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Does chloride channel accessory 3 have a role in arthritis pain? A study on murine antigen-induced arthritis

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HIGHLIGHTS

- In DRGs, a 1724-fold up-regulation of mClca3 was shown in acute arthritis.
- mClca3 knock-out mice showed significantly less swelling in very acute AIA.
- No modification of mechanical hyperalgesia was seen in mClca3 knock-out mice.
- Pharmacological inhibition of CaCCs by niflumic acid did not alter AIA.
- mClca3 does not significantly contribute to arthritic pain.

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ABSTRACT

Calcium-activated chloride channels (CaCCs) are thought to regulate neuronal excitability, and recently chloride (Cl^-) regulation in DRG neurons has attracted much attention in pain research. Furthermore, the activity of CaCCs is modified by a family of CLCA proteins. In acute antigen-induced arthritis (AIA), a remarkable up-regulation of the murine chloride channel accessory 3 (mClca3) was shown in dorsal root ganglion (DRG) neurons. Therefore we tested the hypothesis that mClca3 is involved in arthritic pain perception. In mClca3 knock-out mice and wild-type control mice, AIA was induced and measures of inflammation and pain were assessed. In the very acute phase of AIA, joint swelling was reduced in mClca3 knock-out mice. This effect disappeared during the course of AIA. We could not show significant differences in mechanical hyperalgesia between both groups of mice, neither at the acute nor at the chronic stage (21 days of AIA). Additional experiments on thermal hyperalgesia in wild-type and mClca3 knock-out mice in the first 3 days of AIA did not show a difference either. In addition, niflumic acid, an antagonist at CaCCs, did not significantly influence hyperalgesia during AIA. Thus, we were not able to provide evidence for a role of CaCCs, and in particular of mClca3, on the expression of arthritis or inflammation-evoked hyperalgesia.

1. Introduction

Calcium-activated chloride channels (CaCCs) are a group of ubiquitously expressed ion channels. One important role is their impact in neuronal excitability [1]. They are expressed in spinal cord neurons, autonomic neurons and dorsal root ganglion (DRG) neurons but the functions of CaCCs here remain poorly understood [2–5]. One member of the CaCC family (anoctamin 1) was recently identified to act directly as a heat sensor in mice [6].

CLCA proteins are a family of proteins which are associated with CaCCs. They are thought to act as chloride channel accessory molecules which can modify the currents through CaCCs [7]. In mouse medium-sized DRG neurons, calcium-activated chloride currents were reported, and subsequently transcripts of mClca1 and mClca5 were detected [2,8]. Using microarray analysis we recently detected mClca3 in the DRGs. This protein is the murine

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homolog for human CLCA1, also known as gob-5 [9]. In mice, mClca3 plays a critical role in allergen-induced airway hyperreactivity, goblet cell metaplasia and the regulation of tissue inflammation in the innate immune response [10–12]. Interestingly, we found a 1724-fold up-regulation of the mClca3 gene in the lumbar DRGs in the acute inflammatory phase of murine antigen-induced arthritis (AIA) [13].

Chloride (Cl^-) is in the focus of pain research. It was shown that the effect of GABA in the spinal cord depends on the intracellular Cl^- concentration which is regulated by Cl^- transporters such as NKCC1. If the intracellular Cl^- concentration is elevated, GABA may cause rather an efflux than an influx of Cl^-, and this changes the effect of GABA from inhibition to excitation. Also in DRG neurons changes in the Cl^- regulation were shown to be important in nociceptive processing [14–16]. In vivo, NKCC1 knock-out mice show increased latencies to withdrawal from heat and a reduction in allodynia at the paws [17,18]. In DRG neurons, the chloride gradient is different from that in most other types of neurons such that calcium-activated Cl^- current can be excitatory and that these channels are pronociceptive [1]. Consistent with this view the knockdown of anoctamin 1 (see above) reduced thermal pain [6]. It was suggested that inhibition of CaCCs may be a potential strategy for the development of analgesis [1].

Because we observed an upregulation of mClca3 in the DRGs of mice with AIA [13], we tested the hypothesis that mClca3 is involved in inflammatory pain perception in murine AIA. We used mClca3 knock-out mice and wild-type (WT) control mice, induced AIA and assessed inflammation and pain.

2. Materials and methods

2.1. Animals

Homozygous mClca3$^{-/-}$ mice were generated and kindly provided by Prof. M.J. Holtzman (Washington University School of Medicine, St. Louis, MI, USA) [9]. Female knock-out and WT C57BL/6J control mice (9–13 weeks) were bred and genotyped by the Animal Facility of the University Hospital Jena. All animal studies were approved by the local government commission for animal protection (No. 02-019/12).

2.2. Arthritis induction and pharmacological treatment

Mice were immunized twice at 21 and 14 days before AIA induction with subcutaneous injection of 100 μg of methylated bovine serum albumin (mBSA) (Sigma–Aldrich, Taufkirchen, Germany), emulgated with 50 μl of complete Freund's adjuvant (CFA; Sigma–Aldrich), supplemented to 2 mg/ml *Mycobacterium tuberculosis*, strain H37Ra (Difco, Detroit, USA). Additionally, 5×10^8 heat-inactivated *Bordetella pertussis* germs (Chiron-Behring, Marburg, Germany) were applied intraperitoneally (i.p.). Monoarticular arthritis was induced by injection of 100 μg mBSA in 25 μl 0.9% NaCl into the right knee joint cavity on day 0. A flare-up reaction of inflammation was induced by a second intraarticular injection of 100 μg mBSA in 25 μl saline on day 42 after primary arthritis induction. In WT mice niflumic acid (NA), a potent blocker of CaCCs, was applied i.p. NA was dissolved in 0.4 M $NaHCO_3$ in 5% glucose. Mice received either 10 mg or 30 mg NA/kg body weight one day before arthritis induction for 5 consecutive days as previously described [19].

2.3. Assessment of AIA

Swelling at the knee was assessed by measuring the mediolateral joint diameter using an Oditest vernier caliper (Kroeplin, Schlüchtern, Germany). For histopathological examination knee joints were removed, fixed *in toto* in 4.5% formalin, decalcified in EDTA, embedded in paraffin and cut into 3 μm frontal sections which were stained with hematoxylin–eosin. The pathologist who scored arthritis was unaware of the experimental groups. Acute inflammation (infiltration of the synovial membrane by granulocytes and exudation of granulocytes into the joint space) was scored 0–3: 0 = no, 1 = mild, 2 = moderate, and 3 = severe changes (+1 if fibrin exudation in the joint space). Chronic inflammation (hyperplasia of synovial lining cells, infiltration of the synovial membrane by mononuclear cells, fibrosis of the synovial membrane and the periarticular tissue) was also scored 0–3. Cartilage surface defects with cell necrosis were scored 0–4: 0 = no damage, 1 = <5%, 2 = 5–10%, 3 = 11–50%, and 4 = >50% of the cartilage surface affected. Damage to bone was also evaluated: 0 = no, 1 = mild, 2 = medium, and 3 = severe damage (extensive area of deep invasive destruction of bone).

2.4. Pain-related behavior

Mechanical hyperalgesia at the hindpaws as an indicator of secondary hyperalgesia remote from the inflamed knee joint was assessed before and until 21 days after induction of AIA. Mice were placed into the testing device, and after accommodation to the environment the pain threshold was determined. Two testings before arthritis induction defined the baseline (BL). For mechanical hyperalgesia a dynamic plantar aesthesiometer (Ugo Basile, Comerio, Italy) was used which applied increasing pressure (stimulus increase rate 1 g/s; cut-off value 10 g) to the paw. The latency of the elicited leg withdrawal which reflects the respective mechanical threshold was averaged from three consecutive stimuli. Data are given in gram as alteration related to BL (d(x)–BL). Gait abnormalities of the ipsilateral hindlimb were quantified in the guarding score: 0 = normal walking, 1 = slight limping, 2 = persistent severe limping (still touching floor), 3 = severe limping with partial guarding of ipsilateral hindlimb (sometimes not touching floor), 4 = mainly guarding of ipsilateral hindlimb (most times not touching floor), and 5 = no walking at all.

In other groups of wild-type and mClca3$^{-/-}$ mice thermal hyperalgesia was assessed in the first 3 days of AIA using the Hargreaves plantar test (Ugo Basile) [20]. Two consecutive standardized heat stimuli were applied to the paw for evaluation of a mean latency (cut-off value 20 s).

2.5. Statistical analysis

Data are expressed as mean ± SEM. Differences between groups were calculated using the two-tailed Student's *t*-test for unpaired values. Arthritis score, guarding score and mechanical and thermal thresholds of experimental groups were compared using analysis of variance (ANOVA). Changes in responses within groups (baseline versus after AIA induction) were analyzed using Wilcoxon's matched pairs signed rank test. Statistical significance was calculated with the SPSS software package (v.16.0, Chicago, USA) and accepted for $p < 0.05$.

3. Results

3.1. Slightly decreased severity of AIA in mClca3$^{-/-}$ mice

After induction of AIA, joint swelling in the acute phase of AIA was significantly attenuated in mClca3$^{-/-}$ mice compared to control WT mice (ANOVA day 3: $F[1,25] = 5.441$; $p = 0.028$) (Fig. 1A). This difference between both groups of mice disappeared in the chronic phase of AIA from day 7 on. The second injection of mBSA at day 42 of AIA evoked a strong flare-up reaction in both WT and

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