nNOS in canine lower esophageal sphincter: colocalized with Cav-1 and Ca\(^{2+}\)-handling proteins?

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Daniel, E. E., J. Jury, and Y. F. Wang. nNOS in canine lower esophageal sphincter: colocalized with Cav-1 and Ca\(^{2+}\)-handling proteins? Am J Physiol Gastrointest Liver Physiol 281: G1101–G1114, 2001.—Immunochemical studies with light microscopy, confocal microscopy, and electron microscopy were used to examine proteins associated with caveolin (Cav) in canine lower esophageal sphincter. The main Cav was Cav-1. It appeared to be colocalized at the cell periphery, in punctate sites, with immunoreactivity to antibodies against different COOH- and NH\(_2\)-terminal epitopes of neuronal nitric oxide (NO) synthase (nNOS). One COOH-terminal-directed antibody, made in guinea pig, was used to colocalize other immunoreactivities. Those that apparently colocalized with nNOS were L-Ca\(^{2+}\) channels, the PM Ca\(^{2+}\) pump, and, in part, calreticulin and calsequestrin. The large-conductance Ca\(^{2+}\)-activated K\(^+\) (BK\(_{Ca}\)) channels were located in discrete peripheral sites, some with Cav. Immunoreactivities not fully colocalized with nNOS were to the cated in discrete peripheral sites, some with Cav. Immuno-

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any of these proteins interact in a fashion that would be expected from their association with Cav.

**METHODS**

**Animals and Tissues**

Mongrel dogs, chosen irrespective of gender, were euthanized with an overdose of pentobarbital sodium (100 mg/kg) in accordance with a protocol approved by the McMaster Animal Ethics Committee and the guidelines of the Canadian Council for Animal Care. The gastroesophageal region was then carefully removed from the dog and placed in a cold (4°C) Krebs-Ringer solution composed of (in mM) 115.0 NaCl, 4.6 KCl, 22.0 NaH2PO4, 2.5 CaCl2, and 11.0 glucose. The Krebs-Ringer solution was also equilibrated with 5% CO2-95% O2. The gastroesophageal junction was then opened on the gastric greater curvature side, and the mucosa was removed by sharp dissection. This revealed the LES as a thickened ring of muscle composed of clasp fibers with oblique gastric sling fibers on either side and skeletal muscle in the longitudinal layer above. The LES used for experimentation was taken only from the clasp region of the LES.

**Fixation and Preparation of Tissues for Light Microscopic Immunohistochemistry**

These methods have been described in detail (11, 12, 34, 35, 49). In brief, fixation was usually in 4% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7. When cryostat sections were to be prepared, tissues were cryoprotected with 20% sucrose, frozen and sectioned 10-μm thick, and studied with indirect immunofluorescence with the use of a Leitz LaborLux fluorescence microscope with an I2 filter for Cy3 and an N2 filter for FITC. Laminar preparations were prepared by dissection of the relevant layers after they were stretched by being mounted on Sylgard and cleared with DMSO. Cryostat sections were immunostained by incubation overnight with the appropriate antibody and washed before being treated with a secondary antibody labeled with a fluorescent molecule.

Staining for immunocytochemistry used antibodies to Cav-1 and -3, nNOS, connexins 43, 45, and 40, vinculin, calreticulin, calsequestrin, the SR Ca2+ pumps, and the PM. Table 1 summarizes the primary and secondary (fluophore labeled) antibodies used. Usually, smooth muscle cells were cut in cross sections.

Specificity of staining was determined by a series of tests: omission of primary antibody (to rule out nonspecific staining by secondary antibody), omission of secondary antibody (to rule out autofluorescence), and saturation of primary antibody by antigen when available (to ensure that the antibody stained only specific antigen sites). Additional procedures were carried out to reduce background staining, e.g., pre-treating sections with an antibody made against an immunoglobulin from a species different from that used to raise either the primary or the secondary antibody.

For normal colocalization studies, primary antibodies from different species were used with previously detailed procedures (12, 13, 49), e.g., when colocalization of proteins appeared to be possible based on staining with antibodies against individual proteins. We first carried out colocalization studies at the level of light microscopy. Because orientation or section thickness problems limit resolution in studies of colocalization of proteins with Cav-1, we used both normal immunocytochemistry and dual-laser confocal microscopy (Carl Zeiss, LSM 510) on 1-μm sections. Additional studies were conducted at the ultrastructural level.

**Methods for Fixation and Preparation of Tissues for Ultrastructural Study**

Tissues were fixed after dissection by immersion in 2% glutaraldehyde and 4% paraformaldehyde with 4.5% sucrose, then carefully removed from the dog and placed in a cold (4°C) Krebs-Ringer solution composed of (in mM) 115.0 NaCl, 4.6 KCl, 22.0 NaH2PO4, 2.5 CaCl2, and 11.0 glucose. The Krebs-Ringer solution was also equilibrated with 5% CO2-95% O2. The gastroesophageal junction was then opened on the gastric greater curvature side, and the mucosa was removed by sharp dissection. This revealed the LES as a thickened ring of muscle composed of clasp fibers with oblique gastric sling fibers on either side and skeletal muscle in the longitudinal layer above. The LES used for experimentation was taken only from the clasp region of the LES.

**Table 1. Antibodies**

<table>
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<tr>
<th>Antibody</th>
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<th>Source</th>
<th>Antigen</th>
<th>Dilution</th>
<th>Peptide</th>
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<td>Dr. A. Schwartz</td>
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<td>α1C Subunit</td>
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<td>Affinity BioReagents</td>
<td></td>
<td>1:400</td>
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**Secondary**

- Anti-guinea pig in donkey with Cy3
- Anti-rabbit in donkey with FITC
- Anti-mouse in donkey with Cy3
- Anti-guinea pig in goat with FITC
- Anti-rabbit in donkey with Cy3
- Anti-mouse in goat with FITC

*a All secondary antibodies from BIO/CAN Scientific.
crose in 0.75% cacodylate buffer at pH 7.4. The methods have been described in detail (3, 4). After fixation, the tissues were washed, stained en bloc with uranyl acetate, dehydrated in graded ethanol and propylene glycol, and embedded in Spurr resin. After ultrathin sections were cut on a Reichert Ultracut E microtome, they were stained in grids with lead citrate and studied.

Fixation for ultrastructural immunohistochemistry was carried out by immersion in 0.1% glutaraldehyde with 4% paraformaldehyde and 3% sucrose in 0.1 M phosphate buffer (pH 7.4) followed by washing, dehydration in ethanol, infiltration with LR White acrylic resin, polymerization at −20°C, and sectioning. Labeling was with protein A gold or related methods. Silver enhancement was carried out with a silver-enhancing kit (British Biocell, Cardiff, UK). Ultrastructural studies were carried out on a JOEL-1200 EX Biosystem electron microscope at 80 kV.

Patch-Clamp Techniques

Cell isolation. The LES was dissected as described in Methods for Fixation and Preparation of Tissues for Ultrastructural Study, and strips were cut into 1- to 2-mm² pieces and placed in the dissociation solution. Cells were dissociated in (mM) 0.25 EDTA, 125 NaCl, 4.8 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose for 30 min. An enzyme solution containing papain (130 mg/ml), (−)-1,4 dithio-L-threitol (DTT, 15.4 mg/ml), BSA (100 mg/ml), and Sigma collagenase blend H (occasionally F) was added to the tissue pieces for 30–60 min. After incubation, the enzyme solution was decanted off, and the tissue pieces were rinsed in enzyme-free dissociation solution. Single cells were gently agitated mechanically with siliconized Pasteur pipettes to disperse and isolate single smooth muscle cells. Cells used in this study were patch clamped at room temperature (22–24°C), usually within 8 h of isolation.

**Fig. 1.** Light microscopic immunohistochemical locations of caveolins (Cav) in canine lower esophageal sphincter (LES). A: low-magnification view of section of canine LES including overlying skeletal muscle (ske) immunostained for Cav-1. All LES smooth muscle cells are stained in their periphery; skeletal muscle is stained in a few discrete sites. B: higher-magnification view of immunostaining of LES muscle. Note punctate staining in the periphery. C: immunostaining of skeletal muscle by antibody against Cav-3. Note staining of all muscle bundles at the periphery. In some regions where the muscle bundle membrane is cut obliquely, punctate staining is found. D: immunostaining of LES sphincter (sph) by Cav-3. Note inconsistent scattered staining, rarely complete, around the cell periphery. E: immunostaining of LES skeletal muscle with antibody against Cav-3 after saturation with the peptide epitope used to produce it. Compare with C. Bars: 50, 12.5, 25, 12.5, and 25 μm for A–E, respectively.
Patch-clamp methodology. Cells from the suspension were placed in a glass-bottomed dish. Within 30 min, cells adhered to the dish. The cells were then washed by perfusion with Ca\textsuperscript{2+}-containing external solution containing (in mM) 140 NaCl, 4.5 KCl, 2.5 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 HEPES, and 5.5 glucose (pH adjusted to 7.35 with NaOH). Patch electrodes were made from borosilicate glass capillary tubes with a Flaming/Brown micropipette puller (Sutter Instruments). After being polished with a microforge (Narishige MF-83) and being filled, the pipettes had resistances of 2–5 M\textOmega. High-Ca\textsuperscript{2+} pipette solution contained (in mM) 2.5 CaCl\textsubscript{2}, 140 KCl, 1 MgCl\textsubscript{2}, 10 HEPES, 4 Na-ATP, and 0.3 EGTA to obtain free [Ca\textsuperscript{2+}] of 8 \textmu M. CaCl\textsubscript{2}, KCl, and EGTA levels were adjusted to obtain 50 or 200 nM free Ca\textsuperscript{2+} levels as calculated with MAX Chelator software (version 6.72) by Bers et al. (5).

A standardized stimulation protocol was used to evoke currents from isolated smooth muscle cells, which were studied without leak subtraction or capacity compensation. All cells had access resistance <25 M\textOmega. Cell capici-

Fig. 2. Electron micrographs of immunostaining of ultrastructural sections from canine LES. a: Immunostaining of LES sphincter muscle with Cav-1 (immunogold followed by silver enhancement). Nearly all visible caveolae are stained. b: Immunostaining of LES skeletal muscle with Cav-3 (immunogold followed by silver enhancement). Extensive staining is evident. c: Immunostaining of canine LES with Cav-3. Most visible caveolae are unstained. Only a few sites (arrowheads) are stained.
Fig. 3. Immunostaining of LES with antibodies against neuronal nitric oxide synthase (nNOS) as visualized with light microscopy. A–C: antibody against COOH-terminal (C) epitope of nNOS raised in guinea pig (GP). A: low-magnification view of LES sphincter and skeletal muscles. Note staining in periphery of smooth muscle cells and occasional staining near skeletal muscle (likely nerves). B: immunostaining of nerves in the myenteric plexus (Myp) of LES. C: higher magnification of immunostaining of LES smooth muscle showing punctate immunostaining of cell peripheries. D–F: antibody against C epitope of nNOS raised in rabbit (Rab-nNOS-C). D: immunostaining of Myp region of LES shows strong staining of nerves and weak staining of muscle (not discernible because exposure time was too short to allow clear visualization of nerve staining). E: immunostaining of LES muscle. Less intense and more nonspecific staining than in C but stain can be found in periphery and is punctate in many areas. F: staining of skeletal muscle bundle cut in cross section. Note staining of periphery. G and H: antibody against NH₂-terminal epitope of nNOS raised in rabbit. G: immunostaining of skeletal muscle bundles in various orientations, showing that the periphery and Z lines appeared to be stained. H: in contrast to other nNOS antibodies, this one stains the whole surface of sphincter cells. Bars: A, B, D, and G = 50 μm; C, E, F, and H = 12.5 μm.
Fig. 4. Electron micrographs of immunostaining of ultrastructural sections from canine LES. 
a: Immunogold particles (10 nm) localized nNOS (arrows; antibody raised in guinea pig against nNOS) and are associated with caveolae. Not all caveolae were stained (*). Note the gap junction (open arrow). 
b: Immunogold particles (5 nm) localized Cav-1 to cell surface (double arrows), usually associated with visible caveolae. Larger particles (10 nm) also localized nNOS to the cell surface, often near caveolae (larger arrows). 
c: Gold (5-nm) particles localized nNOS mostly to caveolae (double arrows) but sometimes near 10-nm gold particles (large arrows) labeling Cav-1. A caveolae, cut tangentially at the cell surface, is indicated by ▲.

Fig. 5. Localization of Ca\textsuperscript{2+}-handling proteins in LES. 
A: localization of calreticulin (Ca-R) in LES. Calreticulin immunostaining was predominantly found at punctate sites at cell peripheries and centrally located in the region of the cell nucleus as either a ring or a round spot. 
B: localization of immunostaining to a polyclonal antibody to the cardiac L-Ca channel. What appears to be nonspecific staining obscures part of the section, but in other regions, punctate staining at the cell periphery was found. 
C: localization of the sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} pump in LES (sph). Note bundle of skeletal muscle intensely labeled and a small blood vessel (bv) largely unlabeled. In LES cells, labeling was mostly punctate at the cell periphery except for centrally located sites as with calreticulin. 
D: localization of the plasma membrane (PM) Ca\textsuperscript{2+} pump in LES. Immunostaining was located at cell peripheries and was usually punctate. 
E: localization of calsequestrin (Ca-q) in LES. Calsequestrin immunostaining was located predominantly at cell periphery, sometimes punctate, and in some cells, covering the entire cell profile (likely nonspecific staining). 
F: localization of L-Ca\textsuperscript{2+} channel α\textsubscript{1C}-subunit (α\textsubscript{1C}) in canine LES, predominantly at the cell periphery. 
G: localization of the large-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} (BK\textsubscript{Ca}) channel in LES. Localization of immunostaining for this protein was clearly different from that of the other proteins depicted in this figure. Immunostaining occurred at punctate sites scattered over cell surfaces. Bar in B provides calibration for all panels, 12.5 μm.
tances were \(58 \pm 5\) pF \((n = 6\) cells\) for canine LES cells. Cells were held at \(-50\) mV and subsequently depolarized in seven cumulative steps, each of 250 ms duration, of 20–90 mV. Current-voltage curves were constructed with the use of the maximum current value \(I\) measured at \(t = 200\) ms in the pulse. Membrane currents were measured with an Axopatch 1C voltage-clamp amplifier, filtered with a 0.3-dB Bessel filter at 1 kHz, and recorded online with pClamp version 5.5.1 software.

RESULTS

Nature and Distribution of Caveolins in Canine LES

Cav-1 is present in endothelial cells but Cav-3 is considered to be the Cav present in muscle cells \((13, 41, 43, 46, 50)\), but Segal et al. \((40)\) found Cav-1 in vascular smooth muscle, accompanied by Cav-3 in arterial smooth muscle. Only Cav-3 was found in the skeletal muscle
overlying the LES. In our tissue, a sparse distribution of immunoreactivity against an antibody to Cav-3 was found in canine LES when evaluated both at the light and ultrastructural levels (Figs. 1D and 2c). Positive controls, skeletal muscle that overlies the LES, showed heavy staining for Cav-3 (Figs. 1C and 2b). In contrast, an antibody to Cav-1 found dense immunoreactivity at the cell periphery of LES cells and little in skeletal muscle (Fig. 1, A and B, and Fig. 2a). The ultrastructural studies showed this immunoreactivity to be located in or near caveolae. As expected from the difficulty in maintaining proteins without excessive denaturation during fixation, embedding, sectioning, and staining, labeling did not occur at all caveolae. Additional studies found that Cav-1 was the primary Cav in other canine gastrointestinal, airway, and vascular smooth muscle (Ref. 12 and C. Y. Kwan, E. E. Daniel, and Y. F. Wang, unpublished observations).

**Localization of nNOS in Canine LES**

Intestinal muscle cells contain several isoforms of nNOS (20, 37), one likely targeted to the membrane by an NH₂-terminal extension (31). A previous study (40) reported that nNOS was present in vascular smooth muscle. Canine LES cells show immunoreactivity to three nNOS antibodies, one of which was raised in guinea pig (See Table 1), was directed against an epitope to the COOH-terminal end of nNOS. Figure 3, A and C, shows that this immunoreactivity, like that to Cav-1, was punctate and located at the cell periphery of LES cells. Immunoreactivity was also present in many myenteric neurons in the myenteric plexus (Fig. 3B). Occasional sites of immunoreactivity were present on skeletal muscle. One antibody, directed against an NH₂-terminal epitope in rabbit, recognized a site in the periphery of skeletal muscle as well as Z lines (Fig. 3, F and G), where nNOS may be located. It stained LES muscle diffusely (Fig. 3H). An antibody raised against a COOH-terminal peptide also recognized immunoreactivity in nerve cells of the myenteric plexus (Fig. 3D) and stained the periphery of LES muscle cells (Fig. 3E) but with less fluorescence intensity than the antibody raised in guinea pig.

Figure 4a shows that, ultrastructurally, nNOS immunoreactivity was, like Cav-1, often located in or near caveolae. Not all caveolae were immunostained, possibly because of technical limitations in preserving antigenic sites. Cav-1 immunoreactivity was similarly located and appeared, like nNOS, to be associated with caveolae as expected (Fig. 4, b and c). The absence of the colocalization of immunoreactivity to Cav-1 and to nNOS at a molecular level is considered later.

**Localization of Ca²⁺-Handling Proteins in LES**

Figure 5, A–G, shows the localization of calreticulin (Ca-R), the L-Ca²⁺ channel, the SR Ca²⁺ pump, the PM Ca²⁺ pump, calsequestrin, the α₁C-subunit of the L-Ca²⁺ channel, and the BKCa channel, respectively. In LES cells, all these protein antigens appeared to be located, at least in part, at punctate sites in the cell periphery. Calreticulin had an additional central location (a ring or a central sphere) in some cells, probably endoplasmic reticulum around the nucleus. Calsequestrin immunoreactivity was located peripherally in most cells, but sometimes covered an entire cell. The polyclonal antibody against the L-Ca²⁺ channel and the antibody against the α₁C-subunit of that channel both stained the cell periphery, punctate in some areas, but there was extensive nonspecific staining with the polyclonal antibody. The immunoreactivity to the SR Ca²⁺ pump was similar in all respects to that of calreticulin and stained skeletal muscle deeply. Immunoreactivity to the BKCa channel was differently located in that, although peripheral and punctate in cells, it did not regularly outline the cell periphery as did Cav and nNOS immunoreactivities.

**Colocalizations of Cav-1 and nNOS with Ca²⁺ Handling Proteins**

In light microscopy studies with confocal microscopy, Cav-1 and nNOS were clearly colocalized in LES, as expected from their common presence in caveolae (Fig. 6, A–C). Similarly, nNOS and calreticulin appeared to be colocalized (Fig. 6, D–F). Moreover, the PM Ca²⁺ pump appeared to colocalize closely with both Cav-1 (Fig. 6, J–L) and with nNOS (Fig. 6, G–I), in agreement with previous biochemical studies (39).

**Proteins Not Colocalized with nNOS or Cav-1**

We compared the localization of PM proteins, vinculin, and connexins 43, 40, and 45 as well as the SR Ca²⁺ pump to nNOS and Cav-1. Figure 7, A–C, illustrates for connexin 43 that these proteins, none of which is expected to be present in caveolae, were usually not colocalized with Cav-1 or nNOS (data not shown). When they were, it is possible that this was a result of inadequate resolution from the technique. Vinculin, expected to be largely located at sites distinct from Cav (30), was expressed at the cell periphery in a nonpunctate fashion and with some overlap to that of Cav (Fig. 7, D–F) or nNOS (data not shown).
COLOCALIZATION OF Ca^{2+}-HANDLING PROTEINS TO CAVEOLAE
Fig. 7. Colocalization of proteins not fully colocalized with Cav-1 or nNOS. A–C: colocalization of Cav-1 (FITC labeled) with connexin 43 (Cx43; Cy3 labeled). Most gap junctions containing Cx 43 were not localized to sites with Cav-1. D–F: colocalization of Cav-1 (FITC labeled) with Vinculin (Vin; Cy3 labeled). There appeared to be colocalization at the periphery in some cases, but Vin and Cav-1 were also located at independent sites. G–I: colocalization of Cav-1 (FITC labeled) with the SR Ca\(^{2+}\) pump (Cy3 labeled). There was colocalization at peripheral sites, but Ca\(^{2+}\)-SR labeling, located centrally in cells, was labeled separately. J–L: colocalization of calreticulin (Ca\(^{2+}\)-R; labeled with FITC) with the SR Ca\(^{2+}\) pump (labeled with Cy3). There was colocalization at peripheral sites as well as at Ca\(^{2+}\)-SR labeling sites located centrally in cells. Compare with G–I.
Colocalization of SR Ca\textsuperscript{2+} Pump and Calreticulin or Cav-1

Calreticulin rather than calsequestrin is reported to be the primary Ca\textsuperscript{2+}-binding site of low affinity and high capacity in SR of smooth muscle (1, 21, 27, 32, 47) but is also distributed outside the SR (24, 44, 45). Figure 7, J–L, shows the colocalization of calreticulin and the SR Ca\textsuperscript{2+} pump. As noted earlier, immunoreactivity to calreticulin and to the SR Ca\textsuperscript{2+} pump is punctate at the periphery of cells but also present in the central portion of some cells, either as a circle or a sphere. Figure 7, J–L, illustrates that both sets of structures have the Ca\textsuperscript{2+} pump and calreticulin colocalized. However, Fig. 7, G–I, shows that Cav-1 is colocalized only with the peripheral, punctate location for the SR Ca\textsuperscript{2+} pump not with locations in the cell centers.

**Patch-Clamp Data**

When the anti-Cav-1 antibody was placed in a patch pipette with 200 nM Ca\textsuperscript{2+} during whole cell patch-clamp studies, it decreased the outward currents. After 20 min, currents induced by depolarization to +10 mV or more were significantly decreased, from \( P < 0.05 \) to \( P < 0.01 \). These decreases were reversed by addition to the bath of 10\textsuperscript{-4} M sodium nitroprusside (SNP). Figure 8 shows representative data and a summary of current-voltage relationships. Parallel studies (data not shown) in tissues not exposed to the antibody showed that over the same time period the outward currents did not decrease significantly from control values. Also in other studies, we showed that calmodulin at 10\textsuperscript{-6} M had no effect on outward currents when the patch pipette contained either 50 or 200 nM Ca\textsuperscript{2+} (data not shown).

**DISCUSSION**

These studies show that nNOS and a number of Ca\textsuperscript{2+}-handling proteins appear colocalized with Cav-1 in punctate sites around the cell periphery of cross-sectioned LES cells. Because caveolae, formed by the presence of Cav, are arranged in rows in the longitudinal axis of smooth muscle cells, punctate sites around the periphery of cross-sectioned cells are the expected arrangement. However, sections for immunohistochemistry were 10-\( \mu \)m-thick, and out-of-register membrane domains can appear colocalized with caveolae. Even in 1-\( \mu \)m sections evaluated with dual laser confocal microscopy, such spurious colocalization can occur. Thus studies done with either light or confocal

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**Fig. 8.** Patch-clamp studies of anti-Cav-1 antibody effects. In whole cell patch-clamp studies with pipettes with a 200 nM intracellular calcium concentration ([Ca\textsuperscript{2+}],), we examined the effects on outward currents over time, shown previously (35) to be \( \sim 80\% \) carried through BKCa channels, of the addition of the Cav antibody (diluted 1:100) to the patch pipette. Time controls in LES cells (data not shown) showed no changes in outward current. Selected current traces from a single experiment are shown at left (A–C). After 20 min (right), these currents were significantly reduced at depolarized potentials (from +10 to +90 mV). These reductions were reversed by addition of sodium nitroprusside (SNP; 10\textsuperscript{-4} M) to the bath.
microscopy cannot establish molecular colocalization but do support or negate such a possibility. Even ultrastructural studies require careful interpretation because many antigenic sites are lost during preparation by fixation, embedding, and processing. Also, false positive sites will inevitably occur.

Loss of normal relations of antigenic sites is likely the case with Cav-1 or -3 in relation to eNOS or nNOS. The association between these molecules is based on the NOS protein binding reversibly to sites on both the NH2- and COOH-terminal cytoplasmic arms of the hairpinlike Cav molecule and becoming inactive (8, 13–15, 25, 26, 46). This binding is thought to be reversed by elevation of Ca2+ levels near the membrane. Because membrane permeabilization occurs during fixation of tissues, Ca2+ levels likely rise and dissociate nNOS from Cav (Fig. 4). However, nNOS appears to remain associated with caveolae under our conditions for ultrastructural immunocytochemistry, as eNOS and nNOS appear to do functionally, allowing rebinding to Cav when Ca2+ levels fall in cells (8, 13–15, 25, 26, 46).

Despite these caveats, the apparent colocalizations we observed may explain earlier studies. In them, L-Ca2+-channel activity was required to support nNOS activity when [Ca2+]pipette was 1,000 nM, and nifedipine reduced the activation of BK Ca channels by SNP when [Ca2+]pipette was 8 nM (35). These studies implied that [Ca2+] near the nNOS, the L-Ca2+-channel, and the BK Ca channel was controlled differently than that in the general cytosolic [Ca2+]i. Subsequently we found that in the absence of extracellular Ca2+, achieved by eliminating it from the medium and adding 100 μM EGTA, the LES could contract tonically and repeatedly to carbachol. However, these contractions were abolished by nifedipine and by high levels of external EGTA and reduced by blockade of the sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA) pump with cyclopiazonic acid (33). These findings, along with similar ones made in canine trachea and bronchi (2, 6, 7, 28) suggested that Ca2+ was bound weakly at an extracellular site near the L-Ca2+-channel from which it was made available by carbachol and recycled by the activity of the SERCA pump activity.

The apparent colocalization of some of these proteins in caveolae may result from their possession of sequences that recognize scaffolding peptides in the cytoplasmic COOH and NH2 termini of Cav-1, as eNOS and nNOS do (8, 9, 13–15, 17–19, 22, 25, 26, 41–43, 46). Whether this or some other mechanist accounts for the apparent colocalization of Ca2+ proteins with Cav, their presence in close proximity helps explain the control of active tone in this sphincter.

Earlier, we showed that there was a spontaneously active cNOS, requiring Ca2+ entry through L-Ca2+ channels and operating to activate iberiotoxin-sensitive BK Ca and other K channels (34, 35). As described above, there was also recycling of Ca2+ between an extracellular site, which retained Ca2+ even when the extracellular medium had no added Ca2+ and contained 100 μM EGTA, and peripheral SR.

These properties are difficult to explain unless there are close spatial relationships between the site of Ca2+ entry, the nNOS, the BK Ca channels, and the peripheral SR. Also, there must be a region between the peripheral SR and the site of extracellular Ca2+ entry in which the [Ca2+] differs from the general cytoplasmic [Ca2+]. This study provides evidence that caveolae, composed primarily of Cav-1 molecules, may provide a biochemical, organizational basis to localize Ca2+-handling proteins close together and near peripheral SR. It provides evidence that an nNOS, possibly nNOS-α, is associated with Cav-1 as it is with Cav-3 in skeletal muscle (40, 46, 50). There is already biochemical evidence that both Cav-1 and -3 can interchangeably bind this nNOS (46). Thus our findings are consistent with expectations from the clear evidence that canine LES has membrane cNOS that is spontaneously active based on Ca2+ entry through L-Ca2+-channels (34, 35). As expected, these channels too were associated with caveolae and nNOS immunoreactivity based on our immunochemical findings. Our data do not exclude that they were present elsewhere in the PMs, as suggested by studies with isolated Cav-rich membranes compared with other membrane fractions in canine trachea (12).

In addition to the L-Ca2+-channel, the PM Ca2+-pump was present associated with caveolae and nNOS. Its presence may be associated with transport of Ca2+ from intracellular sites to the extracellular low-affinity binding sites suggested by our studies showing that repeated contractions to carbachol were possible in Ca2+-free medium provided the L-Ca2+-channels and the SR Ca2+-pump were working.

As demonstrated in this study, the BK Ca channel was colocalized in part with Cav-1. When present, its immunoreactivity was close to that of Cav, but there were regions where Cav reactivity, but not that for BK Ca channels, was present. If this is generally the case, it implies that some caveolae possess and others lack this channel in close proximity.

In the study on Cav-enriched membranes from trachea (12), we found that calreticulin and calsequestrin were associated with the membranes and were immunoprecipitated with Cav-1. Although these proteins, especially calreticulin, are low affinity, high capacity Ca2+-binding sites in the SR interior of smooth muscle (16, 24, 27, 29, 32, 44, 48), there is evidence, at least for calreticulin, that it occurs in low concentrations in plasma and can bind to cell surfaces (45, 51). It is therefore a candidate for the extracellular binding site we require to explain our findings (33). It is also possible that one or both of these Ca2+-binding proteins exist bound to Cav, where they provide a basis to lower the free [Ca2+] in the region between the peripheral SR and caveolae. They would also provide a reservoir of Ca2+ to sustain SR Ca2+ uptake and recycling between SR and the caveolar extracellular space.

It is also possible, of course, that the presence of calreticulin and calsequestrin with Cav in immunoprecipitation experiments in trachea is the result of loss from the SR interior during cell disruption and mem-
brane isolation, followed by binding to a consensus sequence of Cav-1 (12). Furthermore, it is possible that their apparent partial colocalization with Cav-1 or nNOS in this study is a result of lack of resolution in our microscopy methods. Such a possibility is supported by the fact that the SR Ca\(^{2+}\) pump also appears to be in part colocalized with nNOS, suggesting that the peripheral SR is so close to the caveolae as not to be resolved from them. However, our findings suggest explanations for phenomena observed in smooth muscle and raise important questions that open new possibilities for further study.

Finally, the ability of an antibody to Cav-1 to partially inhibit the outward currents when pipettes were filled with Ca\(^{2+}\) buffered at 200 nM requires comment. We cannot exclude that this is an artifact, but this is less likely because SNP restored outward currents. It is also possible that the Cav antibodies occupied the Cav molecules partially occluding sites at which nNOS binds. This could release nNOS into the cytosol making it unavailable in the region near caveolae where [Ca\(^{2+}\)] may be higher and where NOS activation occurs. Provision of NO from an external donor would reverse any decrease in outward currents, as observed. Further studies on the interaction of Cav antibodies and nNOS activity are needed. The failure of calmodulin to affect outward currents significantly may be the result of our use of an inadequate concentration. There was a tendency for a reduction in outward currents both at 50 and 200 nM [Ca\(^{2+}\)], and this might have been the result of a reduction in free [Ca\(^{2+}\)] near nNOS in caveolae as a result of calmodulin binding it. Calmodulin has multiple interaction sites and additional data about its effects on outward current under patch-clamp conditions are needed.

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