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RYANODINE RECEPTORS IN SMOOTH MUSCLE

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TABLE OF CONTENTS

1. ABSTRACT

The sarcoplasmic reticulum (SR) of smooth muscle is endowed with two different types of Ca^{2+} release channels, i.e. inositol 1,4,5-trisphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs). In general, both release channels mobilize Ca^{2+} from the same internal store in smooth muscle. While the importance of IP_3Rs in agonist-induced contraction is well established, the role of RyRs in excitation-contraction coupling of smooth muscle is not clear. The participation of smooth muscle RyRs in the amplification of Ca^{2+} transients induced by either opening of Ca^{2+}-permeable channels or IP_3-triggered Ca^{2+} release has been studied. The efficacy of both processes to activate RyRs by calcium-induced calcium release (CICR) is highly variable and not widely present in smooth muscle. Although RyRs in smooth muscle generate Ca^{2+} sparks that are similar to those observed in striated muscles, the contribution of these local Ca^{2+} events to depolarization-induced global rise in $[Ca^{2+}]_i$ is rather limited. Recent data suggest that RyRs are involved in regulating the luminal $[Ca^{2+}]$ of SR and also in smooth muscle relaxation. This review summarizes studies that were carried out mainly in muscle strips or in freshly isolated myocytes, and that were aimed to determine the physiological role of RyRs in smooth muscle.

2. INTRODUCTION

2.1. Ca^{2+} regulation of smooth muscle contraction

Visceral smooth muscle constitutes one of the layers of numerous hollow organs such as trachea, uterus, intestines, urinary bladder, etc. whereas vascular smooth muscle is present in blood vessels. The mechanical activity of all these organs depends on the contraction-relaxation features of their smooth muscle tissues. Similarly to striated muscle, smooth muscle cells contracts in response to an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

However, significant differences between smooth and striated muscles exist; among them, contraction is slower in the former and myofilaments in smooth muscle do not show the regular pattern of sarcomeric muscles. One of the reasons for the slower mechanical response of smooth muscle is that the Ca^{2+} sensor (calmodulin) is not an integral part of the myofilaments, as is troponin C in striated muscles. The Ca^{2+}-calmodulin complex activates the myosin light chain kinase to phosphorylate serine 19 of myosin light chain, which in turn removes inhibition of the myosin ATPase. This event is followed by ATP hydrolysis and sliding of myosin on actin filaments to generate force (for review, see 1-3).

2.2. Sources of Ca^{2+} for smooth muscle contraction

Elevation of $[Ca^{2+}]_i$ in smooth muscle can be due to Ca^{2+} influx from the external millieu or Ca^{2+} release from internal stores, which are located in the sarcoplasmic reticulum (SR). External Ca^{2+} gains access to the cytoplasm through either voltage-dependent Ca^{2+} channels (VDCCs) or different types of Ca^{2+} permeable cation channels; whereas internal stores provide Ca^{2+} by at least two types of release-channels, the inositol 1,4,5-trisphosphate receptor (IP_3R) and the ryanodine receptor (RyR) (for review see 1, 2). The participation of internal Ca^{2+} stores in smooth muscle contraction is highly variable. In general, internal stores release Ca^{2+} during the initial phase of contraction, but their overall participation is rather small. In some cases, Ca^{2+} internal stores supply basically all Ca^{2+} for agonist-induced contraction, e.g. guinea pig pulmonary artery and porcine coronary artery (4,5). The main mechanism by which neurotransmitters, hormones and other agonists release Ca^{2+} from internal stores involves the activation of phospholipase C, which in turn hydrolyzes phosphatidylinositol bisphosphate to generate both diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). The latter induces the opening of IP_3Rs to produce a global elevation in $[Ca^{2+}]_i$ and contraction in smooth muscle (1). Therefore, IP_3Rs have an essential participation in pharmaco-mechanical coupling (1). By contrast, the role played by RyRs in triggering smooth muscle contraction by physiological stimuli is not clear. This review summarizes studies focused on the physiological role of RyRs in smooth muscle, that have been carried out either in tissue preparations or in freshly isolated myocytes.

3. CHARACTERISTICS AND TYPES OF RYANODINE RECEPTORS IN SMOOTH MUSCLE

Initially, the characterization of RyRs from smooth muscle was carried out with pharmacological tools such as caffeine and ryanodine, similarly to the studies done in striated muscles. Subsequently, biochemical and molecular studies of RyRs from smooth muscles have been reported.

3.1. Pharmacological characterization

The presence of RyRs in smooth muscle was first suggested by studies showing that caffeine induces transient contractures of smooth muscle bundles in the absence of extracellular Ca^{2+} (6). Caffeine works by increasing the Ca^{2+} sensitivity of RyRs such that these release-channels are opened by basal $[Ca^{2+}]_i$ (7). The action of caffeine is to produce a transient increase in $[Ca^{2+}]_i$ that originates from internal stores. However, caution must be exercised since it has been shown that caffeine can also activate a Ca^{2+} permeable cation channel, which is present in the plasma membrane of gastric smooth muscle cells (8-10).

Ryanodine is a plant alkaloid that binds with high affinity and selectivity to RyRs (11). Micromolar concentrations of this alkaloid in combination with caffeine produce a complete depletion of caffeine-sensitive Ca^{2+} stores in skinned smooth muscle of pulmonary artery, portal vein and taenia caeci from guinea pig (5). Similar results have been obtained in freshly isolated smooth muscle cells, including guinea pig urinary bladder (12) and mouse duodenum (13). The effect of either ryanodine or fluorescently-labeled ryanodine on caffeine-sensitive internal Ca^{2+} stores is shown in Figure 1. The application of caffeine to smooth muscle cells incubated with 2 μM ryanodine (concentration that "locks" RyR in a subconductance state) (14, 15) did not appreciably alter the initial $[Ca^{2+}]_i$ response to caffeine. However, internal Ca^{2+} stores were unable to recover in the presence of ryanodine, most likely because the SR was leaky, which is reflected in both a slower rate of recovery of the $[Ca^{2+}]_i$ (indicated by the arrow, Figure 1) and a lack of $[Ca^{2+}]_i$ response to a second application of caffeine (Figure 1). Fluorescently-labeled ryanodine did not behave similarly to parental ryanodine because in the presence of the former, there was a partial recovery of the internal Ca^{2+} store (Figure 1).

The effect of ryanodine on the ion channel activity of RyRs from smooth muscle has also been studied in planar lipid bilayers. The toad stomach RyR displays a subconductance state of high open probability in response to micromolar concentrations of ryanodine (15), similar to the effect described for cardiac and skeletal RyRs (17). However, ryanodine does not induce this subconducting state in RyRs from aorta (18) or from coronary artery smooth muscle (19). In the case of RyRs from aorta, millimolar concentrations of ryanodine induced the fully blocked state of this Ca^{2+} release channel (18), whereas for RyRs from coronary artery, concentrations of ryanodine up to 10 μM increased the ion channel activity, while higher concentrations inhibited this activity (19). It is not clear whether these differences between visceral and vascular RyRs imply the existence of different RyR isoforms or the loss of some regulatory factor during the RyR isolation procedure. Thus, further studies at the single channel level are needed to clarify the effect of ryanodine on RyRs from different types of smooth muscle cells.

3.2. Biochemical characterization

The ryanodine receptor has been localized to the SR of smooth muscle cells and its abundance correlates with the amount of SR, which fluctuates between 1.5 and 7.5 % of the total myocyte volume (20-23). Interestingly, phasic smooth muscle contains less SR than tonic smooth muscle and the SR of the former is preferentially localized close to the plasma membrane (20, 22-24). Apparently, some parts of the SR (peripheral SR) are in close apposition

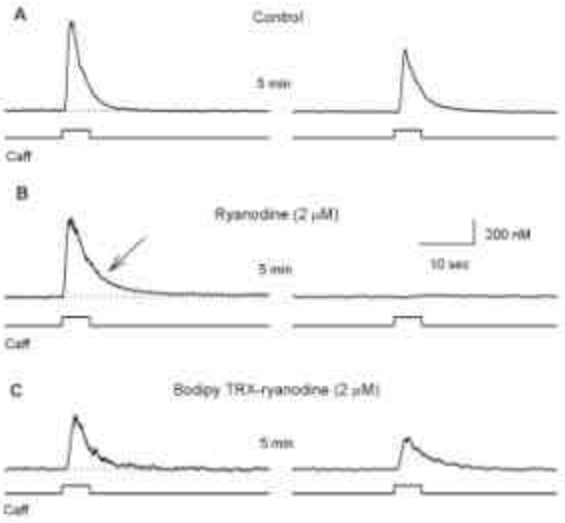

Figure 1. Effect of ryanodine on caffeine-induced Ca^{2+} release from internal stores. Single smooth muscle cells isolated from guinea pig urinary bladder were loaded with fura-2 and changes in $[Ca^{2+}]_i$ were recorded in response to the application of 20 mM caffeine (Caff) with a puffer pipette placed close to the cell (as indicated by the traces below the $[Ca^{2+}]_i$ recordings). Bath solution was Hepes-buffered saline solution (2 mM Ca^{2+}). A. Caffeine induced a transient increase in $[Ca^{2+}]_i$ that returned to the basal level even in the presence of caffeine. A time period of 5 min was allowed to recover internal Ca^{2+} stores. This recovery was not complete based on the smaller amplitude of the $[Ca^{2+}]_i$ transient induced by a second application of caffeine. B. Cells that were incubated with ryanodine responded normally to the first application of caffeine. This was true even when cells were incubated with ryanodine for a prolonged period of time (> 30 min). However, two main differences were evident respect to control responses. First, the rate of decay of $[Ca^{2+}]_i$ was significantly delayed, which is notorious at $[Ca^{2+}]_i$ below 300 nM (arrow). Second, an additional application of caffeine produced no increase in $[Ca^{2+}]_i$. These data and the absence of a significant capacitative Ca^{2+} influx in this type of myocytes (16) suggest that ryanodine and caffeine combined lock the RyR open, leading to a complete depletion of internal Ca^{2+} stores. C. Fluorescently-labeled ryanodine (BODIPY TRX-ryanodine) appears to be incapable of correctly interacting with the open state of RyRs as this derivative did not produce a complete depletion of the internal Ca^{2+} stores. Collectively, these data support the hypothesis that RyRs must be activated before interacting with ryanodine and also that low concentrations of this alkaloid locks the RyRs in an open state, to the extent that internal Ca^{2+} stores cannot be recovered. The dotted line indicates resting $[Ca^{2+}]_i$. Both $[Ca^{2+}]_i$ and time scales apply for all recordings.

(~ 20 nm) to the plasma membrane, generating what is known as junctional gaps. These regions contain structures that resemble the feet described in skeletal muscle (20). Additionally, recent data in myocytes from cerebral arteries (21) and guinea pig urinary bladder (25) indicate that VDCCs co-localize with RyRs.

Further characterization of RyRs has been carried out using binding of [^{3}H]ryanodine to microsomal preparations from different smooth muscles. Overall, these studies have yielded a Kd close to 5 nM and a Hill coefficient of 1 (15, 26-29), which are similar to those obtained for RyRs from striated muscles (30). The density of [^{3}H]ryanodine binding sites is in the vicinity of 100 fmol/mg protein, although a density as high as 5.7 pmol/mg protein has been reported in crude microsomal preparations of smooth muscle from rat portal vein (31). In general, the number of [^{3}H]ryanodine binding sites in smooth muscle is 10 times lower or even less than in striated muscles (18). Presumably, this low density of RyRs is a consequence of the sparse SR in smooth muscle (26). Moreover, the binding of [^{3}H]ryanodine to microsomal membranes from smooth muscle can be increased by the same factors that also modulate the activity of RyRs from striated muscles (32), such as Ca^{2+}, caffeine, ATP, high ionic strength and pH (26,28). Ruthenium red and Mg^{2+} inhibit [^{3}H]ryanodine binding in smooth muscle microsomes (26,28), similar to the effect observed in striated muscles. All these data suggest the presence of typical RyRs in smooth muscle, albeit at a low density.

3.3. Molecular characterization

The ryanodine receptor is a homotetrameric protein of approximately 2 MDa molecular weight. Three isoforms of RyRs that are encoded by different genes (*ryr1*, *ryr2* and *ryr3*) have been identified and cloned (33-36). All three types of RyRs have been detected in RNA extracted from smooth muscle (Table 1). However, these results should be interpreted with some caution, since the detection of different RyR transcripts may reflect contamination from cells other than smooth muscle (e. g. endothelial cells, neurons, etc.). Studies in isolated smooth muscle cells have shown that there is no predominant RyR isoform in smooth muscle (Table 1).

RyR knockout mice have recently emerged as suitable tools to study the role of RyRs in smooth muscle physiology. Arterial smooth muscle from mice lacking RyR3 contracts normally to caffeine and norepinephrine (50). Another study has shown that the frequency of Ca^{2+} sparks (localized $[Ca^{2+}]_i$ events that are produced by the opening of a cluster of RyRs) is significantly increased in RyR3 knockout mice (41). Studies in smooth muscle derived from RyR2 knockout mice are lacking because mutant embryos die at day 10 due to abnormalities in the heart tube (51). In addition, there are no data on smooth muscle function in RyR1 knockout mice (52,53).

Studies in rat portal vein myocytes with antisense oligonucleotides targeting each of the three types of RyRs demonstrated that the presence of both RyR1 and RyR2 is required for myocytes to respond to membrane depolarization with Ca^{2+} sparks and a global increase in $[Ca^{2+}]_i$ (42). The inhibition of RyR3 expression in rat portal vein myocytes did not alter either evoked or spontaneous Ca^{2+} sparks (42). Apparently, RyR3 acquires the ability to

Table 1. Expression of RyR types in different smooth muscles

Smooth muscle source	RyR Isoform			Detection Method	Reference
	RyR1	RyR2	RyR3		
Aorta	N. D.	+ (rt)	+ (rb)	Northern-blot	36, 37
	+ (rt)	+ (p, rt)	+ (p, rt)	RT-PCR	38, 39, 40
Aorta without endothelium	- (rt)	- (rt)	+ (rt)	RT-PCR	40
Cerebral arteries	N.D.	+ (rt)	N. D.	Inmunocitology	21
	+ (m)	+ (m)	+ (m)	RT-PCR	41
Mesenteric arterial vessels	+ (rt)	+ (rt)	+ (rt)	RT-PCR	39
Portal vein myocytes	+ (rt)	+ (rt)	+ (rt)	RT-PCR	42
Bronchi	- (h)	- (h)	+ (h)	RT-PCR	43
Esophagus	N. D.	N. D.	+ (rb)	Northern-blot	36
	+ (p, m)	+ (m)	+ (p,m)	RT-PCR	38, 44
Small intestine	- (p)	- (p)	+ (p)	RT-PCR	38
Duodenum	- (m)	+ (m)	+ (m)	RT-PCR	45
Taenia coli	N. D.	N. D.	+ (rb)	Northern-blot	36
Stomach	+ (m)	+ (m)	+ (m)	RT-PCR	44
Ureter	N. D.	N. D.	+ (rb)	Northern-blot	36
Ureteric myocytes	- (rt)	- (rt)	+ (rt)	RT-PCR	29
Urinary bladder	N. D.	N. D.	+ (rb)	Northern-blot	36
	- (h)	+ (h)	- (h)	RT-PCR	46
Cultured urinary bladder myocytes	- (h)	+ (h)	- (h)	RT-PCR	46
Uterus	N. D.	N. D.	+ (rb)	Northern-blot	36
Non-pregnant myometrium	+ (h)	+ (h)	+ (h)	RT-PCR	47,48
	- (h)	- (h)	+ (h, m)	RT-PCR	49,45
Pregnant myometrium	+ (h)	+ (h)	+ (h)	RT-PCR	48
	- (h)	+ (h)	+ (h)	RT-PCR	49
Cultured myometrial myocytes	- (h)	- (h)	+ (h)	RT-PCR	49

Abbreviations: N.D. not determined; + detected isoform; - not detected isoform; h, human; m, mouse; p, pig; rb, rabbit; rt, rat; RT-PCR, reverse transcriptase-polymerase chain reaction.

respond to caffeine only in conditions of increased SR Ca^{2+} loading in myocytes from both rat portal vein (54) and myometrium of non-pregnant mice (45). However, some of these studies were carried out in cells that had been cultured for several days, which might have changed the type and level of expression of RyRs. Indeed, RyRs cannot be found in rat aortic smooth muscle cells in proliferating conditions, but they are detected when cells reach a non-proliferative state (40). Thus, the type and functional role of RyRs need to be assessed for each type of smooth muscle.

4. PHYSIOLOGICAL ROLE OF RYANODINE RECEPTORS IN SMOOTH MUSCLE

The role of RyRs in smooth muscle cells is not clearly established. This release channel has been involved in the amplification of Ca^{2+} transients that are originated by either opening of VDCCs or IP_3-induced Ca^{2+} release in some smooth muscle cells. Alternatively, RyRs also seem to participate in both the regulation of luminal [Ca^{2+}] and the local activation of large-conductance Ca^{2+}-dependent K^+ channels (BK_{Ca} channels). These roles suggest a more important participation of RyRs in smooth muscle relaxation than in excitation-contraction coupling as summarized below.

4.1. Excitation-contraction coupling

4.1.1. Amplification by RyRs of the Ca^{2+} influx through voltage-dependent Ca^{2+} channels

Membrane depolarization in smooth muscle increases $[Ca^{2+}]_i$ as a consequence of VDCCs opening. However, it has been calculated that the Ca^{2+} coming through these channels, either in a single or a train of 5-10 action potentials, might not be sufficient to induce contraction because the cytoplasmic Ca^{2+} buffer capacity may reduce the activity of Ca^{2+} ions (4,20). Therefore, in this scenario it is obligatory to postulate the existence of an additional source of Ca^{2+}, most likely the SR. The question then turns: How does the SR amplify the Ca^{2+} influx through VDCCs? One possibility could be the activation of IP_3Rs, because smooth muscle produces IP_3 in response to Ca^{2+} influx (55) and membrane depolarization increases the activity of phospholipase C (56,57). However, this scenario seems unlikely as heparin, an antagonist of IP_3Rs, does not reduce the $[Ca^{2+}]_i$ transient induced by membrane depolarization (58,59).

Another possibility for the amplification of Ca^{2+} influx through VDCCs could be the activation of RyR by the calcium-induced calcium release (CICR) mechanism, which is well established for cardiac myocytes (60). The first direct evidence of CICR in smooth muscle was obtained by studies in skinned smooth muscle bundles (61). However, it has been suggested that CICR might not be functioning as the primary physiological Ca^{2+} release mechanism, since higher Ca^{2+} is required to activate CICR than to induce contraction (62). Nevertheless, these results do not completely exclude the participation of CICR in releasing Ca^{2+} during excitation-contraction coupling in smooth muscle, as local elevations of $[Ca^{2+}]_i$ in the vicinity of RyRs may be high enough to activate these release

channels (62). Alternatively, a cytosolic factor regulating CICR may have been lost during the smooth muscle permeabilization procedure (62).

Studies of $[Ca^{2+}]_i$ in single smooth muscle cells under the whole-cell configuration of the patch clamp technique demonstrated that membrane depolarization produces a bell-shape curve of both VDCC currents and changes in $[Ca^{2+}]_i$, with the peak $[Ca^{2+}]_i$ response close to 0 mV (63-68). Similar shape of the voltage-dependent changes in $[Ca^{2+}]_i$ has been described in cardiac myocytes (69-70). This relationship between voltage and $[Ca^{2+}]_i$ implies that Ca^{2+} influx is required in smooth muscle to elevate $[Ca^{2+}]_i$ during membrane depolarization. Different studies looking at $[Ca^{2+}]_i$ (58,65,71,72) or Ca^{2+}-dependent ion channels (73) have suggested the presence of CICR in different smooth muscle cells. However, these studies did not show the extent of CICR contribution to the depolarization-induced $[Ca^{2+}]_i$ transient. In addition, it appears that CICR is not universally present in smooth muscle cells (67,74-76). To further complicate this picture, there are cases where CICR is evident only for the first depolarization pulse (77) or the first train of voltage pulses (68). Studies aimed to quantify the relevance of CICR in the Ca^{2+} transient induced by activation of VDCCs demonstrated that an average of only 20 % of the $[Ca^{2+}]_i$ transient at 500 msec was due to Ca^{2+} release from internal stores (78). The same type of study was carried out to calculate the cytoplasmic Ca^{2+} buffer capacity (79), but in this case the idea was to determine the initiation of CICR in VDCC-induced $[Ca^{2+}]_i$ transient (9, 78). The Ca^{2+} buffer capacity was obtained for the initial 50 msec of membrane depolarization and compared with a late determination from 100 to 200 msec after VDCCs have been activated. In the absence of ryanodine, the initial Ca^{2+} buffer was 87.8 ± 2.7 (n = 10) while the late Ca^{2+} buffer was significantly lowered to 54.1 ± 5.4 (n = 10). This artificial reduction of the cytoplasmic Ca^{2+} buffer implies that Ca^{2+} ions from internal stores contribute to increase $[Ca^{2+}]_i$ but are not part of the integrated voltage-dependent Ca^{2+} current. Indeed, the presence of ryanodine in the internal solution of the patch clamp pipette inhibited this extra source of Ca^{2+}, since the initial and late Ca^{2+} buffer were similar (81.1 ± 10.0 vs 79.2 ± 9.1, n = 8). These data indicate that CICR is a delayed event in smooth muscle (78), as this amplification mechanism was evident only 50 to 100 msec after the activation of VDCCs. This contrast with a time constant of ~ 7 msec between the activation of VDCCs and Ca^{2+} sparks in cardiac cells (80).

Confocal studies of $[Ca^{2+}]_i$ in smooth muscle cells under voltage clamp have also shown delays of tens of msec between the activation of VDCCs and Ca^{2+} sparks (59,81,82). These studies have suggested that smooth muscle RyRs are loosely coupled to VDCCs, implying that it is the bulk $[Ca^{2+}]_i$ that triggers RyRs activation in smooth muscle (59,83). This is a completely different situation to the one described in cardiac cells, where the efficiency of CICR depends to a great extent on the close proximity between RyRs and VDCCs (80, 84). The concept of "loose coupling" of CICR may be in line with the demonstration that Ca^{2+} influx through other channels, e.g. stretch-activated channels, can also trigger CICR (78). One of the problems with the loose coupling hypothesis is that Ca^{2+} sensitivity of RyRs is not high enough (15,18,61) to activate these channels by bulk $[Ca^{2+}]_i$. Another limitation is that CICR should be unstable due to the lack of local control of RyRs.

Additional data undermine the role of CICR in amplifying the Ca^{2+}-influx through VDCCs in smooth muscle cells. For instance, inhibition of SR Ca^{2+} pumps with cyclopiazonic acid, although abolishing Ca^{2+} sparks, it does not reduce the global rise in $[Ca^{2+}]_i$ triggered by membrane depolarization (82). If anything, it increases the elevation in $[Ca^{2+}]_i$ (82). Furthermore, the application of ryanodine to rat gastric myocytes increases the efficiency of VDCCs to elevate $[Ca^{2+}]_i$ (76). The fact that Ca^{2+} influx through VDCCs is able to increase global $[Ca^{2+}]_i$ before triggering Ca^{2+} sparks (59, 81) and that ryanodine does not change the initial Ca^{2+} buffering capacity (78) suggest that RyRs from smooth muscle are insensitive to Ca^{2+} influx through VDCCs, even when the activation of VDCCs generates a strong increase in the subsarcolemmal $[Ca^{2+}]$ (85). Interestingly, line scan recordings of $[Ca^{2+}]_i$ in cardiac cells have shown that when a sparklet (a local $[Ca^{2+}]_i$ event due to the opening of a single VDCC) does not trigger a Ca^{2+} spark, the probability of a second, similar sparklet to induce a Ca^{2+} spark is the same as the probability of the first sparklet that successfully triggered a Ca^{2+} spark (see figure 6 in reference 80). Collectively, these studies suggest that RyRs might be able to switch between Ca^{2+}-sensitive and Ca^{2+}-insensitive states. Thus, beside localization, it appears that there are other factors that determine the ability of RyRs to respond to Ca^{2+}. If this is true, then identifying these factors might explain the variability of CICR in smooth muscle.

4.1.2. Amplification by RyRs of IP_3R-mediated Ca^{2+} release

The sarcoplasmic reticulum of smooth muscle cells is a continuous membrane organelle (1), although only some parts are specialized in storing Ca^{2+} (86-89). Smooth muscle SR can be divided in peripheral and central SR, and both types of release-channels (RyRs and IP_3Rs) are localized in these two sections (1, 22, 90). Conceivably, the activation of IP_3Rs could either stimulate adjacent RyRs by increasing cytoplasmic $[Ca^{2+}]$i or inhibit RyRs by decreasing luminal $[Ca^{2+}]$. Such reduction in luminal [Ca2+] has already been demonstrated to affect the activity of RyRs in smooth muscle (91). We have summarized work done on how these two release channels interact in smooth muscle.

It has been proposed that CICR via RyRs propagates the vasopressin-induced IP_3-initiated Ca^{2+} release in A7r5 cells (92). Recently, further evidence has been reported supporting the participation of RyRs in amplifying the $[Ca^{2+}]_i$ signal initiated by activation of IP_3Rs in smooth muscle cells. Both anti-RyR antibodies and ryanodine strongly inhibit the rate of rise of agonist-induced Ca^{2+} release in myocytes from either portal vein or duodenum (31).

However, there are also many examples where RyRs do not seem to participate in the IP_3-mediated Ca^{2+}

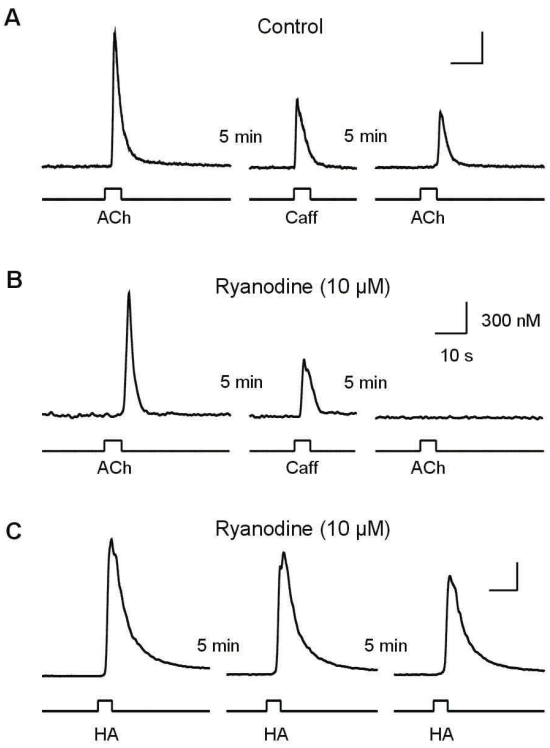

Figure 2. RyRs and IP_3Rs share the same internal Ca^{2+} store and RyRs do not seem to participate in agonist-induced Ca^{2+} release. Single smooth muscle cells from guinea pig urinary bladder were loaded with fura-2 and challenged with either 10 μM Acetylcholine (ACh), or 20 mM caffeine (Caff) or 1 mM histamine (HA) by pressure ejection from a puffer pipette placed close to the cell. The application of agonists are indicated by the traces below the $[Ca^{2+}]_i$ recordings. A. Cells responded to both agonists with transient increases in $[Ca^{2+}]_i$ provided that time periods of 5 min were allowed between the different applications. Note that recovery of internal stores was only partial because cells were not depolarized to rise $[Ca^{2+}]_i$ and facilitate refilling of the stores. B. ACh did not affect caffeine-induced Ca^{2+} release in the presence of 10 μM ryanodine, but Caff inhibited ACh-mediated Ca^{2+} release in this condition. C. The $[Ca^{2+}]_i$ responses to HA were not affected by the presence of ryanodine. These data imply that ACh-induced increase in $[Ca^{2+}]_i$ derives from internal Ca^{2+} stores only, and all agonists release Ca^{2+} from the same internal store. The fact that in the presence of ryanodine, neither ACh nor HA induces an irreversible depletion of internal Ca^{2+} stores suggests that IP_3-mediated Ca^{2+} release does not involve RyRs.

release. The presence of ryanodine does not alter the agonist-induced contraction in portal vein, pulmonary artery and taenia caeci from guinea pig (5). In rat portal vein myocytes, tetracaine, although inhibits Ca^{2+} release mediated by caffeine, does not affect noradrenaline-triggered Ca^{2+} mobilization (93). Acetylcholine-induced Ca^{2+} release is not affected by 50 μM ruthenium red in equine tracheal myocytes (94). In rat ureteric myocytes, neither ryanodine nor anti-RyR antibody modifies acetylcholine-mediated Ca^{2+} waves (29). Additionally, ryanodine does not block either multiple IP_3-triggered Ca^{2+} releases in colonic smooth muscle (95) or acetylcholine-induced Ca^{2+} release in guinea pig urinary bladder myocytes (Figure 2). These data suggest that either RyRs are not opened during IP_3-mediated Ca^{2+} release or if they are, then the open time is too short for ryanodine to recognize the open conformation of RyR. However, the latter seems unlikely, as the opening of RyRs by caffeine does induce a complete depletion of internal stores in the presence of ryanodine (Figure 2). Similarly, ryanodine does not have any effect on the histamine-induced Ca^{2+} release (96 and Figure 2). Possible explanations for the absence of IP_3R-induced activation of RyR could be either that IP_3R does not increase $[Ca^{2+}]_i$ high enough to activate RyRs or that IP_3Rs decrease the Ca^{2+}-sensitivity of RyRs by lowering luminal $[Ca^{2+}]$, a possibility that might be supported by localization of both receptors at the same internal Ca^{2+} store (97). Indeed, different agonists that release Ca^{2+} from internal stores also inhibit the spontaneous transient outward currents (STOCs) (98,99), which are due to Ca^{2+} sparks activating BK_{Ca} channels (100). This inhibition appears to depend on the Ca^{2+}-releasing activity of the agonists, since blocking IP_3Rs with heparin inhibits the action of agonists on STOCs (98,99). Alternatively, it has been suggested that protein kinase C, activated by agonist-induced diacylglycerol, reduces the Ca^{2+} sensitivity of RyRs (101). Thus, although it seems that RyRs can amplify the IP_3R-induced Ca^{2+} signal in smooth muscle cells, this action of RyRs does not appear to be present in all types of smooth muscle.

4.2. cADPR and smooth muscle function

From data summarized in the previous section, it seems that RyRs do not play a strong role in excitation-contraction coupling of smooth muscle. It is feasible that other factors might increase the *in vivo* efficiency of CICR in smooth muscle cells. One candidate is cyclic adenosine diphosphate-ribose (cADPR), a metabolite derived from β-NAD^+ with the ability to induce Ca^{2+} release from internal stores in a wide variety of mammalian cells, including cardiac and smooth muscle myocytes. cADPR is generated by ADP-ribosyl cyclase and degraded by cADPR hydrolase. Both enzyme activities appear to reside in the same protein (102), which was firstly identified in mammalian cells as the lymphocyte antigen CD38 (103).

The Ca^{2+} releasing activity of cADPR is blocked by procaine, ruthenium red (104) and high concentrations of either ryanodine (19,104) or caffeine (104). Moreover, cADPR-induced Ca^{2+} release is not affected by heparin, but it is enhanced by low concentrations of caffeine (104, 105). These data support the notion that cADPR activates RyRs in bovine coronary artery (19) and in smooth muscle from both rabbit longitudinal intestine (106) and porcine trachea (104). Direct evidence that RyR is the target of cADPR comes from a recent work showing that 1 μM cADPR increases 8-fold the activity of bovine coronary artery RyRs incorporated in planar lipid bilayers (19). Nevertheless, it has also been suggested that cADPR may activate a novel and RyR-independent Ca^{2+} release mechanism in vascular smooth muscle (105).

The first report by Kuemmerle and Makhlouf (106) demonstrated that cADPR stimulated Ca^{2+} release in permeabilized longitudinal smooth muscle cells from rabbit ileum. This effect was specific because permeabilized circular smooth muscle cells did not respond to cADPR. Since this report, it has been shown that both visceral and vascular smooth muscles respond to cADPR by releasing Ca^{2+} from internal stores (104,105,107). Moreover, it has been proposed that cADPR-mediated Ca^{2+} signaling participates in the regulation of a variety of functions in smooth muscle such as, agonist-induced contraction (106,108-110) and agonist-induced $[Ca^{2+}]_i$ oscillations (104). It has also been suggested that cADPR controls resting $[Ca^{2+}]_i$ levels (19,109), vascular and visceral tone (110,111) and decreases BK_{Ca} channel activity (112,113). Recently, it has been reported that cADPR participates in hypoxic pulmonary vasoconstriction as well (107,114). Nevertheless, the role of cADPR in inducing contraction does not seem to be universal (86,115). In addition, there are unanswered questions regarding how cADPR works in smooth muscle, e.g. there is no evidence that membrane depolarization increases cADPR and the mechanism that triggers activation of ADP-ribosyl cyclase in smooth muscle cells is unknown.

4.3. Superficial buffer barrier

Another possible physiological function of RyR in smooth muscle cells is the regulation of luminal $[Ca^{2+}]$ of the SR ($[Ca^{2+}]_{SR}$). It has been shown that the activity of RyR from cardiac myocytes is sensitive to the $[Ca^{2+}]_{SR}$ (116), which is also supported by studies in permeabilized cardiac myocytes showing that the frequency of Ca^{2+} sparks increases in response to a higher Ca^{2+} loading of SR. Thus, the modulation of RyR activity by luminal $[Ca^{2+}]$ could be a mechanism to regulate the $[Ca^{2+}]_{SR}$ (117). This mechanism may be present in smooth muscle cells as well, since the frequency of Ca^{2+} sparks is also sensitive to the $[Ca^{2+}]_{SR}$ (91).

In agreement with the superficial buffer barrier hypothesis proposed for smooth muscle (for review see 118), the peripheral SR separates cytoplasm into a subsarcolemmal region and the bulk cytoplasmic compartment. This compartmentalization would permit the buffering by the peripheral SR of Ca^{2+} entering in the subsarcolemmal region. To avoid Ca^{2+} overloading of the SR, the sequestered Ca^{2+} should be vectorially leaked in the subsarcolemmal space to be extruded from the cell (118). It seems feasible that RyRs are the "leak" channels responding to an increase in the $[Ca^{2+}]_{SR}$ of smooth muscle (119). Indeed, the incubation of vascular smooth muscle with ryanodine induces vasoconstriction (100). This could be due to the effect of ryanodine on the superficial buffer barrier, as this alkaloid impedes the function of SR as a Ca^{2+} store by locking RyRs in an open state (5). Considering that a small Ca^{2+} influx through VDCCs is continually sequestered by the activity of peripheral SR Ca^{2+} pumps, and since this action limits Ca^{2+} access to the myofilaments (16,76,119-121), then eliminating this mechanism with ryanodine should result in a higher effect of VDCCs on contraction (119). Thus, ryanodine either induces or facilitates smooth muscle contraction by nullifying the sink activity of peripheral SR. This is completely opposite to the effect of ryanodine in heart since this alkaloid inhibits contraction by depleting the internal Ca^{2+} stores of cardiac myocytes. Nevertheless, stronger or faster Ca^{2+} entries are needed to saturate the buffering activity of peripheral SR and to induce contraction in smooth muscle cells (16). Therefore, RyR activity appears to be involved in the vectorial release of Ca^{2+} to the subsarcolemmal region.

4.4. Smooth muscle relaxation

Recently, it has been proposed that RyRs might participate in smooth muscle relaxation by generating Ca^{2+} sparks (100). In general, Ca^{2+} sparks are localized close to the plasma membrane (82,122) where they activate BK_{Ca} channels in a coordinated fashion to produce STOCs, first described by Benhan and Bolton (123). STOCs in turn induce membrane hyperpolarization with the consequent deactivation of VDCCs. This last action decreases Ca^{2+} influx, which in turn facilitates smooth muscle relaxation (124). Accordingly, it appears that BK_{Ca} channels are functionally associated to RyRs (125). Indeed, a co-localization study with antibodies showed limited zones where BK_{Ca} channels are close to RyRs (122). Thus, a fraction of RyRs in some smooth muscle cells is tuned or organized in a way that Ca^{2+} sparks but not global $[Ca^{2+}]_i$ elevations are generated (126). The importance of RyRs generating only Ca^{2+} sparks is the implication that overloaded SR can be discharged by an increased frequency of Ca^{2+} sparks. These events, as indicated above, generate STOCs with the concomitant hyperpolarization of the cell membrane and deactivation of VDCCs. These effects together with the contribution of the superficial buffer barrier would have as a final result the reduction of Ca^{2+} loading in SR. This dynamic regulation of Ca^{2+} influx and SR Ca^{2+} loading may be responsible for the myogenic tone (124).

However, Ca^{2+} sparks also activate Ca^{2+}-dependent Cl^- channels, which can induce membrane depolarization and smooth muscle contraction (127). Furthermore, BK_{Ca} channels can also be directly activated by local Ca^{2+} entry through VDCCs (128). These data indicate that not all BK_{Ca} channels are strictly associated with RyRs. Indeed, it has been found that a substantial number of Ca^{2+} sparks does not elicit STOCs in myocytes from both feline esophagus (129) and toad stomach (130). In addition, studies in intact cells have shown that BK_{Ca} channels display an extremely high Hill number and a Ca^{2+} sensitivity near 1 μM (131), both of which are higher than the same obtained for these channels in planar lipid bilayers (132). Therefore, Ca^{2+} sparks do not need to be in such close apposition to BK_{Ca} channels, as nearby Ca^{2+} sparks would only require to increase $[Ca^{2+}]_i$ to ~ 1 μM to trigger STOCs.

It has been established that cyclic nucleotides (cAMP and cGMP) play a significant role in smooth muscle relaxation. These second messengers increase the frequency of both Ca^{2+} sparks and STOCs in smooth muscle cells isolated from basilar arteries (133), supporting the role of Ca^{2+} sparks in smooth muscle relaxation. However, the same nucleotides either barely increase the

frequency of STOCs in myocytes from rabbit portal vein (134), or do not have any effect on STOCs frequency in pulmonary artery smooth muscle cells (135). Interestingly, in these three cases cyclic nucleotides appear to increase the $[Ca^{2+}]_{SR}$. Certainly, although the function of RyRs in smooth muscle relaxation seems to be appealing, more studies are needed to establish the actual role of RyRs in terminating smooth muscle mechanical activity.

5. PERSPECTIVES

The role of RyRs in smooth muscle has begun to be unraveled. From the data reviewed here, it appears that unknown factors may regulate the activity of RyRs in this type of cells. This notion is supported by the fact that RyR3 in uterine smooth muscle cannot directly respond to Ca^{2+} or caffeine (45, 47), although they respond to these agents when expressed in HEK293 cells (136). Identifying the nature of these factors is critical to understand the role of RyRs in smooth muscle physiology. However, there are still other questions that need to be addressed, among them: 1) Is there more than one type of RyR expressed in the same smooth muscle cell? 2) What is the intracellular distribution of the different types of RyRs? 3) How tight is the relationship between RyR and BK_{Ca} channels? 4) What is the importance of RyR in regulating the luminal $[Ca^{2+}]$ in smooth muscle cells? These issues are further complicated by the diversity of smooth muscles. Thus, although RyRs are present on the SR of smooth muscle cells, these release channels do not seem to participate in excitation-contraction coupling. Clearly, this is the opposite to the key role played by RyRs in the contraction of striated muscles.

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Abbreviations: ACh, acetylcholine; BK_{Ca} channels, high conductance Ca^{2+}-dependent K^+ channels; cADPR, cyclic adenosine diphosphate-ribose; Caff, caffeine; CICR, calcium-induced calcium release; $[Ca^{2+}]_i$, intracellular calcium concentration; $[Ca^{2+}]_{SR}$, luminal calcium concentration; HA, histamine; IP_3R, inositol 1,4,5-trisphophate receptor; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; VDCC, voltage-dependent calcium channels; STOCs, spontaneous transient outward currents.

Key Words: Smooth Muscle, Ryanodine Receptor, CICR, cADPR, Ca^{2+} Sparks, Sarcoplasmic Reticulum, Internal Ca^{2+} stores, Review

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