AS-MX phosphate, and Calcium Ionophore I [Cocktail A were bought from the Sigma-Aldrich Company (Catalog Number: 21048)]. Lipofectamine 2000 was purchased from Invitrogen. The TRPC1 polyclonal antibody was acquired from the Abnova Company. Peroxidase-conjugated secondary antibody was obtained from the Beijing Zhongshan Golden Bridge Co.. All the other reagents were of reagent grade.

Cell culture, plasmid construction and transfection

The human liver cell line, HL-7702, bought from the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, was maintained in RPMI-1640 containing 10% FBS, 1% Penicillin, and Streptomycin. Plasmid pBS-T-TRPC1 was constructed and verified as previously described[17]. HL-7702 cells were grown to 75%-80% confluence in 35 mm dishes in advance and transfection was carried out with 1 μg/mL of the recombinant plasmid and Lipofectamine 2000 according to the manufacturer's protocol.

Measurements of extracellular Ca^{2+} influxes

Measurements of net influxes of Ca^{2+} were performed using NMT (BIO-001A, Younger USA Sci. & Tech. Co., Amherst, MA, USA; Applicable Electronics Inc., Forestdale, MA, USA; and ScienceWares Inc., East Falmouth, MA, USA). The electrode was controlled to move with an excursion of 10 μm at a programmable frequency in the range of 0.3-0.5 Hz; this minimized mixing of the bathing saline.

To construct the microelectrodes, borosilicate micropipettes (2-4 μm aperture, XYPG120-2, Xuyue (Beijing) Science and Technology Co., Ltd., Beijing, China) were silanized with tributylchlorosilane and the tips filled with Calcium Ionophore I - Cocktail A. An Ag/AgCl wire electrode holder (XYEH01-1) was inserted in the back of the electrode to make electrical contact with the electrolyte solution. Only electrodes with Nernstian slopes > 25 mV were used. Ca^{2+} fluxes were calculated by Fick' law of diffusion: $J_0 = -[D \times (dC/dX)]$ where J_0 represents the net Ca^{2+} flux (in μmol/cm per second), D is the self-diffusion coefficient for Ca^{2+} (in cm^2/s), dC is the difference value of Ca^{2+} concentrations between the two positions, and dX is the 10 μm excursion over which the electrode moved in our experiments. Data and image acquisition, preliminary processing, control of the three-dimensional electrode positioner, and stepper-motor-controlled fine focus of the microscope stage were performed with ASET software.

Single cell reverse transcription-polymerase chain reaction (RT-PCR)

Single cell RT-PCR was performed to determine whether the cells measured by NMT were successfully transfected, using a previously described method, with some modifications[18]. Directly after the Ca^{2+} influx assay, the contents of tested cell were aspirated into a microelectrode. The tip of the electrode was then broken in a PCR tube and stored at -80℃ until use. Reverse transcription was carried out using a kit from TIANGEN (Beijing, China) according to the manufacturer's instructions. The first PCR was performed using specific primers (Forward: GCAATGATACCTTCCATTCGTTC; Reverse: CGATGCACTAGGCAGCAGATC) and the following conditions: pre-denaturation at 94℃ for 3 min; followed by 30 cycles of denaturation at 94℃ for 30 s and annealing at 60℃ for 30 s, then synthesis at 72℃ for 60 s; the last step was extension at 72℃ for 5 min. After the first PCR, 1 μL of the reaction products was used as the template for a secondary PCR with the same conditions as above and 25 cycles. The predicted size of the TRPC1 amplicons were 455 bp and reaction products were confirmed and analyzed by agarose gel electrophoresis.

Western blotting

Total proteins were obtained from cultured cells by using lysis buffer (35 mmol/L Tris at pH 7.4, 0.4 mmol/L EDTA, 10 mmol/L $MgCl_2$, 0.1% protease inhibitor Cocktail). For western blotting analysis, 20 μg proteins were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto to polyvinylidene difluoride membranes for 2 h at 20 V. The membranes were then blocked for 2 h with blocking solution (5% bovine serum albumin) and probed with anti-TRPC1 antibodies. The primary antibodies were incubated for 1 h at room temperature and, after washing, the membranes were incubated with peroxidase-conjugated secondary antibodies for 1 h. Finally, the proteins on the membranes were dyed by staining solution containing Fast Red TR and Naphthol AS-MX phosphate. Immunoblots were then scanned to obtain images.

Statistical analysis

Data were expressed as mean ± SD of n cells from at least six cell culture dishes. The statistical significance of diversities between means was determined using the DUNNET t-test. A value of $P < 0.05$ was considered significant.

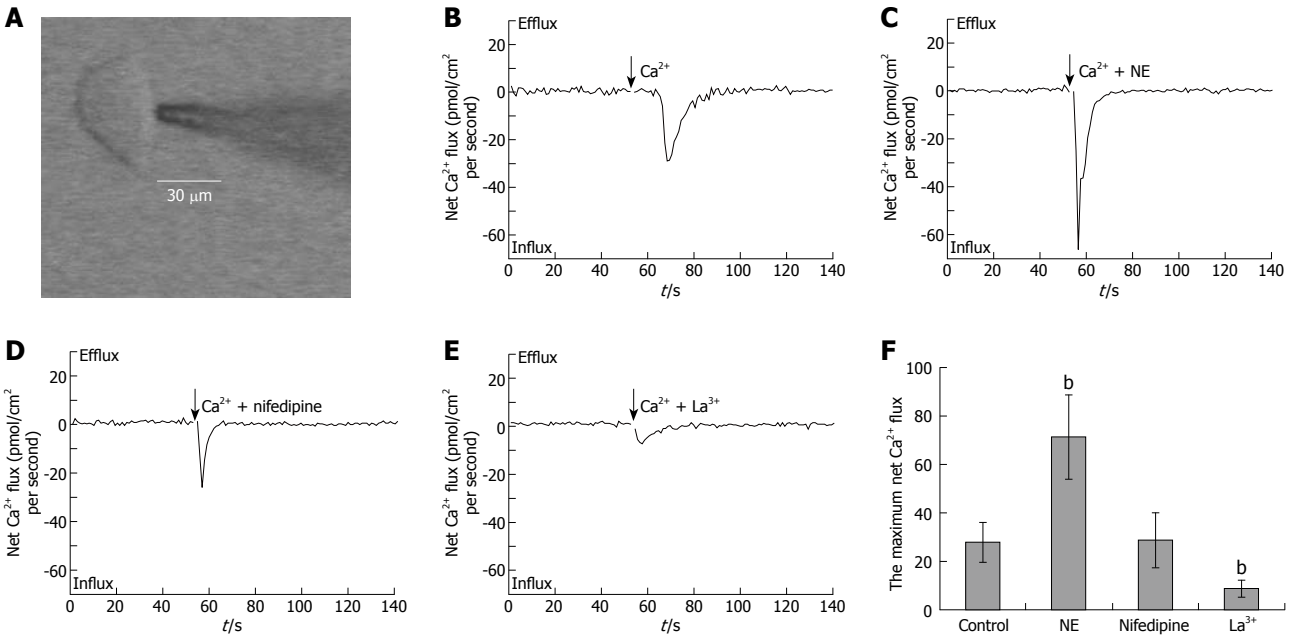

Figure 1 The Ca^{2+} fluxes of HL-7702 cells. A: A screen-printed picture of a cell measured by NMT; B: Net Ca^{2+} fluxes of a HL-7702 cell. The maximum values were 28.2 ± 8.2 pmol/cm^2 per second (n = 6); C: Net Ca^{2+} fluxes of a noradrenalin-treated HL-7702 cell. The maximum values were 71.7 ± 17.5 pmol/cm^2 per second (n = 6); D: Net Ca^{2+} fluxes of a nifedipine-treated HL-7702 cell. The maximum values were 29.0 ± 11.3 pmol/cm^2 per second (n = 6); E: Net Ca^{2+} fluxes of a La^{3+} -treated HL-7702 cell. The maximum values were 9.0 ± 3.5 pmol/cm^2 per second (n = 6); F: Bar graph of the maximum net Ca^{2+} influxes in four groups. $^bP < 0.01$, control group *vs* noradrenalin-treated or La^{3+}-treated group.

RESULTS

The Ca^{2+} influxes of HL-7702 cells were measured by NMT

Before the experiment, 35 mm dishes with pre-dispersed normal HL-7702 cells were perfused with test solution containing (in mmol/L): 2.3 $NaHCO_3$, 27 Na_2SO_4, 9.7 KCl, 61.1 $MgCl_2$, and 366.7 NaCl. The Ca^{2+} influxes were then measured by NMT. The background noise was recorded for three min before 1 mmol/L $CaCl_2$ was added to elicit an inward Ca^{2+} current. As shown in Figure 1A, the Ca^{2+} selective microelectrode moved between two positions close to the tested cells constantly to acquire experimental data. Net Ca^{2+} fluxes are depicted in Figure 1B, a giant wave trough emerged shortly after $CaCl_2$ was added.

Effects of noradrenalin, nifedipine and La^{3+} on Ca^{2+} influxes of HL-7702 cells

To further identify the property of the Ca^{2+} influxes of HL-7702 cells, three drugs were selected as tools in the following experiments. After background noise was recorded for three min, a Ca^{2+} channel agonist, noradrenalin (20 μmol/L), or two types of inhibitors, nifedipine (5 μmol/L) or $LaCl_3$ (100 μmol/L) were added into the test solution together with 1 mmol/L $CaCl_2$. The effects of the three drugs on Ca^{2+} influxes are shown in Figure 1C-E. Net Ca^{2+} fluxes were significantly influenced by noradrenalin and La^{3+}; however, nifedipine did not induce any change. A bar graph of the maximum net Ca^{2+} fluxes in the four groups (three drug-treated groups and control group) is depicted in Figure 1F; these experiments were repeated six times (n = 6).

Effects of TRPC1-transfection on Ca^{2+} influxes of HL-7702 cells

When TRPC1-transfected HL-7702 cells were measured by NMT, the maximum net Ca^{2+} influxes increased to 48.9 ± 6.4 pmol/cm^2 per second (n = 6) and a deeper wave trough was observed (Figure 2A), The bar graph shown in Figure 2B shows that the statistical difference between the TRPC1-transfected group and the control group was significant ($P < 0.01$). Single cell RT-PCR and western blotting were performed after NMT experiments. The results of agarose gel electrophoresis and SDS-PAGE are shown in Figure 2C and D, respectively. They demonstrated that TRPC1-expressions in tested cells were elevated after transfection.

Effects of nifedipine and La^{3+} on Ca^{2+} influxes of TRPC1-transfected HL-7702 cells

TRPC1-transfection induced increases in Ca^{2+} influxes. To investigate the effects of nifedipine and La^{3+} on these increased Ca^{2+} influxes, the two drugs were added into the test solution together with 1 mmol/L $CaCl_2$. As shown in Figure 3A-C, the maximum net Ca^{2+} fluxes maintained an average of 44.0 ± 5.7 pmol/cm^2 per second (n = 6) when 5 μmol/L nifedipine was applied, whereas 100 μmol/L $LaCl_3$ made reduced the maximum value to 7.6 ± 1.9 pmol/cm^2 per second (n = 6). A significant statistical difference only existed between the TRPC1 + La^{3+}-treated group and the TRPC1-transfected group ($P < 0.01$). Single cell RT-PCR and western blotting were performed after NMT experiments to confirm the increased TRPC1 expression (data not shown).

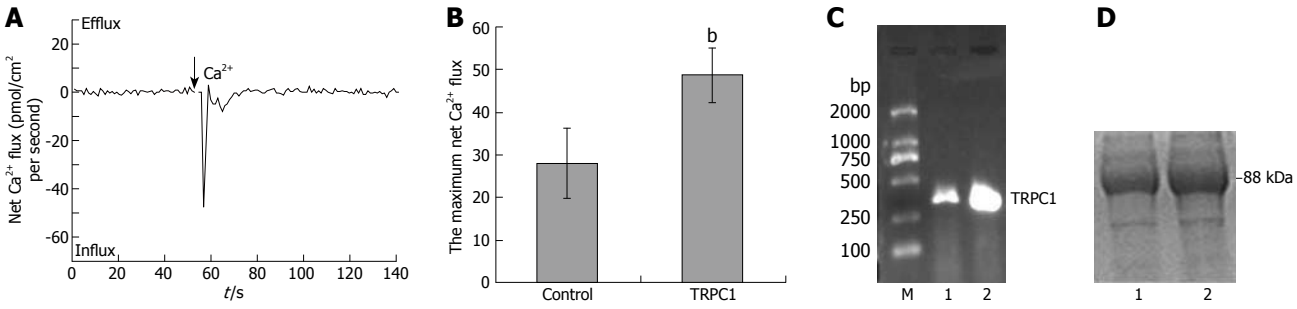

Figure 2 TRPC1-transfection influences Ca^{2+} influxes of HL-7702 cells. A: Net Ca^{2+} fluxes of a TRPC1-transfected cell. The maximum values were 48.9 ± 6.4 pmol/cm^2 per second (n = 6); B: Bar graph of the maximum net Ca^{2+} fluxes in the control group and the TRPC1-transfected group. The statistical difference between the two groups was significant ($^bP < 0.01$); C: Single cell RT-PCR products from cultured HL-7702 cells and TRPC1-transfected cells using primers for human TRPC1 (455 bp); lane M: DNA marker, lane 1: TRPC1 amplified from HL-7702 cells, lane 2: TRPC1 amplified from transfected cells; D: TRPC1 protein was detected in cultured HL-7702 cells and TRPC1-transfected cells using western-blotting analysis; lane 1: TRPC1 protein extracted from HL-7702 cells, lane 2: TRPC1 protein from transfected cells. The molecular mass of TRPC1 is 88 kDa.

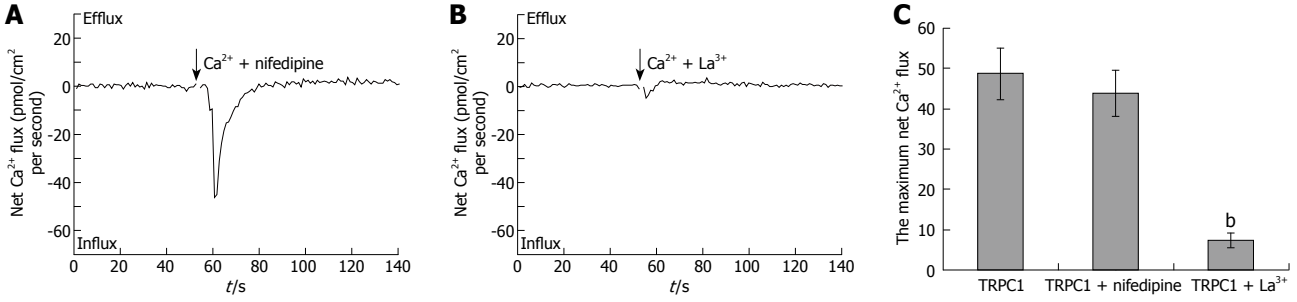

Figure 3 Nifedipine and La^{3+} influence Ca^{2+} influxes of TRPC1-transfected HL-7702 cells. A: Net Ca^{2+} fluxes of a TRPC1 + nifedipine-treated cell. The maximum values were 44.0 ± 5.7 pmol/cm^2 per second (n = 6); B: Net Ca^{2+} fluxes of a TRPC1 + La^{3+}-treated cell. The maximum values were 7.6 ± 1.9 pmol/cm^2 per second (n = 6); C: Bar graph of the maximum net Ca^{2+} fluxes in the TRPC1-transfected group, TRPC1 + nifedipine-treated group, and TRPC1 + La^{3+}-treated group. A significant statistical difference only existed between the TRPC1-transfected group and the TRPC1 + La^{3+}-treated group ($^bP < 0.01$).

DISCUSSION

Initially, we detected Ca^{2+} influxes in HL-7702 cells using NMT when 1 mmol/L $CaCl_2$ was added to the test solution and the extracellular Ca^{2+} concentration markedly changed. Ca^{2+} influx could be influenced by two drugs, noradrenalin, and $LaCl_3$; however nifedipine did not induce any change.

As a known neurotransmitter, noradrenalin can act through α_1-adrenoceptors to activate phospholipase C. This generates inositol 1,4,5-trisphosphate (IP_3) within the cell, which in turn mediates the rise of Ca^{2+} concentration by release from intracellular stores[19,20] and opening of receptor operated Ca^{2+} entry at the plasma membrane[21-23]. Thus, the increase in Ca^{2+} influx induced by noradrenalin together with 1 mmol/L external $CaCl_2$ could be explained if receptor operated Ca^{2+} channels played a central role in these experiments. On the other hand, Ca^{2+} influxes were prominently inhibited by La^{3+}. Analogs often effect their action through competitive inhibition with their common receptors or channels. La^{3+} has a similar size of ionic radius to that of Ca^{2+}, which enables La^{3+} to compete with Ca^{2+}, which makes La^{3+} a non-selective Ca^{2+} channel blocker. In addition, a study showed that La^{3+} could inhibit both influx and efflux of Ca^{2+} in lacrimal cells[24], which was consistent with the present study. Most importantly, nifedipine, as an antagonist of L-type Ca^{2+} channels, did not induce any change in Ca^{2+} influxes, which would indicate that no VOCCs existed or that transmembrane Ca^{2+} influxes elicited by external Ca^{2+} did not pass through VOCCs in HL-7702 cells.

TRPC1 is one of seven members of the TRPC subfamily of non-selective cation channels and is expressed in a wide variety of cell types and tissues[25-27]. It is likely that TRPC1 plays a significant part in intracellular Ca^{2+} homeostasis. In salivary gland cells, the current through the endogenous SOCs was the same as the membrane current which was activated by the depletion of intracellular Ca^{2+} stores in cells in which TRPC1 was ectopically expressed and the endogenous SOCs were decreased by transfection with antisense TRPC1[28]. In kidney epithelial cells, a new receptor-operated channel formed by heteromeric assembly of TRPP2 and TRPC1 subunits was discovered[29]. In T cells, intracellular Ca^{2+} elevation induced by Δ9-tetrahydrocannabinol was attributable entirely to extracellular Ca^{2+} influxes, which were not dependent on store depletion, but mediated through TRPC1 channels[30]. The results of the second part of our study do not conflict with these reports. In HL-7702 cells, overexpression of TRPC1 causes an increase of Ca^{2+} influxes induced by adding external Ca^{2+}, and the non-selective Ca^{2+} channel blocker, La^{3+}, can attenuate this elevation. In summary, there is a TRPC1-dependent Ca^{2+}-type channel(s), either receptor-activated or store-operated present in HL-7702 cells, which can be inhibited by La^{3+}.

Taken together, NMT is a powerful tool for ion

channel research, which has been effectively applied in various systems[31-33]. We used NMT to explore the properties of Ca^{2+} channels, and found that there was a TRPC1-dependent Ca^{2+}-type channel(s), which could be detected *via* NMT and inhibited by La^{3+}, in HL-7702 cells.

COMMENTS

Background

Ca^{2+} plays an important role in intracellular signaling pathways and Transient Receptor Potential Canonical 1 (TRPC1) is considered as one of the most likely candidates in forming Ca^{2+} channels in mammalian cells. As a technology to obtain dynamic information of specific ionic/molecular activities on material surfaces non-invasively, Non-invasive Micro-test Technique (NMT) is being increasingly applied to study characters of ion channels.

Research frontiers

TRPC1 has been verified as a molecular candidate or a regulator of Ca^{2+} channels in several mammalian cells. However, the role of TRPC1 in normal human liver cells has not been elucidated. In this study, the authors demonstrate that a TRPC1-dependent Ca^{2+}-type channel(s) exists in HL-7702 cells, using NMT, a non-invasive technique.

Innovations and breakthroughs

This is the first study to investigate the role of TRPC1 in regulating Ca^{2+} channels in normal human liver cells. Furthermore, measuring Ca^{2+} influxes was performed non-invasively, which cannot be accomplished with other traditional techniques.

Applications

Cytoplasmic Ca^{2+} overloading might cause damage to liver cells. By understanding the role of TRPC1 in mediating extracellular Ca^{2+} influxes, this study might represent a future strategy for preventing or treating diseases induced by dysfunctions of Ca^{2+} channels in the clinic, such as hepatic ischemia-reperfusion injury.

Terminology

The canonical transient receptor potential (TRPC) channel subfamily consists of seven mammalian cation channels and is expressed in almost every tissue, including the liver. The TRPC1 channel is permeable to Ca^{2+} and is the most likely candidate for receptor-operated Ca^{2+} channels. In addition, TRPC1 also plays a dominant role in mediating store-operated Ca^{2+} channels in many types of cells.

Peer review

The authors recorded Ca^{2+} influxes elicited by adding external Ca^{2+} into a test solution in several different conditions. It revealed that there is a TRPC1-dependent Ca^{2+}-type channel(s), which can be detected *via* NMT, in normal human liver cells. This is an interesting study.

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