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TRPC1-mediated Ca^{2+} and Na^{+} signalling in astroglia: Differential filtering of extracellular cations

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Summary

Canonical transient receptor potential 1 (TRPC1) plasmalemmal cation channels mediate Ca^{2+} and Na^{+} fluxes and control respective cytoplasmic ion signals in rat cortical astrocytes. Mechanical stimulation of astrocytes results in increases in cytosolic Ca^{2+} and Na^{+} levels that are in part due to entry of extracellular cations through TRPC1 containing channels. Inhibition of the TRPC1 pore with an antibody against the selective filter of TRPC1 reduced cytosolic Ca^{2+} accumulation caused by mechanical stimulation. In contrast, this immunological treatment increased the cytosolic Na^{+} peak accumulation induced by mechanical stimulation. We propose that TRPC channels are amenable to changes in selective filtering as mutations in previous studies and antibody binding in our present study differentially affect the flux of Ca^{2+} and Na^{+}. TRPC1 containing channels might represent focal points for co-ordination of Ca^{2+} and Na^{+} signalling in astroglia and this can have consequences on Ca^{2+}- and Na^{+}-dependent processes such as regulated exocytosis and lactate production, respectively, which in turn can modulate neuronal synaptic transmission.

Keywords

astrocyte; calcium; differential filtering; sodium; store operate calcium entry

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1. Introduction

Astrocytes, the main homeostatic cells of the central nervous system (CNS) express multiple plasmalemmal ion channels that contribute to cytosolic ion signalling, which is central for astroglial homeostatic functions [1-3]. These channels are activated by multiple stimuli such as neurotransmitters or mechanical stimulation. This latter mechanism is physiologically relevant for astrocytes since they have remarkable morphological plasticity and may rapidly change their volume. The majority of plasmalemmal ion channels expressed in astrocytes belong (with obvious exception of selective K^+ channels) to nonselective cationic channels permeable to Ca^{2+}, Na^+ and K^+. Activation of these channels results in Ca^{2+} and Na^+ signalling events that control various functional responses [4, 5].

Canonical transient receptor potential 1 (TRPC1) channel is one of seven mammalian TRPC subtypes widely expressed in various tissues [6]. TRPC1 is a nonselective cation channel with equal permeability for Ca^{2+} and Na^+ and is different from other TRPC channels some of which are highly Ca^{2+} permeable with $P_{Ca}/P_{monovalent}$ between 2 and 9 [7]. Astroglial cells express several types of TRPC proteins, which, through heteromeric assembly with the TRPC1 subunit (that is known to be obligatory for channel formation), form functional channels [8-11]. TRPC channels in astrocytes are responsible for generation of store-operated Ca^+ entry (SOCE) although the mechanism linking them to the endoplasmic reticulum (ER) Ca^{2+} store remains elusive. Targeting the TRPC1 subunit in astrocytes with a blocking antibody or by reducing TRPC1 expression with a silencing RNA treatment substantially reduced the SOCE in cultured astroglial cells [8, 10]. The TRPC 1-mediated SOCE contributes to astroglial Ca^{2+} signalling and it has been shown to modulate Ca^{2+}-dependent vesicular glutamate release in cortical astrocytes in response to mechanical stimulation [12, 13]. The TRPC channel in astrocytes can also provide substantial Na^+ influx that may occur in response to mechanical stimulation or develop alongside with SOCE following depletion of the ER Ca^{2+} store. This Na^+ influx can be instrumental for local Na^+ signals critical for glial homeostatic response [4, 14]. In this study we characterized TRPC1-mediated Ca^{2+} and Na^+ signalling in mechanically-stimulated cultured astrocytes and demonstrate that Ca^{2+} and Na^+ fluxes can be dissociated following the treatment with a TRPC1 blocking antibody.

2. Materials and methods

2.1. Astrocyte cultures

Solitary astrocytes from visual cortices of 1- to 2- day-old Sprague Dawley rats were maintained *in vitro* as previously described [15]. Briefly, visual cortices were dissected and enzymatically treated with papain (20 i.u./ml, 1 hr at 37 °C) in the presence of L-cysteine (0.2 mg/ml); digestion was arrested by trypsin inhibitor (10 mg/ml; type II-O; 5 min at room temperature). Tissue was mechanically dissociated and neural cells were seeded into culture flasks containing culture medium composed of α-minimum essential medium (α-MEM, without phenol red; Life Technologies Corp. Invitrogen™, Carsbad, CA, USA) supplemented with foetal bovine serum (10% v/v; Thermo Scientific HyClone, Logan, UT, USA), glucose (20 mM), L-glutamine (2 mM), sodium pyruvate (1 mM), sodium bicarbonate (14 mM), penicillin (100 i.u./ml), and streptomycin (100 μg/ml), pH 7.35. After

allowing cells to adhere to the bottom of the flasks for 1 hour, they were washed and provided with new media. Cells were then maintained at 37°C in a 95% air/ 5% CO_2 environment for 5 to 7 days to reach ∼60% confluency. At that juncture, the cell cultures were purified for astrocytes using previously described procedure [16]. Purified astrocytes were detached from the flasks using trypsin (10,000 Nα-benzoyl-arginine ethyl ester hydrochloride units/ml; Sigma-Aldrich, St. Louis, MO, USA). After inhibition of trypsin activity by addition of complete culture medium, cells were pelleted using centrifugation (100 × g for 10 minutes), resuspended and plated onto round (12 mm in diameter) glass coverslips (Thermo Fisher Scientific) pre-coated with polyethyleneimine (1mg/ml; Sigma). Purified astrocytes were kept in culture medium at 37°C in a 95% air/ 5% CO_2 atmosphere incubator for 5 - 8 days when used in experiments. The purity of astrocytic culture (>99%) was confirmed: (i) by indirect immunocytochemistry using anti-glial fibrillary acidic protein antibody and (ii) by visualization of accumulation of a dipeptide, β-Ala-Lys, conjugated to 7-amino-4-methylcoumarin-3-acetic acid as previously described [16]. Astrocytes in our culture system are flat polygonal cells and thus have a simplified morphology compared to astrocytes *in situ* [16, 17].

2.2. Anti-TRPC1 antibody treatment

Astrocytes grown on coverslips were incubated in external solution (pH 7.35) consisting of sodium chloride (140 mM), potassium chloride (5 mM), calcium chloride (2 mM), magnesium chloride (2 mM), HEPES (10 mM), and glucose (5 mM), with or without 30 μg/ml of anti-TRPC1 antibody (cat. No. ACC-010, Alomone labs, Jerusalem, Israel) for 30 min at room temperature (22-25 °C) as described previously [10]. Antibody incubation was performed after loading cells with either the Ca^{2+} indicator fluo-3 acetoxymethyl (AM) ester or the Na^+ indicator CoroNa™Green AM and de-esterification of the indicators. Antibody was kept in solution during the entire imaging procedure lasting ∼200 seconds for Ca^{2+} and Na^+ measurements.

2.3. Intracellular Ca^{2+} imaging

Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in somata of cultured solitary astrocytes were assessed using the Ca^{2+} indicator fluo-3 as described earlier [17]. Briefly, astrocytes were loaded with fluo-3 AM (10 μg/ml; Life Technologies Corp. Invitrogen™) in external solution containing pluronic acid (0.025% w/v) for 30 min at room temperature. To allow de-esterification of fluo-3 AM, cells were subsequently kept in external solution for 30 min at room temperature. Coverslips were transferred into a recording chamber mounted on the inverted microscope, and astrocytes were visualized with a standard fluorescein isothiocyanate (FITC) filter set (Chroma Technology, Rockingham, VT, USA). Fluorescence intensities obtained from somata of indicator-loaded astrocytes were corrected (digital subtraction) for the background fluorescence measured from regions of coverslips containing no cells. Fluorescence data were expressed as $\Delta F/F_0$ (%) with the cell baseline fluorescence (F_0) representing the average of the first 5 images before mechanical stimulation while ΔF represents the change in fluorescence emission. The $[Ca^{2+}]_i$ was determined using calibration of fluo-3 as described elsewhere [18].

2.4. Intracellular Na^+ imaging

Cytosolic Na^+ concentration ($[Na^+]_i$) in somata of cultured solitary astrocytes was monitored using the Na^+ indicator CoroNa™Green AM (10 μM; Life Technologies Corp. Invitrogen™) [15, 19]. Astrocytes were loaded with the indicator, imaged, and data were collected and processed as described above for Ca^{2+} imaging. Because CoroNa™Green tends to leak out of the cell, its intracellular fluorescence intensity substantially decays over time [19]. Consequently, using a linear regression and extrapolation of the baseline fluorescence of individual traces, we corrected them for the leak of the dye. The $[Na^+]_i$ was determined using calibration of CoroNa™Green as described elsewhere [15].

2.5. Image acquisition and processing

An inverted microscope (TE 300; Nikon, Melville, NY, USA), equipped with differential interference contrast and wide-field fluorescence illumination, was used in all experiments. Experiments were performed using a 60X Plan Apo oil-immersion objective (1.4 numerical aperture; Nikon). Images were acquired using a CoolSNAP-HQ cooled charge-coupled device camera (Roper Scientific Inc., Tucson, AZ, USA) driven by V++ imaging software (Digital Optics Ltd., Auckland, New Zealand). All raw data/images had their pixel intensities within the camera's dynamic range (0-4095). The $\Delta F/F_0$ of the control and treatment groups were ranked and normalized to control to accommodate for variations in culture conditions, and to allow comparisons between experimental batches, as we previously described [15].

2.6. Mechanical stimulation

To stimulate a solitary astrocyte of interest, we employed mechanical contact using a glass pipette filled with external solution as we described elsewhere [15, 17]. This approach allows spatio-temporal control of the stimulus application without affecting plasma membrane integrity. The establishment of the patch pipette contact with the plasma membrane was determined by an increase in pipette resistance monitored using a patch-clamp amplifier (PC-ONE; Dagan, Minneapolis, MN, USA) that delivered -20 mV, 10 ms square pulses at 50 Hz. Once established, cell contact was maintained for ∼ 1 s. The strength of the stimulus, expressed as $\Delta R/R_0$ (%), where R_0 represents the pipette resistance (2.6 - 4.8 MΩ) prior to establishing a pipette-astrocyte contact, and ΔR represents the increase in the resistance (0.05 – 0.28 MΩ) during the contact, had comparable intensities under all conditions tested (Mann-Whitney U-test, P = 0.253-0.351).

2.7. Statistical analysis

The comparison of the pipette resistance increases in different conditions and effects of an anti-TRPC1 antibody on mechanically-induced intracellular Ca^{2+} and Na^+ loads were tested using Mann-Whitney U-test. Data are expresses as means ± SEMs.

3. Results

3.1. Cytosolic Ca^{2+} responses to mechanical stimulation in rat cortical astrocytes is reduced by an anti-TRPC1 antibody

Mechanical stimulation of cortical astrocytes generates a rapid increase in cytosolic Ca^{2+} that slowly decays to the basal level [17, 20]. Previous studies conducted by us and others [8, 10] provided conclusive evidence that this increase in part results from Ca^{2+} entry through TRPC1 containing channels, and that application of an anti-TRPC1 antibody directed at amino acid residues 557-571 [21, 22] inhibited this Ca^{2+} entry [10, 12]. In this study mechanical stimulation of solitary astrocytes with glass pipettes induced transient increase in $[Ca^{2+}]_i$ (Fig. 1). Incubation of astrocytes with an anti-TRPC1 antibody significantly reduced the peak of the $[Ca^{2+}]_i$ transient; $\Delta F/F_0$ fell from 408 ± 42 % in control to 320 ± 38% (n = 6, Fig. 1). These changes in fluo-3 fluorescence correspond to cytosolic Ca^{2+} concentrations of ∼1.7 μM and ∼ 860 nM, respectively; resting $[Ca^{2+}]_i$ levels were ∼ 70 nM. Both the peak of $[Ca^{2+}]_i$ transients (Fig. 1C) and cumulative (area under the curve) $[Ca^{2+}]_i$ responses (Fig. 1D) in anti-TRPC1 treated astrocytes were significantly lower than those of control cells (n = 6, Mann-Whitney U-test, $P < 0.01$).

3.2. The peak cytosolic Na^+ response to mechanical stimulation in rat cortical astrocytes is increased by an anti-TRPC1 antibody

Mechanical stimulation of astrocytes triggered a large transient increase in $[Na^+]_i$ (Fig. 2). Changes in peak fluorescence measured in control conditions averaged 58 ± 5 % ($\Delta F/F_0$; n = 18) that correspond to a $[Na^+]_i$ increase to ∼35.5 mM from the resting level of ∼17 mM. Incubation of cultured astrocytes with an anti-TRPC1 antibody increased mechanically-induced $[Na^+]_i$ transients. An increase in the $\Delta F/F_0$ peak averaged 72 ± 7% (Fig. 2; n = 18, Mann-Whitney U-test, $P < 0.01$) that corresponded to the peak $[Na^+]_i$ transient of ∼42.6 mM. At the same time the cumulative $[Na^+]_i$ response measured from anti-TRPC1 antibody treated cells was not significantly different when compared to control cells (Fig. 2D).

4. Discussion

Mounting highly heterogeneous and precisely controlled homeostatic responses to a continuously changing interstitial environment of the CNS is the *raison d'etre* of astroglia. The range of these responses is remarkable as astrocytes regulate fluxes of physiologically relevant ions such as K^+, Na^+, Ca^{2+}, H^+, $C1^-$, HCO_3^-, maintain glutamine-glutamate and glutamine-GABA shuttles, supply lactate to active neuronal compartments, control water movements, secrete scavengers of reactive oxygen species and many more [23, 24]. These responses are critically important for the functional connectivity in the CNS because they maintain neuronal excitability and synaptic transmission. For this purpose astrocytes continuously monitor neuronal activity by multiple neurotransmitter receptors and ion channels [25, 26]. Activation of these channels and receptors triggers often highly localized fluctuations of Ca^{2+} and Na^+ in astroglial cytosol that constitute a substrate for glial excitability [4, 27].

Astrocytes possess a high degree of morphological plasticity, as indeed neuronal activity results in highly dynamic redistribution of water and transient changes in the volume of

perisynaptic astroglial processes [28, 29]; similarly astroglia exhibits a volume response to changes in extracellular osmotic pressure [30]. These dynamic changes in cell volume activate signaling cascades through mechano-sensitive channels that mediate ion fluxes in response to mechano-stimulation of the plasmalemma resulting in cytosolic Ca^{2+} and Na^{+} signals [15]. Array of mechano-sensitive astroglial channels most likely consist of several channel types, of which TRPC1 is a sound candidate. Indeed stretching the membrane of frog oocytes or CHO-K1 cells transfected with TRPC1 activated ionic currents, whereas inhibition of TRPC1 synthesis by antisense RNA reduced these mechano-sensitive current responses [31]. Here we further corroborate this hypothesis by demonstrating that treatment of astrocytes with an antibody against TRPC1 channel inhibited $[Ca^{2+}]_i$ transients triggered by mechanical stimulation. Of note, however, other mechano-sensitive channels may underlie cation fluxes. Recently, channels of Piezo1 and Piezo2 types have been identified as mechano-sensitive channels in dorsal root ganglion neurones [32]. The Piezo1 channel can be selectively inhibited by peptide toxin GsMT×4, isolated from tarantula venom, and GsMT×4 was reported to inhibit stretch-activated channels in astrocyte membranes [33].

We further confirmed that mechanical stimulation of astrocytes triggers large (up to 20 mM in amplitude) increases in $[Na^{+}]_i$ which also were found to be sensitive to anti-TRPC1 antibody. In contrast with $[Ca^{2+}]_i$ responses, however, inhibition of the TRPC1 channels by the anti-TRPC1 antibody resulted in a significant increase in the peak amplitude of $[Na^{+}]_i$ response. Thus binding of the antibody to the TRPC1 channel decreases Ca^{2+} flux with a parallel increase in Na^{+} flux. Somewhat similar observations were gathered in site-directed mutagenesis studies of the TRPC1 and TRPC3 channels. Substitution of seven acidic residues to basic amino acids in the channel region of TRPC1 inhibited Ca^{2+} movement without affecting Na^{+} fluxes [34]. A single mutation (E630Q) of the selective filter of TRPC3 markedly inhibited Ca^{2+} current, whereas increasing Na^{+} currents through the channel especially at negative membrane potentials [35]. Incidentally, the Basic Local Alignment Search Tool (BLAST®) comparison of rat TRPC1 and TRPC3 proteins reveals that the TRPC1 antibody peptide target (amino acids 557-571) and the mutated site of TRPC3 are both located in putative selective filters that are not highly conserved [34, 35]. Taken together, these studies suggest that at least some TRPC channels are amenable to mutations that disrupt Ca^{2+} but preserve (or even increase) Na^{+} entry. This observation may be important to differentially modulate TRP channels in heath and disease.

It should be noted that we have not addressed the ability of anti-TRPC1 antibody to modulate ion dynamics in astrocytes at rest. It is likely that the antibody would require an opening of the channel to bind to the pore region. Such opening events in unstimulated astrocytes at rest are considered rare as TRPC1 channels are activated when the ER store is getting depleted. Nonetheless, binding of the antibodies to TRPC1 channels in astrocytes at rest may cause cell depolarization and reduce the driving force for Ca^{2+} and Na^{+} entry to the cytosol. In such a scenario, in mechanically-stimulated astrocytes treated with the anti-TRPC1 antibody there could be a more profound decrease in $[Ca^{2+}]_i$ and a less prominent increase of $[Na^{+}]_i$ than those we observed. Another intriguing, albeit unlikely, possibility is that the bound antibody, which blocks the Ca^{2+} selectivity filter, could dilate the TRPC1 channel during mechanical stimulation. Such conditions could allow an increase of Na^{+} flux

which would be manifested as an increase in the amplitude of the $[Na^+]_i$ transient as we recorded.

Another fundamental function of TRPC1 containing channels in astroglia lies in their role in SOCE. This mechanism of the Ca^{2+} entry is universally present in neuroglial cells and is ubiquitous in astrocytes. Stimulation of astroglia *in vitro* or *in situ* with neurotransmitters initiates complex $[Ca^{2+}]_i$ responses comprising of the ER Ca^{2+} release-dependent initial peak and the SOCE dependent long-lasting plateau [8, 10, 36-38]; it should be noted that SOCE contributes even to the peak response [10]. Similarly receptor independent depletion of ER Ca^{2+} store results in prominent SOCE in astrocytes [39, 40]. Sites of astroglial SOCE are localized close to the ER thus increasing the efficacy of Ca^{2+} store replenishment [8, 41]. The SOCE pathway is also prominent in pathologically modified glial cells such as for example in glioblastoma [42].

TRPC1 containing channels have been identified as a main mechanism for SOCE in astroglia [5, 8, 10]. This endows them with another highly important role in coordination of ionic signalling in astrocytes. Being store-operated TRPC1 channels establish a functional link between metabotropically induced Ca^{2+} signalling with Na^+ influx thus coordinating Ca^{2+} and Na^+ signalling in astroglial sub-compartments. In this study we found that TRPC1 channels can potentially regulate their permeability to Ca^{2+} and Na^+ thus being capable of dynamic control over local ionic signalling. Such changes of $[Ca^{2+}]_i$ and $[Na^+]_i$ could affect Ca^{2+}- and/or Na^+-dependent processes. Increase of $[Ca^{2+}]_i$ can trigger regulated exocytosis of glutamate [43], while increase of $[Na^+]_i$ can trigger glycolysis leading to lactate production [44]; both events are important for modulation of synaptic transmission and plasticity [45, 46]. Naturally the physiological mechanism of such regulation of TRPC1 permeability remains unknown (and it may include enzymatic or post-translational modification) and requires further investigation.

The TRPC1 channels were also reported to co-localize with astroglial plasmalemmal Na^+/Ca^{2+} exchangers (NCX), which in turn are concentrated in perisynaptic processes being also closely associated with Na^+/K^+ pumps, glutamate ionotropic receptors, and glutamate/GABA Na^+-dependent transporters [4]. This strategic localization allowing focal Na^+ and Ca^{2+} fluxes could be of critical importance for fast neuronal-astroglial signalling at the single synaptic level. For example TRPC1-mediated changes in $[Ca^{2+}]_i$ and $[Na^+]_i$ determined in this study can substantially affect reversal potential of astroglial NCX (E_{NCX}); this has been suggested as a key mechanism underpinning TRPC3 signalling [47]. Indeed, the resting E_{NCX} in our experimental conditions is estimated at -98 mV, it shifts to -79 mV in astrocytes mechanically stimulated in control conditions and to -109 mV in cells stimulated in the presence of an anti-TRPC1 antibody (see Table 1 for details). These fluctuations in E_{NCX} have important functional consequences. In normal conditions mechanical stimulation promotes reversal mode of NCX and Ca^{2+} entry [15], whereas after TRPC1 modification cell stimulation favours forward mode of NCX resulting in Ca^{2+} extrusion and additional Na^+ entry. As outlined above, this can govern Ca^{2+}- and/or Na^+-dependent processes in astrocytes that can in turn modulate the operation of the tripartite synapse.

In conclusion the TRPC1 channels appear as a focal point for co-ordination of Ca^{2+} and Na^{+} signalling in astroglia. The capability of TRPC1 to dynamically modify Ca^{2+} and Na^{+} fluxes may be relevant for this coordination by increasing plastic potential of ionic signalling in astroglia.

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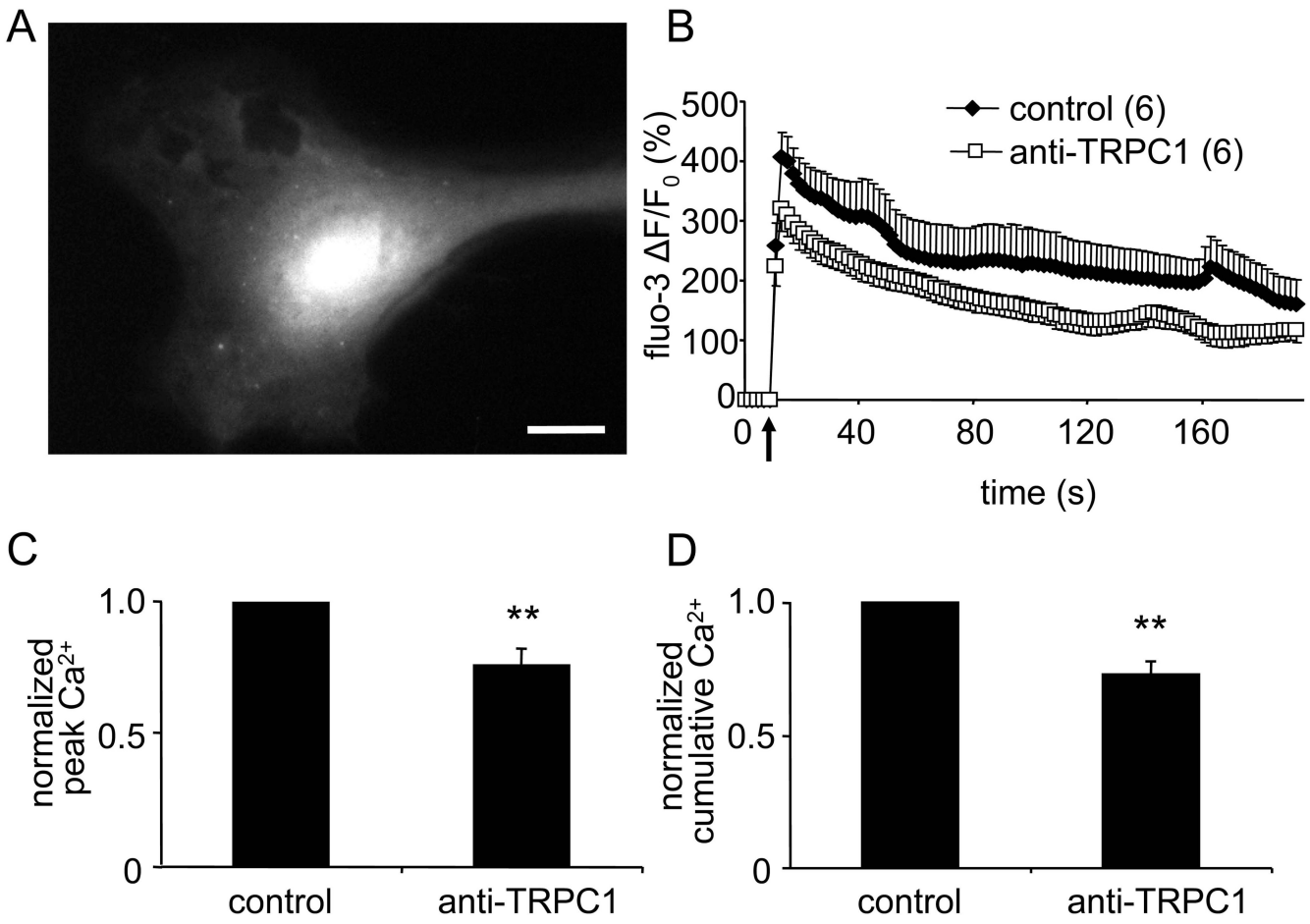

Figure 1.

Cytosolic Ca^{2+} accumulation in astrocytes induced by mechanical stimulation is reduced by an antibody that binds to the pore forming region of TRPC1.

(A) A representative image of a solitary rat cortical astrocyte at rest and loaded with fluo-3. Scale bar = 20 μm. (B) Average kinetics of fluo-3 fluorescence from mechanically stimulated astrocytes. (C,D) Bar graphs showing normalized peak and cumulative cytosolic Ca^{2+} responses to mechanical stimulation. Both responses were significantly reduced when astrocytes were treated with an anti-TRPC1 antibody (30 μg/ml, 30 min). Points and bars represent means ± SEM of measurements. SEMs are shown in single directions for clarity. Arrow indicates the time when the mechanical stimulus occurred. Numbers in parentheses indicate the number of astrocytes studied in each group. Asterisks denote a significant change in measurement when compared to control group, i.e. untreated astrocytes (Mann-Whitney *U*-test; **$p < 0.01$).

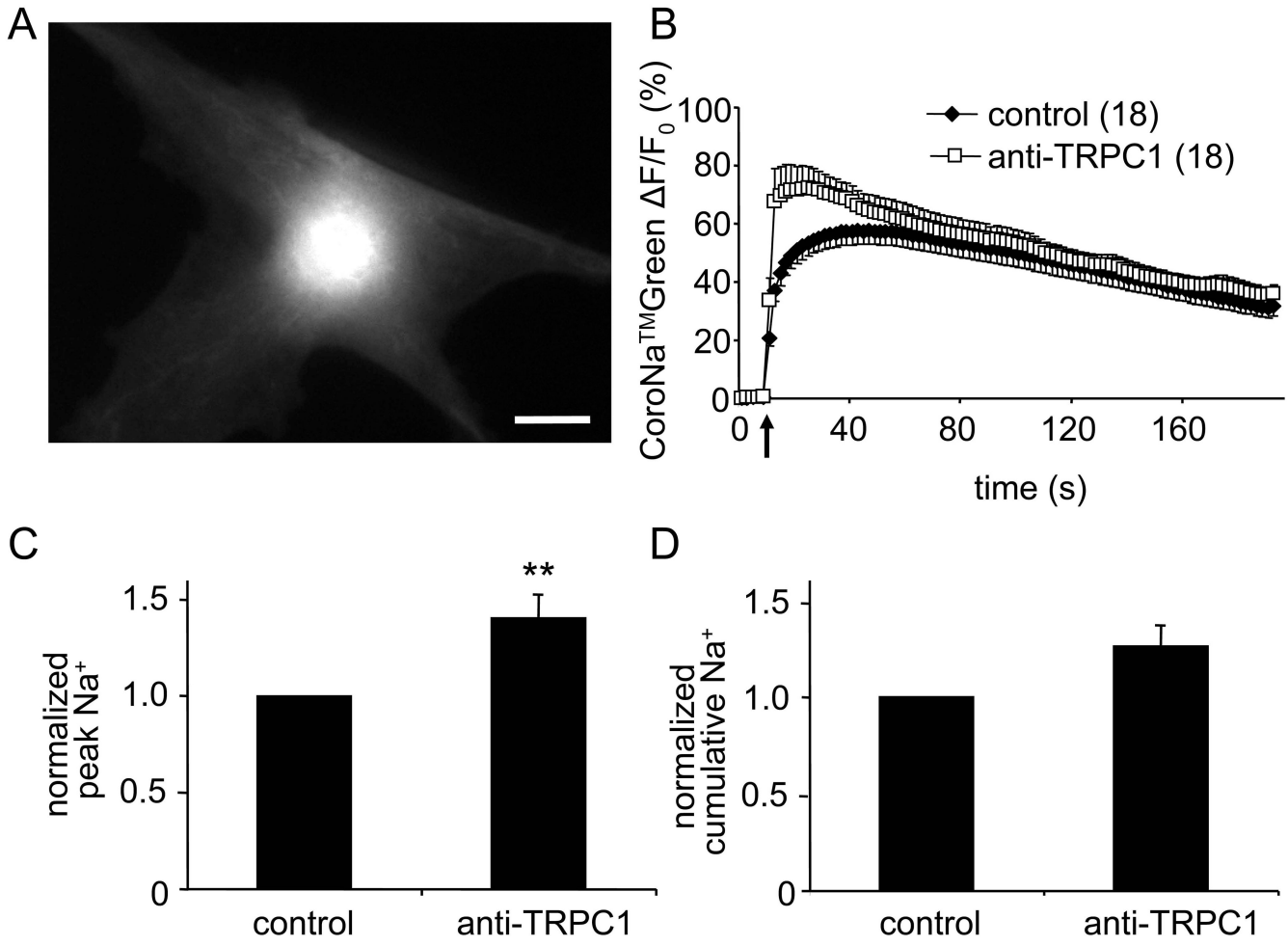

Figure 2.

Cytosolic Na^+ accumulation in astrocytes induced by mechanical stimulation is augmented by an antibody that binds to the pore forming region of TRPC1.

(A) A representative image of a solitary rat cortical astrocyte at rest and loaded with CoroNa™Green. Scale bar = 20 μm. (B) Average kinetics of CoroNa™Green fluorescence from mechanically stimulated astrocytes. (C,D) Bar graphs showing normalized peak and cumulative cytosolic Na^+ responses to mechanical stimulation. The peak value was significantly increased when astrocytes were treated with an anti-TRPC1 antibody (Mann-Whitney *U*-test; **$p < 0.01$). Points, bars, arrow and numbers in parentheses as in Fig 1.

Table 1
Reversal potential of astroglial plasmalemmal NCX

Cytosolic ion concentrations	Condition	E_{NCX}
$[Na^+]_i$= 16.6 mM	resting	-98 mV
$[Ca^{2+}]_i$ = 73 nM		
$[Na^+]_i$=35.5 mM	mechanical stimulation;	-76 mV
$[Ca^{2+}]_i$=1.7 μM	control	
$[Na^+]_i$=42.6 mM	mechanical stimulation;	-107 mV
$[Ca^{2+}]_i$= 861 nM	TRPC1 antibody treatment	

Reversal potential of NCX (E_{NCX}) at 25°C was calculated using our recorded cytosolic Na^+ and Ca^{2+} concentrations together with concentrations of these ions in the external solution ($[Na^+]_O$ = 140 mM and $[Ca^{2+}]_O$ = 2 mM) and presumed NCX 3:1 stoichiometry.