Immunohistochemical localization of a gap junction protein (connexin 43) in the muscularis externa of murine, canine, and human intestine

H.B. Mikkelsen¹, J.D. Huizinga², L. Thuneberg¹, J.J. Rumessen¹

¹ Department of Anatomy, The Panum Institute, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen, Denmark
² Intestinal Disease Research Unit, McMaster University, Hamilton, Ontario, Canada

Received: 8 January 1993 / Accepted: 18 March 1993

Abstract. Electron-microscopic studies have revealed a heterogeneous distribution of gap junctions in the muscularis externa of mammalian intestines. This heterogeneity is observed at four different levels: among species; between small and large intestines; between longitudinal and circular muscle layers; and between subdivisions of the circular muscle layer. We correlated results obtained with two immunomethods, using an antibody to the known gap-junctional protein (connexin 43) with ultrastructural findings, and further evaluated the respective sensitivity of these two approaches. For comparative reasons we also included the vascular smooth muscle of coronary arteries into our study. Two versions of the immunotechnique (peroxidase-antiperoxidase and fluorescence methods) were applied to frozen sections of murine, canine, and human small and large intestines, as well as to pig coronary artery. In the small intestine of all three species a very strong reactivity marked the outer main division of the circular muscle layer, while the longitudinal muscle layer as well as the inner thin division of the circular muscle layer were negative. In murine and human colon both muscle layers were negative, while in canine colon the border layer between the circular muscle and the submucosa reacted strongly, and scattered activity was found in the portion of the circular muscle layer (one tenth of its thickness) closest to the submucosa. The remainder of the circular muscle layer and the entire longitudinal muscle layer were negative in the canine colon. In the coronary artery we could not confirm the positive, specific labeling reported by other investigators (l.c.). In conclusion, we found close correlations at all four above-mentioned levels in the distribution of gap junctions in the gut musculature, as determined by binding of anti-connexin 43 in comparison to conventional ultrastructural studies. Since no significant immunostaining was found in (i) the outer border of the circular muscle layer of the canine colon and (ii) the border layer between the submucosa and the circular muscle layer of human colon, where rare gap junctions have been identified at the ultrastructural level, we conclude that the electron-microscopic analysis is the more sensitive of the two methods.

Key words: Connexin 43 – Gap junction – Muscularis externa – Intestine – Coronary artery – Immunohistochemistry – Mouse – Dog – Man – Pig

Introduction

The observation that the generation of action potentials in intestinal smooth muscle depends on the electrical activity of different cell types emphasizes the importance of a detailed knowledge of cellular coupling in this system. The muscularis externa of the mammalian intestine consists of a longitudinal and circular smooth muscle layer. In the dog colon, slow-wave-type action potentials are generated in the border region between the circular muscle layer and the submucosa where a network of interstitial cells of Cajal is situated (Berezin et al. 1988). This is an area of high gap junction density, possibly because of a particular requirement for metabolic coupling (Huizinga et al. 1991). From here, active propagation occurs into the circular musculature which displays a very low density of ultrastructurally detectable gap junctions. However, a low density of gap junctions is sufficient for efficient electrical coupling when measured as electrotonic coupling (Huizinga and Chow 1988; Huizinga et al. 1988) and synchronization of electrical activities (Smith et al. 1987). In the human colon, the cellular origin of action potentials is not known in detail, but it could be similar to that in the dog colon (Huizinga et al. 1985; Faussone-Pellegrini et al. 1990a, b; Rumessen et al. 1993). The longitudinal muscle layer of the dog and human colon differ in important respects from the circular muscle layer. It does not generate the slow-wave-type action potentials, but instead spike-like action potentials occur at irregular frequency, often in synchrono-
nized bursts (Chow and Huizinga 1987). Despite such evidence for electrical coupling of longitudinal muscle cells (Liu et al. 1993), no gap junctions have been found by means of electron microscopy (Berezin et al. 1990).

In the small intestine, slow-wave-type action potentials originate in the myenteric plexus (Auerbach's plexus), where a network of interstitial cells of Cajal is found (Hara et al. 1986; Thuneberg 1982, 1989). It is believed that from this site active propagation takes place into both muscle layers. The circular muscle layer has an abundance of gap junctions, based on electron-microscopic data from the dog (Dewey and Barr 1962; Henderson et al. 1971; Daniel et al. 1972), mouse (Rumessen et al. 1982; Thuneberg 1982) and human (Rumessen and Thuneberg 1991; Rumessen et al. 1992) with the exception of the inner, thin circular muscle layer. In contrast, in the longitudinal muscle layer no gap junctions were found in the dog, mouse and guinea pig, and in the rabbit and cat only small and very few gap junctions were reported (Taylor et al. 1977; Gabella and Blundell 1979, 1981).

Although gap junctions are probably not essential for electrical communication (Daniel et al. 1976; Mann and Sperelakis 1979), it is common speculation that gap junctions are probably always present but not recognized by conventional electron microscopy (Garfield et al. 1992). It is therefore interesting that a recent report claimed that immunostaining with anti-connexin43 is capable of demonstrating gap junctions in the smooth muscle of pig coronary artery where they were not found by ultrastructural analysis (Bény and Connat 1992).

The aim of the present study was to investigate whether immunohistochemical methods for localization of connexin43 protein are more sensitive than electron microscopy in detecting gap junctions. An attempt is made to detect connexin43 protein using immunohistochemistry in several smooth muscle tissues of the gut, at sites where gap junctions have been reported to be absent by use of electron-microscopic techniques. Furthermore, we re-evaluate immunohistochemical results on the pig coronary artery where gap junctions were demonstrated by use of immunohistochemistry (Bény and Connat 1992) but not by electron microscopy.

Materials and methods

Materials

Small tissue samples including the entire thickness from duodenum, jejunum, ileum and colon were used in the present study. Rat and pig heart were included as positive controls. Two mice (20 g), and 2 rats (100 g) were killed by cervical dislocation. Five dogs were used, 3 of which were killed by intracardial injection of KCl. Prior to sacrifice and while receiving halothane anesthesia, 2 of the dogs had been treated with tumor necrotic factor for 7 h and 1 dog had been treated with an inhibitor of nitric oxide synthase, for unrelated reasons. Two dogs were not subjected to any medication and were killed by an overdose of pentobarbital (100 mg/kg). Freshly resected, unaffected specimens were obtained from 4 human patients undergoing surgery for carcinoma of the pancreas, colon or rectum, and from one patient with carcinoma of the lar-

jejum. Samples of the colon included the ascending and descending portions. The anterior descending ramus of the left coronary artery and samples of heart tissue from two pigs were used for comparative purposes. All animals have been treated according to the Danish requirements for protection of animals. Concerning human material, the study has been approved by the ethical committee of Copenhagen.

Cryotome sections (8 µm) were prepared from unfixed tissue blocks which had been quick-frozen in isopentane cooled to −150 °C with liquid nitrogen. Sections were fixed for 4 min in ice-cold acetone.

Immunohistochemistry

To demonstrate connexin43 (gap junction protein) the sections were covered with solutions of swine serum (X90), Dako) 1:5 for 30 min. Experimental sections were then incubated overnight at +4 °C, the last hour at room temperature, with dilutions of rabbit anti connexin43 1:1000–1:3000 (Beyer et al. 1989; Garfield and Hertzberg 1990; Sakai et al. 1992). Control sections were incubated with rabbit serum (X902, Dako), rabbit IgG (X903, Dako) or rabbit anti-lysozyme (A099, Dako) 1:500–1:1000. The antiserum were diluted with phosphate-buffered saline (PBS) including 0.3% Triton X-114 and 0.1% serum albumin. In some experiments the connexin43 antibodies and control antibodies were absorbed with dog or human serum to reduce cross-reaction.

Immunoreactivity was visualized by means of the streptavidin-biotin/Texas red method; biotin-swine antirabbit (E353, Dako) diluted 1:40, was used as the second layer, followed by streptavidin-Texas red (RPN 1233, Amersham) diluted 1:50 as the third layer. In addition, the peroxidase-antiperoxidase (PAP) technique was employed (Mikkelsen et al. 1990, 1991; Mikkelsen and Rumessen 1992) using swine antirabbit IgG 1:50 (Z196, Dako) and peroxidase-antiperoxidase complex 1:50 (Z113, Dako). This type of immunoreaction was visualized by means of diaminobenzidine.

Results

Small intestine

In the outer, main subdivision of the circular muscle layer of the small intestine, strong punctate staining from the connexin43 antibody was observed in the mouse (Fig. 1), dog (Figs. 2, 3) and man (Figs. 4, 5), with the highest density of staining in dog small intestine, followed by mouse and human. In contrast, this staining

![Fig. 1. Longitudinal section of mouse jejunum. Strong immunoreactivity of connexin43 in the outer division of the circular muscle layer (OCM). The reaction is located at the cell membranes of the smooth muscle cells. The longitudinal muscle layer (LM) and the inner thin circular muscle layer (arrow) are negative, PAP technique. Bar: 20 µm. × 540](image)

![Fig. 2. Longitudinal section of canine jejunum. In the outer division of the circular muscle layer (OCM) immunoreactivity of connexin43 is regularly distributed. The longitudinal muscle layer (LM), the inner circular muscle layer (ICM), and the submucosa (SM) are negative, PAP technique. Bar: 50 µm. × 260](image)

![Fig. 3. Transverse section of canine duodenum. A regular punctate immunostaining is present in muscle cells in the outer circular muscle layer (OCM). The inner circular muscle layer (ICM) is negative, PAP technique. Bar: 50 µm. × 250](image)