# 消化管運動調節機構に与る非神経非筋細胞の細胞組織学的研究

課題番号　１０６７０５１４

平成１２年度　科学研究費補助金（基盤研究Ｃ２）研究成果報告書

平成１４年４月

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平成12年度科学研究費補助金（基盤研究Ｃ２）研究成果報告書

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2、研究課題　：消化管運動調節機構に与る非神経非筋細胞の細胞組織学的研究

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5、研究経費　：

平成１０年度：　９００　千円
平成１１年度：１０００　千円
平成１２年度：　８００　千円
計：２７００　千円

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招待講演

7、研究成果

（ア）研究の背景および目的：

Cajal の初の記載以来、歴史的論争を生んできた Interstitial cells of Cajal (ICC) の細胞学的同定に関しては、古典的染色法に代わり細胞膜上のc-*kit* receptor の検出が ICC の同定に用いられることが分かり、ICC に関わる研究は急速な展開を示し、消化管運動におけるICC のペースメーカー機能については、ほぼ確立された状態となった。
その一方、胃、小腸、大腸では、器官固有の運動を反映して、筋層の配列、神経叢の分布等に違いのあることは周知の事実であるが、ICC の存在様態についても固有の変異があり、これによる消化管各器官各組織層における 観察像の相違は、ICC の細胞学的問題が最近まで混乱していた要因の一つともなっていた。

ところで、ICC は、もともとCajal によって効果器（筋）と自律神経系末梢部とを結ぶ側副刺激伝達路として示唆されたものであるが、神経支配を受ける ICC の gap junction を介しての筋への伝達は、この洞察を証明するものであり、自律神経系末梢部における支配様式に関する新しい視点を示すものである。体性神経系における神経筋結合部との対比の上で曖昧な概念のまま捉えられていた臓性神経系支配領域における支配様式については、その意味で更めて見直す必要がある。非神経、非筋、間質性細胞の刺激伝達機能に関する研究は極く限られており、このような観点からも、ICC の解明は重要である。また、ペースメーカー機能達成の上で、ペースメーカー領域およびこれに続く特殊心筋線維における異種の gap junction 蛋白の役割が心臓では論じられているが、消化管においては明らかにされていない。

以上の問題点を背景として、本研究では、まず第一に、消化管各部各組織層における ICC の分布、神経支配、筋との結合、gap junction 蛋白のタイプ等について明らかにし、次いで、 ICC の器官、部位による細胞学的差異について、微細構造、発生学的由来、免疫組織学的性格、c-*kit* 依存度　などの観点から検索することを企図した。

（イ）研究実施計画と結果：

＊平成１０年度には、神経刺激伝達機能の観点から、ICC の構成する細胞性ネットワークの特性を知るため、同種細胞間（ICC－ICC、筋－筋）、異種細胞間（ICC－筋）における gap junction 蛋白 (connexin : Cx) の性質について免疫組織化学的ならびに電子顕微鏡的に検策することを計画した。その結果、ラット小腸深部筋神経叢では、周囲に位置

する輪走平滑筋細胞相互間、ICC－筋間およびICC－ICC 間の三者のgap junctionには Cx43 が観察されるのに対し、ICC－ICC 間に限って Cx45 の存在することが証明された。このことより、ICCの構成する細胞性ネットワークでは、神経からの信号を筋に伝達する均一なgap junctionによる回路とは別に、ICC 間に限定した信号伝播様式の存在する可能性について考察した。

＊平成１１年度には、消化管各部位、各組織層の違いによるICCの細胞学的特性を系統的に整理するため、従来の研究成果を補完する微細構造学的検索を行った。その結果、ラットおよびモルモットの胃、小腸、結腸に見られるICCは微細構造上、三型に分類された。これらの材料すべてを通して、筋層間神経叢部には線維芽細胞に類似したICC-AP が認められたが、小腸深部筋神経叢部の ICC-DMP と結腸筋層下神経叢部の ICC-SMP にはカヴェオラや明瞭な基底膜が観察され、平滑筋に似た特徴が見られた。また、胃および結腸輪走筋層内の ICC-CM では、連続した基底膜は欠くもののカヴェオラは観察されるなど、両者の中間的な特徴が見られた。 ICC-DMP、ICC-SMPは勿論、胃、結腸の ICC-CM においても、平滑筋と gap junction を形成し、神経終末と密接することから、神経信号の mediator として働くものと推定した。

＊平成１２年度には、消化管のNANC抑制性神経 として知られているNO ニューロンの ICC 支配の有無を検討するため、ラット小腸を材料として、NADPH-diaphorase 組織化学により検索した。またVIP についても免疫組織化学的観察を行った。その結果、小腸輪走筋層の全載伸展標本では、深部筋神経叢に沿ってNADPH-d 組織化学に陽性の強い反応が観察された。同様に処理した標本の電子顕微鏡観察では、ICC に密接してシナップス小胞を含む反応陽性の終末が観察された。また凍結切片によるVIP 免疫染色では、深部筋神経叢に一致して強い陽性反応が規則的に観察された。以上の観察から、ICC は NO ニューロンの運動支配を受け、筋とのgap junction を介して刺激を伝達するものと推定した。また、同一 ニューロンにおけるNO とVIPの混在の可能性についても考察した。

また、*c-kit* receptor の発現に欠陥のあるラット (Ws / Ws)、マウス (W / Wv)、及び その正常対照群を用いた免疫組織化学的および微細構造的検索から、これらの動物の消化管には、ICC とはべつに、gap junctions によって筋と連絡する、*c-kit* receptorには全く依存しない、線維芽細胞様細胞のあることを明らかにした。

8、発表論文

MICROSCOPY RESEARCH AND TECHNIQUE 47:267–285 (1999)

# Comparative Morphology of Interstitial Cells of Cajal: Ultrastructural Characterization

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*KEY WORDS* interstitial cells of Cajal; c-kit; ultrastructure; immunohistochemistry; digestive tract; rat; guinea-pig

*ABSTRACT* The shape, distribution, and ultrastructural features of interstitial cells of Cajal (ICC) of different tissue layers and organs of the rat and guinea-pig digestive tract were described and compared with the corresponding cells in other species including mice, dogs, and humans, as reported in the literature. By light microscopy, the best marker for ICC appeared to be immunoreactivity for c-Kit. Ultrastructurally, ICC were characterized by the presence of many mitochondria, bundles of intermediate filaments, and gap junctions, which linked ICC with each other. However, ICC were morphologically heterogeneous and had particular features, depending on their tissue and organ location and species. ICC in the deep muscular plexus of the small intestine and in the submuscular plexus of the colon were the most like smooth muscle cells, and had a distinct basal lamina and numerous caveolae. In contrast, ICC of Auerbach's plexus at all levels of the gastrointestinal tract were the least like smooth muscle cells. They most closely resembled unremarkable fibroblasts. ICC within the circular muscle layer were intermediate in form. In addition to the tissue specificity, some organ and species specificity could be distinguished. The structural differences between ICC may be determined by their microenvironment, including the effects of mechanical force, type of nerve supply, and spacial relationship with smooth muscle cells. *Microsc. Res. Tech. 47:267–285, 1999.* 

## INTRODUCTION

Among some early ultrastructural studies of interstitial cells of Cajal (ICC) (Richardson, 1958, 1960; Taxi, 1965), Imaizumi and Hama (1969) were the first to describe a special type of cell that was characterized by the presence of a basal lamina, gap junctions with smooth muscle cells, and close contacts with nerve varicosities in the love bird gizzard. Some years later, cells with similar features were also observed in mammals, i.e., the so-called "hybrid cells" of the dog intestine (Duchon et al., 1974), though they did not recognize that these cells corresponded to a type of ICC, at that time. These studies provided the first ultrastructural evidence that ICC were a unique type of cell that was cytologically distinct from fibroblasts, Schwann cells, and smooth muscle cells.

About one century ago, by using methylene blue and Golgi staining methods, Cajal described the intestinal interstitial cells that now bear his name (Cajal, 1911). However, modern research on ICC was not revived until the pacemaker hypothesis was proposed by Thuneberg (1982, 1989). Several authors then described a variety of cells in different tissue layers and regions of the digestive tract of various species (see review by Christensen, 1992). Different studies described remarkably different cytological features of these cells. At that time, it was uncertain whether the differences represented different profiles of the same cell type, morphological variations of the same cell type, or a mixture of different cell types, including cells that were not true ICC. In those studies, any cells or cytoplasmic processes of uncertain origin that were located within or between muscle layers were often regarded as ICC purely on account of their position. This tendency was especially true when the cells were adjacent to nerves and/or had gap junctions. Collection of such fragmented observations resulted in a chimeric image of ICC, which tended to further obscure their true nature. In fact, it was not immediately clear how to distinguish ICC from genuine fibroblasts or fibroblast-like cells, since the latter show a variety of features, including gap junctions, that are generally believed to be atypical for fibroblasts (Komuro, 1990).

Another important reason that the cytological definition and developmental origin of ICC was unsettled for a long time was the lack of a truly specific staining method for these cells. The supravital methylene blue and Golgi staining methods were notoriously capricious in the hands of different investigators. Indeed, the very existence of ICC as a distinct cell type was questioned by a report that postulated ICC were a chimera composed of glial cell bodies and neurites of enteric neurons that were stained simultaneously (Kobayashi et al., 1989). Nevertheless, many investigators developed new histochemical and immunohistochemical methods to identify ICC, including NADH diaphorase histochemistry (Xue et al., 1993), cholera toxin subunit b labelling (Anderson and Edwards, 1993), cyclic GMP immunoreactivity (Shuttleworth et al., 1993; Young et al., 1993),

Abbreviations used: AP: Auerbach's plexus; CM: circular muscle; DMP: deep muscular plexus; FL: fibroblast-like cells; ICC: interstitial cells of Cajal; LM: longitudinal muscle; SMP: submuscular plexus.

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Received 18 February 1999; accepted in revised form 27 May 1999

and nitric oxide synthase immunoreactivity (Xue et al., 1994). However, none of these methods was considered to be truly specific for ICC.

Therefore, it became apparent that it was essential to describe a clear set of cytological criteria for ICC. Furthermore, to avoid ambiguity, the whole shape of a given cell type as well as its relation to nerve and muscle should correspond closely to that originally described by Cajal. Immunohistochemical staining for c-Kit fit these criteria and became accepted as the best marker of ICC at the light microscopic level (Huizinga et al., 1995; Maeda et al., 1992; Ward et al., 1994). Studies using a combination of the zinc iodide-osmic acid method, c-Kit immunostaining, and fine structural observations all contributed to bridge the gap between traditional histological descriptions and more recent finding on ICC (Komuro and Zhou, 1996; Komuro et al., 1996). Cells of the guinea-pig small intestine depicted by the zinc iodide-osmic acid method (Figs. 2, 5) corresponded to c-Kit immunoreactive cells (Figs. 3, 6), which showed almost the same characteristics as ICC illustrated in Cajal's textbook (Figs. 1, 4; Cajal, 1911). These studies confirmed the use of c-Kit immunoreactivity as a specific marker for ICC, and also revealed that ICC were strongly stained by an anti-vimentin antibody (Komuro et al., 1996).

In spite of this progress, it remained unclear whether ICC were a single clearly defined class of cells. As pointed out by Christensen (1992), a variety of ultrastructural features was reported, and it was necessary to decide which features were common or specific for each type of ICC, and how ICC were related cytologically to fibroblasts or smooth muscle cells. Furthermore, it is clear that the basic normal cytology of ICC should be systematically described so that recently published studies of ICC in pathological conditions can be accurately interpreted (Hirota et al., 1998; Kenny et al., 1998; Kindblom et al., 1998; Vanderwinden et al., 1996).

Thus, the aims of this review are first to describe the distribution of c-Kit immunoreactive cells in different levels of the gastrointestinal tract, and then to focus on the ultrastructural features of ICC located in different tissue layers and regions of the rat and guinea-pig gastrointestinal tract. The aim of the last section of this review is to clarify which features are common to all types of ICC, and which features are specific for types of ICC in particular tissue regions, organs, or species. For historical and functional aspects of ICC see several excellent reviews (Christensen, 1992; Huizinga et al., 1997; Sanders, 1996; Thuneberg, 1989) and other articles in this issue.

Regarding terminology, the only cells that are termed ICC are those that have been confirmed as equivalent to the original description, or those cells that can be regarded as species variations of ICC. To avoid further confusion, other cells that have been claimed as being ICC without conclusive evidence, and cells in the interstitial space in a general sense, are described simply as "interstitial cells." The terminology of Thuneberg et al. (1995) is adopted to describe ICC at different locations: i.e., ICC-AP (Auerbach's plexus) located between the circular and longitudinal muscle layers; ICC-DMP (deep muscular plexus) located between the inner thin and outer thick sublayers of the circular smooth muscle of the small intestine; ICC-SMP (submuscular plexus) located at the submucosal border of the colonic circular muscle layer; ICC-CM located within the outer thick circular muscle layer; ICC-LM located within the longitudinal muscle layer. Fibroblast-like cells of each tissue layers are termed FL-AP, -DMP, -SMP, -CM, and -LM, respectively.

## DISTRIBUTION OF C-KIT IMMUNOREACTIVE CELLS

Our previous observations (Seki et al., 1998) showed that c-Kit immunoreactive cells were regularly located

Fig. 1. Part of a drawing by Cajal of ICC-AP of rabbit intestine stained with methylene blue (Cajal, 1911, Fig. 572). Note the close similarity of the cell shape(*) to cells in Figures 2 and 3.

Fig. 2. ICC-AP of the guinea-pig small intestine stained with the zinc-iodide osmium method. These ICC-AP are characterized by slender cytoplasmic processes with a dichotomous branching pattern. Note triangular knots at branching points (arrows). Cell bodies are usually located away from the tertiary nerve bundles of Auerbach's plexus. Reproduced from Komuro and Zhou (1996) with permission of the publisher. ×600.

Fig. 3. Cells with almost the same characteristics as those in Figure 1, demonstrated by immunohistochemical staining for c-Kit. Reproduced from Komuro and Zhou (1996) with permission of the publisher. ×650

Fig. 4. A drawing of ICC-CM within the circular muscle layer of the rabbit stained with methylene blue, adapted from Cajal (1911, Fig. 573). Note similarity of their shape to those in Figures 5 and 6.

Fig. 5. ICC-CM detached from nerve bundles of the guinea-pig intestine stained with the zinc iodide-osmium method. Note the terminal portions of the cytoplasmic processes are almost parallel with each other, and with the axis of the smooth muscle cells of the circular layer. Reproduced from Komuro et al. (1996) with permission of the publisher. ×430.

Fig. 6. ICC-CM of the guinea-pig small intestine stained immunohistochemically for c-Kit. ×550.

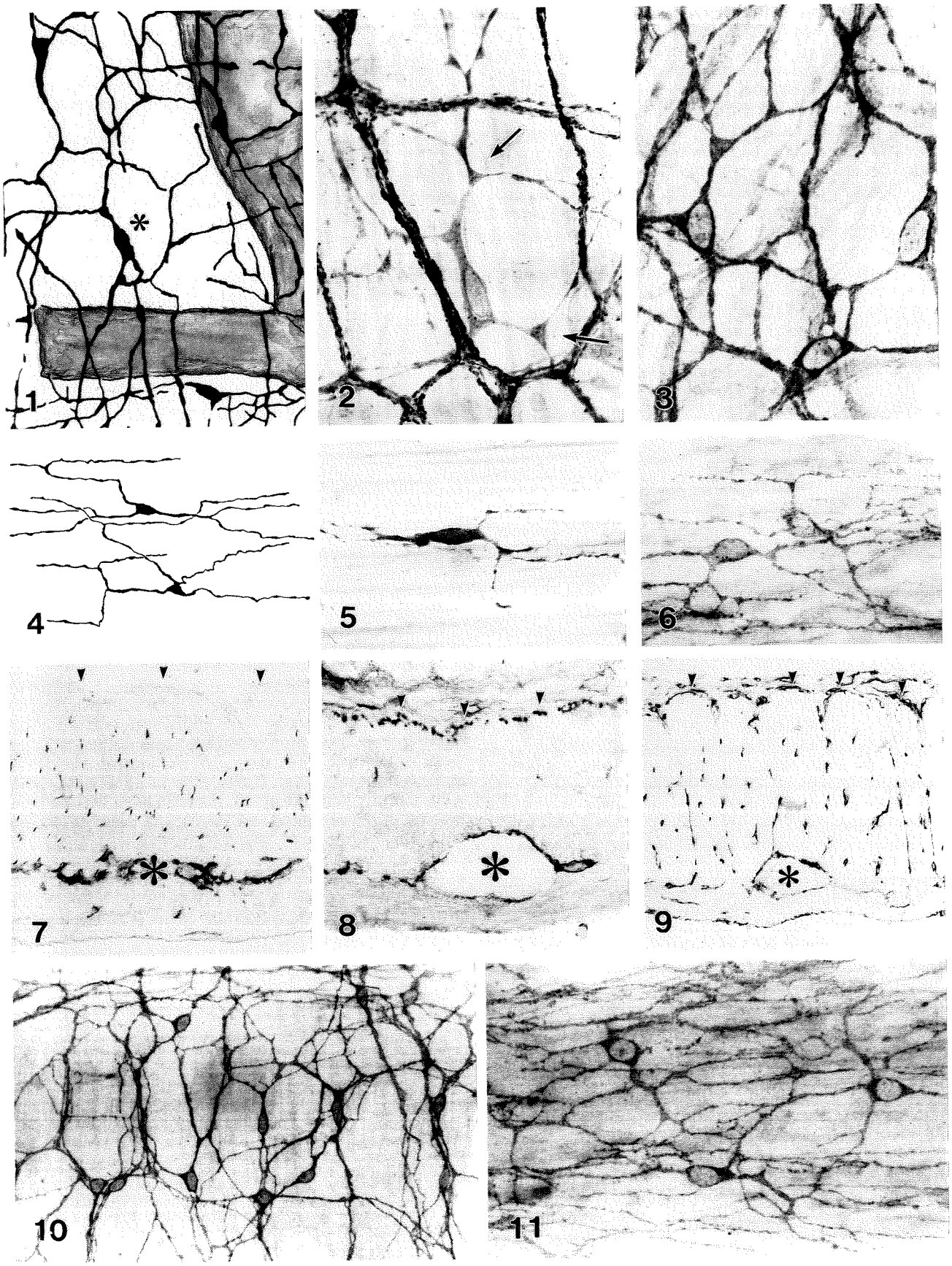
Fig. 7. Longitudinal section of the guinea-pig stomach stained immunohistochemically for c-Kit. The reaction deposits are seen in the myenteric region (*) and within the circular muscle layer excluding the third of the layer at the submucosal surface (arrowheads). Reproduced from Seki et al. (1998) with permission of the publisher. ×100.

Fig. 8. Longitudinal section of the guinea-pig small intestine stained immunohistochemically for c-Kit. Dense reaction deposits are localized in DMP region (arrowheads) and around the myenteric ganglion (*). Reproduced from Seki et al. (1998) with permission of the publisher. ×130.

Fig. 9. Longitudinal section of the guinea-pig colon stained immunohistochemically for c-Kit. Reaction deposits are distributed in SMP region (arrowheads), around a myenetric ganglion (*) and within the circular muscle layer. Reproduced from Seki et al. (1998) with permission of the publisher. ×100.

Fig. 10. Whole mount stretch preparation of the guinea-pig small intestine showing the cellular network of ICC-AP stained immunohistochemically for c-Kit. ×250.

Fig. 11. Cellular network of ICC-DMP of the guinea-pig small intestine stained immunohistochemically for c-Kit. Note nearly parallel arrangement of the cellular processes which reflects the axis of the smooth muscle cells of the circular layer. ×500.




Figs. 1–11.

in the myenteric region of all levels of digestive tract: stomach, small intestine, and colon of the guinea-pigs (Figs. 7–9). However, c-Kit immunoreactive cells in the muscle layers showed different distribution patterns depending on each of three regions. In the stomach, they were observed within the circular muscle layer, excluding about one third or one quarter of the circular layer from the submucosal interface. Additionally, a few immunoreactive cells were found in the longitudinal muscle layer. In the small intestine, c-Kit immunoreactive cells were clearly localized in the region corresponding to the DMP, and only a few immunoreactive cells were detected in the outer subdivision of the circular layer. No immunoreactivity was found in the longitudinal muscle layer. In the colon, c-Kit immunoreactive cells were observed at the interface between the submucosa and the circular muscle layer corresponding to the location of SMP. Sparse immunoreactivity was also seen in the circular muscle layer, often in the connective tissue septa that outline muscle bundles. Some c-Kit immunoreactivity was found in the longitudinal muscle layer.

Extensive cellular networks of c-Kit immunoreactive cells were clearly revealed in the tissue planes containing the AP and DMP in whole mount stretch preparations of the guinea-pig small intestine (Figs. 10, 11). Cells of the AP region were characterized by triangular or spindle cell bodies containing an elongated nucleus and usually with two to five slender primary cytoplasmic processes that can branch further (Fig.10). The processes interconnected with each other to form a dense network. A peculiar feature of these cells was that they formed triangular knots at every branching point of processes (Fig. 3; Komuro and Zhou, 1996). Their cell bodies and processes tended to orientate nearly parallel to the axis of either the circular or longitudinal muscle layer.

The network of c-Kit immunoreactive cells in the DMP region (Fig. 11) was slightly looser than that of the AP region. It showed almost the same pattern as the nerve network in the DMP, i.e., mainly parallel to the axes of muscle cells in the circular layer. The c-Kit immunoreactive cells in this region often had more rounded cell bodies and two to five primary processes (Fig.11) that also branched like those of ICC-AP. Terminal portions of their processes often extended along the axes of muscle cells of the circular layer. Similar cellular networks of ICC were also observed in specimens from the oesophagus to colon of the guinea-pigs (Burns et al., 1997). Different degrees of connection by gap junctions of these ICC were suggested by immunohistochemistry for connexin 43 (Seki et al., 1998).

## ULTRASTRUCTURAL FEATURES OF ICC IN RATS

The availability of Ws/Ws mutant rats, deficient in c-*kit*, has permitted an analysis of the function of interstitial cells of Cajal in rats (Horiguchi and Komuro, 1998) that is currently not possible in guinea-pigs. This mutation further confirms the identity of selected types of interstitial cells and c-Kit immunoreactive cells. Thus, the ultrastructural features of ICC are first described for rats, and then for guinea-pigs.

### Stomach

In the stomach of normal rats, there was no nerve plexus corresponding to either the DMP of the small intestine or the SMP of the colon, and thus a special type of ICC was not found around the most inner region of the circular muscle layer. Instead, ICC were observed in close association with small nerve bundles within the circular muscle layer (ICC-CM) and in the myenteric region (ICC-AP). A similar situation was found for c-Kit immunoreactivity in guinea-pig specimens (Fig. 7).

ICC-CM were frequently observed within the circular muscle layer of the pylorus and corpus of the stomach. They were characterized by electron-dense cytoplasm containing oval nuclei with dense heterochromatin at the periphery, and abundant mitochondria (Figs. 12–14). Golgi apparatus, rough and smooth endoplasmic reticulum were also observed. Cilia, basal bodies, and lipid droplets were occasionally seen. Intermediate filaments were abundant, particularly in the cytoplasmic processes extending in various directions (Ishikawa et al., 1997). A basal lamina could not be clearly identified, but many caveolae were observed on the cell membranes (Fig. 12). The most prominent feature of these cells was their arrangement in a cellular network, interconnecting with each other and with neighbouring smooth muscle cells via many large gap junctions. The possibility of cellular communication via gap junctions between such a series of cells was strongly suggested by a study of serial sections (Figs. 14a,b). ICC-CM also showed close contacts with nerve terminals containing many synaptic vesicles (Fig. 12).

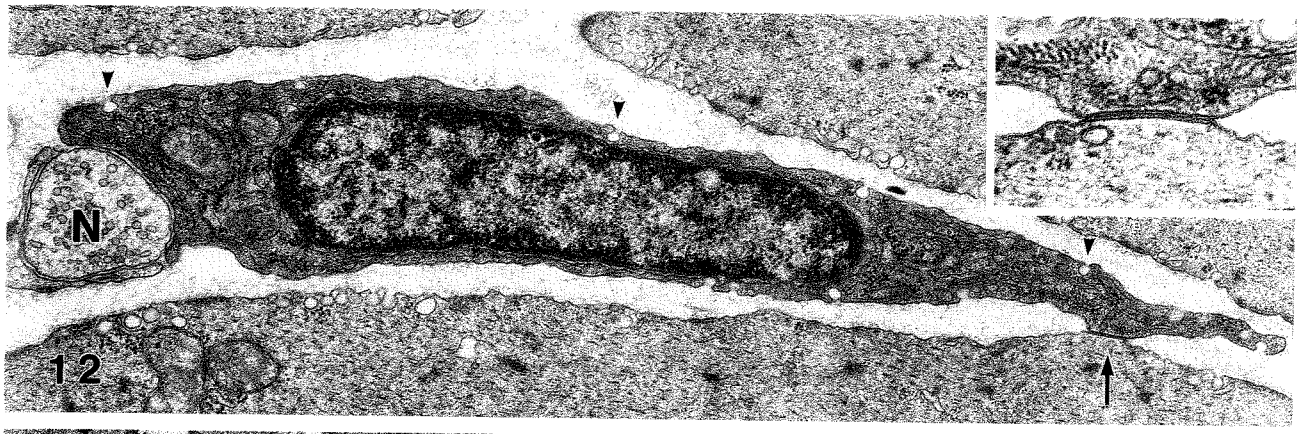
Other simple interstitial cells or fibroblast-like cells (FL-CM) were also observed in the vicinity of nerve bundles in the circular muscle layer (Fig. 15). Their cytoplasm was usually less electron-dense than that of adjacent smooth muscle cells. These cells had typical fibroblast-like features, such as well-developed rough endoplasmic reticulum and Golgi apparatus, but they were unusual in that they formed gap junctions with smooth muscle cells. These gap junctions, however, were usually small (Fig. 15, inset). There was no basal lamina. Evidence as to the identity of these cells came

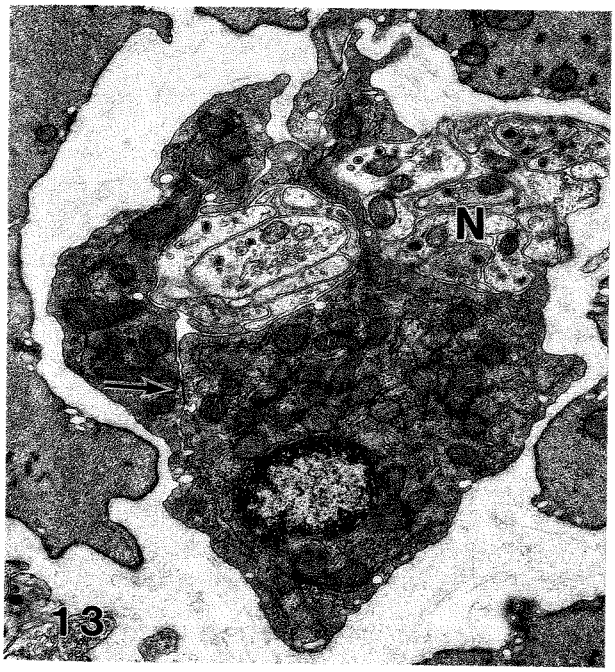
Fig. 12. ICC-CM in the rat stomach (antrum) characterized by electron-dense cytoplasm, caveolae (arrowheads), gap junction with smooth muscle (arrow) and close contact with nerve terminal (N). ×21000. **Inset:** Higher magnification of the gap junction indicated by arrow. Reproduced from Ishikawa et al. (1997) with permission of the publisher. ×40,000.

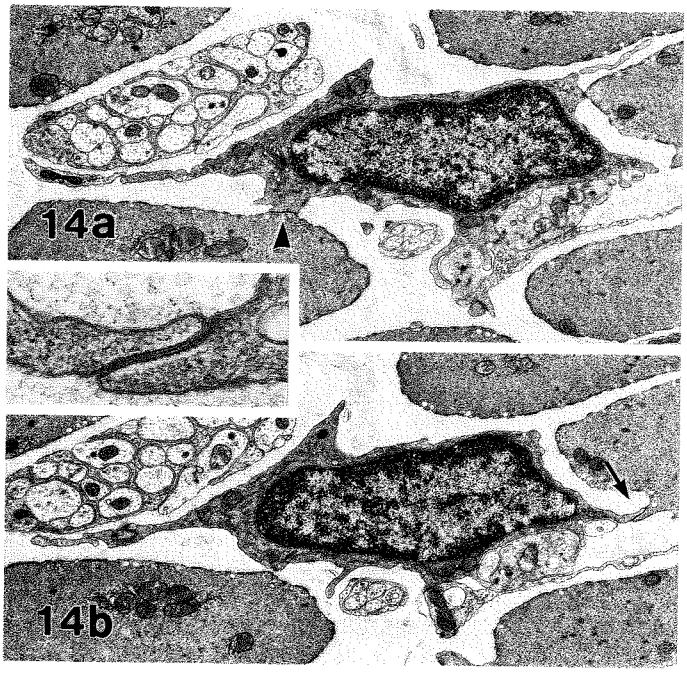
Fig. 13. Cross section of ICC-CM in the rat corpus containing abundant mitochondria, which is closely associated with nerve bundles (N). A gap junction is observed between the same type of cells (arrow). Reproduced from Ishikawa et al. (1997) with permission of the publisher. ×13,000.

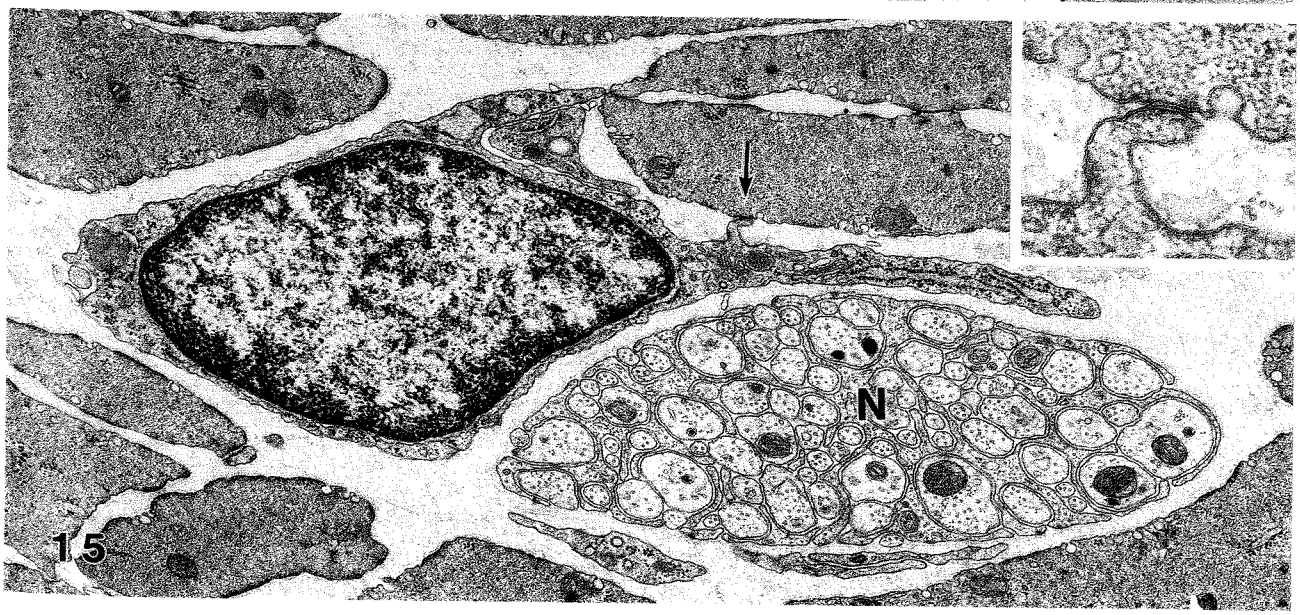
Fig. 14. **a,b:** Serial sections of ICC-CM of the rat stomach showing frequent connections with smooth muscle cells via gap junctions (arrowhead and arrow). ×10,000. **Inset:** Higher magnification of the gap junction indicated by the arrow. (Courtesy of Dr. Ishikawa, K.Waseda University.) ×52,000.

Fig. 15. FL-CM of stomach of Ws/Ws mutant rat, located near a nerve bundle (N). Note the less electron-dense cytoplasm and the cistern of rough endoplasmic reticulum even in the slender process, which forms a small gap junction with a muscle cell (arrow). ×17,000. **Inset:** Higher magnification of the gap junction indicated by the arrow. (Courtesy of Dr. Ishikawa, K.Waseda University.) ×80,000.




Figs. 12–15.

from ultrastructural observations of tissues in Ws/Ws rats. ICC-CM were absent in Ws/Ws rats, but FL-CM could be observed without any substantial changes in the mutant rats (Fig. 15), suggesting that FL-CM were not true ICC.

ICC-AP and FL-AP were distinguished from each other by differences in cytoplasmic electron density, richness of mitochondria, and development of rough endoplasmic reticulum. Similar features were found in the colon, as described below (see Figs. 27, 28).

### Small Intestine

ICC in the small intestine were observed in association with two well-developed nerve plexuses, the DMP and AP, but interstitial cell components were rare within the subdivisions of the outer circular muscle layer.

ICC-DMP were elongated with the same cell orientation as the circular muscle cells (Komuro and Seki, 1995). Their nuclei were oval and had smooth contours, unlike those of the neighbouring smooth muscle cells which had many deep indentations along their long axes. Their cytoplasm was usually less electron-dense than that of the smooth muscle cells (Fig. 16). Golgi apparatus and rough endoplasmic reticulum were mainly located in the perinuclear regions. Abundant mitochondria were observed throughout the perinuclear cytoplasm and in cell processes. Microtubules, thin filaments, and intermediate filaments were abundant in the processes. A basal lamina, caveolae, and subsurface cisterns were observed along the cell membranes (Fig. 17, inset). These cells had many large gap junctions between each other (Fig. 16) and with smooth muscle cells of the outer circular layer. Their gap junctions with muscle cells of the inner layer were also observed occasionally (Fig. 18). These ICC were frequently contacted by nerve varicosities containing accumulations of synaptic vesicles (Horiguchi and Komuro, 1998; Komuro and Seki, 1995; Seki and Komuro, 1998). Three-dimensional examination of serial ultrathin sections indicated that the ICC-DMP could be classified into two subtypes on the basis of their cytoplasmic features (Fig. 17; Seki and Komuro, 1998).

FL-DMP were also observed in this region (Fig. 19). They had close contacts with nerve varicosities and formed small gap junctions with smooth muscle cells (Horiguchi and Komuro, 1998; Komuro and Seki, 1995). Basal lamina and caveolae were absent.

ICC-AP were characterized by less electron-dense cytoplasm and abundant mitochondria (Fig. 20), but unlike ICC-DMP, they had no basal lamina or caveolae. Intermediate filaments and thin filaments were conspicuous in their thin processes. ICC-AP formed large gap junctions with each other mainly along the length of overlapping processes (Fig. 20, inset). In one case, a part of a slender cytoplasmic process, probably originating from an ICC, was observed in close contact with a varicosity of the myenteric ganglion, and another part of the same process was connected to a muscle cell via a gap junction, suggesting a possible route for cell communication (Fig. 21; Komuro, 1989).

FL-AP were characterized by fibroblast-like features, well-developed rough endoplasmic reticulum and Golgi apparatus, and an absence of basal lamina and caveolae (Fig. 22). FL-AP formed small gap junctions with smooth muscle cells of both circular and longitudinal layers (Horiguchi and Komuro, 1998; Komuro, 1989). Their gap junctions with smooth muscle cells were often observed in comparison of those by ICC-AP.

Immunoreactivity for c-Kit was detected in control wild-type rats (Fig. 23), but not in Ws/Ws mutant rats (Fig. 24). Normal-looking FL-AP (Fig. 22), FL-DMP, and ICC-DMP were found, while ICC-AP were not observed in Ws/Ws mutant rats (Horiguchi and Komuro, 1998).

### Colon

ICC of the rat colon were observed in association with the SMP, AP, and small nerve bundles within the circular muscle layer.

ICC-SMP were located at the interface between the submucosa and the circular muscle layer (Fig. 25). They were distinguished from fibroblasts of the submucosa by the presence of abundant mitochondria, a continuous basal lamina and many caveolae along their cell membranes. They were frequently connected to each other and to smooth muscle cells via gap junctions (Fig. 26, inset). Intermediate filaments were conspicuous in the cytoplasmic processes, which often had close contacts with nerve varicosities containing many synaptic vesicles. (Since the ultrastructural features of rat ICC-SMP were similar to those of the guinea-pig ICC-SMP, refer to Figures 40 and 41 for a higher magnification view of these structures.)

ICC-CM and FL-CM of the rat colon were characterized by very similar features to the corresponding cells in the stomach, as described above. The differences between ICC-CM and FL-CM are clearly illustrated in a micrograph of both cell types in one frame (Fig. 26).

ICC-AP were characterized by features similar to those of ICC-CM: electron-dense cytoplasm, the presence of many mitochondria, caveolae, and abundant intermediate filaments (Fig. 27). They formed gap junctions with each other, often at the tips of cytoplasmic processes (Fig. 26, inset), but to date, gap junctions with smooth muscle cells have not been identified.

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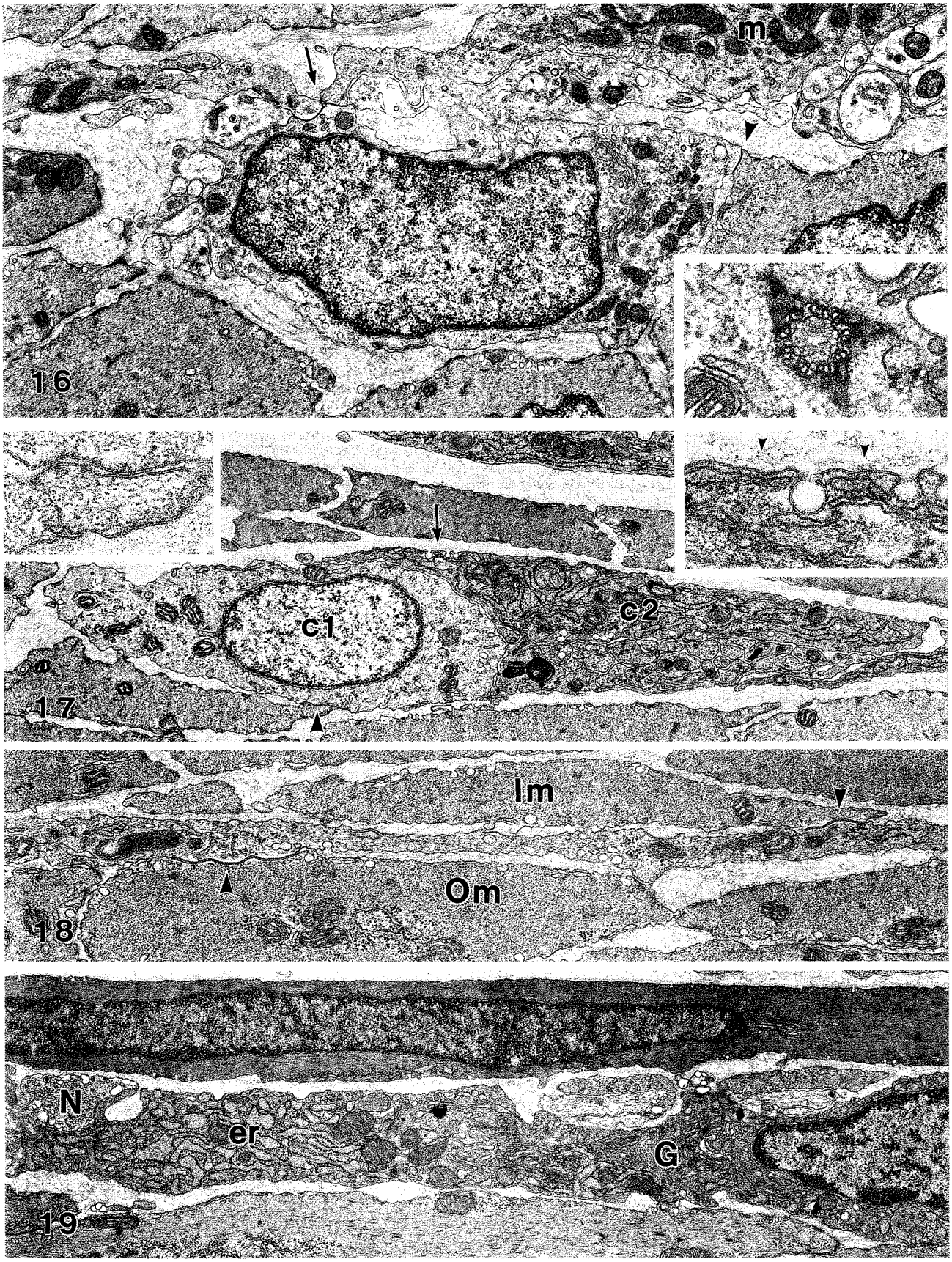
Fig. 16. Cross section of ICC-DMP of the rat small intestine characterized by less electron-dense cytoplasm, and gap junctions with smooth muscle cell (arrowhead) and with processes of the same type of cell (arrow) with many mitochondria (m). ×13,000. **Inset:** A basal body of the cilium observed in ICC-DMP of the rat. Reproduced from Komuro and Seki (1995) with permission of the publisher. ×37,000.

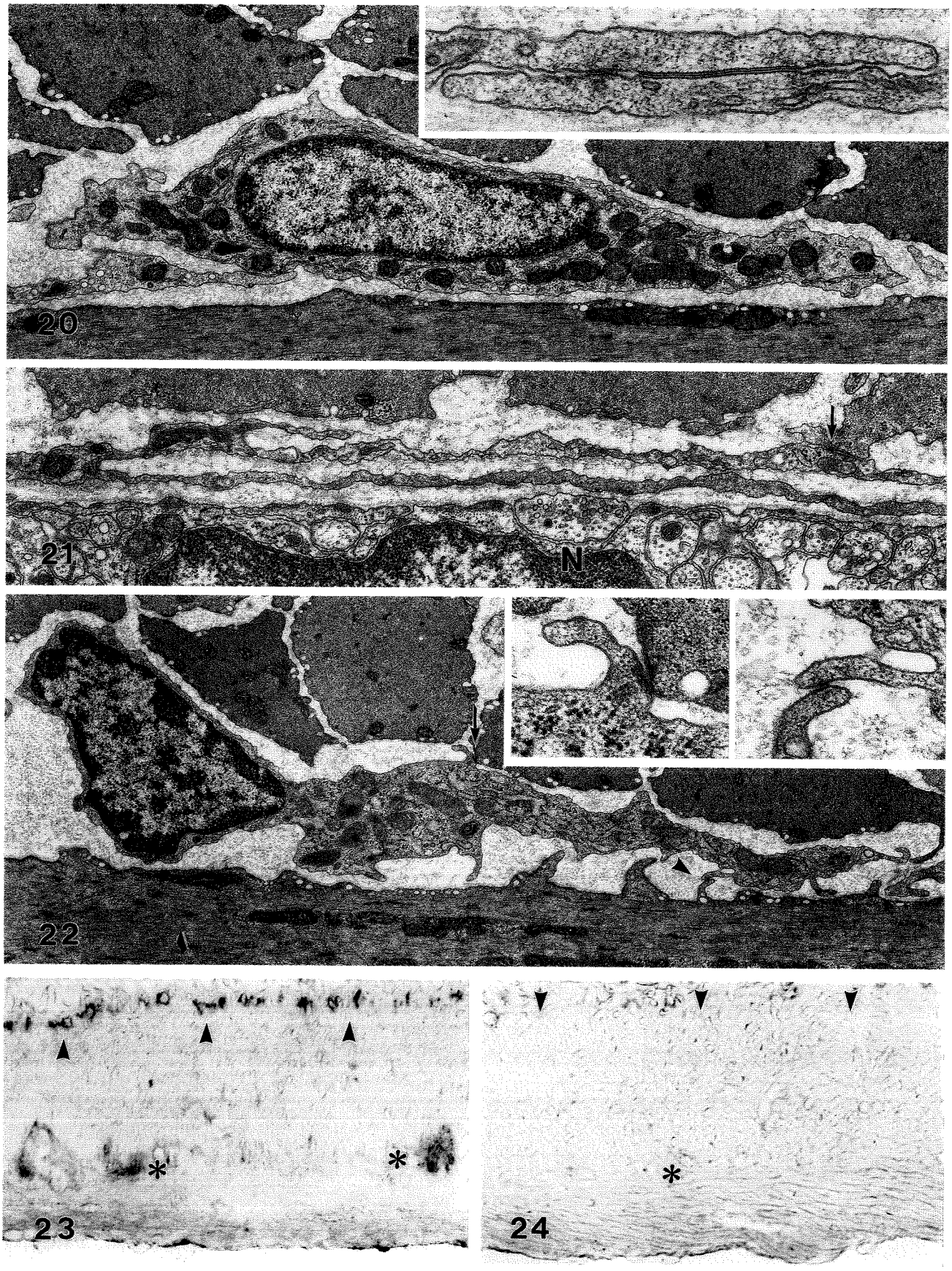
Fig. 17. Two different profiles of ICC-DMP. One (c1) has less electron-dense cytoplasm and the other (c2) has electron-dense cytoplasm and well-developed rough endoplasmic reticulum. A gap junction with the muscle cell (arrowhead) and caveolae (arrow) are observed for each cell, respectively. ×15,000. **Inset:** Higher magnification of the gap junction indicated by the arrowhead (left) and of the caveolae indicated by arrow (right). Note the presence of a basal lamina in the right inset (arrowheads). Reproduced from Seki and Komuro (1998) with permission of the publisher. ×6,000, ×90,000.

Fig. 18. Cytoplasmic process of ICC-DMP forming large gap junctions with muscle cells of the inner (Im) and the outer (Om) sublayers (arrowheads). Reproduced from Komuro and Seki (1995) with permission of the publisher. ×25,000.

Fig. 19. FL-DMP of the rat intestine located between the inner and outer sublayers of muscle cells. The cytoplasm contains well-developed rough endoplasmic reticulum (er) and Golgi apparatus (G). An axon varicosity (N) containing many synaptic vesicles is observed in the surface indentation. Reproduced from Komuro and Seki (1995) with permission of the publisher. ×12,500.




Figs. 16–19.




Figs. 20–24.

FL-AP with similar features to FL-CM were observed in the myenteric region (Fig. 28). Their gap junctions with the same type of cells (Fig. 28, inset) and with smooth muscle cells were observed.

## ULTRASTRUCTURAL FEATURES OF ICC IN GUINEA-PIGS

### Stomach

ICC-CM of guinea-pigs (Fig. 29) were located close to small nerve bundles within the circular muscle layer and showed features very similar to the corresponding cells in rats; electron-dense cytoplasm, abundant mitochondria, many caveolae and bundles of intermediate filaments, particularly rich in the cytoplasmic processes. A basal lamina was not clearly defined along the cell membranes. FL-CM, which resembled those in the rat stomach in many respects, were also identified.

ICC-AP of the guinea-pig stomach differed from those in the rat stomach and had less electron-dense cytoplasm. Thus, they could not be easily identified at lower magnifications (Fig. 30). However, they were distinguished from FL-AP by the presence of many caveolae (Fig. 31) and large gap junctions connecting the same type of cells (Fig. 32). FL-AP were identified by the absence of caveolae (Fig. 30), but they formed small gap junctions with smooth muscle cells, as in the small intestine and colon.

### Small Intestine

ICC-DMP of guinea-pigs were of two sub-types of cells with many large gap junctions, i.e., glycogen-rich cells (Fig. 33), and other (glycogen-deficient) cells rich in gap junctions (Fig. 34), in addition to the non-related fibroblast-like cells (Zhou and Komuro, 1992a,b). Both types of cell with many gap junctions contained numerous mitochondria and had a basal lamina and caveolae along the cell membranes. They were located close to nerve varicosities and formed connections with each other, and with smooth muscle cells. Subsurface cisterns and intermediate filaments were observed in both types of cells. Glycogen-rich cells had many slender processes extending in different directions (Fig. 33, inset), whereas gap junction-rich (glycogen-deficient) cells had elongated cell bodies with few processes, as revealed by serial ultrathin sections (Zhou and Komuro, 1992a,b). In this respect, the shape of glycogen rich cells was comparable to that of ICC-DMP observed in other species, as described below.

FL-DMP, forming small gap junctions with smooth muscle cells, were observed in close contact with nerve varicosities (Zhou and Komuro, 1992a,b).

ICC-AP of the guinea-pig small intestine (Fig. 35) were the most difficult to identify, because of the lack of distinctive features compared to ICC in other regions. However, they were distinguished from fibroblast-like cells by the presence of fairly well developed smooth endoplasmic reticulum, relatively numerous mitochondria, and flattened cisterns of rough endoplasmic reticulum, which were only rarely distended, unlike those in fibroblasts (Komuro and Zhou, 1996). There was no basal lamina. Caveolae were not observed. They formed gap junctions with each other along the length of their processes (Fig. 36).

In contrast, FL-AP often formed small gap junctions with each other (Fig. 37 and inset) and with smooth muscle cells of both circular and longitudinal layers (Komuro et al., 1996).

### Colon

ICC-SMP of guinea-pigs were similar to those of the rat and characterized by the presence of many mitochondria, caveolae, and a basal lamina (Figs. 39–41). Intermediate filaments were particularly rich in the small processes (Fig. 41). They were distinguished from fibroblast-like cells located near the SMP region (Fig. 38), since the latter often contained distended cisterns of rough endoplasmic reticulum even in thin cytoplasmic processes.

ICC-CM and FL-CM (Fig. 42), both of which showed features similar to the corresponding cells in the stomach, were also observed. In the colon, these cells tended to be found in the connective tissue septa between the muscle bundles, whereas, in contrast, in the stomach, these cells were found within muscle bundles.

ICC-AP and FL-AP with features similar to those of the small intestine were observed.

## CLASSIFICATION OF ICC AS A SPECIAL CELL TYPE

Table 1 summarizes the ultrastructural features of a wide variety of ICC from stomach to colon of human and animals (mouse, rat, guinea-pig, and dog). ICC described in the literature under various terms are designated to the categories described in the present review, taking into account their histological location. ICC of other species, including love-bird (Imaizumi and Hama, 1969), bat (Yamamoto, 1977), opossum (Daniel and Posey-Daniel, 1984), hedgehog (Faussone-Pellegrini, 1987b), and monkey (Wong et al., 1990), and ICC of the oesophagus (Daniel and Posey-Daniel, 1984; Faussone-Pellegrini and Cortesini, 1985) are listed separately.

Table 1 shows that although different types of ICC share certain common features with each other, depend-

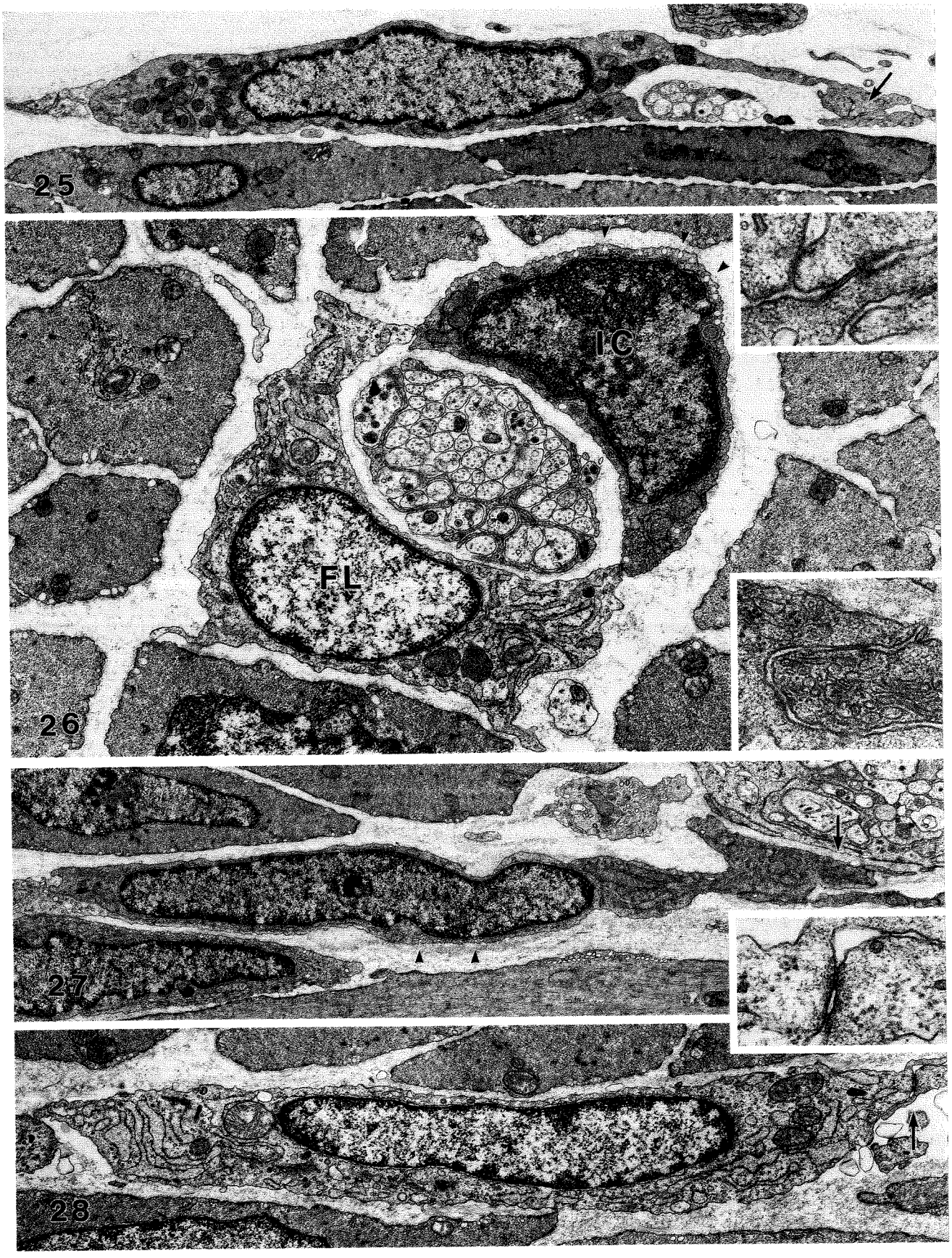
Fig. 20. ICC-AP of rat intestine characterized by many mitochondria and less electron-dense cytoplasm. ×10,000. **Inset:** A gap junction between slender cytoplasmic processes of ICC-AP. Note there is no basal lamina. Reproduced from Horiguchi and Komuro (1998) with permission of the publisher. ×96,000.

Fig. 21. Close relationship between a surface varicosity of the myenteric ganglion (N) and a slender process of ICC-AP, which, in turn, connects with a smooth muscle cell via a gap junction (arrow). Reproduced from Komuro (1989) with permission of the publisher. ×20,000.

Fig. 22. FL-AP of the myenteric region of Ws/Ws rat, which is characterized by well-developed rough endoplasmic reticulum. It forms small gap junctions with muscle cells of both circular and longitudinal layers (arrow and arrowhead, respectively). ×10,000. **Inset:** Higher magnification of the gap junctions indicated by the arrow and arrowhead. Reproduced from Horiguchi and Komuro (1998) with permission of the publisher. ×52,000.

Fig. 23. Immunoreactivity for c-Kit in DMP regions (arrowheads) and the myenteric plexus (*) in a wild-type rat intestine. Reproduced from Horiguchi and Komuro (1998) with permission of the publisher. ×320.

Fig. 24. No immunoreactivity for c-Kit observed in regions corresponding to DMP (arrowheads) and the myenteric plexus (*) in Ws/Ws rat intestine. Reproduced from Horiguchi and Komuro (1998) with permission of the publisher. ×320.




Figs. 25–28.

ing on their location and species, they can resemble either smooth muscle cells and/or fibroblasts ultrastructurally. Christensen (1992) suggested three ultrastructural features for distinguishing ICC from fibroblasts: the presence of many caveolae, the presence of at least a partial basal lamina, and the abundance of cytoplasmic filaments. Huizinga et al. (1997) proposed eight ultrastructural features as the gold standard for the identification of ICC: numerous mitochondria, large bundles of intermediate filaments, absence of thick filaments, presence of surface caveolae, variably developed basal lamina, well-developed rough and smooth endoplasmic reticulum, synapse-like contacts with nerves, and close appositions or gap junction contacts with smooth muscle cells. However, the only ultrastructural features common to the majority of ICC described in Table 1 are the presence of numerous mitochondria, abundant intermediate filaments, and frequent gap junctions, though a question of the presence or absence of a basal lamina is not always clearly defined because of the variability in appearance of a basal lamina caused by different preservation or staining. The exception to this finding is in human specimens, where gap junctions have been reported only rarely, probably because of the extreme difficulties in obtaining and preserving adequate specimens of human intestine. But they may be found more consistently in future studies. So, it is apparent that if all types of ICC are regarded as a single category of cells, they have no single distinct qualitative diagnostic feature. Because it is also obvious that most ICC do not represent typical fibroblasts or smooth muscle cells, it seems desirable to elucidate their cytological features more precisely, and perhaps quantitatively. In this way, it may be possible to reliably distinguish ICC from each other, and from smooth muscle cells and fibroblasts.

In spite of their morphological heterogeneity, all classes of ICC appear to share a common embryological origin from mesenchymal cells. This was clearly demonstrated in birds using chick-quail chimera experiments (Lecoin et al., 1996) and in mammals using transplants of embryonic intestinal segments (Young et al., 1996). A recent developmental study in the mouse intestine indicated that ICC-AP and longitudinal muscle cells are derived from the same mesenchymal progenitor cells expressing c-*kit* (Klüppel et al., 1998).

## TISSUE SPECIFICITY OF ICC

All studies indicate that ICC of different tissue layers are not ultrastructurally identical, but have their own characteristics. Detailed examination of each type of ICC may reveal whether all types of ICC merely represent morphological variations of the same cell type, or are separate categories of cells.

ICC-DMP and ICC-SMP of mice, rats, guinea-pigs, dogs, and humans have a basal lamina, caveolae, and gap junctions, as shown in Table 1. Because these ultrastructural features are generally believed to be features of smooth muscle cells, these interstitial cells were regarded as a special type of immature smooth muscle cell (Yamamoto, 1977). However, as already noted, these features of ICC do not indicate an immature condition, but represent a fully differentiated cell state (Thuneberg, 1989). Nevertheless, because of their ultrastructural features, many investigators have considered that they are modified smooth muscle cells (Christensen, 1992; Faussone-Pellegrini, 1987b, 1992; Komuro et al, 1996; Rumessen and Thuneberg, 1996; Sanders, 1996; Thuneberg, 1982, 1989; Torihashi et al., 1993; Yamamoto, 1977).

Among ICC of the same species, the ones that resemble smooth muscle cells most closely are ICC-DMP and ICC-SMP. The presence or absence of myosin filaments is clearly an important point in considering the cytological relationships between ICC and smooth muscle cells. However, there are some apparent discrepancies between different reports on this point. In immunohistochemical studies of the dog, myosin filaments were reported in ICC-DMP of the small intestine (Torihashi et al., 1993) and in ICC-SMP of the colon (Torihashi et al., 1994). However, other investigators did not observe such filaments in the same cells in the same species (Berezin et al., 1988; Daniel et al., 1998). Clearly, further observations seem necessary, since ICC profiles cannot always be identified for certain, particularly in histochemical preparations, and since myosin filaments have been reported by only one research group.

Another question concerning ICC-DMP is whether this type of ICC can be further subdivided, since two types of ICC-DMP have been distinguished in guinea-pigs and rats (Seki and Komuro, 1998; Zhou and Komuro, 1992a,b). Even if these cells merely represent different physiological states or maturation phases of the same type of ICC, this question should be clarified in future studies in other species.

ICC-CM of the stomach or colon from the rat (Ishikawa et al.,1997; Ishikawa and Komuro, 1998) and guinea-pig (Komuro, present observations) do not have a distinct basal lamina, but they are characterized by an electron-dense cytoplasm, numerous mitochondria, bundles of intermediate filaments, and numerous caveolae. The main distinguishing feature from fibroblast-like cells was the consistent presence of caveolae. However, an incomplete basal lamina was reported in the human small intestine (Rumessen et al., 1993a), in the human stomach (Fausonne-Pellegrini et al., 1989), in the gastro-oesophageal junction of the opposum

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Fig. 25. ICC-SMP of the rat colon located at the submucosal border of the circular muscle layer . It contains many mitochondria and forms a gap junction with the same type of cell at the tip of the cytoplasmic process (arrow). Reproduced from Ishikawa and Komuro (1998) with permission of the publisher. ×9,000 .

Fig. 26. ICC-CM (IC) and FL-CM (FL) of the rat colon. ICC-CM is characterized by electron-dense cytoplasm and caveolae (arrowheads), while FL-CM is characterized by less electron-dense cytoplasm and well developed rough endoplasmic reticulum. (Courtesy of Dr. Ishikawa, K. Waseda University.) ×14,000. **Inset:** Top shows higher magnification of the gap junction indicated by arrow in Figure 25. ×60,000. Bottom shows the gap junction indicated by arrow in Figure 27. ×50,000 .

Fig. 27. ICC-AP of the rat colon showing electron-dense cytoplasm, caveolae (arrowheads) and gap junction with the same type of cell (arrow). (Courtesy of Dr. Ishikawa, K. Waseda University.) ×9,000.

Fig. 28. FL-AP of the rat colon characterized by well-developed rough endoplasmic reticulum and less electron-dense cytoplasm. A small gap junction is observed at the tip of the cytoplasmic process (arrow). (Courtesy of Dr. Ishikawa, K. Waseda University.) ×10,000. **Inset:** Higher magnification of the gap junction indicated by the arrow. ×50,000.

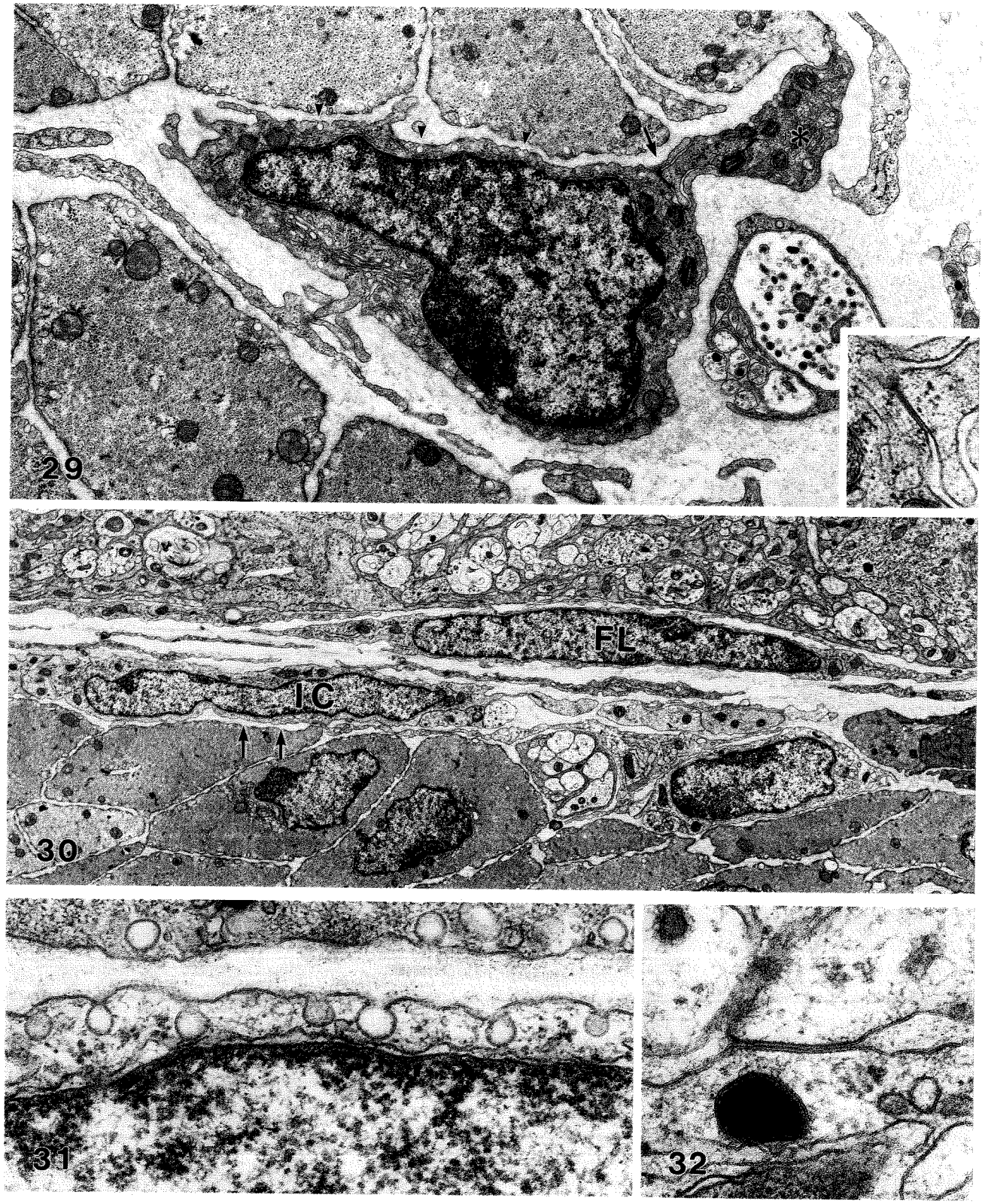



Fig. 29. ICC-CM of the guinea-pig stomach showing electron-dense cytoplasm, caveolae (arrowheads) and gap junction (arrow) with a process of the same type of cell that contains bundles of intermediate filaments (*). (Courtesy of Dr. Ishikawa, K.Waseda University.) ×14,000. **Inset:** Higher magnification of the gap junction indicated by the arrow. ×40,000.

Fig. 30. ICC-AP (IC) and FL-AP (FL) of the guinea-pig stomach located between the myenteric ganglion and a muscle layer. The former is, characterized by less electron-dense cytoplasm and the presence of caveolae (arrows). ×6,000.

Fig. 31. Higher magnification of the caveolae of ICC-AP indicated by the arrows in Figure 30. A basal lamina cannot be clearly identified. ×67,000.

Fig. 32. Gap junction observed between ICC-AP of the guinea-pig stomach. ×80,000.

(Daniel and Posey-Daniel, 1984), and also in the monkey (Wong et al., 1990). Therefore, it appears that ICC-CM are ultrastructurally intermediate between ICC-AP and ICC-DMP or ICC-SMP.

A distinct continuous basal lamina has not been observed around ICC-AP in any level of the digestive tract of any species. However, an ill-defined or incomplete basal lamina was reported for ICC-AP of the small intestine of mouse (Thuneberg, 1982), human (Rumessen and Thuneberg, 1991) and dog (Torihashi et al., 1993; Daniel et al., 1998), and in dog colon (Berezin et al., 1989; Torihashi et al., 1994). A basal lamina could not be detected on ICC-AP of the small intestine of rats and guinea-pigs (Horiguchi and Komuro, 1998; Komuro, 1989; Komuro and Zhou, 1996). A distinct row of caveolae were also not observed on these cells. Therefore, ICC-AP cannot be qualitatively distinguished from the fibroblast-like cells in this location by these features, but instead can be distinguished by the presence of many large gap junctions, abundant mitochondria, bundles of intermediate filaments, well-developed smooth and less well developed rough endoplasmic reticulum. ICC-AP appear to have the least muscle-like features of all types of ICC. The question of which species has a basal lamina on the cell membrane of ICC-AP remains to be investigated further (see note added in proof).

The different electron density of the cytoplasm of ICC has been reported in many studies. Significance of the electron density remains unclear, since differences could result from differences in fixation. However, if such differences can be reproduced under controlled conditions, they might be helpful to distinguish different cell types.

ICC-LM have only been briefly mentioned in a few studies (Komuro et al., 1996; Rumessen and Thuneberg, 1991; Thuneberg, 1982) and thus more attention should be focused on them.

## ORGAN SPECIFICITY OF ICC

One way to determine whether a particular type of ICC has a specific set of cytological features is simply to examine the analogous cells in different organs in the same species. ICC-AP of small intestines from the rats (Horiguchi and Komuro, 1998) and guinea-pigs (Komuro and Zhou, 1996) do not have a distinct row of caveolae, while those of stomach have many caveolae (Ishikawa, 1999; present observations). Daniel et al.(1998) reported that the cell bodies of ICC-AP of the dog small intestine were nearly devoid of caveolae, while Berezin et al. (1989) described that ICC-AP of the dog colon were identified by the presence of caveolae seen in all parts of the cell. In addition, ICC can be very similar to each other in different organs, such as ICC-CM of stomach and colon of both the rats and guinea-pigs.

## SPECIES SPECIFICITY

Numerous caveolae were reported in ICC-AP of the small intestine from mouse (Huizinga et al., 1995; Thuneberg, 1982; Ward et al., 1995) and human (Rumessen and Thuneberg, 1991), while they were rarely observed in rats (Horiguchi and Komuro, 1998; Komuro, 1989) and guinea-pigs (Komuro and Zhou, 1996). ICC-AP of the human small intestine were described as an ultrastructurally distinct cell type, characterized by more pronounced muscle-like features than in other species (Rumessen and Thuneberg, 1996).

Dense bodies were reported in ICC-DMP of the small intestine of dogs (Torihashi et al., 1993) and humans (Rumessen et al., 1992), but they were not observed in those of mouse (Rumessen et al., 1982; Thuneberg, 1982), rats (Komuro and Seki, 1995; Seki and Komuro, 1998) and guinea-pigs (Komuro and Zhou, 1996).

Numerous caveolae were observed in ICC-CM of the stomach and colon of both rats and guinea-pigs, but their absence was reported in ICC-CM of the dog colon (Torihashi et al., 1994).

## FUNCTIONAL SIGNIFICANCE OF ICC

Almost all reports in Table 1 indicate that ICC-DMP have a rich innervation and frequent contacts with smooth muscle cells via gap junctions. Although few gap junctions of ICC with muscle cells of the inner subdivision of the circular muscle have usually been reported in those studies, such gap junctions have been revealed in the rat intestine, in addition to those formed with the outer subdivision (Komuro and Seki, 1995). Therefore, a morphological generalization can be made that ICC-DMP are intercalated between nerves and smooth muscle cells. Furthermore, smooth muscle cells of both subdivisions of the circular layer have very close contacts with nerve varicosities in the guinea-pig small intestine, suggesting neuromuscular transmission (Zhou and Komuro, 1992b). Thus, it is quite likely that ICC-DMP can act as an accessory route for neuromuscular transmission, as originally suggested by Cajal (1911), though structural and functional importance may differ between the inner and outer subdivisions of the circular muscle.

The functional significance of gap junctions in ICC-DMP can be evaluated from evidence that the percentage of the total cell area occupied by gap junctions is 1.3% in rats (Seki and Komuro, 1998) and 4% in guinea-pigs. These values are about 6 and 20 times greater, respectively, than the corresponding percentage area (0. 2%) occupied by gap junctions on smooth muscle cells of the guinea-pig intestine (Gabella and Blundell, 1979). The gap junction proteins connexin 43 and connexin 45 were abundant in ICC-DMP of guinea-pigs, rats, and dogs (Nakamura et al., 1998; Seki et al., 1998). These highly developed gap junctions are consistent with the notion that the well-organized network of ICC-DMP acts as an impulse-conducting system analogous to that in the heart.

In general, ICC-SMP have similar features to those of ICC-DMP. However, the distribution pattern of connexin 43 is totally different between the colon and small intestine of the guinea-pig (Seki et al., 1998). A specialized pacemaker function has been proposed in the colon, with ICC-SMP primarily responsible for generating the slow waves, and ICC-AP act as secondary pacemaker cells (Sanders, 1996).

Since ICC-CM have gap junctions and close contacts with nerve varicosities, this type of ICC probably also functions in neurotransmission to smooth muscle cells. This assumption is compatible with the observation that in the guinea-pig, ICC-CM are abundant in the colon, where muscle cells are not connected by gap junctions, while they are very rare in the small intestine, where muscle cells are well coupled via gap junctions (Seki and Komuro, 1998).

Regarding the function of ICC-AP, it was reported that c-Kit receptor is necessary for the normal development of pacemaker cells (Maeda et al., 1992; Torihashi et al., 1995). Defects in c-*kit* result in a loss of a slow waves in the mouse intestine (Huizinga et al., 1995; Ward et al., 1994). Furthermore, ICC-AP are absent in Ws/Ws rats (Horiguchi and Komuro, 1998), which have a mutant c-Kit receptor and show abnormalities in gut movement and function of the pyloric sphincter (Isozaki et al., 1995). Therefore, it is very likely that ICC-AP act as pacemaker cells, also in the rat intestine.

## FUTURE QUESTIONS REGARDING FIBROBLAST-LIKE CELLS

A major issue is the inconsistent observation of gap junctions on fibroblast-like cells in a variety of specimens. In our observations of rats and guinea-pigs, FL with small gap junctions could also be found in every tissue layer of every organ where ICC are located. All other studies, except one, have not reported gap junctions on FL. The connective tissue cells interconnecting the circular and longitudinal muscle layers of the cat small intestine seem to be fibroblast-like cells rather than ICC (Taylor et al., 1977). These junctions are usually so small and often punctate that they might escape from observation or might not be categorized into gap junctions by many investigators. We identified these structures as gap junctions because of focal closeness of outer leaflets of two membranes, though they often appeared to make focal fusions of outer leaflets rather than with narrow gap. This discrepancy raises several important questions: (1) Are these gap junctions actually present on FL reported in other specimens? If so, (2) Is there any possibility that true ICC and FL were confused in previous studies, particularly when only part of the cytoplasmic processes was observed?

(3) Do FL participate in the regulation of gut contraction as do ICC? If so, (4) How do FL participate in the functions mediated by gap junctions with smooth muscle cells?

A noteworthy feature of FL is that they all show very similar ultrastructural characteristics irrespective of the tissue layer, organ, or species in which they are found. For example, FL of the myenteric region of the rat colon resemble those of the guinea-pig small intestine. These fibroblast-like cells of the myenteric region probably correspond to the flattened cells observed by scanning electron microscopy closely associated with the myenteric plexus in rats and guinea-pigs (Baluk and Gabella, 1987; Jessen and Thuneberg, 1992; Komuro, 1989). Clearly, further systematic, comprehensive studies are required to establish a basic knowledge of FL.

## CONCLUSIONS

It is now generally acknowledged that ICC of different locations have different cytological features. But this conclusion might have been reached a century ago. The familiar drawings of ICC-DMP, ICC-AP, and ICC-CM in Cajal's textbook of 1911 were obtained by staining with the Golgi method or with supravital methylene blue. This authoritative depiction led to the false notion that ICC could be stained by either of these two methods. In reality, however, these early histological methods could have given clues that these cells are different in nature. As Thuneberg (1982) already noticed, ICC-DMP had never been successfully stained, and ICC-AP were frequently stained together with nerves by the supravital methylene blue method.

Further evidence for the heterogeneity of ICC comes from the differences in their dependence on c-*kit* during development. ICC-DMP and -SMP were observed without substantial changes in Ws/Ws mutant rats, whereas ICC-AP and ICC-CM were absent in the stomach, small intestine, and colon (Horiguchi and Komuro, 1998; Ishikawa et al.,1997; Ishikawa and Komuro, 1998). Consistent with this finding, ICC-DMP were present in normal numbers in mice with the W/W$^v$ mutation in the c-Kit receptor (Malysz et al., 1996) and in Sl/Sl$^d$ mice, which have deficient synthesis of stem cell factor (SCF), the ligand for the c-Kit receptor (Ward et al., 1995). It has also been suggested that ICC differ in cyclic GMP immunoreactivity (Shuttleworth et al., 1993; Young et al., 1993). Therefore, it can be concluded that ICC are heterogeneous not only in their morphological features, but also in their physiological functions.

The present review shows that different types of ICC can express a wide range of phenotypes, ranging from those most similar to smooth muscle cells (ICC-DMP and ICC-SMP), to the least myoid features (ICC-AP), with ICC-CM expressing an intermediate character. The different morphological features of ICC can be determined by their microenvironment, including the

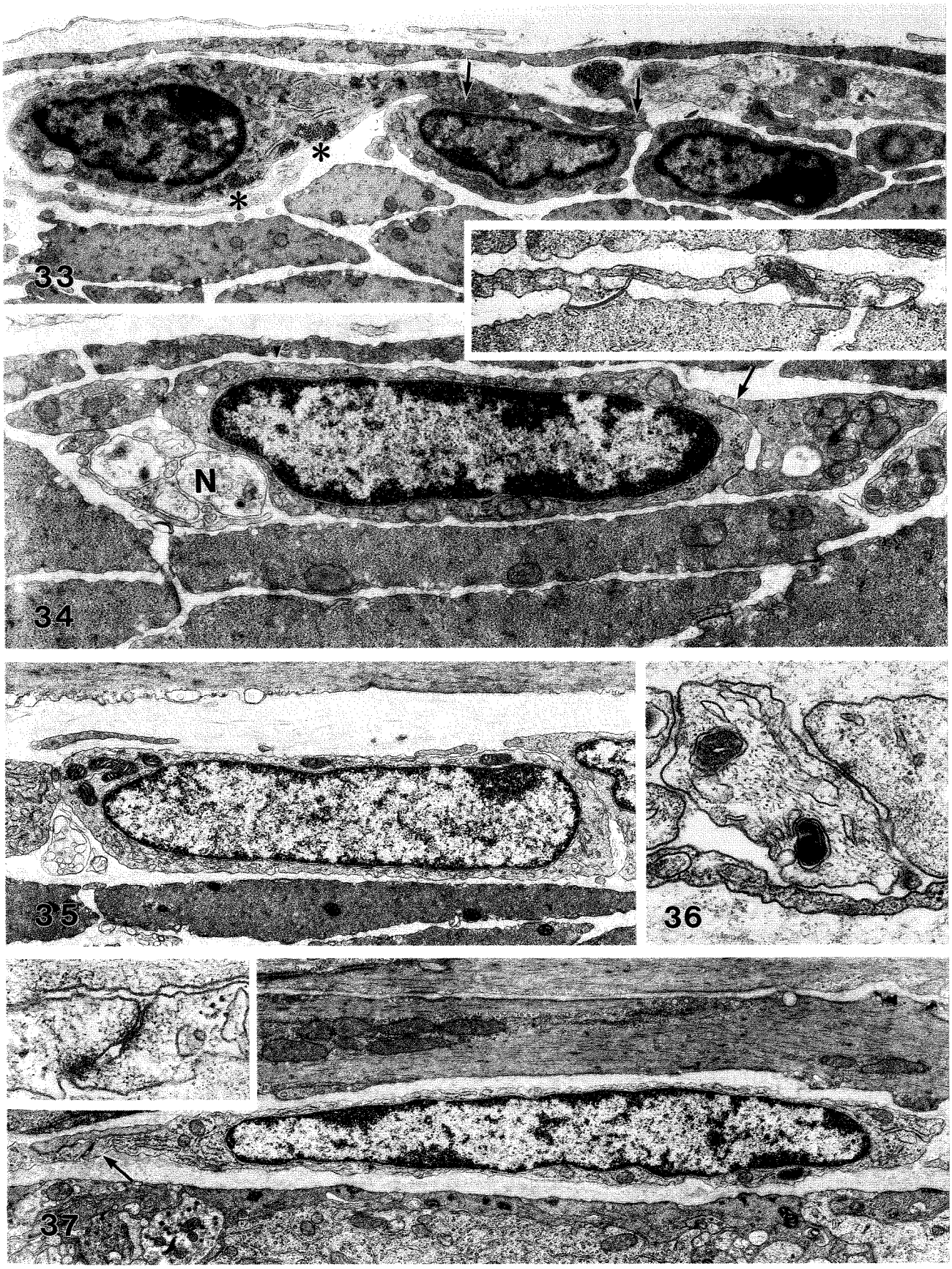
Fig. 33. A glycogen-rich cell of the DMP region of the guinea-pig small intestine, probably corresponding to ICC-DMP. Perinuclear cytoplasm contains many mitochondria and abundant glycogen granules (*), and the slender process forms gap junctions (arrows) with the same type of cells. ×10,000. Reproduced from Komuro et al. (1996). **Inset:** Higher magnification of the gap junction between a process of the glycogen-rich cell and the smooth muscle cells. Pale spaces of the process represent location of glycogen granules extracted during uranyl acetate fixation. ×33,000.

Fig. 34. Gap junction-rich (glycogen-deficient) cell which may be a subtype of ICC-DMP in the guinea-pig small intestine. Closely associated nerves (N) and gap junctions with the same type of cells are observed (arrow). Caveolae are also seen along the cell membrane (arrowhead). (Courtesy of Dr. D. Zhou) ×20,000.

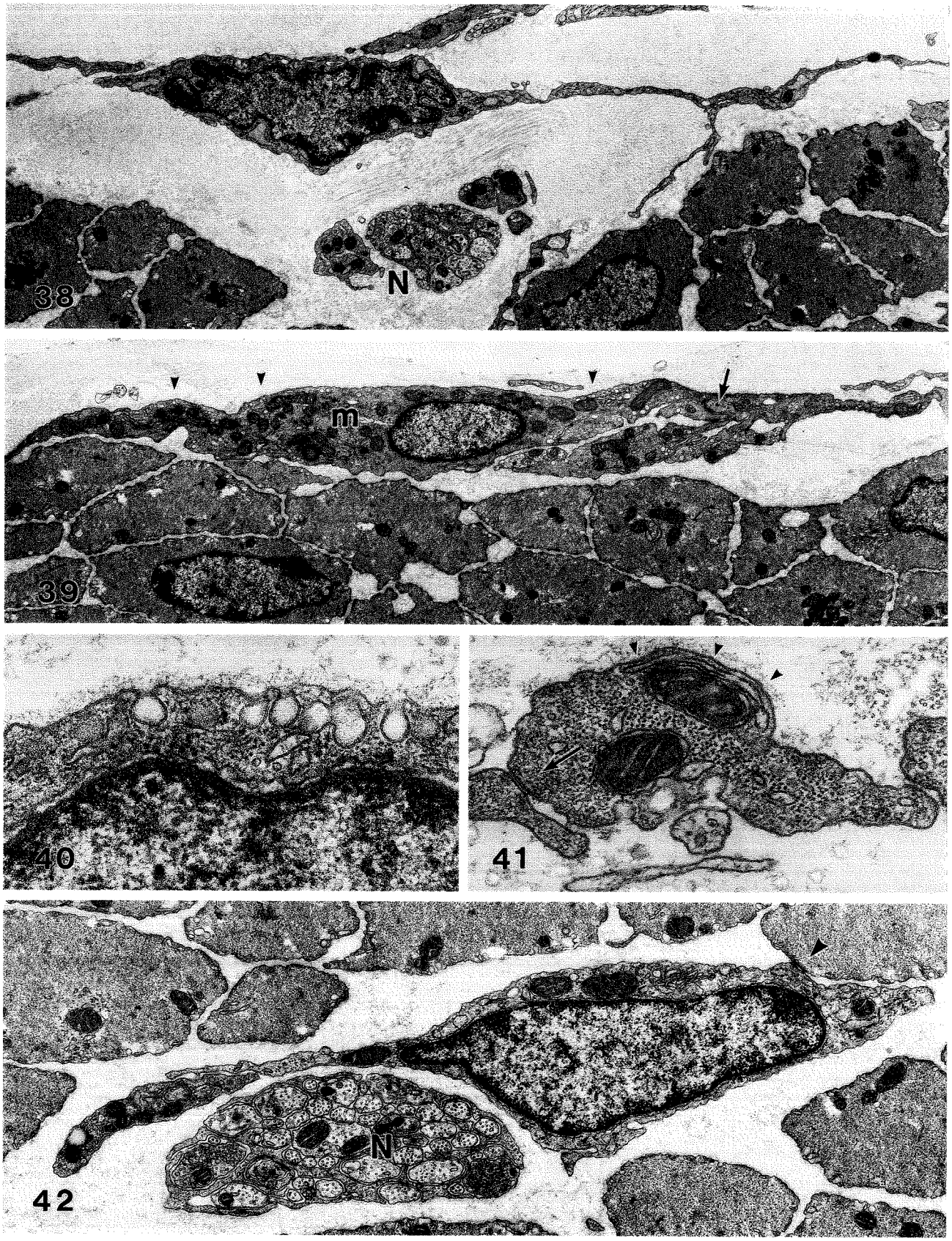
Fig. 35. ICC-AP of the guinea-pig small intestine characterized by less electron-dense cytoplasm containing many mitochondria and cisterns of smooth endoplasmic reticulum. Reproduced from Komuro et al. (1996) with permission of the publisher. ×9,800.

Fig. 36. Gap junction between the small processes of ICC-AP of the guinea-pig small intestine. Many intermediate filaments are also seen. Reproduced from Komuro et al. (1996) with permission of the publisher. ×38,000.

Fig. 37. FL-AP of the guinea-pig small intestine characterized by well-developed rough endoplasmic reticulum. A small gap junction is present at the tip of the process (arrow). ×10,000. **Inset:** Higher magnification of the gap junction indicated by the arrow. ×50,000.




Figs. 33–37.




Figs. 38–42.

*TABLE 1. Ultrastructural comparison of ICC type of different levels of digestive tract of mouse, rat, guinea-pig, dog, and human*

| Species / Organ | Authors | ICC-AP | | | | | | | | DMP-SMP | | | | | | | | CM | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | BL | Cv | GJ | Cy | Mt | IF | DB | NC | BL | Cv | GJ | Cy | Mt | IF | DB | NC | BL | Cv | GJ | Cy | Mt | IF | DB | NC |
| Mouse | | | | | | | | | | | | | | | | | | | | | | | | | |
| Stomach | No report | | | | | | | | | | | | | | | | | | | | | | | | |
| Intestine | Yamamoto 77 | | | | | | | | | + | ++ | ++ | D | ++ | / | + | ++ | | | | | | | | |
| | Thuneberg 82 | + | ++ | ++ | M | ++ | ++ | / | + | ++ | ++ | ++ | D | + | / | / | ++ | − | − | − | M | + | / | − | − |
| | Rumessen et al. 82 | | | | | | | | | ++ | ++ | ++ | L | ++ | + | − | ++ | | | | | | | | |
| | Ward et al. 94 | / | + | / | D | ++ | / | / | + | | | | | | | | | | | | | | | | |
| | Huizinga et al. 95 | / | ++ | / | / | ++ | / | / | + | | | | | | | | | | | | | | | | |
| Colon | F. Pellegrini 87a | | | | | | | | | ++ | ++ | + | / | ++ | / | / | + | | | | | | | | |
| | Hanani et al. 98 | | | | | | | | | + | ++ | / | / | ++ | / | / | / | | | | | | | | |
| Rats | | | | | | | | | | | | | | | | | | | | | | | | | |
| Stomach | Ishikawa et al. 97 | | | | | | | | | | | | | | | | | − | ++ | ++ | D | ++ | ++ | − | ++ |
| | Ishikawa 99 | − | + | ++ | D | ++ | + | − | + | | | | | | | | | | | | | | | | |
| Intestine | Komuro 89 | − | − | ++ | L | ++ | + | − | − | | | | | | | | | | | | | | | | |
| | Komuro, Seki 95 | | | | | | | | | + | + | ++ | L | ++ | ++ | − | ++ | | | | | | | | |
| | Seki, Komuro 98 | | | | | | | | | ++ | ++ | ++ | DL | ++ | + | − | ++ | | | | | | | | |
| | Horiguchi, Komuro 98 | − | − | ++ | L | ++ | / | / | / | ++ | ++ | ++ | L | ++ | + | / | ++ | | | | | | | | |
| Colon | Ishikawa, Komuro 98 | | | | | | | | | ++ | ++ | ++ | D | ++ | ++ | − | ++ | − | ++ | ++ | D | ++ | ++ | − | ++ |
| | Ishikawa 99 | − | + | ++ | D | ++ | + | − | + | | | | | | | | | | | | | | | | |
| Guinea pig | | | | | | | | | | | | | | | | | | | | | | | | | |
| Stomach | This Report | − | ++ | ++ | L | ++ | ++ | − | + | | | | | | | | | − | ++ | ++ | D | ++ | ++ | − | ++ |
| Intestine | Zhou, Komuro 92a,b | | | | | | | | | ++ | ++ | ++ | M | ++ | ++ | − | ++ | | | | | | | | |
| | Komuro, Zhou 96 | − | − | ++ | M | ++ | ++ | − | / | | | | | | | | | | | | | | | | |
| Colon | Ishikawa, Komuro 96 | | | | | | | | | ++ | ++ | ++ | M | ++ | ++ | − | ++ | | | | | | | | |
| | Naher et al. 98 | | | | | | | | | ++ | ++ | + | / | ++ | ++ | + | + | | | | | | | | |
| | This Report | − | − | ++ | L | ++ | + | − | + | | | | | | | | | − | ++ | ++ | D | ++ | ++ | − | ++ |
| Dog | | | | | | | | | | | | | | | | | | | | | | | | | |
| Stomach | Daniel et al. 84 | | | | | | | | | | | | | | | | | / | / | + | D | ++ | / | / | + |
| | Daniel et al. 89 | | | | | | | | | | | | | | | | | / | ++ | ++ | D | ++ | / | / | − |
| Intestine | Duchon et al. 74 | | | | | | | | | / | ++ | ++ | M | + | / | + | + | | | | | | | | |
| | Torihashi et al. 93 | | | | | | | | | + | ++ | ++ | M | ++ | ++ | ++ | ++ | | | | | | | | |
| | Daniel et al. 98 | + | + | + | M | ++ | + | − | + | + | ++ | ++ | M | + | + | / | ++ | | | | | | | | |
| Colon | Berezin et al, 88 | | | | | | | | | + | ++ | ++ | M | ++ | ++ | + | ++ | | | | | | | | |
| | Berezin et al, 89 | + | ++ | ++ | D | ++ | + | + | ++ | + | ++ | ++ | M | ++ | ++ | + | ++ | | | | | | | | |
| | Torihashi et al, 94 | + | + | + | D | ++ | ++ | + | + | ++ | ++ | ++ | D | ++ | ++ | ++ | ++ | − | − | / | D | ++ | ++ | + | + |
| Human | | | | | | | | | | | | | | | | | | | | | | | | | |
| Stomach | F. Pellegrini et al. 89 | + | + | − | / | ++ | + | + | + | | | | | | | | | + | / | + | / | / | + | / | + |
| Intestine | Rumessen, Thuneberg 91 | + | ++ | − | M | ++ | ++ | + | ++ | | | | | | | | | | | | | | | | |
| | Rumessen et al. 92 | | | | | | | | | ++ | ++ | + | M | ++ | ++ | ++ | ++ | | | | | | | | |
| | Rumessen et al. 93a | | | | | | | | | | | | | | | | | + | ++ | ++ | M | / | ++ | + | ++ |
| Colon | F. Pellegrini et al. 90a | | | | | | | | | / | + | + | D | / | + | / | / | | | | | | | | |
| | F. Pellegrini et al. 90b | / | + | / | M | / | + | / | − | | | | | | | | | | | | | | | | |
| | Rumessen et al. 93b | | | | | | | | | ++ | ++ | − | M | ++ | ++ | ++ | − | | | | | | | | |

Key:

| | | | | |
|---|---|---|---|---|
| BL: basal lamina | (++) continuous, distinct | (+) ill-defined, incomplete | (−) absent, lack | (/) no description |
| CV: caveola | (++) abundant, many | (+) a few, | (−) no, rare | (/) no description |
| GJ: gap junction | (++) abundant, many, large | (+) present, small, a few, | (−) no, rare | (/) no description |
| Cy: cytoplasmic density | (D) electron-dense | (L) less electron-dense | (M) moderate | (/) no description |
| Mt: mitochondria | (++) abundant, many, rich | (+) present | (−) no, rare | (/) no description |
| IF: intermediate filament | (++) abundant, many, rich | (+) present | (−) no, rare | (/) no description |
| DB: dense body | (++) distinct, many | (+) present | (−) no, absent | (/) no description |
| NC: close contact with nerve | (++) frequent, many | (+) present, | (−) no, rare | (/) no description |

Fig. 38. Submucosal border of the guinea-pig colon showing a fibroblast with slender processes. Nerve bundles (N) of the SMP are observed close by. ×7,000.

Fig. 39. ICC-SMP of the guinea-pig colon characterized by electron-dense cytoplasm containing many mitochondria (m). Caveolae (arrowheads) and connections with the same type of cells are observed (arrow). ×8,000.

Fig. 40. Higher magnification of the caveolae of the ICC-SMP. Note the presence of the clearly defined basal lamina. Reproduced from Ishikawa and Komuro (1996) with permission of the publisher. ×52,000.

Fig. 41. Process of ICC-SMP with abundant intermediate filaments. A subsurface cistern (arrowheads) and gap junction (arrow) are also seen. Reproduced from Ishikawa and Komuro (1996) with permission of the publisher. ×60,000.

Fig. 42. FL-CM of the guinea-pig colon located near the nerve bundle (N). It forms a small gap junction with a smooth muscle cell (arrowhead), but no caveolae are found along the cell membrane. ×15,000.

effects of mechanical force, type of nerve supply, spatial relationships with muscle cells, which are further affected by the eating habits of animals, movement patterns of the organ proper, and so on. Thus, the organ or species-specificity of corresponding types of ICC may also be interpreted in this context. The heterogeneity of ICC may be understood to originate from the multipotency of a family of mesenchymal cells that display a wide diversity of cytological features depending on the requirements of their tissue or organs (Komuro, 1990).

## ACKNOWLEDGMENTS

The author thanks Dr. Peter Baluk, Cardiovascular Research Institute, University of California, for reading the manuscript. The author also thanks Drs. Keisuke Seki, Koichi Ishikawa, and Shunichi Morikawa and Mr. Kazuhide Horiguchi, Waseda University, Japan, for assistance with electronmicroscopy.

## NOTE ADDED IN PROOF

By reviewing the proofs of this paper, the author revealed the presence of a distinct basal lamina along the cell membrane of ICC-AP in the mouse pylorus (Komuro et al., 2000).

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# Ultrastructural Characterization of the Interstitial Cells of Cajal

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Reprinted from
Arch. Histol. Cytol., Vol. 62, No. 4 (1999)
p. 295-316

*REVIEW*

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# Ultrastructural Characterization of the Interstitial Cells of Cajal

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*Received August 19, 1999*

***Summary.*** **Recent studies on the interstitial cells of Cajal (ICC) have determined ultrastructural criteria for the identification of these previously enigmatic cells. This review deals with the electron microscopic findings obtained by the author's research group in different tissue regions of the gut in mice, rats and guinea-pigs, comparing these with reports from other groups in different species and in humans.**

**ICC are characterized by the following morphological criteria: numerous mitochondria, abundant intermediate filaments and large gap junctions which connect the cells with each other and with smooth muscle cells. Due to their location in the gut and the specific species, the ICC are markedly heterogeneous in appearance, ranging from cells closely resembling smooth muscle cells to those similar to fibroblasts (Table 1). Nevertheless, the above-mentioned morphological features are shared by all types of ICC and serve in identifying them.**

**Recent discoveries on a significant role of c-*kit* in the maturation of the ICC and their specific immunoreactivity to anti-c-Kit antibody have confirmed the view that the ICC comprise an independent and specific entity of cells. This view is reinforced by the findings of the author's group that the ICC characteristically possess vimentin filaments and are stained with the zinc iodide-osmium tetroxide method which provides a staining affinity similar to methylene blue, the dye used in the original work by CAJAL (1911). Developmental studies indicate that the ICC are derived from a non-neuronal, mesenchymal origin.**

**This paper further reviews advances in the physiological studies on the ICC, in support of the hypothesis by THUNEBERG (1982) that they function as a pacemaker in the digestive tract and a mediator transmitting impulses from the nerve terminals to the smooth muscle cells.**

During the last decade it has become widely acknowledged that the interstitial cells of Cajal (ICC) carry out a pacemaking activity and regulatory roles in gastrointestinal muscle movement (see reviews by THUNEBERG, 1989; THUNEBERG et al., 1995; KOMURO et al., 1996; SANDERS, 1996; HUIZINGA et al., 1997). The presence of the ICC has been described in a wide variety of species, including the love-bird (IMAIZUMI and HAMA, 1969), bat (YAMAMOTO, 1977), rabbit (KOMURO, 1982), opposum (DANIEL and POSEY-DANIEL, 1984), hedgehog (FAUSSONE-PELLEGRINI, 1987), monkey (WONG et al., 1990), pig (JIMENEZ et al., 1999) and horse (HUDSON et al., 1999), in addition to humans and conventional experimental animals such as the mouse, rat, guinea-pig and dog.

Recent reports have suggested that the ICC might be involved in the etiology of chronic idiopathic intestinal pseudo-obstructions (ISOZAKI et al., 1997) and of gastrointestinal stromal tumors (HIROTA et al., 1998; KINDBLOM et al., 1998; SAKURAI et al., 1999; SEIDAL and EDVARDSON, 1999). Rapid progress toward an understanding of the ICC is now occurring after a long period of confusion in which even their existence as a special cell type was questioned (KOBAYASHI et al., 1989).

This paper reviews the dramatic changes in our conception of the ICC during the century after its first description by CAJAL (1893, 1911). It will first deal with the distribution of ICC in different regions of the digestive tract mainly in the guinea-pig. Next, the focus will be turned to the ultrastructural features of ICC located in different gut regions in the mouse, rat and guinea-pig. The third section of this review aims to clarify those features which are common to all types of ICC and those which are specific for types of ICC proper to tissue regions, organs, or species. Finally, this paper will discuss whether the ICC can be regarded as an independent cell category unequivocally separate from neurons, smooth muscle cells and fibroblasts.

## The current studies

In the course of his studies to clarify the histological

basis of autonomic innervation, CAJAL (1911) recorded a fine cellular network over the myenteric plexus of the rabbit intestine with the vital methylene blue staining method (Fig. 1). CAJAL considered these cells primitive nerve cells which might mediate impulses from the terminal portions of the sympathetic nerves to smooth muscle cells. These cells, which thereafter came to bear his name, have been the subject of histological controversy; they have been regarded by different microscopists either as neurons, Schwann cells, connective tissue cells or smooth muscle cells (see reviews by BOEKE, 1949, MEYLING, 1953; TAXI, 1965).

With the development of electron microscopy, ultrastructural identification of the ICC was attempted by several investigators (RICHARDSON, 1958, 1960; TAXI 1965; ROGERS and BURNSTOCK, 1966; IMAIZUMI and HAMA, 1969; GABELLA, 1972; YAMAMOTO, 1977), but the cytological definition and the developmental origin of the cells remained unsettled.

A breakthrough in the ICC research was triggered by the novel hypothesis proposed by THUNEBERG (1982), which claimed that ICC might act as pacemaker cells and as an impulse conduction system in the gut musculature in fashion analogous to those in the heart. This hypothesis, which was formulated under the influence of earlier studies by KEITH (1914/15, 1915), greatly stimulated both morphological and physiological studies of ICC.

Subsequently, variety in the structural features of the ICC in different regions of the digestive tract of different species was recorded by many authors (see reviews by THUNEBERG, 1989; CHRISTENSEN, 1992; KOMURO et al., 1996; KOMURO, in press). Again, however, it was uncertain whether the different cytological features of the cells represented different profiles of the same cell type, or morphological variations of the same cell type, or a mixture of different cell types, possibly including cells that were not true ICC.

Part of the reason for this confusion was the lack of a specific staining method for the ICC and the difficulty in performing ultrastructural observation adequately correlated to the traditional histological results by silver-impregnation and methylene blue staining. Therefore, to establish an unambiguous set of cytological criteria, it was essential that the entire structure of a given cell type as well as its relation to nerve and muscle cells should precisely correspond to that originally described by CAJAL.

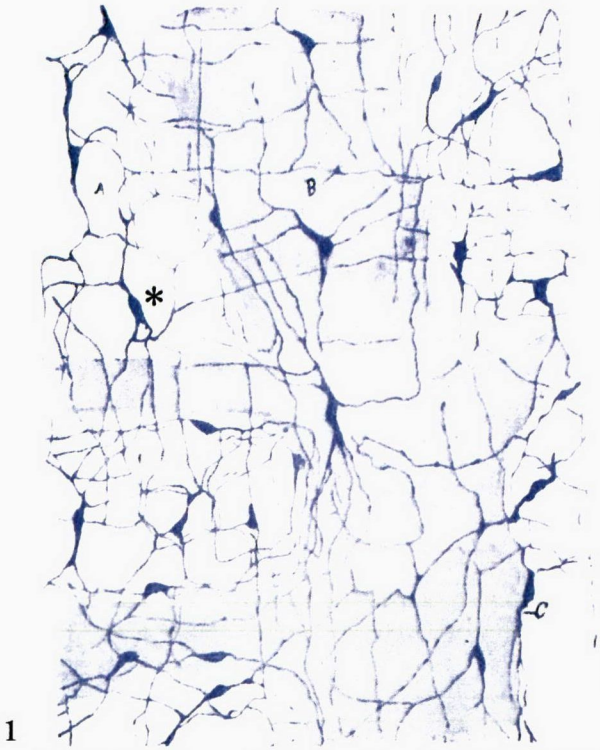
In this context, a major recent advance has been the discovery of a significant role of c-*kit* in the maturation of ICC; it was found that ICC correspond to the cells expressing c-Kit receptor tyrosine kinase. Abnormal development of ICC was demonstrated after an experimental blockade of c-Kit (MAEDA et al., 1992; TORIHASHI et al., 1995) or under genetic defects in its production (WARD et al., 1994; HUIZINGA et al., 1995). Thus, immunohistochemical staining for c-Kit became accepted as the most reliable marker of ICC at the light microscopic level.

Studies using a combination of c-Kit immunostaining, vimentin-immunostaining and ultrastructural observation contributed to bridging the gap between the old histological descriptions and more recent findings of the ICC (KOMURO and ZHOU, 1996). Cells in the guinea-pig small intestine depicted by the zinc iodide-osmium tetroxide (ZIO) method (Fig. 3) corresponded to c-Kit immunoreactive cells (Fig. 4) and vimentin immunoreactive cells (Fig. 5), which showed almost the same characteristics as those of the ICC illustrated by CAJAL with the methylene blue staining (Fig. 1; CAJAL, 1911) and by TAXI with the Bielschowsky-Gros method (Fig. 2; TAXI, 1965). These studies confirmed the c-Kit immunoreactivity as a specific marker for the ICC, and further demonstrated the usefulness of an anti-vimentin antibody to demonstrate the ICC (KOMURO et al., 1996).

In addition to the ICC in the myenteric region, CAJAL (1911) described similar cells in the deep muscular plexus of the guinea-pig intestine (Fig. 6) and within the circular muscle layer of the rabbit (Fig. 7). These cells were also visualized by the c-Kit immunostaining (Fig. 8) or the ZIO method (Fig. 9) and were immunoreactive for vimentin (KOMURO et al., 1996).

In spite of the remarkable progress in the identification of ICC, it remained unclear whether they represented a single, clearly defined class of cells, because a conspicuous variance in their ultrastructural features was reported. Clarification was needed as to which features were common to all or specific for each type of ICC. It was also to be determined whether the ICC were cytologically distinguishable from fibroblasts and from smooth muscle cells.

Regarding the nomenclature, the only cells that are termed ICC in this review are those which have been confirmed as being equivalent to the cells originally described by CAJAL, or those cells which can be regarded as species-specific variations of them. The terminology of THUNEBERG et al. (1995) is adopted to describe ICC in different locations, i.e., ICC-AP (Auerbach's myenteric plexus) located between the circular and longitudinal muscle layers; ICC-DMP (deep muscular plexus) located between the inner thin and outer thick sublayers of the circular smooth muscle of the small intestine; ICC-SMP (submuscular plexus) located at the submucosal border of the colonic

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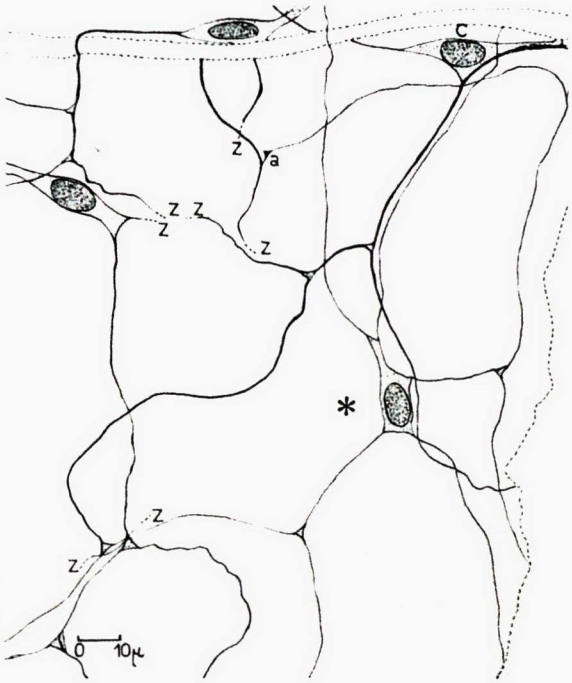


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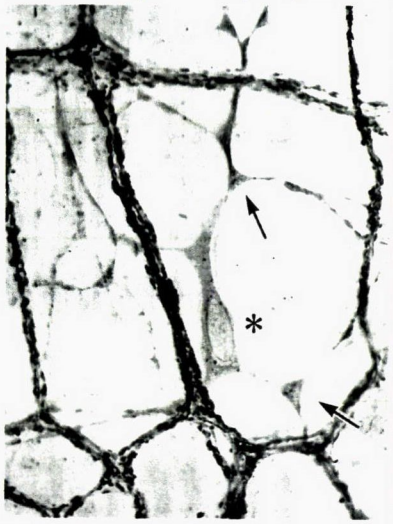


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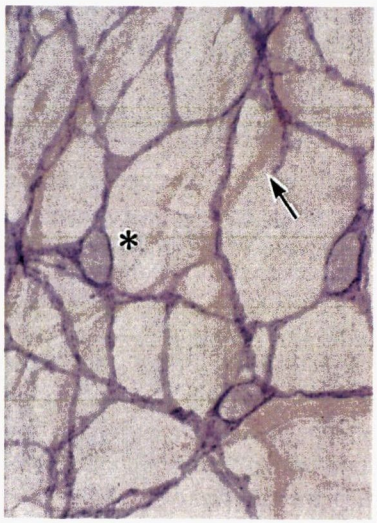


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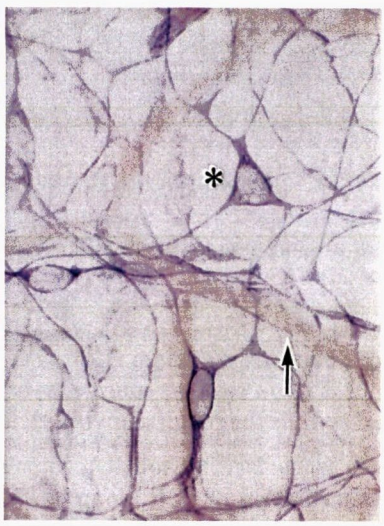


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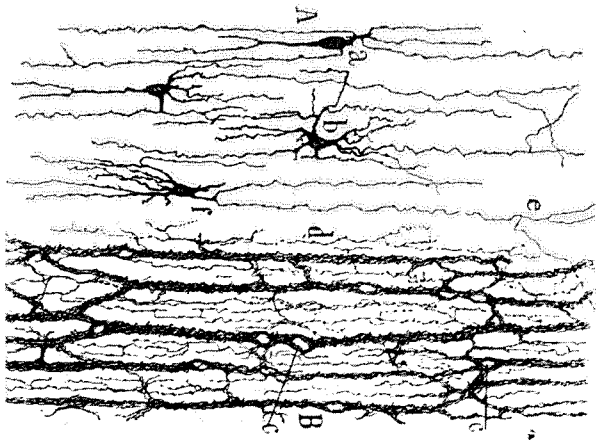
**Fig. 1.** A drawing by CAJAL of ICC-AP in the rabbit intestine stained with methylene blue. (Reproduced from CAJAL, 1911, Fig. 572). Note the close similarity in shape of the cells (*) to the cells in Figures 2-5.

**Fig. 2.** A drawing by TAXI of ICC-AP (*) in the guinea-pig intestine stained with the Bielschowsky-Gross method. (Reproduced under permission from TAXI, Ann. Sci. Nat. Zool., 1965, Fig. 43)

**Fig. 3.** ICC-AP (*) in the guinea-pig small intestine stained with the zinc-iodide osmium tetroxide method, which show almost the same characteristics as those in Figures 1 and 2. These ICC-AP are characterized by slender cytoplasmic processes with a dichotomous branching pattern. Note triangular knots at branching points (*arrows*). Cell bodies are usually located away from the tertiary nerve bundles of the myenteric plexus. ×600. (Figs. 3-5: Reproduced under permission from KOMURO and ZHOU, J. Auton. Nerv. Syst., 1996)

**Fig. 4.** ICC-AP (*) of the guinea-pig small intestine, demonstrated by immunohistochemical staining for c-Kit. The tertiary nerve plexus is brown from the cholinesterase reaction (*arrow*). ×650

**Fig. 5.** ICC-AP (*) of the guinea-pig small intestine, demonstrated by immunohistochemical staining for vimentin. The tertiary nerve plexus is brown from the cholinesterase reaction (*arrow*). ×600



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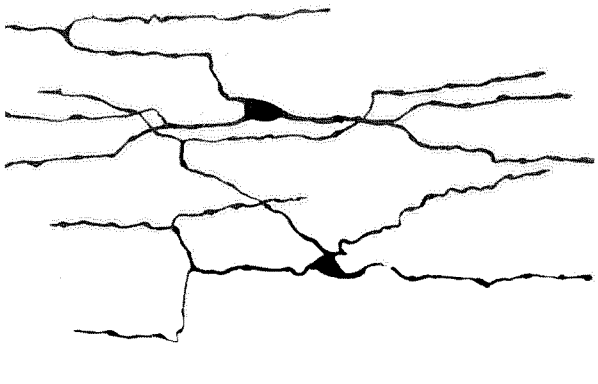

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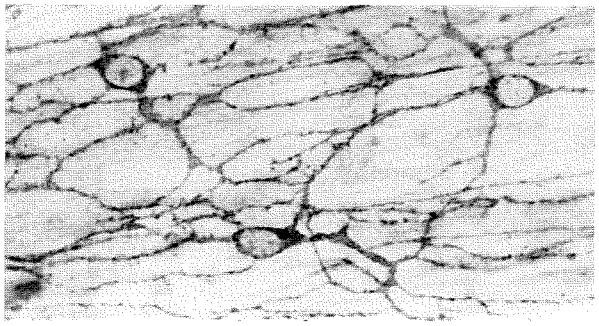

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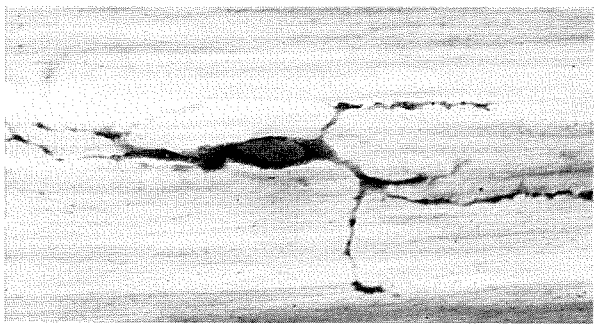

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**Fig. 6.** A drawing by CAJAL of ICC-DMP of the guinea-pig intestine stained with Golgi method. (Reproduced from CAJAL, 1911, Fig. 575)

**Fig. 7.** A drawing by CAJAL of ICC-CM of the rabbit stained with methylene blue. (Reproduced from CAJAL, 1911, Fig. 573)

**Fig. 8.** ICC-DMP in the guinea-pig small intestine demonstrated by c-Kit immunostaining. ×500

**Fig. 9.** ICC-CM of the guinea-pig small intestine stained by zinc iodide-osmium tetroxide method. ×600. (Reproduced under permission from KOMURO et al., Histol. Histopathol. 1996)

circular muscle layer; ICC-CM located within the outer thick circular muscle layer; and ICC-LM located within the longitudinal muscle layer.

### Distribution of ICC along the digestive tract

Since ICC are believed to be involved in the regulatory mechanism of the gut motility, it seems necessary to elucidate their distribution in different organs, together with their coupling pattern by gap junctions in the intestinal musculature.

Immunohistochemical staining clearly demonstrated that c-*kit* expressing ICC were regularly observed in the region of myenteric plexus throughout the gut, including the stomach, small intestine and colon of guinea-pigs (Figs. 10-12; SEKI et al., 1998). In contrast, the ICC in the muscle layers showed different distribution patterns among these three organs. Cells with c-Kit immunoreactivity were sparsely distributed in the circular muscle layer of both the stomach and the colon, but not in the small intestine. Instead, they were abundant in the DMP of the small intestine and SMP of the colon. A few immunoreactive cells were observed in the longitudinal muscle layer of the stomach and colon.

In contrast, only weak immunoreactivity for gap junction protein, connexin 43, was detected in the myenteric region of these three organs (Figs. 13-15). However, fairly strong immunoreactivity was recognized throughout nearly the entire thickness of the circular muscle layer of the stomach and the small intestine, but not in the colon; very strong immunoreactivity was observed in the DMP and SMP.

### Ultrastructural features of ICC in the stomach

In the stomach of the mouse, rat, and guinea-pig, there is no nerve plexus corresponding either to the

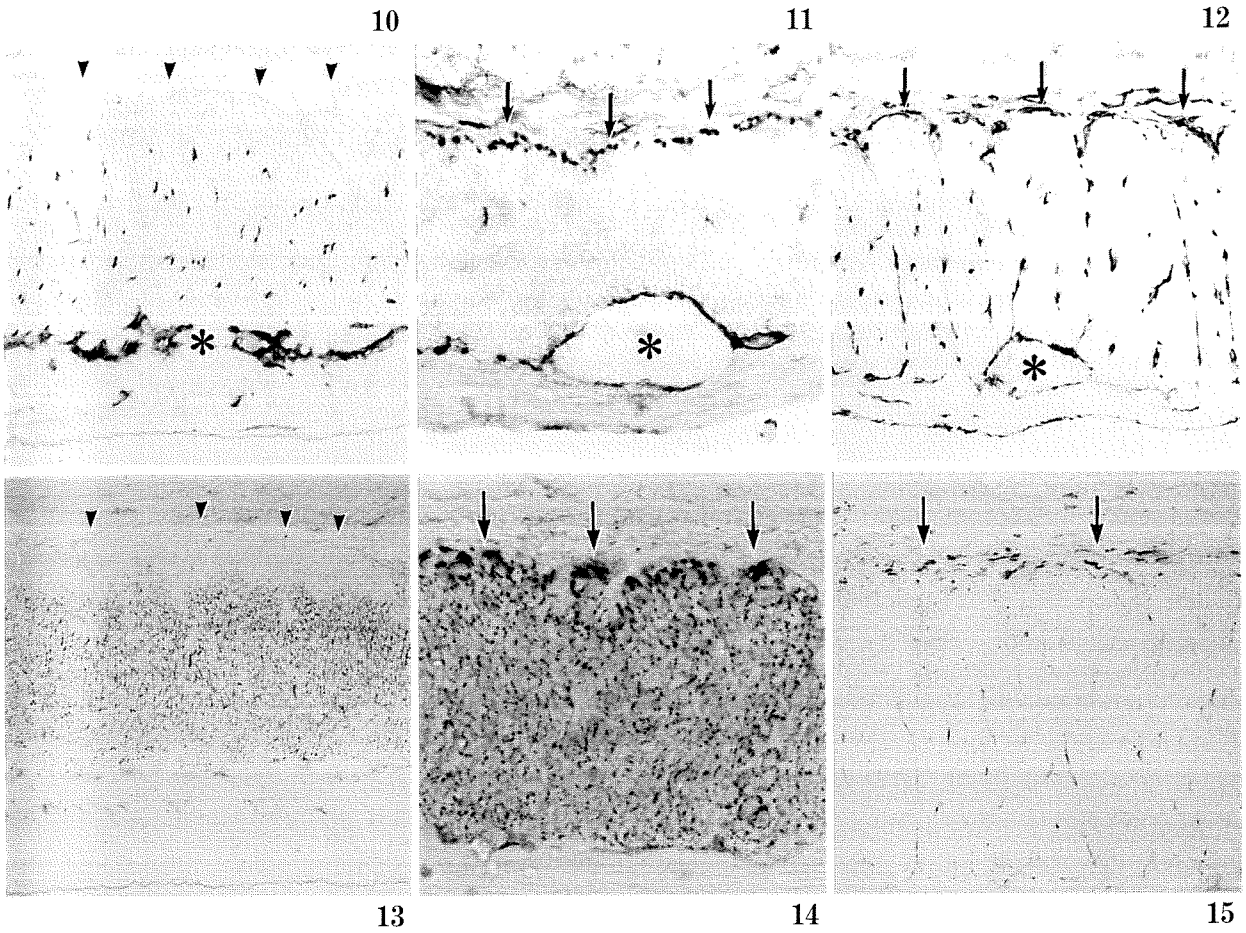



**Fig. 10.** Longitudinal cryosection of the guinea-pig stomach stained by immunohistochemical reaction for c-Kit. The reaction deposits are seen in the myenteric region (*) and within the circular muscle layer excluding one third from the submucosal surface (*arrowheads*). A few deposits are also seen in the longitudinal muscle layer. ×100. (Figs. 10-15: Reproduced under permission from SEKI et al., J. Auton. Nerv. Syst., 1998)

**Fig. 11.** Longitudinal section of the guinea-pig small intestine stained by immunohistochemical reaction for c-Kit. Dense reaction deposits are localized in the DMP region (*arrows*) and around the myenteric ganglion (*). ×120

**Fig. 12.** Longitudinal section of the guinea-pig colon stained by immunohistochemical reaction for c-Kit. Reaction deposits are distributed in the SMP region (*arrows*), around a myenteric ganglion (*) and within the circular muscle layer. A few deposits are found in the longitudinal muscle layer. ×100

**Fig. 13.** A section of the stomach showing immunohistochemical reaction for connexin 43. Immunopositive deposits are densely distributed within the circular muslce layer excluding one third from the submucosal surface (*arrowheads*). ×110

**Fig. 14.** A section of the small intestine showing immunohistochemical reaction for connexin 43. Immunopositive deposits are densely distributed in the whole circular muslce layer. Large deposits are located in the region of DMP (*arrows*). ×120

**Fig. 15.** A section of the colon showing immunohistochemical reaction for connexin 43. Dense deposits are located along the region of SMP, the interface between the submucosa and circular muslce layer (*arrows*). A few weak deposits are observed in the circular muscle layer. ×120

DMP of the small intestine, or to the SMP of the colon. Thus, no special type of ICC was found at the most inner region of the circular muscle layer. Instead, the ICC were observed in close association with small nerve bundles within the circular muscle layer (ICC-CM) and in the myenteric region (ICC-AP).

The ICC-CM were frequently observed within the circular muscle layer of the pylorus and corpus of the rat stomach. They were characterized by electron-dense cytoplasm and abundant mitochondria (Fig. 16). Golgi apparatus, rough (RER) and smooth endoplasmic reticulum (SER) were also observed. Intermediate filaments were abundant, particularly in the cytoplasmic processes extending in various directions (ISHIKAWA et al., 1997). Cilia, basal bodies and lipid droplets were occasionally seen. No basal lamina could be clearly identified, whereas numerous caveolae were observed on the cell membranes (Fig. 16). These cells interconnected with each other and with neighboring smooth muscle cells via a number of large gap junctions (Fig. 16 inset). The ICC-CM showed close contact with nerve terminals containing many synaptic vesicles. Cells with the same features were also observed in the pylorus of mouse and guinea-pig stomach (KOMURO, in press). It is worth noting that a similar type of cell was found in the circular muscle layer of the mouse fundus, in which part of the mucosa was lined with stratified squamous epithelium (unpublished data).

ICC-AP of the mouse stomach were found in the space between the circular and longitudinal muscle layers (Fig. 17) and around the myenteric plexus (Fig. 18). They were characterized by numerous caveolae and a distinct basal lamina (Fig. 17 inset), as well as large gap junctions, many mitochondria, abundant intermediate filaments (Fig. 19) and electron-dense cytoplasm. By these features, the ICC-AP were easily distinguished from fibroblast-like cells which had less electron-dense cytoplasm containing well-developed RER with dilated cisterns (Fig. 17). Part of the cellular network of ICC connected by gap junctions is shown in a single section (Fig. 18). Sites of close contacts between the ICC-AP and nerve terminals were demonstrated in the mouse stomach (Fig. 20). Cells with similar features, but without a clear basal lamina, were also found in the myenteric region of the rat and guinea-pig pylorus (KOMURO, in press).

### Ultrastructural features of ICC in the small intestine

ICC in the small intestine were found in association with well-developed DMP and AP nerve plexuses, but they were rarely seen within the outer subdivision of the circular muscle layer, or in the longitudinal muscle layer. The DMP of the mouse, rat and guinea-pig extends two-dimensionally in a plane between the inner thin (1-3 cell thick) and outer main layers of the circular muscle. The plexus consists of nerve bundles running parallel to the smooth muscle cells of the circular layer and transverse interconnecting bundles (RUMESSEN et al., 1982; ZHOU and KOMURO, 1992a; KOMURO and SEKI, 1995).

The ICC-DMP in the mouse (Fig. 21) and rat (Fig. 22) were elongated cells showing the same orientation of cell axis as the circular muscle cells. Their cytoplasm was usually less electron-dense than that of the neighboring smooth muscle cells. A basal lamina and numerous caveolae were observed along the cell membrane (Fig. 22 inset). Well-developed Golgi apparatus and RER were mainly located in the perinuclear region. Many mitochondria were observed throughout the cytoplasm (Figs. 21, 22). Subsurface cisterns of SER could be found immediately

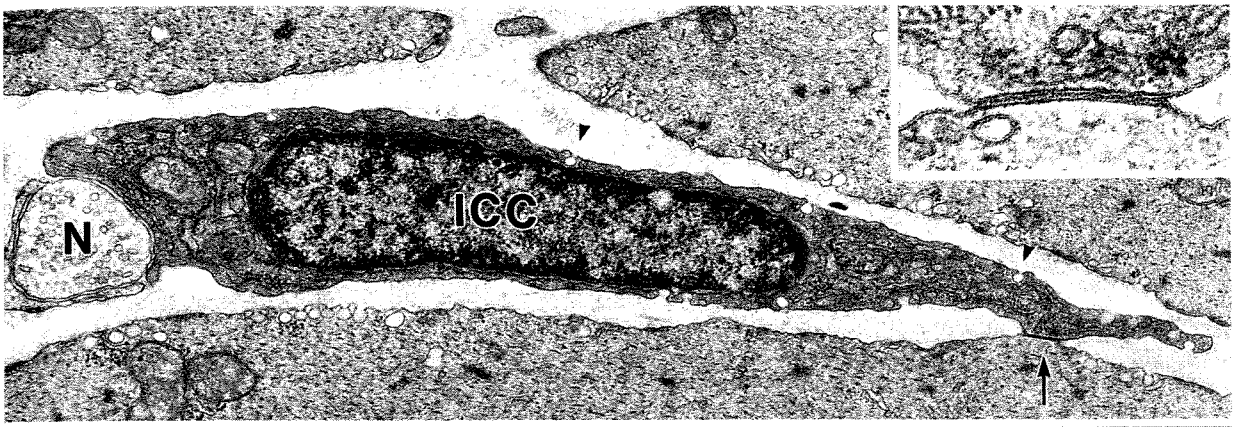
**Fig. 16.** An ICC-CM (*ICC*) in the rat stomach (antrum) characterized by electron-dense cytoplasm, caveolae (*arrowheads*), gap junction (*arrow*) with smooth muscle and close contact with nerve terminal (*N*). ×20,000. **Inset:** Higher magnification of the gap junction indicated by the *arrow*. ×40,000. (Reproduced under permission from ISHIKAWA et al., Cell Tiss. Res., 1997)

**Fig. 17.** An electron micrograph showing an ICC-AP (*ICC*) and a fibroblast-like cell (*FL*) located beside a nerve bundle (*N*) in the space between the circular and longitudinal muscle layers of the mouse pylorus. Note, a large gap junction of the ICC-AP (*arrow*) and the well-developed RER (*) in the *FL*, and difference between electron-density of their cytoplasm. ×8,000. **Inset:** Higher magnification of a part of the ICC-AP showing a continuous layer of basal lamina. ×47,000

**Fig. 18.** Two ICC-AP (*ICC*) located over the myenteric ganglion in the mouse pylorus, which interconnected with each other by gap junctions (*arrows*). Note, electron dense cytoplsm and the presence of many caveolae. ×17,000. **Inset:** Higher magnification of the gap jucntion indicated by the *double arrows*. ×80,000

**Fig. 19.** A bundle of intermediate filaments in the cytoplasmic process of an ICC-AP in the mouse pylorus. The *arrow* indicates a gap junction between thin processes. ×32,000

**Fig. 20.** Close contact between the nerve terminals and a ICC-AP of the mouse stomach. ×23,000

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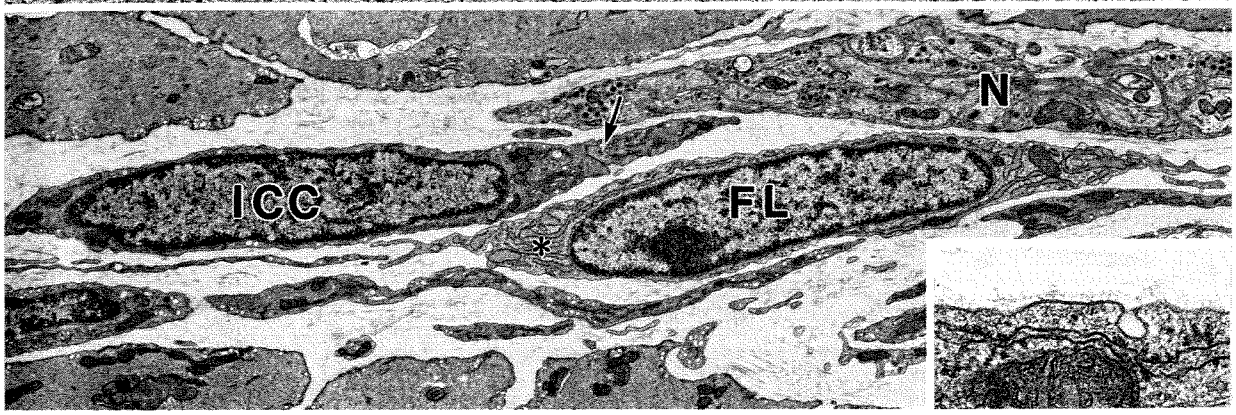


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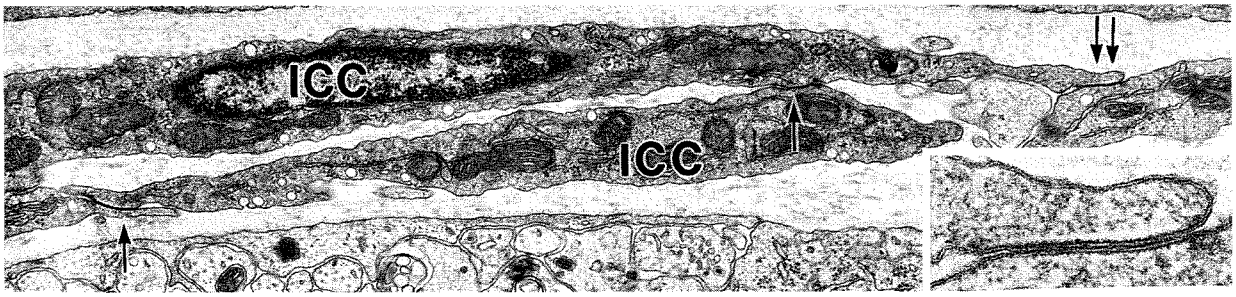


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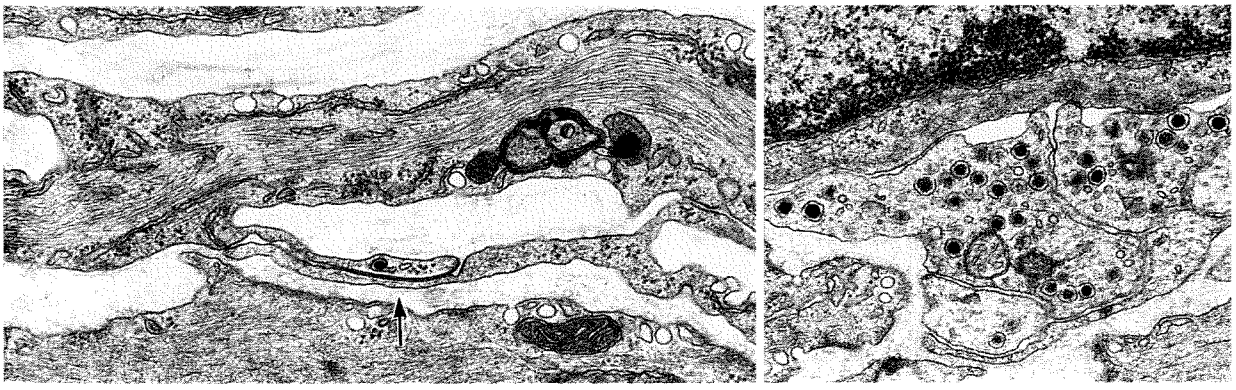

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**Figs. 16–20.** Legends on the opposite page.

beneath the cell membranes (KOMURO and SEKI, 1995; SEKI and KOMURO, 1998). Cytoskeletal elements such as microtubules, microfilaments and intermediate filaments were abundant in the cytoplasmic processes, but myosin filaments and dense bodies were not found.

The most conspicuous feature of the ICC-DMP was the frequent occurrence of large gap junctions that interconnected the cells with each other and also with smooth muscle cells (Figs, 21, 22 and Fig. 21 inset). Their gap junctions with muscle cells were mainly formed with those of the outer subdivision, but some gap junctions with the muscle cells of the inner sublayer were also observed (KOMURO and SEKI, 1995). These ICC had close contact with nerve varicosities containing accumulations of synaptic vesicles (KOMURO and SEKI, 1995; HORIGUCHI and KOMURO, 1998; SEKI and KOMURO, 1998). Three-dimensional examination of serial ultrathin sections indicated that the ICC-DMP of the rat could be classified into two subtypes on the basis of their cytoplasmic features (SEKI and KOMURO, 1998).

On the other hand, the ICC-DMP of the guinea-pig had some peculiar features, while showing certain characteristics common to those of the mouse and rat. In the DMP region of the guinea-pig small intestine, there were two types of large gap junction forming cells, i.e., glycogen-rich cells (Fig. 23) and glycogen-deficient cells (Fig. 24; gap junction-rich cells in ZHOU and KOMURO, 1992a, b). Both types of cells possessed a basal lamina and many caveolae along the cell membrane and contained many mitochondria, Golgi apparatus, RER and subsurface cisterns. Microtubules and intermediate filaments were abundant in the cytoplasmic processes (ZHOU and KOMURO, 1992a). They were located close to nerve varicosities containing many synaptic vesicles. Furthermore, the glycogen-rich cells were characterized by dense deposits of glycogen granules throughout the cytoplasm (Fig. 23). They had rounded cell bodies with a few cytoplasmic processes forming a wide angle with each other, while the glycogen-deficient (gap junction-rich) cells had elongated cell bodies with few processes, as demonstrated by serial ultrathin sections (ZHOU and KOMURO, 1992a).

The ICC-AP were generally characterized by well-demarcated cell bodies with long slender processes which extended several hundreds of microns in length to form a cellular reticulum (Figs. 3–5). These characteristics could be demonstrated three-dimensionally by scanning electron microscopy (Figs. 25, 26). The ICC-AP in the rat small intestine possessed a less-electron dense cytoplasm, numerous mitochondria, and large gap junctions which were mainly involved in the connection at the ends of the thin processes of the cells (Fig. 27 and inset). Golgi apparatus and both SER and RER were also seen in the cytoplasm. However, cisterns of RER were rarely dilated, in contrast to fibroblast-like cells which often showed this form of RER (Fig. 27). Intermediate filaments were abundant in the cytoplasmic processes. A few caveolae could be seen, but no basal lamina was observed. The numbers of this type of cells were greatly reduced in Ws/Ws rats (HORIGUCHI and KOMURO, 1998). The ICC-AP in the mouse small intestine (Fig. 28) were very similar in many respects to those in the rat small intestine.

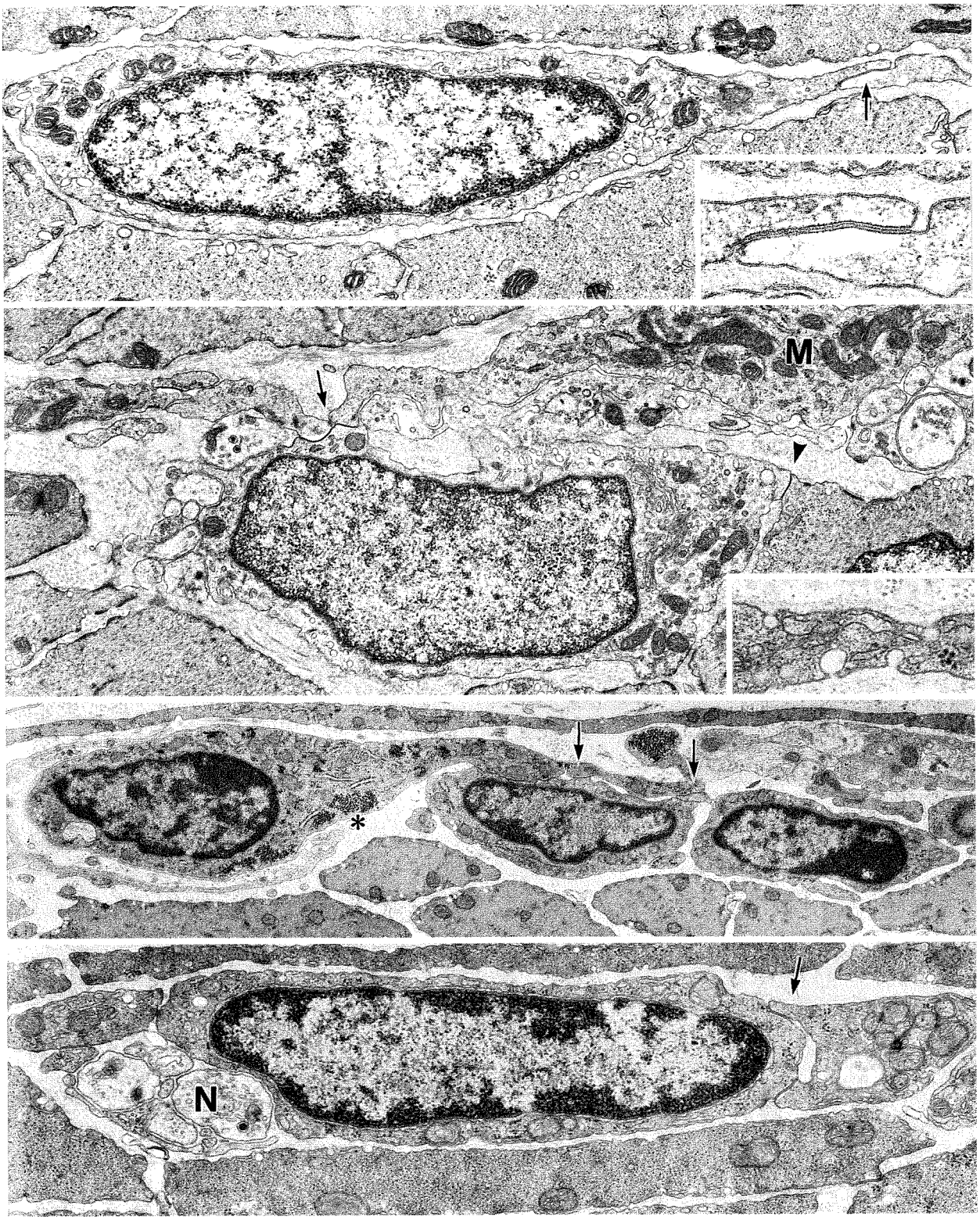
The ICC-AP in the guinea-pig small intestine were similar in appearance to the fibroblast-like cells and were often not easy to distinguish from the latter in a single profile (KOMURO and ZHOU, 1996). However, mitochondria and SER were fairly abundant in the cytoplasm, and these cells only rarely showed dilated cisterns of RER, as did the cells in mice and rats. Large gap junctions and abundant intermediate filaments were observed mainly at the tips of the

**Fig. 21.** ICC-DMP of the mouse small intestine showing less electron-dense cytoplasm containing many mitochondria. An *arrow* indicates a gap junction formed with a smooth muscle cell. ×21,000. **Inset:** Higher magnification of the gap junction indicated by the *arrow*. ×75,000

**Fig. 22.** Cross section of ICC-DMP of the rat small intestine characterized by less electron-dense cytoplasm, and gap junctions with smooth muscle cell (*arrowhead*) and with processes of the same type of cell (*arrow*) with many mitochondria (*M*). ×13,000. (Reproduced under permission from KOMURO and SEKI, Cell Tiss. Res, 1995). **Inset:** A part of ICC-DMP of the rat showing a distinct layer of basal lamina and caveolae. ×42,000

**Fig. 23.** A glycogen-rich cell which is probably a subtype of ICC-DMP of the guinea-pig small intestine. The perinuclear cytoplasm contains many mitochondria and abundant glycogen granules (*), and the slender process forms gap junctions (*arrows*) with cells of the same type. ×10,000. (Reproduced under permission from KOMURO et al., Histol. Histopathol., 1996)

**Fig. 24.** Glycogen-deficient (gap junction-rich) cell which may be another subtype of ICC-DMP in the guinea-pig small intestine. Closely associated nerves (*N*) and gap junctions with a cell of the same type (*arrow*). ×20,000. (Coutesy of Dr. D. S. ZHOU)

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**Figs. 21–24.** Legends on the opposite page.

cytoplasmic processes. A few caveolae could be occasionally seen. They lacked a basal lamina.

### Ultrastructural features of ICC in the colon

ICC in the colon of the mouse, rat and guinea-pig were found in association with the SMP and AP nerve plexuses, and in small nerve bundles within the circular muscle layer.

The ICC-SMP of the rat colon were observed at the interface between the submucosa and the circular muscle layer (Fig. 29). They were characterized by the presence of a basal lamina, caveolae (Fig. 29 left inset), and many mitochondria, and thus were clearly distinguished from fibroblasts. They had gap junctions which connected them with the same type of cells (Fig. 29 right inset) and with smooth muscle cells. Intermediate filaments were particularly abundant in their small processes. They often had close contact with nerve varicosities containing many synaptic vesicles. The cells with almost the same features as these were observed in the guinea-pig colon (ISHIKAWA and KOMURO, 1996). It was demonstrated that ICC-SMP of the guinea-pig were positive for vimentin immunostaining and zinc iodide-osmium tetroxide staining (ISHIKAWA and KOMURO, 1996).

The ICC-CM in the rat colon were characterized by an electron-dense cytoplasm, caveolae and many mitochondria, as those in the stomach. Their differences from fibroblast-like cells were clearly seen in a micrograph showing both types of cells in one frame (Fig. 30). Similar types of cells were observed in the guinea-pig colon (KOMURO, in press).

The ICC-AP in the rat colon were similar to those in the rat pylorus, but they differed from those of the small intestine. They were characterized by a moderate to electron-dense cytoplasm and some caveolae (Fig. 31; ISHIKAWA, 1999). However, no basal lamina were clearly defined, unlike the ICC-AP in the mouse pylorus. Gap-junctions were observed between the processes of the same type of cells (Fig. 31 inset).

### Fibroblast-like cells within the muscle coat of the digestive tract

Fibroblast-like cells were found everywhere ICC were located within the tunica muscularis of the digestive tract (Figs. 32–34). Most interestingly, the fibroblast-like cells had almost uniform features irrespective of the tissue layer, region of the digestive tract, or species in which they were found, in contrast to the ICC which showed a great diversity (KOMURO, 1989; ZHOU and KOMURO, 1992a, b; KOMURO and SEKI 1995; KOMURO et al., 1996; ISHIKAWA et al., 1997; SEKI and KOMURO, 1998; HORIGUCHI and KOMURO, 1998). The fibroblast-like cells were characterized by well-developed RER, often with dilated cisterns containing moderately electron-dense materials (Figs. 17, 27, 30, 32). Golgi apparatus and mitochondria were mainly found in the perinuclear region. In opposition to the general belief about the fibroblast-like cell, these cells formed small gap junctions with the same types of cells and with smooth muscle cells (Figs. 32–34 and insets). These cells were found without noticeable changes in the c-Kit deficient animals, such as Ws/Ws rats (Fig. 32; ISHIKAWA and KOMURO, 1997; HORIGUCHI and KOMURO, 1998) and W/W$^{v}$ mice (Fig. 33; HORIGUCHI and KOMURO, unpublished data).

### Are ICC a specific cell type?

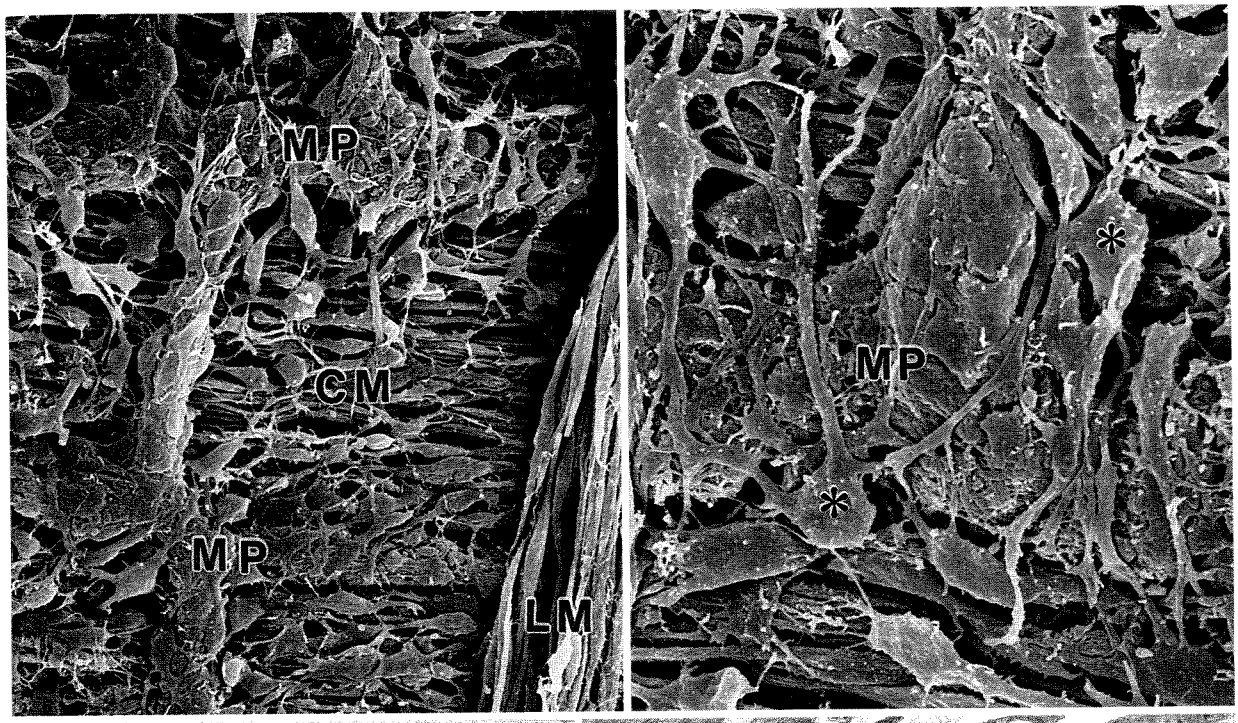
Many early light microscopic observations noted that the ICC-AP, ICC-DMP and ICC-CM all shared extremely long, slender cytoplasmic projections (Figs. 3–5, 8, 9) and suggested that they resembled nerve cells in this respect. It was, however, demonstrated

**Fig. 25.** A scanning electron micrograph showing interstitial cells including ICC over the myenteric plexus (*MP*) located between the circular (*CM*) and longitudinal (*LM*) muscle layers of the rat small intestine. ×700. (Figs. 25, 26: Reproduced under permission from KOMURO, Cell Tiss. Res., 1989)

**Fig. 26.** A scanning electron micrograph of ICC-AP (*) showing well-demarcated cell body with a few slender cytoplasmic processes which extend over the myenteric ganglion (*MP*). ×2,200

**Fig. 27.** An electron micrograph showing an ICC-AP (*ICC*) and a fibroblast-like cell (*FL*) in the space between the circular and longitudinal muscle layers of the rat small intestine. Note the presence of many mitochondria in the electron-lucent cytoplasm of the ICC-AP and well-developed RER in the *FL*. ×10,000. (Reproduced under permission from KOMURO et al., Histol. Histopathol., 1996). **Inset:** A gap junction between the processes of ICC-AP of the rat small intestine. ×40,000. (Reproduced under permission from HORIGUCHI and KOMURO, Cell Tiss. Res., 1998)

**Fig. 28.** ICC-AP (*ICC*) of the mouse small intestine showing electron-lucent cytoplasm containing many mitochondria. It does not show many caveolae and a basal lamina, unlike the ICC-AP of the mouse stomach. ×8,800

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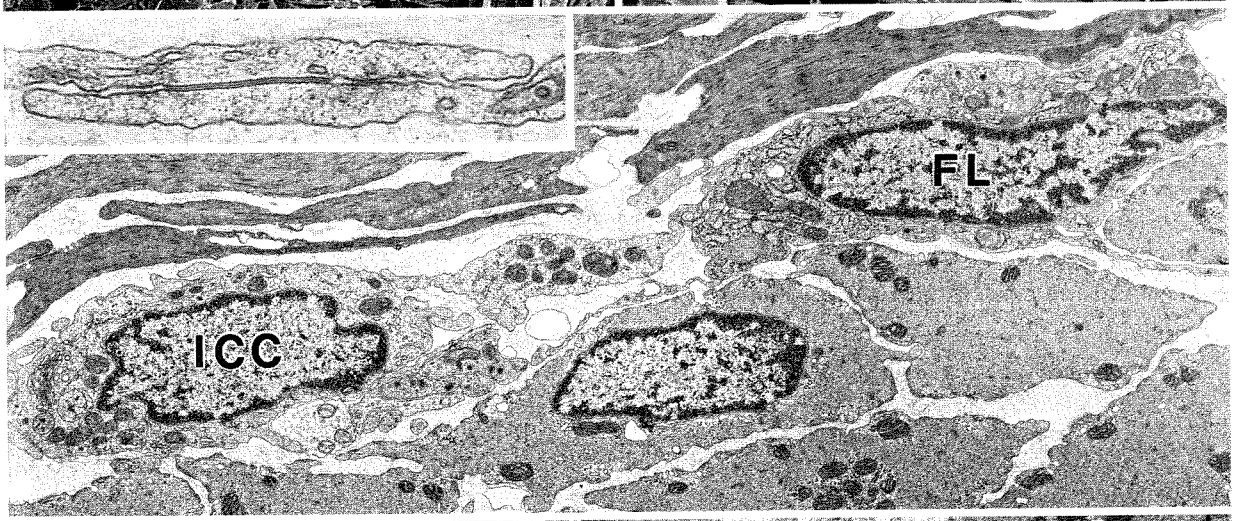


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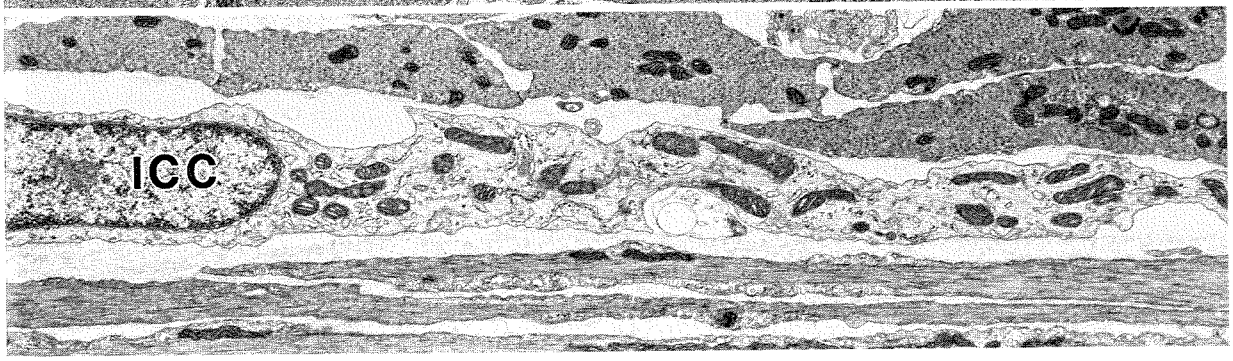


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**Figs. 25-28.** Legends on the opposite page.

that those processes contained abundant vimentin filaments instead of neurofilaments (KOMURO, 1987; KOMURO and ZHOU, 1996; ISHIKAWA and KOMURO, 1996; WANG et al., 1999). Although immunoreactivity for neuron specific enolase (NSE) was reported in the ICC of the rat small intestine (PROSSER et al., 1989), no NSE positive cells were detected in the AP and DMP plexuses of the guinea-pig intestine (TOKUI et al., 1992), and there has been no firm evidence indicating the neuronal nature of the ICC so far.

*Ultrastructural distinction from fibroblasts or smooth muscle cells*

The first ultrastructural evidence that the ICC represented a unique type of cells was obtained in a particular specimen, i.e., the love bird gizzard, in which the ICC-CM were characterized by the presence of a basal lamina, gap junctions with smooth muscle cells, and close contact with nerve varicosities (IMAIZUMI and HAMA, 1969). Cells with similar features in mammals (ICC-DMP) were later described in the dog intestine as "hybrid cells" (DUCHON et al., 1974). As this term suggests, ICC were believed to have mixed features of different types of cells. More recent, careful observation has made it possible to identify ICC as a special cell type (see below).

Ultrastructural studies have revealed that different types of ICC possess more or less manifest features which are usually regarded as the characteristics of smooth muscle cells, such as a basal lamina, caveolae, subsurface cisterns and gap junctions. For this reason, certain populations of ICC have been considered as modified or specialized smooth muscle cells by many investigators (YAMAMOTO, 1977; THUNEBERG, 1982, 1989; RUMESSEN and THUNEBERG, 1991; FAUSSONE-PELLEGRINI, et al., 1989; CHIRISTENSEN, 1992; TORIHASHI et al., 1993; KOMURO et al., 1996; SANDERS, 1996) However, the ICC do not contain the well-organized contractile apparatus characteristic of muscle cells, even in those cases where myosin filaments and dense bodies have been demonstrated (TORIHASHI et al., 1993, 1994). Thus, the ICC appear to be distinct from smooth muscle cells. Although the presence or absence of myosin filaments is an important criterion in determining the cytological relationships between the ICC and smooth muscle cells, there are discrepancies between different reports concerning this point. Myosin filaments were reported in the ICC-DMP of the small intestine (TORIHASHI et al.,1993) and in ICC-SMP of the colon (TORIHASHI et al., 1994) from the dog, whereas there were other reports failing to make note of their occurrence in the same species (BEREZIN et al., 1988; DANIEL et al., 1998). Further studies seem to be required.

Some ICC may have an appearance similar to fibroblasts and lack clear muscle-like features. However, even in these cases, they are distinguishable from fibroblast-like cells by a combination of features including large gap junctions, abundant intermediate filaments, numerous mitochondria, flattened cisterns of RER, well-developed SER and a characteristic electron density of the cytoplasm. In fact, the difference between this type of ICC from the fibroblast-like cells was only confirmed by using animals of c-*kit* deficiency or c-Kit receptor blockade (see below), since the most important difference between both cells is the presence or absence of the c-Kit receptor.

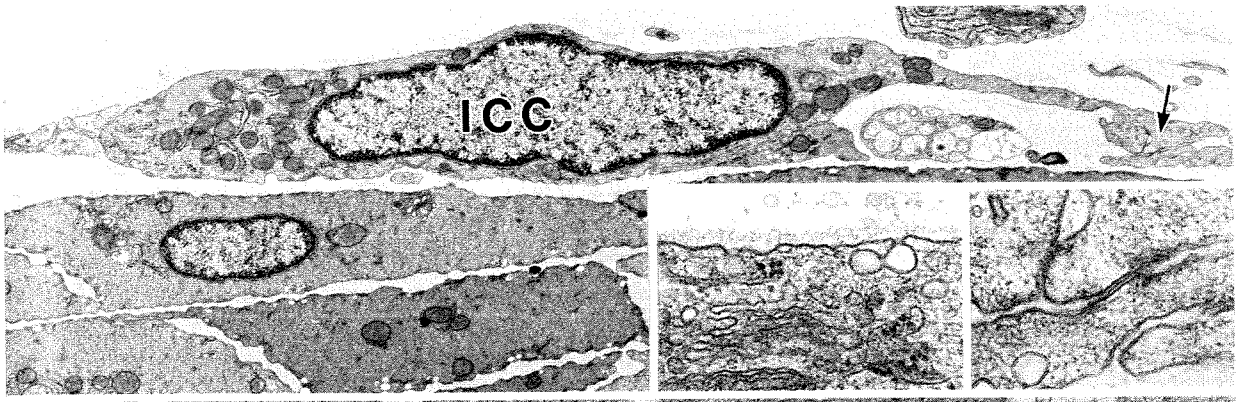
*c-Kit immunoreactivity*

The c-*kit* is a proto-oncogene encoding the receptor tyrosine kinase. Its extracellular domain contains the receptor for a natural ligand, stem cell factor (SCF), and the cytoplasmic tyrosine kinase domain affects important cell behavior such as differentiation via a cascade of cytoplasmic signaling (YARDEN et al., 1987; FANTL et al., 1993). The mouse W locus (CHABOT et al., 1988) and the rat Ws locus (TSUJIMURA et

**Fig. 29.** ICC-SMP (*ICC*) of the rat colon located at the submucosal border of the circular muscle layer. It contains many mitochondria and forms a gap junction with the same type of cell at the tip of a cytoplasmic process (*arrow*). ×9,000. **Left inset** is higher magnification of a part of the ICC-SMP of the rat showing the clearly defined basal lamina and many caveolae. ×52,000. **Right inset** shows higher magnification of the gap junction indicated by *arrow* in the main figuer. ×60,000. (Reproduced under permisson from ISHIKAWA and KOMURO, J. Human Sci., 1998)

**Fig. 30.** ICC-CM (*ICC*) and a fibroblast-like cell (*FL*) in the rat colon. The former is characterized by electron-dense cytoplasm and caveolae (*arrowheads*), while the latter is characterized by electron-lucent cytoplasm and well developed rough endoplasmic reticulum. ×14,000. (Courtesy of Dr. K. ISHIKAWA., Waseda University)

**Fig. 31.** ICC-AP (*ICC*) of the rat colon showing electron-dense cytoplasm, and a gap junction with the same type of cell (*arrow*). ×9,000. **Inset:** Higher magnification of the gap junction indicated by the *arrow*. ×50,000. (Reproduced under permission from ISHIKAWA, Thesis, 1999)

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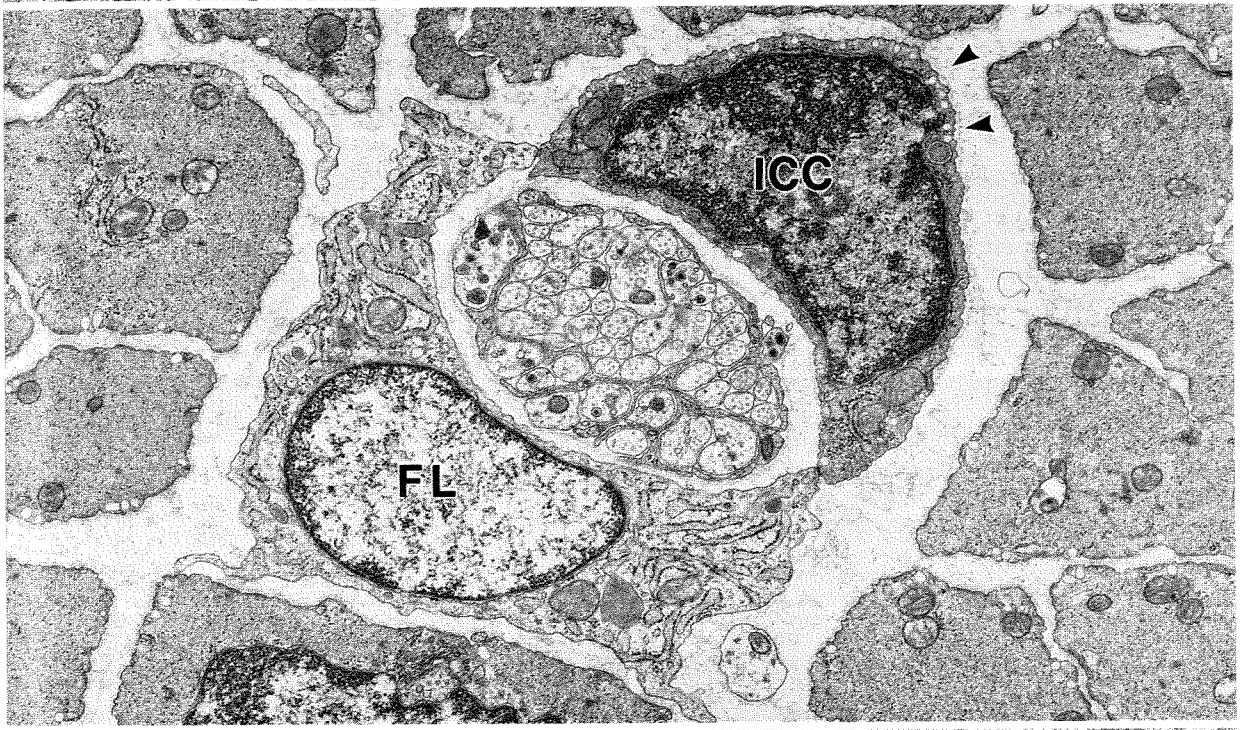


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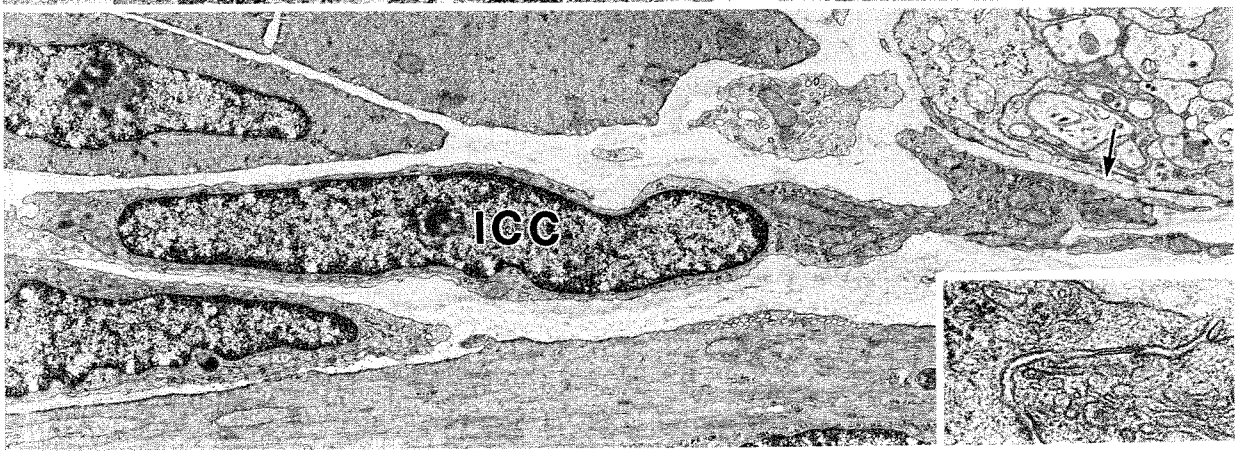


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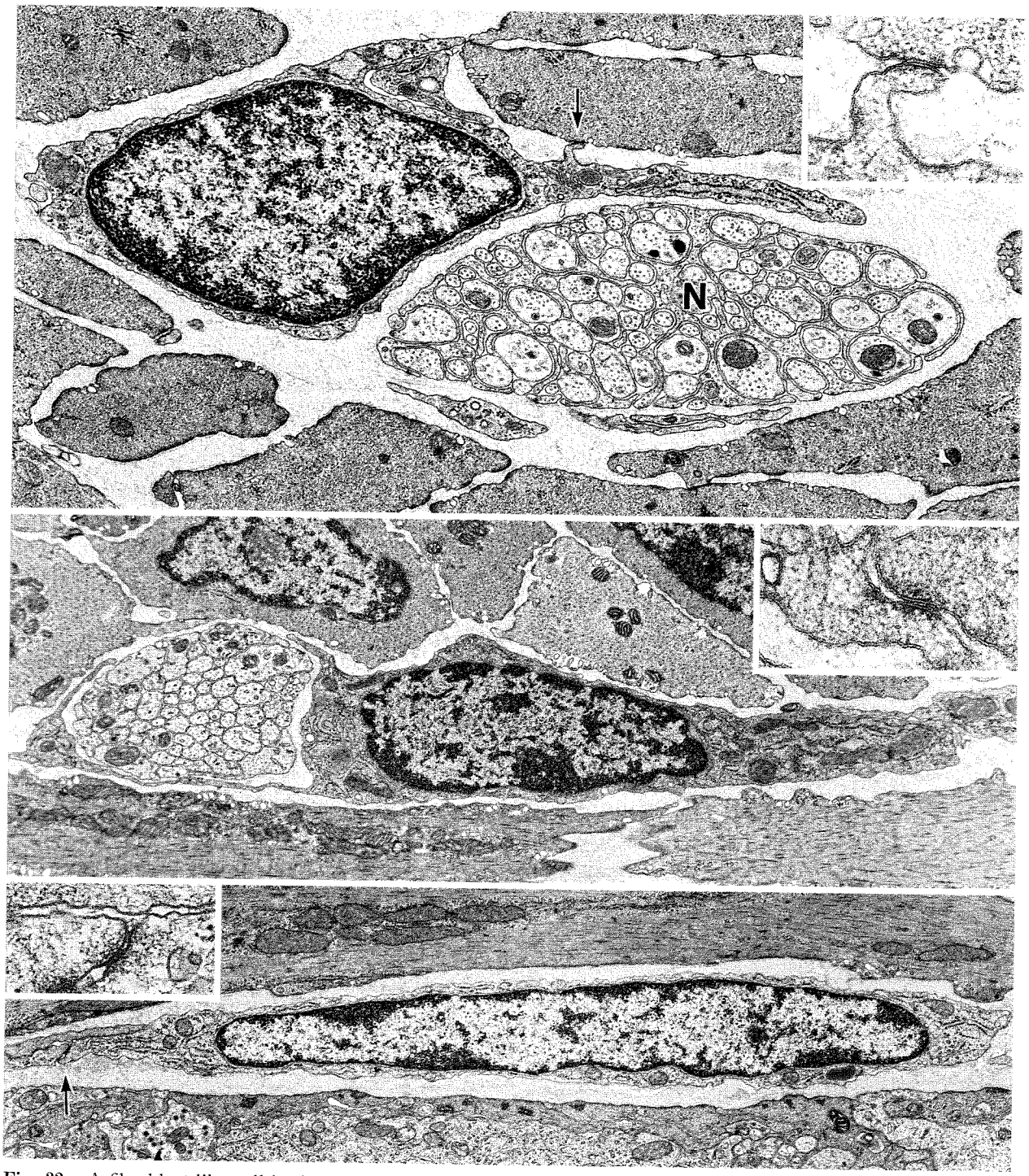


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**Fig. 32.** A fibroblast-like cell in the circular muscle layer of the stomach of Ws/Ws mutant rat, located near a nerve bundle (*N*). Note the less electron-dense cytoplasm and the cistern of RER even in the slender process which forms a small gap junction with a muscle cell (*arrow*). ×17,000. **Inset:** Higher magnification of the gap junction indicated by the *arrow*. ×80,000. (Courtesy of Dr. K. ISHIKAWA, Waseda University)

**Fig. 33.** A fibroblast-like cells in the myenteric region in the small intestine from the W/W$^{v}$ mutant mouse. ×10,500. **Inset:** Higher magnification of a gap junction in the neighboring section of the same cell. ×86,000

**Fig. 34.** A fibroblast-like cell in the myenteric region of the guinea-pig small intestine characterized by well-developed RER. A small gap junction is present at the tip of the process (*arrow*). ×10,000. **Inset:** Higher magnification of the gap junction indicated by the *arrow*. ×50,000

al., 1991) contain the alleles for this receptor. The SCF is known to be encoded by the mouse Sl locus (WILLIAMS et al., 1990; FLANAGAN and LEDER, 1990; ZSEBO et al., 1990).

In studies designed to elucidate the role of c-*kit* in mouse development, MAEDA et al (1992) found that blockade of the function of c-Kit for a few days postnatally with an anti-c-kit antibody resulted in a severe anomally in the gut movement and dramatic reduction in the number of c-*kit* expressing cells. They speculated that c-Kit is required for the development of a pacemaker system for gut motility and that the c-*kit* expressing cells correspond to ICC. Their suggestion was soon strongly supported by combined morphological and physiological studies using mutant $W/W^v$ mice, which have mutations in c-*kit* at the white spotting W locus (WARD et al., 1994; HUIZINGA et al., 1995; MALYSZ et al., 1996; DER-SILAPHET et al., 1998). The absence of a normal network of ICC-AP in the small intestine and loss of slow waves representing pacemaker activity were recognized in $Sl/Sl^d$ mutant mice which lacked the membrane-bound form of SCF (WARD et al., 1995; MIKKELSEN et al., 1998).

Meanwhile, as described above, studies using c-Kit immunostaining and zinc iodide-osmium tetroxide staining, which stain cells in a way similar to methylene blue, demonstrated that the c-*kit* expressing cells in the guinea-pig small intestine actually corresponded to the ICC depicted in the original drawing by CAJAL (KOMURO and ZHOU, 1996; KOMURO et al., 1996). This result clearly indicates that the c-Kit immunostaining is useful and specific for the identification of the ICC.

*Developmental origin:*

Studies using chick-quail chimeras (LECOIN et al., 1996) and studies using transplants of intestinal segments of the mouse embryo (YOUNG et al., 1996) demonstrated that all classes of ICC share a common embryological origin from mesenchymal cells. Recent developmental studies showed that ICC-AP originate from the same mesenchymal progenitor cells expressing c-*kit* as smooth muscle cells of the longitudinal layer (TORIHASHI et al., 1997, 1999b; KLÜPPEL et al., 1998).

## Morphological varieties of ICC

*Tissue specificity*

The present review indicates that ICC in different tissue layers within the same species are not ultrastructurally uniform, but have their own characteristics. The ICC-AP in the small intestine of the rat (Fig. 27; HORIGUCHI and KOMURO, 1998) is classified into the first category of cells which lack both a continuous basal lamina and distinct rows of caveolae, while those structures are clearly seen in the ICC-DMP from the same materials (Fig. 22) which is classified into the second category. As a second category of cells ICC-SMP in both the rat (Fig. 29) and guinea-pig colon (KOMURO, in press) are also characterized by the presence of a basal lamina and many caveolae. Similar features are observed in the ICC-DMP or in the ICC-SMP of the mouse (YAMAMOTO, 1977; THUNEBERG, 1982; RUMESSEN et al., 1982; FAUSSONE-PELLEGRINI et al., 1989; HANANI et al., 1998), dog (BEREZIN et al., 1988, 1989; TORIHASHI et al., 1993, 1994; DANIEL et al., 1998), and of humans (RUMESSEN et al., 1992, 1993a). A third category of cells, the ICC-CM of the rat stomach (Fig. 16; ISHIKAWA et al., 1997) and colon (Fig. 30; KOMURO, in press) are characterized by many caveolae but not by a continuous basal lamina. Thus, the ICC can be classified into three groups in these materials depending on the presence or absence of a basal lamina and distinct rows of caveolae.

*Organ specificity*

The variance in the appearance of ICC in different organs of the same species can be clearly seen by comparing the ICC-AP with a distinct basal lamina in the mouse stomach (Fig. 17 inset) to those of the mouse small intestine which lack a basal lamina (Fig. 28). Similarly, many caveolae are observed in the ICC-AP of the stomach from guinea-pigs and rats (KOMURO, in press) but not in those of the small intestine (KOMURO and ZHOU, 1996; HORIGUCHI and KOMURO, 1998). The cell bodies of the ICC-AP in the dog small intestine were reported to be nearly devoid of caveolae (DANIEL et al., 1998), while those in the dog colon were identified by the presence of caveolae over the entire cell surface (BEREZIN et al., 1989). On the other hand, the ICC-CM in the stomach and colon from both rats and guinea-pigs share very similar features (Figs. 16, 30; KOMURO, in press).

*Species specificity*

Many caveolae and an incomplete basal lamina were observed in the ICC-AP of the small intestine from mice (THUNEBERG, 1982; WARD et al., 1995; HUIZINGA et al., 1995), dogs (TORIHASHI et al., 1993; DANIEL et al., 1998) and humans (RUMESSEN and THUNEBERG, 1991), while they were rarely observed in rats (KOMURO, 1989; HORIGUCHI and KOMURO, 1998) or guinea-pigs (KOMURO and ZHOU, 1996). Prominent accumulations of glycogen granules were observed in

the ICC-DMP of the guinea-pig small intestine, while they have so far never been seen in those of other species. Dense bodies were reported in the ICC-DMP of the small intestine of dogs (TORIHASHI et al., 1993) and humans (RUMESSEN et al., 1992), but so far they have not been demonstrated in those of mice (RUMESSEN et al., 1982; THUNEBERG, 1982), rats (KOMURO and SEKI, 1995; SEKI and KOMURO, 1998) or guinea-pigs (KOMURO and ZHOU, 1996). Many caveolae were observed in the ICC-CM of the stomach and colon in both rats and guinea-pigs (ISHIKAWA et al., 1997; KOMURO, in press), but their absence was reported in the ICC-CM of the dog colon (TORIHASHI et al., 1994).

*Dependency on c-Kit/SCF system of cell maturation*

HORIGUCHI et al. (1995, 1998) demonstrated that ICC-DMP in the Ws/Ws rat small intestine showed no difference in ultrastructure and distribution from their wild-type siblings. The unaffected organization and population of the cells were also reported in the $W/W^v$ mouse (MALYSZ et al., 1996). Similar observations, i. e., a normal distribution of the ICC-DMP, in contrast to the absence or marked reduction in the ICC-AP, were also reported in $Sl/Sl^d$ mice (WARD et al., 1995). It was thus suggested that the ICC-DMP in these animals, could develop independently of the c-Kit/SCF system, unlike the ICC-AP.

## Cytological characterization of ICC

The foregoing remarks stress that the ICC show a certain range of morphological heterogeneity due to different tissue layers, different levels of the digestive tract and different species. However, it is also apparent that all types of ICC comprise a unique entity of cells which could not be classified into any category of cell in traditional histological doctorine. Instead, it can be summarized that the ICC are cells clearly characterized by the presence of numerous mitochondria, abundant intermediate filaments (vimentin) and

**Table 1.** Cytological and ultrastructural features of the three types of ICC, as compared with those of fibroblast-like cells (FL).

| Cell type | c-Kit | BL | CV | GJ | IF | MIT | NC | RER | Examples |
|---|---|---|---|---|---|---|---|---|---|
| **Type 1** Least muscle-like ICC | ++ | − | +− | ++ | ++ | ++ | ++ | + | **ICC-AP** in rat (KOMURO 89; HORIGUCHI & KOMURO 98) and guinea-pig (KOMURO & ZHOU 96) small intestine. |
| **Type 2** Intermediate type ICC | ++ | +− | ++ | ++ | ++ | ++ | ++ | + | **ICC-CM** in rat stomach (ISHIKAWA et al. 97) and colon (ISHIKAWA & KOMURO 98) and guinea-pig stomach (KOMURO 99 in press)<br>**ICC-AP** in mouse small intestine (THUNEBERG 82) |
| **Type 3** Most muscle-like ICC | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | **ICC-DMP** in mouse (THUNEBERG 82; RUMESSEN et al. 82), rat (SEKI & KOMURO 98; HORIGUCHI & KOMURO 98), guinea-pig (ZHOU & KOMURO 92ab), dog (TORIHASHI et al. 93; DANIEL et al. 98) and human (RUMESSEN et al. 92)<br>**ICC-SMP** in mouse (F-PELLEGRINI 87), rat (ISHIKAWA & KOMURO 98), guinea-pig (ISHIKAWA & KOMURO 96) and dog (BEREZIN et al. 88, 89; TORIHASHI et al. 94)<br>**ICC-AP** in mouse pylorus (KOMURO et al., present observation) |
| **FL** | − | − | − | + | + | + | + | ++ | **FL-AP** in rat small intestine (KOMURO 89; HORIGUCHI & KOMURO 98)<br>**FL-DMP** in rat small intestine (KOMURO & SEKI 95; HORIGUCHI & KOMURO 98)<br>**FL-CM** in rat stomach (ISHIKAWA et al. 97), and colon (ISHIKAWA & KOMURO 98) |

c-Kit: immunoreactivity for c-Kit staining, BL: basal lamina, CV: caveolae, GJ: gap junction, IF: intermediate filaments, MIT: mitochondria, NC: close nerve contact, RER: rough endoplasmic reticulum

frequent contacts with each other and with smooth muscle cells by gap junctions, combined with different sets of muscle-like features including a basal lamina, caveolae, subsurface cisterns and dense bodies. Although their differences from other types of cells may occasionally appear rather vague, the ICC are identified without difficulty when the quantitative aspects of cytoplasmic features rather than the qualitative data are considered.

Table 1 summarizes the cytological properties of the three types of ICC and their differences from those of fibroblast-like cells.

## Functional role of ICC

### *ICC-AP*

Following the proposal of the pacemaker hypothesis (THUNEBERG, 1982), evidence gradually accumulated from physiological studies supporting a pacemaker function for ICC-AP or the generation of slow waves from them (HARA et al., 1986; SUZUKI et al., 1986; SMITH et al., 1989). More recently, it has been discovered that c-Kit receptor is essential for the normal development of ICC-AP and for the initiation of slow waves in the mouse small intestine. Experiments using mice with a genetic defect in c-*kit* (WARD et al., 1994; HUIZINGA et al., 1995) or with a postnatal blockade of the receptor with its antibody (MAEDA et al., 1992; TORIHASHI et al., 1995) resulted in a loss of the pacemaker activity. In addition to the small intestine, the pacemaker activity of the ICC-AP was recently shown in the stomach from the mouse (ÖRDÖG et al., 1999) and guinea-pig (DICKENS et al., 1999).

### *ICC-DMP*

Every report indicates that ICC-DMP have a rich innervation and frequent contact with smooth muscle cells via gap junctions. It appears that they are intercalated between nerves and smooth muscle cells. In particular, an intimate relationship between the ICC-DMP and nitrergic neurons was demonstrated in the guinea-pig small intestine (TOMA et al., 1998; WANG et al., 1999). Therefore, it is quite possible that ICC-DMP may act as an accessory route for neuromuscular transmission, as originally suggested by CAJAL (1911).

The functional significance of gap junctions in the ICC-DMP can be evaluated from the evidence that the percentage of the total cell area occupied by gap junctions is 1.3% of the cell surface in rats (SEKI and KOMURO, 1998) and 4% in guinea-pigs (ZHOU and KOMURO, 1992a). These values are about 6 and 20 times higher respectively in comparison with the ratio of the area (0.2%) occupied by gap junctions in smooth muscle cells of the guinea-pig intestine (GABELLA and BLUNDELL, 1979). The gap junction proteins connexin 43 and connexin 45 were abundantly demonstrated in the ICC-DMP of guinea-pigs, rats, and dogs (SEKI et al., 1998; NAKAMURA et al., 1998). The presence of these highly developed gap junctions also seem to be consistent with the notion that the well organized network of ICC-DMP acts as an impulse-conducting system analogous to that in the heart.

### *ICC-SMP*

ICC-SMP have features similar to those of the ICC-DMP. However, the distribution pattern of connexin 43 within the circular muscle layer is totally different between the colon and small intestine in the guinea-pig (Figs. 14, 15; SEKI et al., 1998). SANDERS (1996) proposed a specialized pacemaker function in the colon, with the ICC-SMP primarily responsible for generating the slow waves, and ICC-AP acting as secondary pacemaker cells. RAE et al. (1998) demonstrated a pacemaker activity in human colonic circular muscle layer similar to that observed in dogs.

### *ICC-CM*

This type of ICC probably also functions in neurotransmission to smooth muscle cells, since the ICC-CM possess gap junctions and are in close contact with nerve varicosities. This assumption is compatible with the observation that, in the guinea-pig, the ICC-CM are abundant in the colon, where smooth muscle cells are not connected by gap junctions, while they are very rare in the small intestine, where muscle cells are well coupled via gap junctions (SEKI et al., 1998). WARD et al. (1998) reported that the ICC-CM play an important role in NO-dependent neurotransmission in the lower esophageal and pyloric sphincters in the mouse.

### *ICC-LM*

As only limitted observations are available on the ICC-LM (THUNEBERG, 1982; RUMESSEN and THUNEBERG, 1991; KOMURO et al., 1996; BURNS et al., 1997), their physiological significance remain to be investigated.

## Clinical and etiological aspects of ICC

Some recent studies have investigated the distribution of ICC in the aganglionic segments with abnormal contractility in Hirschsprung's disease, but unequivocal results have not yet emerged. In one study, no differences were observed between the aganglionic

segments and the corresponding areas of intact tissue (HORISAWA et al., 1998), whereas in other studies marked reductions in the number of c-Kit immunoreactive cells were recorded in the aganglionic segments of the human colon (YAMATAKA et al., 1995; VANDERWINDEN et al., 1996). It must be considered, however, that the baseline distribution of the ICC in normal human colon is not yet clear; TORIHASHI et al. (1999a) described large regional differences, whereas HAGGER et al. (1998) a constant distribution throughout the entire colon. Clearly, it is necessary to resolve these discrepancies and to properly determine the precise distribution of ICC in control specimens before we are able to interpret the significance of the data obtained from the pathological specimens.

Defects in ICC have been recorded in other gastrointestinal disorders. In human infantile pyloric stenosis, they were reported to be absent by both electron microscopy (LANGER et al., 1995) and immunohistochemistry (VANDERWINDEN et al., 1996). Marked reduction in number of c-Kit immunoreactive cells was also reported in patients with a myopathic form of chronic idiopathic intestinal pseudo-obstruction (ISOZAKI et al., 1997).

Furthermore, recent evidence has strongly suggested that gastrointestinal stromal tumors are caused by the proliferation of ICC (HIROTA et al., 1998; KINDBLOM et al., 1998; SAKURAI et al., 1999; SEIDAL and EDVARDSSON, 1999). However, VANDERWINDEN et al. (1999) denied this origin on the basis of the ICC lacking any immunoreactivity for sialomucin CD34 which is considered as a marker for the tumors.

## Significance of fibroblast-like cells

Fibroblast-like cells have been reported in the tunica muscularis at different levels of the digestive tract of many species, including the mouse small intestine (THUNEBERG, 1982; RUMESSEN et al., 1982), dog colon (BEREZIN et al., 1990, 1994), and human small intestine and colon (FAUSSONE-PELLEGRINI and CORTESINI, 1983; FAUSSONE-PELLEGRINI et al., 1990; RUMESSEN et al., 1992, 1993a, b). These studies indicated that a specialized membrane contact was lacking between the fibroblast-like cells and smooth muscle cells, and the absence of the contacts was regarded as a useful feature to discriminate them from the ICC.

However, in our observations in mice, rats, and guinea-pigs (HORIGUCHI and KOMURO, unpublished data; KOMURO, 1989; KOMURO and SEKI, 1995; ZHOU and KOMURO, 1992a, b), fibroblast-like cells equipped with small gap junctions could be found in every tissue layer of every organ where ICC were located. It is not yet clear whether the discrepancies between the observations represent differences between species or whether the specialized membrane contact is a general feature of the fibroblast-like cells overlooked by other research groups.

Providing that the fibroblast-like cells in the gut musculature generally form gap junctions with surrounding smooth muscle cells, they probably are involved in the gastrointestinal motility. It seems reasonable to suggest that their gap junctions act as routes for intercellular communication, passing electrical or molecular signals for muscle contraction, as suggested previously (KOMURO, 1989, 1990; KOMURO and SEKI, 1995; HORIGUCHI and KOMURO, 1998). Synapse-like close contact between the fibroblast-like cells and nerve varicosities containing many synaptic vesicles was demonstrated in the guinea-pig small intestine (ZHOU and KOMURO, 1992b).

A remarkable feature of the fibroblast-like cells is their ultrastructure being rather uniform irrespective of the tissue layer, organ, or species in which they are found. This fact suggests that they may have a fundamental, and possibly more general role than ICC in the regulation of the gut motility.

The fibroblast-like cells of the myenteric region probably correspond to the flattened cells closely associated with the myenteric plexus in rats and guinea-pigs demonstrated by scanning electron microscopy (Figs. 25, 26; BALUK and GABELLA, 1987; KOMURO, 1989; JESSEN and THUNEBERG, 1991). Further studies are required to investigate the possible contribution of the cells to the entire regulatory system of the gut musculature, which is currently ascribed mainly to nerves and ICC.

## Concluding remarks

The present review indicates a marked heterogeneity in phenotypes of the ICC, ranging from those most similar to smooth muscle cells (ICC-DMP and ICC-SMP) to those with the least muscle-like features (ICC-AP), an intermediate type of cells (ICC-CM) being added. Yet, all types of the ICC differ in ultrastructure from typical fibroblasts and from smooth muscle cells. In spite of their heterogeneity in ultrastructure and dependency on the c-Kit/SCF system for cell maturation, ICC share a common embryological origin from mesenchymal cells (LECOIN et al., 1996; YOUNG et al., 1996). ICC are identified as such and differ from fibroblast-like cells and smooth muscle cells by expressing c-Kit receptors on their cell surface in normal adult animals. Part of their heterogeneity is apparently due to the microenvironment that they live in, including the effects of mechanical force, type of nerve supply, spatial relationships with

muscle cells, the feeding habits of the animal and the movement patterns of the gut. Overall, the data suggest that there is more to unite rather than to divide the various types of ICC. Thus, although ICC have had a long and chequered history since their first description, it is now high time to deal with them as a structurally and functionally specialized family of cells involved in such functions as pacemaking, intercellular communication and mediation of nerve impulses.

**Acknowledgments.** The author wishes to thank Dr. Peter BALUK, Cardiovascular Research Institute, University of California, San Francisco, USA for reading the manuscript.

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# Properties of gastric smooth muscles obtained from mice which lack inositol trisphosphate receptor

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(Received 23 December 1999; accepted after revision 1 March 2000)

1. Membrane potential recordings, made from the circular smooth muscle layer of the gastric antrum taken from mutant mice which lacked the inositol trisphosphate ($Ins P_3$) type 1 receptor, were compared with those obtained from the stomach of control (wild-type) mice.

2. Immunostaining of gastric muscles indicated that the distribution and form of c-kit positive cells were similar in wild-type and mutant mice.

3. Smooth muscles from wild-type mice generated slow waves that in turn initiated spike potentials, while those from mutant mice were either quiescent or generated irregular bursts of spike potentials. In the presence of nifedipine, slow waves with reduced amplitude were generated in wild-type mice, while all electrical activity was abolished in mutant mice.

4. Acetylcholine depolarized and sodium nitroprusside hyperpolarized the membrane in muscles from both types of mice, being more effective in wild-type mice. Noradrenaline produced similar hyperpolarizations in both types of mice.

5. Transmural nerve stimulation evoked inhibitory junction potentials (IJPs) in both wild-type and mutant mice. In wild-type mice, the IJPs were reduced in amplitude by nitroarginine and converted to a cholinergic excitatory junction potential (EJP) by apamin. In mutant mice, the IJPs were unaffected by nitroarginine or atropine but were abolished by apamin.

6. It is concluded that in antral smooth muscle, the expression of $Ins P_3$ type 1 receptors may be causally related to the generation of slow waves but not to the generation of action potentials. A lack of $Ins P_3$ receptors attenuates cholinergic excitatory and nitrergic inhibitory responses but does not alter the response to noradrenaline.

Gastric smooth muscles are spontaneously active, and they rhythmically generate slow waves and action potentials (Tomita, 1981). The action potentials are inhibited by organic L-type $Ca^{2+}$-channel antagonists such as nifedipine and diltiazem, whilst the slow waves are insensitive to $Ca^{2+}$-channel antagonists (Ishikawa *et al.* 1985; Dickens *et al.* 1999). Interstitial cells of Cajal (ICC) are considered to trigger rhythmical activity, since animals which lack ICC have digestive disorders and lack rhythmic contractile activity in the gatrointestinal tract (Sanders, 1996; Huizinga *et al.* 1997).

Gastrointestinal smooth muscles receive cholinergic and non-adrenergic non-cholinergic (NANC) projections, as well as on some occasions an adrenergic projection (Furness & Costa, 1987). Transmural nerve stimulation (TNS) evokes cholinergic excitatory junction potentials (EJPs) and non-adrenergic, non-cholinergic (NANC) inhibitory junction potentials (IJPs) in isolated preparations of rat and guinea-pig stomach muscle (Komori & Suzuki, 1986; Xue *et al.* 1996). The cholinergic depolarization is thought to result from the activation of cation selective channels following the activation of a muscarinic receptor that is coupled to a

G-protein. At some point the pathway involves the second messenger inositol trisphosphate ($InsP_3$) (Bolton & Large, 1986). IJPs appear to involve the co-release of nitric oxide (NO) and a second unidentified substance which activates apamin-sensitive $K^+$ channels (Ohno *et al.* 1996).

The present experiments were carried out to find out how the properties of gastric smooth muscle and the functioning of its different innervations were changed in mutant mice which lacked $InsP_3$ type 1 receptors.

## METHODS

Mice lacking type 1 $InsP_3$ receptors (mutant mice) were bred using the method reported previously (Matsumoto *et al.* 1996). All experiments were carried out using 18- to 22-day-old mutant and age-matched wild-type littermates. All animals were handled and treated according to the rules of the JST Animal Use and Care Committee. Mice were anaesthetized with ethyl ether, and exsanguinated by cutting the femoral artery. The stomach was excised and, after opening, the mucosal layer was removed. Pieces of muscle wall, cut in a circular orientation (about 1 mm width, 5 mm long) were dissected from the antrum region. As such the preparations contained both the circular and longitudinal muscle layers.

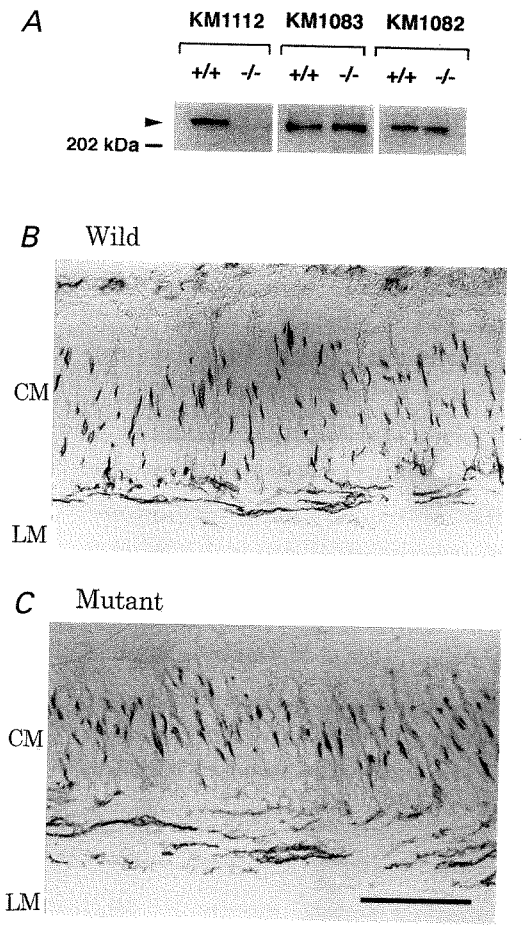


**Figure 1. Expression of $InsP_3$ receptor proteins and c-kit positive cells in the antrum**

*A*, Western blot analysis showing the expression of proteins for type 1 (KM 1112), type 2 (KM 1083) and type 3 (KM 1082) $InsP_3$ receptors in the antrum muscles from wild-type (+/+) and mutant mice (−/−) (arrowhead indicates 250 kDa level). *B* and *C*, longitudinal sections of the stomach antrum obtained from 18-day-old wild-type and mutant mice, respectively, showing cells stained by immuno-histochemical reactions for c-kit. CM, circular muscle layer; LM, longitudinal muscle layer. Scale bar, 100 $\mu$m.

The presence or absence of $InsP_3$ receptors in antral muscles was checked by the methods reported previously (Li *et al.* 1996). Briefly, segments of the antrum were pulverized in liquid nitrogen, and then homogenized in 5 ml of 10 mM ice-cold Tris-HCl (pH 8·0 at 0 °C) containing 0·32 M sucrose, 1 mM EDTA, 0·1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM pepstatin A, 10 mM leupeptin and 1 mM 2-mercaptoethanol. The homogenates were centrifuged at 2000 *g* for 5 min at 4 °C. The supernatants were further centrifuged at 90 000 *g* for 20 min at 2 °C. The pellets were resuspended in 50 mM of Tris-HCl (pH 8·0 at 0 °C) containing 1 mM EDTA and 1 mM 2-mercaptoethanol. The membrane fraction dissolved in the sample buffer (final concentrations: 2% SDS, 10% 2-mercaptoethanol, 6·25 mM Tris-HCl, 0·02% Bromophenol Blue and 10% glycerol; pH 6·8) was subjected to 5% SDS-PAGE, followed by electroblotting onto nitrocellulose membranes (Hybond TM-ECL, Amersham, UK). The blots were blocked with skimmed milk and immunoreacted with the mouse antibody KM 1112 for type 1, KM 1083 for type 2 and KM 1082 for type 3 (Sugiyama *et al.* 1994), and then with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Amersham).

Immunostaining for c-kit was performed by the methods reported previously (Komuro & Zhou, 1996; Seki *et al.* 1998). Briefly, segments of antrum muscles were moderately inflated with injections of OCT compounds and immediately frozen with liquid nitrogen in the embedding medium. Sections (10 $\mu$m thick) were cut with a Micron HM 505E cryostat, and mounted on glass slides. The specimens were fixed with acetone for 10 min at room temperature, rinsed in phosphate-buffered saline (PBS) several times and incubated with 4% Block Ace solution (Dainippon Seiyaku, Osaka, Japan) for 20 min at room temperature to prevent non-specific antibody binding. Then, the specimens were incubated overnight at 4 °C with the rat monocronal antibody against c-kit (ACK-2, Gibco BRL, Gaitherburg, MD, USA) at a dilution ratio of 1:200. After rinsing in PBS several times, the specimens were incubated overnight at 4 °C with peroxidase-conjugated secondary antibody (rabbit anti-rat IgG; DAKO, Glostrup, Denmark) at a dilution ratio of 1:80. Horseradish peroxidase reaction was developed in 50 ml 0·1 M Tris-HCl buffer (pH = 7·4) solution containing 6 mg 4-chloro-naphthol (Sigma, USA) and 8 $\mu$l 30% $H_2O_2$. Control tissues were processed in a similar manner, but the primary incubation solution did not contain ACK-2.

For the electrophysiological experiments, the preparations were pinned on a rubber plate using tiny pins in the recording chamber with the mucosal layer uppermost. The tissue was superfused with warmed (36 °C) oxygenated Krebs solution (composition (mM): $Na^+$ 137·4, $K^+$ 5·9, $Ca^{2+}$ 2·5, $Mg^{2+}$ 1·2, $HCO_3^-$ 15·5, $H_2PO_4^-$ 1·2, glucose 11·5, $Cl^-$ 135) at a constant flow rate (about 3 ml $min^{-1}$). Solutions were aerated with 95% $O_2$–5% $CO_2$, which maintained the pH of the solution at 7·2–7·3. Intracellular recordings were made using conventional microelectrode techniques. Intramural nerves were stimulated using brief electrical pulses transmurally using methods described previously (Komori & Suzuki, 1986). The selectivity of nerve stimulation was routinely confirmed by checking that the responses could be abolished by adding tetrodotoxin (0·5 $\mu$M) to the physiological saline. Drugs used were acetylcholine chloride (ACh),

apamin, guanethidine sulfate, nifedipine, noradrenaline hydrochloride (NA), propranolol hydrochloride, tetrodotoxin (Sigma Chemical Co, St Louis, MO, USA), atropine sulfate (Merck, Germany), $N^{\omega}$-nitro-L-arginine (nitroarginine, Peptide Institute, Osaka, Japan) and phentolamine mesylate (CIBA Geigy, Basel, Switzerland).

All measured values were expressed as the means ± standard deviation (S.D.). The statistical difference between the measured values was tested using Student's $t$ test, and probabilities less than 5% were considered significant.

## RESULTS

Western blot analysis performed to verify the expression of $InsP_3$ receptor proteins using type-specific mouse antibodies, KM 1112 for type 1, KM 1083 for type 2 and KM 1082 for type 3, indicated that the immunoreactive band for KM 1112 was detected at 250 kDa with cell fragments obtained from wild-type mice, but this band was absent in mutant mice. Proteins for KM 1083 and KM 1082 were found expressed, in both wild-type and mutant mice (Fig. 1*A*). These observations show that whilst $InsP_3$ type 1 receptors are present in the antral muscles of wild-type mice they are absent in those taken from mutant mice. Type 2 and type 3 $InsP_3$ receptor proteins were expressed in both wild-type and mutant mice.

Immunostaining for c-kit, which is a reliable marker for ICC (Komuro, 1996), indicated that in the antrum from wild-type mice the c-kit positive cells were distributed in the myenteric plexus and within both the circular and longitudinal muscle layers (Fig. 1*B*). The cells characteristically had elongated cell bodies with a few long cytoplasmic processes (Komuro, 1996). Immunoreactive cells were also found in the antrum from the mutant mice without detectable differences in the structure and distribution (Fig. 1*C*). These results indicate that the development of ICC is not impaired in the absence of $InsP_3$ type 1 receptors.

The circular smooth muscle isolated from the antrum of wild-type mice generated rhythmic oscillations of the membrane potential, termed slow waves (Tomita, 1981) (Fig. 2*A*). The amplitude of slow waves varied between 4·1 and 21·2 mV (mean, 12·9 ± 0·5 mV, $n = 24$) and their frequency varied between 3·0 and 9·8 waves $min^{-1}$ (mean, 5·5 ± 1·6 waves $min^{-1}$, $n = 24$). The membrane potential at its most negative value (the resting membrane potential) ranged between −50 and −70 mV (mean, −57·4 ± 0·8 mV, $n = 14$). Often bursts of spike potentials with amplitudes of 5–20 mV were generated during the peak of the slow wave. These membrane potential changes persisted when tetrodotoxin (0·3 μM) was added to the physiological saline ($n = 3$, data not shown), indicating that they were myogenic in origin. Nifedipine (1 μM) abolished the spike potentials and reduced the amplitude of slow waves, without changing the resting membrane potential (Fig. 2*B*).

In mutant mice, the resting membrane potential ranged between −48 and −58 mV (mean, −51·7 ± 4·5 mV, $n = 11$), with the values being significantly different from those of

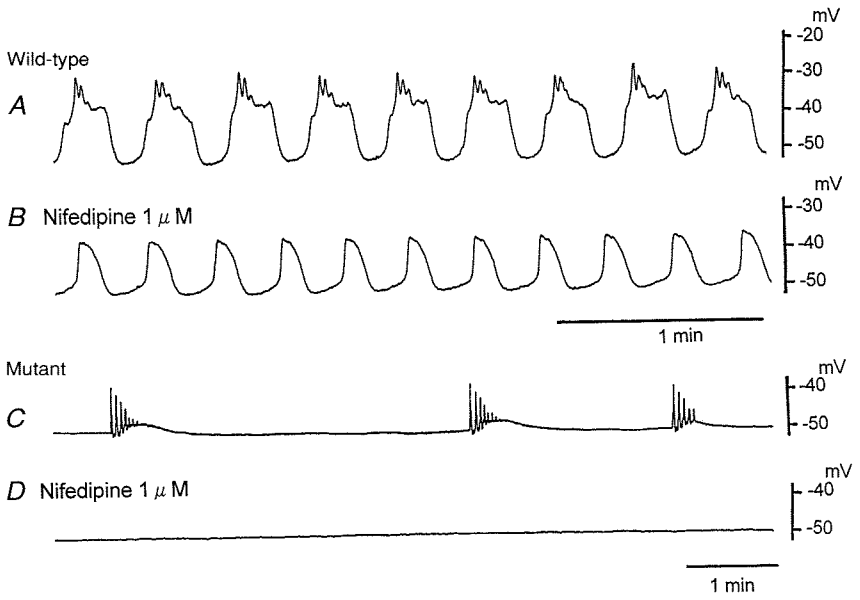


**Figure 2. Spontaneous activity of antrum smooth muscle cells**

Spontaneous activities recorded from the antral smooth muscle of wild-type and mutant mice, before (*A* and *C*, respectively) and after (*B* and *D*, respectively) application of nifedipine (1 μM). *A* and *B* were recorded from a single cell in the antral region of a 20-day-old wild-type mouse, *C* and *D* from a single cell of a 19-day-old mutant mouse. Amplitude of slow wave in wild-type mice: control, 12·3 ± 0·5 mV, $n = 16$; in nifedipine, 10·8 ± 0·4 mV, $n = 19$ ($P < 0·05$). Membrane potential in wild-type mice: control, −56·8 ± 2·2 mV, $n = 12$; in nifedipine, −57·1 ± 2·8 mV, $n = 7$ ($P > 0·05$). Membrane potential in mutant mice: control, −53·2 ± 1·8 mV, $n = 8$; in nifedipine, −53·0 ± 2·2 mV, $n = 5$ ($P > 0·05$).

wild-type mice ($P < 0{\cdot}05$). Antral smooth muscles from mutant mice failed to generate slow waves. In about half the preparations, the membrane potential was stable ($n = 5$); in the others ($n = 6$), groups of spike potentials were superimposed on irregularly occurring small depolarization (Fig. 2*C*). All spontaneous activity, recorded from antral muscle of the mutant mice, was abolished by nifedipine (Fig. 2*D*), with no significant alteration of the membrane potential.

Stimulation of antral muscles isolated from wild-type mice with a brief electrical pulses, in the interval between slow waves, evoked an inhibitory junction potential (IJP) (Fig. 3*A*). Nitroarginine (10 $\mu$M) reduced the amplitude of IJP by about 30 % (Fig. 3*B*). The IJPs were unchanged when either propranolol (1 $\mu$M), phentolamine (1 $\mu$M) or guanethidine (5 $\mu$M) was added to the physiological saline (data not shown). Apamin (0·1 $\mu$M) converted the IJP to an excitatory junction potential (EJP) (Fig. 3*C*). The membrane depolarizations evoked in the presence of apamin were abolished by atropine (1 $\mu$M, Fig. 3*D*). These results indicate that gastric smooth muscle of wild-type mice is innervated by cholinergic excitatory and non-adrenergic non-cholinergic (NANC) inhibitory nerves. One of the inhibitory transmitters may be nitric oxide (NO). Both NO and the unidentified NANC inhibitory transmitter activate an apamin sensitive increase in potassium conductance. When the effects of the inhibitory transmitters are blocked then the effect of stimulating the cholinergic excitatory projection is apparent, EJPs are detected and these are abolished by atropine.

In mutant mice, IJPs were also evoked by nerve stimulation. The IJPs were abolished in the presence of apamin (0·1 $\mu$M). Unlike the recordings from wild-type mice, abolishing the IJP did not reveal an EJP (Fig. 3*E* and *F*). Furthermore neither nitroarginine (10 $\mu$M) nor atropine (1 $\mu$M) altered the

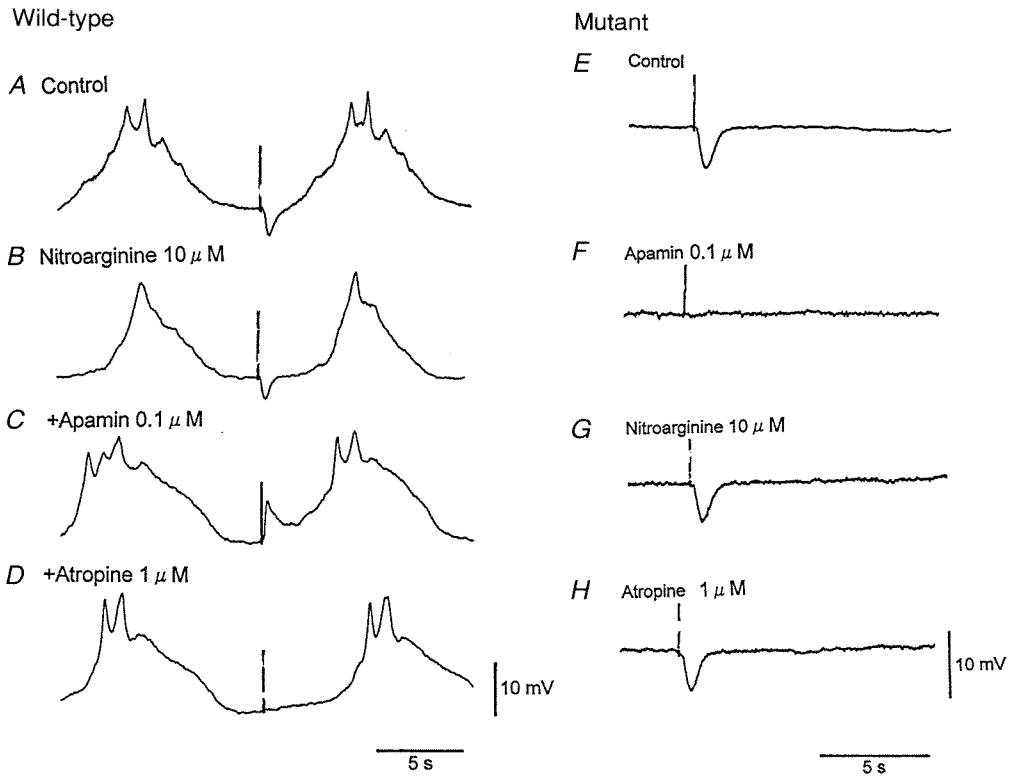


**Figure 3. Junction potentials recorded from antrum smooth muscles**

*A–D*, recordings from an 18-day-old wild-type mouse; *E–H*, recordings from a 20-day-old mutant mouse. In wild-type mice, transmural nerve stimulation (0·05 ms duration, 30 V intensity, 3 stimuli at 20 Hz frequency) was applied. Junction potentials evoked in control solution (*A*) and in the presence of 10 $\mu$M nitroarginine (*B*), 0·1 $\mu$M apamin (*C*), and 1 $\mu$M atropine (*D*). After the addition of each drug the cell remained in the physiological saline (membrane potential, −58 mV). Mean amplitude of IJPs ($n = 8$); control, 5·7 ± 1·8 mV; in nitroarginine, 4·2 ± 1·0 mV, $P < 0{\cdot}05$; in atropine, 6·1 ± 1·3 mV, $P > 0{\cdot}05$. Mean amplitude of EJPs: in apamin, 6·5 ± 2·5 mV; in atropine with apamin, 0 mV. In mutant mice, transmural stimulation (0·05 ms duration, 30 V intensity, 2 stimuli at 20 Hz frequency) was applied before (*E*) and after (*F*) application of 0·1 $\mu$M apamin, 10 $\mu$M nitroarginine (*G*) or 1 $\mu$M atropine (*H*). Responses *E–H* were recorded from the same cell; membrane potential, −50 mV. Amplitude of IJPs ($n = 5$); control, 4·3 ± 1·7 mV, in apamin, 0 mV; in nitroarginine, 4·1 ± 1·7 mV ($P > 0{\cdot}05$); in atropine, 4·5 ± 1·2 mV ($P > 0{\cdot}05$).

IJPs (Fig. 3*G* and *H*, respectively), indicating that the contribution of nitrergic and cholinergic projections was insignificant. IJPs were also unaffected by guanethidine (5 $\mu$M).

The membrane potential changes elicited by acetylcholine (ACh), noradrenaline (NA) and sodium nitroprusside (SNP) were measured in antrum smooth muscles. In both wild-type and mutant mice, ACh (0·1–10 $\mu$M) depolarized the membrane in a concentration-dependent manner; the depolarization in each concentration was significantly smaller in mutant mice than in wild-type mice (Fig. 4*A*, *C* and *E*). In mutant mice, the depolarization was invariably associated with the discharge of spike potentials (Fig. 4*C*). The ACh-induced depolarization was abolished by 1 $\mu$M atropine (data not shown). NA in concentrations of 1 $\mu$M or higher hyperpolarized the membrane in wild-type (Fig. 4*B*) and mutant mice (Fig. 4*D*), to a similar extent (Fig. 4*F*). The responses to NA were inhibited by phentolamine (1 $\mu$M) but not by propranolol (1 $\mu$M) (data not shown). In wild-type mice, SNP (1 $\mu$M), an NO donor, hyperpolarized the membrane by 6·4 ± 1·4 mV ($n = 7$) and prevented the generation of slow waves. However, the SNP-induced hyperpolarization was significantly smaller in mutant mice (2·7 ± 0·7 mV, $n = 6$, $P < 0·05$).

## DISCUSSION

The present experiments show that antral smooth muscle of mice lacking $InsP_3$ type 1 receptors differ from those of the wild-type in three ways. The preparations lacked slow waves, and appeared to lack a cholinergic transmission and the response to NANC inhibitory nerve stimulation was changed in that a nitrergic component was absent. These changes were not associated with a detectable change in sensitivity to applied NA but were associated with a reduced responsiveness to applied ACh and SNP.

The cellular mechanism underlying the spontaneous activity of gastrointestinal smooth muscle cells remains unclear. In mice, inhibiting the development of ICC by injecting antibodies for the Kit receptor protein or breeding mutant mice which lack ICC disrupts the normal pattern of spontaneous activity (Maeda *et al.* 1992; Ward *et al.* 1994; Torihashi *et al.* 1995). Cultured ICC show spontaneous activity (Tokutomi *et al.* 1995; Thomsen *et al.* 1998). These observations suggest that the pacemaker cells responsible for the spontaneous activity may be the ICC (Sanders, 1996; Huizinga *et al.* 1997). In the guinea-pig stomach antrum, ICC appear to fulfil the role of pacemaker cells (Dickens *et al.* 1999). However, the antrum smooth muscles also possess the ability of generating spontaneous activity (Suzuki & Hirst, 1999).

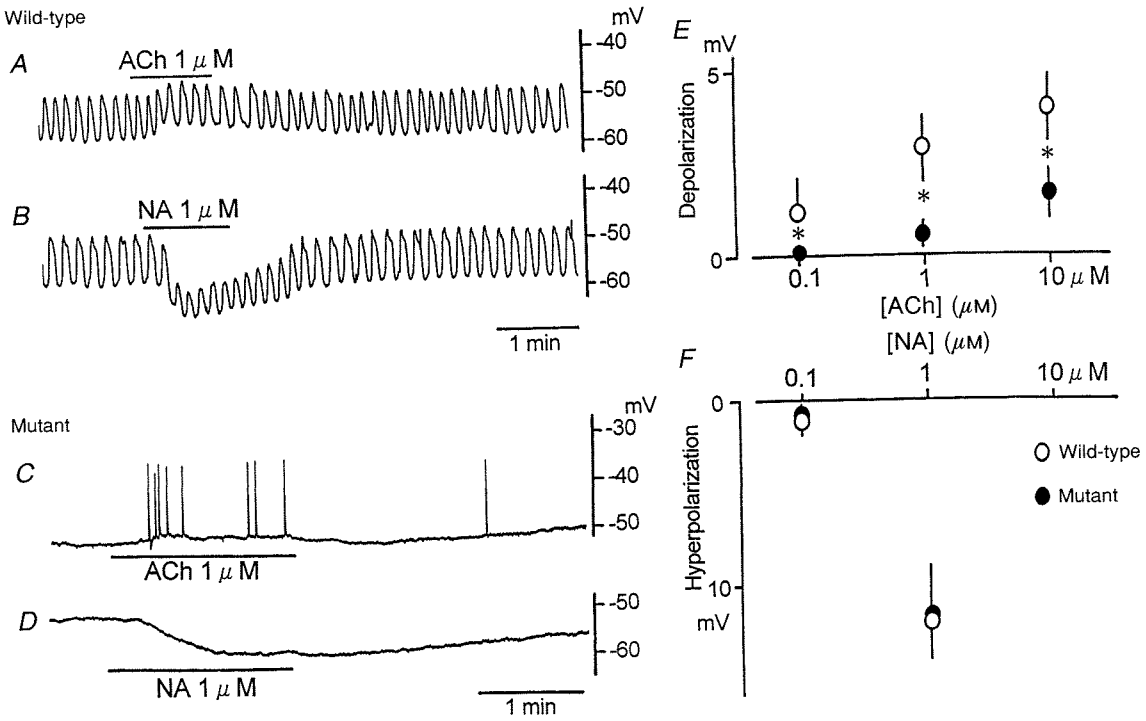


**Figure 4. Membrane responses evoked by acetylcholine and noradrenaline in antral smooth muscles**

Membrane potential changes recorded in antral muscle of 19-day-old wild-type (*A* and *B*) and mutant (*C* and *D*) mice, in response to acetylcholine (ACh) or noradrenaline (NA). ACh (1 $\mu$M) was applied in *A* and *C*. NA (1 $\mu$M) was applied in *B* and *D*. The resting membrane potential: *A* and *B*, −58 mV; *C* and *D*, −50 mV. The graphs show the mean responses to ACh (*E*) and NA (*F*) when applied to antral smooth muscles of wild-type (○) or mutant mice (●). * Significant difference between wild-type and mutant mice ($P < 0·05$).

The present experiments indicated that slow waves are absent in gastric muscle of the mutant mice. As the development of ICC is not impaired in the mutant mice, Ins$P_3$ type 1 receptors may play a key role in the generation of slow waves, either within ICC or in smooth muscle cells. In the guinea-pig stomach, the generation of much of the slow wave is inhibited by depleting $Ca^{2+}$ from the internal store with caffeine, inhibiting $Ca^{2+}$-ATPase at the sarcoplasmic reticulum (SR) membrane with cyclopiazonic acid or chelating intracellular $Ca^{2+}$ with BAPTA (Dickens *et al.* 1999; Suzuki & Hirst, 1999). These observations suggest that the release of $Ca^{2+}$ from internal stores is involved in the generation of slow waves. Similarly the activity of smooth muscle in lymphatic vessels (Van Helden, 1993) or the urethra (Hashitani *et al.* 1996) is initiated by depolarization of the membrane through the opening of $Ca^{2+}$-activated $Cl^-$ channels after the release of $Ca^{2+}$ from intracellular stores. Although $Cl^-$ channels do not seem to be involved in the generation of slow waves in the guinea-pig stomach (Suzuki *et al.* 1999), the release of $Ca^{2+}$ from internal stores may be a common factor in the initiation of activity in several smooth muscle cells. The release of $Ca^{2+}$ from internal stores frequently involves the activation of Ins$P_3$ receptors on the SR (Berridge, 1993). Thus we suggest that the generation of slow waves is initiated by a release of $Ca^{2+}$ from internal stores following the activation of Ins$P_3$ receptors. A similar suggestion has been made on circular smooth muscle of the antrum in which depolarization of the membrane elicits regenerative potentials after a long latency (about 1 s) only when functioning of SR has not been disrupted (Suzuki & Hirst, 1999).

Gastric smooth muscle receives excitatory and inhibitory innervations, and stimulation of these nerves evokes cholinergic EJPs, nitrergic IJPs and NANC IJPs in smooth muscles (Komori & Suzuki, 1986; Ohno *et al.* 1994; Xue *et al.* 1996). The present experiments showed that the lack of Ins$P_3$ type 1 receptors is accompanied by impaired cholinergic and nitrergic transmission in the mouse stomach. The sensitivity of smooth muscles to both the stimulating effect of ACh and the inhibitory effect of NO was reduced in mutant mice, and these alterations could partly explain the changes which appeared in the junction potential. However it remains unclear whether the impaired junctional transmission in the mutant mice reflects the impaired development of cholinergic and nitrergic nerves. The finding that the NANC component of the inhibitory response was unaltered suggests that this is not the case.

It is concluded that in gastric smooth muscle of mice, a lack of type 1 Ins$P_3$ receptors is associated with an inability to generate slow waves and an impairment of cholinergic and nitrergic transmissions, with no significant change in the adrenergic pathway. There are three subtypes of Ins$P_3$ receptors which are distributed heterogeneously in many cells (Berridge, 1993; Furuichi *et al.* 1994; Mikoshiba *et al.* 1996). Each of these subtypes of Ins$P_3$ receptors differs in its functions and properties for the release of $Ca^{2+}$ from internal stores (Miyakawa *et al.* 1996). It remains unclear whether the alteration of membrane properties appearing in gastric smooth muscle of the mutant mice has a direct causal relationship with the absence of type 1 Ins$P_3$ receptors or a quantitative imbalance of Ins$P_3$ receptor subtypes.


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## Acknowledgements

The authors are grateful to Miss A. Hoshino and M. Saito for identifying the genotypes of the mutant mice and care of the animals. The authors are also grateful to Professor G. D. S. Hirst, The University of Melbourne, for critical reading of the manuscript.

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論　文

# ラット小腸深部筋神経叢カハールの介在細胞のNO作動性神経支配

川　瀬　里　美*・小　室　輝　昌**

本研究では，小腸深部筋神経叢ICC（ICC-DMP）の刺激伝達機能を検討するため，NADPH-diaphorase（NADPH-d）組織化学を用い，ラット小腸の微細形態学的検索を行った。その結果，ラットの深部筋神経叢ICC-DMPにはNADPH-d陽性の神経終末が密接している像が多数観察された。NADPH-d陽性反応は神経終末の細胞膜上に発現し，終末内には多数のシナプス小胞が認められた。シナプス小胞の種類としては直径40～50 nmの円形の無芯小胞と直径100～140 nmの大型の有芯小胞が観察された。また，NADPH-d陽性の神経終末には平滑筋に直接密接しているものも観察された。これらの観察をもとにICC-DMPのNO作動性神経による消化管運動調節機構について考察した。

**キーワード**：カハールの介在細胞（ICC），NO，微細構造，小腸，ラット

## 1. 緒　　言

胃，小腸など消化管に見られる蠕動運動は，外来性の交感・副交感神経の影響下に，内在する腸管神経系の無数のニューロンによって調節されていることが明らかにされてきたが（Costa, et al. 1987），これに加えて注目を集めてきたカハールの介在細胞（interstitial cells of Cajal; ICC）の関与についての解明も進み，最近では，小腸アウエルバッハ神経叢部（Auerbach's plexus; AP）のICC-APに関しては消化管ペースメーカーとして働くことが定説となりつつある（Thuneberg, et al. 1995；Sanders 1996；Komuro 1999）。

このICC研究の急速な進展は，造血細胞や肥満細胞の分化，増殖に重要な役割を果たすことが知られていたc-kit遺伝子が，ICCの細胞分化にも関与していることが報告され（Maeda, et al. 1992；Ward, et al. 1994），細胞膜上に発現されるc-Kit受容体がICCの同定上，信頼性の高い細胞学的標識として使用可能となったことが挙げられる（Komuro, et al. 1996）。

一方，アウエルバッハ神経叢と並んでICCのよく発達した部位として知られる小腸深部筋神経叢（deep muscular plexus; DMP）のICC-DMPは，ICC-APが欠損し，消化管運動に異常の見られる *c-kit* 突然変異動物でもほぼ正常に存在することから（Malysz, et al. 1996; Horiguchi and Komuro 1998），ペースメーカー機構への直接の関与は否定的である。他方，ICC-DMPは同種細胞相互，または平滑筋との間で多数のgap junctionを形成することや，腸管神経の軸索膨大部に密接していることから（Rumessen, et al. 1982；Zhou and Komuro 1992；Komuro and Seki 1995；Seki and Komuro 1998），腸管神経系のmediatorとして機能することが推測されている（Komuro, et al. 1996；Sanders 1996）。

ところで，消化管平滑筋の収縮を抑制する非アドレナリン非コリン作動性（NANC）神経伝達物質の一つとしてNO（一酸化窒素）が知られているが，小腸深部筋神経叢ではICC-DMPを介しての支配も推定されている（Desai, et al. 1991；Sanders and Ward 1992；Waterman and Costa 1994）。

そこで，本研究では，同定上NO synthase（NOS）組織化学と等価であることが認められている，

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NADPH-diaphorase（NADPH-d）組織化学（Brookes 1993）を用い，ラット小腸深部筋神経叢ICC-DMPのNO神経支配について微細形態学的検索を行った。

## 2. 材料および方法

生後4週齢の雌Wistarラット10匹を用いた。

**（1） 電子顕微鏡試料作製法**

エーテル麻酔下に近位空腸を摘出し，腸間膜付着部に沿って切開し，中程度に伸展した状態にピンでとめた後，0.1 M燐酸緩衝液（pH 7.3）で緩衝した4％ホルムアルデヒド・3％グルタールアルデヒドを含む固定液に浸漬して固定を行った。約10分後，2×3 mmほどの小片に細切後，同じ固定液でさらに固定した（4°C，2 h）。次いで，試料を0.1 M燐酸緩衝液で洗浄し，1％$OsO_4$により後固定（4°C，2 h），蒸留水にて洗浄後，3％酢酸ウラン水溶液でブロック染色を行った（室温，一晩）。エタノール系列で脱水後，酸化プロピレンによる浸透を行い，試料をエポキシ樹脂に包埋した。Reichert超ミクロトームにより厚さ50 nmの超薄切片を作製し，3％酢酸ウラン水溶液，クエン酸鉛（Reynolds 1963）による電子染色を施した後，日本電子1200-EX II型電子顕微鏡により観察および撮影を行った。

**（2） 電子顕微鏡的組織化学**

エーテル麻酔下に近位空腸の両端を結紮し内腔に燐酸緩衝生理食塩水を注入して適度に膨らませた後，摘出，0.1 M燐酸緩衝液（pH 7.3）で緩衝した4％ホルムアルデヒド・0.4％グルタールアルデヒドを含む固定液に浸漬して固定を行った（4°C，2 h）。粘膜を除去し，筋層のみにした後，5％ショ糖を含む0.1 M燐酸緩衝液（pH 7.3）にて洗浄した（4°C，一晩）。試料を基質液［1.2 mM β-NADPH（オリエンタル酵母），1.2 mM BSPT（Sigma）を含む0.1 M燐酸緩衝液（pH 8.0）］で反応させた（37°C，90 min）（Llewellyn-Smith, I. J., et al. 1992）。0.1 M燐酸緩衝液（pH 7.3）で洗浄した後，2×3 mmほどの小片に細切，1％$OsO_4$により後固定（4°C，2 h），蒸留水にて洗浄後，3％酢酸ウラン水溶液でブロック染色を行った（室温，1 h）。エタノール系列で脱水後，試料をエポキシ樹脂に包埋し，Reichert超ミクロトームにより厚さ50 nmの超薄切片を作製した。3％酢酸ウラン水溶液，クエン酸鉛（Reynolds 1963）による電子染色を施した後，日本電子1200-EX II型電子顕微鏡により観察および撮影を行った。

## 3. 結　　果

ラット小腸筋層は内輪走筋と外縦走筋からなり，ICC-DMPの存在する深部筋神経叢はおよそ20～30層の平滑筋細胞から構成される輪走筋層の，最内側1～3層とこれより外側の主要筋層との間に位置する（**図1**）。

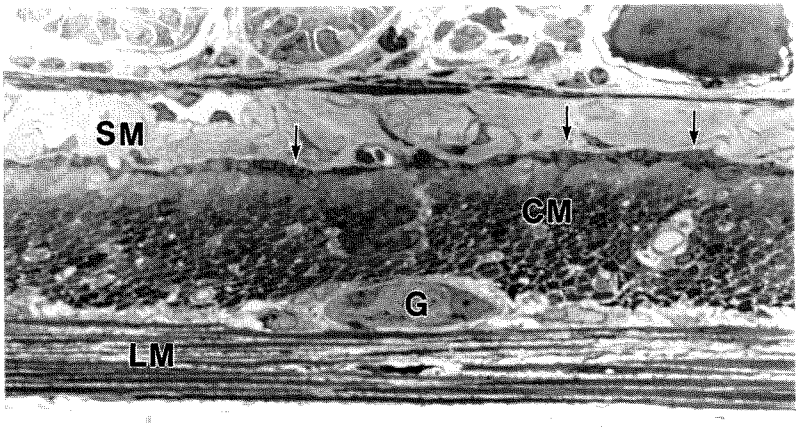


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**図1** ラット小腸筋層の縦断像。深部筋神経叢（矢印）は輪走筋層内の粘膜下結合組織（SM）に近い部位に位置する。輪走筋層（CM）と縦走筋層（LM）の間には筋層間神経節（G）が認められる（×490）。



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電子顕微鏡的観察では，ラットのICC-DMPは，通常，平滑筋より電子密度の低い，ミトコンドリアの豊富な細胞として特徴づけられる（**図2**）。粗面小胞体，ゴルジ装置などの細胞内小器官も比較的よく発達している。細胞膜にはcaveolaeが多数存在し，基底膜も観察される。中でも，この細胞の最も大きな特徴は同種細胞間，周辺平滑筋細胞との間に多くのgap junctionを形成することで，超薄切片の一断面にも数個を数えることができるほどである（図2）。また，この細胞と密接して観察される深部筋神経叢の神経終末には，およそ短径20～30 nm，長径50～60 nmの扁平な小胞を含む終末（**図3**），直径40～50 nmの円形の無芯小胞に直径100～140 nmの大型有芯小胞を含む終末（**図4**）が高頻度に観察された。

NADPH-d組織化学反応を行った試料では，細胞組織の保存状態に若干の違いは認められるものの，全体として微細構造はよく保たれており，細胞学的にICC-DMPと明瞭に同定される細胞にNADPH-d陽性の神経線維が多数密接している像が観察された（**図5**）。NADPH-d陽性の神経終末では，陽性反応は神経終末の細胞膜上に発現し，終末内には多数のシナプス小胞が認められた。シナプス小胞の種類としては直径40～50 nmの円形の無芯小胞と直径100～140 nmの大型の有芯小胞（**図6**）がおもに観察された。また，NADPH-d陽性の神経終末には平滑筋に直接密接しているものも観察された（**図7**）。NADPH-d陽

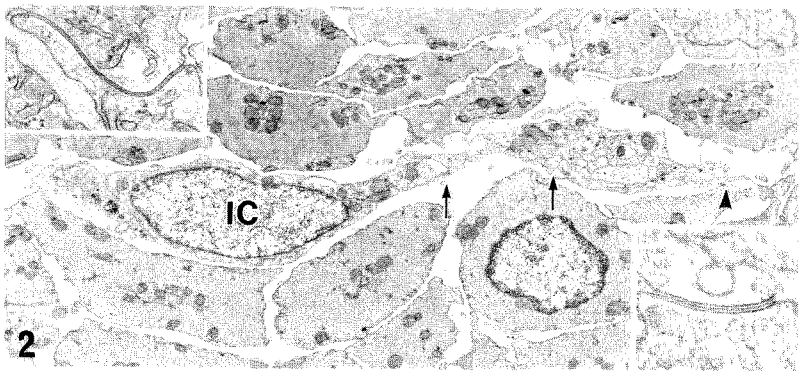


**図2** 深部筋神経叢のICC-DMP(IC)。同種細胞間(矢印)，平滑筋(矢頭)との間にgap juctionが認められる（×8 400）。左上の挿入図：矢印で示されたICC突起間のjap junctionの拡大図（×64 000）。右下の挿入図：矢頭で示されたICCと平滑筋のjap junctionの拡大図（×70 000）。

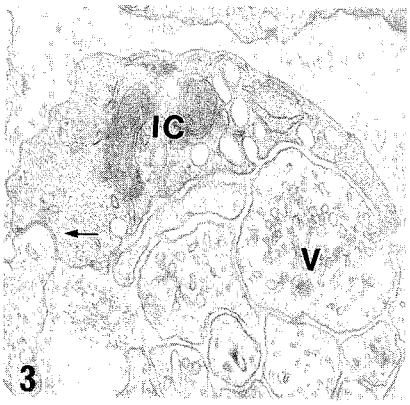


**図3** ICC-DMPの突起(IC)に密接する軸索膨大部(V)。終末内には，短径20～30 nm，長径50～60 nmの扁平な小胞が観察される。矢印はこの細胞と平滑筋との間のgap junctionを示す(×26 500)。

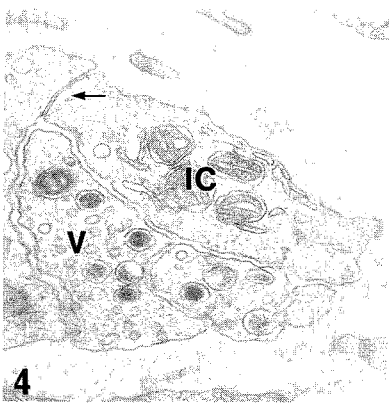


**図4** ICC-DMPの突起(IC)に密接する軸索膨大部(V)。直径40～50 nmの円形の無芯小胞に直径100～140 nmの大型有芯小胞を含む終末が観察される。矢印は同種細胞間のgap junctionを示す(×30 000)。

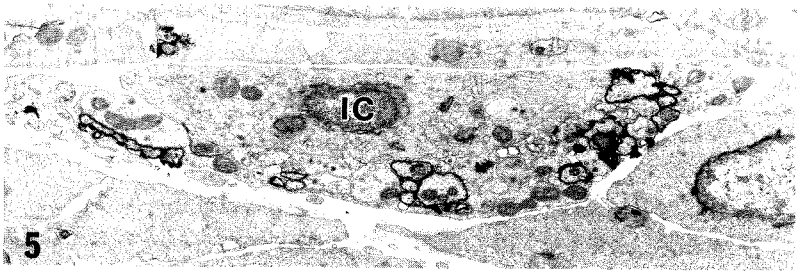


図5　NADPH-d組織化学を施したラット小腸深部筋神経叢部。ICC-DMP(IC)に密接してNADPH-d陽性の神経束が観察される(×5 900)。

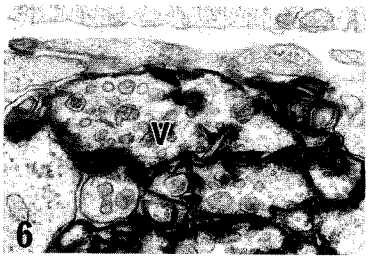


図6　ICC-DMPに密接するNADPH-d陽性の軸索膨大部(V)。直径40～55 nmの円形の無芯小胞が観察される(×26 000)。

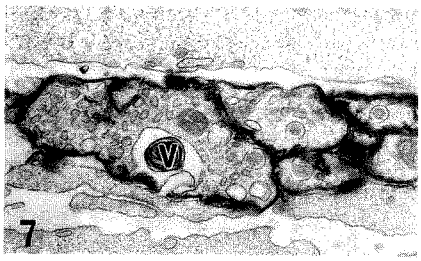


図7　平滑筋に密接するNADPH-d陽性の軸索膨大部(V)(×38 000)。

性終末とICC-DMPおよび平滑筋細胞との間隙は最も狭いところで約20～25 nmであった。

## 4. 考　　察

ICC-DMPとNO作動性神経との関係を示す形態学的研究については，NOS陽性線維との密接な像を示すモルモット小腸での光学顕微鏡的（Wang, et al. 1999），電子顕微鏡的観察（Toma, et al. 1999）がこれまでにも報告されているが，陽性反応を示す線維が機能的な伝達部位としての終末部に相当するか否かは明らかではなかった。

本研究では，神経終末の特徴であるシナプス小胞を多数含む軸索膨大部にNADPH-d陽性反応が認められ，ICC-DMPに密接することが観察されており，このことは平滑筋細胞との間に形成される多数のgap junctionの存在と合わせて，小腸ICC-DMPの刺激伝達上の介在機能を強く支持するものと考えられる。ICC-DMPのNO作動性神経支配が形態学的に示されたといえよう。

NOが消化管におけるNANC抑制性神経伝達物質として推定されてきたことは緒言に記したとおりであるが（Waterman, et al. 1994；Ward, et al. 1996），ICCの欠損しているW/Wvマウスの胃輪走筋ではNOの抑制効果減少が観察されており，ICCのNO作動性神経mediatorとしての機能が報告されている(Burns, et al. 1996)。

ところで，NADPH-d反応を示す終末部には円形の無芯小胞が観察されたが，NOの神経終末の微細形態については，モルモット小腸の粘膜下層においても，同様に大型の有芯小胞と円形の無芯小胞が観察されている（Llewellyn-Smith, et al. 1992)。これらのシナプス小胞にNOが貯蔵されていることは考え難く，両者の関係については不明であるが，その一方で，NO作動性神経の終末部には他の神経伝達物質との混在が知られており，モルモットの小腸ではVIP (Costa, et al. 1992；Ekblad, et al. 1994；Ward, et al. 1994)，GABA (Williamson, et al. 1995)，ラットの回腸，結腸では，ATP (Belai and Burnstok 1994) が，またラットの食道では，galanin (Worl, et al. 1998) などが報告がされている。VIPを含むシナプス小胞の形態としては，large granular vesicle，ATPに関してはlarge opaque vesicleと報告されており（Burnstok 1981)，ラット小腸深部筋神経叢の

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NO作動性神経にどのような神経伝達物質が混在し，NOとどのような機能的関係を示すかについては今後の検討を重ねて結論を出したいと考えている。

また，本研究では，深部筋神経叢のNADPH-d陽性終末がICC-DMP，平滑筋のそれぞれに密接している像が観察されたが，同様の所見はモルモット小腸におけるSubstance PやNOS陽性の軸索膨大部についても観察されており（Wang, et al. 1999），NO作動性神経の作用経路としてICCを介するものと平滑筋に直接作用するものがあることを示すものであろう。

元来，ICCはカハール（Cajal 1893, 1911）により，交感神経と効果器の間をつなぐ可能性を有するものとして位置付けられた細胞であり，今，ICCのsubtypeの刺激伝達機能が明らかになりつつあることは，自律神経支配下にある他の臓器についても，あらためて見直す時期にあるものと考えられる。

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# Fine Structural Observation of NADPH-d Positive Nerve Terminals on Interstitial Cells of Cajal in Rat Small Intestine

Satomi KAWASE* and Terumasa KOMURO**

Abstract

Nitrergic innervation of the interstitial cells of Cajal (ICC-DMP) in the rat small intestine was examined by using NADPH-d histochemistry. Electron microscopic observation revealed that NADPH-d positive nerve terminals were closely apposed to the cell body of ICC-DMP. These NADPH-d positive terminals contained small round clear synaptic vesicles with diameter of 40-50 nm and large granular vesicles with diameter 100-140nm. NADPH-d positive terminals were also observed in close vicinity of smooth muscle cells of outer circular muscle layer.

Keywords : interstitial cells of Cajal (ICC), NO, ultrastructure, small intestine, rat

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Journal of the
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Journal of the Autonomic Nervous System 80 (2000) 142–147

# Ultrastructural observations of fibroblast-like cells forming gap junctions in the $W/W^{v}$ mouse small intestine

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Accepted 3 February 2000

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**Publication information:** *Journal of the Autonomic Nervous System* (ISSN 0165-1838). For 1999, volumes 75–81 are scheduled for publication. Subscription prices are available upon request from the Publisher or from the Regional Sales Office nearest you or from this journal's website (http://www.elsevier.com/locate/jans). Further information is available on this journal and other Elsevier Science products through Elsevier's website: (http://www.elsevier.nl). Subscriptions are accepted on a prepaid basis only and are entered on a calendar year basis. Issues are sent by standard mail (surface within Europe, air delivery outside Europe). Priority rates are available upon request. Claims for missing issues should be made within six months of the date of dispatch.

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Printed in The Netherlands

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Journal of the Autonomic Nervous System 80 (2000) 142–147

Journal of the Autonomic Nervous System

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# Ultrastructural observations of fibroblast-like cells forming gap junctions in the $W/W^v$ mouse small intestine

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Accepted 3 February 2000

**Abstract**

The ultrastructure of the wild-type (+/+) mice small intestine was compared with c-*kit* mutant ($W/W^v$) mice which only have few interstitial cells of Cajal (ICC) associated with Auerbach's plexus, in order to elucidate whether the specialized membrane contacts are general features of so-called fibroblast-like cells that are widely distributed in the tunica muscularis of the alimentary tract. Fibroblast-like cells in the Auerbach region were found in approximately equal number in $W/W^v$ mice as in +/+ mice, while ICC associated with Auerbach's plexus (ICC-AP) could not be demonstrated in $W/W^v$ mice in the present investigation. Fibroblast-like cells were characterized by cytoplasm of moderate to high electron density, well developed rough endoplasmic reticulum and nuclei with thick peripheral accumulations of heterochromatin. There were no basal lamina and caveolae along the cell membrane. It was observed that single fibroblast-like cells formed probable small gap junctions with muscle cells of both circular and longitudinal layers. Fibroblast-like cells with the same features were also observed in the region of the deep muscular plexus in both +/+ and $W/W^v$ mice. The present observation, together with our previous studies on rats and guinea-pigs, suggest the common presence of gap junctions or gap junction-like structures on fibroblast-like cells in the gastrointestinal musculature and their involvement in the regulatory system of gastrointestinal motility by passing electrical or molecular signals to influence the state of muscle tonus. 

*Keywords:* Gut motility; Fibroblast; Gap junction; Interstitial cells of Cajal; Alimentary tract; c-*kit*

## 1. Introduction

During the last two decades, a wide range of studies has disclosed the pacemaking activity of the interstitial cells of Cajal (ICC) in gastrointestinal motility (see reviews by Thuneberg, 1982, 1989; Sanders, 1996). However, 'interstitial cells' with a fibroblast-like ultrastructure have attracted only little interest and their biological significance has remained obscure.

These so-called fibroblast-like cells are widely distributed in the tunica muscularis at different levels of the alimentary tract of many species, including mouse small intestine (Rumessen et al., 1982; Thuneberg, 1982), dog distal esophagus and colon (Berezin et al., 1990, 1994), and human small intestine and colon (Faussone-Pellegrini and Cortesini, 1983, 1984; Faussone-Pellegrini et al., 1990; Rumessen et al., 1992, 1993a,b). These studies describe the absence of specialized membrane contacts between the fibroblast-like cells and smooth muscle cells, which were often regarded as a discriminating feature from ICC. Because of the lack of evidence regarding their intercellular communication, the functional involvement of fibroblast-like cells in gut movement has rarely been discussed.

In other studies, small gap junctions of fibroblast-like cells with smooth muscle cells and close contacts with nerve varicosities have been observed in the small intestine of rats (Komuro, 1989; Komuro and Seki, 1995) and guinea-pigs (Zhou and Komuro, 1992a,b) and their participation in intercellular communication has been suggested.

It is not yet clear whether the discrepancy between these observations represents differences between species or whether the specialized membrane contacts are general features of the fibroblast-like cells in the gastrointestinal tract, since recent studies have revealed that ICC are heterogeneous in their ultrastructure depending on species or tissue layer or level of the gastrointestinal tract (Christensen, 1992; Rumessen, 1994; Komuro et al., 1996).

In order to further elucidate the regulatory system of gut

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0165-1838/00/$ – see front matter 
PII: S0165-1838(00)00089-8

motility it seems desirable to clarify the morphological basis for the possible involvement of fibroblast-like cells in muscle contraction. Therefore, the goal of the present study was to determine whether gap junctions exist between fibroblast-like cells and smooth muscle cells in the mouse small intestine, an organ that has frequently been used in the study of ICC. To clearly distinguish fibroblast-like cells from ICC, our strategy was to compare the ultrastructure of the small intestine in wild-type (+/+) mice with c-*kit* mutant ($W/W^v$) mice that have few ICC associated with Auerbach's plexus (Ward et al., 1994; Huizinga et al., 1995).

## 2. Materials and methods

### 2.1. c-Kit immunohistochemistry

Homozygous WBB6F1-$W/W^v$ and +/+ mice (aged 4–6 weeks) were purchased from Japan SLC (Shizuoka, Japan).

Short segments of proximal jejunum were removed from animals under terminal anesthesia with ether and were immediately frozen with liquid nitrogen in the embedding medium. Sections (10 μm thick) were cut with a MICROM HM 505E cryostat, and mounted on glass slides. The specimens were fixed with acetone for 10 min at room temperature, rinsed in phosphate-buffered saline (PBS) several times and incubated with 4% Block Ace solution (Dainippon Seiyaku, Osaka, Japan) for 20 min at room temperature to prevent non-specific antibody binding. The specimens were then incubated overnight at 4°C with the rat monoclonal antibody raised against c-Kit (ACK2; Gibco BRL, Gaithersburg, MD, USA) at a dilution ratio of 1:200. After rinsing in PBS several times, the specimens were incubated overnight at 4°C with peroxidase-conjugated secondary antibody (rabbit anti-rat IgG; Dako, Gloslrup, Denmark) at a dilution ratio of 1:80. Horseradish peroxidase reaction was developed in 50 ml 0.1 M Tris–HCl buffer (pH 7.4) solution containing 6 mg 4-chloro-1-naphthol (Sigma, St. Louis, MO, USA) and 8 μl 30% $H_2O_2$.

### 2.2. Transmission electron microscopy

Short segments of proximal jejunum were removed from $W/W^v$ and +/+ mice (aged 4–6 weeks) under ether anesthesia and placed in a fixative containing 3% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3, for 2 h at room temperature. After rinsing in the same buffer, the specimens were post-fixed in 1% osmium tetroxide for 2 h at 4°C. The specimens were rinsed in distilled water, block-stained with saturated aqueous uranyl acetate solution for 3 h, dehydrated in a graded series of ethyl alcohols and embedded in Epon epoxy resin. Ultrathin sections were cut with a Reichert microtome, double stained with uranyl acetate and lead tartrate, and were observed with a JEM 1200 EX II electron microscope.

## 3. Results

### 3.1. c-Kit immunohistochemistry

Strong immunoreactivity to anti-c-Kit antibody was clearly localized in two regions corresponding to the deep muscular plexus and the Auerbach's plexus in the jejunum of +/+ mice (Fig. 1). In contrast, no immunoreactivity was detected in the regions of both the Auerbach's plexus and the deep muscular plexus of $W/W^v$ mice (Fig. 2).

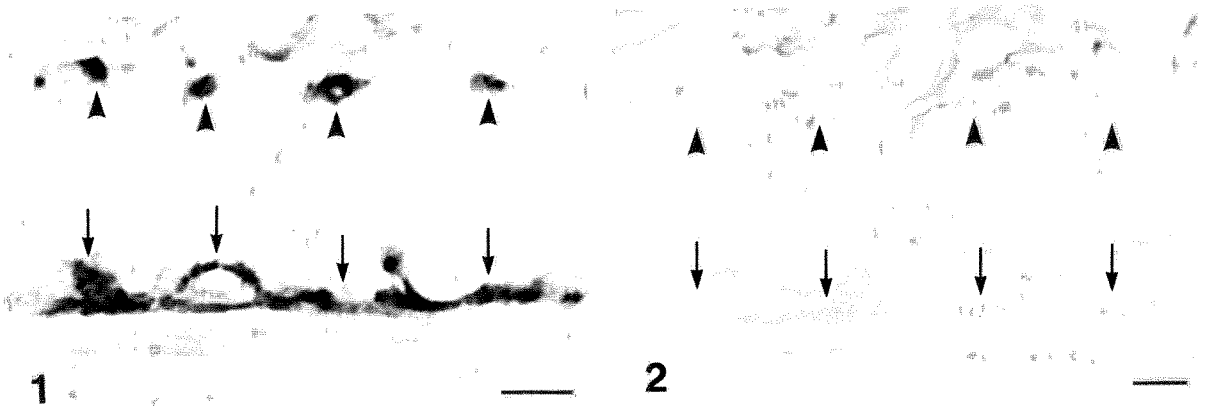


Fig. 1. Longitudinal cryosection of the +/+ mouse small intestine showing the c-Kit immunoreactive cells. c-Kit immunoreactive cells are observed in the region corresponding to both the Auerbach's plexus (arrows) and the deep muscular plexus (arrowheads). bar, 10 μm.

Fig. 2. Section of the $W/W^v$ mouse small intestine showing immunoreactivity for c-Kit. No immunoreactive cell is observed in the region corresponding to both the Auerbach's plexus (arrows) and the deep muscular plexus (arrowheads). bar, 10 μm.

### 3.2. Interstitial cells associated with Auerbach's plexus

ICC associated with Auerbach's plexus (ICC-AP), characterized by numerous mitochondria, a cytoplasm of low electron density and large gap junctions, were observed in +/+ mice (Fig. 3 and inset). However, the cells characterized by these features could not be demonstrated in $W/W^v$ mice in the present study, so far as we observed 10 grids containing about 10 sections from each of 10 blocks from $W/W^v$ mice as for +/+ mice.

On the other hand, fibroblast-like cells were found in approximately equal numbers in $W/W^v$ mice as in +/+ mice in the observations described above. These cells were irregular in shape and often had several cytoplasmic processes extending in different directions (Fig. 4). They were characterized by cytoplasm of moderate to high electron density and had nuclei with thick peripheral accumulations of heterochromatin. In the cytoplasm, there was well-developed rough endoplasmic reticulum which usually contained flocculent materials in dilated cisterns. Golgi apparatus and mitochondria were also seen. There was no basal lamina and caveolae were not found along the cell membrane. Fibroblast-like cells were often located near nerve bundles and partially surrounded them by thin processes (Fig. 4). Single fibroblast-like cells formed probable small gap junctions with muscle cells of both circular and longitudinal layers (Fig. 4). Although these junctions were usually very small and punctate, occasional large junctions were observed between this type of cell and smooth muscle cells of the circular layer (Fig. 5 and inset). The contact length of this particular junction measures 140 nm along the two membranes. These probable gap junctions of fibroblast-like cells were far fewer than those of ICC or smooth muscle cells.

### 3.3. Interstitial cells associated with the deep muscular plexus

ICC associated with the deep muscular plexus (ICC-DMP) were present in both +/+ and $W/W^v$ mice in spite of the absence of immunoreactivity to c-Kit in the latter. The ICC-DMP were characterized by numerous mitochondria, a less electron dense cytoplasm (Fig. 6), caveolae, a basal lamina and large gap junctions that interconnected the same type of cells, and also connected them with smooth muscle cells (Fig. 6, inset). The issue of the normal distribution of ICC-DMP in the *c-kit* deficient animals has been discussed elsewhere (Ward et al., 1995; Malysz et al., 1996; Sanders, 1996; Horiguchi and Komuro, 1998).

Fibroblast-like cells could easily be distinguished from the ICC-DMP. Fibroblast-like cells in this region were characterized by the same features as fibroblast-like cells associated with Auerbach's plexus, such as a moderately electron dense cytoplasm and a nucleus with thick accumulation of heterochromatin (Fig. 7). These cells were often found in the close vicinity of nerve bundles. They formed probable small gap junctions with surrounding smooth muscle cells at the tips of cytoplasmic projections.

## 4. Discussion

The present study provides for the first time probable evidence that fibroblast-like cells in the mouse small intestine form gap junctions with smooth muscle cells. In particular, the present study demonstrates fairly large probable gap junctions on fibroblast-like cells of $W/W^v$ mice which have few ICC-AP. Gap junctions of fibroblast-like cells in the c-*kit* deficient animals have also been observed in the stomach of *Ws/Ws* rats (Ishikawa et al., 1997). This evidence, together with our previous observations in the small intestine of rats (Komuro, 1989; Komuro and Seki, 1995; Horiguchi and Komuro, 1998) and guinea-pigs (Zhou and Komuro, 1992a,b; Komuro et al., 1996), suggest the common presence of gap junctions on fibroblast like cells in the gastrointestinal musculature. Further observations on a wide variety of specimens are needed to establish how general this finding may be.

It is worth discussing the identification of small gap junctions of fibroblast-like cells, since some of them do not show sufficient length to judge a parallel seven layered structure with an intercellular gap of less than 3 nm along opposing membranes (Bennett et al., 1991). The best way to identify them is to demonstrate the accumulation of connexin molecules on freeze-fractured surfaces of target structures, athough this is not always practical. However, common junctional specializations found between mammalian cells other than gap junctions are classified into two types. One type is characterized by the fusion of the outer leaflets of adjoining cell membranes (*zonula occludens* or tight junction) and the other type has an intercellular gap of 15–20 nm (*zonula adherens*, *macula adherens*, and chemical synapses). The contact regions between the fibroblast-like cells and muscle cells were often punctate in profile, but were extensive enough to reveal that their intercellular clefts were far less than 15–20 nm and, in fact, were more on the order of a few nanometers. Therefore, we believe that the junctions between fibroblast-like cells and smooth muscle cells are true gap junctions, albeit small in size. It should be emphasized here again that Fig. 5 seems to offer clear evidence for a gap junction between a fibroblast-like cell and a smooth muscle cell, even though other small gap junctions in punctate form remain to be demonstrated.

Provided that the fibroblast-like cells in the gut musculature generally form gap junctions with surrounding smooth muscle cells, they probably have some functional role in gastrointestinal motility. Possibly, their gap junctions act as routes for intercellular communication, i.e. passing electrical or molecular signals that regulate the state of muscle tonus, as suggested by previous reports (Komuro, 1989; Komuro and Seki, 1995; Horiguchi and Komuro,

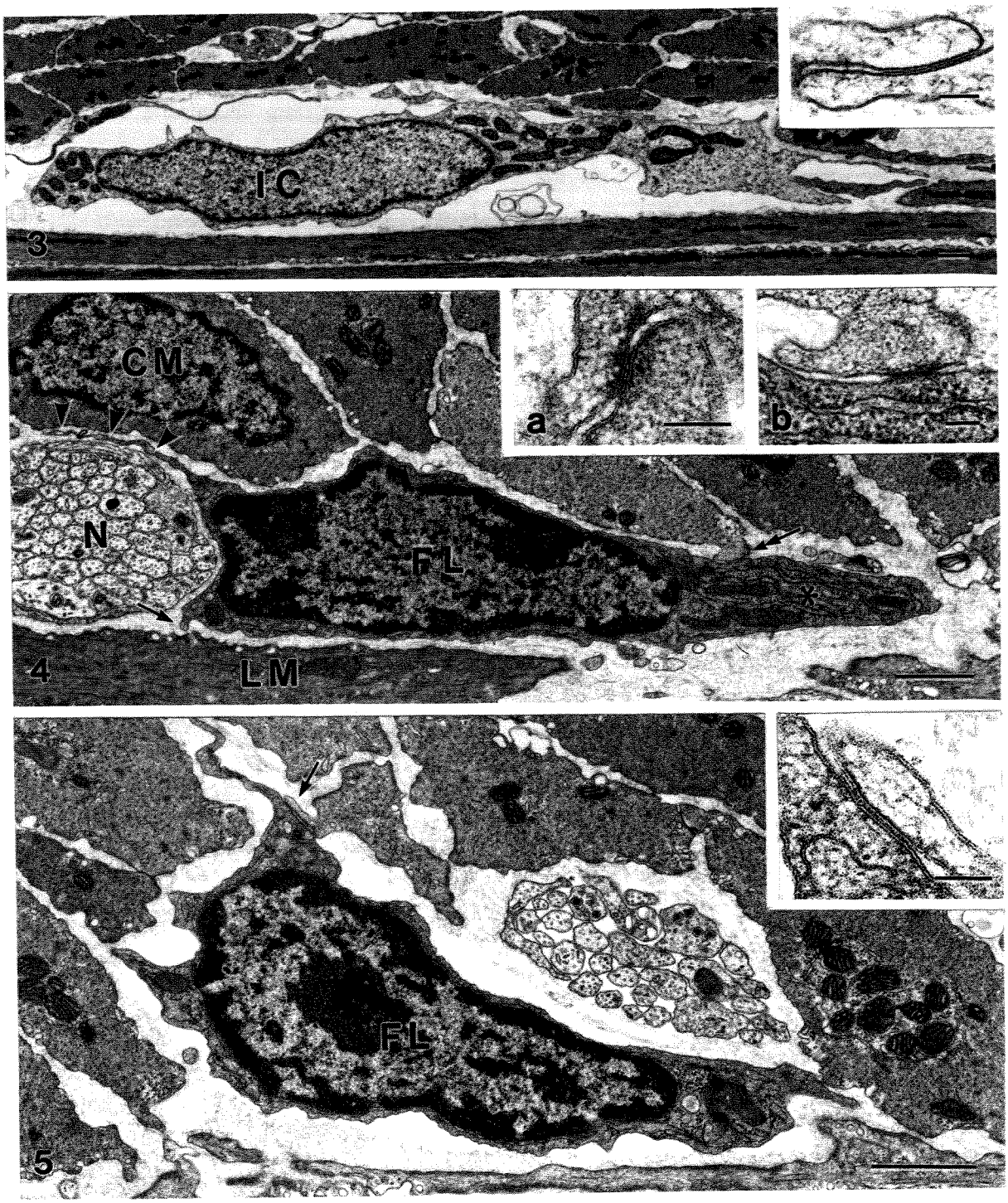


Fig. 3. ICC-AP (IC) of the + / + mouse small intestine, characterized by numerous mitochondria and cytoplasm of low electron density. Bar, 1 μm. Inset: a gap junction between the processes of ICC-AP. Bar, 0.1 μm.

Fig. 4. Fibroblast-like cell (FL) located between the circular (CM) and longitudinal (LM) muscle layers of the $W/W^v$ mouse small intestine. It is characterized by a nucleus contoured with dense heterochromatin and rough endoplasmic reticulum (*). Its thin process (arrowheads) partially surrounds nerve bundles (N) of the Auerbach's plexus. Bar, 1 μm. Insets: probable small gap junctions between FL and longitudinal (a) and circular (b) smooth muscle cells indicated by the double-headed arrow and arrow, respectively, in neighboring sections. Bar, 0.1 μm.

Fig. 5. Fibroblast-like cell (FL) in the region of Auerbach's plexus in $W/W^v$ mouse small intestine. It forms a probable gap junction with a small process of a circular smooth muscle cell (arrow). Bar, 1 μm. Inset: higher magnification of the junction indicated by the arrow in a neighboring section. Bar, 0.1 μm.

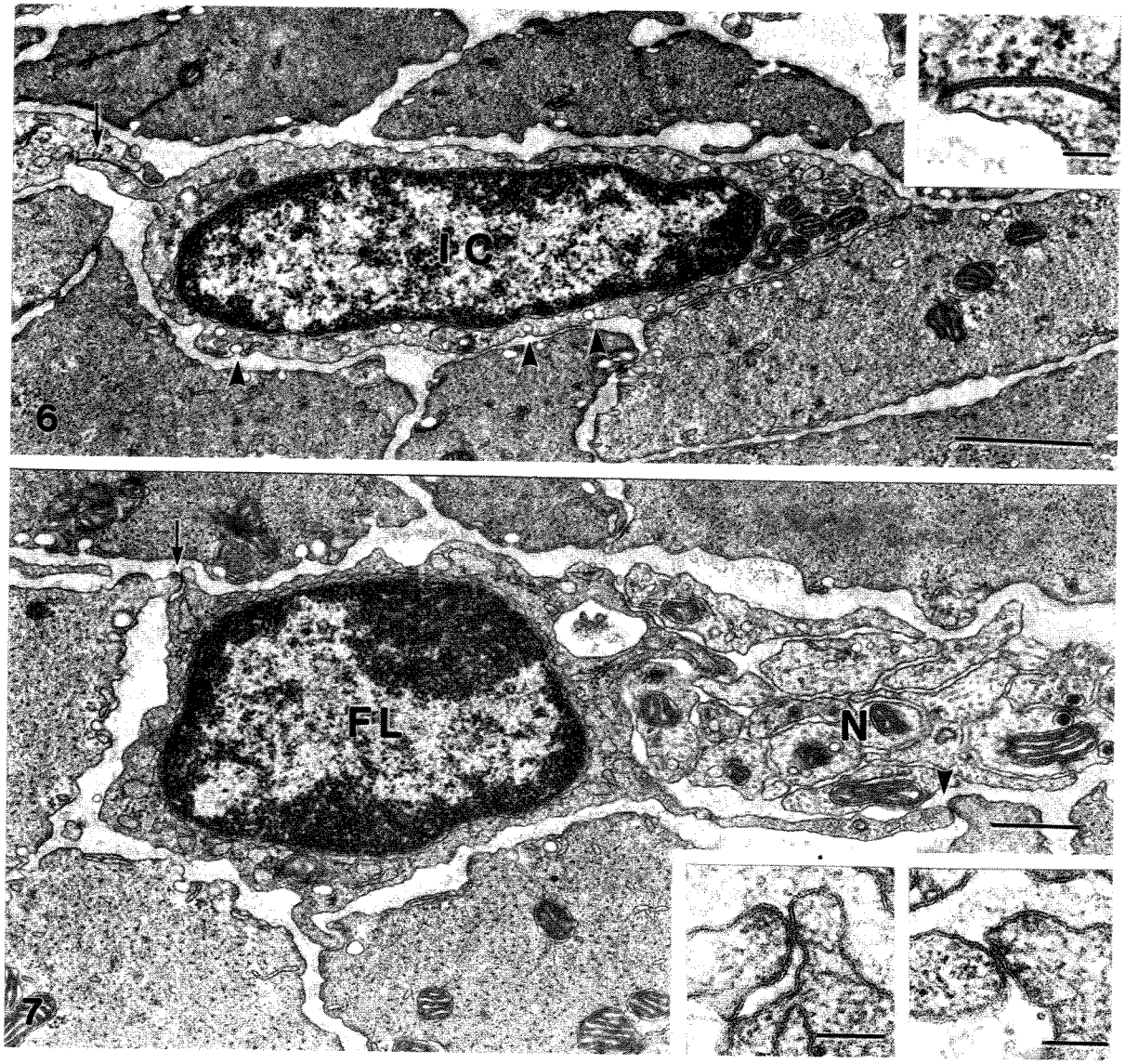


Fig. 6. ICC-DMP (IC) of the *W/W*$^{v}$ mouse characterized by numerous mitochondria, less electron dense cytoplasm, caveolae (arrowheads) and large gap junction interconnect the same type of cell (arrow). Bar, 1 μm. Inset: higher magnification of the gap junction indicated by the arrow. Bar, 0.1 μm.

Fig. 7. Fibroblast-like cell (FL) associated with the deep muscular plexus (N) of the *W/W*$^{v}$ mouse small intestine. Basal lamina and caveolae are absent along the cell membrane. Bar, 0.5 μm. Insets: higher magnifications of the probable small gap junctions indicated by the arrow (left) and the arrowhead (right). Bar, 0.1 μm.

1998). Synapse-like close contacts between fibroblast-like cells and nerve varicosities containing many synaptic vesicles have been observed in the guinea-pig small intestine (Zhou and Komuro, 1992b).

Recent immunohistochemical (Portbury et al., 1996) and pharmacological (Burns et al., 1996) studies strongly support the concept that ICC-DMP serve as mediators of neurotransmission (Thuneberg, 1982; Komuro et al., 1996; Sanders, 1996). Moreover, fibroblast-like cells may act as another mediator of neural inputs to muscle cells. In this context, it is worth noting that fibroblast-like cells show almost uniform ultrastructural features, irrespective of species or organ or tissue layer (Komuro, 1989; Zhou and Komuro, 1992a,b; Ishikawa et al., 1997; Horiguchi and Komuro, 1998), while ICC show ultrastructural diversity dependent on the tissue and species (Faussone-Pellegrini, 1987; Thuneberg, 1989; Christensen, 1992; Komuro et al., 1996). Common features of fibroblast-like cells may indicate that they have a rather more fundamental role than ICC regarding mediator function in the regulatory system for muscle contraction.

Furthermore, the circular and longitudinal muscle layers of the intestine have been reported to be electrically coupled (Smith et al., 1987), but the morphological basis for such a connection between the two muscle layers has remained obscure. The observation of probable gap junctions between the fibroblast-like cells and both circular and longitudinal muscles appears to provide such a connection.

Bridging of the two muscle layers by a single fibroblast-like cell via gap junctions has also been observed in the rat small intestine (Komuro, 1989; Horiguchi and Komuro, 1998).

In conclusion, we provide evidence based on ultrastructural observations of wild type and $W/W^{v}$ mice that fibroblast-like cells, structurally distinct from interstitial cells of Cajal, form probable gap junctions with both circular and longitudinal smooth muscle layers and have a role in gastrointestinal motility. The question raised by recent reports of whether CD-34 was expressed by ICC (Hirota et al., 1998) or fibroblast-like cells (Vanderwinden et al., 1999) may offer another interesting viewpoint in the future study of the physiological significance of fibroblast-like cells.

## Acknowledgements

The authors thank Dr. P. Baluk, Cardiovascular Research Institute, University of California, San Francisco, CA, USA, for reading the manuscript. This work was supported by a grant from the Ministry of Education, Science and Culture of Japan (10670514) and by a Waseda University Grant for Special Research Projects (99A-572).

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Cell Tissue Res (2001) 306:417–422
DOI 10.1007/s00441-001-0470-2

REGULAR ARTICLE

Keisuke Seki · Terumasa Komuro

# Immunocytochemical demonstration of the gap junction proteins connexin 43 and connexin 45 in the musculature of the rat small intestine

Received: 18 June 2001 / Accepted: 29 August 2001 / Published online: 10 October 2001


**Abstract** The immunohistochemical localization of connexin (Cx) 43 and Cx 45 in the musculature of the rat small intestine was studied at the ultrastructural level, with special reference to the interstitial cells of Cajal in the deep muscular plexus region (ICC-DMP). Cx 43 was localized at gap junctions formed between every group of cells, i.e., smooth muscle cell~smooth muscle cell, smooth muscle cell~ICC-DMP and ICC-DMP~ICC-DMP. In contrast, Cx 45 immunoreactivity was only detected at gap junctions between ICC-DMP~ICC-DMP. Since different types of Cx molecules have different properties for electrical and chemical coupling of cells, it is suggested that the homotypic network of ICC-DMP connected with Cx 45 gap junctions may function as an independent compartment segregated from the whole cellular network including the smooth muscle cells connected with Cx 43 gap junctions. It is further speculated that the ICC-DMP of the rat small intestine communicate with each other and with smooth muscle cells via the passage of messenger molecules through Cx 43, but they may use an additional mechanism, as yet unknown, for communications restricted to other ICC-DMP.

**Keywords** Interstitial cells of Cajal (ICC) · Gap junction · Connexin (Cx) · Small intestine · Rat (Wistar)

## Introduction

Recent reports indicate that certain types of interstitial cells of Cajal (ICC) can mediate neural activity in the gastrointestinal musculature through their cellular network of connection of gap junctions with each other and with smooth muscle cells (Komuro et al. 1996; Komuro 1999; Sanders 1996; Sanders et al. 1999). Indeed, both inhibitory and excitatory motor innervations have been reported for the ICC within the circular muscle layer (Burns et al. 1996; Wang et al. 2000; Ward et al. 1998, 2000). Neurokinin 1 receptors, representing the target sites for substance P released from nerve terminals, were also demonstrated on ICC in the deep muscular plexus (DMP) (Portbury et al. 1996; Vannucci et al. 1997, 2000).

However, it is not yet clear whether ICC propagate nerve signals to neighboring ICC and/or smooth muscle cells through gap junctions by simple spread of electrical current or by using some messenger molecules. To obtain a clue for answers to this question it is desirable to elucidate at the molecular and ultrastructural level what types of gap junctions are localized on different sets of cells.

Gap junctions are composed of pairs of hemichannels or connexons which are formed by oligomerization of the structural protein subunits, connexins. These connexins (Cx) show a specific distribution pattern depending on the tissue, organ and species (Bennet 1991). At least 13 different isoforms have been identified in rodents (White and Bruzonne 1996). Different connexins possess their own characteristics and display a variable degree of ionic selectivity (Veenstra et al. 1994a, 1994b), different molecular size permeability (Steinberg et al. 1994) and sensitivity to messenger molecules (Kwak et al. 1995; Moreno et al. 1994).

The presence of Cx 43 in the intestinal musculature was observed in the mouse, dog and human by immunohistochemical methods (Mikkelsen et al. 1993) and in the dog with Western and Northern blotting (Li et al. 1993). More recently, Nakamura et al. (1998) demonstrated Cx 45 immunohistochemically in the deep muscular plexus of both the dog and rat small intestine, in addition to Cx 43 within the circular muscle layer, and suggested that Cx 45 is localized at gap junctions formed by ICC-DMP in these animals. However, the precise ultrastructural localization of the connexins remains to

This study was partly supported by a Waseda University Grant for Special Research Projects (99A-893)

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be elucidated. Furthermore, the differences between the gap junctions formed by three different sets of cells, ICC-DMP~ICC-DMP, ICC-DMP~smooth muscle cell and smooth muscle cell~smooth muscle cell, have not been clarified.

The present study aimed to reveal the immunohistochemical localization of Cx 43 and Cx 45 in the musculature of the rat small intestine at the ultrastructural level, with special reference to the ICC-DMP. This knowledge will lead to a better understanding of the role of ICC in regulating contraction or relaxation of the intestinal musculature.

## Materials and methods

### Animals

Young adult Wistar rats (weighing 120–150 g) of both sexes were used in the present study. Short segments of proximal jejunum were removed from the animals under deep ether anesthesia. All procedures were performed in accordance with the guidelines for the care and use of laboratory animals in the School of Human Sciences, Waseda University.

### Transmission electron microscopy

The specimens were fixed in a fixative containing 4% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer, pH 7.3, for 2 h at 4°C. The specimens were rinsed with the same buffer and postfixed in 1% osmium tetroxide for 2 h at 4°C. Then the specimens were rinsed with distilled water, block-stained with saturated uranyl acetate solution for 3 h, dehydrated in a graded series of ethyl alcohol and embedded in Epon epoxy resin. Ultrathin sections were cut using a Reichert microtome and double stained with uranyl acetate and lead citrate for observation with a JEM 1200 EX II electron microscope.

### Immunohistochemistry

Short segments of proximal jejunum were rinsed in phosphate-buffered saline (PBS) and immersed in Tissue-Tek OCT compound (No. 4583; Sakura Fine Technical Co., Tokyo). The specimens were immediately frozen with liquid nitrogen and sectioned longitudinally with a Microm HM 505-E cryostat. These frozen sections were mounted on poly-L-lysine-coated glass slides and fixed for 20 min at –20°C with absolute acetone, and then first incubated with 4% Block Ace solution (Dainippon Seiyaku, Osaka, Japan) for 20 min at room temperature to reduce non-specific background staining. Since our preliminary experiments indicated that Cx 45 immunoreactivity was very weak in the present specimens, additional experiments for immunohistochemistry were performed separately for the different antibodies as follows: (1) Specimens were incubated with the mouse monoclonal antibodies against Cx 43 (Chemicon International Inc., Temecula, CA, No. MAB 3068) at a dilution of 1:500. The peroxidase-conjugated secondary antibodies (rabbit anti-mouse IgG, DAKO, Glostrup, Denmark, No. P161) containing 0.1% normal rat serum were used at a dilution of 1:100 and horseradish peroxidase reaction was developed in 50 ml 0.1 M TRIS-HCl buffer containing 6 mg 4-chloro-1-naphthol (Sigma) and 8 µl 30% $H_2O_2$. (2) Sections were incubated with the rabbit polyclonal antibodies against Cx 45 (Chemicon, No. AB 1745) at a dilution of 1:100. The fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (swine anti-rabbit IgG, DAKO No. F205) were used at a dilution of 1:100. After a brief rinse with PBS, the specimens were mounted with Mount-Quick or Vectashield mounting medium (Vector, Burlingame, CA) and photographed with a Nikon microscope equipped with appropriate fluorescence filters and transmitted light optics. Black and white fluorescent images were reversed and printed with a digital photographic device (Pictrostat digital 400, Fujifilm, Tokyo) to show the location of ICC-DMP in the tissue structure.

### Immunoelectron microscopy

Short segments of proximal jejunum were inflated and fixed for 2 h at 4°C with 0.1 M phosphate buffer containing 3% formaldehyde. After rinsing in PBS, the mucosa was removed and the circular muscle layer was isolated. The specimens containing the whole circular muscle layer were stretched on slide glasses and were placed in PBS containing 0.3% Triton X-100 at 4°C for 20 min. After incubation with Block Ace solution, the specimens were incubated with the primary and secondary antibodies according to the same procedures described above, except peroxidase-conjugated swine anti-rabbit IgG (DAKO No. P217) (1:100) was used as the secondary antibody for Cx 45 immunostaining. Horseradish peroxidase reaction was developed with diaminobenzidine (DAB). After a brief rinse with PBS, the specimens were fixed with 3% glutaraldehyde, postfixed with 1% $OsO_4$. Then the specimens were placed in saturated uranyl acetate solution for 30 min, and processed according to the procedures for electron microscopy described above.

## Results

In frozen sections of the rat small intestine immunoreactivity to Cx 43 was densely distributed throughout the whole circular muscle layer in an almost homogeneous fashion (Fig. 1). Immunoreactivity to Cx 43 was not observed in the longitudinal muscle layer. In contrast to Cx43, immunoreactivity to Cx 45 was only observed at the innermost part of the circular muscle, corresponding to the region of the deep muscular plexus (Fig. 2).

Under the electron microscope, ICC-DMP of the rat small intestine were observed between the inner thin layer (one to three cells thick) and the outer main division of the circular muscle layer and were closely associated

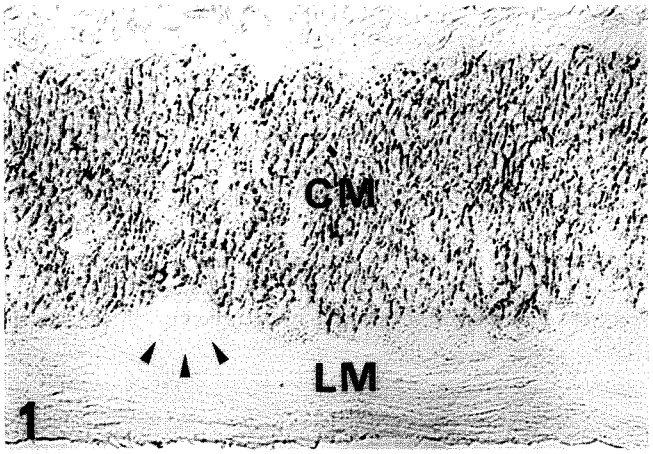
**Fig. 1** A frozen section showing the Cx 43 immunoreactivities distributed within the outer circular muscle layer (*CM*) of the rat small intestine. Reaction deposits were not observed at the myenteric ganglion (*arrowheads*) and in the longitudinal muscle layer (*LM*). ×750

**Fig. 2** Cx 45 immunoreactivity limited to the DMP region (*arrows*). The circular (*CM*) and longitudinal (*LM*) muscle layers were distinguished by the location of the myenteric ganglion (*arrowheads*). ×750

**Fig. 3** ICC-DMP (*ICC*) associated with the deep muscular plexus. A gap junction is observed between two ICC (*arrow*) (*N* nerve bundle of the DMP). ×15,000. *Inset*: A gap junction between ICC and smooth muscle cell. ×40,000

**Fig. 4** An immunoelectron micrograph showing Cx 43 immunoreactivity at gap junctions between smooth muscle cells in the circular muscle layer. ×12,000. *Inset*: A higher magnification of the gap junction indicated by *the arrow*. ×38,000

**Fig. 5** Cx 43 immunoreactivity localized at gap junctions between ICC-DMP (*ICC*) and smooth muscle cell (*arrow*), and between the processes of ICC-DMP (*double-headed arrow*). ×22,000. *Inset*: Cx 43 immunoreactivity localized at gap junction between the processes of ICC-DMP indicated by *double-headed arrow in a neighboring section*. ×63,000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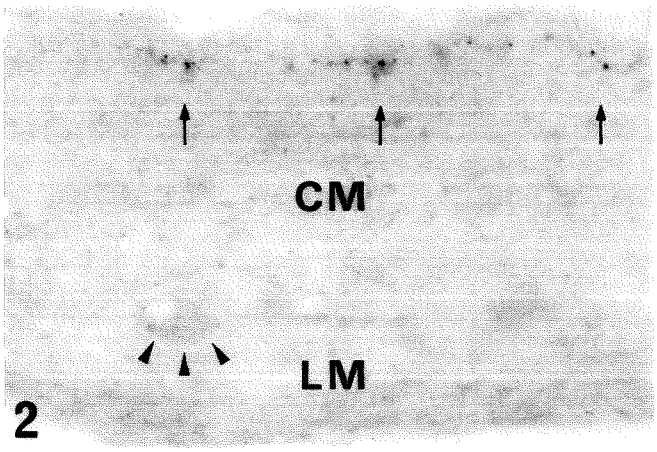
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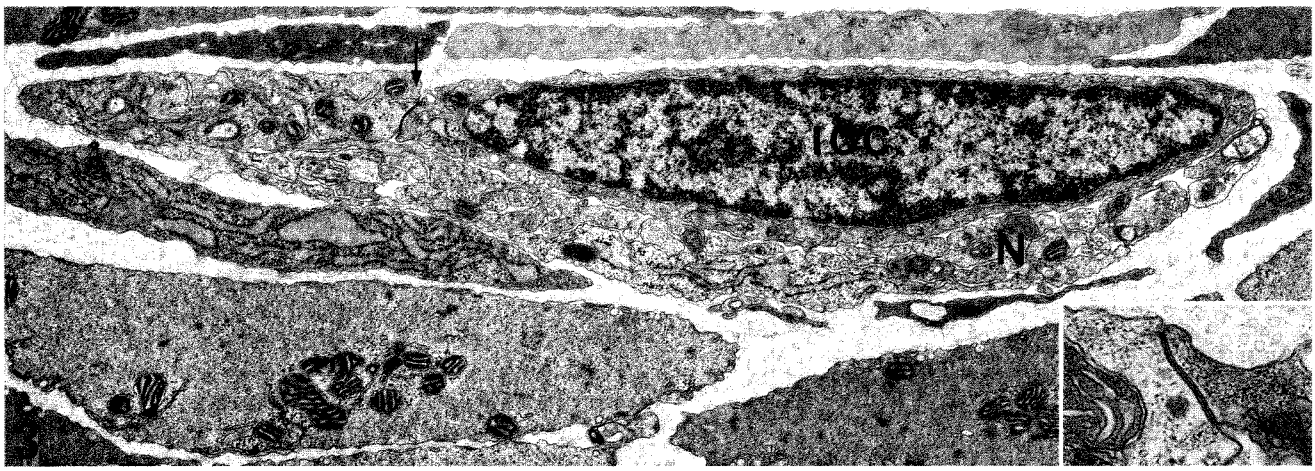
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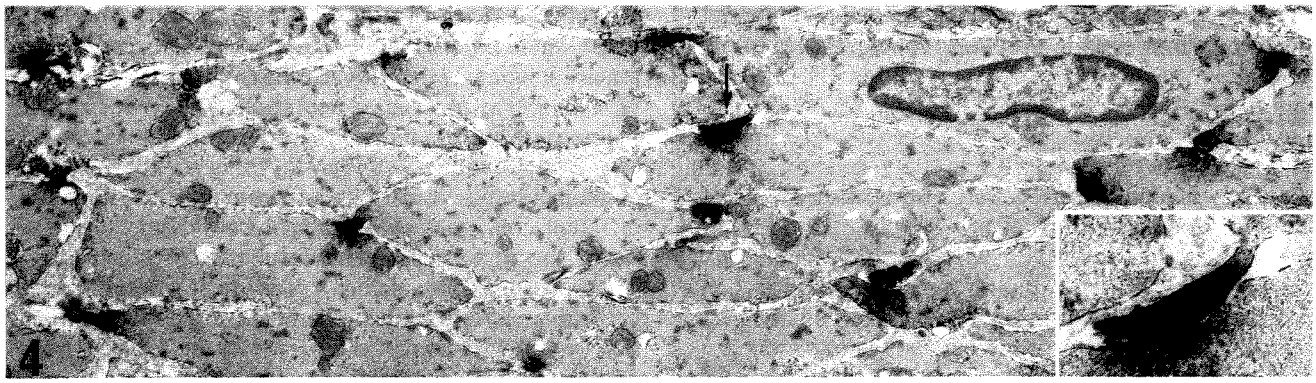
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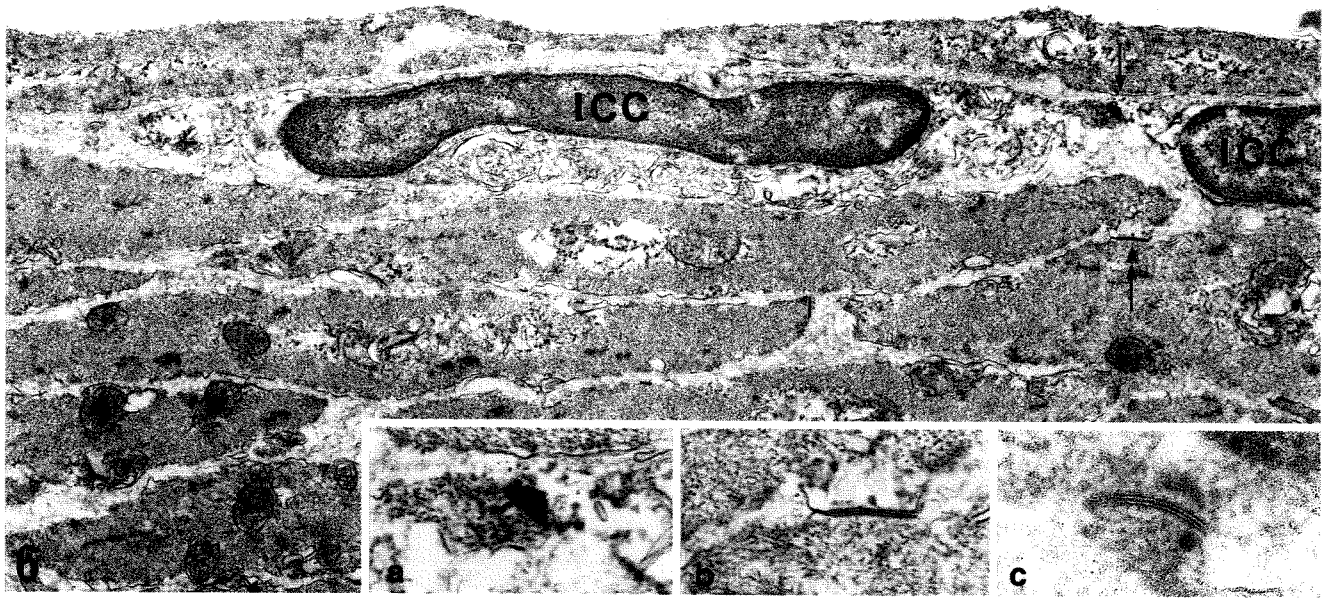


**Fig. 6** Cx 45 immunoreactivity of ICC-DMP (*ICC*). The gap junction between two ICC-DMP shows Cx 45 immunoreactivity (*arrow*), but the junction between smooth muscle cells (*double-headed arrow*) does not. ×14,000. *Inset a*: A higher magnification of the gap junction indicated by *the arrow*. ×60,000. *Inset b*: The gap junction indicated by *the double-headed arrow*. ×60,000. *Inset c*: A gap junction between ICC-DMP and smooth muscle cell has no Cx 45 immunoreactivity. ×70,000

with nerve bundles (Fig. 3). The ICC-DMP were characterized by the presence of well-developed cell organelles including mitochondria, Golgi apparatus and both smooth and rough endoplasmic reticulum. The organized contractile machinery that is normally found in smooth muscle cells was absent in ICC-DMP. The most important feature of this type of cell was the formation of many large gap junctions with cells of the same type (Fig. 3) and with smooth muscle cells (Fig. 3, inset).

Cx 43 immunoreactivity was clearly localized at the gap junctions between smooth muscle cells in the outer division of the circular muscle layer (Fig. 4). The reaction deposits were usually observed at the cell membranes forming gap junctions and within the very confined area of the cytoplasm adjacent to the junctions (Fig. 4, inset). They were never detected along the other part of the cell membrane. The immunoreactive deposits were also observed at the gap junctions between smooth muscle cells and ICC-DMP (Fig. 5), and between the processes of ICC-DMP (Fig. 5, inset). Cx 43 immunonegative gap junctions were not observed between smooth muscle cells, and between smooth muscle cells and ICC-DMP.

On the other hand, anti-Cx 45 immunoreactivity was only observed at the gap junctions formed between ICC-DMP (Fig. 6, inset a). These reaction deposits were not observed at gap junctions formed between smooth muscle cells (Fig. 6, inset b) and between ICC-DMP and smooth muscle cells (Fig. 6, inset c).

## Discussion

The present study clearly demonstrated for the first time that Cx 43 immunoreactivity was localized at gap junctions formed between every group of cells, i.e., smooth muscle cell~smooth muscle cell, smooth muscle cell~ICC-DMP and ICC-DMP~ICC-DMP. In contrast, Cx 45 immunoreactivity was only detected at gap junctions between ICC-DMP~ICC-DMP. These results are compatible with the observations of Cx 43 immunoreactivities in the outer subdivision of the circular muscle layer of the small intestine in the mouse, dog and human (Mikkelsen et al. 1993), and the observation of Cx 45 immunoreactivities in the DMP region of the dogs and rats (Nakamura et al. 1998).

The presence of different types of ICC equipped with different connexins in different locations of the intestine raises the important functional question of whether the gastrointestinal musculature has different types of signaling pathways, as is found in the heart. It has been reported that Purkinje fibers of the cardiac conduction system have mainly Cx 40, and, in contrast, the ordinary cardiac muscles contain Cx 43 (Kanter et al. 1993). These two connexins seem to contribute to the compartmentalization of communication pathway allowing selective communication. Thus, unexpected excitation of the cardiac muscle along the length of the conducting fibers can be minimized and terminal branches expressing Cx 43 allow the coordinated propagation of stimuli to precisely defined regions of the cardiac muscle (Bruzzone et al. 1996).

On the other hand, Coppen et al. (1998) have demonstrated that Cx 45 has a restricted distribution in rat and mouse heart, localized to Cx 40-expressing endocardial zones of the ventricular conduction system. They assumed for Cx 45 a specific functional role that may facilitate continuity of functional linkage of gap junctional channels in those regions.

In the rat small intestine, the present study suggests that the homotypic network of ICC-DMP may function as an independent compartment segregated from the whole cellular network including the smooth muscle cells. Communication within these compartments may be mediated by Cx 45, which was only detected at the gap junctions between ICC-DMP and the same type of cell. The gap junctions between ICC-DMP of the rat small intestine occupy about 10~40% of the total area of gap junctions formed by ICC-DMP (Seki and Komuro 1998), but they may effectively communicate with each other by pathways separate from those used for communicating to the smooth muscle cells.

Different types of connexin molecules have different properties for electrical and chemical coupling of cells. By using two osteoblastic cell lines, Steinberg et al. (1994) revealed that one type of cell expressing only Cx 43 allowed the passage of Lucifer yellow dye to many neighboring cells, and that another cell type expressing Cx 45 was poorly coupled according to that criterion, though both cell lines showed good electrical coupling. Based on these results, these authors suggested that low molecular weight intracellular second messenger molecules such as cyclic nucleotides and inositol phosphates can diffuse between cells coupled by Cx 43, but not between cells coupled by Cx 45.

In the intestine, the presence of the second messenger system responding to nitrergic stimulation was suggested for ICC within the circular muscle layer of both the lower esophageal and pyloric sphincters (Ward et al. 1998) and of the colon in the mouse (Wang et al. 2000). An increase of intracellular cGMP was reported in ICC in response to nitric oxide donors (Shuttleworth et al. 1993; Young et al. 1993). Therefore, it is probable that the ICC-DMP of the rat small intestine communicate with each other and with smooth muscle cells via the passage of messenger molecules through Cx 43, but they may use an additional mechanism, as yet unknown, for communications restricted to other ICC-DMP.

It has been well documented that heterotypic channels can mediate a distinctive unidirectional cell-to-cell communication. For instance, in the retina, intercellular channels between one type of amacrine cell and bipolar cell, presumably heterotypic, are impermeable to molecules that can diffuse through gap junction channels between the amacrine cells, presumably homotypic (Mills and Massey 1995). The unidirectional nature of heterotypic channels has also been studied with dye movement occurring from endothelial cells to smooth muscle cells (Little et al. 1995). It remains to be seen whether ICC-DMP form mixtures of two types of homotypic channels or heterotypic channels in the same gap junctions which connect ICC-DMP with each other, though the colocalization of Cx 43 and Cx 45 at the rat DMP region has been observed (Nakamura et al. 1998; Seki and Komuro, unpublished data). The answer to this question would provide an important clue to understanding flow of stimuli regulating the contraction and relaxation of the intestinal musculature. These possible differences and/or the different efficiency of communication systems may also contribute to the differentiation of signal pathways between the circumferential and longitudinal axis of the intestinal wall, so as to enable segmental movement.

**Acknowledgements** The authors would like to thank Dr. Peter Baluk, Cardiovascular Research Institute, University of California, San Francisco, USA, for reading the manuscript.

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