Experimental Neurology 238 (2012) 149–155

Contents lists available at SciVerse ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

The TASK1 channel inhibitor A293 shows efficacy in a mouse model of multiple sclerosis

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ARTICLE INFO

Article history:
Received 4 May 2012
Revised 14 August 2012
Accepted 21 August 2012
Available online 27 August 2012

Keywords:
Multiple sclerosis
Experimental autoimmune encephalomyelitis
EAE
Potassium channels
K2P channels
TASK1
A293
Ion channel blocker

ABSTRACT

The two-pore domain potassium channel TASK1 (KCNK3) has recently emerged as an important modulator in autoimmune CNS inflammation. Previously, it was shown that T lymphocytes obtained from TASK1$^{-/-}$ mice display impaired T cell effector functions and that TASK1$^{-/-}$ mice show a significantly reduced disease severity in myelin oligodendrocyte glycoprotein (MOG_{35-55}) peptide induced experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. We here evaluate a potent and specific TASK1 channel inhibitor, A293, which caused a dose-dependent reduction of T cell effector functions (cytokine production and proliferation). This effect was abolished in CD4$^+$ T cells from TASK1$^{-/-}$ mice but not in cells from TASK3$^{-/-}$ mice. In electrophysiological measurements, A293 application induced a significant reduction of the outward current of wildtype T lymphocytes, while there was no effect in TASK1$^{-/-}$ cells. Preventive and therapeutic application of A293 significantly ameliorated the EAE disease course in wildtype mice while it had no significant effect in TASK1$^{-/-}$ mice and was still partly effective in TASK3$^{-/-}$ mice. In summary, our findings support the concept of TASK1 as an attractive drug target for autoimmune disorders.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disorder of putative autoimmune origin with a heterogeneous clinical presentation (Frohman et al., 2006; Lassmann et al., 2007). Autoreactive T lymphocytes are thought to play a central role in the pathophysiology of MS and existing therapies mostly target the inflammatory component of the disease. Strategies aiming at the development of neuroprotective strategies have not yet reached clinical standards despite intensive research efforts (Kleinschnitz et al., 2007).

The importance of members of the two-pore domain potassium (K_{2P}) channel family, especially TASK1–3, for the activation and effector functions of T lymphocytes *in vitro* and in EAE models was shown recently (Bittner et al., 2009, 2010a,b; Meuth et al., 2008). After induction of EAE with MOG-peptide, TASK1$^{-/-}$ mice showed a significantly ameliorated disease course which was accompanied by a reduced activation of immune cells and less axonal damage compared to wildtype (WT) mice (Bittner et al., 2009). Application of the endocannabinoid anandamide, a semi-selective inhibitor of TASK1/3 channels, resulted in a comparable phenotype. Pharmacological blockade of TASK channels could therefore interfere with the peripheral T cell activation and represents a novel attractive strategy for the treatment of T cell-mediated autoimmune diseases (Bittner et al., 2010a,b). However, the further therapeutic translation of this pathophysiological concept was hampered by a lack of highly selective, clinically administrable TASK channel inhibitors.

We here evaluate the recently described TASK1 channel inhibitor A293 (Bista et al., 2012; Decher et al., 2011; Putzke et al., 2007) in the EAE model which could represent a major pharmacological advancement for the future development of treatment strategies.

Material and methods

Induction and assessment of experimental autoimmune encephalomyelitis (EAE)

All animal experiments were approved by local authorities and conducted according to the German law of animal protection (87-

Abbreviations: CFA, complete Freund's adjuvant; DMEM, Dulbecco's Modified Eagle's Medium; EAE, experimental autoimmune encephalomyelitis; FCS, fetal calf serum; K_{2P} channel, two-pore domain potassium channel; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PMSF, phenylmethanesulfonylfluoride; WT, wildtype.

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http://dx.doi.org/10.1016/j.expneurol.2012.08.021

51.04.2010.A325). EAE was induced by immunization of female 8–10 week old C57BL/6 mice (Charles River, Sulzfeld, Germany), TASK1$^{-/-}$ or TASK3$^{-/-}$ mice (Mulkey et al., 2007) with 200 μg myelin oligodendrocyte glycoprotein (MOG_{35-55}) peptide (Biotrend, Cologne, Germany (Mendel et al., 1995)). MOG peptide was added to complete Freund's adjuvant (CFA) to obtain a 1 mg/ml emulsion and 2×100 μl was injected subcutaneously at both sites of the flank of deeply anesthetized mice using isoflurane. Pertussis toxin was injected at the day of immunization and two days later at a dose of 400 ng (Alexis, San Diego, USA). In a number of experiments, the TASK1 channel inhibitor A293 (2-(butane-1-sulfonyl-amino)-N-[1-(R)-(6-methoxypyridin-3-yl)-propyl]-benz-amide, supplied by Sanofi-Aventis, Frankfurt, see Decher et al. (2011) for structure of the compound) was applied intraperitoneally (100 μl of 10 μM daily) either from the day of immunization (preventive treatment) or starting when animals showed clinical signs of EAE (EAE score = 1; therapeutic regimen). Groups were age-, weight-, and sex-matched and mice were randomly assigned to control *vs.* treatment groups. The clinical course of EAE was monitored using the following score system: grade 0, no abnormality; grade 1, limp tail; grade 2, moderate hind limb weakness; grade 3, complete hind limb paralysis; grade 4, quadriplegia or premoribund state; grade 5, death. Animals were scored by a blinded observer.

Proliferation assay

Spleen tissue was homogenized and strained through a 40 μm nylon filter (BD Biosciences, Heidelberg, Germany). The homogenates were rinsed with washing medium Dulbecco's Modified Eagle's Medium (DMEM; containing 1% fetal calf serum (FCS), 1% glutamine, 1% antibiotics) and resuspended in erythrocyte lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA; pH 7.3) for 2 min. Splenocytes from WT and knockout mice were cultured for 2 days after isolation and stimulated with CD3/CD28 beads (T cell to bead ratio: 2:1; Dynal Biotech, Hamburg, Germany). The amount of ATP in the supernatant following cell lysis was assessed as a parameter of cell proliferation using the ATPLite™ Luminescence Assay System (PerkinElmer) according to the manufacturer's instructions. Luminescence was measured on a Topcount NXT. All experiments were performed in triplicate.

Assessment of cytokine secretion

Splenocytes were isolated as described above and $3*10^6$ splenocytes were plated in 1 ml DMEM containing 10 mM HEPES, 25 μg/ml gentamicin, 50 μM β-mercaptoethanol, 5% FCS, 2 mM glutamine and 1% non-essential amino acids (NEAA; Cambrex, Verviers, Belgium). Splenocytes were either left unstimulated or were stimulated with CD3/CD28 mouse dynabeads (2:1) in the absence or presence of A293 (0.1–10 μM). The solvent in the final experimental solution did not exceed 0.1% and application of the solvent alone had no impact on read-out parameters. Cells were cultured for 2 days at 37 °C and 5% CO_2 and supernatants were assessed for IFNγ and IL17 protein levels by ELISA (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions. Viability of cells incubated with A293 was assessed by flow cytometric stainings for annexin V and propidium iodide as described before (Meuth et al., 2008). For MOG recall assays, splenocytes were restimulated with 10 μg/ml MOG peptide (same stock as used for immunization). Cytokine levels were assessed after two days as described above. In one set of experiments, ELISPOT assays were performed using the same experimental conditions. Briefly, $1*10^5$ splenocytes per well were stimulated for 48 h with CD3/CD28 mouse dynabeads (2:1) in the presence or absence of A293 (0.1–10 μM). Cells incubated with medium alone were used as negative control. ELISPOT assays for IFNγ were performed according to the manufacturer's instructions (Mabtech). Spots were counted using an Immunospot S5 Versa Analyzer (Cellular Technology Ltd.) and analyzed with ImmunoSpot 5.0.41 software.

Immunohistochemistry

Mice were transcardially perfused with phosphate-buffered saline (PBS). Spinal cords were carefully excised from the brainstem to lumbar region and embedded in Tissue-Tek OCT compound (Miles Laboratories, Elkhart, USA). Ensuring that the same lumbar region was analyzed for all mice, 10 μm cross cryosections of the spinal cord were cut. Haemalaun-staining (0.1% hematoxylin (Sigma-Aldrich, München, Germany) in distilled water containing 0.02% $NaIO_3$ (Fluka, Sigma-Aldrich), 5% $KAlSO_3$ (Merck, Darmstadt, Germany), 5% chloral hydrate (Merck, Darmstadt Germany) and 0.1% citric acid (Serva, Heidelberg, Germany)) was performed for 1 min. For detection of myelin, slides were incubated with Luxol fast blue solution for 12 h at 60 °C (0.1%, Sigma-Aldrich, München, Germany), washed in 95% ethanol, and then placed in lithium carbonate (0.05%, Sigma-Aldrich, München, Germany). Analysis was done in 6 tissue sections, each section being at least 40 μm apart in 5 animals per group. Histological quantifications were visually counted by a blinded investigator (Bittner et al., 2009). For quantification, staining was examined under a microscope (Axiophot 2, Zeiss, Germany) equipped with a charge-coupled device camera and analyzed using MetaVue Software (Molecular Devices, Downington, USA).

Flow cytometry of CNS-invading cells

Mice were perfused transcardially with PBS to diminish contamination by leukocytes located within blood vessels. CNS tissue from immunized mice was dissociated mechanically and mononuclear cells from the interface of a 30–50% Percoll (Amersham, Piscataway, NJ, USA) density centrifugation were counted by Casy Model TT (Innovatis AG, Reutlingen, Germany), stained with rat anti-mouse CD4-PerCP, rat anti-mouse CD8a-PE and rat anti-mouse CD11b-APC (all from BD Biosciences, Heidelberg, Germany) following standard protocols. Afterwards, Calibrite™ beads (BD Biosciences) were added and relative cell numbers were determined by flow-assisted cell analysis (FACS-Calibur, BD Biosciences).

Electrophysiological measurements

Splenocytes were isolated as described above, cells were stimulated for 24 to 48 h, and $CD4^+$ T cells were isolated using the $CD4^+$ T cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). All measurements were conducted in the whole-cell configuration of the patch-clamp technique. Recording pipettes were fabricated from borosilicate glass (GT150TF-10, Clark Electromedical Instruments, Pangbourne, UK; typical resistance was 6–7 MΩ) and filled with an intracellular solution containing (in mM): K-gluconate, 88; K_3-citrate, 20; phosphocreatine, 15; NaCl, 10; HEPES, 10; $MgCl_2$, 1; $CaCl_2$, 0.5; BAPTA, 3; Mg-ATP, 3; Na_3-GTP, 0.5. The internal solution was set to a pH of 7.25 with KOH and an osmolarity of 295 mOsm/kg. Extracellular solution contained (in mM): NaCl, 125; KCl, 2.5; NaH_2PO_4, 1.25; HEPES, 30; $MgSO_4$, 2; $CaCl_2$, 2; glucose, 10; pH 7.35 and osmolarity was set to 305 mOsm/kg. Membrane currents were recorded using an EPC-10 double amplifier, and digital analysis was done using Fitmaster software (HEKA Elektronik, Lamprecht, Germany (Meuth et al., 2005)). After a hyperpolarizing pulse at −70 mV and a conditioning step at −40 mV, outward currents were elicited by 1 s rectangular pulses at +40 mV, applied at 10 s intervals (0.1 Hz). Outward currents under control conditions and A293 application were recorded from identical cells. Mean outward currents were analyzed using Origin 8.5G software (Origin Lab, Northampton, MA, USA). Statistical analysis for paired observations was done using Prism 5 (Graph Pad, La Jolla, CA, USA).