Bestrophin-1 Enables Ca\(^{2+}\)-activated Cl\(^{-}\) Conductance in Epithelia*

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Epithelial cells express calcium-activated Cl\(^{-}\) channels of unknown molecular identity. These Cl\(^{-}\) channels play a central role in diseases such as secretory diarrhea, polycystic kidney disease, and cystic fibrosis. The family of bestrophins has been suggested to form calcium-activated Cl\(^{-}\) channels. Here, we demonstrate molecular and functional expression of bestrophin-1 (BEST1) in mouse and human airways, colon, and kidney. Endogenous calcium-activated whole cell Cl\(^{-}\) currents coincide with endogenous expression of the Vmd2 gene product BEST1 in murine and human epithelial cells, whereas calcium-activated Cl\(^{-}\) currents are absent in epithelial tissues lacking BEST1 expression. Blocking expression of BEST1 with short interfering RNA or applying an anti-BEST1 antibody to a patch pipette suppressed ATP-induced whole cell Cl\(^{-}\) currents. Calcium-dependent Cl\(^{-}\) currents were activated by ATP in HEK293 cells expressing BEST1. Thus, BEST1 may form the Ca\(^{2+}\)-activated Cl\(^{-}\) current, or it may be a component of a Cl\(^{-}\) channel complex in epithelial tissues.

Ca\(^{2+}\)-activated Cl\(^{-}\) channels (CaCC)\(^{2}\) are present in almost every cell type examined. Although this type of channel has been studied extensively, the molecular identity of CaCC in epithelial tissues remains a mystery (1, 2). The channels are of small conductance, show outward rectification upon moderate increases in intracellular Ca\(^{2+}\), and have an anion selectivity of $I_\text{sc} > Cl^-$. Both direct activation of the channel by increases in intracellular Ca\(^{2+}\) and indirect activation through Ca\(^{2+}\)-dependent phosphorylation by calmodulin kinase II have been described (2, 5). Small conductance CaCC in excised membrane patches of human pancreatic CFPAC cells have been shown to be regulated by Ca\(^{2+}\), calmodulin kinase II, and inositol 3,4,5,6-tetrakisphosphate (6). CaCC are inhibited by compounds like DIDS and niflumic acid, although these pharmacological tools lack specificity (7).

Molecular candidates for CaCC have been proposed in the past. A family of Ca\(^{2+}\)-activated Cl\(^{-}\) channels (CLCA) has been identified (8, 9). These proteins are involved not only in chloride conductance and epithelial secretion but also in cell-cell adhesion, apoptosis, and cell cycle control. However, detailed structure-function analysis is still missing, and these proteins have a surprisingly low Ca\(^{2+}\) sensitivity (1). Thus, CLCA could be part of a higher Cl\(^{-}\) channel complex (10). The CIC-3 channel has been shown to be regulated by calmodulin kinase II (11). However, CIC-3 is a cytosolic Cl\(^{-}\) channel, located in endosomes, and CIC-3 knock-out animals apparently do not lack CaCC (1).

Previous studies demonstrated that bestrophin-1, the product of the vitelliform macular dystrophy (VMD2) gene, is able to form CaCC (12). Mutations in the VMD2 gene cause early-onset autosomal dominant macular dystrophy of the retina, the so-called Best disease (13). Previous studies detected this channel in the basolateral membrane of the retinal pigment epithelium (14), where it controls the light-peak amplitude in the electrooculogram. Expression of BEST1 was reported to be limited to the retinal pigment epithelium, although it has also been detected in cultured airway epithelial cells (15, 16). For the first time, we will provide evidence that endogenously expressed BEST1 causes Ca\(^{2+}\)-activated Cl\(^{-}\) conductance in epithelial tissues.

**EXPERIMENTAL PROCEDURES**

_Ussing Chamber Recordings_—Mice (C57BL/6, Charles Rivers Laboratories) were killed under CO\(_2\) narcosis by cervical dislocation. Tissues were put immediately into an ice-cold buffer solution containing 145 mmol/liter NaCl, 3.8 mmol/liter KCl, 5 mmol/liter D-glucose, 1 mmol/liter MgCl\(_2\), 5 mmol/liter HEPES, and 1.3 mmol/liter calcium gluconate (pH 7.4). After mounting into a perfused micro-Ussing chamber, apical and basolateral surfaces of the epithelium were perfused continuously with buffer solution at a rate of 5–10 ml/min (chamber volume of 2 ml). All experiments were carried out at 37 °C under open circuit conditions. Transepithelial resistance ($R_{te}$) was determined by applying short (1 s) current pulses ($I = 0.5 \mu A$), and the corresponding changes in transepithelial voltage ($V_{te}$) and basal $V_{te}$ were recorded continuously. $V_{te}$ values refer to the serosal side of the epithelium. The equivalent short circuit current ($I_{sc}$) was calculated according to Ohm’s law from $V_{te}$ and $R_{te}$ ($I_{sc} = V_{te}/R_{te}$).

_Patch Clamp_—Cell culture dishes were mounted on the stage of a Zeiss IM35 inverted microscope and kept at 37 °C. The bath was perfused continuously with Ringer’s solution at ~10 ml/min. Patch clamp experiments were performed in the fast whole cell configuration. Patch pipettes had an input resistance of 2–4 megaohms when filled with a solution containing 30 mm KCl, 95 mm potassium gluconate, 1.2 mm Na\(_2\)HPO\(_4\), 4.8 mm

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2 The abbreviations used are: CaCC, Ca\(^{2+}\)-activated Cl\(^{-}\) channel(s); DIDS, 4,4'-disothiocyanostilbene-2,2'-disulfonic acid; RT, reverse transcription; siRNA, small interfering RNA; EGFP, enhanced green fluorescent protein; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N',N'-tetraacetic acid; CFTR, cystic fibrosis transmembrane conductance regulator.
Oligonucleotide primers for both human CTGGG-3′ mBEST2, Vmd2l1 mBEST1, and mBEST4, GAGG-3′ were designed and synthesized by Invitrogen. The sense strand of the siRNA served as a control. Transfection of HT29, T84, and 16HBE cells was carried out 1 day after seeding (Lipoectamine 2000, Invitrogen) in Opti-MEM I. After 24–48 h, cells were used for patch clamping and protein isolation.

Expression of Human BEST1 in HEK293 Cells—pRK5 vector carrying cDNA for human BEST1 was kindly provided by Dr. Hugh Cahill (The Johns Hopkins University, Baltimore, MD). The plasmid was cotransfected (Lipofectamine 2000) in Opti-MEM I into HEK293 cells together with pEGFP-1 (Clontech) at a ratio of 10:1. One day after transfection, the cells were replated on 4-cm2 glass coverslips. Transfected cells were identified by enhanced green fluorescent protein (EGFP) fluorescence and used for patch clamp experiments within 3 days.

Antibodies—Affinity-purified polyclonal antiserum were produced in rabbits immunized with the peptide carrying either mouse BEST1 (AESYPYRDEAGTKP/LYE) or human BEST1 (KDHMDPYWALENDEAHS) coupled to keyhole limpet hemocyanin (Davids Biotechnologie, Regensburg, Germany).

Immunohistochemistry—Tissues were fixed for 2 h with 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). Tracheas and colons were dehydrated and embedded in paraffin. Paraffin-embedded tissues were cut at 4 μm on a rotary microtome (RM 2165, Leica, Wetzlar, Germany). Sections were dewaxed and rehydrated. In tracheal sections, endogenous peroxide activity was eliminated by incubation in methanol with 3% H2O2 for 20 min. Sections were incubated overnight at 4 °C with rabbit anti-mouse bestrophin-1 antibodies diluted 1:10,000 in Tris buffer containing Triton X-100 (0.8%) and goat serum to prevent nonspecific binding. Subsequently, sections were incubated with horseradish peroxidase-linked goat anti-rabbit secondary antibodies (Amersham Biosciences), and the ABC (avidin-biotin-peroxidase complex) technique was used to visualize labeling with 3,3-diaminobenzidine. The ABC technique involves application of a biotin-labeled secondary antibody, followed by the addition of an avidin-biotin-peroxidase complex (17). Sections were counterstained with Mayer’s hematoxylin.

Detection of Mouse and Human BEST1 Proteins by Western Blotting—Protein was isolated from mouse nose, trachea, and kidney epithelium and from cultured cells using buffer containing 150 mmol of NaCl, 50 mmol of Tris, 100 mmol of dithiothreitol, 1% Nonidet P-40, and 1% protease inhibitor mixture (Sigma). Equal amounts of total protein (20 μg) were separated on a 7% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences), which was blocked with 3% nonfat dried milk in phosphate-buffered saline/Tween 20 (0.1%) buffer for 1 h at room temperature. The membrane was incubated with a rabbit anti-mouse or rabbit anti-human BEST1 antibody (1:5000) overnight at 4 °C. Proteins were visualized using a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:30,000; Acris Antibodies GmbH, Hiddenhausen, Germany) and an ECL Advance Western blotting detection kit (Amersham Biosciences). Signal detection was done using a Fluor-S Multimager system (Bio-Rad).

Cell Culture—9HTE and 16HBE-14o cells (bronchial epithelium; kindly provided by Prof. D. Gruenert, California Pacific Medical Research Institute, San Francisco), Calu-3 cells (pulmonary adenocarcinoma), H441 cells (lung adenocarcinoma; kindly provided by Dr. S. Wilson, University of Dundee,
Dundee, Scotland, United Kingdom), HT29 cells (colorectal carcinoma epithelial), T84 cells (colorectal carcinoma), and M1 cells (mouse collecting duct; kindly provided by C. Korbmacher, Physiologisches Institut, Universität Erlangen, Erlangen, Germany) were grown in Dulbecco’s modified Eagle’s/Ham’s F-12 medium (1:1). HEK293 cells (embryonic kidney; kindly provided by Dr. Ralph Witzgall, Institute of Anatomy, University of Regensburg) and 9HTE/16HBE cells were grown in Dulbecco’s modified Eagle’s medium and minimum Eagle’s medium, respectively. H441 cells were grown in RPMI 1640 medium. All media were supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. Cells were incubated in 5% CO2 at 37 °C. Media were substituted with 2 mM glutamine (Calu-3, H441, T84, and 9HTE), 20 mg/ml galactose (HT29), 10 mM HEPES (Calu-3), and 10 μg/ml insulin, 5.5 μg/ml transferrin, 6.7 μg/ml selenium, and 0.1 μM dexamethasone (H441 and M1). Cells were seeded on plastic dishes or glass coverslips coated with bovine plasma fibronectin (Invitrogen) and bovine dermal collagen (Cellon, Bereldange, Luxembourg).

Materials and Statistical Analysis—All compounds used were of the highest available grade of purity and were from Sigma or Merck. All cell culture reagents were from Invitrogen. Student’s t test (for paired or unpaired samples as appropriate) and analysis of variance were used for statistical analysis. p < 0.05 was accepted as significant.

RESULTS

Ca2+-activated Cl− Secretion in Mouse Airways—In mouse trachea, stimulation of luminal purinergic P2Y receptors by ATP (100 μM) increases intracellular Ca2+ and stimulates Cl− secretion (18, 19). ATP induced a negative voltage deflection in Ussing chamber experiments and activated a short circuit current (Isc) (Fig. 1). Application of a CaCC inhibitor, DIDS (100 μM) or niflumic acid (10 μM), inhibited ATP-induced transport in the trachea (Fig. 1, A and B). Activation of Cl− secretion was suppressed by the Ca2+ chelator BAPTA (10 μM) or by depletion of intracellular endoplasmic reticulum Ca2+ stores with cyclopiazonic acid (10 μM) (Fig. 1, C and D). CaCC have been found in primary murine nasal cell cultures and in cystic fibrosis nasal epithelium but not in normal murine nasal tissue (20–22). We occasionally detected a small Cl− secretory response upon ATP stimulation, which was insignificant, however (Fig. 2, A and B). mRNA analysis demonstrated the presence of mouse bestrophin-1, -2, and -4 in both the trachea and nose (Fig. 2C). However, Western blot analysis detected BEST1 protein only in the trachea but not in the nasal epithelium (Fig. 2D). Expression of BEST1 was further demonstrated by immunohistochemistry using horseradish peroxidase-conjugated rabbit IgG. BEST1 was detected in the tracheal epithelium at lower and higher magnifications, whereas control stains without primary antibody were negative (Fig. 2, E and F). Thus, expression of BEST1 correlates with the appearance of Ca2+-activated Cl− currents in mouse trachea.

Expression of Endogenous BEST1 Induces CaCC in Airway Cells—we examined CaCC in different human airway epithelial cell lines. In patch clamp experiments with human bronchial epithelial cells (16HBE), a whole cell Cl− current was activated by 100 μM ATP, which was inhibited by 100 μM DIDS (Fig. 3). Expression of BEST1 protein in 16HBE cells was verified by Western blotting (data not shown). We suppressed expression of BEST1 in 16HBE cells by incubation with BEST1 siRNA. Successful suppression was verified by semiquantitative RT-PCR, BEST1 immunocytochemistry, and Western blotting (Fig. 3E). ATP-induced whole cell conductances were significantly reduced in siRNA-treated cells compared with control cells or cells transfected with scrambled RNA (Fig. 3D). Ca2+-activated whole cell conductances were compared in four different human airway epithelial cell lines grown on glass coverslips (Fig. 4). Both Cl− and K+ currents may be activated by increases in intracellular Ca2+, which we stimulated the cells in the presence of the K+ channel blockers Ba2+ (5 mM) and tetraethylammonium (10 mM). RT-PCR analysis of the four human bestrophin isoforms demonstrated expression of BEST1 in 16HBE and Calu-3 cells but not in H441 and 9HTE cells (Fig. 4). Accordingly, ATP-activated Cl− currents were found only in 16HBE and Calu-3 cells but not in H441 and...
9HTE cells. This further suggests that expression of endogenous BEST1 allows for Ca\(^{2+}\)/H1001-activated Cl\(^{-}\)/H1002 currents in airway epithelial cells.

Expression of BEST1 in Colonic Epithelial Cells—We also detected expression of BEST1 along with BEST4 in the human colonic cancer cell line HT29 (Fig. 5A). These cells activated a Ca\(^{2+}\)/H1001-dependent Cl\(^{-}\)/H1002 conductance upon stimulation with ATP (Fig. 5B). Similar results, e.g. expression of BEST1 mRNA and protein along with Ca\(^{2+}\)/H1001-activated Cl\(^{-}\) currents, were obtained in the colonic cancer cell line T84 (data not shown). We suppressed expression of BEST1 in HT29 cells by BEST1 siRNA. BEST1 knockdown was 61–78% according to densitometric analysis of Western blots (Fig. 5D). Incubation of the cells with scrambled unrelated RNA showed no effects. Whole cell patch clamp analysis demonstrated largely reduced Ca\(^{2+}\)/H1001 (ATP)-activated whole cell currents in siRNA-treated cells compared with control cells or cells transfected with scrambled RNA. Of 23 siRNA-incubated cells, none showed a current similar in size to control cells (Fig. 5, C and E).

We further examined expression of BEST1 in native mouse colonic epithelium. To this end, we isolated proximal and distal colonic epithelia and determined ion transport activated by carbachol stimulation (100 \(\mu\)M) of basolateral M3 receptors. In the proximal colon, carbachol induced a fast and negative voltage deflection due to activation of a transient Cl\(^{-}\) secretion. In contrast, stimulation of the distal colon activated a delayed
buminal K⁺ secretion (Fig. 6, A and B). Expression of both BEST1 mRNA and protein was more prominent in isolated colonic crypts of the proximal colon (data not shown). Immunohistochemistry clearly detected BEST1 expression in the mouse proximal colon, whereas only a few cells stained positive for BEST1 in the distal colonic epithelium (Fig. 6C). Taken together, these results suggest that BEST1 also participates in Ca²⁺+-dependent Cl⁻ secretion in the proximal colonic epithelium.

Expression of BEST1 in Renal Epithelial Cells—In mouse collecting duct cells, ATP activated a whole cell conductance, which was inhibited by 100 μM DIDS but not by 5 mM Ba²⁺ (Fig. 7A). The corresponding I/V curves were linear (Fig. 7B), probably due to high intracellular Ca²⁺ levels induced by purinergic stimulation (data not shown). We added an anti-mouse BEST1 antibody (1:500) to the patch pipette filling solution, which binds to the C-terminal end of BEST1. Inclusion of this anti-body significantly reduced the whole cell conductance activated by 100 μM ATP (Fig. 7, C and D). M1 cells expressed mRNAs for the two isoforms BEST1 and BEST2 (Fig. 7E). Expression of BEST1 protein was detected in lysates from M1 cells and mouse kidney (Fig. 7F). In mouse kidney, immunohistochemistry detected expression of BEST1 throughout the medulla and cortex, with the strongest expression in the region of the papilla. However, not all cells within a tubular segment expressed BEST1, and thus, a rather spotted staining was obtained. Fig. 7G shows co-staining of the cytoskeleton (red) and BEST1 (green). In agreement with this result, CaCC have been found in cells from almost all renal tubular segments (4).

Expression of Human BEST1 Induces CaCC in HEK293 Cells—To further confirm a role for BEST1 as a CaCC, we expressed human BEST1 in HEK293 cells. Expression was verified by Western blot analysis and immunocytochemistry (data not shown). HEK293 cells coexpressing BEST1 and EGFP activated a whole cell conductance upon stimulation with 100 μM ATP. This was not observed in HEK293 cells expressing only EGFP (Fig. 8, A and B). Coexpressing cells demonstrated an enhanced base-line conductance and a reduced membrane voltage, probably due to enhanced base-line Cl⁻ conductance. Membrane voltage was further depolarized, and the whole cell conductance was inhibited by reducing the bath Cl⁻ concentration to 25 mM (Fig. 8, C and D).
30 mM (Fig. 8, B and C). The ATP-activated whole cell conductance was inhibited by 100 μM DIDS (Fig. 8D). Taken together, these results strongly suggest that Ca²⁺-dependent Cl⁻ currents in epithelial cells from airways, colon, and kidney are due to expression of BEST1.

**DISCUSSION**

**CaCC in Epithelial Tissues**—CaCC are fundamental for fluid secretion in acini of exocrine tissues, such as lachrymal, parotid, mandibular, and pancreatic glands (23). In airways and in the colonic epithelium, electrolyte secretion is dominated by cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channels (24). Apart from CFTR, Cl⁻ secretion is activated through stimulation of CaCC present in the apical membranes of airways and other organs. Previous studies showed that (i) application of the Ca²⁺ ionophore ionomycin also activates Cl⁻ secretion; (ii) in the presence of low (1 μM) extracellular Ca²⁺, activation of Cl⁻ secretion is very transient; (iii) after inhibition of phospholipase C, purinergic Cl⁻ secretion is reduced; and (iv) DIDS inhibits Ca²⁺-activated Cl⁻ secretion in mouse airways (18). These and other experiments (19) strongly suggest that secretion occurs through Ca²⁺-dependent activation of Cl⁻ channels. Outwardly rectifying Cl⁻ channels are unlikely candidates because Ca²⁺ activates small conductance Cl⁻ channels in HT29 and 16HBE cells (25, 26).
Both CFTR and CaCC control airway surface liquid, which is essential for mucociliary clearance (27). Purinergic activation of CaCC in the airways resembles an important therapeutic principle for cystic fibrosis (28, 29). Cl\(^-\) secretion in mouse airways is dominated by CaCC, which explains why CFTR knock-out animals do not develop a cystic fibrosis lung disease (21). Interestingly, a functional relationship exists between CaCC and CFTR (30, 31), and Ca\(^{2+}\)-mediated Cl\(^-\) secretion is enhanced in cystic fibrosis airways (19). Thus, Ca\(^{2+}\)-activated Cl\(^-\) secretion is found in cystic fibrosis but not normal murine nasal epithelium (21). After pre-stimulation of CFTR, CaCC was also activated in normal nasal epithelium. However, this is probably due to activation of basolateral K\(^+\) channels rather than activation of luminal CaCC (32). Although transcripts for BEST1 were detected in the nasal epithelium, no significant levels of BEST protein were found. This is probably not explained by mRNA instability because no AU-rich elements could be detected.

In the human colonic epithelia of older infants and adults, CaCC-mediated Cl\(^-\) secretion is less important. Thus, no Cl\(^-\) secretion is found in the absence of functional CFTR, e.g. in the colonic epithelia of cystic fibrosis patients (33–35). However, younger infants may express CaCC in the colonic epithelium and therefore be susceptible to virus-induced diarrhea. In fact, age-dependent diarrhea was induced in mice by the rotavirus toxin NSP4 in a previous study (36). NSP4 increases intracellular Ca\(^{2+}\) and activates Cl\(^-\) secretion in newborn mouse pups (37). In the present study, we detected Ca\(^{2+}\)-activated Cl\(^-\) secretion in the proximal but not distal colon. Accordingly, BEST1 was well detected in the proximal but not distal colon. The role of CaCC in the kidney is controversial (38, 39). However, in studies with primary and permanent cultures of proximal and distal tubules, thick ascending limb, and collecting ducts, CaCC was identified (40–43). Also in the present experiments with M1 collecting duct cells, CaCC was detected. Thus, renal epithelial cells may retain the ability to induce Ca\(^{2+}\)-activated Cl\(^-\) transport.

**Bestrophin: Cl\(^-\) Channel or Regulator of Ca\(^{2+}\) Signaling?**—Sulphydryl scanning enabled structure-function analysis and demonstrated that bestrophins form bona fide Cl\(^-\) channels (44, 45). Direct activation of BEST4 by Ca\(^{2+}\) was demonstrated in excised membrane patches (46). Nevertheless, it has been proposed that BEST1 is a regulator of voltage-gated L-type Ca\(^{2+}\) channels rather than a Ca\(^{2+}\)-regulated Cl\(^-\) channel (47, 48). Purinergic increase in [Ca\(^{2+}\)]\(_{i}\) was augmented in retinal pigment epithelium cells of Vmd2\(^-/-\) mice. In this study, the [Ca\(^{2+}\)]\(_{i}\) increase was remarkably slow. This is in sharp contrast to Ca\(^{2+}\) responses observed in the epithelial tissues used here: the [Ca\(^{2+}\)]\(_{i}\) increase occurred within <1 s and was typically 4–10-fold (data not shown). Moreover, the ATP-induced Ca\(^{2+}\) increase is due to Ca\(^{2+}\) release from intracellular stores (peak) and Ca\(^{2+}\) influx, probably through transient receptor potential-related Ca\(^{2+}\) channels (plateau) (49). Preliminary unpublished measurements show only insignificant changes in Ca\(^{2+}\) signaling upon manipulation of BEST1 expression.

**BEST1 Mediates Ca\(^{2+}\)-activated Cl\(^-\) Secretion in Epithelial Tissues**—The present data show endogenous expression of BEST1 in mouse and human epithelial cells from airways, colon, and kidney. BEST1 expression clearly correlated with the appearance of CaCC. Rectification of the Cl\(^-\) currents varied between the different cell lines. I/V curves were almost linear for M1 and HEK293 cells but were rectifying for 16HBE cells. Rectification depends on the level of [Ca\(^{2+}\)]\(_{i}\), and disappears at high (>1 \(\mu M\)) concentrations (3, 42, 50, 51). ATP increased [Ca\(^{2+}\)]\(_{i}\), to values above 1 \(\mu M\) in M1 cells but to values below 1 \(\mu M\) in 16HBE cells (data not shown), which may explain the variability. In HT-29 and 16HBE cells, suppression of BEST1 by siRNA inhibited CaCC. These results clearly indicate that BEST1 is necessary for Ca\(^{2+}\)-activated Cl\(^-\) secretion. However, they do not rule out the possibility that BEST1 is a regulator of a still unidentified CaCC or that it is part of a Cl\(^-\) channel complex. Only a fraction of BEST1 appears to be localized in the plasma membrane, whereas most is found in the cytosol, probably bound to vesicular membranes (15, 45). The spotted expression of BEST1 in some tissues suggests that CaCC is not equally present within the epithelium. Thus, expression may
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depend on additional factors, which should be the subject of subsequent studies.

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