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Astrocytes express functional TRPV2 ion channels

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ABSTRACT

Thermosensitive transient receptor potential (thermo TRP) channels are important for sensory transduction. Among them, TRPV2 has an interesting characteristic of being activated by very high temperature (>52 °C). In addition to the heat sensor function, TRPV2 also acts as a mechanosensor, an osomosensor and a lipid sensor. It has been reported that TRPV2 is expressed in heart, intestine, pancreas and sensory nerves. In the central nervous system, neuronal TRPV2 expression was reported, however, glial expression and the precise roles of TRPV2 have not been determined. To explore the functional expression of TRPV2 in astrocytes, the expression was determined by histological and physiological methods. Interestingly, TRPV2 expression was detected in plasma membrane of astrocytes, and the astrocytic TRPV2 was activated by very high temperature (>50 °C) consistent with the reported characteristic. We revealed that the astrocytic TRPV2 was also activated by lysophosphatidylcholine, a known endogenous lipid ligand for TRPV2, suggesting that astrocytic TRPV2 might regulate neuronal activities in response to lipid metabolism. Thus, for the first time we revealed that TRPV2 is functionally expressed in astrocytes in addition to neurons.

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1. Introduction

TRPV2 was originally cloned as a heat sensor and is well known to be activated by very high temperature (>52 °C) [1]. TRPV2 was also reported to be a mechano-sensor [2–5]. Recently, we reported that the mechanosenitive characteristics of TRPV2 regulated axonal outgrowth in developing neurons [4], and also regulated peristalsis of intestine [5]. It was also reported that insulin like growth factor I (IGF-I) promoted TRPV2 surface expression and activity [6]. Chemical ligands such as 2-aminoethoxydiphenyl borate (2-APB) and probenecid also activate TRPV2 [7,8]. Lysophosphatidylcholine (LPC) acts as an endogenous ligand for activation of TRPV2 [9]. In central nervous system, TRPV2 expression was reported in striatal, hippocampal and hypothalamic neurons [10–14].

In addition to neurons, glial cells are important to maintain our brain function. Notably, astrocytes provide metabolic support and eliminate waste products, such as neurotransmitters, from extracellular spaces [15]. They also regulate blood flow depending on neuronal activity [16,17]. Furthermore, astrocytes are essential for bidirectional communication with neurons, and thus can modulate neuronal activity [18,19]. Although TRPV2 expression was confirmed in neurons, the expressions in astrocytes remain poorly understood. In this study, we examined whether functional TRPV2 expressed in astrocytes by using a combination of histological and physiological methods.

2. Materials and methods

2.1. Animals

C57BL/6J mice were used for the study. All animal care and procedures were performed according to Gunma University guidelines.

2.2. Immunohistochemical analysis

Immunohistochemistry were performed as previously described [20,21]. The following antibodies were used; rabbit polyclonal anti-TRPV2 antibody (1:200, Transgenic [4]), mouse anti-GFAP (1:500, Sigma) antibody.

2.3. Reverse transcription PCR

The *TRPV channels* expressions were examined by reverse transcription PCR (RT-PCR). Total RNA was prepared from the DRG of adult ICR mice using TRIzol reagent (Invitrogen). Total RNA (1 μg) was converted to cDNA using SuperScriptII RNaseH (−) Reverse Transcriptase (Invitrogen). The *TRPV channels* were PCR-amplified from cDNA with reported PCR primer sets [22].

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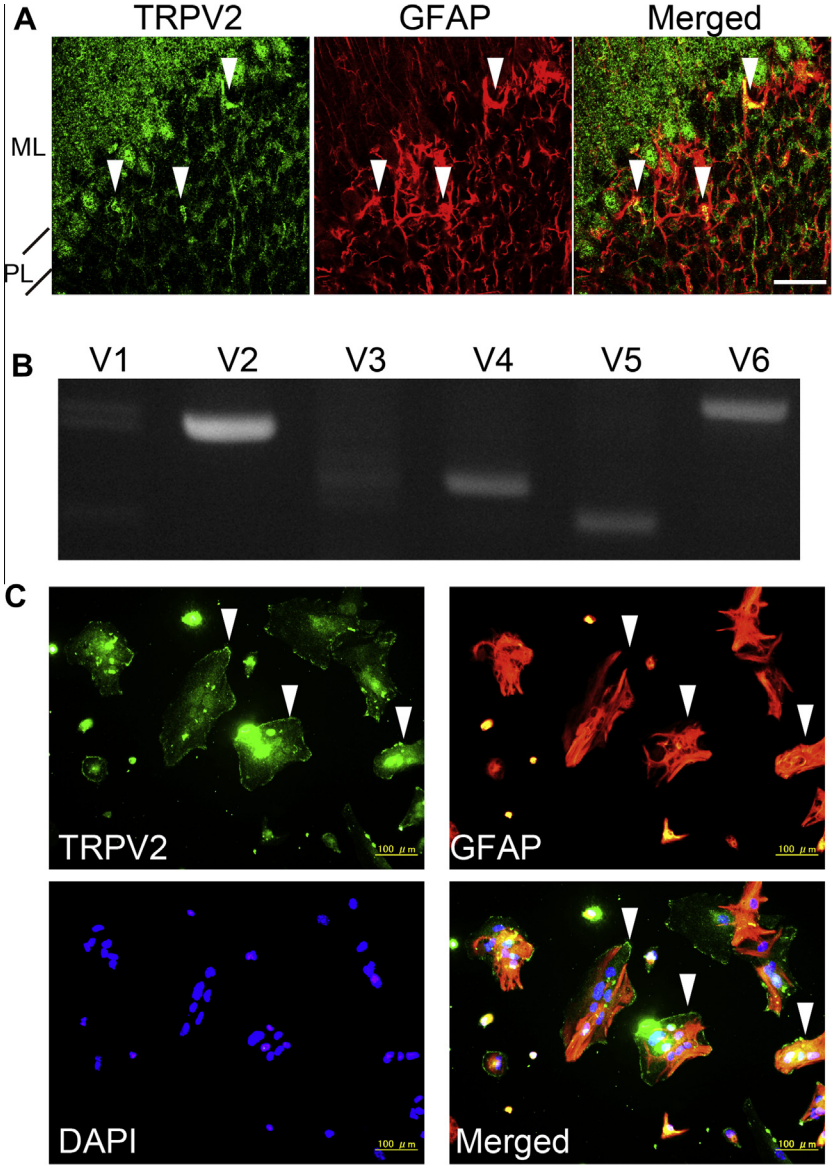

Fig. 1. TRPV2 is expressed in neurons and astrocytes. (A) Immunostaining of TRPV2 (green) and GFAP (red) in adult mouse cerebellum. ML; molecular layer. PL; Purkinje cell layer. TRPV2 expressions were observed in ML, PL and internal granular layers. Arrowheads represent TRPV2-expressing GFAP-positive astrocytes. Scale bar; 100 μm. (B) RT-PCR was performed from total RNA of cultured cerebellar astrocytes by each TRPV channel primer sets. (C) Immunostaining of TRPV2 (green) and GFAP (red) in cultured cerebellar astrocytes. Those cells were counter stained by DAPI (blue). Arrowheads represent TRPV2-expressing GFAP-positive astrocytes. Scale bars; 100 μm.

2.4. Cultivation of dissociated astrocytes

Cerebellar astrocytes were prepared from P0 to P2 mice as previously described [21]. The astrocytes were cultured in MEM + 10% FBS + 0.6% D-glucose + 100 U/mL penicillin + 100 μg/mL streptomycin.

2.5. Fluorescent measurements and electrophysiology

Fura2 fluorescence was measured by Fura2-AM (Molecular Probes, Carlsbad, CA) in a standard bath solution containing 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4. The Fura2 ratiometric fluorescence (340:380 nm) measurements were recorded. The standard bath solution for the patch–clamp experiments was the same as that used for fluorescence measurements. Reversal potential was measured using voltage-ramps (−100 to +100 mV in each 5 s interval). Pipette solutions for whole-cell recordings contained 120 mM K-gluconate, 20 mM KCl, 0.5 mM EGTA, 2 mM Mg-ATP, 2 mM K_2-GTP, and 10 mM HEPES, pH 7.4. Whole-cell recording data were sampled at 10 kHz and filtered at 5 kHz for analysis (Axon 200B amplifier with pCLAMP software, Axon Instruments, Foster City, CA).

3. Results and discussion

We first determined whether TRPV2 expression was observed in brain sections by immunohistochemical analysis utilizing reported specific anti-TRPV2 antibody [4]. Specificity of the antibody was confirmed as described by our previous work [4]. In cerebellum, strong TRPV2 expressions were observed in molecular layer (ML) and Purkinje cell layer (PL) consistent with previous reports (Fig. 1A), which neurons expressed TRPV2 [10–14]. In addition to the cerebellar neurons, GFAP, a specific marker for astrocytes, positive cells were also expressed TRPV2 (Fig. 1A, arrowheads), indicating that astrocytes express TRPV2 protein. To further confirm

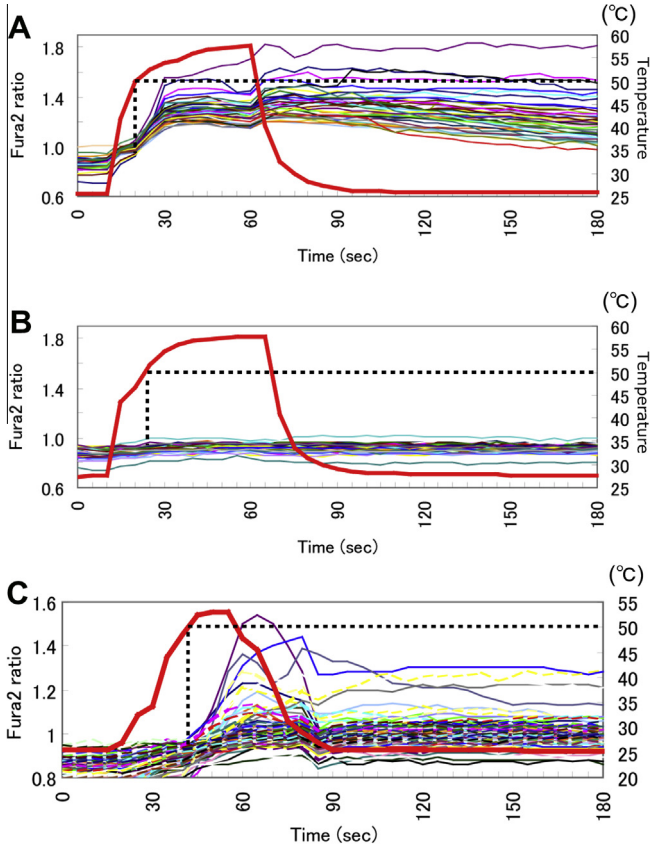

Fig. 2. Astrocytic TRPV2 respond to heat stimulus. (A and B) Quantification of Ca^{2+}-imaging experiments in cultured cerebellar astrocytes. We applied heat stimulus from room temperature to near 60 °C. Red thick traces represent the heat changes. Other thin traces represent changes of $[Ca^{2+}]_i$. Heat application (A) evoked steep rises of $[Ca^{2+}]_i$, however, heat application in the presence of 10 μM ruthenium red (B) inhibited the rises of $[Ca^{2+}]_i$. Dashed lines represent the temperature threshold for rises of $[Ca^{2+}]_i$. (C) Quantification of Ca^{2+}-imaging experiments in cultured cerebellar astrocytes. We applied short time course of heat stimulus from room temperature to near 55 °C. Red thick trace represents the heat changes. Other thin traces represent changes of $[Ca^{2+}]_i$. Dashed line represents the temperature threshold for rises of $[Ca^{2+}]_i$.

the astrocytic expression of TRPV2, we cultured astrocytes from neonatal cerebellum, and performed RT-PCR or immunostaining by anti-TRPV2 antibody. RT-PCR revealed that *TRPV2*, *TRPV4*, *TRPV5* and *TRPV6* expressions in cultured cerebellar astrocytes, but *TRPV1* and *TRPV3* expressions were not detected (Fig. 1B). Immunostaining revealed that TRPV2 proteins were localized in plasma membranes of astrocytes, which could be classified by GFAP-staining (Fig. 1C, arrowheads) in cultured cells.

The above results on the expression of TRPV2 in the cerebellar astrocytes prompted us to conduct studies to show functional expression of the TRPV2. Hence, we performed Ca^{2+}-imaging experiments and used heat stimulus (>52 °C) as reported [1]. Acute heat stimulus increased the intracellular Ca^{2+} ($[Ca^{2+}]_i$) level in cultured astrocytes (Fig. 2A). Application of ruthenium red, a broad TRP channel inhibitor, with heat stimulus abolished the increase of $[Ca^{2+}]_i$, indicating that TRPV2 involvement for increase of $[Ca^{2+}]_i$ by heat (Fig. 2B). Since our heat stimulus was very high temperature and strong, the $[Ca^{2+}]_i$ increases did not recover to baseline levels following removal of heat stimulus by cooling (Fig. 2A). We considered that these might due to the long exposure to such very high temperature. Therefore, we used shorter exposure for heat stimulus to confirm the recovery of the $[Ca^{2+}]_i$ (Fig. 2C). In the case of short time exposure of the cells to heat stimulus, the $[Ca^{2+}]_i$ level increased similar to that observed during the longer heat exposure (Fig. 2A). However, following the removal of the shorter exposure to heat stimulus, most cells showed recoveries of $[Ca^{2+}]_i$ to baseline levels (Fig. 2C). These results indicated that the Ca^{2+}-responses were dependent on exposure time to heat. It remains inconclusive that the response of astrocytes to heat and its inhibition by ruthenium red is suggestive of functional expression of TRPV2, since ruthenium red can inhibit other thermo TRP channels. In order to confirm the functional expression of TRPV2 in the astrocytes, we used electrophysiological approaches in astrocytes, which were ectopically transfected dominant-negative form of TRPV2 (DN-TRPV2) [4]. For transfection of DN-TRPV2, we co-transfected EGFP expression vector into cultured astrocytes under human GFAP-promoter (hGFAP promoter) control to visualize DN-TRPV2 expressing/GFAP-positive astrocytes (Fig. 3A). To evaluate the dominant negative effects, we co-transfected pCAGGS expression vector (DN-TRPV2 was subcloned into this vector) with hGFAP-EGFP vector as mock transfections. To perform whole cell patch clamp recordings, firstly we dissociated cultured astrocytes (transfected vectors) by trypsin/EDTA, and then placed coverslips into the chamber for the electrophysiological recording. After achieving the whole cell configurations, we lifted up the round shape astrocytes from the surface of coverslips. This was done to avoid the breaking of the whole cell configurations due to acute heat application to the cells on the coverslips. The acute heat application near to ~52 °C into mock transfectants evoked a large inward current at −60 mV holding potential (Fig. 3B). Upon whole cell patch clamp recordings, we applied ramp pulses from −100 to +100 mV (Fig. 3B) at every 5 s intervals. Heat application over 50 °C evoked outward rectified current–voltage relationship (Fig. 3B, red arrowhead and Fig. 3C, red traces), although the basal current–voltage relationship was linear pattern (Fig. 3B, black arrowhead and Fig. 3C, black traces). These current properties by heat stimulus were perfectly matched with reported TRPV2 current property [1]. DN-TRPV2 transfections were almost perfectly inhibited the generation of heat-evoked current compared with mock transfections (Fig. 3D and E). Occasionally, lower temperature threshold heat-evoked current was observed (arrowhead in Fig. 3E), as we found some of specific astrocytes rarely expressed TRPV4 (under submission). These results clearly demonstrated that functional TRPV2 was expressed in cultured astrocytes. Notably, the temperature thresholds of astrocytes by heat stimulus (>48 °C) were less than reported one (>52 °C). There could be two possibilities to explain the lower threshold; one is that trypsin treatment could have affected the TRPV2 protein structure. The other is that astrocytic TRPV2 might have different characteristics compared with sensory neuronal TRPV2. The first possibility was unlikely, because no trypsin treatment cells had similar temperature thresholds in our Ca^{2+}-imaging experiments (Fig. 2). Generally, TRP channels have many binding partners. In astrocytes, the TRPV2 might have specific binding proteins, and the binding might affect the temperature threshold. In this study, we applied the heat stimulus as a tool to evaluate whether functional TRPV2 expressed in astrocytes, since brain astrocytes does not have any opportunities to expose to very high temperature (>48 °C). Next, we applied LPC as a known endogenous ligand for TRPV2 (Fig. 4). LPC application (30 μM) evoked a large inward current at −60 mV holding potential in mock transfected astrocyte (Fig. 4A), although the inward current was significantly reduced in DN-TRPV2 transfected astrocytes (Fig. 4B). In the mock and DN-TRPV2 transfectants, LPC applications evoked outward rectified current–voltage relationship (Fig. 4A and B, red and green arrowheads and Fig. 4C, red and green traces), although the basal current–voltage relationships were linear patterns (Fig. 4A and B, black and light green arrowheads and Fig. 4C, black and light green traces). These current properties by LPC were perfectly matched with previously reported TRPV2 current property [9]. Quantification of current densities evoked by

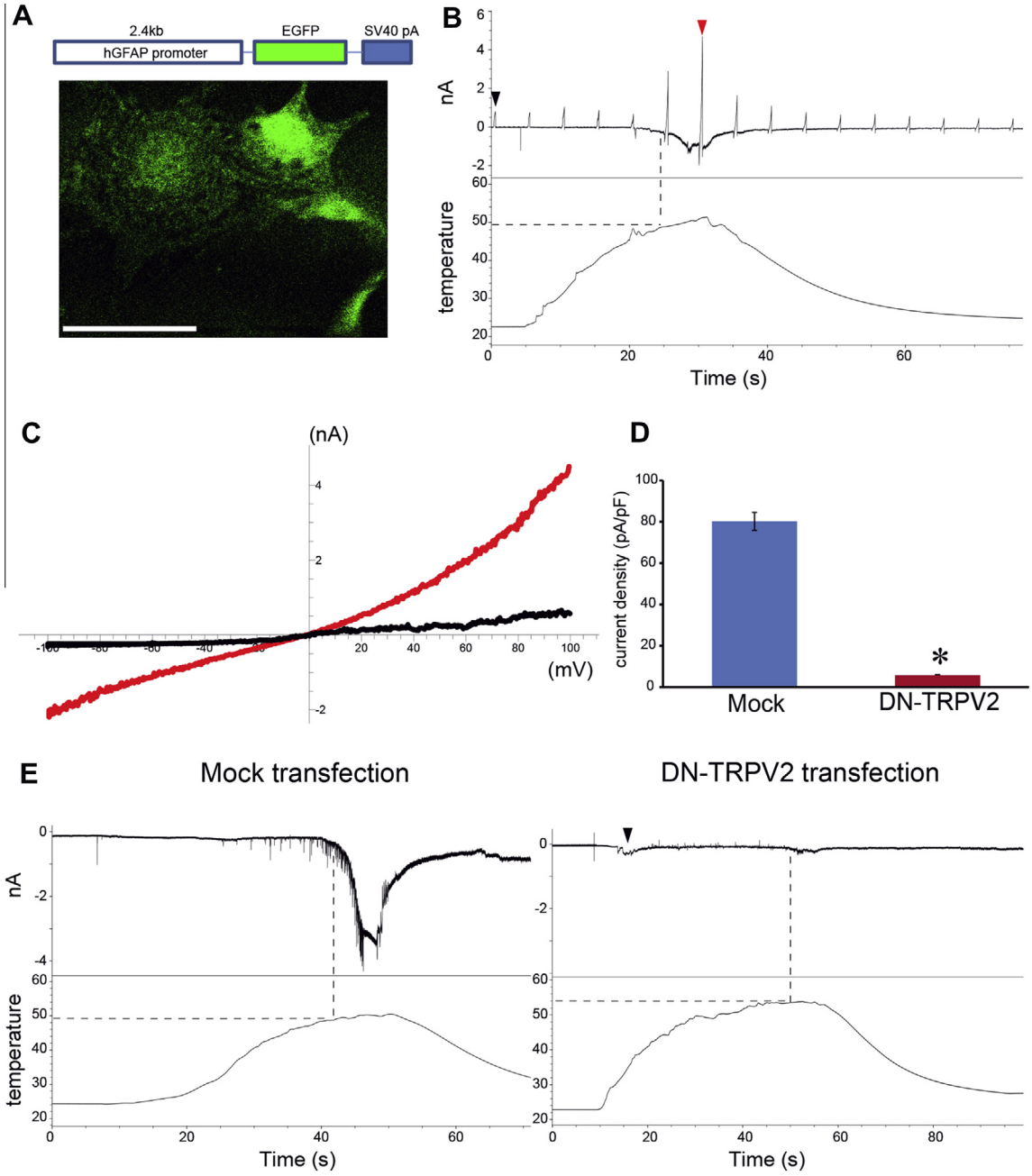

Fig. 3. Astrocytes respond to heat stimulus by activation of TRPV2. (A) A schematic drawing of EGFP expression vector under hGFAP promoter control. The representative picture was taken after the vector was expressed in cultured cerebellar asterocytes (2 days after). Scale bar; 50 μm. (B) A representative trace of heat-evoked current in cultured cerebellar astrocyte. The current was recorded in EGFP-expressing GFAP positive astrocyte. Holding potential was at −60 mV. Dashed line represents the temperature threshold of heat-evoked current. C; The outward rectified current–voltage relationship of heat-evoked current (red trace) corresponding to red arrowhead point in panel **B**. Black trace represents linear basal current–voltage relationship corresponding to black arrowhead point in panel B. (D and E) Comparison of current densities between mock or DN-TRPV2 expressing astrocytes. Quantified current density results were shown as bar graphs (D). Asterisk represents statistical significance at $p < 0.01$. Representative traces of heat-evoked current in cultured cerebellar astrocyte expressing mock or DN-TRPV2 (E). Holding potential was at −60 mV. Dashed line represents the temperature threshold of heat-evoked current.

LPC application between mock and DN-TRPV2 transfectants revealed that the LPC currents were evoked by TRPV2 activations in GFAP-positive astrocytes (Fig. 4D). These results clearly demonstrate that astrocytes express functional TRPV2, and the channel can respond to very high temperature and LPC. LPC is generated by metabolism of phosphatidylcholine (PC). Degradation of PC by phospholipase A2 generated arachidonic acid and LPC [23]. It is reported that arachidonic acid was generated in postsynaptic sites, and affect neuronal activities [23]. In a separate study, we have shown that another thermo TRP member namely TRPV4 was expressed in astrocytes [21], and the astrocytic TRPV4 responded to arachidonic acid, and modulated neuronal excitabilities through releases of gliotransmitters (under submission). In the case of generation of arachidonic acid in postsynaptic sites, LPC is also generated at the same time, and might affect the excitation of astrocytes through TRPV2 activation. Therefore, astrocytic TRPV2 might be activated responded to increase of lipid metabolism in synaptic sites. Our upcoming study will identify important physiological roles of astrocytic TRPV2 and their contribution to synaptic and brain function.

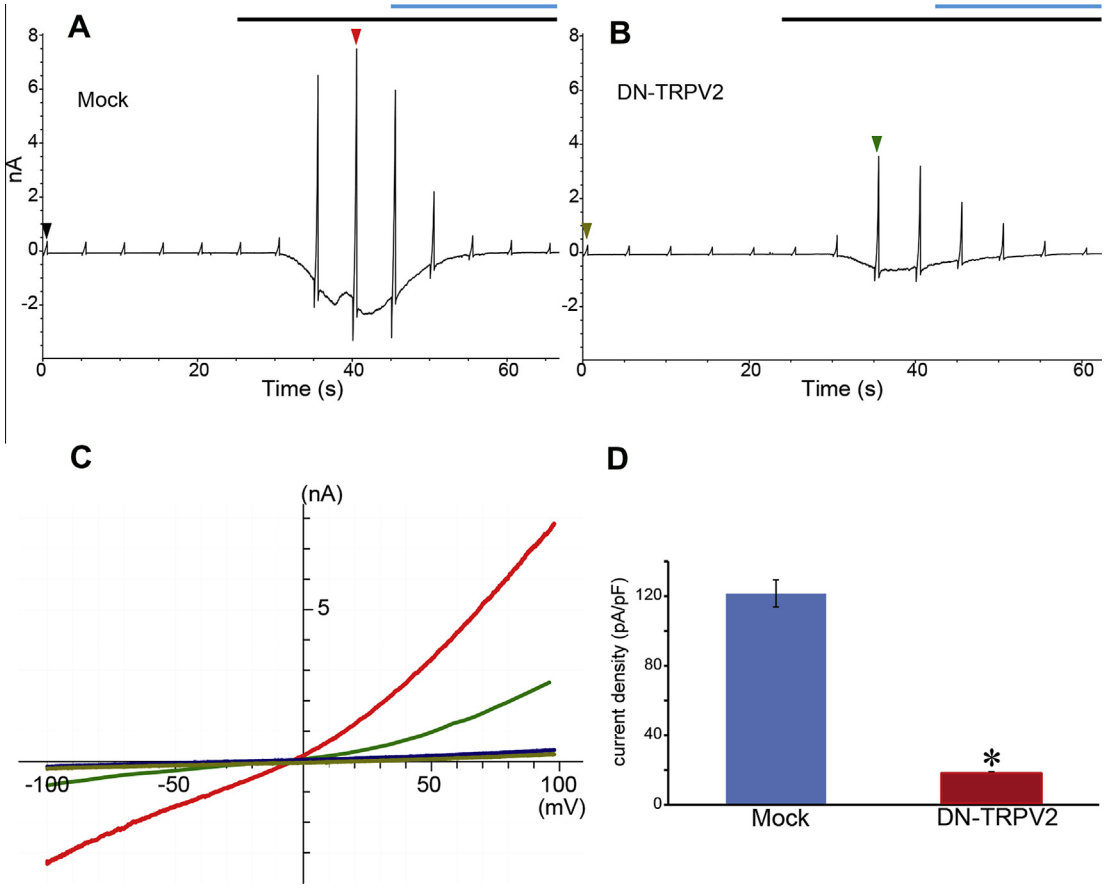

Fig. 4. Activation of TRPV2 currents in astrocytes by its endogenous ligand LPC. (A and B) Representative traces of LPC-evoked current in cultured cerebellar astrocyte transfected with mock or DN-TRPV2. Holding potential was at −60 mV. Ramp pulses from −100 to +100 mV were applied at every 5 s intervals. The black line represents the application of LPC (30 μM), and blue line represents the application of tranilast (75 μM), which is a TRPV2 inhibitor. (C) The outward rectified current–voltage relationship (red trace) of LPC-evoked current corresponding to the red arrowhead in panel A or outward rectified current–voltage relationship (dark green trace) of LPC-evoked current corresponding the dark green arrowhead in panel B. Black trace represents basal current–voltage relationship of mock astrocyte corresponding to the black arrowhead in panel A. Light green trace represents the basal current–voltage relationship of DN-TRPV2 astrocyte corresponding to the light green arrowhead in panel B. (D) Quantified current density results were shown as bar graphs. Asterisk represents statistical significance at $p < 0.01$.

Acknowledgments

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