Chapter 11
Calcium Mobilization via Intracellular Ion Channels, Store Organization and Mitochondria in Smooth Muscle

John G. McCarron, Susan Chalmers, Calum Wilson, and Mairi E. Sandison

Abstract In smooth muscle, Ca^{2+} release from the internal store into the cytoplasm occurs via inositol trisphosphate (IP_3R) and ryanodine receptors (RyR). The internal Ca^{2+} stores containing IP_3R and RyR may be arranged as multiple separate compartments with various IP_3R and RyR arrangements, or there may be a single structure containing both receptors. The existence of multiple stores is proposed to explain several physiological responses which include the progression of Ca^{2+} waves, graded Ca^{2+} release from the store and various local responses and sensitivities. We suggest that, rather than multiple stores, a single luminally-continuous store exists in which Ca^{2+} is in free diffusional equilibrium throughout. Regulation of Ca^{2+} release via IP_3R and RyR by the local Ca^{2+} concentration within the stores explains the apparent existence of multiple stores and physiological processes such as graded Ca^{2+} release and Ca^{2+} waves. Close positioning of IP_3R on the store with mitochondria or with receptors on the plasma membrane creates 'IP_3 junctions' to generate local responses on the luminally-continuous store.

Keywords Smooth muscle • Calcium signalling • Calcium stores • IP3 receptors • Ryanodine receptors • Quantal calcium release • Mitochondria

Introduction

Ca^{2+} regulates several smooth muscle functions including contraction, proliferation and the changes in muscle performance that accompanies disease [1]. The characteristics of the Ca^{2+} signal (e.g. the amplitude, duration, frequency and location) determine the nature of the biological response. A major Ca^{2+} source in smooth muscle is

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J.G. McCarron (✉) • S. Chalmers • C. Wilson • M.E. Sandison
Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, SIPBS Building, 161 Cathedral Street, Glasgow G4 0RE, UK
e-mail: john.mccarron@strath.ac.uk

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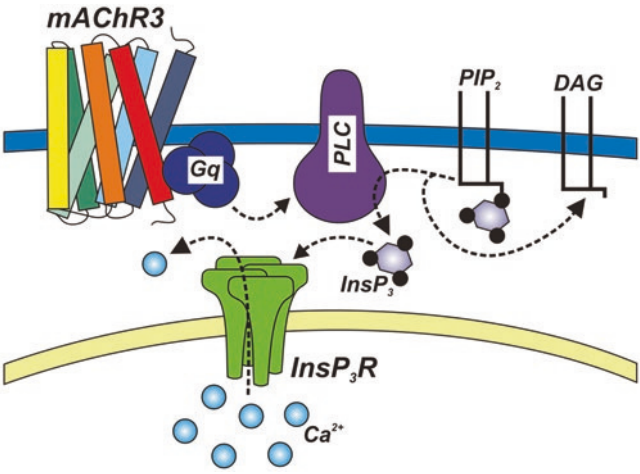

Fig. 11.1 Receptor activation and generation of IP_3 and Ca^{2+} release. Muscarinic receptors (mAChR3), phospholipase C (PLC) and IP_3R may be co-localized to create junctions in which IP_3 acts as a highly localized signal by being rapidly delivered to IP_3R. PIP2, phosphatidylinositol 4,5-bisphosphate; DAG diacylglycerol

an internal storage compartment which accumulates Ca^{2+} via sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCA). Ca^{2+} is released from the store into the cytoplasm via the ligand-gated channel/receptor complexes, the inositol trisphosphate (IP_3R) and ryanodine receptors. Release of Ca^{2+} via IP_3R is activated by IP_3 generated in response to many G-protein or tyrosine kinase-linked receptor activators including drugs (Fig. 11.1). RyR may be activated pharmacologically (e.g. caffeine), by Ca^{2+} influx from outside the cell in the process of Ca^{2+}-induced Ca^{2+} release (CICR), or when the stores's Ca^{2+} content exceeds normal physiological values, i.e. in 'store overload' [2–6]. Activation of either receptor allows diffusion of Ca^{2+} from the store to increase the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) from the resting value of ~100 nM to ~1 μM for many seconds throughout the cell and briefly (e.g. 100 ms) to much higher values (e.g. 50 μM) in small parts of the cytoplasm.

Physiological Functions Proposed to Be Explained by the Structure of the Store

The amplitude and duration of the Ca^{2+} signal depends on the quantity of Ca^{2+} available for release, which is determined in large part by the structural arrangement of the store. The store appears as an interconnected network of tubules [7] with a single lumen in which Ca^{2+} is in free diffusional equilibrium throughout (Fig. 11.2) [e.g. 8, 9]. However, considerable controversy persists about the stores structural and functional continuity or discontinuity. Rather than a store with a single lumen, multiple separate smaller Ca^{2+} storage units may exist (Fig. 11.2) [e.g. 7, 10–12]. Although the structure is unresolved, the arrangement of the store is proposed to account for several characteristics of Ca^{2+}

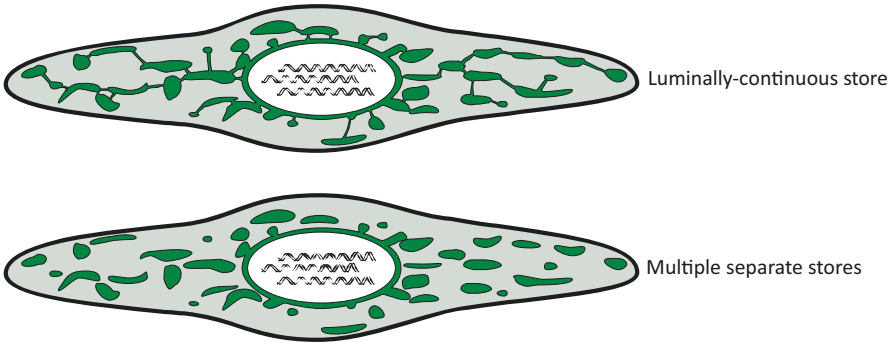

Fig. 11.2 Arrangement of the store. The store may be a single luminally-continuous structure with Ca^{2+}in free diffusional equilibrium throughout (*top*) or a series of multiple separate elements (*bottom*)

signals, such as the graded concentration-dependence of IP_3-mediated Ca^{2+} release, the variation in sensitivity in different parts of the cell to generate local responses and the progression of Ca^{2+} signals through the cell. For example, while Ca^{2+} entry via voltage-dependent Ca^{2+} channels generates quite uniform rises in Ca^{2+} (Fig. 11.3; [13, 14]), Ca^{2+} release from internal stores may generate complex patterns, such as travelling spatial gradients of Ca^{2+} ('Ca^{2+} waves'; Fig. 11.3). For Ca^{2+} waves to progress through the cell, sequential activation of IP_3R [13], by Ca^{2+} itself, occur in a repeating positive feedback CICR-like process [15, 16], i.e. Ca^{2+} release from one IP_3R activates neighbouring receptors to progress the wave. An explanation put forward to explain wave movement, rather than there being a persistent Ca^{2+} release at one site on the cell, is that store is arranged as several stores along the length of the cell, each with a limited amount of Ca^{2+}. Each store is activated and depleted in turn (Fig. 11.4a).

A discontinuous structure of the store has also been proposed to explain the graded IP_3 concentration-dependent Ca^{2+} release process [17, 18]. Low concentrations of IP_3 release only part of the overall available Ca^{2+} content of the store [17, 19–22]. As the IP_3 concentration increases, a further release of Ca^{2+} occurs [reviewed 23]. Such a graded release seems incompatible with the positive feedback CICR-like facility at IP_3R [24], which would be anticipated to fully deplete the store when activated. To explain graded Ca^{2+} release, the store has been proposed to assemble in multiple separate units, each endowed with a finite Ca^{2+} storage capacity and sensitivity to IP_3 (Fig. 11.4b). At any given concentration of IP_3 only some stores will be activated to release Ca^{2+} [17, 18, 25] (Fig. 11.4b). This same feature of the store may also explain the reported variations in sensitivity different parts of the cell to IP_3 [19, 26, 27].

Structure of the Ca^{2+} Stores

There are several different RyR and IP_3R arrangements which may exist on each of the proposed separate stores to explain the various experimental observations. Indeed, the Ca^{2+} stores have been classified on the arrangement of IP_3R and RyR and proposals for one, two, or more, stores with a variety of complex receptor

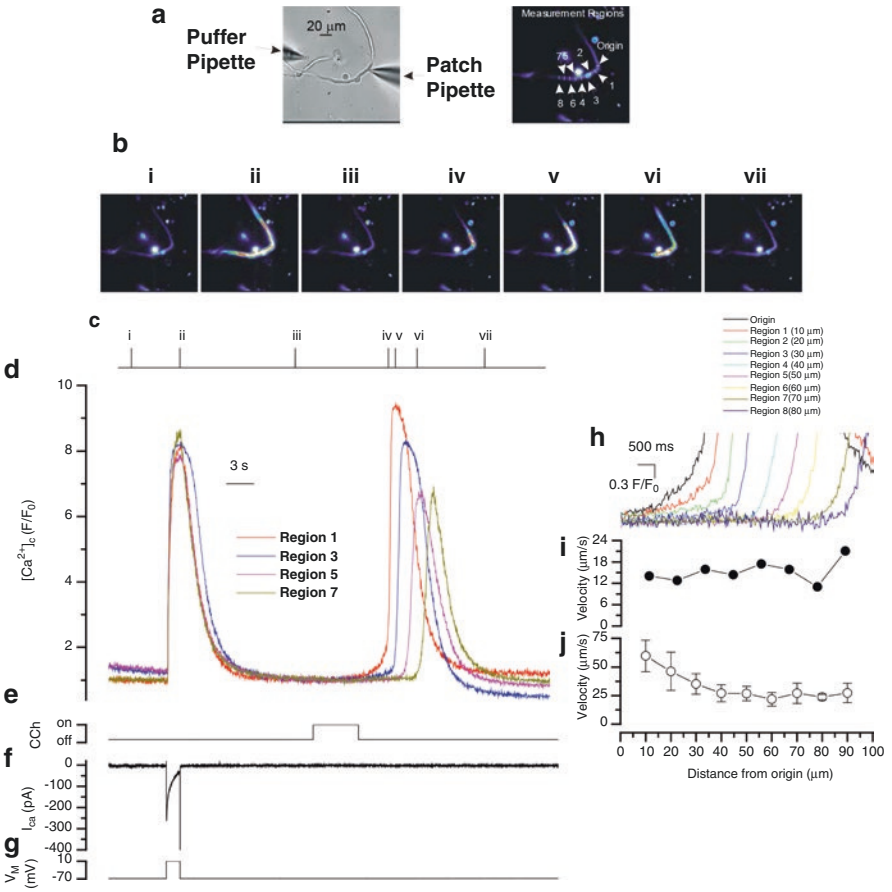

Fig. 11.3 Depolarization and IP_3-evoked increases in $[Ca^{2+}]_c$. Depolarization (−70 mV to +10 mV; **g**), activated a voltage-dependent Ca^{2+} current (I_{Ca}; **f**) to evoke a relatively uniform rise in $[Ca^{2+}]_c$ (**b**, **d**). In contrast, $[Ca^{2+}]_c$ increases in response to and IP_3-generating agonist began in one part of the cell and progressed from that site (**b**, **d** and expanded time base **h**). The $[Ca^{2+}]_c$ images (**b**) are derived from the time points indicated by the corresponding *numerals* in **c**. $[Ca^{2+}]_c$ changes in **b** are represented by *colour*; *blue* low and *red*/*white* high $[Ca^{2+}]_c$. Changes in the fluorescence ratio with time (**d**, **h**) are derived from 1 pixel lines ('origin' and regions 1–8 in **a**, *right* panel; drawn at a 3 pixel width to facilitate visualization). (**a**) *Left* panel shows a bright field image of the cell; see also whole cell electrode (*right* side) and puffer pipette containing agonist (*left* side). The velocity of wave progression is shown in **i** for the data presented in (**d**, **h**). Summarized velocity data is presented (**j** n = 5). From McCarron et al. 2010 [13] with permission

arrangements have been made (Fig. 11.5). There may be multiple stores each containing both IP_3R and RyR [28–32], or there may be stores which contain only RyR and separate stores only IP_3R [12, 28, 32–34] (e.g. basilar mesenteric or pulmonary arteries; Fig. 11.5i, ii). In other studies, there may be Ca^{2+} stores containing IP_3R and RyR together on some stores along with other separate stores in the same cell with either IP_3R alone (e.g. pulmonary artery and aorta [29, 35]; Fig. 11.5iii) or RyR

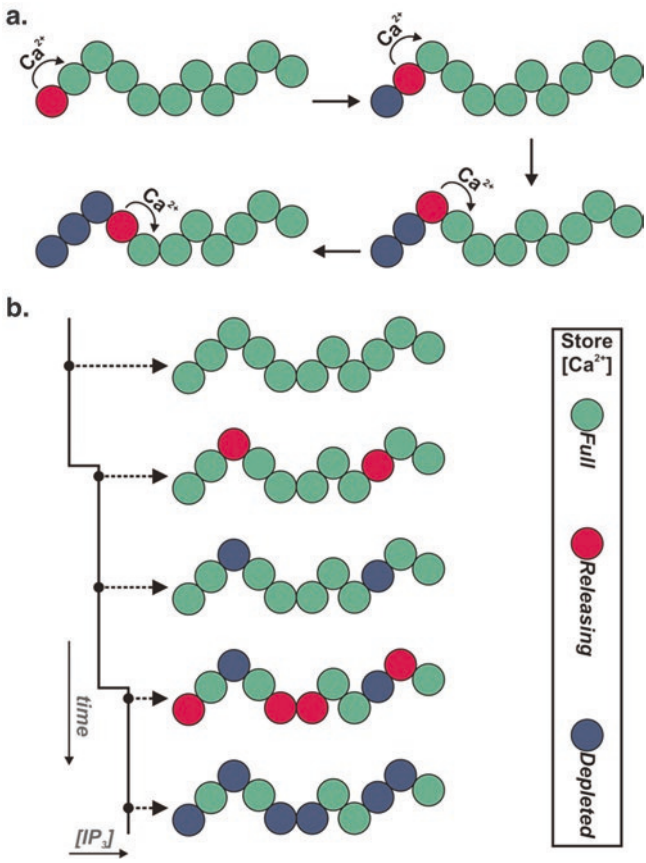

Fig. 11.4 Wave progression and store arrangement. (**a**) The store may function as a series of discontinuous compartments that are activated and depleted in turn to explain wave progression. (**b**) Separate stores with various sensitivities to IP_3 are activated and depleted as the IP_3 concentration (*left*-side) increases

alone (e.g. mesenteric artery [30]; Fig. 11.5iv). Stores have also been differentiated by their sensitivity to the SERCA pump inhibitors cyclopiazonic acid (CPA) and thapsigargin. In A7r5 cells (a cell line derived from thoracic aorta tissue) there are stores containing RyR that are insensitive to thapsigargin and separate stores in the same cells (also with RyR) that are sensitive to thapsigargin [12]. In an alternative proposal for store arrangement in A7r5 cells, a thapsigargin-insensitive store with IP_3R but not RyR may exist [36]. In murine bladder smooth muscle, three types of Ca^{2+} store are proposed: two sensitive to thapsigargin, one with IP_3R and one without, and a third store insensitive to IP_3 and thapsigargin [37]. In tracheal myocytes three types of Ca^{2+} stores are proposed which were refilled by different pathways. Ca^{2+} influx through voltage-dependent Ca^{2+} channels and CPA sensitive pumps refilled 80 % of the IP_3R-containing stores. The remaining 20 % were not refilled by CPA-sensitive pumps or Ca^{2+} influx through voltage-dependent Ca^{2+} channels and

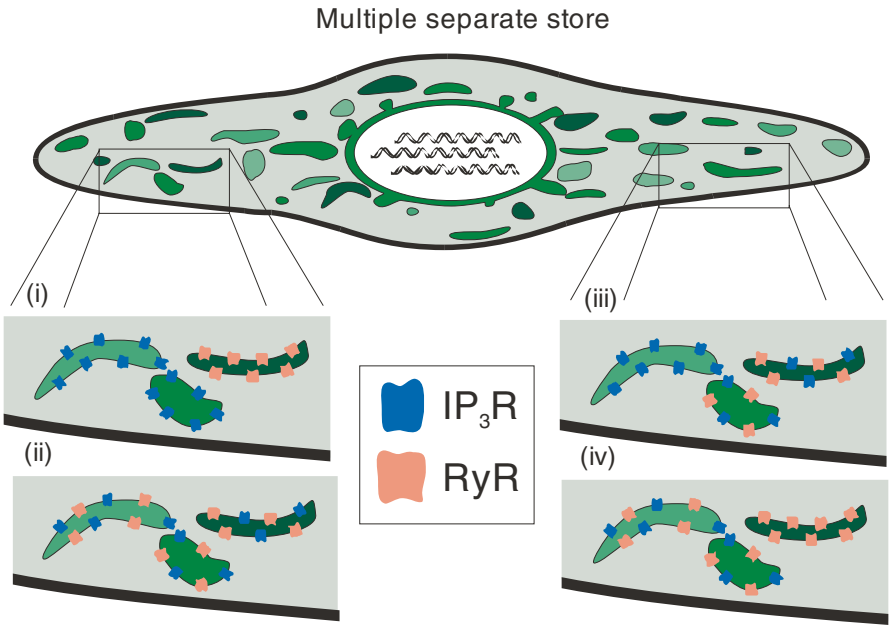

Fig. 11.5 Arrangement of RyR and IP_3R on the store(s). There may be store with RyR (*blue*) alone or IP_3R (*red*) alone (i), or stores with both receptors (ii) or a combination of the two (iii, iv). Although the cartoon shows the different proposed store receptor arrangements in the same cell, the proposed stores have been described for different cell types

neither was the RyR-containing store. Instead, thapsigargin depleted the CPA/voltage-dependent Ca^{2+} channels insensitive IP_3R store fully and the RyR store by more than 50 % [38]. These differences in refilling mechanisms of the stores are proposed to demonstrate pharmacologically distinct Ca^{2+} stores which play an important role in the generation of Ca^{2+}signals in airway smooth muscle cells [38].

Thus, data from various functional studies suggest there may be structural discontinuities in the store and that different types of receptor arrangements on those stores exist. Proposals for stores which contain only IP_3R or RyR exist as do proposals for stores with RyR and IP_3R together and in combination with additional separate stores in the same cells containing only either IP_3R or RyR. The questions arise, why is such a diversity of stores and receptor arrangement required and do functional experiments unambiguously reveal structural discontinuities in the store?

Methods Used to Investigate Stores May Create the Appearance of Multiple Stores

It could be the case that the experimental conditions used to investigate the stores may contribute to the diversity of proposals on arrangement. In native cells, methods for studying Ca^{2+} store subcompartments are limited. The main experimental

approach is to define the structural organisation of the Ca^{2+} stores from functional (Ca^{2+} response) data. To do this, the store is depleted typically via one receptor (RyR or IP_3R) by repeated activation with a *single* concentration of either IP_3 or caffeine under conditions which prevent store refilling with Ca^{2+}. After depletion via one receptor (e.g. RyR), whether or not Ca^{2+} is available to be released via the other receptor (e.g. IP_3R) is then determined. If depletion via one receptor abolishes Ca^{2+} release from the other, the receptors are suggested to be co-localized on a single store and access a common Ca^{2+} source. However, if depletion of the stores from one receptor leaves the other receptor's response largely unaffected, the two channels are suggested to be localized on different stores. With this approach, some investigations (e.g. on portal vein and pulmonary artery) have shown a single store containing both RyR and IP_3R, since depletion of the Ca^{2+} store by caffeine (which activates RyR) prevented IP_3-mediated Ca^{2+} release [31, 32, 39, 40]. On the other hand, other studies on pulmonary artery have suggested there may be separate stores for each receptor since depletion of the RyR-containing store did not abolish agonist-evoked IP_3-mediated Ca^{2+} release and *vice versa* [41]. In yet other studies (e.g. portal vein, pulmonary artery and taenia caeci), one store may express RyR and IP_3R and other stores, in the same cell, only IP_3R [11, 35, 42]. This conclusion came from the finding that depletion of the IP_3R-containing store abolished Ca^{2+} release via RyR, while depletion of the RyR-containing store did not abolish Ca^{2+} release via IP_3R. In further studies in other cell types (mesenteric artery) and in our own investigations in colonic smooth muscle [43], some stores may express both RyR and IP_3R while others only RyR [30, 43]. In this case, depletion of the RyR-containing store abolished Ca^{2+} release via IP_3R, while depletion of the IP_3R-containing store did not abolish Ca^{2+} release via RyR—a result apparently consistent with there being a store which contained RyR alone.

However, in our own later experiments examining the structure of the store [44–46] we found unexpectedly that the entire store appeared to be a single luminally-continuous entity rather than a series of separate stores. In these later experiments, to examine luminal continuity, the store was depleted at *one small site* in the cell by repetitively applying IP_3 to a small (10 μm) region under conditions preventing store refilling. Even though only a small site in the cell was activated, the store depleted throughout the cell [44]. This result suggested that Ca^{2+} was in free diffusional equilibrium in the store (Fig. 11.6) i.e. a luminally-continuous store. In keeping with these findings, the IP_3-sensitive store also could be refilled from one small site on the cell (Fig. 11.7); a result suggesting there was a single store in which Ca^{2+} was able to diffuse freely throughout.

Depletion of the RyR-sensitive store at one site also depleted the entire store [44, 46]. In this case the RyR-containing store was depleted by attaching a pipette containing ryanodine to one small site of the cell to deplete the store there. Caffeine was applied to the entire cell. If the RyR containing store comprised separate elements, depletion of one aspect of the store should not affect the Ca^{2+} available to be released in another area of the store. However, caffeine-evoked Ca^{2+} transients decreased uniformly throughout the cell [44, 46] suggesting that ryanodine, acting at one part of the cell, had depleted the entire store i.e. a single luminally-continuous store exists.

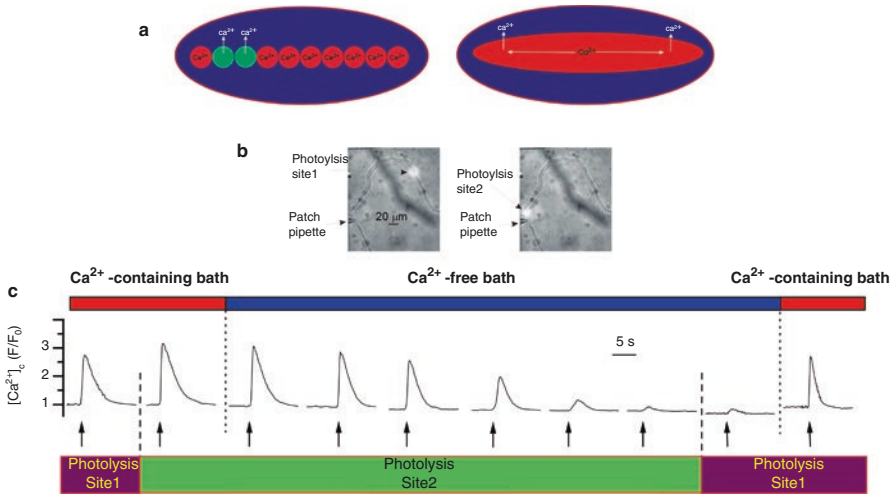

Fig. 11.6 Store luminal continuity:depletion of the IP_3-sensitive Ca^{2+} store in a localized area depletes the entire store of Ca^{2+}. (**a**) If the store was a series of luminal discontinuous elements (*left*) then Ca^{2+} release at one site would not alter the Ca^{2+} available for release from another. However if the store was luminally continuous, then Ca^{2+} release from one site would decrease the Ca^{2+} available for release from another site. To test this, at −70 mV, locally-photolyzed IP_3 (↑, **c**) in a 10 μm diameter region, (photolysis site 1; *bright spot* in **b** *left-hand* panel; see also patch electrode, *left* side) evoked Ca^{2+} transients (**c**). Results from photolysis site 1 are indicated by the *magenta bar* below the $[Ca^{2+}]_c$ trace in **c**. When repositioned to photolysis site 2 (**b**; *right hand* panel) subsequent photolysis ~90 s later produced a $[Ca^{2+}]_c$ increase (**c**). Photolysis site 2 is indicated by the *green line* below the $[Ca^{2+}]_c$ trace (**c**). In a Ca^{2+} free solution (containing EGTA (1 mM) and $MgCl_2$ (3 mM); *blue bar* above the $[Ca^{2+}]_c$ trace) the $[Ca^{2+}]_c$ increase evoked by IP_3 at photolysis site 2 declined in amplitude as the store was depleted of Ca^{2+} (**c**). When the store content had been substantially reduced at photolysis site 2 (**b**) (as revealed by the smaller Ca^{2+} transients **c**) IP_3 was liberated by photolysis at site 1 (**b**). Again as at photolysis site 2 the response was now almost abolished compared to control. On restoring external Ca^{2+} (**c**, *right hand* side) the Ca^{2+} increase evoked by IP_3 at photolysis site 1 was restored towards control values. These results suggest that the SR is luminally-continuous and within it Ca^{2+} is freely diffusible. $[Ca^{2+}]_c$ measurements were made from a 5 μm diameter circle at the photolysis site. Thus when photolysis occurred at photolysis site 1 $[Ca^{2+}]_c$, measurements were made from a 5 μm diameter circle at the photolysis site 1. When photolysis occurred at photolysis site 2, $[Ca^{2+}]_c$ measurements were made from a 5 μm diameter circle at the photolysis site 2. (**b**, **c**) These results were original published in McCarron & Olson 2008 [44]

The question of whether there is a single store with luminal continuity or multiple stores has also been addressed in other cell types (HeLa, RBL, CHO) using a Ca^{2+} store-located green fluorescent protein (GFP) [47, 48]. Prolonged GFP photobleaching in a small restricted region of the cell resulted in the disappearance of fluorescence throughout store, suggesting GFP could move freely around the store to be eventually photobleached. Short periods of photobleaching were followed by a rapid restoration of fluorescence by the diffusion of GFP from sites neighbouring the photobleached region [47, 49]. A single store with luminal continuity throughout was also suggested by the diffusion of Ca^{2+} in pancreatic acinar cells [8].

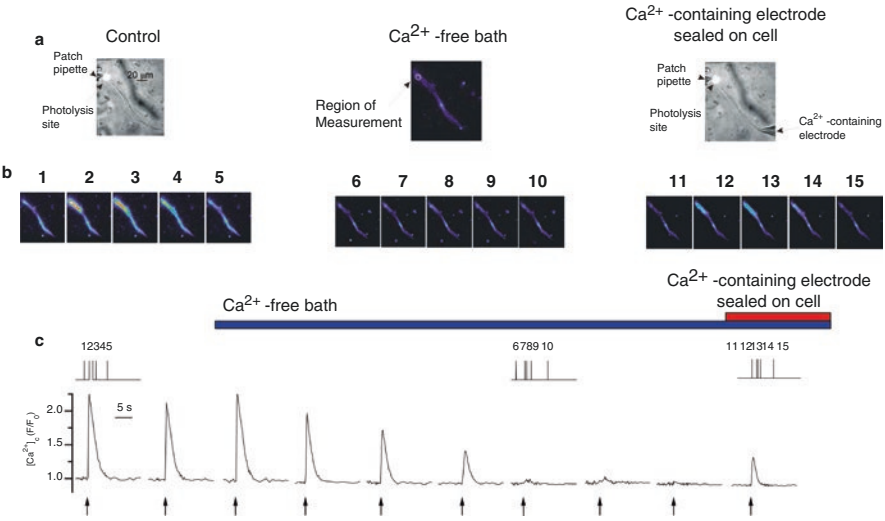

Fig. 11.7 Ca^{2+} can move through the SR to replenish a site previously depleted of the ion. At −70 mV, locally-photolyzed IP_3 (↑, **c**) in a 10 μm diameter region (*bright spot* in **a** *left-hand* panel; see also whole cell patch electrode (*left* side)) increased $[Ca^{2+}]_c$ (**b** and **c**). The $[Ca^{2+}]_c$ images (**b**) are derived from the time points indicated by the corresponding numbers in C. $[Ca^{2+}]_c$ changes in **b** are represented by colour; blue low and red high $[Ca^{2+}]_c$ A second photolysis of IP_3 ~ 60 s later at the same site (**c**) generated an approximately comparable $[Ca^{2+}]_c$ increase. In a Ca^{2+} free solution (containing 1 mM EGTA and 3 mM $MgCl_2$; *blue bar above* the trace) the $[Ca^{2+}]_c$ increase evoked by IP_3 declined and was abolished as the store became depleted of Ca^{2+}. When the Ca^{2+} containing patch electrode was subsequently sealed onto the cell in 'cell-attached' mode (**a** *right hand* panel; **c** *red bar*) there was no measurable increase in $[Ca^{2+}]_c$ yet the Ca^{2+} increase to IP_3 at the photolysis region (**a**) was subsequently restored partially (**c**). This result suggests that Ca^{2+} had diffused through the store lumen to replenish the store. The position of the region of $[Ca^{2+}]_c$ measurement is shown as a *white circle* in **a**, *center* panel. These results were original published in McCarron & Olson 2008 [44]

The Ca^{2+} store in the apical region was refilled with Ca^{2+} originating from a pipette attached to the opposite side of the cell on the basolateral membrane [see also 9]. Together, these experiments suggest the store is a luminally-continuous entity in which Ca^{2+} can diffuse freely throughout. How then does the appearance of multiple stores [43] occur on a single luminally-continuous store structure?

Complex RyR and IP_3R Regulation Characteristics and Apparent Store Configuration

IP_3R and RyR are each regulated by the Ca^{2+}concentration within the lumen of the store ('luminal Ca^{2+} regulation') [4, 45]. As the luminal Ca^{2+} concentration increases so does the activity of the store release channels [3–6]. Conversely, the activity of

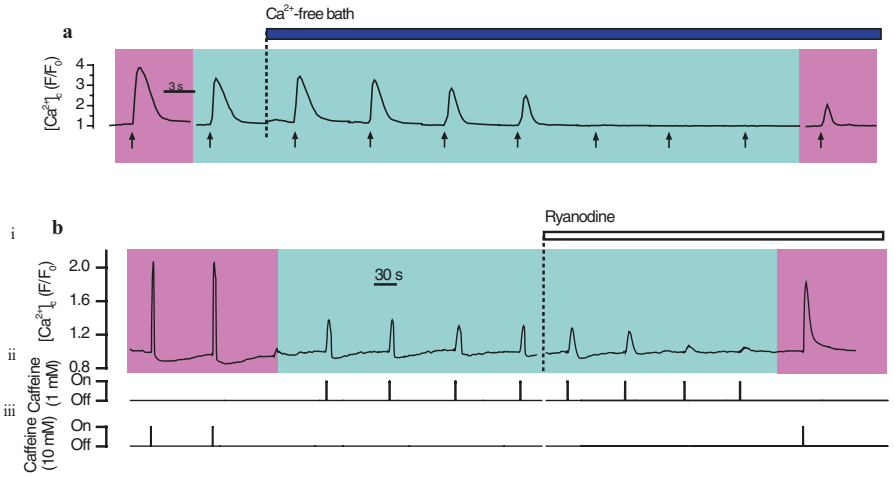

Fig. 11.8 The store may contain substantial residual Ca^{2+}after apparently being depleted. (**a**) At −70 mV high $[IP_3]$ (*pink*; photolysed using a high lamp intensity; ↑) increased $[Ca^{2+}]_c$. A lower $[IP_3]$ (*light blue*; ↑) evoked a submaximal $[Ca^{2+}]_c$ increase. In a Ca^{2+} free bath solution (containing 1 mM EGTA and 3 mM $MgCl_2$; *dark blue bar*) these increases declined then disappeared. The absence of a response to $[IP_3]$ was not due to depletion of the store. Increasing $[IP_3]$ (*pink*; *right side*; ↑) evoked further Ca^{2+} release. A mechanism, other than depletion of the store of Ca^{2+}, e.g. 'luminal' regulation of IP_3R, may have accounted for the loss of response to IP_3. The time between each IP_3 challenge was approximately 1 min. (**b**) Caffeine (10 mM; iii) indicated by *pink* (i) evoked approximately reproducible increases in $[Ca^{2+}]_c$ (i). Caffeine (1 mM; ii) indicated by *light blue* (i) evoked submaximal $[Ca^{2+}]_c$ increases (i). In ryanodine (50 μM; for the duration of the unfilled bar) these increases declined to 12 % of their control value (i). However, after the substantial reduction in response to submaximal caffeine (1 mM; ii), caffeine (10 mM; iii) evoked a $[Ca^{2+}]_c$ rise of 77 % of its control value. The break in the record is ~90 s in which a new data recording file was established. These results were original published in McCarron & Olson 2008 [44]

RyR and IP_3R each decrease as the store Ca^{2+} content declines. Ca^{2+} release evoked by IP_3 or caffeine may substantially decline or stop as the store content falls, even when this store retains a significant residual quantity of Ca^{2+}. To examine this possibility, a series of experiments were carried out in which the store was depleted of Ca^{2+} (Fig. 11.8). When the store had been 'depleted', as revealed by the inhibition of response to IP_3 or caffeine, the concentration of each activator was increased and a substantial Ca^{2+} release occurred [44]. These experiments suggest that after apparent depletion the store retained significant quantities of Ca^{2+} and that residual Ca^{2+} is available for release with increased concentrations of IP_3 or caffeine.

Interpreting the amplitude of a Ca^{2+} response to a *single* repeatedly applied concentration of either IP_3 or caffeine as the store content declines is problematic as the amplitude of the response depends (1) on the position of the activator concentration on the concentration-response relationship curve and (2) the store luminal Ca^{2+} concentration. The absence of a response to a single concentration of IP_3 or caffeine, therefore, may not reflect an absence of available Ca^{2+} within the store but rather

termination of channel activity by luminal regulation of the store release channels as the store Ca^{2+} content declines.

Luminal regulation may explain the appearance of multiple stores when pharmacological agents and functional data are used to define store subcompartments. Indeed, we reproduced data previously interpreted as various different store arrangements in a single smooth muscle cell type. For example, after depletion of the Ca^{2+} stores with caffeine and ryanodine, the response to IP_3 disappeared (Fig. 11.9a). This result suggest RyR and IP_3R access a single Ca^{2+} pool. However, in the same cell type, after depletion of the Ca^{2+} stores with caffeine and ryanodine, when a higher concentration of IP_3 (125 μM vs. 250 μM) was subsequently applied, a substantial Ca^{2+} increase occurred (Fig. 11.9b). This result suggests IP_3R accesses a different Ca^{2+} pool from RyR. On the other hand, after the store had been apparently depleted of Ca^{2+} by IP_3 (at a concentration which produced a maximal response) a substantial response to caffeine persisted (Fig. 11.9c), suggesting there was a store which only contains RyR [30, 43]. In yet other experiments, in the same cell type, when the concentration of IP_3 used to deplete the store of Ca^{2+} was increased, no Ca^{2+} response to caffeine occurred i.e. the apparently separate stores for RyR disappeared (Fig. 11.9d).

Rather than there being various separate stores with different receptor arrangements, these results suggests that partial depletion of the store terminates activity of the channels by luminal channel regulation by $[Ca^{2+}]$ within the store.

These results (Figs. 11.6, 11.8, 11.9) do not dispute the existence of multiple stores but suggest that care is required when interpreting results from functional data in terms of store structure. In some cells, multiple stores do exist unequivocally. Different Ca^{2+}concentrations have been measured in various regions of the store using recombinant aequorin [47], electron microscopic determination of Ca^{2+} content [50] or fluorescent indicators loaded into the cell [34], suggesting that discontinuities exist within the structures surrounding the lumen itself. The store [34] may adopt different configurations within the cell and components may even detach and reattach, so influencing the pattern and distribution of Ca^{2+} release channel [51]. In Purkinje neurons, for example, IP_3R-expressing regions may separate off from other internal store elements [52]. Store compartments exist which accumulate and release Ca^{2+} but are luminally-discontinuous from the bulk of the store have been observed in cultured hippocampal dendrites [53]. Life cycle stage or prior experimental conditions of the cell may influence the appearance of subcompartments. $[Ca^{2+}]_c$ increases which persisted for at least 10 min, led to the breakdown of the Ca^{2+} store into subcompartments in rat basophilic leukaemia cells [49]. Store structural changes are also associated with fertilization and mitosis [54]. Fertilization leads to a reorganization of the store, measured as a slowing of the diffusion of membrane probes and luminal proteins, in sea urchin eggs [55, 56]. In mitosis, significant Ca^{2+} store changes also occur, which include the structure itself fragmenting into subcompartments [57, 58].

Other structures within the cell such as Golgi, mitochondria, granules and the nucleus may also contribute to Ca^{2+} storage [59–63] and generate subregions which appear to have various Ca^{2+} concentrations, especially when lipophilic Ca^{2+} indicators are used to image the distribution of $[Ca^{2+}]$ through the cell.

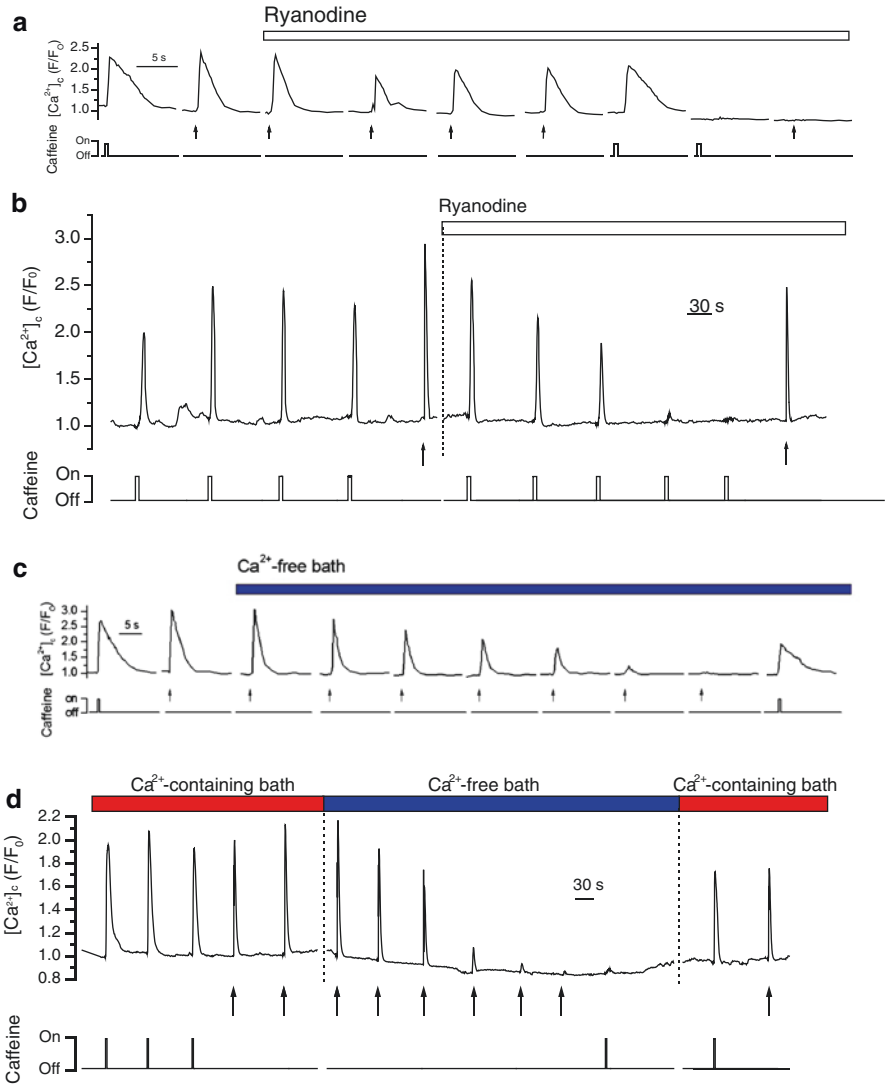

Fig. 11.9 Various apparent SR receptor arrangements. All the following experiments were performed on the same cell type (colonic smooth muscle) (**a**) *IP_3R and RyR access a single Ca^{2+}pool*. Caffeine (10 mM by pressure ejection lower trace) evoked a rise in Ca^{2+}. IP_3-evoked Ca^{2+} increases (125 μM; ↑) were not significantly reduced by ryanodine (50 μM; *open bar* above the trace). Activation of RyR by caffeine (10 mM), in the continued presence of ryanodine, initially increased $[Ca^{2+}]_c$. A second application of caffeine to the same cell however some 90 s later, generated little increase in $[Ca^{2+}]_c$ presumably because of SR store depletion; ryanodine's effects on RyR require prior channel activation. The IP_3 response was also subsequently inhibited (↑). Because the IP_3-evoked Ca^{2+} transient was not blocked by ryanodine alone (only after RyR activation with caffeine), IP_3-mediated Ca^{2+} release did not activate RyR. IP_3R and RyR may share a common Ca^{2+} store; this is depleted of Ca^{2+} by ryanodine, after activation of RyR by caffeine, to reduce the Ca^{2+} available for IP_3-mediated Ca^{2+} release to occur. (**b**) *IP_3R accesses a separate Ca^{2+}pool from RyR*.

Graded Ca^{2+} Release, Ca^{2+} Waves and Local Ca^{2+} Events from a Luminally-Continuous Store

If the Ca^{2+} store in smooth muscle is indeed a single, luminally-continuous entity, how do the various physiological events (waves, graded release, local responses) previously explained with multiple separate stores occur?

Ca2+waves: Ca^{2+}waves are the progressive movement of Ca^{2+} through the cell following Ca^{2+} release from the internal store. Using localized activation of IP_3R, the forward movement of the Ca^{2+} wave was shown to arise from CICR at the IP_3R [13, 16]. The decline in $[Ca^{2+}]_c$—the back of the wave—occurred not because of depletion of separate stores but from a functional compartmentalization of the store which rendered the site of IP_3-mediated Ca^{2+} release—and only this site—refractory to IP_3 after Ca^{2+} release . A localized feedback deactivation of IP_3R produced by an increased $[Ca^{2+}]_c$ caused the functional compartmentalization [16]. The deactivation of the IP_3R was delayed in onset, compared with the time of the rise in $[Ca^{2+}]_c$ and persisted (>30 s) even when $[Ca^{2+}]_c$ had been restored to resting levels [13, 16]. This feedback deactivation ensures the wave's progressive movement in a single direction [16].

Graded Ca2+release: There are several proposals for graded IP_3-mediated Ca^{2+} release that do not require the presence of numerous stores with various sensitivities to IP_3. Rather, at any given $[IP_3]$ the entire Ca^{2+} store is activated and releases a fraction of its content, becoming partially depleted. Partial depletion may deactivate Ca^{2+} release [64, 65]. Raising the $[IP_3]$ reactivates IP_3R to renew the Ca^{2+} release process. This proposal does not require multiple stores but a complex adaptive change in IP_3R activity. Negative feedback processes operating either at the cytoplasmic or the luminal aspects of IP_3R may explain the adaptive behaviour. In one proposal the binding of IP_3 to IP_3R may initially activate, then partially inactivate IP_3R in a concentration-dependent way to produce graded Ca^{2+} release [66–68]. To test this proposal we examined the time course of IP_3R activation at a constant $[IP_3]$ but under conditions in which there was varying amplitude of Ca^{2+} release [45]. The latter was achieved by buffering the cytoplasmic Ca^{2+} concentration (BAPTA) or partial depletion of the store (Ca^{2+} free bath solution). If IP_3 inactivated IP_3R to prevent release, then at

Fig. 11.9 (continued) Caffeine (1 mM; by pressure ejection, lower trace) evoked approximately reproducible increases in $[Ca^{2+}]_c$. IP_3 (250 μM; ↑) also increased $[Ca^{2+}]_c$. Ryanodine (50 μM; *open bar*) inhibited caffeine-evoked $[Ca^{2+}]_c$ increases by depletion of the SR. After the apparent depletion of caffeine-sensitive Ca^{2+} store, IP_3-evoked a substantial $[Ca^{2+}]_c$ increase (in contrast to the results in **a**).(**c**) *RyR accesses a different Ca^{2+}pool from IP_3R*. Caffeine (10 mM) and photolyzed IP_3 (↑) increased $[Ca^{2+}]_c$. In a Ca^{2+} free solution (containing 1 mM EGTA and 3 mM $MgCl_2$; *blue bar* above the trace) the IP_3-evoked Ca^{2+} transient decrease as the store was depleted of Ca^{2+}. Following depletion of the IP_3-sensitive store, caffeine evoked a substantial Ca^{2+} transient. (**d**) *RyR and IP_3R access a single Ca^{2+}pool*. Caffeine (2 mM) and IP_3 (125 μM) each evoked approximately reproducible increases in $[Ca^{2+}]_c$. Removal of external Ca^{2+} (and addition of 1 mM EGTA and 3 mM $MgCl_2$; *blue bar*) reduced the IP_3-evoked Ca^{2+} transient. Following depletion of the IP_3-sensitive store, the caffeine-evoked $[Ca^{2+}]_c$ transient was inhibited (in contrast to the results in **c**). Reintroduction of Ca^{2+} (*red bar*) restored the IP_3- and caffeine-evoked Ca^{2+} transients towards control values. These results were original published in McCarron & Olson 2008 [44]

constant [IP_3], release should stop at approximately the same time regardless of the amplitude of the $[Ca^{2+}]_c$ rise. However, as the amplitude of the $[Ca^{2+}]_c$ rise declined (in either BAPTA or in Ca^{2+}-free solution) the time course of release became more prolonged [45]. This result suggests that mechanisms other than IP_3 inactivation of IP_3R would appear responsible for terminating IP_3-mediated Ca^{2+} release.

In another proposal, the sensitivity of IP_3R to IP_3 is controlled by the luminal $[Ca^{2+}]$ so that as the concentration of the ion within the store lumen falls so does IP_3R activity [e.g. 65, 69]. For example, decreasing the store $[Ca^{2+}]$ to below 80 % of the steady-state level abolished IP_3-mediated Ca^{2+}release in rat uterine myoctes [70] [see also 65, 69]. However, it is unclear whether or not the control of IP_3R activity by luminal Ca^{2+} operates over the store's physiological Ca^{2+} concentration range. The threshold for luminal regulation to begin altering the activity of IP_3R is depletion of the store by >70 % of the steady-state luminal Ca^{2+} concentration (500–600 μM; [71]) in HeLa cells. The store $[Ca^{2+}]$ must also be substantially depleted in hepatocytes (>45 or 95 %) [72, 73] and in A7r5 cells by >70 % [74] before IP_3R sensitivity changes are detected. In each case, control of IP_3R activity by Ca^{2+} binding to the luminal aspect of the receptor, is unlikely to explain 'quantal' Ca^{2+} release when store $[Ca^{2+}]$ exceeds 55, 5, or 30 % of the normal steady-state value respectively in these cells [72–74].

On the other hand, IP_3R might not be controlled by luminal Ca^{2+} at all. Single channel IP_3R activity, measured in planar lipid bilayers, *increased* when the $[Ca^{2+}]$ at the luminal aspect of the channel declined [75]. In the latter study a luminal $[Ca^{2+}]$ exceeding 1 mM inhibited IP_3R activity [75] (see also [76]). In other studies in permeabilized cells (e.g. portal vein; [18] or hepatocytes; [77]), decreases in store $[Ca^{2+}]$ failed to reduce the sensitivity of IP_3-mediated Ca^{2+} release or alter Ca^{2+} leak when pumps were blocked in permeabilized avian supraorbital nasal gland cells [78]. Together, these results suggest that regulation of IP_3R by Ca^{2+} at the luminal aspect of the channel may, at best, operate over a limited range of store $[Ca^{2+}]$.

Our results (Fig. 11.8) [44–46] suggest that as the store content falls IP_3R become less responsive to IP_3. However, rather than luminal regulation being expressed from within the store at the luminal aspect of IP_3R, detection of $[Ca^{2+}]$ within the store may lie at the *cytoplasmic aspect of* IP_3R [45]. The Ca^{2+} current flowing through IP_3R evokes further release by a positive feedback effect of the ion at the cytoplasmic aspect of the channel, i.e. a Ca^{2+}-dependent positive feedback loop. Reduction of the store Ca^{2+} content reduces the Ca^{2+} current flowing through IP_3R and will result in a falling positive feedback at the cytoplasmic aspect of IP_3R until release eventually stops. Ca^{2+} release is renewed by an increased $[IP_3]$. In this case, the co-incidental activation of several neighboring IP_3Rs within a cluster offsets the declining IP_3R Ca^{2+} current to renew positive feedback and Ca^{2+} release and accounts for graded IP_3-mediated Ca^{2+} release.

Alternatively, the rise in cytoplasmic $[Ca^{2+}]_c$, which derives from the activity of IP_3R, may itself inactivate the receptor [79–81]. However, if Ca^{2+}-dependent inactivation terminated release [16, 79] to explain the graded IP_3-mediated Ca^{2+} release, the Ca^{2+} chelator BAPTA, would have been expected to have potentiated IP_3-evoked $[Ca^{2+}]_c$ increase; BAPTA decreased IP_3-mediated Ca^{2+} release [45].

Localized Ca2+responses IP_3 is a rapidly diffusing messenger and IP_3R are subject to positive feedback CICR on a single luminally-continuous entity, so how do highly-localized Ca^{2+} changes occur? In heart cells, the store is also a continuous network [82] in which Ca^{2+} can rapidly redistribute [83, 84] and positive feedback CICR occurs at RyR, yet highly localized Ca^{2+} release events occur. The highly localized responses arise in specialized domains formed by a junction of the store with the plasmalemma ('peripheral couplings') or the store and transverse (T)-tubules ('Dyads'). A number of proteins accrue at these specialized store domains: the L-type channel dihydropyridine receptors of the plasmalemma and T-tubules; the RyRs of store; triadin and junctin, of the store membrane; and calsequestrin (CSQ), the internal calcium binding protein [82]. The close coupling of dihydropyridine receptors and RyR provides control of Ca^{2+} release by Ca^{2+} influx. The quaternary complexes between triadin, junctin, RyR, and CSQ provides the luminal Ca^{2+} sensing capabilities that regulates RyR activity[85].

IP_3-mediated Ca^{2+} signaling may also generate highly localized responses even though IP_3 is a messenger that can diffuse quickly to evoke activity throughout the cell. To do this, certain receptors co-localize with IP_3R to form a local signalling complex [86–89]. In cultured sympathetic neurons, although muscarinic and bradykinin receptors each stimulate phospholipase C, only bradykinin receptors co-immunoprecipitate with, and activate, IP_3R to evoke Ca^{2+} release [86]. The arrangement enables PLC activation by muscarinic and bradykinin receptors to evoke different cellular responses. In SH-SY5Y cells the positioning of IP_3R near the plasma membrane provides a mechanism which may enable agonist activation, acting via IP_3, to target specific types of cellular response i.e. by generating Ca^{2+} rises in specific regions of the cell [90]. The clustering of agonist-activated surface receptors in certain regions on the plasma membrane (e.g. the *Escherichia coli* chemotaxis receptor) may contribute further, by providing areas with increased sensitivity to extracellular stimuli [91].

Smooth muscle also assembles IP_3 Ca^{2+} release components into specialized Ca^{2+} domains [92] (Fig. 11.1). This conclusion came initially from the observation that Ca^{2+} waves, triggered by agonists applied to the entire cell, began consistently at the same site on successive activations in smooth muscle i.e. there appeared to be regions with preferential IP_3-mediated Ca^{2+} release. Using centre of mass co-localization analysis of the distribution of the surface membrane receptors (for ACh) and IP_3R, a small percentage (~10 %) of sites showed co-localization. Significantly, the extent of co-localization was greatest at the Ca^{2+} wave initiation site. At these sites of co-localization, wave initiation may arise from a preferential delivery of IP_3 from mAChR3 activity to particular IP_3R clusters to generate faster local $[Ca^{2+}]_c$ increases. When the Ca^{2+} rise at the initiation site was rapidly and selectively attenuated (using photolysis of the caged Ca^{2+} buffer diazo-2) the Ca^{2+} wave shifted and initiated at a new site. Conversely, when a localized subthreshold 'priming' IP_3 concentration was applied rapidly to regions distant from the initiation site, the wave initiation site shifted to the site of priming IP_3 release. These results indicate that Ca^{2+} waves initiate where the most rapid Ca^{2+} change occurs at sites in which there is a structural and functional coupling of ACh receptors and IP_3R (Fig. 11.1). The coupling generates junctions in which IP_3 acts as a highly localized signal by being rapidly and selectively delivered to IP_3R.

Role of Mitochondria in Modulating Ca^{2+}Signals

Away from the plasma membrane, IP_3R activity in smooth muscle is also tightly regulated by mitochondria. Mitochondria have a well-developed Ca^{2+} uptake facility and may modulate bulk cytoplasmic Ca^{2+} signals [93–96] derived from Ca^{2+} entry and release [97]. Mitochondria also provide tight local control of Ca^{2+} release via IP_3R [93, 94, 98] but Ca^{2+} influx via voltage-dependent Ca^{2+} channels or release via RyR appears to be less tightly controlled at a local level by mitochondria [93, 94].

Mitochondrial control of IP_3R arises at IP_3-mediated release sites. IP_3-sensitive Ca^{2+} release initiates at discrete sites on the store that contain a few tens of IP_3R from which the local increase in $[Ca^{2+}]$ is called a 'puff'. Ca^{2+} puffs are spatially restricted events and of short duration but may interact and coalesce to generate a global release in Ca^{2+}. Mitochondria are positioned close to IP_3R and regulate activity of the channels [99]; inhibition of mitochondrial Ca^{2+} uptake attenuated the magnitude of Ca^{2+} puffs [100]. Indeed mitochondrial Ca^{2+} uptake was rapid enough to influence Ca^{2+} communication within an IP_3R cluster. Mitochondrial Ca^{2+} uptake appears to prevent the negative feedback effect of high $[Ca^{2+}]_c$ on IP_3R activity within a cluster to prolong Ca^{2+} release from the store [100]. As a consequence of the control at IP_3R, mitochondrial Ca^{2+} uptake exerts a pronounced effect on IP_3-mediated Ca^{2+} release throughout the cell [93, 94, 98, 101].

Mitochondria and IP_3R appear to be close, and perhaps tethered, to allow mitochondrial Ca^{2+} uptake, ATP supply, ROS production and or redox/antioxidant control to influence IP_3R activity. Conversely, mitochondrial division (required to maintain mitochondrial population health and allow cell proliferation) involves encircling of the dividing mitochondria by a store membrane tubule at the point of mitochondrial constriction [102]. During smooth muscle proliferation IP_3R expression and activity are increased [103–105] and there is a marked switch in mitochondrial phenotype from stationary to highly motile [106]. Inhibiting either IP_3R activity [104, 107] or mitochondrial motility and division [106, 108] inhibits smooth muscle proliferation. The interplay between mitochondria and IP_3R in smooth muscle thus presents an interesting potential therapeutic avenue by which pathological smooth muscle proliferation in vascular disease may be targeted.

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