Proteins of interstitial cells of Cajal and intestinal smooth muscle, colocalized with caveolin-1

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Cho, Woo Jung, and E. E. Daniel. Proteins of interstitial cells of Cajal and intestinal smooth muscle, colocalized with caveolin-1. Am J Physiol Gastrointest Liver Physiol 288: G571–G585, 2005. First published October 7, 2004; doi:10.1152/ajpgi.00222.2004.—The murine jejunum and lower esophageal sphincter (LES) were examined to determine the locations of various signaling molecules and their colocalization with caveolin-1 and one another. Caveolin-1 was present in punctate sites of the plasma membranes (PM) of all smooth muscles and diffusely in all classes of interstitial cells of Cajal (ICC; identified by c-kit immunoreactivity), ICC-myenteric plexus (MP), ICC-deep muscular plexus (DMP), ICC-serosa (ICC-S), and ICC-intramuscularis (IM). In general, all ICC also contained the L-type Ca\(^{2+}\) (L-Ca\(^{2+}\)) channel, the PM Ca\(^{2+}\) pump, and the Na\(^+/\)Ca\(^{2+}\) exchanger-1 localized with caveolin-1. ICC in various sites also contained Ca\(^{2+}\)-sequestering molecules such as calreticulin and calsequestrin. Calreticulin was present also in smooth muscle, frequently in the cytosol, whereas calsequestrin was present in skeletal muscle of the esophagus. Gap junction proteins connexin-43 and -40 were present in circular muscle of jejunum but not in longitudinal muscle or in LES. In some cases, these proteins were associated with ICC-DMP. The large-conductance Ca\(^{2+}\)-activated K\(^+\) channel was present in smooth muscle and skeletal muscle of esophagus and some ICC but was not colocalized with caveolin-1. These findings suggest that all ICC have several Ca\(^{2+}\)-handling and -sequestering molecules, although the functions of only the L-Ca\(^{2+}\) channel are currently known. They also suggest that gap junction proteins are located at sites where ultrastructural gap junctions are known to exist in circular muscle of intestine but not in other smooth muscles. These findings also point to the need to evaluate the function of Ca\(^{2+}\) sequestration in ICC.

Ca\(^{2+}\)-handling proteins; immunocytochemistry; colocalization with caveolin-1; interstitial cells of Cajal pacing

IN THE INTESTINE, interstitial cells of Cajal (ICC) play crucial roles in control of motor function. These include pacing of excitation and contraction of both muscle layers and intermediate of neurotransmission to cholinergic and nitricergic nerves (7, 15, 16, 26, 28–30, 32). ICC as well as smooth muscle have caveolae, small (40- to 60-nm diameter) invaginations in the plasma membrane (PM). These depend on the presence of caveolin (Cav) proteins. In smooth muscle and ICC, the important Cav is Cav1 (4, 8, 11). Cav1 insert into the inner leaflet of the PM but leave both COOH- and NH\(_2\)-terminal ends in the cytosol. These termini have binding sites for numerous signaling molecules including nitric oxide synthase (NOS), G proteins, kinases, and receptors. Earlier, we showed that a number of important signaling molecules appears colocalized with Cav1 in smooth muscle of lower esophageal sphincter (LES) (4) and in canine airway smooth muscle (11).

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MATERIALS AND METHODS

Tissue Preparation

Mice (Balb/c, 8–10 wk) were killed by cervical dislocation in accordance with a protocol approved by the University of Alberta Animal Care Committee and following the guidelines of the Canada Council on Animal Care. The abdomen was opened along the median line. The lower esophagus containing the stomach and the small intestine were removed and put into ice-cold oxygenated (95% O2-5% CO2) Krebs-Ringer buffer (pH 7.4) containing (in mM) 115.5 NaCl, 4.6 KCl, 1.16 MgSO4·7H2O, 21.9 NaHCO3, 2.5 CaCl2·2H2O, 1.16 Na2HPO4·H2O, and 11.1 glucose. To isolate the LES, the stomach was opened along the greater curvature and the lesser curvature to reveal the junction between the lower esophagus and the fundus of the stomach. We studied both the LES and the region just proximal, which has mixed smooth and striated muscle bundles. The jejunum was opened along the mesenteric border and pinned on a petri dish of Sylgard silicon rubber, mucosa side down.

For cryosection preparation, the jejunum and LES were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 4 h at room temperature. The fixed tissues were washed in PB for 30 min × 8 and were cryoprotected in graded sucrose solution (10, 20% sucrose in PB) for 2 h each, placed in 30% sucrose in PB overnight at 4°C, and were then stored at −80°C until sectioned. For whole mount preparation, the jejunum was microdissescted, fixed, and treated as in the cryosection preparation for 4 h at room temperature. The fixed tissues were washed in PB for 30 min eight times, dehydrated and cleared in DMSO for 10 min three times, and were rehydrated in PB for 15 min four times at room temperature.

Double-Immunofluorescent Labeling for Cryosection

Frozen tissues were sectioned by a cryostat (Leitz 1720 digital cryostat, Germany) to make sections of 10-μm thickness. The sections were attached on slide glasses coated with 2% 3-amino propyltriethoxysilane (Sigma, St. Louis, MO) in acetone, and were dried overnight at 4°C. The sections were washed in PBS (pH 7.0) containing 0.4% Triton X-100 (TX-100; 0.4% in PBS) for 15 min three times. To reduce nonspecific binding proteins, the sections were blocked with 10% normal sera that were raised in the host of PBS) for 2 h each, placed in 30% sucrose in PB overnight at 4°C, and were then stored at −80°C until sectioned. For whole mount preparation, the jejunum was microdissescted, fixed, and treated as in the cryosection preparation for 4 h at room temperature. The fixed tissues were washed in PB for 30 min eight times, dehydrated and cleared in DMSO for 10 min three times, and were rehydrated in PB for 15 min four times at room temperature.

Immunofluorescent Labeling for Whole Mount Preparation

Muscle (circular and longitudinal muscle) layers of the jejunum were separated from the mucosa and submucosa layers, and then the circular muscle layer was separated from the longitudinal muscle layer with the MP under the dissection microscope. The muscle layers were washed vigorously in PBS containing 0.5% TX-100 (0.5% in PBS) for 15 min four times on orbital shaker. The muscle layers were blocked with 10% normal sera that were raised in the host of secondary antibody for 1.5 h at room temperature. For immunohistochemistry, primary antibodies used in the cryosection were used for 48 h at 4°C. The muscle layers were washed in 0.5% TX-100 in PBS for 15 min four times. For immunofluorescent labeling secondary antibodies, immunoglobulins conjugated with Cy3, FITC, or Alexa488 were used for 1.5 h at room temperature. The sections were washed in 0.5% TX-100 in PBS for 15 min three times and were then washed in PBS for 15 min one time. The sections were mounted with aqua-mount medium.

To determine specificity of immunostaining, primary antibody was omitted or when the antigen was available, it was used to saturate the primary antibody. The primary and secondary antibodies as well as the antigens used are summarized in Table 1.

For double staining with two primary antibodies, which had different hosts, the two antibodies were mixed and incubated. The secondary antibodies conjugated with Cy3, FITC, or Alexa Fluor 488 were also applied mixed in PBS. On the other hand, two primary antibodies, which had the same host, were incubated one by one, with washing between and applying sequentially each secondary conjugated with the fluorescence molecule. In all cases reported here, the following control was carried out: the secondary antibody for the second primary antibody was added without exposure to that antibody. No staining by that secondary antibody was taken to indicate that no artificial binding to sites on the first primary antibody or its secondary antibody occurred.

Single and double-immunolabeled sections were observed with a confocal laser scanning microscope (Zeiss CLSM 1500) equipped with an argon and helium/neon laser. Most of the images obtained were adjusted by brightness and contrast, and a few were reconstructed to three-dimensional image with the scanning of 1-μm serial images in depth. All results are based on studies from three or more animals.

Evaluation of Extent of Colocalization

All images for the colocalization study of proteins in ICC and smooth muscle of mouse jejunal tissue and the LES study were originally 512 × 512 pixels of image size obtained from Carl Zeiss confocal laser scanning microscope 1500 and LSM 510 software. The images have not only well-focused interesting areas such as ICC and smooth muscle membrane, but also uninteresting areas such as mucosa and submucosa or unfocused interesting areas due to differences of tissue thickness, immunostaining, or artifacts. Almost every image was cut and adjusted to be enhanced using brightness and contrast of LSM 510, and they were also edited using Adobe PhotoShop to organize for submission. Thus sizes of the images were decreased, and brightness and contrast were changed.

Analysis of the colocalized proteins in ICC and smooth muscle membrane was shown by scatter diagram. The scatter diagram was obtained from the modified image, but not 512 × 512 pixels of the original image. With the use of the colocalization toolbar of the laser scanning microscope software (Zeiss LSM 510), each scatter diagram was created and displayed. Consequently, initial points of two intensity channels on the scatter diagram appear sometimes moved to the extent that the brightness and contrast were changed. However, the analyzed scatter diagram shows the general pattern of colocalization distributing along the diagonal line running from the bottom to the top when colocalization was good.

RESULTS

General

Distribution of Cav1 in smooth muscle and ICC of jejunum, LES. Cav1 was present in both circular and longitudinal smooth muscle of jejunum. Cav1 immunoreactivity (IR) was punctate at the cell periphery of the cross-sectioned circular muscle cells and appeared along the periphery of longitudinal-sectioned longitudinal muscle cells (Fig. 1A). In LES, Cav1
was also present in the cell periphery of the sphincter muscle cells but was almost absent in skeletal muscle cells (Fig. 1, B and C). In addition to its presence at the periphery of smooth muscle cells of the jejunum and LES, Cav1-IR was present at the cell periphery of ICC in MP (ICC-MP), ICC in DMP (ICC-DMP), and ICC-S (Fig. 1A) but was not clearly punctate in its distribution in ICC. These cells marked with asterisks are ICC based on colocalization studies.

Table 1. Antibodies

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<th>Antibody</th>
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Secondary

- Anti-rat IgG in goat with FITC
- Anti-guinea pig IgG in donkey with FITC
- Anti-mouse IgG in donkey with Cy3
- Anti-mouse IgG in rabbit with FITC
- Anti-rabbit IgG in goat with Cy3
- Anti-rabbit IgG in goat with Alexa Fluor 488
- Anti-chicken IgG in goat with Alexa Fluor 488

aa, amino acid; Cx, connexin; nNOS, neuronal nitric oxide synthase; L-Ca2+, L-type Ca2+; PM, plasma membrane; Ca-r, calreticulin; BK, large conductance; Ca-q, calsequestrin; Cav1, Caveolin 1.

Fig. 1. This figure shows the general distribution of caveolin-1 in murine intestine (A), in lower esophageal sphincter (LES) muscle (B), and in adjacent esophageal skeletal muscle with intermixed small smooth muscle bundles (C). Caveolin-1 was located at the cell membranes of all smooth muscle cells, punctate when these were in cross section, and diffuse in interstitial cells of Cajal (ICC). ICC in A at the ICC-myenteric plexus (MP) network and the serosal ICC network as well as ICC-intramuscularis (IM) in C are labeled with asterisks. ICC-deep muscular plexus (DMP) are labeled in A with triangles. Length bars are 20 μm in A and 10 μm in B and C, respectively. sphm, Sphincter; skm, skeletal muscle; CM, circular muscle; LM, longitudinal muscle.
Fig. 2. This figure shows nearly complete colocalization of NCX1 with Cav1 (D–F) but not, in general, with Cx40 in A–C. Cells believed to be ICC in Fig. 2 are labeled as in Fig. 1. G–L show that Cx43 and Cx40 are present at punctate site on the cell periphery, primarily in CM, none in LM, and generally not colocalized with Cav1. Some exceptions, labeled with triangles, occur in ICC-DMP (I and L) and ICC-MP (L). M–O show the complete colocalization of Connexin (Cx)40 and -43 in CM. P–R show the general lack of colocalization of Cx40 and neuronal nitric oxide synthase (nNOS) labeled with an antibody against the COOH-terminal epitope (nNOS-C). nNOS appears to be present in the membranes of smooth muscle and some ICC. In Fig. 2, cells believed to be ICC are labeled with asterisks. Length bars are 20 μm in all cases. G, myenteric ganglia.
Fig. 3. A–C show that the nNOS is colocalized with caveolin (Cav1) throughout the muscularis externae and also with ICC-DMP at sites with triangles. Voids in these figures are due to regions out of focus. D–F show that Cav1 and c-kit are colocalized in ICC-MP (asterisks) and ICC-DMP (triangles). Note that Cav-1 in ICC does not appear punctate. G–I show that c-kit immunoreactivity is colocalized with Na\(^+/\)Ca\(^{2+}\) exchanger 1 (NCX1) in ICC-MP, ICC-serosa, and ICC-DMP (triangles). J–M are from whole mount preparations. J shows that immunoreactivity to the NH\(_2\) terminal epitope of nNOS, which recognizes neural NOS of nerves but neural NOS of other cells, is not colocalized with c-kit in ICC-MP. K shows c-kit immunoreactivity in the DMP. L shows that immunoreactivity to the \(\alpha_{1c}\) subunit of the L-Ca\(^{2+}\) channel was present in the gangionated plexus and in ICC and muscle, whereas M shows the same for the ICC of DMP and muscle. N–P and Q–S show the colocalizations of Cav1 with the \(\alpha_{1c}\) subunit of the L-Ca\(^{2+}\) channel and with the plasma membrane (PM) Ca\(^{2+}\) pump, respectively. These colocalize both with smooth muscle and with ICC-MP (asterisks) and ICC-DMP (triangles). Length bars are 20 \(\mu\)m in A–K and N–S; 50 \(\mu\)m in L and M. G, myenteric ganglia.
Jejunum

NCX1, colocalized with Cav1, not with Connexin-40. The NCX1-IR was punctate in the cell periphery of the circular muscle cells and located densely along PM of the longitudinal smooth muscle cells. Connexin (Cx)40-IR was distributed in the circular muscle but was not colocalized with NCX1 (Fig. 2, A–C). NCX1 was previously reported to be distributed in smooth muscles, ICC-MP, ICC-DMP, and ICC-S of jejunum (see Fig. 2A). Apparent colocalization of Cav1 and NCX1 appeared also in some ICC around myenteric ganglia and a few ICC-DMP of jejunum (Fig. 2, D–F).

Little colocalization of Cav1 and Cx43 or Cx40. Cx43 and Cx40 were distributed in punctate sites at the cell periphery of jejunum circular muscle cells but were not found in longitudinal muscle. Neither Cx43 nor Cx40 was colocalized with Cav1. However, a few Cav1-IR cells adjacent to myenteric ganglia and DMP showed yellow dots colocalized with Cx40 or Cx43 (Fig. 2, G–L). These may represent gap junctions on ICC or smooth muscle.

Colocalization of Cx43 and Cx40. Cx43 was colocalized with Cx40 in circular muscle cell membranes of jejunum. Some Cx43-IR was sparsely distributed at the inner circular

Fig. 4. A–C show that the PM Ca\(^{2+}\) pump and c-kit are colocalized in some ICC-MP and and ICC-serosa (asterisks) and ICC-DMP (triangles). D–F show that calreticulin (Ca-r) is present in smooth muscle, mostly in the cytosol, and also colocalized with Cav1 in ICC-MP and serosa (asterisks) and some ICC-DMP (triangle). G–I show that the large-conductance Ca\(^{2+}\)-activated (BK) channels are present in smooth muscle and and ICC but colocalized with Cav1 only in some ICC-MP and serosa (asterisks) and ICC-DMP (triangle). J–O show the locations of calsequestrin (Ca-q), rare in smooth muscle but primarily in ICC-MP and serosa (asterisks), and ICC-DMP and ICC serosa (triangles). Length bars are all 20 \(\mu\)m.
muscle cell layer without colocalization with Cx40, but it was distributed and colocalized with Cx40 in the main circular muscle cell layer (Fig. 2, M–O).

Colocalization of nNOS-C and Cx40. nNOS identified when the antibody against the epitope to the COOH-terminal end (nNOS-C) was applied, was colocalized occasionally with Cx40 in jejunum circular muscle. Cx40 was located in the cell periphery of the circular muscle cells immunostained with nNOS-C and showed significant colocalization, based on the presence of punctate yellow dots. Interestingly, nNOS-C-IR was localized in ICC-S of the jejunum (Fig. 2, P–R).

Colocalization of Cav1 and nNOS-C. Cav1 appeared to be colocalized with the nNOS-C in smooth muscle of jejunum. In studies using double immunofluorescences, Cav1 antibody conjugated with Cy3 and nNOS-C antibody conjugated with FITC showed nearly complete colocalization, based on the presence of punctate yellow coloring in the cell periphery of both circular muscle and longitudinal muscle of jejunum. Similar colocalization also appeared as dense yellow IR in ICC-DMP (Fig. 3, A–C).

Colocalization of Cav1 and c-kit and NCX1 in ICC of jejunum. Figure 3, D–F, shows that Cav1 is colocalized with c-kit in the cell periphery of ICC-MP and ICC-DMP of the jejunum. ICC-MP sometimes appeared to connect to ICC-DMP by an ICC process. NCX1 was colocalized with c-kit in ICC-MP, ICC-DMP, and ICC-S of jejunum. ICC immunostained to c-kit were located underneath and above myenteric ganglia, in DMP and in serosa, and were also colocalized with NCX1. NCX1-IR was also present in myenteric ganglia of jejunum (Fig. 3, G–I).
Three-dimensional images of ICC-MP and ICC-DMP. Figures 3, J and K, show three-dimensional confocal images of ICC-MP and ICC-DMP of jejunum. Cell bodies of ICC-MP were distributed near and under myenteric neurons immunostained with an antibody against an epitope to the NH2-terminal end (nNOS-N), and cell processes of ICC-MP were connected to other ICC-MP. ICC-DMP were distributed along the circular muscle layer.

Colocalization of Cav1 and L-Ca$^{2+}$/H11001 channel. In whole mount preparations of smooth muscle of jejunum, the L-Ca$^{2+}$/H11001, $\alpha_{1c}$-subunit, channel was distributed in ICC-MP and ICC-DMP and also in MP and DMP (Fig. 3, L and M). Immunoreactivity to the L-Ca$^{2+}$ channel was distributed over smooth muscle, MP, DMP, and serosa of jejunum. In smooth muscle, L-Ca$^{2+}$ channel was present not only in cell periphery but also in cell center of the circular and longitudinal muscle and was colocalized with Cav1 in the cell periphery. Dense immunoreactivity to the L-Ca$^{2+}$ channel was colocalized with Cav1 in LES muscle and ICC-IM. Note that L-Ca$^{2+}$ channel immunoreactivity is also present in the cytosol of LES muscle. G–I show the excellent colocalization of the PM-Ca$^{2+}$ pump with Cav1 in LES. J–L show that the PM-Ca$^{2+}$ pump is present in ICC-IM of LES (asterisks) as well as in muscle cells. Length bars are 20 µm for A–C and G–I but 10 µm for D–F and J–L.

Fig. 6. A–C show the colocalization of NCX1 and c-kit in ICC-IM (asterisks) of sphincter muscle. D–F show the colocalization of Cav1 and the L-Ca$^{2+}$/H11001 channel in LES muscle and ICC-IM. Note that L-Ca$^{2+}$ channel immunoreactivity is also present in the cytosol of LES muscle. G–I show the excellent colocalization of the PM-Ca$^{2+}$ pump with Cav1 in LES. J–L show that the PM-Ca$^{2+}$ pump is present in ICC-IM of LES (asterisks) as well as in muscle cells. Length bars are 20 µm for A–C and G–I but 10 µm for D–F and J–L.
and serosa of jejunum and some ICC. Colocalization with Cav1 occurred in a few circular muscle cells cut in cross section, ICC-MP, ICC-DMP, and serosal ICC (Fig. 4, D–F). The BK Ca\(^{2+}\) channel was found distributed in smooth muscle cells, myenteric ganglia, DMP, and serosa of jejunum, but colocalization with Cav1 was found only in ICC-MP, ICC-DMP, and ICC-S (Fig. 4, G–I).

**Colocalization of Cav1 and calsequestrin (Ca-q) in jejunum.** Only weak immunoreactivity to Ca-q appeared in smooth muscle cells, but dense immunoreactivity was observed in some ICC-MP, a few ICC-DMP, and ICC-S of jejunum. Colocalization with Cav1 was noted in ICC-MP, ICC-DMP, and ICC-S, and colocalization with c-kit was found in ICC-MP and ICC-S (Fig. 4, J–O).

**LES Smooth Muscle**

nNOS-C and NCX1 in smooth muscle of LES, colocalized with smooth muscle Cav1. nNOS-C-IR and NCX1 were densely present in the cell periphery of LES cells. nNOS-C appeared to be colocalized with Cav1 in the cell periphery of smooth muscle cells (Fig. 5, A–C), as was NCX1 (Fig. 5, D–F).

**c-kit Present in ICC-IM and colocalized with NCX1, but Cx absent from LES.** As in jejunum, c-kit-IR appeared in the cell periphery of the ICC-IM of LES (Fig. 5, G–I). Surprisingly, both Cx43 and Cx40 were absent in LES. Cx43 was absent (Fig. 5J) as was Cx40 (Fig. 5, K and L). Also, we failed to find gap junctions in LES in ultrastructural studies (not shown). NCX1, similar to Cav1, was colocalized with c-kit in ICC-IM in sphincter muscle bundles of LES (Fig. 6, A–C).
Colocalization of L-Ca\(^{2+}\) channel and PM-Ca\(^{2+}\) pump with Cav1 in LES. As in smooth muscle cells of jejunum, the L-Ca\(^{2+}\) channel was located at both the cell periphery and the cell center of LES and was colocalized with Cav1 in the cell periphery (Fig. 6, D–F). It was also colocalized with cells thought to be ICC-IM adjacent to LES muscle bundles. As also in jejunum, the PM-Ca\(^{2+}\) pump was similarly colocalized with Cav1 and at cells appearing to be ICC-IM (Fig. 6, G–I). This was confirmed in Fig. 6, J–L, which show that these cells are c-kit immunoreactive.

Colocalization of Cav1 and Ca-r and Ca-q in LES. In the LES, Ca-r-IR was distributed in the cell membrane and cytoplasm of sphincter muscle cells and colocalized, in part, with Cav1 in the cell periphery (Fig. 7, A–C). In skeletal muscle, there was Ca-r-IR in sarcolemma and the Z-line of skeletal muscle cell as well as in ICC-IM in associated small smooth muscle bundles. When ICC-IM were present, Ca-r was colocalized, in part, with Cav1.

Colocalization of Cav1 and BK-Ca\(^{2+}\) channel in LES. In LES, BK-Ca\(^{2+}\) channels appeared to be distributed in cytoplasm of sphincter muscle cell, but only in ICC-IM was it colocalized with Cav1 around sphincter muscle bundle (Fig. 7, D–F). The BK-Ca\(^{2+}\) channel appeared in sarcolemma and the Z-line of skeletal muscle cell but was colocalized with Cav1 in sarcolemma of ICC-IM associated with smooth muscle bundles intermingled with skeletal muscle fibers.

Colocalization of Cav1 and Ca-q and Ca-r. In LES, very weak immunoreactivity to Ca-q was present in sphincter muscle, but ICC-IM showed not only dense immunoreactivity to...
Ca-q but also colocalization with Cav1 (Fig. 7, G and I). Colocalization with Cav1 appeared to be with ICC-IM, and this was confirmed by findings of colocalization with c-kit in ICC-IM of LES (Fig. 7, J–L). In contrast, Ca-r was present as in smooth muscle of jejunum, as shown above, in the cytoplasm of smooth muscle bundles amidst skeletal muscle. It, similar to Ca-q, was colocalized with Cav1 in what appear to be ICC-IM (Fig. 7, A–C).

Fig. 9. A–C show that L-Ca^2+ channels and Cav1 were colocalized at the plasmalemma in some skeletal muscle bundles. Other unidentified cells were also heavily immunoreactive to L-Ca^2+ channel antibodies. D–F illustrate the close colocalization of Cav1 and the PM-Ca^2+ pump in smooth muscle bundles and ICC-IM (asterisks). G–I illustrate that Ca-r is present in smooth muscle but not skeletal muscle bundles in this region and also in ICC-IM associated with smooth muscle (asterisks). J–L show that BK-Ca^2+ channels are extensively present in skeletal muscle bundles and colocalized with Cav1 at the plasmalemma of some bundles. In contrast to Ca-r (G–I), Ca-q is present in skeletal muscle bundles often associated with Z-lines and colocalized with Cav1 at the plasmalemma.
LES Region with Mixed Smooth and Skeletal Muscle

In general, the region just proximal to the LES had smooth muscle bundles intermixed with skeletal muscle, and ICC-IM had the same associated proteins as in LES proper. The smooth muscle cells had Cav1, but skeletal muscle cells lacked immunoreactivity to both Cav1 and nNOS-C (Fig. 8, A–C). Small smooth muscle bundles among skeletal muscle often had c-kit immunoreactive cells, presumably ICC-IM, which also were Cav1 immunoreactive (Fig. 8, D–F).

In skeletal muscle, NCX1-IR was present in ICC-IM associated with small muscle bundles near skeletal muscles (Fig. 8, G–I). Neither Cx43 (Fig. 8J) nor Cx40 (Fig. 8, K and L) were present in this region. In skeletal muscle, the L-Ca2+ channel was present in skeletal muscle bundles, apparently in Z-line, and possibly in the plasmalemma associated with Cav1 (Fig. 9, A–C). Other deeply stained cells in these figures may be ICC-IM, however, there was no immunoreactivity to the PM-Ca2+ pump, but the PM-Ca2+ pump in ICC-IM and smooth muscle was colocalized with Cav1 (Fig. 9, D–F). Ca-r was nearly absent from skeletal muscle bundles, but it was present colocalized with Cav1 in smooth muscle and their ICC-IM (Fig. 9, G–I). The BK-Ca2+ channel had a distribution similar to the L-Ca2+ channel in skeletal muscle bundles (Fig. 9, J–L) and was present also in small muscle bundles and their associated ICC-IM, where it was sometimes colocalized with Cav1. However, Ca-q was abundantly present in skeletal muscle and colocalized with Cav1 in sarcolemma (Fig. 9, M–O).

Expression Level Analysis with the Scatter Diagram

Images of Cav1 and NCX1 (Fig. 10, A and E), Cav1 and nNOS-C (Fig. 10, B and F), Cav1 and L-Ca2+ (Fig. 10, C and G), and Cav1 and PM-Ca2+ pump (Fig. 10, D and H) were analyzed to visualize intensity distributions of colocalization in both jejunum and LES. Scatter diagrams of colocalization of Cav1 with NCX1, nNOS, and PM-Ca2+ were centered around the diagonal, indicating good general colocalization. In contrast, the scatter diagram for Cav1 with the L-Ca2+ channel was poorly centered, suggesting less good colocalization. Studies using the inverted ellipse function indicated that L-Ca2+ channels were best colocalized with Cav1 in smooth muscle membranes and ICC (not shown).

DISCUSSION

This study has defined the distribution of several proteins and signaling molecules that are or are not colocalized with Cav1 in ICC and smooth muscle, of mouse intestine, and LES. Previous studies showed that cholesterol depletion diminished both Cav1 and caveolae while reducing pacing frequencies (6). This suggested that pacing might depend on proteins associated with Cav1. Our further goal was to evaluate proteins involved in signal transduction, which might depend on their location with Cav1 for normal function. Our findings regarding ICC of various classes are summarized in Table 2. Note that many of

Fig. 10. A–H show scatter diagrams of Cav1 and NCX1, Cav1 and nNOS-C, Cav1 and L-Ca2+ channel, and Cav1 and PM-Ca2+ pump in jejunum and LES. A, B, D–F, and H show the scatter spots distributed along the diagonal line running from bottom left to top right producing identical colocalization image. C and G show the irregular spots in general, but the scatter spots for CM membrane, ICC-DMP, and ICC-MP in C and for LES muscle membrane in G are distributed along the diagonal line. In A and E, Intensity Ch1 displays Cy3 as source 1 for NCX1 and Intensity Ch2 displays FITC as source 2 for Cav1. In B–D and F–H, Intensity Ch1 displays Cy3 as source 1 for Cav1, and Intensity Ch2 displays FITC or Alexa488 as source 2 for nNOS-C, L-Ca2+ channel, or PM-Ca2+ pump.

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G582 CAVEOLIN-1 AS SITE OF SIGNALING
the proteins associated with Cav1 in the various classes of ICC are the same despite some differences in functions among these classes: ICC-MP pace slow waves and are possible sites of enteric innervation, ICC-DMP in intestine are sites of enteric innervation, and ICC-IM in LES are also sites of enteric innervation (3, 32). A few differences were found, e.g., only the ICC-DMP had significant immunoreactivity to Cx43 and 40, consistent with the known presence of gap junctions among these (26). Aside from our study, there is little information about differences in proteins in various ICC classes. However, the proteins found in ICC-MP from intestine and ICC-IM from fundus of mice using PCR (12) also had some differences in protein expression, but few of those studied were involved in signal transduction.

One surprising result was the failure to find either Cx40 or 43 associated with ICC-MP or smooth muscle of LES. In other species, ICC of LES are connected to smooth muscle and to one another by gap junctions. Gap junctions also interconnect smooth muscle cells. In canine LES, gap junctions as well as Cx40 and Cx43 were present in abundance (4, 7, 9, 29). And gap junctions are present in opossum, feline, human LES, and other mammalian species (29). The gap junctions between ICC and smooth muscle in mouse LES are believed to be the means of transmitting enteric signals delivered to ICC-MP to muscle and those between smooth muscle cells are believed to allow spread of enteric messages throughout the LES. Other connexins may compose gap junctions of murine LES, but we found none in a preliminary ultrastructural study. Smooth muscle cells had proteins similar to those in ICC, consistent with the origin of both from common mesenchymal precursors.

Many findings were consistent with the structural and functional data we have obtained. For example, we found that the L-Ca\(^{2+}\) channels appeared to be associated with Cav1 in ICC, consistent with the important role we have recently found that these channels play in allowing a gradient of frequencies, higher proximally, along the intestine (G. Boddy and E. E. Daniel, unpublished observation). The apparent colocalization of these channels with Cav1 in smooth muscle and ICC is also consistent with the effects we observed when caveolae and Cav1 distribution were affected by manipulation of cholesterol using cyclodextrin6. We found that removal of cholesterol affected Cav1 and caveolae in ICC and smooth muscle, an effect associated with a decrease in ICC pacing frequencies and contractions. These changes largely reversed on restoring cholesterol. Conversely, adding excess cholesterol affected Cav1 distribution, but not the numbers of caveolae in ICC and muscle, and also decreased pacing frequency and affected contractions. We suspect that these changes were related to the altered functions of L-Ca\(^{2+}\) channels when Cav1 and L-Ca\(^{2+}\) channels are disassociated. However, the coincidence between L-type Ca\(^{2+}\) channels and Cav in ICC and smooth muscle was only partial, as shown by quantitative evaluation (see Fig. 10). This hypothesis can be tested functionally now that Cav1-deficient mice are available.

In general, our findings strongly suggest that, in canine intestine, LES, as in airway smooth muscle (7, 11), besides L-Ca\(^{2+}\) channels, other Ca\(^{2+}\)-handling molecules, PM-Ca\(^{2+}\) pump, NCX1, and sometimes Ca-r, were near or colocalized with Cav1. When we examined the degree of colocalization semiquantitatively, it was apparent that the PM-Ca\(^{2+}\) channel, nNOS, and NCX1 were closely colocalized with Cav1 in smooth muscle and ICC, whereas neither Ca-r or Ca-q was closely colocalized with Cav1 in smooth muscle (Fig. 10). Given these findings, it is probable that Cav1 and Ca-r are side by side rather than in the same organelle in LES and intestine. However, the close overlap of Cav1 and the PM-Ca\(^{2+}\) pump, the nNOS and the NCX-IR with Cav1, leads to the hypothesis that they are all associated with caveolae of both smooth muscle and ICC. For the L-Ca\(^{2+}\) channel, the colocalization is quantitatively less good than for other Ca\(^{2+}\)-handling molecules, and this may mean that is near but not bound to Cav1. This allows the further hypothesis that the L-Ca\(^{2+}\) channel near caveolae of ICC is very close to the ER and functions to restore ER-Ca\(^{2+}\) when Ca\(^{2+}\) is released from ER and lost from the ICC or smooth muscle cells via the PM-Ca\(^{2+}\) pump or NCx. We have recently reported that the L-Ca\(^{2+}\) channel plays a crucial role in pacing and is essential for the intrinsic frequency gradient (G. Boddy and E. E. Daniel, unpublished data).

Other findings raised important questions about why certain signaling molecules are present associated with Cav1. In smooth muscle of jejunum and LES, as in canine jejunum and LES and canine airway smooth muscle, Cav1 was associated with nNOS using an antibody (7, 11) that recognized an epitope located in the COOH-terminal end of the molecule. Earlier in canine (7) LES, we found that nNOS appeared to function to modulate tone (21, 22) by releasing NO in response to increased intracellular Ca\(^{2+}\) concentration. The role of Cav1 in control of eNOS in endothelium has been described as inhibition of its function by binding it until Ca\(^{2+}\)-calmodulin occupies the NOS molecule and releases it. A similar role can be proposed for nNOS in smooth muscle (12) and ICC. However, we found no change in frequency of pacing in longitudinal muscle segments of mouse intestine when L-NNA was applied either alone or with TTX. In circular muscle segments (5, 6), when the tonic neural inhibition was abolished either by TTX or L-NNA, frequency increased, but not to the level of adjacent longitudinal muscle segments. Studies are required of a possible role of this nNOS in modulating con-

Table 2. Immunohistochemical distribution of proteins, channels, and receptors in the different ICC of the mouse jejunum and LES

<table>
<thead>
<tr>
<th>Protein</th>
<th>ICC-IM</th>
<th>ICC-S</th>
<th>ICC-MP</th>
<th>ICC-DMP</th>
<th>L-Ca channel</th>
<th>PM-Ca</th>
<th>BK-Ca</th>
<th>Ca-r</th>
<th>Ca-q</th>
<th>Cx40</th>
<th>Cx43</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cav1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NCX1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>nNOS-C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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+, ++, and +++ indicate intensity of staining; − means absent or inconspicuous. ICC, interstitial cells of Cajal; LES, lower esophageal sphincter; DMP, deep muscular plexus; MP, myenteric plexus; S, serosa; IM, intramuscular; NCX1, Na\(^+/\)Ca\(^{2+}\) exchanger 1; nNOS-C, neuronal nitric oxide synthase with an antibody against the COOH-terminal epitope.
tractions in response to agonists that raise Ca\(^{2+}\) levels near the membrane.

In the ICC of the DMP, Cav1 was also colocalized with nNOS. Again, the implications of this for function of these ICC as secondary pacing molecules, as in the canine intestine, or as intermediaries in neurotransmission are unexplored.

In the case of NCX1, the sodium-calcium exchanger, we found it colocalized semiquantitatively with Cav1 in smooth muscle of jejunum and LES and in some ICC-MP and ICC-DMP. However, so far we have not been able to define a role for this exchanger. Removal or marked reduction of external sodium levels by replacement with LiCl markedly reduced pacing frequency but did not usually cause tone increase as expected on abolition of the Na\(^+\) gradient if the exchanger was a major pathway for Ca\(^{2+}\) removal after contraction (E. E. Daniel, G. Boddy, W. J. Cho, and A. Willis, unpublished observation). The effect on frequency could be an effect on inward current in ICC carried mainly by sodium. A selective inhibitor of NCX1 had no effect on the frequency of pacing in longitudinal or circular muscle segments (2).

We found that Cx43 and -40 were closely colocalized with each other but were not quantitatively associated with Cav1 in circular muscle of the jejunum. This was similar to our findings in canine intestine (7, 29). Both were absent from longitudinal muscle, consistent with ultrastructural data. We have found that block of formation of functional gap junctions using peptides (4, 26) from the external loops of Cx40 and -43 had no effect on pacing frequencies driven from ICC (M. Mannarino, W. J. Cho, G. Boddy, and E. E. Daniel, unpublished observation). The last finding is consistent with the lack of evidence that low-resistance contacts through gap junctions are essential for transmission of slow waves and pacing from ICC to muscle (4, 8–10, 24). Although occasional punctate sites of immunoreactivity to Cx40 appeared to be colocalized with c-kit in ICC-S and very rarely in ICC-MP, there was no evidence that gap junction proteins were present between ICC and smooth muscle. The absence of either Cx40 or Cx43 from LES was expected on abolition of the Na\(^+\) pump was present and colocalized with Cav1 in smooth muscle, consistent with ultrastructural data. We have found that block of formation of functional gap junctions using peptides (4, 26) from the external loops of Cx40 and -43 had no effect on pacing frequencies driven from ICC (M. Mannarino, W. J. Cho, G. Boddy, and E. E. Daniel, unpublished observation). The last finding is consistent with the lack of evidence that low-resistance contacts through gap junctions are essential for transmission of slow waves and pacing from ICC to muscle (4, 8–10, 24). Although occasional punctate sites of immunoreactivity to Cx40 appeared to be colocalized with c-kit in ICC-S and very rarely in ICC-MP, there was no evidence that gap junction proteins were present between ICC and smooth muscle. The absence of either Cx40 or Cx43 from LES was expected on abolition of the Na\(^+\) gradient if the exchanger was a major pathway for Ca\(^{2+}\) removal after contraction (E. E. Daniel, G. Boddy, W. J. Cho, and A. Willis, unpublished observation). The effect on frequency could be an effect on inward current in ICC carried mainly by sodium. A selective inhibitor of NCX1 had no effect on the frequency of pacing in longitudinal or circular muscle segments (2).

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