Evidence against Functional Interaction between Aquaporin-4 Water Channels and Kir4.1 Potassium Channels in Retinal Müller Cells

Javier Ruiz-Ederra, Hua Zhang, and A. S. Verkman

From the Departments of Medicine and Physiology, Cardiovascular Research Institute, University of California, San Francisco, California, 94143-0521

Indirect evidence suggests that the Müller/glial cell water channel aquaporin-4 (AQP4) modulates K⁺ channel function of the closely associated Kir4.1 protein. We used patch clamp to compare Kir4.1 K⁺ channel function in freshly isolated Müller cells from retinas of wild-type (+/+) and AQP4 knock-out (−/−) mice. Immunocytochemistry showed a comparable Kir4.1 protein expression pattern in Müller cells from +/+ and −/− retinas, with greatest expression at their end feet. Osmotic water permeability was >4-fold reduced in −/− than in +/+ Müller cells. Resting membrane potential did not differ significantly in +/+ versus −/− Müller cells (−64 ± 1 versus −64 ± 1 mV, S.E., n = 24). Whole-cell K⁺ currents recorded with a micropipette inserted into the cell soma were Ba²⁺-sensitive and showed no significant differences in magnitude in +/+ versus −/− Müller cells (1.3 ± 0.1 versus 1.2 ± 0.1 nA at −160 mV) or in inwardly rectifying current-voltage relationships. Spatially resolved K⁺ currents generated by pulsed K⁺ injections along Müller cell bodies were also comparable in +/+ versus −/− Müller cells. Single-channel cell-attached patch clamp showed comparable unitary conductance, current-voltage data, and open probability in +/+ versus −/− Müller cells. Thus, contrary to the generally accepted view, our results provide direct evidence against functionally significant AQP4 modulation of Müller cell Kir4.1 K⁺ channel function.

Aquaporin-4 (AQP4) is a water-selective transport protein expressed strongly in plasma membranes of supporting cells that are closely associated with electrically excitable cells, including Müller cells in retina (associated with bipolar cells), astroglial cells in the central nervous system (associated with neurons), and Hensen’s/Claudius’ cells in cochlea (associated with hair cells) (1–3). At the light microscopic level AQP4 colocalizes with the inwardly rectifying K⁺ channel Kir4.1 (4) and has been shown by immunoprecipitation to interact with AQP4 (5), likely as part of a macromolecular complex that includes α-syntrophin and dystrophin (6). It is, thus, generally believed without direct evidence that AQP4 modulates Kir4.1 K⁺ channel function.

Phenotype studies of mice lacking AQP4 support the possibility of functionally significant AQP4-Kir4.1 interactions. AQP4 null mice manifest altered seizure susceptibility and duration (7, 8) and reduced evoked potential responses to sound (9) and light (10). After neuronal stimulation in mouse models of spreading cortical depression and electrical seizure induction associated with increased extracellular space (ECS) K⁺ (8, 11), mice lacking AQP4 have remarkably delayed cellular reuptake of K⁺ from the ECS. Similar alterations in seizure and ECS K⁺ dynamics have been reported in α-syntrophin knock-out mice that manifest cellular mislocalization in glial cell AQP4 (12). Impaired Kir4.1-dependent cellular K⁺ uptake in AQP4 deficiency/mislocalization has been proposed to account for these phenotype findings.

The purpose of this study was to test the hypothesis that AQP4 expression modulates Kir4.1 K⁺ channel function in retinal Müller cells. For these studies we used patch clamp to characterize Kir4.1 K⁺ channel function in freshly isolated Müller cells from retinas of wild-type and AQP4 knock-out mice and immunofluorescence to compare Kir4.1 protein expression. Contrary to expectations, we found, based on multiple criteria, no influence of AQP4 knock-out on Müller cell Kir4.1 expression or function.

EXPERIMENTAL PROCEDURES

Müller Cell Isolation—Adult wild-type and AQP4 null mice in a CD1 genetic background were used as described (13). After mouse sacrifice, enucleation, and removal of the anterior segment, retinas were carefully dissected and incubated at 37 °C for 30–40 min in Ringer solution containing 0.3 mg/ml papain and 2.5 mM L-cysteine (both from Sigma). Retinas were then briefly incubated in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Invitrogen) and 0.1 mg/ml DNase I (Sigma) and gently triturated using a Pasteur pipette. To enrich for Müller cells, the triturated material was transferred to a 15-ml tube containing a continuous density gradient composed of 0–30% Percoll (GE Healthcare) in Dulbecco’s modified Eagle’s medium and centrifuged at 1500 × g for 5 min. The band located in the middle of the tube, which was enriched...
in Müller cells, was transferred with a Pasteur pipette to a polylysine-laminin-coated plastic plate in CO₂-independent medium and kept for ~30 min at room temperature to allow cells to adhere.

**Immunofluorescence**—Eye globes were fixed in 4% paraformaldehyde for 24 h at 4 °C, processed in increasing concentrations of ethanol and then in a clearing agent (Citrisolv, Fisher), and embedded in paraffin. Five-micrometer-thick sagittal sections from the central eye were used, taking the optic nerve and the pupil as reference points. Sections were deparaffinized in ethanol and then in a clearing agent (Citrisolv, Fisher), and embedded in paraffin. Five-micrometer-thick sagittal sections of ethanol and then in a clearing agent (Citrisolv, Fisher), and embedded in paraffin. Five-micrometer-thick sagittal sections from the central eye were used, taking the optic nerve and the pupil as reference points. Sections were deparaffinized in the clearing agent and rehydrated in graded ethanol. After epitope retrieval with citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0, 30 min, 95–100 °C), sections were blocked with bovine serum and incubated with rabbit anti-AQP4 or anti-Kir4.1 antibodies (at 1:500 and 1:250, respectively; Alomone Labs, Jerusalem, Israel). Primary antibodies were detected with Texas Red goat-anti-rabbit and Alexa Fluor 488 goat anti-rat secondary antibodies (1:200; Molecular Probes, Eugene, OR).

For immunostaining of isolated cells (as prepared for electrophysiological measurements), adherent cells were fixed for 15 min at room temperature, blocked with bovine serum, and incubated with primary and secondary antibodies as described above. Colocalization studies were done using Zenon Technology (Molecular Probes) according to manufacturer’s instructions. Kir4.1 immunofluorescence was quantified using a Leica DM4000B epifluorescence microscope equipped with a 40× objective lens (Leica, Heidelberg, Germany) and cooled CCD camera (Spot, Sterling Heights, MI). Fluorescence intensities were measured using NIH Image software in eight square regions-of-interest, each ~50 μm², selected along the longitudinal axis of a total of 18 Müller cells (as shown in Fig. 1B). Area-integrated fluorescence intensities were background-corrected and normalized to the fluorescence in the cell soma.

**Immunoblot Analysis**—Retinas were isolated and homogenized in 250 mM sucrose, 1 mM EDTA, 2 μg/ml aprotinin, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, and 100 μg/ml serine protease inhibitor (Sigma). Homogenates were centrifuged at 1500 × g for 10 min, and the supernatant was loaded onto a 4–12% sodium dodecyl sulfate-polyacrylamide gel (20 μg of protein/lane). Proteins were transferred to a polyvinylidene difluoride membrane and incubated with rabbit anti-AQP4 or anti-Kir4.1 antibody (1:1000 and 1:250, respectively; Alomone Labs) or anti-β-actin antibody (1:2000; GE Healthcare) followed by horseradish peroxidase-linked anti-rabbit IgG (1:10,000; GE Healthcare) and visualized by enhanced chemiluminescence (Roche Diagnostics). Protein band densitometry was performed (Scion Image for Macintosh; Scion, Frederick, MD) normalizing to β-actin immunoreactivity.

**Osmotic Water Permeability**—After immobilization of retinal cell suspensions on a plastic dish as described above, cell cytoplasm was labeled with calcein by 30 min of incubation with 10 μM calcein-AM (Molecular Probes) at 37 °C. Müller cells were identified by their characteristic morphology. Osmotic water permeability was measured by calcein fluorescence in response to changing perfusate osmolalities from 300 (phosphate-buffered saline (PBS)) to 150 mosM (diluted PBS) as described (14). Calcein fluorescence was measured on a Nikon TE-2000 inverted fluorescence microscope equipped with ×40 oil immersion objective and cooled CCD camera.

**Whole-cell Patch Clamp**—After cell immobilization as described above, Müller cells were identified by their characteristic morphology. Whole-cell patch clamp recording was done at room temperature (20–22 °C) using an Axon 200B amplifier (Molecular Devices, Union City, CA). All recordings were done within 4 h after plating. Standard external recording solution contained 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM EGTA, and 10 mM dextrose, pH 7.4, adjusted with NaOH. In some experiments, BaCl₂ (final concentration 100 μM) was added to the bath solution. Exchange of perfusion solutions was done using a DAD-8VC superfusion drug application system (ALA Scientific Instruments, Westbury, NY). Micro-electrodes were fabricated using a P-97 Micropipette Puller (Sutter Instruments, Novato, CA). A small tip with resistance of 6–10 MΩ was used for the relatively small soma of mouse Müller cells. The intracellular (pipette) solution contained 10 mM NaCl, 130 mM KCl, 0.5 mM CaCl₂, 2 mM MgCl₂, 5 mM EGTA, 10 mM HEPES, and 3 mM Na₂ATP, pH 7.2, adjusted with NaOH. Data were acquired using a Digidata 1440A digitizer (Molecular Devices) with Clampex 10.0 software (Molecular Devices). Data were sampled at 5 kHz and low-passed at 5 kHz. Series resistance and capacitance compensation were not used during recording. Data were analyzed by Clampfit 10.0 (Molecular Devices) and Sigmaplot (SPSS, Chicago, IL).

For K⁺ injection experiments, Müller cells with two long arms were identified and voltage-clamped by micropipette puncture of the soma as described above. A second pipette with a tip diameter of 3 μm was backfilled with high K⁺ solution containing 90 mM NaCl, 50 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM dextrose, and 10 mM HEPES, pH 7.4. Injection was controlled by a Nanoliter 2000 injector (World Precision Instruments, Sarasota, FL) mounted on a high-resolution micromanipulator (New Focus, San Jose, CA). The tip was positioned at specified regions of the cell, as visualized by phase-contrast microscopy at 400× magnification. Cell-micropipette images were recorded at the site of each current recording. Data were recorded as described above after 100-ms injections of 2.3 nl of high K⁺ solution at a cell holding potential of ~−80 mV. All electrophysiological studies were done with the experimenter blinded to information about mouse genotype.

**Single Channel Recordings**—Cell-attached single-channel patch recordings were done at room temperature. Pipettes were pulled from borosilicate tubing (Sutter Instruments, Novato, CA). The tips of the pipettes were coated with sticky wax (Pourette Candlemaking Supplies, Seattle, WA) and fire-polished with a MF-830 Microforge (Narishige, Japan). Pipettes were filled with a solution containing 145 mM KCl, 0.2 mM CaCl₂, 1 mM MgCl₂, 5 mM EGTA, and 10 mM HEPES, pH 7.3, adjusted with KOH. The bath solution contained 145 mM KCl, 5 mM EGTA, 1 mM MgCl₂, and 10 mM HEPES, pH 7.4, adjusted with KOH. Data were sampled at 5 kHz and low-passed at 1 kHz using an Axon 200B amplifier. Data were analyzed using Clampfit 10.0 software.

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RESULTS

Kir4.1 and AQP4 Expression in Müller Cells—Kir4.1 and AQP4 expression were studied by immunofluorescence in isolated cells and retinal sections. Fig. 1A shows representative fluorescence micrographs of two Müller cells from wild-type (+/+) and AQP4 null (−/−) mice. Kir4.1 and AQP4 showed similar distributions in wild-type Müller cells, with protein expression throughout the membrane and a higher concentration in the end feet and to a lesser extent in the soma and the apical region. As expected, no specific staining for AQP4 was observed in Müller cells from AQP4 null mice. The distribution of Kir4.1 was not affected by AQP4 deletion, as seen qualitatively in Fig. 1A and by quantitative image analysis in Fig. 1B, where background-subtracted, area-integrated fluorescence intensity was determined along the axis of many isolated Müller cells and normalized to fluorescence in the soma. Reduced osmotic water permeability in the AQP4 null Müller cells was verified using a calcein fluorescence quenching method in which immobilized cells were labeled with calcein (Fig. 1C, top) and subjected to an osmotic gradient (Fig. 1C, center). Osmotic equilibration was very fast in wild-type Müller cells, with the measurement limited by solution mixing. Relative water permeability, which is proportional to the reciprocal half-time for cell osmotic equilibration, was more than 4-fold reduced in the AQP4 null Müller cells (Fig. 1C, bottom).

In retinal sections from wild-type mice, Kir4.1 and AQP4 showed a polarized distribution, with greatest expression in inner retina containing the end foot processes (Fig. 2A). Immunofluorescence labeling was also seen in perivascular processes (arrows), and strong expression of AQP4 was also detected in the outer plexiform layer. The distribution of Kir4.1 was similar in wild-type and AQP4 null retinas.

Immunoblot analysis with AQP4 antibody of homogenates of retinas from wild-type mice showed a band at ~32 kDa (Fig. 2B, top) that was absent in retinas from AQP4 null mice. Kir4.1 expression was seen as an ~85-kDa band corresponding to the dimeric form of the channel that was similar in retinas from wild-type and AQP4 null mice as seen qualitatively and by quantitative analysis after normalization to β-actin expression (Fig. 2B, bottom).

Electrophysiological Properties of Müller Cell K⁺ Channels—Whole-cell currents were characterized in Müller cells isolated from retinas of wild-type mice. The isolated cells had the typical morphological characteristics of Müller cells as seen in Fig. 1A. The resting membrane potential was −64.2 ± 1.1 mV (S.E., n = 24). Under whole-cell voltage-clamp conditions (~80 mV holding potential), a series of voltage steps from −160 to +40 mV with 10-mV increments produced inward and outward currents as shown in Fig. 3A. The currents were strongly inhibited by 100 μM Ba²⁺ added to the bathing solution. After subtraction, the Ba²⁺-insensitive currents showed weak inward rectification (Fig. 3B) with a reversal potential of −81.6 ± 0.3 mV (n = 21), in good agreement with the calculated K⁺ reversal potential of 82.8 mV. As shown in Fig. 3B, increasing the extracellular K⁺ concentration to 20 mM (by replacing NaCl) shifted the reversal potential to −48.6 ± 1.7 mV (n = 8), in agreement with the calculated reversal potential of −47.6 mV. We conclude that the Ba²⁺-sensitive current component in Müller cells from wild-type mice is carried mainly by K⁺ ions. All of
these characteristics are consistent with those reported for Kir4.1 channel-mediated currents (15, 16).

**Kir4.1 Currents in Wild-type and AQP4-deficient Müller Cells**—To determine whether AQP4 expression alters Kir4.1 function, K+ currents were measured in Müller cells isolated from retinas of wild-type and AQP4 null mice. Representative current recordings are shown in Fig. 4A. All currents were sensitive to 100 μM Ba2+ (data not shown). Fig. 4B summarizes averaged data from many cells, showing no significant difference in current magnitude or current-voltage relationships in wild-type versus AQP4 null Müller cells. We also used a voltage-ramp protocol to characterize Kir4.1 K+ currents. Fig. 4C summarizes data from many cells, showing weak inward rectification with no significant differences in wild-type versus AQP4 null Müller cells. Fig. 4D shows a single component distribution of resting membrane potentials, with no significant difference in mean resting potential in wild-type versus AQP4 null Müller cells.

Prior studies of Müller cells in different mammalian species have suggested a non-uniform spatial distribution of K+ conductance along their long axis, with the highest conductance at their end feet and in the central region (17, 18). Cell regional conductance is dependent on the product of specific K+ membrane conductance and regional cell surface area. To determine the cellular distribution of Kir4.1, cells were voltage-clamped to −80 mV, and induced currents were recorded after focal injection of a high K+ solution. Currents were recorded from eight distinct regions of Müller cells, from the end feet to the apical end. Fig. 5A shows representative currents evoked from different regions in wild-type and AQP4 null Müller cells. There were relatively large currents at the end feet, soma, and apical end, with lower current in the proximal process (Fig. 5A). A qualitatively similar pattern was seen in AQP4 null Müller cells. Fig. 5B summarizes the data, with current maxima normalized to currents in the soma of the same cells. There was no significant difference in K+ currents in wild-type versus AQP4 null Müller cells, which is consistent with the similar spatial distribution of Kir4.1 protein as shown in Fig. 1B.

The single-channel properties of Kir4.1 in Müller cells were examined by the cell-attached patch clamp technique with pipette and bath solutions containing 145 mM K+. A single population of Kir channel currents was seen in ~30 successful cell-attached patch recordings for each type, with ~40% of recordings showing single channels. Representative single-channel recordings at different holding potentials are shown in Fig. 6A. The recordings were qualitatively similar. Single-channel current-voltage analysis showed no significant differences in wild-type versus AQP4-deficient Müller cells (Fig. 6B). The unitary conductances of Kir4.1 (in the inward direction) were 21 ± 4 pS (n = 8, wild-type) and 20 ± 4 pS (n = 8, AQP4-deficient). The
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channel opened in bursts with relatively high open probabilities at negative holding potentials as summarized in Fig. 6C. The open probabilities were 0.8–1.0 at −40 to −100 mV, with no significant difference in wild-type versus AQP4-deficient Müller cells.

DISCUSSION

We found no influence of AQP4 deletion on Müller cell Kir4.1 function or distribution as measured by whole-cell current and single channel patch clamp recordings in freshly isolated Müller cells as well as by immunofluorescence in isolated cells and retinal sections. These results contradict the widely accepted notion that glial/Müller cell AQP4 modulates Kir4.1 function. Indirect evidence that had supported a functional AQP4-Kir4.1 interaction includes membrane co-localization of AQP4 and Kir4.1, studies of changes in the extracellular space volume in acute cortical slices, where K⁺ spatial buffering paralleled changes in extracellular space volume, and delayed K⁺ clearance in α-synaptophin null mice (16, 19, 20). From these findings it was proposed that AQP4 and Kir4.1 work in concert so that AQP4 itself or water flux through AQP4 contributes to Kir4.1-dependent volume changes in the extracellular space (12, 20, 21). More recently, using immunoprecipitation, evidence for a direct physical interaction between AQP4 and Kir4.1 through the dystrophia-glycoprotein complex was reported in retinal Müller cells (6).

K⁺ channels in retinal Müller cells are believed to play a crucial role in extracellular K⁺ homeostasis. Of the various K⁺ channels, inwardly rectifying K⁺ (Kir) channels are considered most important for spatial buffering of K⁺ (17). In retinal Müller cells in mammals, the weakly rectifying Kir4.1 channel is the predominant Kir-type K⁺ channel (15, 17). Using similar methods as reported before (15), we recorded Kir4.1 currents from adult mouse Müller cells. The membrane conductance of Müller cells is dominated by K⁺ currents with mild inward rectification as seen in whole-cell currents recorded in physiological solutions. The resting reversal potential was close to the calculated K⁺ equilibrium potential as reported previously to be typical for retinal Müller cells in rat, mouse, and rabbit (15, 17) as well as Kir4.1 currents in heterologously expressing oocytes and HEK293 cells (15, 22). In our experiment the majority of whole-cell current was sensitive to a low extracellular concentration of Ba²⁺, as expected from prior data (18, 23). Thus, as concluded previously (24), other voltage-dependent K⁺ (Kv) currents play at most a minor role in Müller cell K⁺ conductance.

The single-channel properties of Kir4.1 have been studied from different mammals and in transfected cell lines (15, 22, 25–29). All studies indicated a similar inward rectification with a unitary conductance of 20–30 pS, in agreement with our data in Fig. 6. We found no significant difference in the Kir4.1 single channel properties in wild-type versus AQP4-deficient Müller cells in unitary conductance, voltage-dependent open probability, and current-voltage relationships. These results support the conclusion that AQP4 expression does not affect Kir4.1 K⁺ channel function. In contrast to most measurements in mammalian cells, two distinct conductances of 21 and 36 pS were reported in *Xenopus* oocytes expressing Kir4.1. In isolated mouse Müller cells we recorded only a single population of Kir4.1 channels, which likely corresponds to the low conductance channel seen in *Xenopus* oocytes (22) and on HEK293 cells (15).

There are a number of possible explanations to reconcile our data showing no effect of AQP4 expression on Kir4.1 function with the prior immunolocalization and immunoprecipitation studies suggesting a possible AQP4-Kir4.1 interaction. AQP4 and Kir4.1 may indeed interact physically, although physical association does not necessarily imply that they form a functional unit. Alternatively, there may be little AQP4-Kir4.1 interaction. Immunogold evidence indicates co-enrichment of AQP4 and Kir4.1 on Müller cell vitreal and perivascular end feet membranes, although only small regions show good colocalization (4). Another concern in postulating AQP4-Kir4.1 interactions is the quite different membrane densities of these proteins, with AQP4s generally expressed at a density of >10⁹/μm² of membrane compared...
FIGURE 6. Single-channel patch clamp of Kir4.1 K⁺ channels in Müller cells. A, single-channel current traces from cell-attached membrane patches of wild-type and AQP4-deficient Müller cells with 145 mM K⁺ in the pipette. Holding potentials are indicated to the left of each trace (c, channel closed level; o, channel open level). B, unitary current-voltage data averaged from eight patches of wild-type and AQP4-deficient Müller cells (S.E.). Differences are not significant. The dashed line is a linear regression of the inward current with mean inward single-channel conductances of 21 ± 4 pS (wild type) and 20 ± 4 pS (AQP4 null). C, open probabilities (Pₒ) of the Kir4.1 channels from wild-type (S.E., n = 5) and AQP4-deficient (n = 4) Müller cells. Differences are not significant.

with less than 1 K⁺ channel/μm². The reported immunoprecipitation studies supporting AQP4-Kir4.1 interaction are non-quantitative and required non-physiological detergent solubilization conditions and covalent cross-linking that do not mimic native protein interactions (6). A final possibility to reconcile our results with prior data is that a different K⁺ channel becomes up-regulated in AQP4 deficiency to restore native K⁺ conductance. However, this possibility is unlikely given the essentially identical electrophysiological properties and Kir4.1 expression pattern in wild-type versus AQP4-deficient Müller cells.

As described in the Introduction, several types of studies in living mice indicate slowed K⁺ uptake from the ECS in brain in AQP4 deficiency or dysfunction as well as altered seizure dynamics. The absence of functionally significant Kir4.1-AQP4 interactions as found here, thus, requires identification of alternative mechanism(s) to account for the phenotype observations in mice. One possibility proposed by our laboratory is ECS expansion in AQP4 deficiency, which is supported by a variety of biophysical measurements of macromolecule diffusion in the ECS (30). However, the mechanism of ECS expansion in AQP4 deficiency remains unknown, nor is it known whether the ECS expansion in AQP4 deficiency is sufficient to account quantitatively for the delayed K⁺ reuptake. An alternative explanation is that compensatory changes in other channels expressed in Müller cell membranes in AQP4 null mice contribute to water influx, such as the Na⁺/HCO₃⁻ cotransporter (31). Increases in ECS K⁺ cause Müller cell depolarization, which favors the complex that includes AQP4 and Kir4.1 and, thus, indicate the need to identify alternative mechanisms to account for altered K⁺ and seizure dynamics in AQP4 deficiency.

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In conclusion, we found that Kir4.1 K⁺ channel function and expression in retinal Müller cells is independent of AQP4 water channel expression. Our data contradict a widely accepted view of a functionally significant macromolecular cellular uptake of Na⁺ and bicarbonate, producing an osmotic gradient driving water influx. We cannot at this time exclude the possibility of such compensatory changes in AQP4 deficiency. Small-molecule AQP4 inhibitors, when available, are needed to explore this possibility. Yet another possible explanation is that reduced water permeability by itself in AQP4 null mice might account for the impaired K⁺ reuptake and seizure phenotype differences in AQP4 deficiency. Perhaps local osmotic effects or highly polarized water flow might influence K⁺ transport by electro-osmotic or pseudosolvent drag phenomena. However, these possibilities remain speculative and will be quite challenging to verify experimentally.
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