The role of caveolae and caveolin 1 in calcium handling in pacing and contraction of mouse intestine

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Abstract

In mouse intestine, caveolae and caveolin-1 (Cav-1) are present in smooth muscle (responsible for executing contractions) and in interstitial cells of Cajal (ICC; responsible for pacing contractions). We found that a number of calcium handling/dependent molecules are associated with caveolae, including L-type Ca^{2+} channels, Na^+-Ca^{2+} exchanger type 1 (NCX1), plasma membrane Ca^{2+} pumps and neural nitric oxide synthase (nNOS), and that caveolae are close to the peripheral endo-sarcoplasmic reticulum (ER-SR). Also we found that this assemblage may account for recycling of calcium from caveolar domains to SR through L-type Ca^{2+} channels to sustain pacing and contractions. Here we test this hypothesis further comparing pacing and contractions under various conditions in longitudinal muscle of Cav-1 knockout mice (lacking caveolae) and in their genetic controls. We used a procedure in which pacing frequencies (indicative of functioning of ICC) and contraction amplitudes (indicative of functioning of smooth muscle) were studied in calcium-free media with 100 mM ethylene glycol tetra-acetic acid (EGTA). The absence of caveolae in ICC inhibited the ability of ICC to maintain frequencies of contraction in the calcium-free medium by reducing recycling of calcium from caveolar plasma membrane to SR when the calcium stores were initially full. This recycling to ICC involved primarily L-type Ca^{2+} channels; *i.e.* pacing frequencies were enhanced by opening and inhibited by closing these channels. However, when these stores were depleted by block of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump or calcium release was activated by carbachol, the absence of Cav-1 or caveolae had little or no effect. The absence of caveolae had little impact on contraction amplitudes, indicative of recycling of calcium to SR in smooth muscle. However, the absence of caveolae slowed the rate of loss of calcium from SR under some conditions in both ICC and smooth muscle, which may reflect the loss of proximity to store operated Ca^{2+} channels. We found evidence that these channels were associated with Cav-1. These changes were all consistent with the hypothesis that a reduction of the extracellular calcium associated with caveolae in ICC of the myenteric plexus, the state of L-type Ca^{2+} channels or an increase in the distance between caveolae and SR affected calcium handling.

Keywords: interstitial cells of Cajal ● pacing of intestinal contractions ● L-type Ca^{2+} channels ● carbachol ● cyclopiazonic acid ● EGTA

Introduction

Popescu and colleagues [1] studied the distribution of calcium in smooth muscle of guinea-pig taenia coli using ultrastructural methods over 30 years ago and suggested that calcium found in an unexpected place, 'surface microvesicles' was a source of calcium for contraction. They estimated that the calcium in these microvesicles could supply all the calcium needed for a contraction. These vesicles often intrude close to the sarcoplasmic reticulum (SR). These vesicles are now recognized to be caveolae. This group also showed the very high incidence of a close relationship between caveolae and SR in smooth muscle and suggested that caveolae play an important role in excitation contraction coupling by Ca^{2+} translocation [2]. Since the seminal work of Popescu and colleagues, knowledge about calcium handling in smooth muscle has expanded exponentially. Recently we made the hypothesis that caveolae were sites for recycling of calcium between an external source and SR through L-type Ca^{2+} channels [3–6]. This recycling

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was most easily demonstrated when external calcium was absent and a low concentration of EGTA was present, proving that an external store of calcium existed. This external store of calcium may support contractions and pacing when L-type Ca^{2+} channels were available. We postulated that this calcium was associated with caveolae [5, 6].

Caveolae are found in many cell types and are abundant in smooth muscle and interstitial cells of Cajal (ICC) in the intestine [4, 7–10]. Under the electron microscope, caveolae are characterized as 'smooth coated' or 'non-coated' omega – or flask-shaped invaginations (diameter approximately 50–90 nm at the widest point) connected to the plasmalemma by a neck-like structure and separated from the general extracellular space by the basement membrane. The principal component of caveolae is the cytoplasmically orientated integral membrane protein, caveolin, a term encompassing a family of proteins. These membrane structures are involved in variety of cellular processes that include signal transduction pathways, membrane organization and protein trafficking [11–13]. Caveolins are membrane proteins which insert in the inner leaflet of the plasma membrane. There are three caveolins: caveolin-1 (Cav-1), -2 and -3. In smooth muscle and other cells except striated muscle, Cav-1 is the crucial caveolin for formation of caveolae [14]. Cav-1 is inserted by palmitoylated membrane attachment domains into the inner leaflet of the plasma membrane of smooth muscle and ICC of the intestine. Caveolae are formed by homo- and hetero-oligomerization of various caveolins through binding to peptide components in the N−terminal ends of the molecule, which extend into the cytosol. Residues 61-101 on the N terminus of Cav-1, allow homo− and hetero-oligomerization with Cav-1 or 3 molecules, respectively [reviewed in 11–13].

Cav-1 organizes signal transduction through a 20 aa domain, 82–101, which binds a variety of signalling proteins including c-SRC, insulin receptor, eNOS, H-Ras, and $G\alpha$ subtypes. Binding to Cav-1 usually inactivates downstream signalling, and this has been demonstrated clearly for eNOS in endothelium. eNOS remains inactive until released from binding by formation of Ca^{2+} calmodulin [15, 16].

We recently demonstrated that a splice variant of nNOS is closely associated with Cav-1 in the lower oesophageal sphincter [17–19] and responds to release of Ca^{2+} from SR by releasing NO to modulate contraction. A similar nNOS isoform exists in intestinal smooth muscle where it modulates contractions from Ca^{2+} entry through L-type Ca^{2+} channels. In intestine from Cav-1 knockout, this nNOS isoform is absent from the membrane and its modulating function on contractions when Ca^{2+} enters through L-type Ca^{2+} channels, is abrogated [20]. However, L-type Ca^{2+} channels, NCX1 exchangers and plasma membrane (PM) Ca^{2+} pumps (PMCA) persist [21]. These findings raised the question: what are the effects of Cav-1 knockout and associated loss of caveolae on calcium handling and putative recycling between caveolar domains and SR?

Thus, our aim was to determine the consequences of the absence of caveolae and Cav-1 on calcium handling. We postulated that the loss of close association between caveolae and SR would reduce recycling of calcium in both directions.

Materials and methods

Animals

Male Cav-1 knockout mice, 6–10 weeks of age, (cav<tm 1 M ls>/J) were obtained from a colony bred in a our Animal Facility. Their genetic controls, male F2 hybrid (B6 129 SF2/J) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Animals were killed by cervical dislocation following a protocol approved by our Animal Policy and Welfare Committee.

Tissue preparation

After opening the abdominal wall, the gastrointestinal tract, starting from the stomach to the rectum, was removed from the mouse and immediately placed into a beaker of Krebs-Ringer (KR) solution containing (in mM): 115.5 NaCl, 21.9 $NaHCO_3$, 11.1 D-glucose, 4.6 KCL, 1.16 $MgSO_4$, 1.16 $NaHPO_4$ and 2.5 $CaCl_2$, at room temperature (21–22°C), and pre-equilibrated with carbogen (95% O_2 and 5% CO_2). In a dissection dish filled with KR solution and continuously bubbled with carbogen, small intestinal tissue was isolated and cut into approximately 1.0–1.5-cm longitudinal muscle (LM) segments. The intestinal content, if any, was pushed out by gently rubbing the segments with dissection forceps. To study LM contraction the tissue segment was placed between two platinum concentric electrodes and tied to a hook at the bottom of the electrode holder with silk suture thread. The top of the tissue was also tied with thread and attached to a strain gauge (Grass FT-03; West Warwick, RI, USA). The muscle preparations were placed into muscle baths filled with 10 ml KR solution, bubbled continuously through the experiment, and maintained at a temperature of 37°C. The tension was increased or decreased slowly till the tension that produced the maximum amplitude of spontaneous phasic activity was reached. Tissue contractile activities were recorded on a Grass Model 7 Polygraph.

Tissues were allowed to recover from handling for 30 min. Then electrical field stimulation (EFS) was applied for 10 sec. at 50 V/cm, 5 square wave pulses per second of 0.5 ms duration. Tetrodotoxin or TTX (10^{-6} M) was applied for 10 min. and EFS repeated. It there was any response additional TTX was applied until EFS produced no response.

Data analysis

Amplitudes of the spontaneous contractions were measured as the values above the passive tension, determined at the end of the experiments by applying Ca^{2+}-free Krebs solution containing 1 mM EGTA. They were measured as the mean of individual contractions over at least 10 contractions. Frequencies of spontaneous muscle contractions were measured and averaged over a period of at least 20 sec. Frequency and amplitude data were normalized to the control values after nerve were blocked. When carbachol (CCH) was administered in normal media, the values were used to normalize subsequent responses in Ca^{2+}-free media. The measurements were entered into GraphPad Instat and statistically tested using ANOVA with the Bonferroni *post hoc* test, paired t-test, or unpaired t-test, whichever was appropriate. If data were non-Gaussian or sample sizes were small, we used a Kruskal–Wallis evaluation. To compare changes from control values in a given set of tissues, we used the Wilcoxen signed rank test. A *P*-value <0.05 was considered to be statistically significant. The *n*-values represent the number of mice whose intestine provided

segments for study. In the figures, significant differences with a group from initial control values are indicated by the symbols: #, $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ and #### $P < 0.0001$. Differences between groups in comparable experiments are shown as: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ and ****, $P < 0.0001$. Significant differences between segments of Cav$^{+/+}$ and Cav$^{-/-}$ tissues are highlighted by colouring the relevant bars (those with larger means) red.

Comparisons were usually made between values obtained in direct head to head comparisons between tissues with and without caveolae. Secondary comparisons were made to experiments after prior depletion of calcium (Fig. 3A and B) in which very large differences were observed.

Procedures

Most comparisons between Cav-1 knockout and control mouse intestine LM were conducted in Ca^{2+}-free medium with 100 μM EGTA. This focused the responses on the extracellular calcium component which we suggest is associated with caveolae. We used frequency of paced contractions to determine the activity of the ICC and the amplitude of these paced contractions to determine the ability of smooth muscle to respond to paced slow waves by opening L-type Ca^{2+} channels and contracting [22].

Immunohistochemistry

Animals

Cav-1 knockouts (three Cav-1$^{-/-}$ mice) from a colony bred in our Animal Facility and their controls (three Cav-1$^{+/+}$ mice) from Jackson Laboratories were examined for immunohistochemical/confocal microscopic study.

Tissue preparation and cryosection

The small intestine of Cav-1$^{+/+}$ and Cav-1$^{-/-}$ mice were fixed with 4% paraformaldehyde and cryoprotected with 30% sucrose for cryosection. Five micrometre thickness of cryosection was performed with a cryostat as we previously described in detail [20].

Double immunolabelling

For co-localization study of Cav-1 and store operated Ca^{2+} channel (Orai-2) mouse anti-Cav-1 IgG (Cat. No. 160406, BD Transduction Labs; San Jose, CA, USA) and rabbit anti-Orai-2 IgG (Cat. No. ACC-061, Alomone Labs; Jerusalem, Israel) were used as primary antibodies. The 5-μm cryosections were pre-incubated with normal donkey serum (NDS) to block non-specific binding proteins before applying the primary antibodies. It was performed with 10% NDS in 0.1 M phosphate buffered saline (pH 7.2–7.4) containing with 0.3% Triton-X 100 for 1 hr. As a first primary antibody, Cav-1 (1:200 of working concentration) was incubated for 16 hrs followed by Cy3-conjugated donkey antimouse IgG (Cat. No. 715–165–151, Jackson ImmunoResearch; West Grove, PA, USA) was used. Continuously, as a second primary antibody, Orai-2 (1:200 of working concentration) was incubated for 16 hrs followed by Alexa488-conjugated donkey anti-rabbit IgG (Cat. No. A-21206, Invitrogen; Carlsbad, CA, USA) was used.

All experimental procedures were performed at room temperature, 22 $\pm$ 1 °C. During incubation of all antibodies 2% NDS of total incubation volume was added for stabilization of antibodies and reducing of non-specific binding. To determine specificity of immunolabelling three different negative controls were made by: (*i*) using blocking peptide for Orai-2, (*ii*) omitting primary antibodies and (*iii*) omitting secondary antibodies.

Laser lines and emission filters for confocal microscopic study

The immuno-fluorescence labelled cryosections were observed by single photon confocal microscope (LSM 510, Carl Zeiss Co., Jene, Germany). Cy3 (red) was scanned by helium / neon laser (wavelength: 543 nm) with long pass 560 nm emission filter. Alexa488 (green) was scanned by Argon laser (wavelength: 488 nm) with bandpass 500–550 nm emission filter. Images obtained from confocal microscope were enhanced their quality by brightness, contrast and gamma too of LSM 5 image (Carl Zeiss Co.) and by level tool of Adobe PhotoShop (Version 7.0, Adobe, San Jose, CA, USA).

Materials

Chemicals were purchased from Sigma Aldrich (Mississauga, Ontario, Canada) and antibodies were obtained as described above.

Results and discussion

Effects of 0.1 *versus* 1 mM EGTA in Ca^{2+} free ringer

Five minutes after two washes in nominally Ca^{2+}-free KR solution with 0.1 or 1 mM EGTA (Fig. 1A), Cav$^{+/+}$ segments of LM maintained frequencies significantly ($P < 0.05$) better than did Cav$^{-/-}$ segments, in both concentrations of EGTA. These higher frequencies persisted after an additional two washes for a total of 10 min. in Cav$^{+/+}$ compared to Cav$^{-/-}$ segments in 0.1 mM EGTA. Contractions were abolished in both strains after 10 min. at 1 mM EGTA. These results imply that the absence of caveolae diminishes the supply of calcium or the ability of this extracellular calcium source to recycle calcium to ICC SR. This source survives in 0.1 mM EGTA for 10 min., but not in 1.0 mM EGTA.

There were no significant differences in the amplitudes of spontaneous contractions between the two strains after exposures
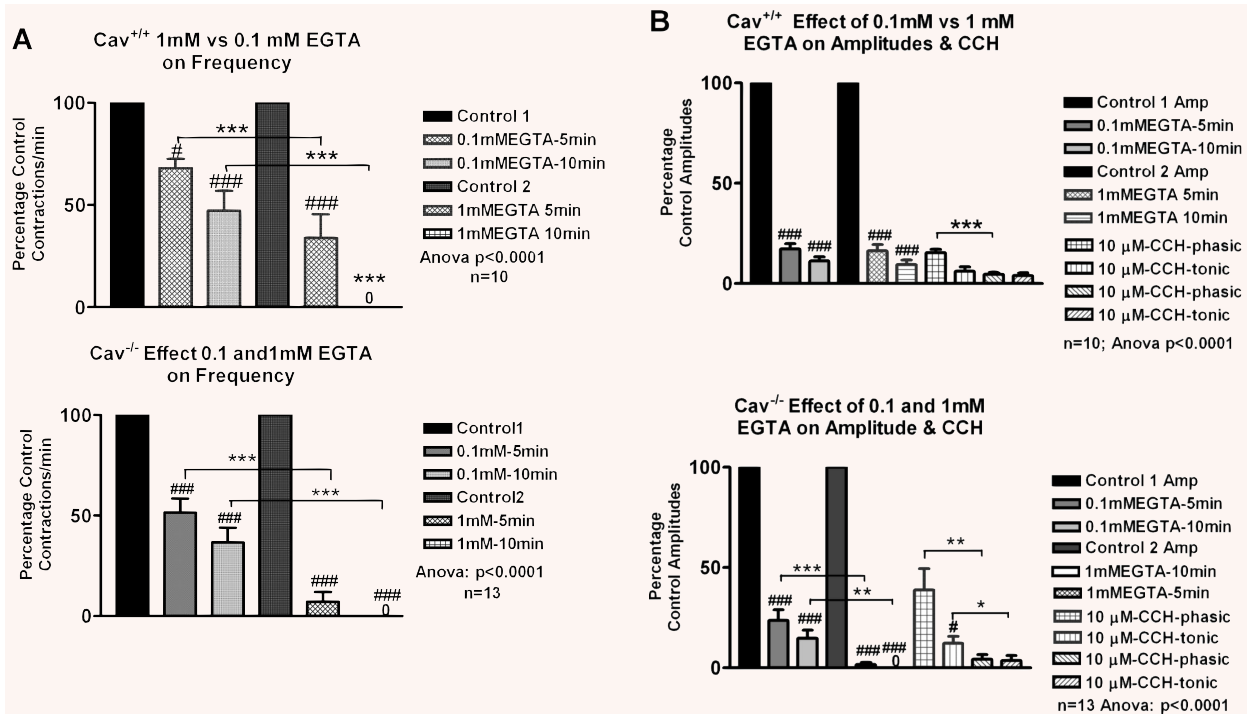
Fig. 1 (**A**) Effects on contraction frequencies of LM segments from two successive exposures of control (Cav$^{+/+}$) and Cav-1 knockout (Cav$^{-/-}$) to Ca^{2+} Krebs solution with 0.1 mM EGTA. Significant differences between the first and second exposures and initial values are indicated by #, the number symbol, and between comparable tissues by asterisks. When there were significant differences between Cav$^{+/+}$ and Cav$^{-/-}$ segments the bars for the Cav$^{+/+}$ tissues are red. (**B**) Effect on contraction amplitudes in the experiments shown in Fig. 1A, with data from the final responses to 10^{-5} M CCH. . Significant differences between the first and second exposures and initial values are indicated by #, the number symbol, and between comparable tissues by asterisks. When there were significant differences between Cav$^{+/+}$ and Cav$^{-/-}$ segments in spontaneous contraction amplitudes the bars for the Cav$^{+/+}$ tissues are red but difference in amplitudes of contractions to CCH are in red for Cav$^{-/-}$ tissues. Responses to CCH after exposure to 0.1 mM EGTA are shown by perpendicular hatching and responses after exposure to 1 mM EGTA are shown with diagonal hatching.

to 0 Ca^{2+} KR with 0.1 mM EGTA prior to addition of CCH (Fig. 1B). However, when 10^{-5} M CCH was added after 10 min. and four washes in 0.1 mM EGTA, phasic and tonic responses were greater in Cav$^{-/-}$ segments (p ≤ 0.05). These data imply that caveolae in smooth muscle are not essential for recycling calcium to SR to support contraction when 0.1 mM EGTA is present. Also, they imply that in the absence of caveolae there is a slower loss of calcium from SR, leaving more calcium to be released by CCH to induce phasic contractions.

These data imply that Cav-1 and caveolae are not absolutely necessary for recycling Ca^{2+} from the extracellular space to SR in ICC or smooth muscle since recycling still occurred in their absence. This is consistent with the observations that L-type Ca^{2+} channels in ICC, which may bind calcium [23], persist in Cav-1 knockout animals even though apparently associated with Cav-1 based on immunochemistry [21] and that some SR persists close to the plasma membrane even when caveolae are absent [24].

Effects of manipulation of the L-type Ca^{2+} channels in Ca^{2+} free KR with 0.1 mM EGTA

The absence of caveolae in LM segments significantly slowed (ANOVA: $P < 0.03$) the decline in frequencies relative to segments with caveolae when 10^{-6} M BayK 8644 was applied in order to open L-type Ca^{2+} channels during exposure to Ca^{2+} KR with 0.1 mM EGTA for 5 ($P = 0.0024$; two tailed) and 10 min. ($P = 0.0069$; two tailed) (Fig. 2A). Irrespective of the presence or absence of caveolae, closure of L-type Ca^{2+} channels prevented recycling of calcium to ICC. Nicardipine abolished spontaneous contractions after 10 min. in 0.1 mM EGTA. As a result, there were no significant differences between frequencies in Cav$^{-/-}$ and Cav$^{+/+}$ segments.

These data may imply that recycling is more conservative of calcium in ICC in medium with 0.1 mM EGTA when L-type Ca^{2+} channels are open and caveolae are absent. Alternately, they may imply that loss of calcium from the endoplasmic reticulum (ER)
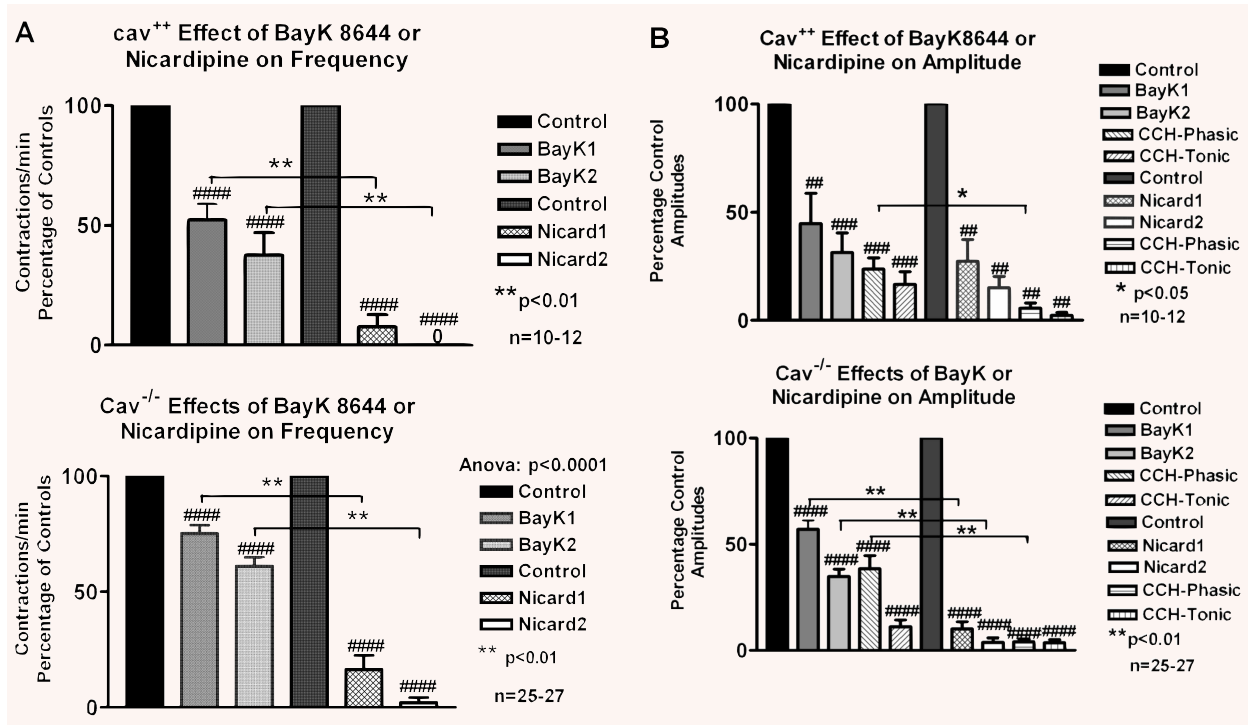
Fig. 2 (**A**) Effects on contraction frequencies of LM segments from two successive exposures of control ($Cav^{+/+}$) and Cav-1 knockout ($Cav^{-/-}$) to Ca^{2+} Krebs solution with 0.1 mM EGTA. In these experiments, either BayK 8644 or nicardipine (both at 10^{-6} M) was added with the Ca^{2+}-free medium. Significant differences between the first and second exposures and initial values are indicated by #, the number symbol, and between comparable tissues by asterisks. The significantly higher frequencies in $Cav^{-/-}$ tissues are indicated by red bars. (**B**) Effect on contraction amplitudes in the experiments shown in (**A**), with data from the final responses to 10^{-5} M CCH. Significant differences between the first and second exposures and initial values are indicated by #, the number symbol, and between comparable tissues by asterisks. When there were significant differences between $Cav^{+/+}$ and $Cav^{-/-}$ segments in spontaneous contraction amplitudes after block of L-type Ca^{2+} channels the bars are in red for $Cav^{+/+}$ tissues.

and the intracellular space into the extracellular medium is less rapid when caveolae are absent and L-type Ca^{2+} channels are open to allow recycling. During pacing in ICC, Ca^{2+} is reported to be released regularly from the ER and recovered in part [25–27]. We showed [28] that in medium with 2.5 mM Ca^{2+}, nicardipine reduced slightly but significantly the frequencies of segments of tissue and eliminated the pacing frequency gradient (jejunum frequency no longer higher than ileum frequency). BayK 8644 did not increase the normal frequencies but opposed the effects of nicardipine. These findings collectively suggest that L-type Ca^{2+} channels open intermittently during pacing, more when BayK is present, and when caveolae are absent, calcium loss through them is less in ICC.

There were no significant differences between $Cav^{-/-}$ and $Cav^{+/+}$ LM segments in amplitudes of spontaneous contractions in BayK 8644 or in the responses to CCH (Fig. 2B). However, spontaneous contractions were lower in the absence of caveolae when L-type Ca^{2+} channels were blocked (two tailed $P = 0.043$ at 5 and 0.026 at 10 min.). This implies that smooth muscle of LM segments recycle calcium to and from an

extracellular source in medium with 0.1 mM EGTA similarly in the presence of open L-type Ca^{2+} channels whether caveolae are present on not. However, in the absence of open L-type Ca^{2+} channels, the presence of caveolae enabled recycling and contractions to persist longer. One possible explanation is that when caveolae are present, another route of calcium entry is present allowing recycling to occur despite closed L-type Ca^{2+} channels. Smooth muscle contractions are postulated to occur passively in response to slow waves initiated by ICC without any spontaneous pacing events intrinsic to smooth muscle [29]. Also store operated Ca^{2+} channels may be associated with Cav-1 as recently suggested [30]. We therefore tested the presence of such channels and observed them co-localized with Cav-1 in mouse intestine (Fig. 3). Since store operated channels did not disappear from $Cav-1^{-/-}$ intestine, the difference likely relates to the loss of proximity between these channels and SR.

These data support the previous suggestions that caveolae are not an absolute requirement for recycling calcium in ICC or smooth muscle, but that they play a role in modulating Ca^{2+} handling in ICC and allow recycling to smooth muscle even when
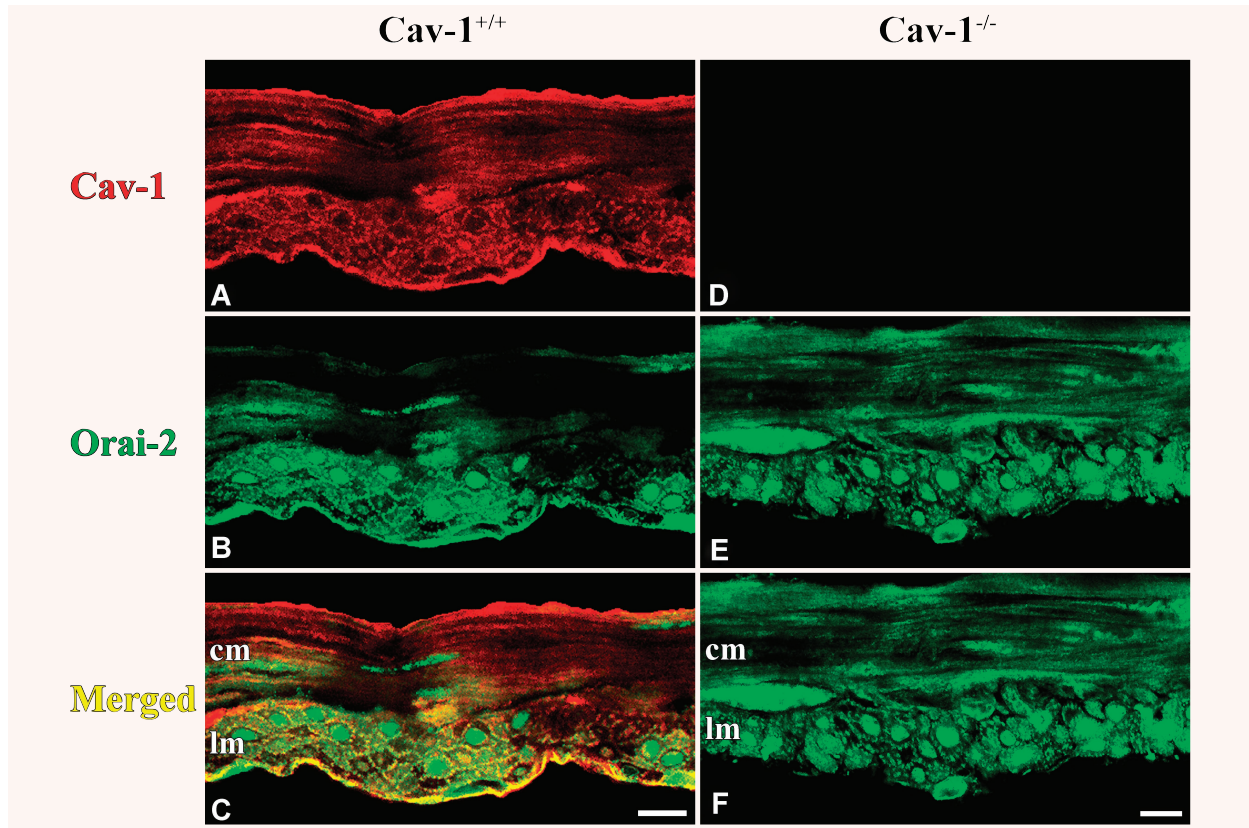
Fig. 3 Distribution of store operated calcium channels in Cav$^{+/+}$ and Cav$^{-/-}$ tissues. At left are micrographs of Cav$^{+/+}$ and at right of Cav$^{-/-}$ tissues, cut with the LM in cross section. The top row of sections shows staining for Cav-1 and the second row shows staining for Orai1 in store operated channels. The third row shows the merged images. Note that Cav-1 Orai 2 stainings in Cav$^{+/+}$ tissues are punctate at the cell membrane and are well co-localized. In Cav$^{-/-}$ tissues Cav-1 is absent and Orai 2 staining appears less clearly punctate. Length bars are 10 μM.

L-type Ca^{2+} channels are closed, likely through store operated channels. They also suggest that the role of caveolae in calcium handling differs in ICC, where caveolae are important in modulating pacing activity, and in smooth muscle, where they are of lesser importance.

Effects of initial depletion of SR Ca^{2+} stores on responses in Ca^{2+}-free KR with 0.1 mM EGTA

Tissues were exposed to 10 μM cyclopiazonic acid for 30 min. in KR, and then placed in Ca2+ KR with 0.1 mM EGTA. Then they were given either BayK 8644, nicardipine or nothing and frequencies and amplitudes of paced contractions were measured after 5 and 10 min. Contractions to 10–5 M CCH were evaluated afterwards. CPA reduced frequencies equivalently in both types of segments similar to the changes previously reported [22] in gut segments from BalbC mice. However, when stores were depleted of calcium and L-type Ca^{2+} channels were manipulated, the presence or absence of caveolae made no significant difference (Fig. 4A and B). Also, after CPA, there was no difference in responses to 10–5 M CCH between LM segments from Cav$^{+/+}$ and Cav$^{-/-}$ mice (data not shown). These findings suggest that availability of calcium in SR is essential for realization of differences in calcium handling between LM segments with or without caveolae.

Frequencies and amplitudes of spontaneous contractions were significantly less ($P < 0.01$–0.001) after depletion of SR calcium compared to findings in the experiments in Fig. 1A (with 0.1 mM EGTA alone). Although this difference was not obtained in a direct comparison, it is likely real. Since release of calcium from the SR is supposed to be an essential component of each paced event in ICC but not an essential component of each smooth muscle contraction, depletion of SR calcium may eliminate the special role of caveolae in ICC pacing. However, in the presence of caveolae, when BayK and 0.1 mM EGTA was added, prior depletion of calcium stores by CPA did not affect frequencies compared to no depletion (Fig. 2A). When caveolae were absent and BayK and
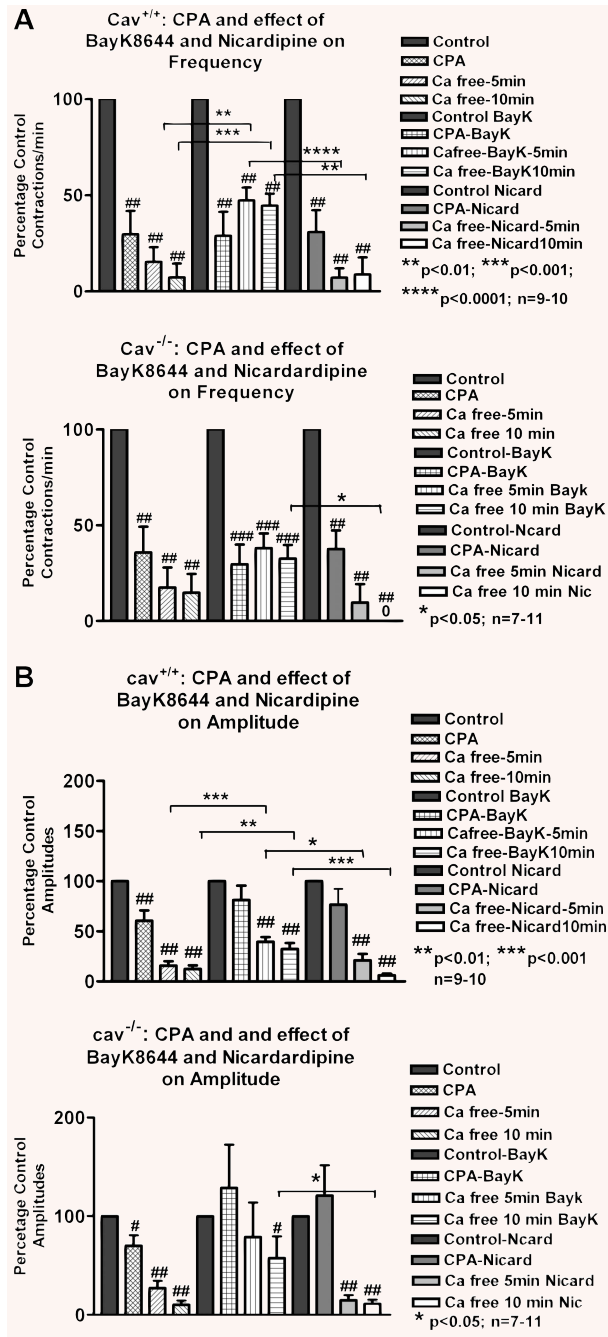
Fig. 4 (**A**) and (**B**) Effects on contraction frequencies (**A**) and amplitudes (**B**) of LM segments, during two successive exposures of control Effects on contraction frequencies (**A**) and amplitudes (**B**) of LM segments, during two successive exposures of control (Cav$^{+/+}$) and Cav-1 knockout (Cav$^{-/-}$) to Ca^{2+} Krebs solution with 0.1 mM EGTA. Some tissues had received an exposure to 10 μM CCH just prior to the first wash in Ca^{2+} solution. There were no significant differences between Cav$^{+/+}$ and Cav$^{-/-}$ tissues.

0.1 mM EGTA were present, depletion of SR calcium decreased frequencies significantly ($P < 0.01$) compared to no depletion. These findings are consistent with the suggestions that opening of L-type Ca^{2+} channels leads to less rapid loss of calcium when caveolae are absent and that SR is the source of this poorly eliminated calcium. In its absence the less efficient recycling of calcium leads to greater net loss for pacing. Further, it suggests that, as expected, store operated Ca^{2+} channels can play no effective role in recycling Ca^{2+} when SR stores are depleted and there is no external calcium.

After calcium depletion by CPA, there were few differences related to caveolin on smooth muscle contractile functions. The only significant differences were in tissue treated with nicardipine when caveolae were present. In these tissues, contractions were decreased by calcium depletion ($P = 0.021$ at 5 min. and 0.036 at 10 min; two-tailed t-tests). As nicardipine decreases calcium recycling, this difference may reflect the need for SR calcium for recycling.

Effects of exposure to CCH on frequency and amplitudes in Cav$^{+/+}$ and Cav$^{-/-}$ on LM - segments in Ca^{2+} free KR with 0.1 mM EGTA

Tissues were washed in Ca^{2+}-free KR with 0.1 mM EGTA, with or without an immediately previous control contraction to 10^{-5} M CCH. Then after 5 min. frequencies and amplitudes of contractions were measured and this procedure was repeated. Note that these experiments differed from those in Fig. 2A and B by the initial exposure of some segments to CCH, which presumably mobilized calcium from SR and opened L-type Ca^{2+} channels [31–32].

There were no significant differences in frequencies between controls and Cav-1 knockout LM segments when CCH or no pre-treatment occurred prior to exposure to the Ca^{2+}-free KR (data not shown). However, after the second exposure to calcium-free medium, control segments exposed initially to CCH had significantly lower frequencies than did these same segments after the first exposure. There was no such difference in frequencies in segments from knockout animals. The amplitudes of contraction were not different in segments from Cav$^{+/+}$ and Cav$^{-/-}$ animals in this experiment (data not shown).

When this experiment was repeated, but the CCH was added after the segments had been in Ca^{2+}-free medium for 5 min., both Cav$^{+/+}$ and Cav$^{-/-}$ segments had decreased frequencies after the second post-CCH wash, but there were no differences between the two strains (data not shown). In these experiments, amplitudes of spontaneous contractions also did not differ within or between strains (data not shown).

The initial responses to CCH in the experiments described in the above studies were also compared. Both the initial phasic (P) responses and subsequent tonic (T) responses after 2 min. were measured. Moreover, the responses were determined both in relation to final passive tone and in relation to the increase in amplitudes over the tone present when CCH was applied (ΔP and ΔT). Figure 5A shows that administrations of CCH in Krebs solution resulted in

normalized contractions which were not different in $Cav^{+/+}$ and $Cav^{-/-}$ segments. However, as illustrated in Fig. 5B, the results when CCH was only applied after exposure to Ca^{2+}-free medium differed markedly between strains. $Cav^{+/+}$ segments responses were smaller in every measure than responses in $Cav^{-/-}$ segments. This may result from the previously proposed slower extrusion of calcium from the space near SR when caveolae are absent.

Effects of repetitive exposure to CCH with manipulation of L-type Ca^{2+} channels on frequency and amplitudes in $Cav^{+/+}$ and $Cav^{-/-}$ on LM segments in Ca^{2+} free KR with 0.1 mM EGTA

These tissues were washed in Ca^{2+}-free KR with 0.1 mM EGTA, after a control contraction to 10^{-5} M CCH. The medium contained in addition in some cases either 10^{-6} M BayK 8644 or 10^{-6} M nicardipine. Then after 5 min., frequencies and amplitudes of contractions were measured and they were exposed again to 10^{-5} M CCH for 2 min.

Effects on frequencies in these experiments are summarized in Fig. 6A. In both $Cav^{+/+}$ and in $Cav^{-/-}$ segments the frequencies decreased with each successive exposure to Ca^{2+}-free medium and in all conditions; control, BayK 8644 and nicardipine. In $Cav^{+/+}$ segments, as expected the addition of BayK 8644 enhanced frequencies significantly during all three exposures in Ca^{2+}-free solutions compared to controls without BayK 8644. However, nicardipine had no significant effect to reduce frequencies significantly compared to controls or to BayK 8644. Thus exposure to CCH made the segments apparently less susceptible to block of L-type Ca^{2+} channels (compare Figs 2A and 6A). In $Cav^{-/-}$ segments, BayK 8644 increased frequencies significantly compared to controls during the second and third exposures to Ca^{2+} medium, while nicardipine reduced frequencies during the third exposure compared to controls and during the second and third exposures compared to BayK 8644. The only conditions in which $Cav^{+/+}$ segments behaved significantly differently from $Cav^{-/-}$ segments were during the third exposure in BayK 8644 and during the second and third exposures in nicardipine. In all cases responses of $Cav^{+/+}$ were larger. These results imply that $Cav^{+/+}$ segments recycle the Ca^{2+} released by CCH from SR on each wash more efficiently than Cav^{B-} segments. This was true whether L-type Ca^{2+} channels were either open or closed, especially when closed. Also, the exposures to CCH facilitated recycling notably when L-type Ca^{2+} channels were closed by nicardipine. This suggests that there is an alternate path for recycling calcium other than the L-type Ca^{2+} channels when caveolae are present and enhanced by exposure to CCH, presumably a store operated channel (Fig. 3). This recycling appears more effective when these channels are open.

There were also significant differences in amplitudes of spontaneous contractions between controls and Cav-1 knockout animals in this experiment (Fig. 6B). As expected the ampli-
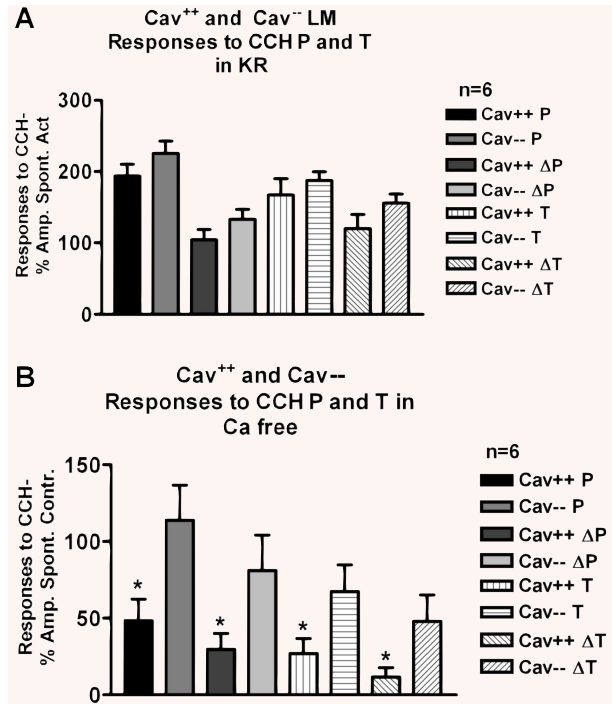
Fig. 5 (**A**) and (**B**) Responses to 10 μM CCH of $Cav^{+/+}$ and $Cav^{-/-}$ tissues exposed to CCH in normal Krebs solution (**A**) and in Ca^{2+}-free solution (**B**). Phasic responses were measured at the maximum and tonic responses were measured 2 min. after the addition of CCH. In medium with normal Ca^{2+} concentrations there were no significant differences in responses to CCH, but in Ca^{2+}-free medium responses of tissues without caveolae were all significantly greater.

tudes of spontaneous contractions declined with repeated washes in both $Cav^{+/+}$ and $Cav^{-/-}$ segments significantly. However, the decline was significantly less compared to control segments in those exposed to BayK in some cases, but was without significant differences from controls in segments exposed to nicardipine except after the third exposure to Ca^{2+}-free medium in $Cav^{-/-}$ segments. During the second and third exposures without additions, control segments with caveolae had significantly higher amplitudes. Also, although several of the means were higher in controls compared to knockout animals when tissues were exposed to nicardipine or BayK 8644, only those in nicardipine were significantly higher for the first and second exposures. Again, these results suggest that in the absence of caveolae, closure of L-type Ca^{2+} channels has more significance for recycling of calcium for smooth muscle spontaneous contractions than when caveolae are present. This is consistent with the existence of another different route for calcium recycling when caveolae are present. However, the ability of spontaneous contractions to continue after closure of L-type Ca^{2+} channels appeared to be unaffected by CCH exposure, unlike the effect on ICC.
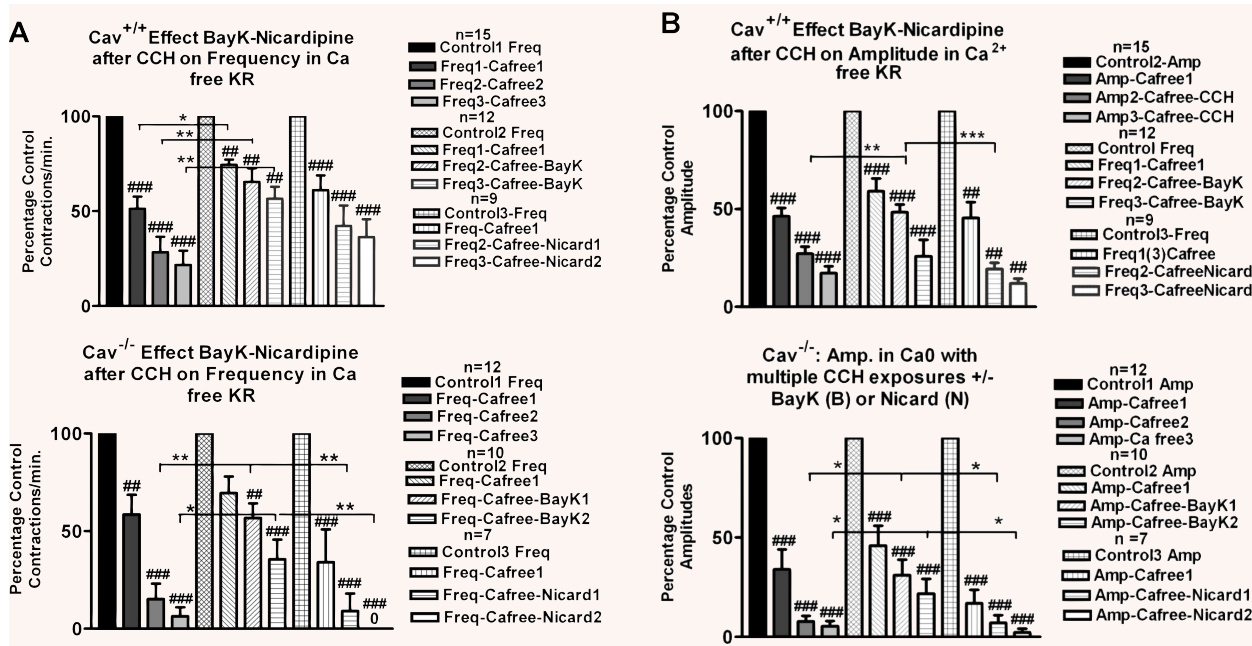
Fig. 6 (**A**) Effects on contraction frequencies of LM segments, during three successive exposures of control (Cav$^{+/+}$) (top panel) and Cav-1 knockout (Cav$^{-/-}$) (bottom panel) tissues to Ca^{2+} Krebs solution with 0.1 mM EGTA. Before the first exposure, 10 μM CCH was applied to all tissues for 2 min. and reapplied for 2 min. after each wash in Ca^{2+}-free medium. CCH was washed out before the following exposure to this medium. In some tissues, either BayK 8644 or nicardipine (both 10^{-6} M) were added with the calcium-free solution. Significant differences between the first and second exposures and initial values are indicated by #, the number symbol, and between comparable tissues by asterisks. Although nearly all mean values in Ca^{2+}-free medium were higher in Cav$^{+/+}$ tissues than in Cav$^{-/-}$ tissues, only those measured during the third wash with BayK 8644 and the second and third washes with nicardipine were significantly so, indicated by red bars. (**B**) Effects on contraction amplitudes of LM segments, during three successive exposures of control (Cav$^{+/+}$) (top panel) and Cav-1 knockout (Cav$^{-/-}$) (bottom panel) tissues to Ca^{2+} Krebs solution with 0.1 mM EGTA. Before the first exposure, 10 μM CCH was applied to all tissues for 2 min. and reapplied for 2 min. after each wash in Ca^{2+}-free medium. CCH was washed out before the following exposure to this medium. In some tissues, either BayK 8644 or nicardipine (both 10^{-6} M) were added with the calcium-free solution. Significant differences between the first and second exposures and initial values are indicated by #, the number symbol, and between comparable tissues by asterisks. Although nearly all mean values in Ca^{2+}-free medium were higher in Cav$^{+/+}$ tissues than in Cav$^{-/-}$ tissues, only those measured during the second and third washes with nicardipine were significantly so, indicated by red bars.

These data, along with those from segments treated with CPA, demonstrate the general importance of SR calcium in the function of caveolae in recycling and retention of calcium. When calcium release from SR is not activated, the absence of caveolae leads to slower depletion of pacing frequency, but when calcium release is activated, the absence of caveolae speeds recycling or delays loss of calcium for contraction.

ICC than they play in control of contractions by LM. These findings also show that recycling of calcium in calcium-free KR with 100 μM EGTA does not depend absolutely on the presence of caveolae. The maintenance of pacing in calcium-free media supports Popescu 's evidence [1, 2] that these stores contain enough calcium to sustain contraction and also, as we know for the first time, for recycling in ICC. Our findings indicate that maintenance of contractions with that calcium depends on recycling it to and from the SR.

General discussion

Our analyses are based on the understanding that the frequencies of contraction are controlled primarily by calcium handling in ICC of the myenteric plexus (ICC-MP), while the contractile force of contractions depends primarily on calcium handling in the longitudinal smooth muscle. Our findings show that caveolae play a different and more important role in calcium handling in the control of pacing by

Caveolae and calcium handling by ICC-MP

Effects on frequency of the absence of caveolae have to be considered in light of the fact that L-type Ca^{2+} channels play a major role in maintaining frequencies in Ca^{2+}-free media with 0.1 mM EGTA, but not in ICC when supplied with extracellular calcium [25–27].

The role of these channels under our conditions is shown by the observation that, in nearly all cases, addition of nicardipine to the medium either abolished or markedly diminished the frequencies within 5–10 min. (See Figs 1A, 2A and 4A). Thus the L-type Ca^{2+} channels is a major route of recycling of Ca^{2+} to SR from caveolae, necessary for pacing to continue, in ICC under our conditions.

However, when CCH was given after each 5-min. exposure to Ca^{2+}-free Krebs with 0.1 mM EGTA, segments with caveolae maintained frequencies as effectively when exposed to nicardipine as in they did in its absence (Fig. 4). In contrast, segments without caveolae failed to maintain frequencies either in the absence or in the presence of nicardipine. CCH-induced release of calcium from the SR stores in ICC may open store operated channels, allowing the calcium lost to the extracellular space to be recycled back to the stores through non-L-type Ca^{2+} channels when caveolae are present, but not when they are absent. Our findings support recent evidence that expression of store operated calcium channels is associated with the expression of caveolae in we have found evidence to support their existence with Cav-1 in intestinal smooth muscle (Fig. 3). Opening of L-type Ca^{2+} channels with BayK 8644 improved the maintenance of frequencies in the absence of caveolae, but was not as effectively as when caveolae were present (Fig. 4).

As shown in Fig. 1A, the absence of caveolae resulted in a lessened ability to maintain frequencies when segments were exposed to Ca^{2+}-free media with 0.1 mM EGTA. Decreased ability to recycle calcium in the absence of caveolae could be a result of loss of a close proximity between Ca^{2+} entering through L-type Ca^{2+} channels and SR/ER causing greater dilution of recycling calcium in the cytosol. Alternatively, it could be from less Ca^{2+} in an extracellular caveolar store when caveolae were absent. We are not aware of quantitative studies of the relationships between caveolae and ER in ICC.

However, when L-type Ca^{2+} channels were opened by BayK 8644, segments lacking caveolae maintained their frequencies better than segments with caveolae (Fig. 2A). Under our condition of no external calcium and close proximity between SR and caveolae, open L-type Ca^{2+} channels could function as sites of calcium efflux. Efflux through them is less effective when caveolae are absent. As suggested above, this could be a result of channels and caveolae being in close proximity (leading to a higher concentration of calcium leaking from the SR into the space between SR and caveolae) when caveolae are present than when they are absent.

The calcium involved in ongoing recycling in ICC appears to leak from the SR. When the SR calcium pump was inhibited and the calcium in these stores was reduced, frequencies were less well maintained in Ca^{2+}-free media in segments both with and without caveolae and there was no difference between them. When L-type Ca^{2+} channels were opened by BayK 8644 after blocking of the SERCA pump, there were no significant differences between segments with or without caveolae in frequencies. Thus Ca^{2+} from the SERCA pump is crucial to maintain recycling of caveolar calcium.

Opening L-type Ca^{2+} channels with BayK 8644 also helped maintain frequencies/contractions in segments with and without caveolae after reduction of Ca^{2+} stores. Secondary analyses suggest that this improvement was as effective as when the SR/ER pumps were working in the segments with caveolae, but not in those without. These findings might result from the ability of open L-type Ca^{2+} channels to supply some calcium for contraction even when caveolae are absent. Less effective recycling from opening of L-type Ca^{2+} channels after store depletion in the absence of caveolae may result from the calcium leaking into the an enlarged space between ER/SR and the caveolar membrane.

In experiments keeping Ca^{2+} stores intact and in which CCH was applied repeatedly (Fig. 4A), the presence of caveolae allowed improved recycling to ICC-MP to maintain frequencies whether L-type Ca^{2+} channels were opened or closed. This suggested an additional efficient route for calcium recycling when caveolae were present, consistent with the hypothesis about store operated channels noted above.

Caveolae and calcium handling by longitudinal smooth muscle

We assume that contractile amplitudes reflect calcium levels available to smooth muscle, allowing contractile responses to slow wave depolarizations. We also assume that the phasic response to CCH reflects the residual calcium in smooth muscle stores. If contractions of smooth muscle reflect primarily the calcium available to enter through L-type Ca^{2+} channels or be released from SR when slow waves arrive [29], then the role of caveolae should be revealed by comparing contractile responses in the presence and absence of caveolae. Figure 1B shows that the absence of caveolae, unlike the result in pacing frequency in Fig. 1A, made no significant differences in contractile responses 5 and 10 min. after exposure to Ca^{2+}-free medium with 0.1 mM EGTA. However, when exposure was to Ca^{2+}-free medium with 1 mM EGTA contractile responses, although much less than in a medium with 0.1 mM EGTA, were better maintained in tissues with caveolae at both 5 and 10 min. Moreover, tissues with caveolae maintained contractions equally well when the medium had either 0.1 or 1 mM EGTA. A possible explanation is that calcium in caveolae is less accessible to EGTA than when caveolae are absent.

There were no significant differences between the relative contractile strengths of $Cav^{+/+}$ and Cav^B LM segments (Fig. 2B) when BayK 8644 was added to the Ca^{2+} medium. When nicardipine was present, there was a significantly increased contraction strengths of $Cav^{+/+}$ segments compared to segments without caveolae. This again implies an alternate route to L-type Ca^{2+} channels for recycling calcium when caveolae are present, possibly a store operated channel (Fig. 3). As this membrane channel persisted in $Cav^{-/-}$ tissues, loss of a close relationship may explain our result.

When LM tissue segments were pre-treated with cyclopiazonic acid, there was no significant differences between the relative contractile strengths of $Cav^{+/+}$ and Cav^B segments when BayK 8644, nicardipine or nothing were added to the Ca^{2+}-free medium with 0.1 mM EGTA. Addition of BayK 8644 increased relative contractile strengths in both the presence and absence of caveolae,
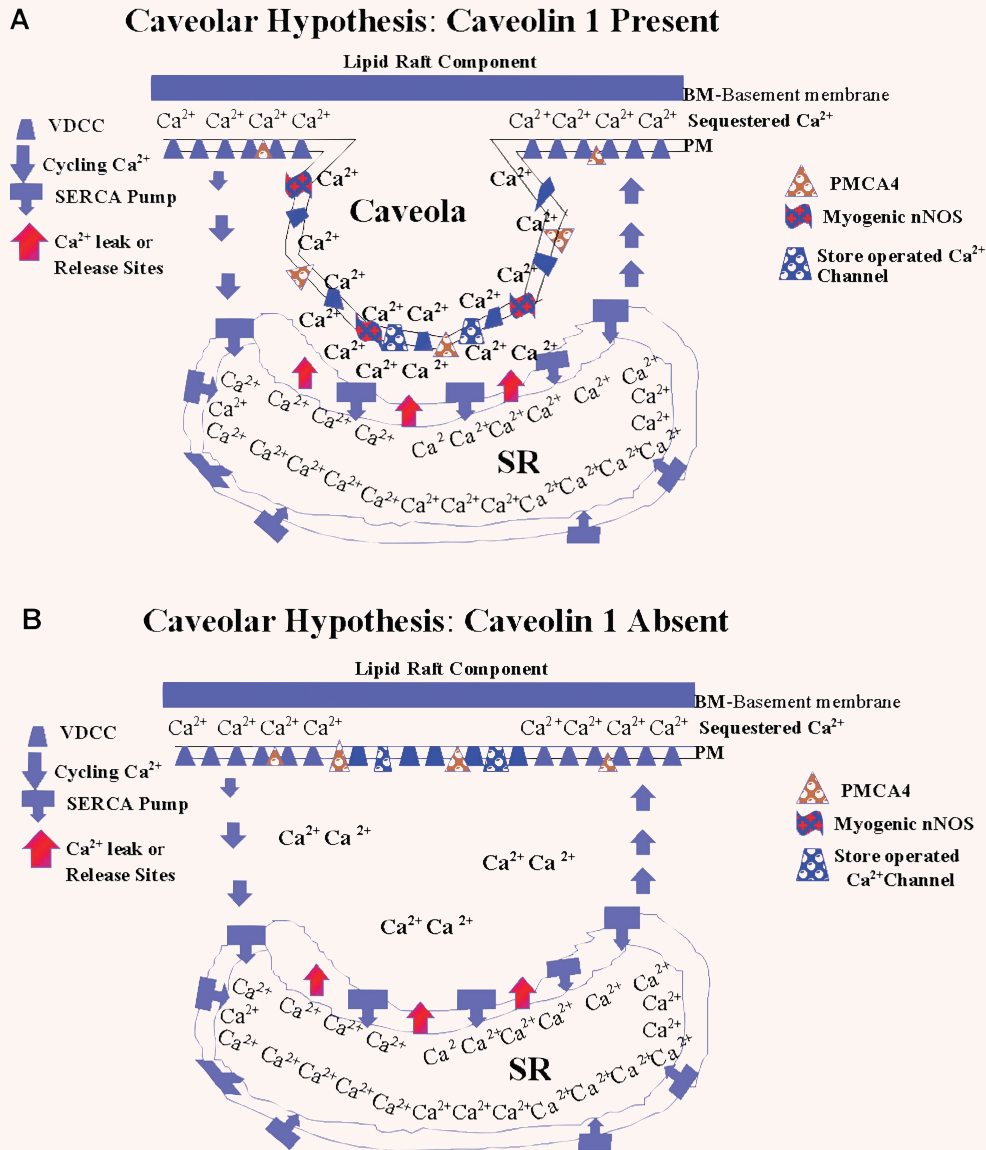
Fig. 7 (**A**) This cartoon represents a possible distribution if Ca^{2+} handling proteins related to caveolae and Cav-1 based on our findings. The peripheral ER/SR is shown near a caveola, with a restricted space in between them, containing a higher Ca^{2+} concentration than in the general cytosol. Sequestered Ca^{2+} is also shown associated with the caveolar extracellular membrane and adjacent raft tissue. This is the Ca^{2+} which is protected from the Ca^{2+}-free medium we used and provides ions for recycling to the restricted space and the ER/SR (and in Cav-1 knockout tissue provides some Ca^{2+} for recycling). Owing to space limitations this recycling Ca^{2+} is depicted adjacent to the restricted space. Proteins apparently co-localized with Cav-1 including VDCC, PMCA4, myogenic nNOS, and store operated Ca^{2+} Channels are shown associated with the caveola membrane. Under the control conditions in Ca^{2+}-free medium, the VDCC were the main entry route for Ca^{2+} to be recycled, but store operated channels contribute when the VDCC are blocked. The SERCA pump and Leak or Release Sites in SR are also depicted. The Ca^{2+} stored in the ER/SR is the other main source of Ca^{2+} to maintain recycling and when it is emptied, recycling stops quickly. (**B**) This cartoon depicts the proposed changes in the Cav-1 knockout tissues. The restricted space is lost as well as the higher local Ca^{2+} concentration which promotes its recycling to the ER/SR. VDCC, PMCA and store operated Ca^{2+} Channels persist, but the myogenic nNOS is missing. The proximity of the remaining Ca^{2+} molecules to the ER/SR is lost. There is also less sequestered Ca^{2+} available for recycling. The result is more rapid loss of pacing in Ca^{2+}-free medium. There is also less rapid clearing of Ca^{2+} released or leaked from ER/SR by the PMCA and other clearance mechanisms. The main route for Ca^{2+} entry remains the VDCC with a possible contribution from the store operated channel.

but only the increases in $Cav^{-/-}$ segments were significant ($P < 0.01$).

In a secondary comparison, calcium depletion from SR did not significantly affect relative contractile strengths of $Cav^{+/+}$ or Cav^B segments compared to controls when the SR was replete. However, after depletion of calcium stores in $Cav^{-/-}$ segments, the presence of nicardipine left greater contraction amplitudes than when stores were not depleted: $P = 0.0212$ at 5 min. and $P = 0.0355$ at 10 min. The most likely explanation was that calcium leaked out less when nicardipine was present and did not get taken up by SR after CPA when caveolae were absent. There were no apparent differences in residual calcium in SR in the presence and absence of caveolae after CPA, as judged by the phasic responses to CCH (Fig. 5).

When CCH was applied to activate calcium release from SR and open L-type Ca^{2+} channels (Fig. 5B), the presence of caveolae resulted in higher contraction amplitudes in most conditions, especially when the L-type Ca^{2+} channels were closed by nicardipine or left unopened. Thus these data also imply an alternate route to L-type Ca^{2+} channels for calcium recycling when caveolae are present, as already discussed. This hypothesis was supported by the fact that CCH phasic responses were higher in all conditions of the L-type Ca^{2+} channels in these experiments, but especially when these channels were opened with BayK 8644. In that case, both L-type Ca^{2+} channels and an alternate route for recycling were presumably available.

In other studies [33], we showed that block of the PMCA4 pump, the main plasma membrane pump of smooth muscle, resulted in an increase in contractile responses to CCH when caveolae and Cav-1 were present, but not when they were absent. There was also probable loss of one isoform of the PMCA4 protein. This implies that the proximity of caveolae with PMCA4 to the peripheral SR is important to removal of elevated calcium and/or that the one PMCA4 isoform plays a special role in removing calcium form the caveolar-SR interspace. Further, we found that Cav-1 knockout animals lacked a myogenic nNOS [20], and as a consequence could no longer release NO when calcium entered through L-type Ca^{2+} channels to modulate contraction.

contractions could only be estimated if contractions persisted. ICC activity might have continued after the muscle was unable to contract and this would have gone unrecorded in our experiments. However, we do not think that this possible error affected our findings about the recycling of calcium by ICC. Most of the differences observed were between frequencies of persisting contractions, not between a zero frequency and positive one.

This possible problem also applies to contractile functions of smooth muscles. They might fail to contract because no pacing signal was delivered from ICC. This cannot be ruled out. Responses to CCH were independent of the arrival of a pacing signal form the ICC and they seem to be consistent with our findings with spontaneous contractions.

A modified caveolar hypothesis

Figure 7A and B summarize the current status of this hypothesis [3] and propose the differences between cell functions with and without caveolae.

In conclusion

When extracellular calcium levels are restricted by removing calcium from the medium and adding 0.1 mM EGTA, a source of calcium resistant to removal and available for recycling to SR stores in ICC-MP and longitudinal smooth muscle is revealed. Availability of this source for recycling depends in part on the presence of Cav-1 and caveolae. The main route for recycling is through L-type Ca^{2+} channels. It is still present, but operates less effectively, in the absence of caveolae. When caveolae are present and L-type Ca^{2+} channels are blocked, an additional route for recycling of calcium to SR is revealed, possibly a store operated channel. The differences which depend on the presence of caveolae are more prominent in the functioning of ICC-MP than in functioning of longitudinal smooth muscle, possibly because pacing by these ICC requires calcium recycling continuously.

Limitations of this study

One obvious limitation is that our findings depended on the ability of the muscle to respond by initiating contractions. Both the effects of experimental procedures on pacing frequencies and on

Acknowledgements

References

1. **Popescu LM, Diculescu I, Zeick U, Ionescu N**. Ultrastructural distribution of calcium in smooth muscle cells of guinea-pig taenia coli. A correlated electron microscopic and quantitative study. *Cell Tissue Res.* 1974; 184: 357–78.

2. **Popescu LM.** Conceptual model of the excitation contraction coupling in smooth muscle, the possible role of the surface microvesicles. *Studia biophysica. Berlin*, *Band.* 1974; 44: 141–53.

3. **Daniel EE, El-Yazbi A, Cho WJ.** Caveolae and calcium handling, a review and a hypothesis. *J Cell Mol Med.* 2006; 10: 529–44.

4. **Daniel EE, Jury J, Wang YF.** nNOS in canine lower esophageal sphincter: colocalized with Cav-1 and Ca2+-handling

proteins? *Am J Physiol Gastrointest Liver Physiol.* 2001; 281: G1101–14.

5. **Darby PJ, Kwan CY, Daniel EE.** Caveolae from canine airway smooth muscle contain the necessary components for a role in Ca^{2+} handling. *Am J Physiol Lung Cell Mol Physiol.* 2000; 279: L1226–35.

6. **Salapatek AM, Lam A, Daniel EE.** Calcium source diversity in canine lower esophageal sphincter muscle. *J Pharmacol Exp Ther.* 1998;287: 98–106.

7. **Montano LM, Barajas-Lopez C, Daniel EE.** Canine bronchial sustained contraction in Ca^{2+}-free medium: role of intracellular Ca2+. *Can J Physiol Pharmacol.* 1996; 74: 1236–48.

8. **Thuneberg L**. Interstitial cells of Cajal: intestinal pacemaker cells? *Adv Anat Embryol Cell Biol.* 1982; 71: 1–130.

9. **Berezin I, Huizinga JD, Daniel EE.** Interstitial cells of Cajal in canine colon: A special communication network at the inner border of the circular muscle. *J Comp Neurol.* 1988; 273: 42–51.

10. **Faussone-Pellegrini MS, Thuneberg L.** Guide to the identification of interstitial cells of Cajal. *Microsc Res Tech.* 1999; 47: 248–66.

11. **Hnasko R, Lisanti MP.** The biology of caveolae: lessons from caveolin knockout mice and implications for human disease. *Mol Interv.* 2003; 3: 445–64.

12. **Cohen AW, Hnasko R, Schubert W, Lisanti MP.** Role of caveolae and caveolins in health and disease. *Physiol Rev.* 2004; 84: 1341–79.

13. **Williams TM, Lisanti MP.** The caveolin genes: from cell biology to medicine. *Ann Med.* 2004; 36: 584–95.

14. **Kogo H, Ito SY, Moritoki Y, Kurahashi H, Fujimoto T.** Differential expression of caveolin-3 in mouse smooth muscle cells in vivo. *Cell Tissue Res.* 2006; 324: 291–300.

15. **Michel JB, Feron O, Sacks D, Michel T**. Reciprocal regulation of endothelial nitric-oxide synthase by Ca2+-calmodulin and caveolin. *J Biol Chem.*1997; 272: 15583–6.

16. **Feron O, Saldana F, Michel JB, Michel T.** The endothelial nitric-oxide synthase-caveolin regulatory cycle. *J Biol Chem.* 1998; 273: 3125–8.

17. **Salapatek AM, Wang YF, Mao YK, Lam A, Daniel EE.** Myogenic nitric oxide synthase activity in canine lower oesophageal sphincter: morphological and functional evidence. *Br J Pharmacol.* 1998; 123: 1055–64.

18. **Salapatek AM, Wang YF, Mao YK, Mori M, Daniel EE.** Myogenic NOS in canine lower esophageal sphincter: enzyme activation, substrate recycling, and product actions. *Am J Physiol.* 1998; 274: C1145–57.

19. **Daniel EE, Jury J, Salapatek AM, Bowes T, Lam A, Thomas S, Ramnarain M, Nguyen V, Mistry V.** Nitric oxide from enteric nerves acts by a different mechanism from myogenic nitric oxide in canine lower esophageal sphincter. *J Pharmacol Exp Ther.* 2000; 294: 270–9.

20. **Ahmed F, El-Yazbi, Woo Jung Cho, Jonathan Cena, Richard Schulz, Edwin E. Daniel**. Smooth muscle nitric oxide synthase, co-localized with caveolin-1, modulates depolarization-induced contraction in mouse small intestine. *J Cell Mol Med.* 2008; 12: 1404–15.

21. **Cho WJ, Daniel EE.** Proteins of interstitial cells of Cajal and intestinal smooth muscle, co-localized with Caveolin 1. *Amer J Physiol (Gastrointest Liver Physiol).* 2005; 288: 571–85.

22. **Boddy G, Bong A, Cho WJ, Daniel EE.** ICC pacing mechanisms in intact mouse intestine differ from those in cultured or dissected intestine. *Am J Physiol (Gastrointestinal Liver Physiol).* 2004; 286: G653–62.

23. **Hagiwara S, Takahashi K.** Surface density and of calcium ions and calcium spikes in the barnacle muscle. *J Gen Physiol.* 1967; 50: 583–601.

24. **Shaw L, Sweeney MA, O'Neill SC, Jones CJ, Austin C, Taggart MJ.** Caveolae and sarcoplasmic reticular coupling in smooth muscle cells of pressurised arteries: the relevance for Ca^{2+} oscillations and tone. *Cardiovasc Res.* 2006; 69: 825–35.

25. **Ward SM, Ordog T, Koh SD, Baker SA, Jun JY, Amberg G, Monaghan K, Sanders KM.** Pacemaking in interstitial cells of Cajal depends upon calcium handling by endoplasmic reticulum and mitochondria. *J Physiol.* 2000; 525: 355–61.

26. **Ward SM, Baker SA, de Faoite A, Sanders KM.** Propagation of slow waves requires IP3 receptors and mitochondrial Ca^{2+} uptake in canine colonic muscles. *J Physiol.* 2003; 549: 207–18.

27. **Park KJ, Hennig GW, Lee HT, Spencer NJ, Ward SM, Smith TK, Sanders KM.** Spatial and temporal mapping of pacemaker activity in interstitial cells of Cajal in mouse ileum in situ. *Am J Physiol Cell Physiol.* 2006; 290: C1411–27.

28. **Boddy G, Daniel EE.** The role of L-Ca^{2+}channels in intestinal pacing in wild-type and W/W^V mice. *Amer. J. Physiol (GI Liver Physiol).* 2005; 288: G439–46.

29. **Sanders KM, Ordog T, Koh SD, Ward SM.** A novel pacemaker mechanism drives gastrointestinal rhythmicity. *News Physiol Sci.* 2000; 15: 291–8.

30. **Patel HH, Zhang S, Murray F, Suda RYS, Head BP, Yokoyama U, Swaney JS, Niesman IR, Schermuly RT, Pullamsetti SS, Thistlewaite PA, Miyanohara A, Farquhar MG, Yuan JX-Y, Insel PA.** Increased smooth muscle expression of caveolin-1 and caveolae contribute to the pathophysiology of idiopathic pulmonary arterial hypertension. *FASEB J.* 2007; 21: 2970–9.

31. **Wegener JW, Schulla V, Koller A, Klugbauer N, Feil R, Hofmann J.** Control of intestinal motiltiy by the Ca 1.2 L-type calcium in mice. *FASEB J.* 2006; 20: E566–73.

32. **Unno T, Matsuyama H, Sakamoto T, Uchiyama M, Izumi Y, Okamoto H, Yamada M, Wess J, Komori S.** M$_2$ and M$_3$ muscarinic receptor-mediated contractions in longitudinal smooth muscle of the ileum studied with receptor knockout mice. *Br J Pharmacol.* 2005; 146: 98–108.

33. **El-Yazbi A, Cho WJ, Schulz R, Daniel EE.** Calcium extrusion by plasma membrane calcium pump is impaired in caveolin-1 knockout mouse small intestine. *Eur J Pharmacol.* 2008; 591: 80–7.