Gap junctions in gastrointestinal muscle contain multiple connexins

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Wang, Y. F., and E. E. Daniel. Gap junctions in gastrointestinal muscle contain multiple connexins. Am J Physiol Gastrointest Liver Physiol 281: G533–G543, 2001.—In the canine gastrointestinal tract, the roles that gap junctions play in pacemaking and neurotransmission are unclear. Using antibodies to connexin (Cx)43, Cx45, and Cx40, we determined the distribution of these connexins. Cx43 was present in all locations where structural gap junctions occur. Cx40 was also widely distributed in the circular muscle of the lower esophageal sphincter (LES), stomach, and ileum. Cx45 was sparsely distributed in circular muscle of the LES. In the interstitial cells of Cajal (ICC) networks of myenteric plexus, in the deep muscular and submucosal plexuses, sparse Cx45 and Cx40 immunoreactivity was present. In colon, immunoreactivity was found only in the myenteric and submucosal plexus and nearby circular muscle cells. No immunoreactivity was found in sites lacking structural gap junctions (longitudinal muscle, inner circular muscle of the intestine, and most circular muscle of the colon). Studies of colocalization of connexins suggested that in the ICC networks, some colocalization of Cx43 with Cx40 and/or Cx45 occurred. Thus gap junctions in canine intestine may be heterotypic or heteromeric and have different conductance properties in different regions based on different connexin compositions.

IN CANINE INTESTINE, GAP JUNCTIONS have been observed with electron microscopy between circular muscle (CM) cells, except cells of the inner CM (iCM) of intestine and most cells of CM of colon, between interstitial cells of Cajal (ICC) in the myenteric plexus everywhere, the deep muscular plexus (DMP) of intestine, and the submucosal plexus of colon (1–5, 9–17, 20). Good electrical coupling has been observed or inferred between CM cells (3, 8–11, 13–17, 19, 20, 22, 29, 31, 32) and assumed but not established experimentally between ICC and between ICC and CM. Even though longitudinal muscle cells have no gap junctions visible by electron microscopy except near the myenteric plexus of colon (5, 8, 9, 11, 13, 15–17), some electrical coupling has been observed and deduced between them because they have regular slow waves coupled to those in CM in intestine (8, 9, 15, 19), and in several species, space constants longer than the cell length have been observed (see Refs. 9, 15).

Recently, strong evidence has accumulated that slow waves throughout the gastrointestinal tract are paced by the networks of ICC in the myenteric plexus of stomach and intestine and in the submucosal plexus of colon (9, 23, 29, 30, 42), as originally proposed by Thuneberg (35). In the intestine, a network of ICC in the DMP plays a subsidiary role (7, 22) as does the ICC network in the myenteric plexus in the colon (31, 32). However, gap junctions visible with electron microscopy between ICC in the myenteric plexus and CM are rare and small (5, 15, 16) and, except in the colon, nonexistent between ICC and longitudinal muscle (5). In contrast, there are numerous gap junctions between the ICC of the submucosal plexus of colon and the adjacent CM (2, 4) and between the DMP and adjacent outer CM (oCM) (9, 16, 17, 20). Recently, accumulated evidence has been interpreted to imply that slow waves on gastrointestinal muscle are driven passively by current flow from the ICC networks in the myenteric plexus or submucosal plexus of colon (19, 29–30).

Furthermore, evidence has accumulated that the intramuscular ICC play an essential role in inhibitory neurotransmission (6, 30, 43). This was originally suggested because of the regular occurrence of nerve endings very close to intramuscular ICC, which are in gap junction contact with CM (12). Additional observations in canine gastrointestinal CM have shown similar structural relationships (1–5, 10, 13–16, 20). How the intramuscular ICC may amplify and transmit neural inhibitory information to the muscle is unclear, but the gap junctions connecting them are considered likely to be essential (15).

These observations raise several structural paradoxes. 1) How can the rare (to CM) or nonexistent (to longitudinal muscle) gap junctions between the myenteric plexus ICC of the myenteric plexus of the stomach and small intestine pass sufficient current to fill the large capacities and drive slow waves of these muscle layers that appear to be syncytia? 2) How can the numerous gap junctions between ICC networks of the
submuscular plexus of colon and DMP of intestine provide current to drive slow waves in the adjacent syncytial CMs and still maintain independent pacemaking activities? One possibility is that these different gap junctions have different current-passing properties, including possible rectification of current flow, because they may contain different connexins, may be heteromeric with more than one connexin in each connexon, or may be heterotypic with a different connexin comprising each connexon to form a channel (44).

The objective of this study was to use immunocytochemistry to evaluate whether any gap junctions of canine gastrointestinal tissues are composed exclusively of Cx43, as is sometimes implied (24, 26), or whether they contain other connexins such as Cx45, which has recently been reported in canine DMP (28), or Cx40, which has been reported in other smooth muscles (25). Although Cx37 has recently been reported to be present in airway smooth muscle (27) and sparsely present in vascular smooth muscle (39), it was not studied. Immunohistochemistry with a light microscope lacks sufficient resolution to determine if the presence of more than one connexin in a given region implies that gap junctions there are heteromeric or heterotypic, but it does allow their localization to different sites where gap junctions occur.

MATERIALS AND METHODS

Tissue preparation. Four mongrel dogs of either sex were euthanized with an overdose of pentobarbital sodium (100 mg/kg) in accordance with a protocol approved by the McMaster University Animal Care Committee and following the guidelines of the Canadian Council on Animal Care. The abdomen was opened along the midline; segments of lower esophagus, gastric antrum, ileum, and colon were excised and immediately put into oxygenated Krebs Ringer solution containing (in mM) 115.0 NaCl, 4.6 KCl, 1.2 MgSO4, 22.0 NaHCO3, 2.5 CaCl2, and 11.0 glucose. Tissues were opened along the gastroesophageal junction and mesenteric border and pinned on a piece of Sylgard silicon rubber (mucosa side facing down) and immersed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) overnight at 4°C. The fixed tissues were rinsed several times with PB, were then placed in PB containing 30% sucrose as a cryoprotection agent for 24 h, and were stored at −70°C until used.

Immunofluorescent labeling. Frozen sections of 8-μm thickness were cut using a Leitz 1720 digital cryostat, mounted on glass slides coated with gelatin, and dried at room temperature overnight. The steps for immunoreaction with the antibodies (shown in Table 1) were performed as follows. Blocking was carried out for 2 h with 3% BSA and 10% normal goat serum in 0.1 M PB at room temperature.

Specimens were separately incubated with the mouse monoclonal antibody against gap junction protein Cx43 and the rabbit polyclonal antibody against gap junction protein Cx40 and Cx45 (Chemicon International) overnight at 4°C; antibodies were diluted 1:400 (Cx43), 1:300 (Cx40), and 1:100 (Cx45). The sections were washed three times with PB and then incubated for 60–120 min with fluorescein-cyanine-3 (Cy3)-labeled goat anti-rabbit (for Cx40 and Cx45) or antimouse (for Cx43) IgG (BIO/CAN Scientific) diluted 1:80. After being washed with PB, the specimens were then mounted in 80% glycerol in PB and viewed with a Leitz microscope equipped with a fluorescence epiluminator. Kodak T-MAX 400 film was used for black and white photography.

For double labeling of Cx43 and Cx40 or Cx45, the sections were incubated with a solution containing a mixture of the two primary antibodies. The secondary antibodies, Cy3-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG, were also applied in a mixed solution.

For peptide inhibition experiments, the antibodies and antigen were incubated together at 1:4 (1 part antibody to 4 parts peptide) for 24 h at 4°C in a cold room with agitation and were then spun using an air centrifuge for 20 min before application to the tissue sections for replacement of the primary antibodies.

Table 1. Antibodies used for immunoreaction

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Catalog No.</th>
<th>Dilution</th>
<th>Peptide</th>
<th>Ratio, Ab:Pep</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx43</td>
<td>MAb 3067</td>
<td>1:400</td>
<td>aa252−270</td>
<td>1:4</td>
<td>Transduction</td>
</tr>
<tr>
<td>Cx45</td>
<td>MAb 3100</td>
<td>1:100</td>
<td>aa354−367</td>
<td>1:4</td>
<td>Chemicon</td>
</tr>
<tr>
<td>Cx45*</td>
<td>MAb 3101</td>
<td>1:100</td>
<td>aa354−367</td>
<td>1:4</td>
<td>Chemicon</td>
</tr>
<tr>
<td>Cx40*</td>
<td>Ab 1745</td>
<td>1:100</td>
<td></td>
<td></td>
<td>Chemicon</td>
</tr>
<tr>
<td>Cx40*</td>
<td>Ab 1726</td>
<td>1:300</td>
<td></td>
<td></td>
<td>Chemicon</td>
</tr>
<tr>
<td>Cx43*†</td>
<td>Ab 71-0700</td>
<td>1:400</td>
<td></td>
<td></td>
<td>Zymed</td>
</tr>
</tbody>
</table>

Cx, connexin; MAb, monoclonal antibody; Ab, antibody; Ab:Pep, antibody-to-peptide ratio. *Raised in rabbit; †staining identical to MAb 3067.
Labeling by this antibody and by a polyclonal antibody to Cx43 (data not shown) was similar at all sites. Very rare labeling (Fig. 2c) of colon longitudinal muscle and of CM of colon was found near the myenteric plexus. Near the submuscular plexus, within CM, very rare labeling was also seen. Preincubation of the antibody with the peptide antigen used to raise the Cx45 antibody reduced, whereas the peptide used to raise the Cx43 antibody abolished, immunostaining against the Cx43 antibody (Fig. 1, b and d).

Distribution of Cx40 immunoreactivity. Dense immunoreactivity to Cx40, like that to Cx43, was observed in the CM of the LES, gastric antrum, and oCM of ileum near the DMP (Fig. 3, a, c, and f). This was not affected by preabsorption with peptide antigen from Cx45 (Fig. 3b). Sparse immunoreactivity to Cx40 was also observed in myenteric plexus between longitudinal muscle and CM and in the DMP in ileum (Fig. 3, e and f) as well as in the submuscular plexus of colon (Fig. 3d). These may represent gap junctions on ICC.
networks located there. There was an occasional occurrence of immunoreactivity to Cx40 in the CM of colon (Fig. 3d). In the antral myenteric plexus, Cx40 antibody also occasionally labeled putative ICC (see sections on colocalization studies).

**Distribution of Cx45 immunoreactivity.** In contrast to Cx43 and Cx40, very sparse or no immunostaining of Cx45 was found in the CM of the LES, antrum, and ileum both near the myenteric plexus and near the DMP (Fig. 4, a–d). Rare Cx45 immunoreactivity was found (Fig. 4, b and c) around the periphery of antral and ileal myenteric plexuses (for colon, see colocalization studies), near the DMP of ileum, and in the submuscular plexus of colon (Fig. 4, d and e). The immunoreactivity to Cx45 was abolished by preabsorption of the Cx45 antibody with its specific peptide (Fig. 4f).

**Immunoreactivity in regions without structural gap junctions.** No immunoreactivity to gap junctions 43, 40, or 45 was consistently found in longitudinal muscle throughout the digestive tract or in iCM in ileum. As illustrated above, very sparse immunoreactivity was found in colon CM, consistent with the ultrastructural findings (6, 8). It was not possible to clearly distinguish immunoreactivity within CM of the various tissues associated with smooth muscle from that associated with intramuscular ICC, but some larger cells, possibly ICC, had Cx43 and Cx40.

**Colocalization: Cx43 and Cx40.** In these studies (Figs. 5–7), Cx43 antibodies were labeled with secondary antibodies conjugated to FITC (green), and Cx40 antibodies were labeled with secondary antibodies conjugated to Cy3 (red). Thus colocalization is indicated by yellow. In CM throughout the gastrointestinal tract, immunoreactivity to Cx43 and Cx40 was closely colocalized in the LES (Fig. 5a).

In antrum, too (Fig. 6, a and b), there was extensive colocalization of Cx43 and Cx40, but note the predominance of Cx40 staining in blood vessel endothelium near the myenteric plexus and the occurrence of Cx40.
staining with and without Cx43 in deeper CM (Fig. 6b).

Staining of Cx43 independently of Cx40 was rare. In ileum (Fig. 6, c and d), there was extensive colocalization of Cx40 and Cx43, but Cx40 also occurred free of Cx43 staining, notably near the plexuses, and, rarely, Cx43 immunoreactivity occurred independently of Cx40. Occasionally, clustered colocalized staining occurred. Comparison of Fig. 6, c and d, suggests that Cx40 was predominant in CM near the myenteric plexus but not near the DMP. In the portion of the oCM near the DMP, the immunoreactivity to both Cx43 and Cx40 was strong, and they were nearly completely colocalized except for a few sites that appeared to contain only Cx40 (Fig. 6d).

Figure 6, e and f, shows that immunolabeling of neither Cx43 nor Cx40 was prominent in circular or longitudinal muscle in colon. There was some colocalized staining near the myenteric plexus (Fig. 6e) and near the submucosal plexus (Fig. 6f). In the latter case, occasional sites had either Cx40 or Cx43 alone.

**Colocalization of Cx43 and Cx45.** Again, Cx43 antibodies were labeled with secondary antibodies conjugated to FITC, and Cx45 antibodies were labeled with secondary antibodies conjugated to Cy3. In LES (Fig. 5b), there was clear labeling of Cx45 as well as Cx43 sites. Some colocalization occurred, predominantly in sites where immunoreactivity was aggregated. These may be intramuscular ICC. These were similar to sites at which immunoreactivity to Cx43 and Cx40 was aggregated.

In antrum (Fig. 7, a and b), there was sparse labeling in the CM with Cx45 compared with Cx43. The exception was within the myenteric plexus, which was predominantly labeled by antibodies to Cx45. Colocalization, when it occurred, was in or near the myenteric plexus.
In ileum (Fig. 7, c and d), myenteric plexus, and oCM, sparse labeling with Cx45 was found compared with that of Cx43, but occasional colocalization occurred on both inner and outer aspects of the myenteric plexus. In the muscle, rare sites of Cx45 labeling as well as numerous sites of Cx43 labeling were seen. Note that in the DMP and oCM (Fig. 6d), the labeling of Cx45 was very sparse compared with that of Cx43, and colocalization was very rare.

In colon (Fig. 7e), myenteric plexus, and submuscular plexus (Fig. 7f), there was sparse labeling with Cx45, which was partially colocalized with Cx43. Near the submuscular plexus, some sites of colocalization appeared to be aggregated. Note the absence of staining to either Cx43 or 45 in most colon circular or longitudinal muscles.

DISCUSSION

This study shows that canine gastrointestinal smooth muscle and the associated ICC have gap junctions that are frequently composed of multiple connexins. Cx43 and Cx40 are most prominent in CM of the LES, antrum, and ileum. Their immunoreactivities were present but more sparse in regions where ICC are located, such as the myenteric plexus of antrum, intestine, and colon and in the deep muscular and submuscular plexuses of ileum and colon, respectively. Immunoreactivity to Cx45 was sparse everywhere but present in LES muscle and all plexuses. There was no special concentration in the DMP of ileum, in contrast to a previous report (28). However, regions containing all ICC networks had some immunoreactivity from Cx40, Cx45, and Cx43, partly but not fully colocalized. Table 2 summarizes our findings.

Another general observation was that immunoreactivity to these connexins was never found in regions that lack structural gap junctions as observed by electron microscopy (1–5, 9–17, 20). Thus longitudinal smooth muscle, iCM of intestine, and the longitudinal and CMs of colon, except near ICC networks, were nearly devoid of immunoreactivity.

If no additional gap junctions exist, it is unclear that gap junctions provide for pacemaking in the gastrointestinal tract. Modes of electrical coupling alternative to gap junctions do exist, and theoretical modeling suggests that they may be effective; field coupling...
between cell membranes closely apposed (33) can occur, and has its ability to transmit electrical events enhanced if there is an intrusion of a projection of one cell into another (40) or if potassium accumulates in the cleft between cells (33, 41). A special type of intrusion, the peg and socket joint, has recently been described in detail in mouse intestine (36–38, 41) and shown to vary with the incidence of physiological events. Peg and socket connections are postulated to function as stretch sensors, leading to activation of...
Table 2. Summary of locations of connexins in canine intestine

<table>
<thead>
<tr>
<th></th>
<th>Connexin 43</th>
<th>Connexin 40</th>
<th>Connexin 45</th>
</tr>
</thead>
<tbody>
<tr>
<td>LES</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Antrum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LM</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CM</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Myenteric plex</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Ileum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LM</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CM (outer)</td>
<td>++</td>
<td>++</td>
<td>+/-</td>
</tr>
<tr>
<td>CM (inner)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Myenteric plex</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>DMP</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Submuscular plex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LM</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>CM</td>
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<td></td>
</tr>
<tr>
<td>Myenteric plex</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
</tr>
</tbody>
</table>

+++ , +, +/-, +/-, +/-, - refer, in decreasing order, to the density of connexin labeling. LES, lower esophageal sphincter; LM, longitudinal muscle; CM, circular muscle; DMP, deep muscular plexus; Cx, connexin; ICC, interstitial cells of Cajal. CM of antrum and ileum near the myenteric plexus had a lower density of Cx43 and a higher density of Cx40, with the reverse occurring in the inner CM. Average overall densities were similar. Immunoreactivity near the plexuses could have been between ICC, between ICC and CM, or between adjacent CM cells. Anti-c-kit antibodies are ineffective in the dog, so these 2 sites could not be distinguished.

stretch-sensitive channels during segmenting and sleeve contractions (38).

It seems unlikely that field coupling of ICC networks to muscle layers could provide sufficient currents to drive slow waves passively throughout the muscularis externae. However, stretch coupling during shortening by ICC or muscle might transmit pacemaking activity throughout the muscle layer by inducing coupled smooth muscle cells to initiate their own currents, which then spread to other smooth cells by a similar mechanism. Moreover, the unidirectional relation between peg and socket joints between ICC and adjacent smooth muscle, the ICC that receive the peg and socket projection from smooth muscle cells, suggests that smooth muscle cells may have activity initiated by stretch during contraction (38).

If pacemaking currents flow through gap junctions, there are two paradoxes. One is that it is difficult to accept that the very low number of small gap junctions coupling ICC of the myenteric plexus to CM and the negligible number coupling them to longitudinal muscle could pass sufficient current to drive these smooth muscle syncytia passively. This study does not help resolve that paradox because no additional large gap junctions formed by connexins connecting ICC to muscle were found. The possibility that other modes of coupling contribute to the pacemaking by ICC networks in the myenteric plexus needs careful evaluation.

The other paradox is that the primary pacemaking region of the colon, the ICC network of the submucosal plexus, has numerous gap junctions to adjacent CM as does the secondary pacemaking area of the intestine, the ICC network of the DMP (4, 15, 17). Yet these pacemaking regions appear to be able to function independently of their coupling to large syncytia of muscle cells. It has been shown, for example, that isolated ICC cells produce pacemaking currents whether connected to smooth muscle or not (23, 30). The findings of this study may help explain those observations by showing that gap junctions in both these regions may contain both Cx45 and Cx40 as well as Cx43, all of which appear to be colocalized in part. We were unable to determine whether these gap junctions were heterotypic or heteromorphic. However, it has been shown by expression studies that channels that are heterotypic for Cx43 and Cx40, as well as for Cx43 and Cx45, have altered conductance, greater sensitivity to transjunctional voltage differences, and altered pH sensitivity (18, 21, 34). Thus these channels may allow rectification of currents, allowing current passing primarily from the ICC network to smooth muscle.

Some caveats to our findings and suggested interpretations must be noted. Our findings, in addition to previous electron microscopy studies (9, 11, 13, 15-17, 24, 35), suggest that gap junctions are unlikely to mediate pacing by ICC or coupling between longitudinal muscle cells of the canine gastrointestinal tract or...


