Available online at www.sciencedirect.com
SCIENCE DIRECT
Peptides 26 (2005) 1567–1572
PEPTIDES
www.elsevier.com/locate/peptides

Potential role of the neuropeptide CGRP in the induction of differentiation of rat hepatic portal vein wall

A. Thiévent [a], S. Sena [b], A. Parlakian [c], G. Breuzard [a], A. Beley [d], L. Rochette [a], J.L. Connat [a,*]

[a] *Université de Bourgogne, Biologie Animale Cellulaire et Moléculaire, LPPCE, IFR 100, 6 Bvd. Gabriel, 21000 Dijon, France*
[b] *INSERM Unité 441, Avenue du Haut Lévêque, 33600 Pessac, France*
[c] *Laboratoire de biologie moléculaire de la différenciation, Université Denis Diderot, Paris 7, Case 7136, 2 place Jussieu, 75005 Paris, France*
[d] *Laboratoire de pharmacodynamie, Université de Bourgogne, UFR Pharmacie, BP 87900, 21079 Dijon, Cedex, France*

Received 29 October 2004; received in revised form 16 February 2005; accepted 16 February 2005
Available online 19 March 2005

Abstract

The media of the rat hepatic portal vein is composed of an internal circular muscular layer (CL) and an external longitudinal muscular layer (LL). These two perpendicular layers differentiate progressively from mesenchymal cells within the first month after birth. In this paper, we studied the development of calcitonin gene-related peptide (CGRP) innervation during post-natal differentiation of the vessel. We show that CGRP innervation is already present around the vessel at birth in the future adventitia but far from the lumen of the vessel. Progressively, CGRP immunoreactive fibers reached first LL then CL. CL by itself become only innervated at day 14 after birth. This corresponds to the time at which thick filaments (myosin) are visible in electron microscopy and desmin visualisable by immunocytochemistry. Furthermore, we provide evidence by autoradiography, that binding sites for CGRP are transiently expressed on the portal vein media at day 1 and 14 after birth. Vascular smooth muscle cells were transfected with constructs containing promoters for desmin or smooth muscle myosin heavy chain (smMHC). CGRP treatment of the cells significantly increased the expression of smMHC. Overall these results suggest that CGRP can potentially influence the differentiation of smooth muscle cells from the vessel wall.

Keywords: Peptidergic innervation; Vascular development; Desmine; Smooth muscle myosin heavy chain (sm-MHC); Binding sites

1. Introduction

Correlations have been found between initiation of smooth muscle development in the mouse genital tract and occurrence of Wolffian nerve terminals [4] and it is now well established that denervation of vascular smooth muscle cells causes not only functional but also structural changes [3,8]. Similarly, in skeletal muscle, denervation induces an increase of the mitotic index of satellite cells and a disorganization of the sarcomeric structure of the fibres [11]. In addition, denervation of skeletal fibres indicate that nerves influence the expression of the different isoforms of actin [18,21] a marker of muscle differentiation. However, the role of neuropeptides in these processes is still poorly understood.

Hepatic portal vein is an atypical vein that presents spontaneous rhythmic contractions due to two perpendicular smooth muscle layers in its media. Both the internal circular muscular layer (CL) and external longitudinal muscular layer (LL) are innervated by sympathetic and parasympathetic nerves [2,15]. In previous studies, we have shown that achievement of the differentiation of the vein wall is post-natal [14,20]. According to cytological and biochemical criterions, the LL seemed to differentiate earlier than CL. In addition, we have shown that there is a fluctuation in neuropeptide content in the nerve terminals [14] concomitant with the vein differentiation. Among the different peptides investigated, CGRP was found to be present at birth and in high quantity during the differentiation of the vessel. However, due to the technique used in the previous study (immunocytochemistry on whole

* Corresponding author. Tel.: +33 380 396216; fax: +33 380 393825.
E-mail addresses: jean-louis.connat@u-bourgogne.fr, connatjl@u-bourgogne.fr (J.L. Connat).

0196-9781/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2005.02.015

mount preparations of vessels) the target of the immunoreactive fibers could not be determined precisely. We thus decided to re-investigate the relationship between CGRP immunoreactive fibers location and differentiation of the two different muscular layers of the vein on transverse sections. Furthermore, we also looked for the presence of binding sites for CGRP on the media of the vessel during its development, and tested the hypothesis that desmin and smooth muscle myosin heavy chain (sm-MHC), two markers of muscle differentiation, could be induced by CGRP in cultured cells.

2. Material and methods

2.1. Animals

Portal veins from 1 day to 7-weeks-old Wistar rats were dissected out and rapidly rinsed in PBS.

2.2. Immunocytochemistry

Tissues were fixed at room temperature (RT), for 1.5–2 h, in 0.01 M phosphate buffered saline (PBS) pH 7.4, containing 0.4% (w/v) parabenzoquinone (Fluka, Buchs, Switzerland), rinsed for 2 h in PBS, deepen overnight at 4 °C in PBS containing 15% sucrose and then frozen in liquid nitrogen until use. Four to six serial 20 μm sections were cut with a cryo-microtome every 0.5 mm along the vessel. Twelve to 60 sections were thus obtained on gelatin-coated slides for each vessel, depending on its size. Sections were dried at room temperature for 1–3 h, re-hydrated in PBS (5 min), permeabilized in 0.8% Triton X-100 in PBS (1 h) and rinsed for 5 min, 3 times in PBS. Sections were then incubated with CGRP antiserum (Amersham) diluted to 1:500 into PBS (pH 7.2) containing 0.5% BSA and 0.02% NaN3 for 24 h at 4 °C in a humid chamber. After 3 washes (15 min each) in PBS, they were incubated for 90 min in the dark with FITC-conjugated goat anti-rabbit IgG (Sigma) diluted 1:50, washed (2 × 10 min) in PBS, and stained for 7 min in 0.05% Pontamine blue 5 BX (BDH Chemicals) in PBS containing 1% DMSO. After 2 last 5 min rinses, slides were mounted in PVA medium. To assess the specificity of the immunostaining, pre-absorption of primary antiserum with excess of 10^{-6} M CGRP (Bachem) or replacement of this serum with non-immune rabbit serum (Nordic Immunology) diluted 1:500 was done. These controls showed the absence of immunostaining.

2.3. Quantification of the innervation

The abundance of innervation was estimated blind at five different locations on each section by counting the number of nerve fibres detected within each field of view. The nerve fibres were counted directly from immunohistochemically labeled sections. Depending upon the number of fibres detected a score was attributed (no fibers, score = 0; 1–2 fibers, score = 1; 3–5 fibers, score = 2; 6–10 fibers, score = 3; and greater than 10 fibers, score given was 4). All of the scoring was carried out by a single observer (A.T.). The data corresponds to the mean of the different scores obtained from between 12 and 60 sections for each age investigated from four different animals at each age.

2.4. Autoradiography

Autoradiography was carried out on 20 μm thick cryostat sections of rat portal veins according to Thiévent and Connat [19]. Incubation was with 0.1 nM (250,000 cpm/ml) human 2-[^{125}I]iodohistidyl CGRP, 2000 Ci/mmol (Amersham International, England) immediately following sectioning. 1 μM un-labeled human αCGRP (Bachem, Bubendorf, Switzerland) was used for estimation of non-specific binding. Pig coronary artery or rat cerebellum sections were included in each autoradiographic experiment as a positive control.

Competition experiments were carried out with 1 μM of 8–37 h CGRP, salmon calcitonin, Amylin or Adrenomedullin (all purchased from Bachem).

Labeled sections were exposed to BIOMAX-MS film with an intensifying screen (Kodak) for 3–7 days at −70 °C and developed in Kodak D19. Results were expressed as intensity of dark on the film by using a scale from 0, pixel intensity (PI) < 15 when measured with ImageJ software; +, week signal, PI from 30 to 50; ++, light grey signal, PI from 70 to 100; +++, dark grey signal, PI from 200 to 230; ++++, black signal, PI > 250.

2.5. Cell culture

AU1, a stable smooth muscle cell (SMC) line derived from human aorta whose cells contain desmin and smooth muscle α-actin, was grown in DMEM/15% FCS. U8A4, a stable SMC line derived from rabbit aortic SMC expressing smooth muscle α-actin and smooth muscle myosin heavy chain (sm-MHC), was cultured at 33 °C in a defined serum-free medium containing a 1:1 mixture of Dulbecco's Modified Eagle's Medium (Life Technologies) and Ham's F10 plus transferrin (5 μg/mL, Sigma), ascorbate (200 μM, Sigma) and sodium selenite (6.25 ng/mL, Sigma).

2.6. Transfection assays

Luciferase-reporter constructs containing the mouse 4 kb-desmin or human 2.8 kb-sm-MHC promoter were transfected into human AU1 aortic smooth muscle cells and rabbit aortic smooth muscle cells U8A4, respectively. AU1 cells were transfected with Superfect reagent (Qiagen, Hilden, Germany) according to the manufacturers' protocol. Luciferase activity was assayed as described in Mericskay et al. [12]. An empty pGL3 plasmid was used to determine the basal level of Luciferase activity. Equal molar ratios of luciferase plasmids equilibrated with pBluescript to ensure an equal amount of DNA was used to transfect each well. CGRP (0.5 μg/mL medium, corresponding to a final concentration of 10^{-7} M)

was added 3 h after transfection. Luciferase assay was performed 48 h later on two independent experiments performed in triplicate. Relative luciferase activity was expressed as fold-activation compared to pGL3-basic basal activity.

U8A4 smooth muscle cells transient transfection was accomplished with cationic lipids using Transfast reagent according to the manufacturer's protocol (Promega). In brief, U8A4 cells were seeded in 6-well dishes at 35,000 cells per cm^2 the day before transfection. Cells were then washed twice with PBS and transfected (1 h, 37 °C) in Opti-MEM (Life Technologies), with 9 μL of Transfast and 3 μg of DNA per well. Cells were then re-fed with 2 mL of serum-free defined medium containing CGRP (0.5 μg/mL medium), cultured at 33 °C and harvested 2 days later. Cell extracts were prepared by use of the Luciferase Assay System (Promega) and levels of luciferase activity were measured with a liquid scintillation analyzer (TRI-CARB 2100 TR, Packard). β-Galactosidase assays were performed by using *o*-nitrophenyl β-D-galactopyranoside (ONPG, Sigma). The luciferase activity was normalized to β-galactosidase activity generated from co-transfected pCH110. An empty pGL3-basic plasmid (Promega) was used to determine the basal level of luciferase activity. The experiments were realized twice in triplicate. Results are expressed as the fold-activation compared to pGL3-basic basal activity.

3. Results

3.1. CGRP innervation of the rat portal vein during development

Transverse cryo-sections of portal veins were performed at 11 different ages of the post-natal development of the vessel and stained with an anti-CGRP antibody. Immunoreactive fibers were counted at 5 different levels of the vessel wall from 4 individuals at each age: within adventitia, in close contact with the external border of LL, into the LL, at the level of the intra medial connective layer iCM and into the CL, (see Fig. 1). Although a scoring system was used, the mean of the different scores was calculated to better visualize the results (Fig. 2). CGRP immunoreactive fibers were always found from birth at the outside of the developing vessel in the adventitia (Fig. 2A). The number of fibers in close contact with the external border of LL slightly increased during development (Fig. 2B). Beginning at days 5–8, CGRP-immunoreactive fibers penetrate the LL (Fig. 2C) to reach iCM (Fig. 2D), then invade CL at days 17 (Fig. 2E).

Table 1
Autoradiographic study of the binding sites for [^{125}I]-CGRP present on transverse sections of portal veins of different ages

Rat age (days)	Nb of vessels	Total binding	Non-specific	Specific binding
1	2	++++	0	++++
5	6	+	+	0
7	3	+	+	0
8	3	+	+	0
10	3	++	+	+
14	6	+++	++	+
19	3	++	+	+
21	3	++	++	0
28	3	++	++	0
31	2	++	++	0
7 weeks	6	++	++	0
Coronary artery	12	++++	0	++++

Number of + in the first two columns corresponds to the intensity of the signal observed on the film (for Pixel intensity see Section 2). The last column corresponds to the estimation made from the 2 previous columns.

3.2. Binding sites to CGRP on rat portal vein wall during development

We examined the presence of binding sites to ^{125}I-CGRP using classical autoradiography on sections of the portal vein at 1, 5, 7, 8, 10, 14, 19, 21, 28 and 31 days after birth. Table 1 summarizes results obtained on portal veins. The hepatic artery (HA), which is very near the portal vein, was also included in the transverse sections and pig coronary arteries were used as positive controls in each experiment. Pig coronary artery was always strongly labeled and displacement was total when un-labeled CGRP was added. Portal vein and HA were both labeled on sections from one day old rats (Fig. 3A). At day 5, only the hepatic artery wall remained radiolabeled (Fig. 3B). At these two stages, the radiolabeling

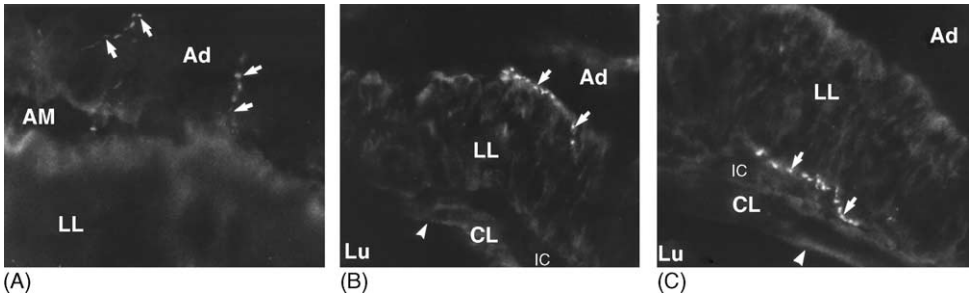

Fig. 1. Immunostaining for CGRP on transverse sections of adult portal vein showing the different levels at which immunoreactive fibers are found (arrows). A: immunoreactive fibers located in the Adventitia (Ad). B: immunoreactive fibers located at the adventitial-medial border. C: immunoreactive fibers located in the intra medial connective layer (ic). LL: longitudinal layer of smooth muscle cells; CL: circular layer of smooth muscle cells; AM: adventitial-medial border; Lu: lumen of the vessel.

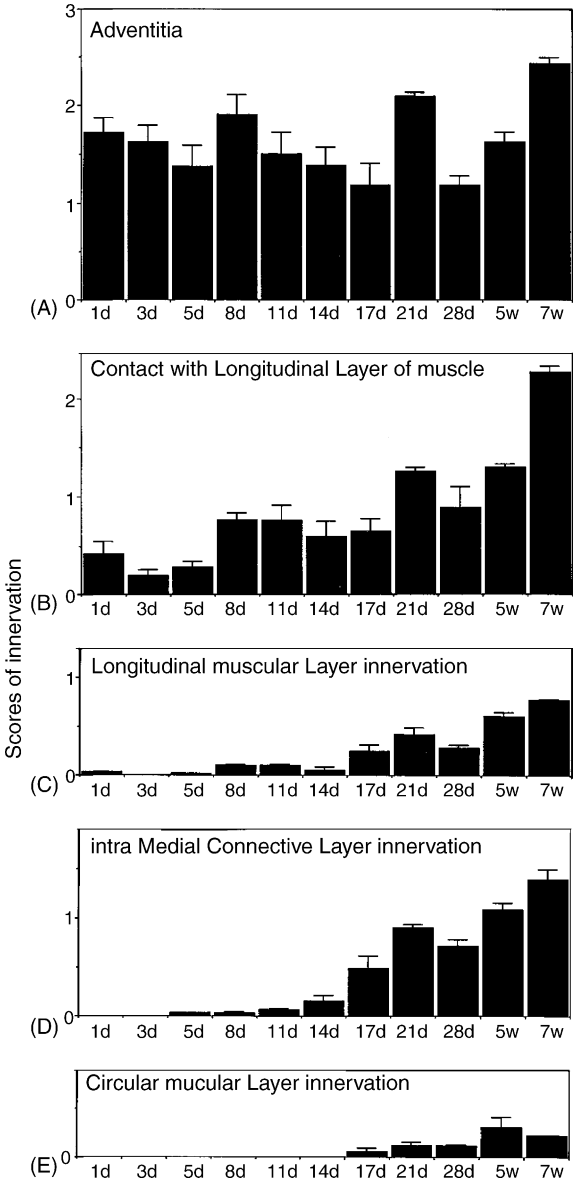

Fig. 2. Variation in the density of CGRP immunoreactive fibers found at different levels of transverse sections from portal veins of different ages. Vertical bars represent S.D. calculated on the averaged scores.

was totally displaced by 1 μM non-labeled peptide. At day 10, 14 (Fig. 3C) and 19, both vessels walls still presented a labeling. However, especially in the case of portal vein, this radiolabeling was not totally displaced by an excess of peptide. Later, on days 28, 31 and at 7 weeks (Fig. 3D), the labeling from HA was displaced only.

3.3. Role of CGRP in the induction of differentiation markers genes

Transfection experiments were performed with pGL3 plasmid constructs containing either the 4 kb desmin promoter or the human 2.8 kb smooth muscle-myosin heavy chain (sm-MHC) promoter, both driving luciferase reporter gene. Transfected cells were incubated with CGRP (0.5 μg/mL) or ethanol 50% as a control. In the case of desmin, no significative changes were noted (data not shown). However, the sm-MHC promoter was significantly stimulated in the presence of CGRP (Fig. 4). Indeed, the 2.8 kb sm-MHC promoter was activated by 24.49 ± 2.65-fold compared to the pGL3-basic plasmid containing no promoter in the presence of ethanol 50%. The addition of CGRP in the culture medium stimulates the sm-MHC promoter activity to 31.74 ± 3.22-fold increase compared to pGL3-basic. These values represent a stimulation of the sm-MHC promoter activity by 129.6% in the presence of CGRP compared to the ethanol condition.

4. Discussion

In the present work, we investigated the time course of CGRP immunoreactive fibers penetration in the hepatic portal vein during its development. Our previous studies [14,20] demonstrated that at birth, the rat portal vein is immature and composed of an endothelium surrounded by mesenchymal cells. These cells progressively organize into two concentric muscular layers. The inner circular layer (CL) is separated from an external longitudinal layer (LL) by a thin intra-medial connective layer (iMC). The adult morphology is reached within 28 days after birth. Using immunohistochemistry, we established that desmine positive cells occur first in LL at day 8 and then progressively invade all the LL's cells [20]. The first desmin-positive cells are found in the CL later, at day 14, this layer being fully labeled at day 21. The same pattern was observed with electron microscopy for occurrence of thick filaments, corresponding to myosin [20]. At day 8, thick filaments occurred and were only located in small areas of many LL's cells. It is only at days 14–21 that myosin was clearly visible in both CL and LL's cells. Present results clearly indicate that the penetration timing of CGRP immunoreactive fibers into the portal vein wall perfectly coincides with the differentiation pattern of the smooth muscle cells.

In parallel, binding sites for iodinated CGRP were found on the vessel wall in these early stages of development. Total displacement of the binding by CGRP was only obtained at day 1, suggesting that a "true" CGRP receptor is present at this time. This is in agreement with studies conducted on cultured smooth muscle cells, which showed such CGRP receptors [5]. Later, displacement was either not possible or partial, suggesting a different characteristic of the receptor, which become much more similar to those found on aortic wall (see [19]). The exact nature of these binding sites remains unclear since displacement with amylin, calcitonin or adrenomedullin did not gave better results (data not shown).

Due to the fact that penetration of CGRP immunoreactive fibers correlates well with occurrence of smooth muscle markers, we used in vitro cultured cells to investigate the effect of this peptide on the induction of desmin and smooth muscle myosin heavy chain genes. Smooth muscle cells

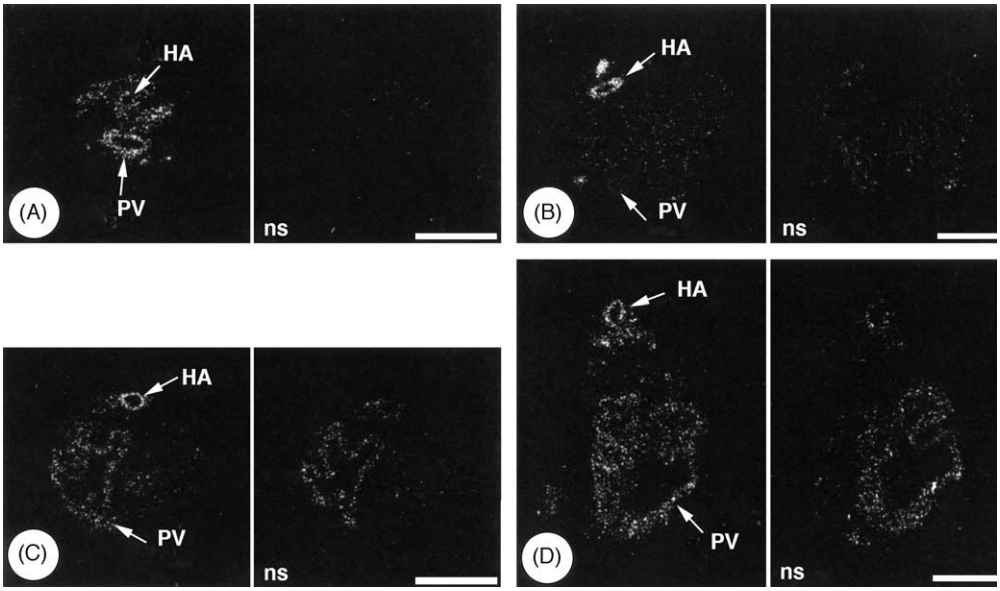

Fig. 3. Binding sites for ^{125}I-CGRP evidenced on transverse sections of portal veins (PV) and hepatic artery (HA) at different time of development. Left panel of each picture: total binding; right panel: non-specific binding (ns). Bars indicate 1 mm.

(SMC) of two different types were transfected with constructs allowing quantification of expression via luciferase activity. Results demonstrated a significant stimulation of the sm-MHC promoter activity in the presence of CGRP compared to the ethanol 50% control. In contrast, the expression of desmin was not significantly affected. This may be due to the high basal expression of the promoter in this type of smooth muscle cells. Our results suggest, however, that CGRP would be implicated in the sm-MHC promoter regulation. We have previously demonstrated that CGRP was able to modulate the expression of alpha smooth actin in cultured rat aorta SMC [6]. The present report provides second evidence

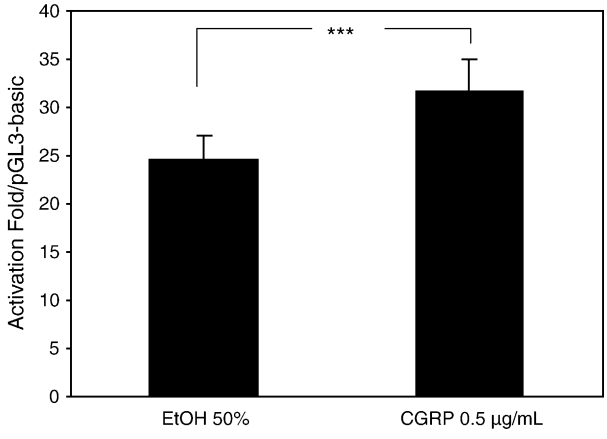

Fig. 4. Stimulation of the human sm-MHC promoter activity by human α-CGRP. U8A4 SMC were transfected with 1.5 μg of pCH 110 plasmid and 1.5 μg of the 2.8 kb-smMHC-Luciferase promoter construct by lipofection. The graph represents the activation fold of the promoter compared to the pGL3-basic vector that is assigned to a value of 1. The values of two separate experiments were averaged, and each error bar represents the standard error of the mean. Differences are statistically significant ($p = 0.0046$).

pointing out the fact that CGRP could influence expression of smooth muscle differentiation markers. Other studies point out similar possibilities for skeletal muscle. CGRP has been shown to slowly stimulate the differentiation of neonatal rat myoblast cultures or L6 cells and creatine kinase activity was enhanced after treatment with 10^{-7} M CGRP [1,13]. A role in the acquisition of the muscle fiber morphology was also suggested by a study realized on dysgenic mdg/mdg myotubes [10]. Finally, CGRP acts as an anterograde trophic agent involved in the control of acetylcholine receptor synthesis and function at the neuromuscular junction [9].

In conclusion, together with our former data indicating that: (1) CGRP influences proliferation and differentiation of SMC in vitro [6]; (2) CGRP prevents apoptosis [16,17]; and (3) that a relationship exists between the loss of CGRP immunoreactive fibers and loss of muscular characters as desmin-positive cells during aging in aortic wall [7], the present study provides further evidence that CGRP innervation plays an important role in the control of vessel wall organization and homeostasis.

Acknowledgements

The Swiss National Foundation (grant 31000, 26610-89/I/II), the Conseil Régional de Bourgogne and the FRM participated to the financial support of this project.

We thank Prof. BRUNOTTE and Prof. HORIOT for hospitality/facilities at the “Centre Leclerc” for working with isotopes. We are indebted to Prof. RENAUD de la Favière (Hôpital Marie Lannelongue) for financial help via iodinated CGRP purchasing, and to Prof. Denise PAULIN (Univ. Paris VI) and Prof. Jacques BONNET (Univ. Bordeaux) for allowing collaborative studies. We gratefully acknowl-

edge Philippe MICHAUT for his help in cryo-sectioning and Stéphanie SUEUR for help in statistical analysis. Finally Prof. MICHAILLE (Univ. Bourgogne) and Prof. GREEN (Univ. of Auckland) kindly accepted to read the manuscript and helped to improve it.

References

[1] Arii Y, Kawai H, Sato K, Yamaguchi H, Saito S. Effects of calcitonin gene-related peptide on muscle cell differentiation and development. Rinsho Shinkeigaku 1993;33:595–9 (Japanese).
[2] Barja F, Mathison R. Sensory innervation of the rat portal vein and the hepatic artery. J Auton Nerv Syst 1984;10:117–25.
[3] Bevan RD, Tsuru H. Long-term denervation of vascular smooth muscle causes not only functional but structural change. Blood Vessels 1979;16:109–12.
[4] Brauer MM, Passaro M, Casanova G, Abeledo G, Barreiro P. Differentiation of smooth muscle in the genital tract of the female mouse and its temporal relation with the development of the Wolffian nerve. Anat Embryol 1989;179:403–10 (Berl).
[5] Connat JL, Thiévent A, Carrier N, Huggel H. Occurrence of CGRP receptors on dedifferentiated smooth muscle cells from media of rat thoracic aorta. Ann N Y Acad Sci 1992;657:444–8.
[6] Connat JL, Schnüriger V, Zanone R, Schaeffer C, Gaillard M, Faivre B, et al. The neuropeptide calcitonin gene-related peptide differently modulates proliferation and differentiation of smooth muscle cells in culture depending on the cell type. Regul Pept 2001;101:169–78.
[7] Connat JL, Busseuil D, Gambert S, Ody M, Tebaldini M, Gamboni S, et al. Modification of the rat aortic wall during ageing; possible relation with decrease of peptidergic innervation. Anat Embryol 2001;204:455–68 (Berl).
[8] Dimitriadou V, Aubineau P, Taxi J, Seylaz J. Ultrastructural changes in the cerebral artery wall induced by long-term sympathetic denervation. Blood Vessels 1988;25:122–43.
[9] Fontaine B, Klarsfeld A, Changeux JP. Calcitonin gene-related peptide and muscle activity regulate acetylcholine receptor alpha-subunit mRNA levels by distinct intracellular pathways. J Cell Biol 1987;105:1337–42.
[10] Garcia L, Pincon-Raymond M, Romey G, Changeux JP, Lazdunski M, Rieger F. Induction of normal ultrastructure by CGRP treatment in dysgenic myotubes. FEBS Lett 1990;263:147–52.
[11] Gosselin LE, Brice G, Carlson B, Prakash YS, Sieck GC. Changes in satellite cell mitotic activity during acute period of unilateral diaphragm denervation. J Appl Physiol 1994;77:1128–34.
[12] Mericskay M, Parlakian A, Porteu A, Dandre F, Bonnet J, Paulin D, et al. An overlapping CArG/octamer element is required for regulation of desmin gene transcription in arterial smooth muscle cells. Dev Biol 2000;226:192–208.
[13] Noble BS, McMillan DN, Maltin CA. Calcitonin gene related peptide stimulates differentiation of neonatal rat myogenic cultures. Growth Regul 1993;3:245–8.
[14] Ody M, Thiévent A, Millet M, Connat JL. Postnatal development of the rat portal vein: correlation with occurence of peptidergic innervation. Cell Tissue Res 1993;272:303–14.
[15] Sasaki Y, Hayashi N, Kasahara A, Matsuda H, Fusamoto H, Sato N, et al. Calcitonin gene-related peptide in the hepatic and splanchnic vascular systems of the rat. Hepatology 1986;6:676–81.
[16] Schaeffer C, Thomassin L, Rochette L, Connat JL. Apoptosis induced in vascular smooth muscle cells by an oxidative stress is partly prevented by a pretreatment with CGRP. Ann N Y Acad Sci 2003;1010:733–7.
[17] Schaeffer C, Vandroux D, Thomassin L, Athias P, Rochette L, Connat JL. Calcitonin gene-related peptide (CGRP) partly protects cultured smooth muscle cells from apoptosis induced by an oxidative stress via activation of ERK1/2 MAPK. Biochim Biophys Acta 2003;1643:65–73.
[18] Shimizu N, Kamel-Reid S, Zak R. Expression of actin mRNAs in denervated chicken skeletal muscle. Dev Biol 1988;128:435–40.
[19] Thiévent A, Connat JL. Calcitonin gene-related peptide innervation and binding sites in rat aorta during development. J Auton Nerv Syst 1993;44:233–41.
[20] Thiévent A, Connat JL. Cytoskeletal features in longitudinal and circular smooth muscle during development of the rat portal vein. Cell Tissue Res 1995;279:199–208.
[21] Toyofuku T, Hoffman JR, Zak R, Carlson BM. Expression of alpha-cardiac and alpha-skeletal actin mRNAs in relation to innervation in regenerating and non-regenerating rat skeletal muscles. Dev Dyn 1992;193:332–9.