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Expression of three distinct families of calcium-activated chloride channel genes in the mouse dorsal root ganglion

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Abstract: Objective A calcium-activated chloride current ($I_{Cl(Ca)}$) has been observed in medium-sized sensory neurons of the dorsal root ganglion (DRG). Axotomy of the sciatic nerve induces a similar current in the majority of medium and large diameter neurons. Our aim is to identify the molecule(s) underlying this current. **Methods** Using conventional and quantitative RT-PCR, we examined the expression in DRG of members of three families of genes, which have been shown to have $I_{Cl(Ca)}$ current inducing properties. **Results** We showed the detection of transcripts representing several members of these families, i.e. chloride channel calciumactivated (CLCA), Bestrophin and Tweety gene families in adult DRG, in the normal state and 3 d after sciatic nerve section, a model for peripheral nerve injury. **Conclusion** Our analysis revealed that that mBest1 and Tweety2 appear as the best candidates to play a role in the injury-induced $I_{Cl(Ca)}$ in DRG neurons.

Keywords: chloride channel calcium activated; bestrophin; tweety; chloride channel; dorsal root ganglion

1 Introduction

Sensory neurons of the dorsal root ganglia (DRG) convey information concerning the sensations of touch, temperature, muscle contraction, limb position and painful stimuli to the spinal cord. These neurons express many types of ion channels, some of which have been shown to be responsible for the specific functional properties of particular neuronal sub-types responsible for defined sensory modalities. We recently reported the induction of a calcium-activated chloride current ($I_{Cl(Ca)}$) in a discrete population of sensory neurons of the dorsal root ganglion[1]. Axotomy of the sciatic nerve led to the massive induction of $I_{Cl(Ca)}$ in the vast majority of neurons of medium and large cell body diameter, generally thought to be low-threshold skin and muscle innervating neurons conveying touch and proprioceptive information. At present, neither the functional relevance of this current in normal or injury-related processes nor its molecular nature is known. In the absence of specific pharmacological modulators, the identification of the ion channel(s) underlying this current is an important first step necessary for the development of tools designed to study this phenomenon. Members of three families of proteins have been shown to induce $I_{Cl(Ca)}$ when expressed in heterologous cellular systems: calcium-activated chloride channels (CLCAs), bestrophins and members of the tweety gene family (for reviews see [4,7-9,16]). In order to assess these molecules as candidates underlying sensory neuron $I_{Cl(Ca)}$ we analyzed expression of members of the 3 families by RT-PCR on normal and axotomized DRGs.

2 Materials and methods

2.1 Bioinformatic analysis The CLCA family. Mouse homologues of the human CLCA genes were found using the National Library of Medicine BLAST program [http://www.Genbank.nlm.nih.gov/BLAST/]. DNA and protein alignments were carried out using the Needleman-Wunch program at EMBM-EBI. Clustal analysis[10] was carried out using the program available at [http://www.ebi.ac.uk/clustalw/]. In a recent publication[22], compared the

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organisation of the human CLCA and mouse Clca (mClca) gene clusters, and identified 6 mouse genes. However, the mouse genome is being continually revised and updated by new transcript information, permitting more accurate gene prediction. By screening of the mouse genome sequence database (NCBI build 35.1, MapViewer) with the 4 human CLCAs using the Blast programme, we found 8 loci containing CLCA-like sequences on the short arm of mouse chromosome 3 in the region between 151 170 Kb and 151 544 Kb. In both species, the genes are arranged as a cluster. Detailed analysis revealed that, in mouse, these 8 mouse loci were represented by full-length cDNA sequences in murine non-redundant and expressed sequence databases (nr/mouse EST), and thus are likely to represent transcribed genes. Reference accession numbers (Refseq) for these sequences and the oligonucleotide primer pairs used to amplify their transcripts, are shown in Tab.1 (s: sense primer; as: antisense primer). The CLCA family is presented in the order in which the genes are located on chromosome 3.

2.2 Animals and surgery Procedures involving animals and their care were conducted in agreement with the French Ministry of Agriculture and the European Community Council Directive No. 86/609/EEC, OJL 358, 18 December 1986. Swiss mice (CERJ, Le Genest St. Isle, France) were housed in cages with a 12/12 h light/dark cycle and fed food and water *ad libitum*. Axotomy was performed on mice deeply anaesthetized by intraperitoneal injection of equithesin [0.6% pentobarbital sodium and chloral hydrate (0.4 mL/100 g body wt)]. The left sciatic nerve was exposed at the mid-thigh level and sectioned. A 3- to 5-mm fragment of nerve was removed. Mice were kept alive for 3 d before sacrifice and dissection of DRG.

2.3 Isolation of total RNA from mouse DRG Adult mice were euthanized by CO_2 inhalation followed by cervical dislocation. Lumbar L4-L5 ganglia from adult and post-axotomy stages were acutely dissected in ice cold phosphate-buffered saline (PBS) and rinsed in the same buffer, then frozen dry at -80 °C until used. Total RNA was isolated using the RNeasy RNA isolation kit (Qiagen) and homogenization of the tissue by passage through a syringe. The integrity of the RNA was checked by the presence of bands representing 28 S and 18 S RNA after migration on an agarose gels using TBE buffer.

2.4 Analysis of gene expression by reverse transcription-PCR Primers for PCR amplification were designed using the (Primer 3) program of MIT Centre for Genetic

Tab. 1 Sequences of PCR primers used in this study

Name	Accession No.	Sense primer	Antisense primer	Product (bps)
mClca5	NM_178697	AAGATGACCCACAGGGACAG	TGGGGGTGAAGTGGATGTAT	428
mClca5		AAGATGACCCACAGGGACAG	TACAGTTCGGCTGCTTGTTG	170
QRT-PCR				
mClca3	NM_017474	TCAGTGATCGTGGACAGCTC	AGGTGTTGAAGTGGTCCCTG	900
mClca6	NM_207208	CTACAGCGCTTCATCAAAGAAA	CTTTGCATGAACATTATGGAACGG	303
mClca7	NM_001033199	AACCCACCCAGACCCGAAATG	TGGATGGTCCCGAACTCAAATTGT	538
mClca4	NM_139148	GATACAGCTTAAAAGTCAATGC	GGTGCTGGGTGTCAAATAAACTTC	613
mClca2	NM_030601	GGTAGATACAGCCTAAAAGTGC	GCAGAAATATCATCACCCAGTGC	634
mClca1	NM_009899	ACCCAGACCAGATGTCCAAG	GCCGTCCATGTAAGGTGAAT	193
QRT-PCR				
mBest1	NM_011913	AGTTGGGCCTACCACACAAC	GCTGCCTCGTTCCAGTACAT	509
mBest2	NM_145388	CTGTGGCGAGAGCTGTTATG	GGAACCGTTTGAAGACTGCT	362
mBest4	NM_001007583	CGCTGGTTTACACACAGGTC	GAGCCCAGAAAGGACGGTA	388
Ttyh1	NM_001001454	GGCTCAGCTCAGACATCCTC	CAGCCAGGAAAGGAGCATAG	533
QRT-PCR		as above	as above	
Ttyh2	NM_053273	GTTCCAGCAGTCACTGACCA	GTATTTCCAGGCACGAGGTC	328
QRT-PCR		as above	as above	
Ttyh3	NM_175274	TGGAGATTCACTTCCGTTCC	ATCCGAAGCTCCAAGGGTAT	380
QRT-PCR		as above	as above	

Research and were synthesized by Sigma-Aldrich SARL, France. The extracted total RNA was treated with RQ1 DNase 1 (Promega) to destroy genomic DNA. cDNA was synthesized in a 20 μL reaction system containing (final concentration indicated): 10 units/μL Superscript II (Invitrogen), 0.5 mmol/L dNTPs (Invitrogen), RNase inhibitor (1 unit/μL, Promega), 12.5 pg/μL oligo (dT) primer and 1-5 μg total RNA. Reverse transcription (RT) was performed at 42 °C for 1 h. The reaction was stopped by heating at 70 °C for 15 min. Polymerase chain reaction (PCR) amplification using 1 μL cDNA was performed in a final volume of 20 μL under standard conditions using 0.03 units/μL Platinum Taq polymerase (Invitrogen) and 0.25 μmol/L of each primer. PCR cycles (35) were performed. Each cycle comprised the following steps: denaturation at 95 °C, 15 s; annealing, 30 s; extension 72 °C, 1.5 min. PCR products were analysed by electrophoresis in 1% agarose gels containing 0.5 μg/mL ethidium bromide. Inserts were sequenced by Qbiogene, Strasbourg, France. For all cDNA synthesis experiments, a control reaction was performed without the addition of reverse transcriptase. No PCR products were generated from this material.

The restriction enzyme *Eco*RI was used to cut the mClca2 into two fragments of approximately the same size to distinguish it from mClca1.

2.5 Quantitative RT-PCR Collection of lumbar DRG was carried out as described above. Total RNA extractions from adult and adult post-sciatic nerve section were performed in TriReagent solution (Sigma) and DNase treated with RQ1 DNase (Promega) according to manufacturer's instructions. Real time PCR was performed as previously described[19]. Briefly, 1 μg of total RNA was reverse-transcribed and real time PCR was performed by using SYBR Green I dye detection on the LightCycler system (Roche Molecular Biochemicals) in a 10 μL volume containing 3 μL of RT product (dilution 1/30), 0.5 μmol/L of forward and reverse primers, and 5 μL of QuantiTect SYBR Green PCR Master Mix (Qiagen). Negative controls using water instead of template and/or RNA non-reverse-transcribed were performed simultaneously. After an initial activation step of 15 min at 95 °C, 45 cycles consisting of 94 °C for 15 s, 55 °C for 20 s and 72 °C for 35 s were performed. The identity of RT-PCR amplified products was confirmed by sequencing (Genome express, France) and routinely by finishing PCR runs with a melting curve analysis (70 °C to 95 °C at 0.2 °C increments) allowing us to check the melting behaviour of amplicons.

The relative amounts of specifically amplified cDNAs were calculated using the delta-CT method[25,12] on three independent experimental replicates. Raw quantities were subsequently normalised by dividing with a proper normalisation factor representing the geometric mean of two stable control genes [polymerase (RNA) II polypeptide J (*Polr2j*) and DEAD box polypeptide 48 (*Ddx48*)]. The Mann Whitney U-test was used for comparison between groups. $P < 0.05$ was considered statistically significant.

3 Results

3.1 ClCA family expression in adult DRG By comparing human and mouse genomic and cDNA sequences[22] 8 loci identified in the mouse contains sequences similar to the 4 human CLCA cDNAs previously identified. These 8 mouse genes are located in a cluster on chromosome 3. We designed oligonucleotide primers to specifically amplify each of these sequences, except for mClca1 [GENBANK: NM_009899] and mClca2 [GENBANK: NM_030601] whose sequences are so similar that specific PCR primers could not be designed. PCR was carried out on cDNA generated from adult mouse DRG tissue. The results are shown in Fig. 1A. It can be observed that mClca1/2 and mClca5 are expressed in the DRG. For the other members of the CLCA family, transcripts were detected in tissues other than the DRG, except for the case of mClca8 which has only been identified as a putative transcript and is probably a pseudogene. It can be noted that our results show the first demonstration of the expression of mClca7, a member of the family that has not yet been studied in any detail. mClca1 and mClca2 can be distinguished by digestion of the PCR product with the restriction enzyme *Eco*RI[15]. In the adult mouse DRG, both transcripts were expressed, however the band representing mClca1 was more intense (Fig. 1B).

3.2 Bestrophin family expression in adult DRG Bestrophins are also called VMDs because mutations in the human vitelliform macular dystrophy type 2 (VMD2) gene are known to cause autosomal dominant Best macular dystrophy (BMD), a degenerative disorder of the central retina. The four human VMD-like proteins are thought to encode a novel family of anion channels. The mouse bestrophin gene family contains 4 members scattered throughout the genome, one of which, mBest3 or mVMD2l2, appears to be a non-transcribed pseudogene[14]. Using specific primer pairs we could detect expression of mBest1 [GENBANK: NM_011913] and mBest 4 [GENBANK:

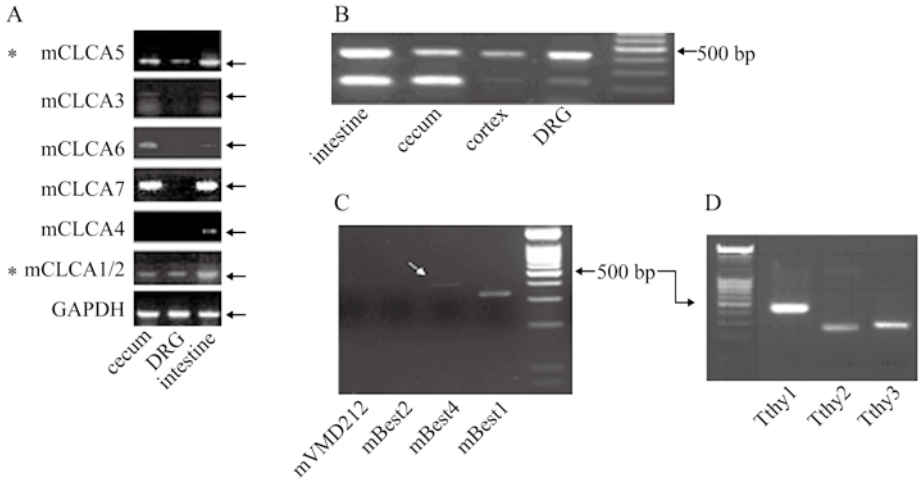

Fig. 1 RT-PCR showing the expression of mouse Clca mRNAs in adult mouse. A: Expression of members of the CLCA family in adult mouse intestine, cecum and DRG. Asterisks in A indicate that mClca5 and mClca1/2 are expressed in DRG. B: Digestion of mClca1/2 PCR products by *Eco*RI distinguishes mClca1 and mClca2 expression. The upper band represents mClca1 and lower band is a doublet representing mClca2. The relative expression levels varied in different indicated tissues, mClca1 was more abundant in the DRG. C: Of the members of the Bestrophin family, only mBest1 and mBest4 show mRNA expression in mouse DRG. D: All three members of the Tweety family (Tthy 1, Tthy2 and Tthy3) show expression in adult mouse DRG.

NM_001007583] in adult mouse DRG (Fig. 1C). For mBest2 [GENBANK: NM_145388] no expression was observed in the DRG, however a band was detected in cecum cDNA thus validating the primers (data not shown).

3.3 Tweety family expression in adult DRG Three genes homologous to the *Drosophila* Tweety gene have been iden-tified in the human genome[23]. Highly-conserved orthologues can be identified in the mouse genome: Ttyh1 [GENBANK: NM_001001454][2],Ttyh2 [GENBANK: NM_053273] and Ttyh3 [GENBANK: NM_175274]. Transcripts representing Ttyh1, Ttyh2 and Ttyh3 were found in adult mouse DRG (Fig. 1D). Thus, members of all three candidate gene families are expressed in the adult mouse DRG.

3.4 Quantitative RT-PCR analysis of expression in the DRG pre- and post axotomy In order to see whether these genes are regulated by sciatic nerve axotomy we carried out quantitative RT-PCR on normal and 72 h post-axotomy DRGs. This time-point corresponds with the increased expression of $I_{Cl(Ca)}$ in axotomized DRG neurons[1]. The results, shown in Fig. 2, demonstrate that axotomy did not change the expression levels of mClca1/2, Tthy2 or Tthy3. However, a statistically significant reduction in the expression levels of mClca5 and Tthy1 was observed. We could not generate reliable results for mBest1 or mBest4. The reason may be that expression levels are very low or that the gene is expressed in very discrete sub-populations of cells.

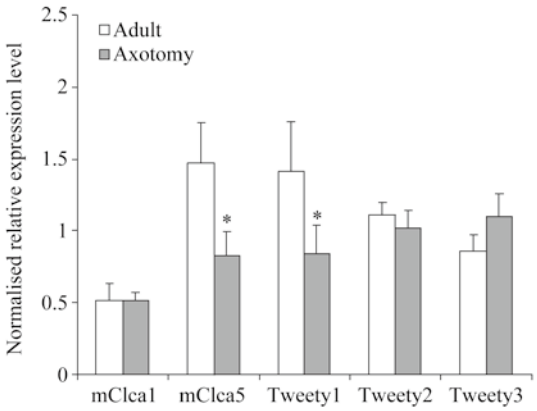

Fig. 2 $I_{Cl(Ca)}$ candidate genes expression determined by real-time PCR on adult (control) and post-axotomy adult lumbar (L4, L5) DRG. Data (means ± SEM) were calculated by the delta-CT method on four independent experimental replicates. The arithmetic means of the expression levels of two genes (*Polr2j*, *Ddx48*) whose expression do not change in the two conditions were used to normalize the expression levels. Data were analyzed using the Mann Whitney U-test. * $P < 0.05$.

4 Discussion

We have previously shown that a population of DRG neurons express $I_{Cl(Ca)}$ current and that this proportion is augmented after sciatic nerve axotomy[1] in particular in medium and large diameter neurons and that this current may play a role in the excitability of axotomized sensory neurons. We have also shown an important role of calcium

homeostasis in DRG neurons excitability[11], thus the regulation of chloride and calcium ionic movement is likely to be interlinked and of importance for the understanding of the response of somatosensory neurons to injury.

Experiments during the past two decades have established the existence of at least five distinct families of Cl channels: the ClCs, the aminobutyric acid and glycine receptors, the CLCAs, the Bestrophins, and the Tweety proteins[13,23]. Of them, members of three families: CLCAs, Bestrophins and the Tweety proteins have been suggested to be calcium-activated chloride channels. To quantify mRNA of candidate genes, we used real time PCR as it is now recognised as the most sensitive and consistent method to study relative gene expression. Moreover to encompass the fact that there is no ideal housekeeping gene to normalise expression levels especially after axotomy which induce major transcriptional modifications, we took advantage of the normalisation method which allows an accurate normalisation of data by using multiple internal control genes[25]. Our analysis by RT-PCR shows the expression of members of all three families in normal and axotomized DRGs. Quantititative RT-PCR analysis did not reveal any augmentation in the expression of these genes after axotomy.

Analysis of the structure of the mouse CLCAs by protein domain prediction programmes suggests that all these proteins harbour a von Willebrand A-like domain and possibly fibronectin domains[6,26]. While several studies of CLCAs expressed in heterologous expression systems suggested that they display chloride channels properties[5], more recent studies have cast doubt on this idea. In particular, it has been shown that all CLCAs studied so far are secreted, a behaviour incompatible with ion channel functions[6,20]. In the light of these new arguments, the implication of CLCAs in the $I_{Cl(Ca)}$ observed in sensory neurons should be reconsidered[7].

The Bestrophin and Tweety proteins can thus be considered as more promising candidates to underlie sensory neuron $I_{Cl(Ca)}$. Firstly, in the case of Bestrophins, membrane topology studies showed that Bestrophin1 is localized in the basolateral membrane of the retinal pigmented epithelium[17]. Secondly, the mutation in hBest1 responsible for the disease vitelliform macular dystrophy impairs its calcium-activated chloride channel function[27]. In addition, suppression of the native *Drosophila* orthologues of Bestrophin molecules eliminated endogenous Ca^{2+}-activated chloride currents[3]. Our expression results suggest that if Bestrophins are the molecules responsible for $I_{Cl(Ca)}$ in DRG neurons, then mBest1 and mBest4, as the only members expressed, are likely candidates.

Similarly, there is evidence suggesting that Tweety family members are calcium-activated chloride channels[23]. Expression of hTTYH3 in CHO cells generated a unique Cl- current activated by an increase in the intracellular Ca^{2+} concentration. Overexpression of Tthy1 shows that it is a membrane protein[18]. Using constructs for overexpression of Httyh-1, -2 and-3 in CHO and HEK cell lines[23,24], Suzuki *et al*. suggested that hTTYH1 activation is calcium-independent but that of hTTYH2 and hTTHY3 are strongly calcium-dependent. If the mouse orthologues of these proteins retain the same properties, mTtyh2 and mTtyh3 would be reasonable candidates to underlie Ca^{2+}-activated chloride currents in DRG neurons.

Since the $I_{Cl(Ca)}$ current in cultured DRG neurons is markedly enhanced by a prior conditioning lesion of the sciatic nerve[1] we tested by quantitative RT-PCR if this induction is reflected in the expression levels of our candidate genes. The expression of the CLCA and Tweety candidate genes remained constant or in the case of mClca5 and Tthy1 decreased under these conditions, suggesting either that members of these families do not play a role in this current, or that post-transcriptional changes in existing proteins (e.g. membrane targeting, activation by other proteins) underlie the current. In fact, we have observed very rapid induction of $I_{Cl(Ca)}$ current in non-axotomized DRG neurons in certain conditions (F.S, unpublished results), thus lending some credence to the latter hypothesis. For the reasons mentioned above, mCLCAs and Tthy1 are unlikely to be involved in $I_{Cl(Ca)}$ induction in DRG neurons leaving the remaining Tweety proteins and two Bestrophins as possible mediators.

In conclusion, members of the CLCA, Bestrophin and Tweety families of proteins are expressed in the mouse DRG; however their implications in the $I_{Cl(Ca)}$ remains to be clarified. Future work, using single cell PCR and siRNA mediated inhibition will concentrate on the most likely candidates revealed by this analysis, i.e. mBest1, mBest4, Tweety2 and Tweety3.

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小鼠背根神经节中三个不同的钙激活氯离子通道基因家族的表达

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摘要 目的 在背根神经节(dorsal root ganglion, DRG)中等大小感觉神经元中可以观察到钙激活氯离子流($I_{Cl(Ca)}$)。在坐骨神经损伤模型中，在大多数大中神经元上诱导出类似的氯离子流。本文旨在探讨引起这个离子流的分子基础。**方法** 使用常规的定量RT-PCR方法检测在DRG中三个基因家族的表达，这三个基因家族都具有诱导$I_{Cl(Ca)}$的特点。**结果** 在成年小鼠的 DRG 中，分别显示了在正常状态和坐骨神经损伤 3 天后 CLCA，Bestrophin 和Tweety基因家族成员的转录产物。**结论** mBestl和Tweety2可能在损伤诱导的DRG神经元$I_{Cl(Ca)}$中发挥作用。

关键词：CLCA 基因 ；bestrophin 基因；tweety 基因；氯离子通道；背根神经节