Caveolae and calcium handling, a review and a hypothesis

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Abstract

Caveolae are associated with molecules crucial for calcium handling. This review considers the roles of caveolae in calcium handling for *smooth muscle* and *interstitial cells of Cajal* (ICC). Structural studies showed that the plasma membrane calcium pump (PMCA), a sodium-calcium exchanger (NCX1), and a myogenic nNOS appear to be co-localized with caveolin 1, the main constituent of these caveolae. Voltage dependent calcium channels (VDCC) are associated but not co-localized with caveolin 1, as are proteins of the peripheral sarcoplasmic reticulum (SR) such as calreticulin. Only the nNOS is absent from caveolin 1 knockout animals. Functional studies in calcium free media suggest that a source of calcium in tonic smooth muscles exists, partly sequestered from extracellular EGTA. This source supported sustained contractions to carbachol using VDCC and dependent on activity of the SERCA pump. This source is postulated to be caveolae, near peripheral SR. New evidence, presented here, suggests that a similar source exists in phasic smooth muscle of the intestine and its ICC. These results suggest that caveolae and peripheral SR are a functional unit recycling calcium through VDCC and controlling its local concentration. Calcium handling molecules associated with caveolae in smooth muscle and ICC were identified and their possible functions also reviewed.

Keywords: caveolin 1 • VDCC • PMCA • SERCA • pacing • calcium heterogeneity • smooth muscle • interstitial cell of Cajal • sarcoplasmic reticulum

Introduction

Over 30 years ago, Popescu and colleagues [1] studied the distribution of calcium in smooth muscle of guinea-pig taenia coli using ultrastructural methods. They used oxalate precipitation to show that calcium was found in three expected places, the sarcoplasmic reticulum, mitochondria, and nucleus, and in an unexpected place, "surface microvesicles". They estimated, from the volumes of caveolae that the calcium passively present in surface microvesicles of guinea pig taenia coli could supply all the calcium needed for contraction [2]. These vesicles were often found close (within 10 to 40 nm) to the peripheral sarcoplasmic reticulum. Since the seminal work of Popescu and colleagues what we know about the role of caveolae and calcium handling in smooth muscle has expanded exponentially.

What are caveolae?

Caveolae are vesicular membrane structures that are found in many cell types and are abundant in the cardiac, striated and smooth muscle [3–5]. They were called "plasmalemmal vesicles" in 1953 by Palade [6] and "caveolae" ('little caves') in 1955 by

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Yamada [7], the name in current use. Under the electron microscope (EM), caveolae are characterized as "smooth coated" or "non-coated" omega- or flask-shaped invaginations (diameter approximately 70 nm at the widest point) connected to the plasmalemma by a neck-like structure. The terms "smooth coated" or "non-coated" vesicles contrast them with the electron-dense cytoplasmic coat that can be seen associated with clathrin-coated pits using EM techniques. A principal component constituting the membranes of caveolae is the cytoplasmically orientated integral membrane protein, caveolin, a term encompassing a family of proteins. These membrane structures are now known to be critically important for a variety of cellular processes that include signal transduction pathways, membrane organization and protein trafficking.

Caveolins

Caveolae exist because of the presence of caveolins, membrane proteins which insert in the inner leaflet of the plasma membrane. There are three caveolins: caveolin 1, 2 and 3. Caveolins 1 or 3 can form caveolae alone or in association with other caveolins, caveolin 2 apparently cannot. In smooth muscle and many other cells, caveolin 1 is the crucial caveolin while in striated muscle, it is caveolin 3. Caveolin 1 is a protein with palmitoylated membrane attachment domains inserted into the inner leaflet of the plasma membrane of many cells, including smooth muscle and interstitial cells of Cajal (ICC) of the intestine. Caveolae are formed by homo- and heterooligomerization of 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 caveolin 1, allow homo- and hetero-oligomerization with caveolin 1 or 3 molecules respectively. Membrane spanning sites are involved in binding between caveolins 1 and 2 [reviewed in 8, 9].

In the plasma membrane there are liquid ordered and disordered domains and caveolae are associated with the liquid ordered domains having a more rigid bilayer assembly because of the coalescence of cholesterol, glycosphingolipids, and sphingomyelin into "lipid rafts" [10, 11]. Cholesterol is essential for caveolae formation and transcription of caveolin 1 is activated by cholesterol and suppressed by oxysterols. Caveolin1 binds cholesterol and cholesterol

depletion, by agents like methyl-β-cylcodextrin, diminishes caveolae by removing caveolin 1 from the membrane [8, 9]. Whether removal of caveolin 1 by cholesterol depletion always gives identical functional changes to those from caveolin 1 genetic knockout is unclear and unlikely (unpublished observations).

Caveolin 1 is involved in signal transduction in large part because of the presence of a 20 aa domain, 82–101, which can bind a variety of signaling proteins including c-SRC, other kinases, the insulin receptor, eNOS, H-Ras, and Gα subtypes. Binding to caveolin 1 usually inactivates downstream signaling, and this has been demonstrated clearly for eNOS in endothelium in $vitro$. eNOS remains inactive until released from binding by formation of Ca^{2+} calmodulin [8, 9]. In caveolin 1 knockout animals, unbound eNOS is persistently active.

Caveolin 1 knockout animals (cav1$^{-/-}$) are viable, but have several abnormalities: absence ofcaveolae from all tissues which express caveolin 1, loss of hypodermal fat and mammary adipocytes, resistance to diet-induced obesity, insulin resistance on a normal diet and postprandial hyper-insulinemia on a high-fat diet, marked reduction of insulin receptor-β levels in adipose tissues, altered lipid metabolism, premature lactation, increased occurrence of urinary Ca^{2+} stones, altered bladder function associated with increased wall thickness along with similar changes in the prostate, altered left ventricular function associated with myocyte hypertrophy and fibrosis, increased and uncontrolled production of NO from endothelial NOS which is no longer bound to caveolin 1, decreased transcytosis of endothelium, decreased angiogenesis, and decreased life span [reviewed in 8, 9].

Caveolin 2 is missing from caveolin 1 knockout mice, but selective caveolin 2 knockout mice have been produced [12]. They, like caveolin 1 knockout mice, have lung hypercellularity with thickened alveolar septa, and increased endothelial cells. These changes are associated with severe exercise intolerance. Caveolin 1 (cav1) and caveolae are still expressed in lungs of caveolin 2 knockout mice. Apparently, the lung effects are the consequence of caveolin 2 deficiency in both cases.

What are Interstitial Cells of Cajal?

Interstitial cells of Cajal (ICC) have been characterized by their appearance in electron microscopy and

by the presence of the *c-kit* kinase, also present in mast cells [13–22]. In ultrastructural studies, ICC differ from fibroblasts by possession of multiple processes, which have many mitochondria, by having extensive smooth endoplasmic reticulum and caveolae. ICC also have an incomplete basement membrane. They have relatively little rough endoplasmic reticulum, but demonstrate a relatively electron dense cytosol with widely distributed bundles of intermediate filaments. ICC in the gut are frequently coupled to one another by gap junctions and are in close proximity to nerves [1–16, 23]. Most ICC can also be identified by their possession of the *c-kit* antigen [17–19, 22]. In the gut, this antigen is crucial for differentiation of cells from mesoderm into ICC instead of smooth muscle. Mutations in it have led to the absence of ICC in the myenteric plexus and neutralizing antibodies to *c-kit* can delay or inhibit ICC differentiation [17, 18, 22]. ICC function as pacemakers for contractions and as targets for enteric nerves in the gut [19, 23].

Objectives

This review will consider caveolae in smooth muscle and associated cells of the gastrointestinal tract, called interstitial cells of Cajal (ICC). Interstitial cells of Cajal (ICC) are also found in other tissues such as the urinary tract [15], but in the gastrointestinal, different networks of ICC pace intestinal slow waves and provide targets for enteric nerves [19, 23]. Recently, ICC identified by their ultrastructural characteristics or their possession of the *c-kit* antigen have been identified in a wide variety of other tissues: heart, uterus, fallopian tube and mammary gland [24–27]. This review will focus on those in the gut, in which some of their functions are known.

Materials and methods

Functional studies of contraction have been carried out with intact strips segments of canine or small segments of mouse gastrointestinal tract and described in detail elsewhere [28–33]. In most cases, nerve functions were blocked with tetrodotoxin (10 M). When Ca^{2+} handling was studied to evaluate caveolae as calcium reservoirs, physiological salines made from distilled water and subsequently de-ionized with Millipore Filtration were used.

When no calcium was added, these solutions were nominally calcium free and contained a calcium concentration estimated to be less than 10 µM calcium. Then, to estimate a possible contribution of Ca^{2+} sequestered in caveolae to contraction and pacing, we added either 0.1 or 1 mM EGTA. In some studies, isolated canine smooth muscle cells were used for some patch clamp studies [29]. Morphological studies were carried out using ultrastructural analysis and immunohistochemical analysis of canine and mouse gastrointestinal tracts [13, 35–41].

Results

Caveolae and caveolins and Ca handling proteins

Structural relationships in smooth muscle

In smooth muscle of the intestine, caveolin 1 is present, along with smaller amounts of caveolins 2 and 3, and is responsible for the formation of most caveolae there and in ICC [4–9]. In caveolin 1 knockout mice, we recently found that caveolin 3 persists in outer circular muscle along with a few caveolae [41]. Figs. 1 elaborates those findings. When both caveolin 1 and 3 are absent, smooth muscle and ICC as well as cardiac, skeletal muscle, endothelial cells in mouse lack caveolae [8, 9]. Several Ca^{2+} handling molecules appear to be co-localized with or associated with caveolin 1 in smooth muscle and ICC using immunohistochemistry (Fig. 2). These include the plasma membrane calcium pump (PMCA), sodium calcium exchanger (NCX1) and an isoform of nNOS. The L-type Ca^{2+} channel also appears to be partially co-localized with caveolin 1 [41]. Moreover, in the canine bronchi, this channel co-immunoprecipitated with caveolin 1 [42]. However, in caveolin 1 knockout animals, among molecules apparently associated with caveolin 1, only the nNOS isoform is missing (Fig. 2A, B). So far it is unclear how the molecules which persist in the absence of caveolin 1 are associated with the lipid raft and whether their activities are influenced by the presence or absence of caveolin 1.

Functional experiments

For my laboratory, the possibility that caveolae played a role in excitation-contraction coupling emerged
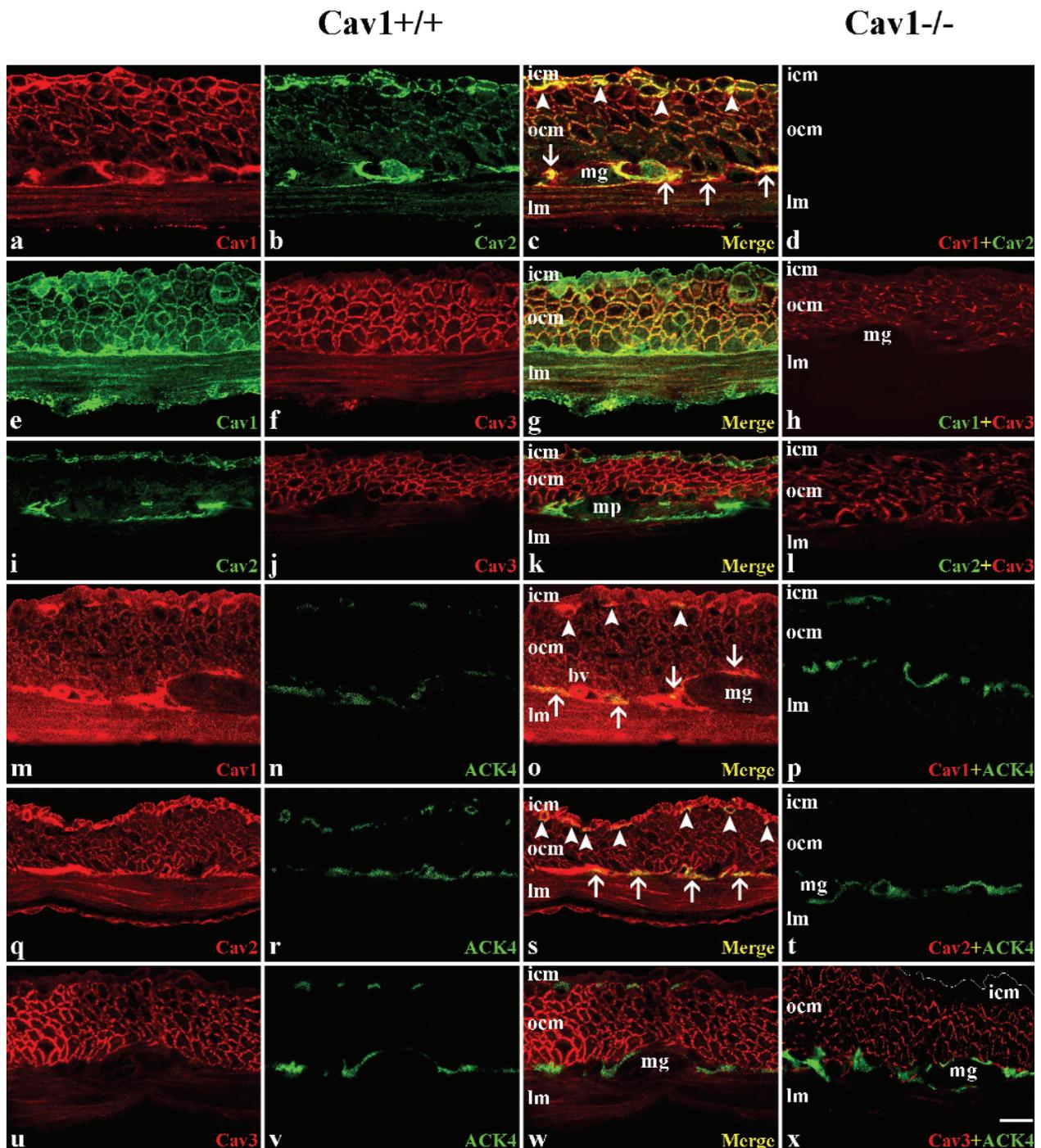
Fig. 1 This figure shows the relationships between caveolin 1, 2 and 3 in smooth muscle and ICC of the mouse intestine. At left, are shown results for intestine from caveolin $1^{+/+}$ animals and on the right are shown the results for intestine from caveolin 1 knockout animals. Panels a-d show that caveolin 2 is present in caveolin $1^{+/+}$ intestine in all muscle layers, apparently co-localized with caveolin 1 in ICC of the myenteric plexus (arrows) and deep muscular plexus (arrowheads), but both ate totally absent in caveolin $1^{-/-}$ intestine. Panels e-h show that caveolin 3 is also present apparently co-localized with caveolin 1 in all these sites in caveolin $1^{+/+}$ intestine, but it persists in the outer circular muscle after caveolin 1 knockout. Panels i-l show the relations between caveolins 2 and 3. Apparently the presence of caveolin 2 depends on caveolin 1 expression as it is absent after knock-out even though caveolin 3 persists in the outer circular muscle layer. The remaining panels demonstrate, using ACK4, an anti-body against c-kit in ICC that the ICC cells of the myenteric plexus (labeled with arrows) and the deep muscular plexus (labeled with arrowheads) contain caveolins 1, 2 and 3 in caveolin $1^{+/+}$ intestine but not after knockout. Length bars are 10 microM.
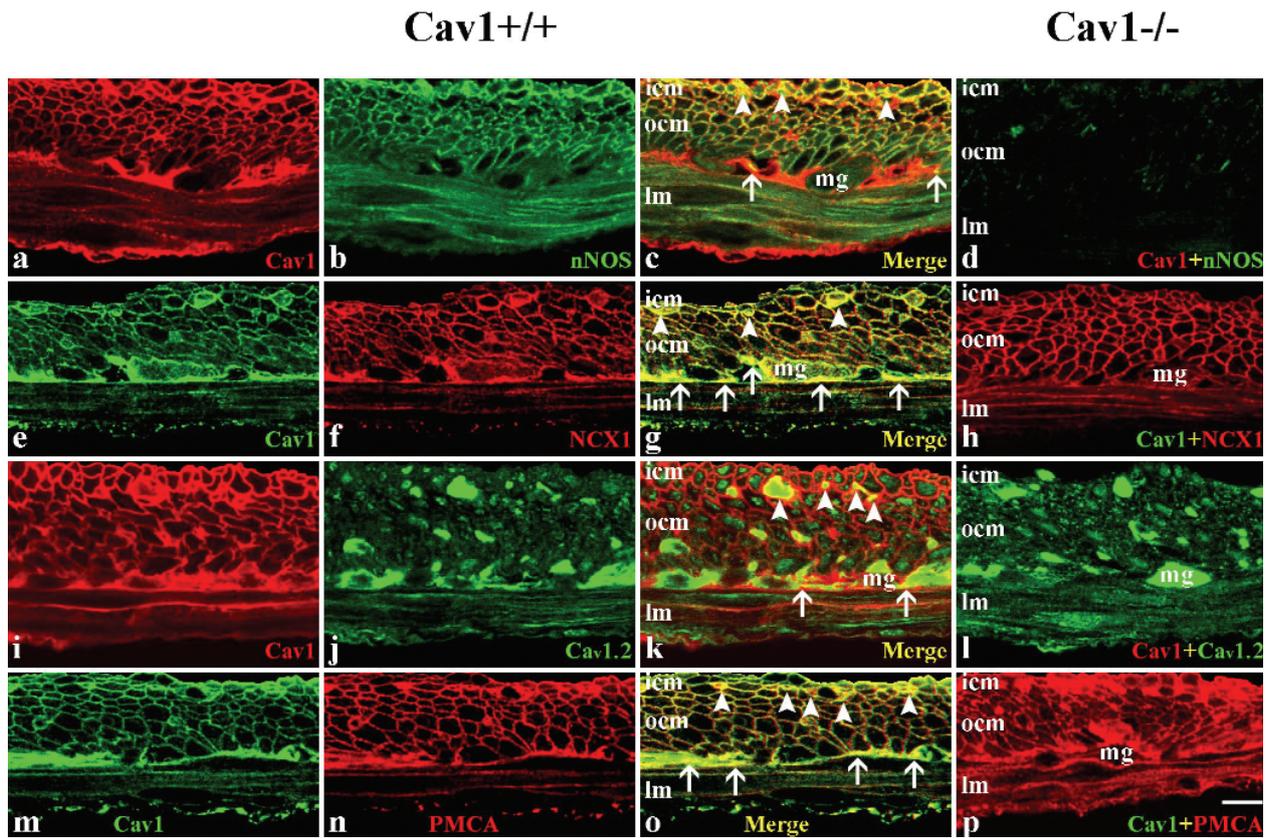
Cav1+/+   Cav1-/-

Fig. 2A This figure shows the apparent co-localization of Ca^{2+} handling molecules (nNOS, NCX1, CaV1.2 and PMCA) with caveolin 1 in ICC and all smooth muscle layers and the persistence of all these except nNOS in knock-out animals. CaV.1.2 antibody recognizes the the L-type Ca^{2+} channels. Length bars are 10 microM.

from unexpected functional findings. We found that smooth muscle of the canine lower esophageal sphincter, studied *in vitro*, underwent tonic contractions repeatedly to carbachol in nominally Ca^{2+} free media containing EGTA at 100 mM or lower concentrations [28]. These sustained contractions to carbachol were prevented/abolished by nifedipine or by increased Ca^{2+} chelation (1mM EGTA) and enhanced by BayK8644. Inhibition of sarcoplasmic reticulum Ca^{2+} pumps (SERCA) by cyclopiazonic acid (CPA) reduced Ca^{2+} free contractions to carbachol, but BayK8644 restored cyclopiazonic acid-reduced Ca^{2+} free contractions to carbachol. We concluded that some Ca^{2+} stores can be refilled by mechanisms not requiring activity of the sarcoplasmic reticulum Ca^{2+} pump. A preferred pathway may exist whereby Ca^{2+} enters stores directly or indirectly through L-Ca^{2+} channels. The proposed Ca^{2+} store refilling mechanism involves Ca^{2+} entry through L-Ca^{2+} channels from sites not equilibrated with external Ca^{2+}.

We suggested that caveolae near peripheral sarcoplasmic reticulum (SR) was the protected source of Ca^{2+} recycled from an extracellular site to SR. With a colleague, Luis Montano, we made similar observations about contractions to carbachol in canine bronchial smooth muscle [43]. Further study [44] revealed that carbachol utilized Ca^{2+} from two different sources during the sustained bronchial contraction in Ca^{2+}-free medium: from a CPA-sensitive source presumably sarcoplasmic reticulum (SR) and from a extracellular membrane Ca^{2+} pool sensitive to 1 mM EGTA but partly sequestered from 0.1mM EGTA. Neither process involved PKC activation. Histamine appeared to produce sustained contractions utilizing only the extracellular membrane pool. We suggested that the extracellular pool was located in caveolae and that the caveolae and the peripheral SR can function together to recycle Ca^{2+} between them.
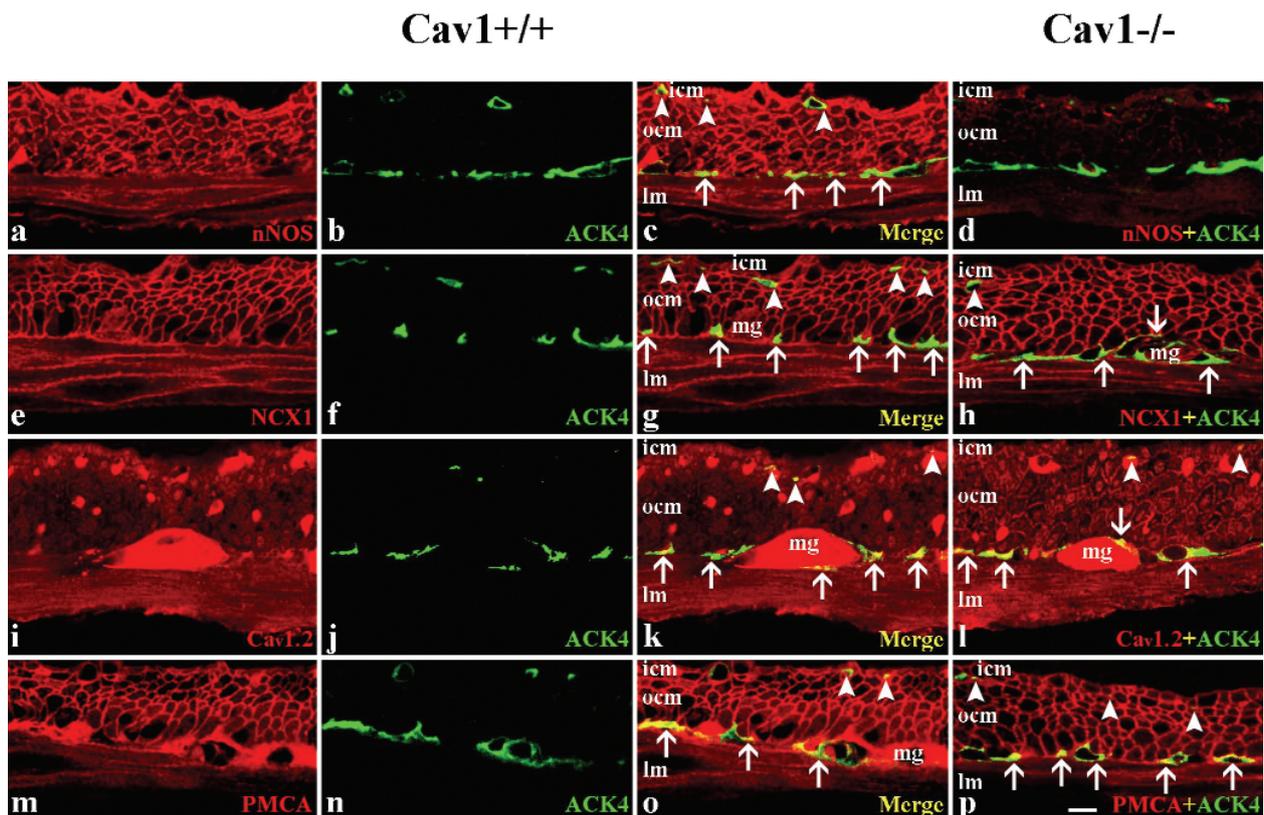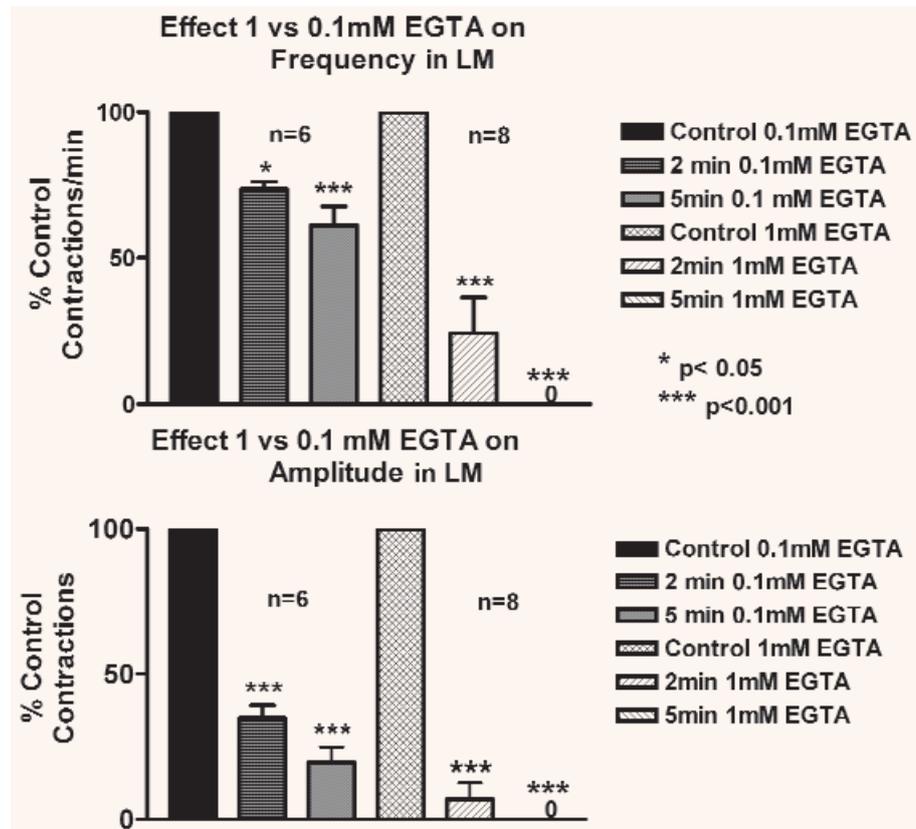
Fig. 2B This figure shows the co-localization of Ca^{2+} handling molecules (nNOS, NCX1, Cav1.2 and PMCA) with ACK4 in ICC of the myenteric plexus and deep muscular plexus and the persistence of all these except nNOS in knockout animals. Cav.1.2 antibody recognizes the L-type Ca^{2+} channels. Length bars are 10 microM.

These findings were observed under Ca^{2+} free conditions, but they suggest that caveolae may play a role under physiological conditions to recycle Ca^{2+} through L-Ca^{2+} channels to SR and to support contractions by virtue of their close physical juxtaposition, as Popescu's structural findings suggested 32 years ago. This implies that the sub-sarcolemmal space between caveolae and peripheral SR has a different Ca^{2+} concentration than the general cytosol and does not equilibrate fully with the cytosol. We went on to show that, in canine LES and bronchi, several Ca^{2+} handling molecules appeared to be co-localized with or associated with caveolin 1 using immunohistochemistry and co-immunoprecipitation [37, 42]. As summarized above, these included the PMCA, the L-type Ca^{2+} channel and to some degree calreticulin.

In contrast to the LES and bronchi which usually undergo tonic contractions, the intestine functions primarily using phasic contractions paced by ICC-driven slow waves [23, 31–34]. In the intact segments of mouse intestine which we have studied, contraction amplitudes reflect local responses to slow waves by smooth muscle as well as effects on slow wave currents from pacing cells. Frequencies of contractions, after TTX, reflect primarily pacing activities by ICC-MP, but also may be influenced by failures of transmission of pacing currents to muscle. Just maximal contractions to 10^{-5} M carbachol in mouse intestine have an initial phasic followed by a decaying tonic contraction. In intact intestinal segments, we found that contractions of longitudinal muscle segments paced by ICC continued for at least 10 minutes after multiple washes in Ca^{2+} free Ringer with 0.1 mM EGTA. In contrast, Ca^{2+} free Ringer with 1 mM EGTA abolished paced contractions within 2–5 minutes (Fig. 3). Carbachol (10^{-5} M) added after 5 minutes Ca^{2+} free media with either 0.1 or 1mM EGTA, produced markedly different contractile responses. The response to 10^{-5} M carbachol (n=7) after 5 minutes in Ca^{2+} free media was 2.86 ± sem2.86 % of control pacing contraction

Fig. 3 In this and subsequent Fig. 4–7, longitudinal muscle segments 1–1.5 cm in length were studied in Krebs Ringer solution at 37° C while oxygenated with 95% O_2 and 5% CO_2. After 30 minutes pre-equilibrations, TTX (1 micro Molar) was added and nerve function blocked. The frequencies and amplitudes of ICC-paced spontaneous contractions were measured and values normalized to the control values. Then various procedures were introduced to evaluate the role of partly sequestered calcium. In Fig. 3, segments were exposed to 2 washes with nominally Ca^{2+} free Ringer with either 0.1 or 1 mM EGTA. After 2 minutes, the frequencies and amplitudes of contractions were remeasured and two further washes with the same solutions were executed. After 5 minutes the tissues were exposed to 10 microMolar carbachol (no responses after 1 mM EGTA exposure and small responses after 0.1 mM EGTA exposure). The results showed that even after 4 washes in Ca^{2+} free Ringer with 0.1 mM EGTA smaller and less frequent spontaneous contractions persisted, but by 5 minutes all contractions were abolished in Ringer with 1 mM EGTA. All values in 0.1 mM EGTA were significantly different from those in 1 mM EGTA and responses to carbachol were also abolished in 1 mM EGTA, but diminished responses persisted in 0.1 mM EGTA. These results showed that a source of Ca^{2+} partly sequestered from extracellular EGTA existed in ICC and smooth muscle.

when the exposure was to 1 mM EGTA and 97.6 ± sem19.54% of control contractions when exposure was to 0.1 mM EGTA; $p<0.001$. This result was likely due to interruption of recycling of Ca^{2+} between the caveolae and stores, since EGTA does not penetrate the cell. Pretreatment for 30 minutes with CPA, to block the SERCA pump and prevent uptake into Ca^{2+} stores also abolished paced contractions in Ca^{2+} free Ringer with 100 μM EGTA (Fig. 4). After exposure to Ca^{2+} free Ringer with 0.1 mM EGTA, nicardipine (10^{-6} M), an L-type Ca^{2+} channel blocker, quickly abolished paced contractions, whereas BayK 8644, the L-type Ca^{2+} channel agonist, maintained them in frequency and amplitude, compared to controls (Fig. 5, compare to Fig. 3). These two agents also affected both phasic and

tonic responses to carbachol; BayK 8644 maintained while nicardipine abolished them (Fig. 6). This suggests that calcium is recycled to SR from a sequestered store even when there is negligible extracellular calcium. Carbachol accelerated loss of frequency of paced contractions (Fig. 7), presumably by increasing loss of ER calcium in ICC. The findings are similar to those in canine LES and bronchi and suggest that a limited store of Ca^{2+}, protected, in part, from extracellularEGTA, and available to support pacing by ICC and contractions of smooth muscle is present in the intestine.

These data are consistent with the hypothesis that calcium is sequestered in caveolae in smooth muscle and ICC and recycles through L-type Ca^{2+} channels in caveolae and lipid rafts to SR. If recycling is abol-

Effect of Cyclopiazonic Acid (10 μM) on Frequency in 0 Ca^{2+} KR with 0.1mM EGTA

Legend:
- Time Control 0m
- Time control 30m
- Control before CPA
- After CPA 30m
- Time Control in 0Ca
- After CPA in 0Ca
- Time Control 0Ca 5m
- After CPA 0Ca5m
- Time Control 0Ca 10
- After CPA 0Ca 10m

*** p<0.001 compared to their controls

Effect of Cyclopiazonic Acid (10 μM) on Amplitude in 0 Ca^{2+} KR with 0.1mM EGTA

Legend:
- Time Control 0m
- Time control 30m
- Control before CPA
- After CPA 30m
- Time Control in 0Ca
- After CPA in 0Ca
- Time Control 0Ca 5m
- After CPA 0Ca5m
- Time Control 0Ca 10
- After CPA 0Ca 10m
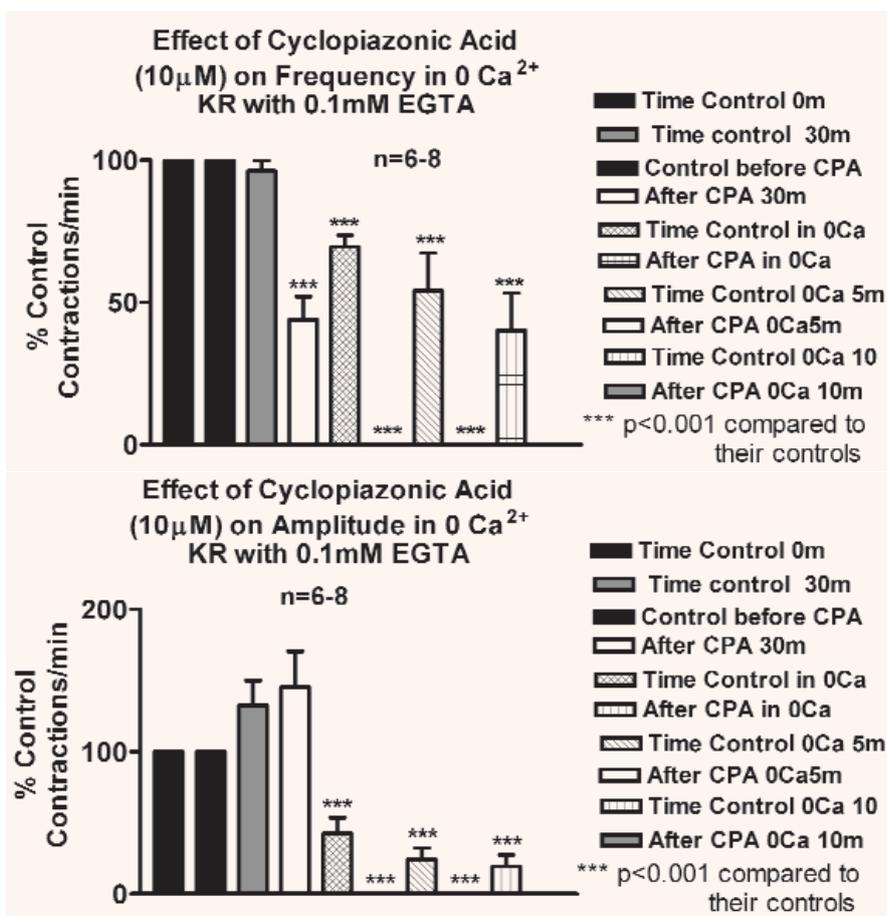
*** p<0.001 compared to their controls

Fig. 4 After equilibration and block of nerves, half the tissues were treated with 10 microMolar CPA for 30 minutes in Krebs Ringer (to inhibit the SERCA pump and deplete the Ca^{2+} stores), and other half were kept as time controls (to ensure that changes were due to the experimental variable rather than time). CPA reduced the frequency of contractions as measured in the presence of CPA and increased their amplitudes, as expected from previous studies [19, 20]. Then all tissues were washed twice in Ca^{2+} free Ringer with 0.1 mM EGTA and effects on frequencies and amplitudes of ICC-paced contractions were measured. Washes in Ca^{2+} free Ringer with 0.1 mM EGTA were repeated after 5 minutes and measurements were made before and then after the final set of washes at 10 minutes. Pretreatment with CPA depleted the Ca^{2+} stores and caused disappearance of paced contractions. These results showed that persistence of paced contractions in in Ca^{2+} free Ringer with 0.1 mM EGTA depended on the availability of Ca^{2+} from SR stores, presumably by supplying Ca^{2+} to the site of sequestration.

ished because high EGTA concentrations deplete caveolar stores, recycling will stop. If the SERCA pump is inhibited, SR stores will be depleted and recycling will also stop. Recycling can be enhanced by opening and prevented by closing L-type Ca^{2+} channels. Fig. 8 diagrams our hypothesis. The availability of caveolin 1 knockout animals will enable us to test our hypothesis. Already we know that these animals lack caveolae in smooth muscle and ICC except for the smooth muscle of the outer circular muscle which retains some caveolin 3 and a few caveolae [40, 41, 53 and Figs. 1 and 2].

Our hypothesis is related to and derived from an earlier one, the Superficial Buffer Barrier Hypothesis of Casey Van Breemen [45–49]. He postulated that the peripheral SR in vascular smooth muscle near caveolae releases Ca^{2+} vectorially toward the nearby plasma membrane using ryanodine receptors in Ca-induced Ca-release (CICR) or IP$_3$ induced release and that Ca^{2+} was removed from that space by Na$^+$-Ca^{2+} exchange. He envisaged this arrangement as buffering Ca^{2+} entry through VDCC or other channels and creating heterogeneity of cytosolic [Ca.$^{2+}$].

A recent review also suggests that caveolae play a role in calcium handling in myometrial smooth muscle by recruiting and inhibiting activities of PKC-α and *rhoA* to caveolae [50]. In addition, PKA as well as PKC γ and τ have been found associated with ICC throughout guinea pig intestine, though their association with caveolae or caveolin 1 was not determined [51]. Thus caveolae of smooth muscle and ICC may regulate calcium by affecting activation of calcium release from SR and calcium sensitivity as well as by allowing recycling between SR and caveolae.

We are currently studying the role of the nNOS isoform co-localized with caveolin 1 and lost in
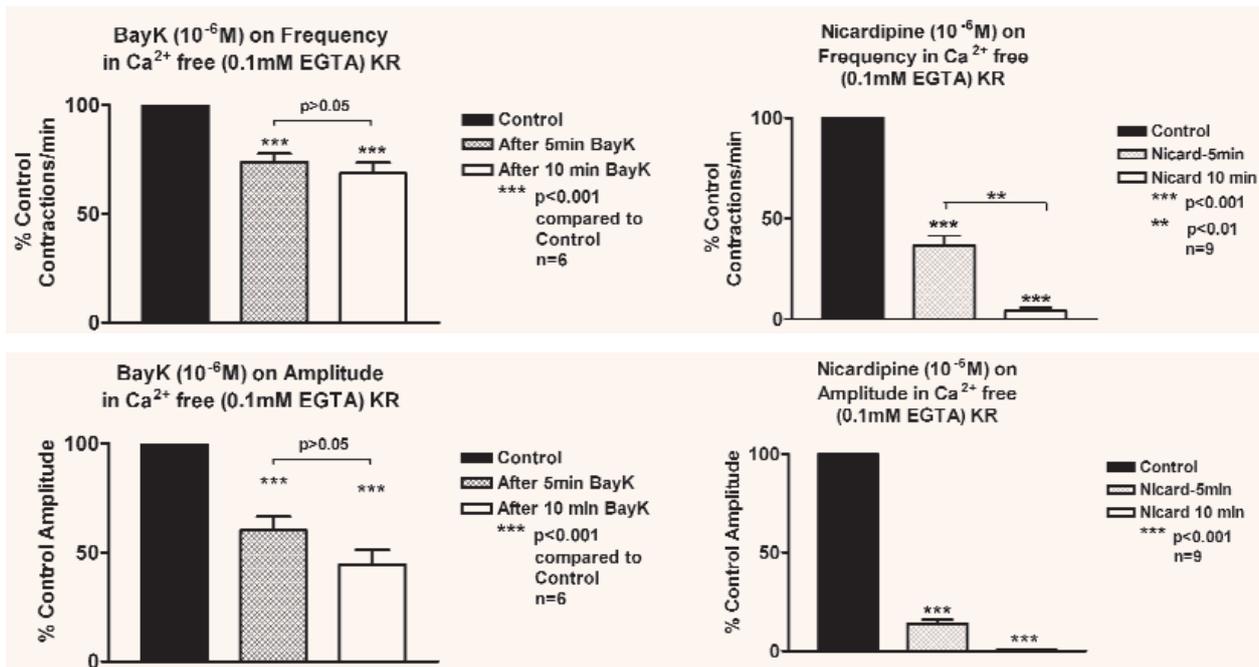
Fig. 5 After equilibration and block of nerves, tissues were placed in Ca^{2+} free Ringer with 0.1 mM EGTA and either the VDCC opener BayK 8644 or the VDCC closer nicardipine was added immediately, both at 1 microMolar. Further washes and additions of BayK 8644 and nicardipine were executed after 5 minutes and the experiments continued for 10 minutes. Measurements of frequencies and amplitudes showed that BayK preserved frequencies and amplitudes of paced contractions (compared to tissues with nothing added as Fig. 3-not shown) while nicardipine nearly abolished within 10 minutes. These results showed that VDCC were essential means by which Ca^{2+} was restored to support contractions.

knockout mice. We postulate that it plays a modulating role. Ca^{2+} entering through L-type Ca^{2+} channels may activate this NOS to release NO, which in turn activates BK_{Ca} channels to limit contraction. In earlier studies in canine LES, we showed that a similar myogenic nNOS existed associated with caveolin 1 [29, 30]. Its role was to limit spontaneous myogenic tone by releasing NO continually in response to Ca^{2+} entering through VDCC to activate BK_{Ca} channels. These channels were also associated with caveolae [37]. Myogenic nNOS was capable of continuous formation of NO by possessing the enzymes which resynthesized L-arginine, the NOS substrate, from L-citrulline, the NOS product [38]. In the mouse intestine, it appears, based on preliminary studies, that Ca^{2+} entry through L-type Ca^{2+} channels is also required for activation of this enzyme.

In caveolin1 knockout animals, there is both a loss of the myogenic nNOS isoform and also a reduced NO mediated relaxation to nerve stimulation, compensated in part by an increased response of apamin sensitive inhibitory mediators [52, 53]. The site of failure appears to be downstream from mediator release and associated with decreased responses to NO donors, sodium nitroprusside and S-nitroso-N-acetyl penicillamine. Responses of both longitudinal and circular muscle to nerve stimulation of nitrergic nerves were diminished, but there were differences in the extent of decrease and the inhibition of responses to NO donors by ODQ and apamin [52].

Other Ca^{2+} molecules associated with caveolin

Current status in smooth muscle and ICC

The plasma membrane Ca^{2+} pump
Although the PMCA appears closely co-localized in immunohistochemistry analyses with caveolin 1 in
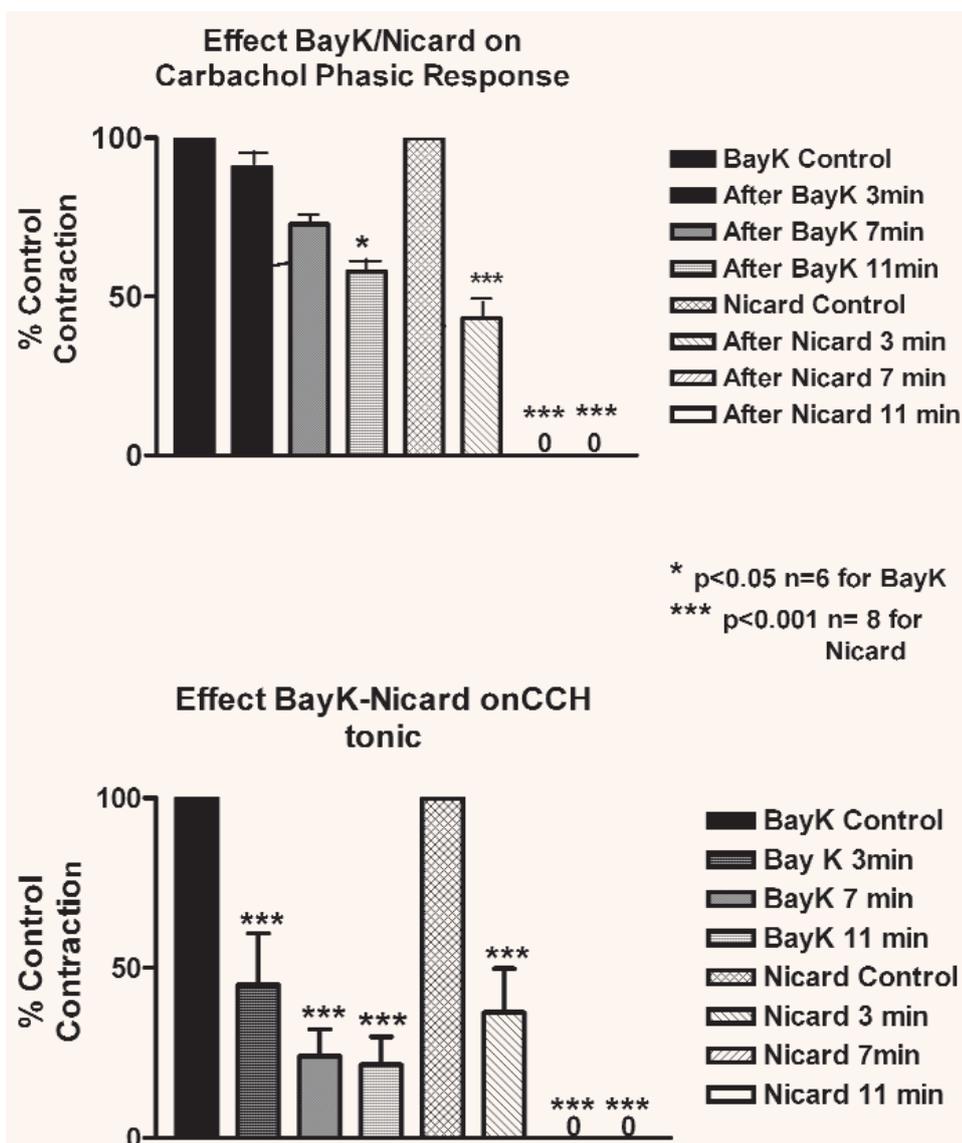
Fig. 6 After equilibration and block of nerves, tissues were contracted with 10 microMolar carbachol and left for 2 minutes. Phasic contractions were taken as the maximum amplitude reached in the first minute and the tonic responses was the tone after 2 minutes. Then all were placed in Ca^{2+} free Ringer with 0.1 mM EGTA with either BayK or nicardipine. After 3, 5 and 9 minutes carbachol was added again followed in each case by washes in 0.1 mM EGTA Ringer and restoration of their BayK or nicardipine. The results showed that opening VDCC with BayK preserved, while closing them with nicardipine abolished, carbachol contractions. This suggest that Ca^{2+} is restored to the SR by VDCC.

smooth muscle and ICC, it is not lost from the plasmalemma in caveolin 1 knockout [40–42]. There are several isoforms of the PMCA and the PMCA 4 isoforms that are important in smooth muscle [54–57]. In the absence of caveolins, PMCA remains, perhaps located in the lipid raft component. Whether PMCA is normally complexed to caveolin 1 is unknown as is also whether it's function is altered in the absence of caveolin 1. So far we lack the means to manipulate its activity pharmacologically. A selective inhibitor of the PMCA is urgently needed. Fulfilling this need may be complex owing to the presence of multiple isoforms of this Ca^{2+} pump, but recently a selective peptide inhibitor of PMCA4 has been reported [58]. So far,

no role for any PMCA has been suggested for calcium handling in ICC.

The Na^{+}-Ca^{2+} exchanger
NCX1, the Na^{+}-Ca^{2+} exchanger predominant in smooth muscle and ICC, also appears closely co-localized with caveolins 1 and 3 [37, 40–42]. However, it is reported to co-localize specifically with caveolin 3, but not caveolin 1, in cardiac muscle [59]. This cannot fully explain why, in caveolin 1 knockout mouse intestine, it persists in the plasma membrane. In outer circular muscle, caveolin 3 persists to some degree after caveolin 1 knockout, but NCX1 is present and nNOS is absent. Further, it persists in longitudinal muscle after both caveolin 1

Fig. 7 After equilibration and block of nerves, tissues were exposed to 0.1 mM EGTA in Ca^{2+} free Ringer for 3 minutes and then some were exposed to 10 microMolar carbachol for 2 minutes and then amplitudes and frequencies of contractions were measured at 3 minutes.
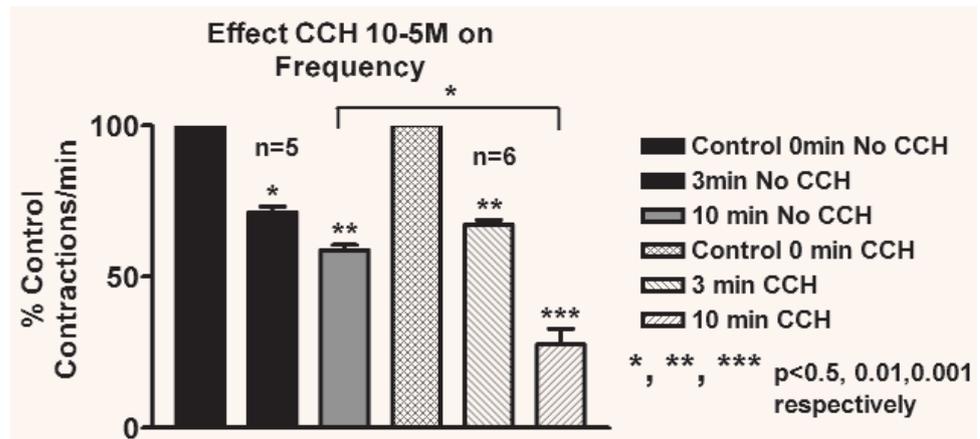
A subsequent wash in 0.1 mM EGTA in Ca^{2+} free Ringer was followed by a second exposure to carbachol for 2 minutes. After 5 minutes (10 in all). The second Carbachol exposure resulted in a significant decrease in frequency compared to tissues not exposed. These results suggest that depletion of ER Ca^{2+} stores inhibits recycling of calcium to stores in ICC to support pacing.

and 3 are lost. Whether its functions are regulated by caveolin 1 has not been tested in knockout animals. Some inhibitors of the NCX1 function are more selective than others. The selective ones include KB-R7943 and SEA0400 [60, 61]. These are more effective against the reverse mode of the exchange, driving Ca^{2+} into the cell, than against the forward mode [60]. KB-R7943 is poorly selective compared to SEA0400, having actions on NMDA channels, nicotinic receptors and store-operated Ca^{2+} entry.

However, SEA0400 has some non-specific actions as it still affects Ca^{2+} handling even in cardiac cells in which the exchanger has been knocked out [63]. This exchanger can also be manipulated by modifying the Na gradient which drives forward exchange [57] or the Ca^{2+} gradient which can drive reverse exchange [46].

In mouse intestine longitudinal muscle, SEA400 blocks the contraction induced by block of the SERCA pump with cyclopiazonic acid or thapsigargin [31, 32]. So far there is little information about effects of caveolin 1 knockout on functions of NCX1 in smooth muscle and no role for NCX1 has been suggested for calcium handling in ICC.

Caveolar Na^+/K^+-ATPase
Maintenance of the Na^+ gradient which drives forward mode Na^+-Ca^{2+} exchange, requires the activity of the sodium pump. Although there is no evidence so far from smooth muscle, there is evidence

in cardiac muscle that subunits of this pump are also associated with caveolae, where it binds and inactivates the tyrosine kinase, *src*, as well as IP_3 receptors [64–68]. Ouabain, which inhibits the sodium pump, activates *src* and brings about an increase in intracellular Ca^{2+} [64, 68]. The main caveolin in cardiac muscle is caveolin 3, so it is not clear that caveolin 1 in smooth muscle will also bind Na^+/K^+-ATPase. However, this seems likely owing to the structural similarity of caveolins 1 and 3. The existence of caveolar Na^+/K^+-ATPase raises the important possibility that there is a local Na^+ gradient as well as a local Ca^{2+} in the space between SR and caveolae. So far, no role for the Na^+/K^+-ATPase has been suggested for calcium handling in ICC or smooth muscle.

L-type Ca^{2+} channels
Voltage dependent Ca^{2+} channels appear partly, but not closely, co-localized with the caveolin 1 in intestinal smooth muscle and traches and persist in the plasma membrane after caveolin 1 knockout [37, 40, 41]. Despite this, these channels co-immuno-precipitated with caveolin 1 in membranes from the canine trachea [43]. Although VDCC are not necessary for pacing by ICC in the mouse intestine, their inhibition decreases pacing frequency and abolishes the frequency gradient (duodenum>jejunum>ileum) [31–33]. This frequency gradient persists in caveolin 1 knockout animals along with the L-type Ca^{2+} channel [32, 33].
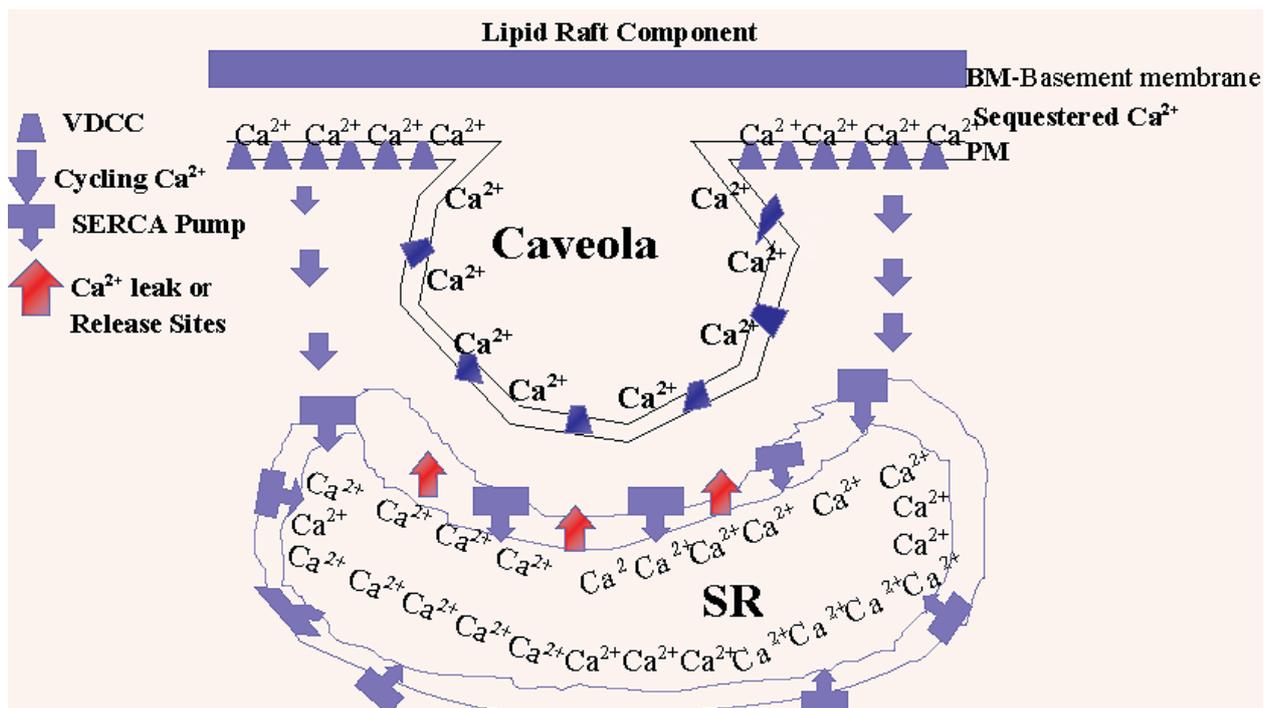
Fig. 8 A schematic cartoon of the Caveolar Hypothesis for Ca^{2+} Handling. This cartoon depicts the elements about which we have some evidence at the present time. L-type Ca^{2+} channels are localized in and near the caveolae. As demonstrated by van Breemen [32–34, 37], the peripheral sarcoplasmic reticulum near caveolae releases Ca^{2+} vectorially toward the caveolar when ryanodine receptors (not shown) are stimulated. Ca^{2+} in the space between caveolae and SR can be supplied from SR or from the caveolae/lipid raft, in the latter case by way of L-type Ca^{2+} channels. Calcium can be removed from the space between caveolae and SR to the caveolae/lipid raft by either the Na^+-Ca^{2+} exchanger (not shown) or the plasma membrane Ca^{2+} pump (not shown). It can be restored to the SR by the SERCA pump. Removal of extracellular Ca^{2+}, as in our experiments, demonstrated that the system can recyle Ca^{2+} efficiently between the caveolae/lipid raft membrane and the SR using L-type Ca^{2+} channels and IP mediated Ca^{2+} release from the SR (by carbachol). We have not so far demonstrated a role for Store-operated Channels in this complex, but it likely exists. It is obvious that more quantitative information about the structural relations is needed: the locations of various Ca handling proteins is needed as well as more quantitative information about the structures (*e.g.*, the extent to which SR surrounds caveolae and abuts the lipid raft). We have not incorporated mitochondria, which also participate in Ca^{2+} handling, but they also play a role and may be close both to caveolae and to SR..

As noted above, in canine LES, these channels seemed to have a special relationship to the SR, providing a mode of refilling before and after block of the SERCA pump. They also played a role in maintaining pacing frequency and contraction amplitudes of mouse intestinal segments when extracellular Ca^{2+} had been eliminated. They also helped maintain these activities even when the SERCA pump was blocked.

Non-specific cation channels

In ICC, a non-specific cation channel opened by Ca^{2+} depletion near the membrane, after uptake into mitochondria, is postulated to play an essential role

in the production of slow waves by ICC [69]. In a study of cell clusters from mouse intestine containing smooth muscle and ICC, oscillation of cell Ca^{2+} were observed in ICC after block of L-type Ca^{2+} channels [70]. These oscillations were linked to ICC slow waves, and blocked by thapsigargin and by SK&F 96365, a putative blocker of Store-operated cation channels. Although SK&F 96365 is also a known blocker of L-type Ca^{2+} channels, but these were already inhibited. Therefore SKF 96365 may have been acting on store-operated channels. Caveolae of ICC were also shown to have transient receptor potential-like channel 4 (TRP4), co-localized with caveolin 1. The TRP4 channel was sug-

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gested to be the non-specific cation channel. Thus Ca^{2+} handling by caveolae in ICC may be crucial to its pacing function. Whether or not the store-operated channels, the TRP4 channels and the cation channels opened by Ca^{2+} depletion are the same and related to caveolae of smooth muscle remains unclear [see Reference 72 for a recent review]. In some cells [71, 72], including endothelial cells, TRP channels appears to be associated with or depend on caveolin 1.

In rat caudal artery, cholesterol depletion with methyl-β-cyclodextrin disrupted caveolae and reduced caveolin 1 levels [73]. Cholesterol depletion also reduced receptors and responses to endothelin 1, 5-HT and vasopressin and these were restored by repletion of cholesterol. However, in cultured cerebral arterial smooth muscle, it reduced responses to endothelin1, not by affecting the receptors located in caveolae, but by reducing store operated Ca^{2+} entry, utilized by ET1. The expression of TRP1 in caveolae was also reduced [74]. Restoration of cholesterol restored the protein and its functions.

K^+ channels

BK_{Ca} channels respond to Ca^{2+} released locally from ryanodine channels in arterial and other smooth muscle during Ca^{2+} sparks and inhibit contraction. Caveolae appear to be sites where Ca^{2+} sparks occur in arterial muscle and cholesterol depletion inhibited the occurrence of Ca^{2+} sparks [76–78]. Although not closely associated -with caveolin 1 in intestinal muscle [40], BK_{Ca} channels appear associated and co-localized with caveolar structures in myometrium [79]. In caveolin 1 knockout mice cerebral arteries lacked caveolae and had Ca^{2+} spark frequency increased [78], but their regulation by voltage-dependent calcium channels was nearly abolished. BK_{Ca} channels were increased in density and this may have allowed Ca^{2+} sparks to activate BK_{Ca} channel current in caveolin 1 knockout arteries similarly to control arteries. The authors attributed the loss of regulation of Ca^{2+} sparks by voltage-dependent calcium channels to an increase in the distance between ryanodine and voltage-dependent calcium channels associated with the disappearance of caveolae [78]. In canine LES, NO released from myogenic nNOS modulated calcium entry by acting on BK_{Ca} channels [29, 30].

Type 1 IP3 Receptors

These have been identified by ultrastructural immunohistochemistry in ICC of smooth muscle. So far, its possible functions in Ca^{2+} handling are unknown [40, 80, 81].

Discussion and future directions

An obvious conclusion from the facts that multiple Ca^{2+} handling molecules are localized or associated with caveolin 1 and caveolae of smooth muscle and ICC is that they work together to mediate/regulate control of cell functions by calcium. These include contraction, relaxation, spark production, and likely Ca^{2+} waves in smooth muscle as well as pacing by ICC. We now have two methods to modify these relationships: caveolin 1 knockout and cholesterol depletion. Outcomes from use of these tools are not identical and more work is needed to elucidate the differences. It should be clear that these reductionist approaches alone will not suffice to clarify the working of caveolar complexes. After all, knockout of a protein also affects its roles in development and allows multiple compensatory responses. Also cholesterol depletion affects the structure and function of the lipid raft as well as the caveolar complexes. Biochemical techniques are available to isolate lipid raft and caveolar complexes and determine what molecules are or are not apparently associated with them and how these interactions affect activities. However, these suffer from the disruption of the structural arrangements between caveolar complexes and consequences of this disruption for ionic and metabolic gradients.

In order to understand fully the details of the organization of these molecules and how caveolin 1 interacts with them, additional approaches are needed. These include structural approaches to determine the locations of the Ca^{2+} handling molecules in the complex, especially those like the L-type Ca^{2+} channels, NCX1 and the PMCA, which are not lost when caveolin 1 is knocked out and are not exclusively associated with it. The use of ultrastructural immunocytochemistry may allow the locations of these molecules in or nearby caveolae to be determined. Structural studies of the relationships between caveolae and other cytosolic tethering and organizing molecules such as dystroglycan,

dystrophin, syntrophin are also needed. The altered distances between SR and PM lipid rafts when caveolae are abolished need study in order to estimate the consequences for their interactions. The ability to manipulate the activities of molecules associated with the caveolar complex pharmacologically is also essential. As indicated above, tools are available for many of these components, but not for the PMCA. Hopefully these will arrive soon.

Acknowledgments

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