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

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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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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

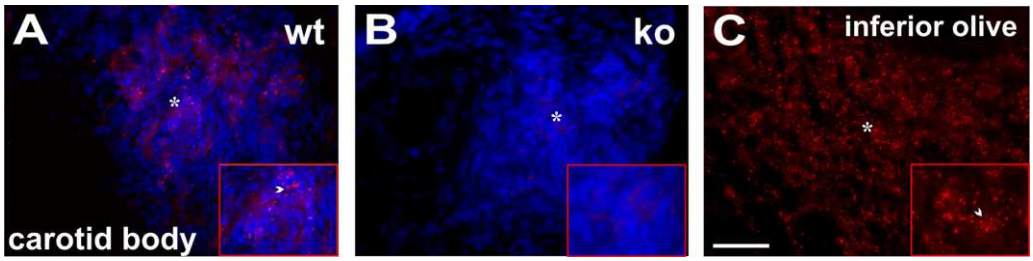

Fig. 1. Microphotographs from tissue sections of mouse carotid body processed for Cx36 by immunohistochemical analysis with specific antibodies. Note that Cx36 is detected as punctate immunostaining (arrow) in the carotid body of wild type (wt) mouse (A) but not in the $Cx36^{del(CFP)/del(CFP)}$ knockout 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.

13,000 rpm for 30 min at 4 °C. The supernatants were stored at −20 °C and aliquots were taken for protein determination by the method of Lowry et al. (1951). The samples (50 μg per lane) and molecular weight markers (Precision Plus Protein Standards, 161-0375, Bio-Rad) were run on an 8% polyacrylamide gel at 100 V and electrophoretically transferred onto nitrocellulose membranes (RPN303E, Hybond-C-extra, GE Healthcare Europe, Milan Italy). Following 1 h of incubation with 5% non-fat milk, the membranes were incubated overnight at 4 °C with goat anti-Cx36 used at a dilution of 1:1000. After washing, the membranes were incubated for 1 h at room temperature respectively with anti-goat IgG horseradish peroxidase-conjugated diluted 1:5000 (Sc 2768, Santa Cruz Biotechnology, Heidelberg, Germany) and anti-rabbit IgG horseradish peroxidase-conjugated diluted 1:5000 (Sc 2054, Santa Cruz). Immuno-complexes were visualized with chemiluminescence reagent (RPN2108, GE Healthcare Europe, Milan, Italy) according to the manufacturer's instructions. The ECL-films were developed using Kodak developer and fixer (Eastman-Kodak, Rochester, NY, USA).

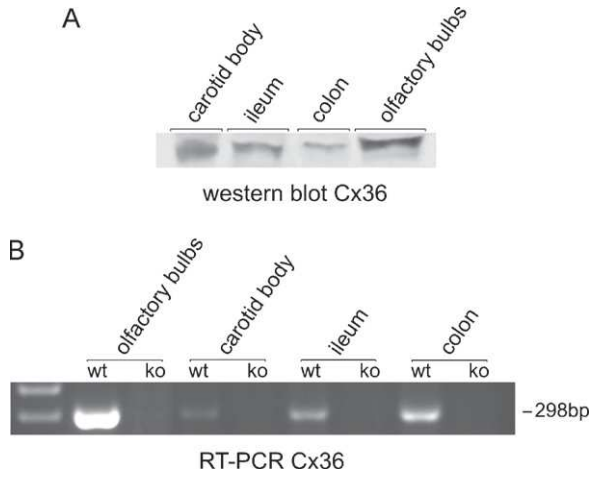

Fig. 2. Western blotting (A) and RT-PCR (B) analysis of Cx36 levels in mouse carotid body, colon and ileum. Olfactory bulbs were used as internal positive control. For Western blots of carotid body, ileum and colon 100 μg of proteins was loaded, whereas for olfactory bulbs 50 μg of proteins was used. For details see the respective results section. wt, wild type mice; ko, $Cx36^{del(CFP)/del(CFP)}$ knockout mice.

Results

Cx36 detection in the carotid body

The specificity of the Cx36 antibody used was tested in brain sections at the level of the inferior olive (Fig. 1C), where Cx36 is expressed at very high levels according to our previous work (Belluardo et al., 2000). Immunohistochemically, the Cx36 was detected in the mouse carotid body and showed diffuse punctate immunostaining (Fig. 1A). The immunohistochemical detection of Cx36 was confirmed by Western blotting analysis (Fig. 2A) and by RT-PCR analysis (Fig. 2B). The specificity of Cx36 in the mouse glomus was further validated by the absence of Cx36 detection, by immunostaining (Fig. 1B) and RT-PCR (Fig. 2B) in the glomus of $CX36^{del(CFP)/del(CFP)}$ knockout mice.

Cx36 detection in the intestine

We restricted this investigation to colon and ileum regions of the intestine. The immunohistochemistry analysis showed both in the colon and ileum punctate Cx36 immunostaining localized in the mouse myenteric plexus (Fig. 3A and C). Using Western blotting and RT-PCR analysis we could confirm the presence of Cx36 both at the level of protein and mRNA (Fig. 2A and B). The specificity of immunohistochemical detection and expression of Cx36 was validated by the disappearance of immunostaining (Fig. 3B and D) and mRNA (Fig. 2B) detection of Cx36 in the myenteric plexus of $CX36^{del(CFP)/del(CFP)}$ knockout mice.

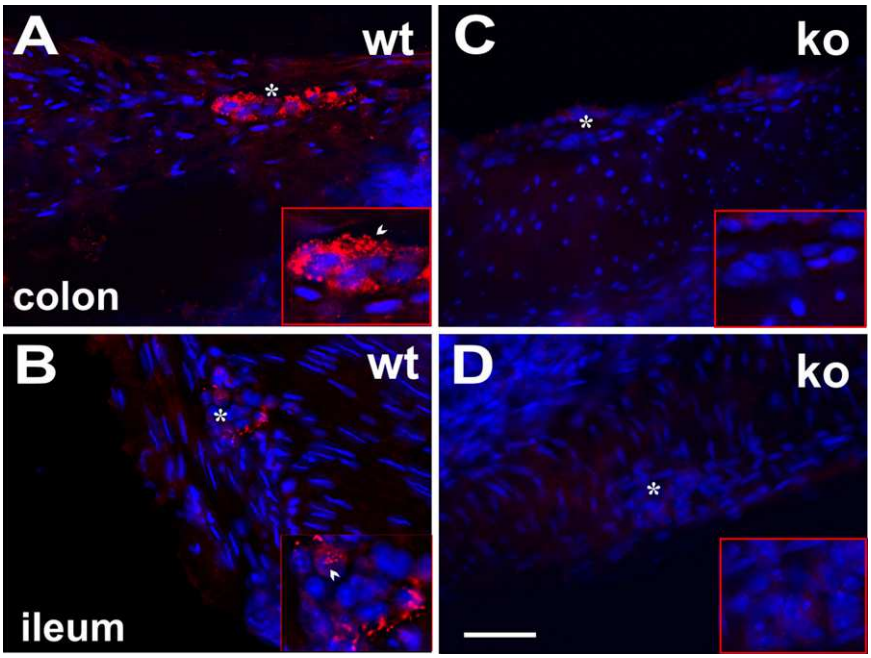

Fig. 3. Micrographs from tissue sections of mouse colon and ileum processed for Cx36 immunohistochemical analysis with specific antibodies. Note Cx36 punctate immunostaining (arrow) in the myenteric plexus (sampled area) of wild type (wt) mouse colon and ileum (A and B), whereas in the same condition no Cx36 was detected in the myenteric plexus (sampled area) of colon and ileum of $Cx36^{del(CFP)/del(CFP)}$ knockout mouse (ko) in (C and D). Asterisk indicates the sampled area. Cell nuclei (blue) were stained with fluorescent nuclear dye Hoechst-33258. Scale bar = 50 μm.

Discussion

The present study has demonstrated for the first time the presence of Cx36 in the myenteric plexus and carotid body. The specificity of Cx36 detection and expression was also corroborated by the disappearance of the Cx36 signal in the $Cx36^{del(CFP)/del(CFP)}$ knockout mice. Additionally, it is notable that, together with previous data on the adrenal medulla (Martin et al., 2001; Degen et al., 2004), the present study revealed that Cx36 is present in three organs with homologous cytological features (Martin et al., 2001). In fact, the sympatho-adrenal system lineage gives rise to the enteric nervous system, the autonomic ganglia, and several associated paraneural organs such as the carotid body and medulla of the adrenal gland (Le Douarin, 1986).

Carotid body

The carotid body parenchyma is organized in clusters (glomeruli) of glomus cells or type I cells similar to sympathetic neurons that are electrically excitable and contain dense-core vesicles with neurotransmitters secreted in response to membrane depolarization. Glomus cell clusters are enveloped by processes of glia-like, sustentacular type II cells, which are non-excitable and still lacking a structural role.

In the present study, although the expression and detection of Cx36 in the carotid body was clearly shown, we did not identify the cell type expressing the Cx36. However, despite our efforts, this failure may probably have been because tissue fixation for cells markers was incompatible with that of CX36 immunostaining. Previously, a variety of electrophysiological techniques have evaluated electrical coupling in the carotid body and have found that coupling is common among dopaminergic neurons or type I cells (Monti-Bloch et al., 1993; Abudara and Eyzaguirre, 1994, 1998). It has been suggested that coupling and uncoupling may be integral processes in the secretion of transmitters by the glomus cells (Eyzaguirre and Abudara, 1995, 1999) or intercellular coupling between glomus cells and carotid nerve terminals (Eyzaguirre, 2000). This coupling between neuronal cells of the carotid body potentially could depend on the presence of Cx36, which among the known Cxs is currently considered the main neuronal connexin (Condorelli et al., 1998, 2000, 2003; Sohl et al., 1998; Belluardo et al., 1999, 2000; Rash et al., 2000; Serre-Beinier et al., 2000; Trovato-Salinaro et al., 2009). Overall, the present data show for the first time the expression of Cx36 in glomus cells and open new possibilities for future functional studies on carotid body-cell coupling.

Myenteric plexus

The analysis of Cx36 in the mouse colon and ileum reveals for the first time Cx36 punctate immunostaining restricted to the myenteric plexus. It is known that the Cajal interstitial cells of the myenteric plexus are considered to serve as pacemakers of the gastrointestinal muscle by initiating slow waves in both muscle layers and that the myenteric plexus can mediate neural activity in the gastrointestinal musculature through intercellular communication by gap junctions (Sanders, 1996; Daniel and Wang, 1999; Komuro, 1999; Daniel, 2004). This immunolocalization of Cx36 is in agreement with the current hypothesis that the presence of very few small gap junctions in the myenteric plexus transmits enough currents and drive slow waves throughout the longitudinal or circular muscular layers (Daniel and Wang, 1999). This observation of Cx36 located in the myenteric plexus opens the opportunity for functional studies to determine how critical Cx36 gap junctions are in the electrical coupling between neuronal cells and/or neuronal cells and smooth muscle cells.

Overall, the present data revealed Cx36 gap-junctions in two tissues of the neural crest-derived neuroendocrine organs and we believe may provide a basis for future functional investigations.

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