Acta Histochemica 115 (2013) 252–256

Contents lists available at SciVerse ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Connexin36 (Cx36) expression and protein detection in the mouse carotid body and myenteric plexus

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ARTICLE INFO

Article history:
Received 8 July 2012
Received in revised form 17 July 2012
Accepted 18 July 2012

Keywords:
Gap junctions
Carotid body
Myenteric plexus
Connexin
Cx36
Mouse

ABSTRACT

Although connexin36 (Cx36) has been studied in several tissues, it is notable that no data are available on Cx36 expression in the carotid body and the intestine. The present study was undertaken to evaluate using immunohistochemistry, PCR and Western blotting procedures, whether Cx36 was expressed in the mouse carotid body and in the intestine at ileum and colon level. In the carotid body, Cx36 was detected as diffuse punctate immunostaining and as protein by Western blotting and mRNA by RT-PCR. Cx36 punctate immunostaining was also evident in the intestine with localization restricted to the myenteric plexus of both the ileum and the colon, and this detection was also confirmed by Western blotting and RT-PCR. All the data obtained were validated using Cx36 knockout mice. Taken together the present data on localization of Cx36 gap-junctions in two tissues of neural crest-derived neuroendocrine organs may provide an anatomical basis for future functional investigations.

Introduction

Gap junctions are specialized cell-to-cell contacts allowing direct intercellular communication through so-called gap-junctional channels (Kumar and Gilula, 1996) that consist of two hemi-channels (connexons), each composed of six connexin (Cxs) proteins. Generally, gap junction channels allow the passive diffusion of small molecules, metabolites, second messengers, cations or anions, facilitating electrical and metabolic communication between coupled cells (Willecke et al., 2002). At present, 20 different connexin genes have been described in the mouse and 21 in the human genome (Sohl et al., 2004). Among these Cxs, Cx36 is considered the main Cx expressed in neuronal cells of the central nervous system (Condorelli et al., 1998, 2003; Sohl et al., 1998; Belluardo et al., 2000; Rash et al., 2005), but it is notable that, with the exclusion of Cx36 found expressed in the mouse and rat adrenal medulla (Martin et al., 2001; Degen et al., 2004), no data are available on Cx36 expression in other neural crest-derived neuroendocrine organs, such as the carotid body and the enteric nervous system (Le Douarin, 1986; Pearse et al., 1973).

The carotid body, a chemoreceptor regulating ventilation (Milsom and Burleson, 2007), is situated at the carotid bifurcation and is composed of two main cell types: type I or glomus cells, which are secretory granule-containing cells similar to adrenal chromaffin cells, and type II or sustentacular cells, which are supporting glial-like cells (Kondo et al., 1982; Pallot, 1987). The glomus cells of the rat carotid body are gap-junction coupled as demonstrated by ultrastructural analysis (Kondo and Iwasa, 1996) and by electrophysiological studies with dye- and electrotonic coupling (Monti-Bloch and Eyzaguirre, 1980; Monti-Bloch et al., 1993; Abudara and Eyzaguirre, 1994; Eyzaguirre and Abudara, 1996, 1999). With regard to the molecular evidence of Cxs in the carotid body, only Cx43 has been identified in the carotid body glomus cells (Abudara et al., 1999, 2000; Kondo, 2002), and no data are available on Cx36 expression.

In the intestine, previous investigations reported sparse Cx45 immunoreactivity in the deep muscular and submuscular plexuses in the dog (Nakamura et al., 1998; Wang and Daniel, 2001) and rat (Nakamura et al., 1998; Seki and Komuro, 2001), and Cx43 in the dog and rat (Seki and Komuro, 2001; Wang and Daniel, 2001). By contrast no data are available for Cx36 expression in the intestine, although it is known for a long time that the myenteric plexus can mediate neural activity in the gastrointestinal musculature

Abbreviations: Cxs, connexins; DTT, dithiothreitol; PBS, phosphate buffered saline; GFAP, glial fibrillary acidic protein; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; TH, thyroxin hydroxylase.

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http://dx.doi.org/10.1016/j.acthis.2012.07.005

through interneuronal communication by gap junctions (Sanders, 1996; Daniel and Wang, 1999; Komuro, 1999).

The present study was undertaken to evaluate, using immunohistochemistry, RT-PCR and Western blotting procedures, whether Cx36 was expressed in the mouse carotid body and intestine at ileum and colon level. The results obtained were validated by analysis of knockout mice lacking the Cx36 gene.

Materials and methods

The present study was performed in adult male C57BL mice and in ubiquitously Cx36 deficient mice $Cx36^{del(CFP)/del(CFP)}$ (Wellershaus et al., 2008) housed under alternating 12 h periods of light and darkness in a temperature (24 ± 2 °C) and humidity-controlled room. The experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local ethical committee.

Immunohistochemistry

The mice were sacrificed by an excess of chloral hydrate anesthesia, and the abdomen was immediately opened to dissect the distal colon and distal ileum (5 cm proximal to the cecum). The carotid body was dissected using a stereomicroscope (MS5, Leica Microsystems, Wetzlar, Germany). All dissected tissues were covered with OCT (Sakura TissueTek, Torrance, CA, USA) and frozen in isopentane precooled in liquid nitrogen, and stored at −80 °C until use.

Cryostat sections of 10 μm thickness of the carotid body and intestine (colon and ileum) were thawed onto gelatin coated slides, fixed in absolute ethanol for 5 min at −20 °C, air-dried for 60 min, rinsed with PBS and, after preincubation in blocking solution (0.5% BSA, triton 0.1% in PBS) for 30 min incubated overnight at 4 °C with goat polyclonal anti-Cx36 (SC-14904, Santa Cruz Biotechnology, Santa Cruz, CA, USA) affinity purified antibodies raised against an epitope mapping at the C-terminus of Cx36 of human origin, diluted 1:500 in blocking solution. After two washing steps with PBS for 5 min, the sections were incubated at RT for 1 h with specific Cy2-coniugated secondary antibodies, diluted 1:2000 (711-225-152; Jackson Immuno Research, West Grove, PA, USA). Following two washing steps with PBS, the sections were counterstained by incubation for 10 min in 0.5 μg/ml of the fluorescent nuclear dye Hoechst-33258 (bisbenzimide, Sigma–Aldrich, Seelze, Germany). Following a short washing with PBS, sections were coverslipped in a glycerol-based medium and slides were examined under a fluorescence microscope (DMRBE, Leica Microsystems, Wetzlar, Germany). Carotid body, colon and ileum tissue from Cx36 knockout mice were used as internal negative controls, whereas brain sections at inferior olive level, where Cx36 is expressed at very high levels, were used as internal positive controls.

RT-PCR analysis

Mouse tissues (carotid body, colon, ileum, olfactory bulbs) were homogenized each in 1 ml of QIAzol Lysis Reagent (Cat. No. 79306, Qiagen, Hilden, Germany) with a plastic pestle. The samples were passed through a 27 G needle until no more visible clumps were observed. Total RNA was isolated from the samples using the Qiagen RNeasy mini kit (Cat. No. 74104, Qiagen, Hilden, Germany). Two micrograms of RNA were reverse transcribed with a mixture containing: 5× first strand buffer (Cat. No. 18080-044, Invitrogen, Carlsbad, CA, USA), random hexamers 0.4 μM (N1034731001, Roche Applied Science, Penzberg, Germany), dithiothreitol (DTT) 100 mM (Cat. No. 18080-044, Invitrogen), dNTPs 0.5 mM (20-3011, Peqlab Biotechnologie, Erlangen, Germany), 40 U of RNAse inhibitor (03335402001, Roche), 200 U of Superscript III Reverse Transcriptase (Cat. No. 18080-044, Invitrogen). Reaction mixtures (20 μl) were incubated for 2 h at 50 °C and then for 15 min at 70 °C. Aliquots of the transcribed cDNA (1/20 of tissue reaction mix) were amplified using the following combination of Cx36 specific primers: upstream primer: 5′-TACTGCCCAGTCTTTGTCTGCTGC-3′, downstream primer: 5′-CACACCATTATGATCTGGAAGACC-3′. Reaction mixtures (25 μl) contained: 5× GoTaq® Flexi Green Buffer, $MgCl_2$ 1 mM, dNTPs 5 mM, 1.2 μM of each primer and 2 U GoTaq® Flexi DNA-polymerase (M8301, Promega, Madison, WI, USA). Reverse transcriptase-PCR (RT-PCR) was carried out using a PTC-200 Peltier Thermal Gradient Cycler (Bio-Rad, Hercules, CA, USA) with the following program: first denaturation step at 94 °C for 3 min, then a cyclic denaturation at 94 °C for 30 s, annealing at 64.5 °C for 30 s, elongation at 72 °C for 1 min repeated for 25 cycles and a cyclic denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 1 min repeated for 25 cycles and a final elongation for 10 min. After gel electrophoresis, the samples were run on a 2% agarose gel and the separated DNA fragments of 298 bp were visualized using a gel documentation system (Syngene, Cambridge, UK).

Western blotting

Tissues (carotid body, colon, ileum, olfactory bulbs) were rapidly dissected under a stereomicroscope, frozen and processed for Western blotting. In order to increase the probability of Cx36 detection in the intestine the muscular tissue was dissected from the mucosa and 100 μg of proteins was loaded per lane. The tissue was homogenized in cold buffer containing 12.5 mM Tris–HCl pH 7.4 and SDS 10% in the presence of protease inhibitors (P8340, Sigma–Aldrich, St. Louis, MO, USA). The homogenate was left at RT for 30 min and then centrifuged at

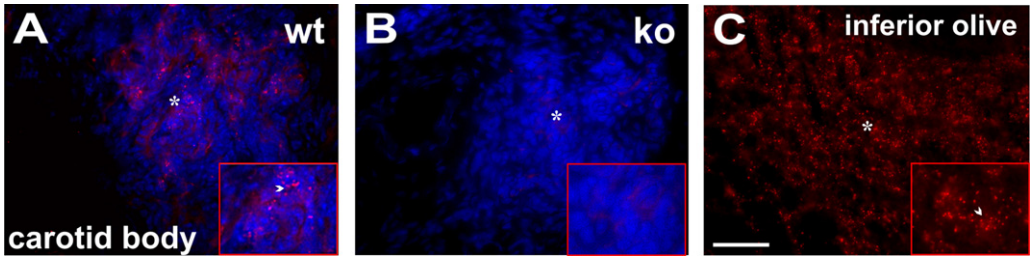

Fig. 1. Microphotographs from tissue sections of mouse carotid body processed for Cx36 by immunohistochemical analysis with specific antibodies. Note that Cx36 is detected as punctate immunostaining (arrow) in the carotid body of wild type (wt) mouse (A) but not in the $Cx36^{del(CFP)/del(CFP)}$ knockout mouse (ko) in (B). In (C) brain section showing, as positive control, Cx36 punctate immunostaining (arrow) at inferior olive nuclei level of wild type mouse. Asterisk indicates the sampled area. Cell nuclei (blue) were stained with fluorescent nuclear dye Hoechst-33258. Scale bar = 50 μm.