

Basic properties of a novel ryanodine-sensitive, caffeine-insensitive calcium-induced calcium release mechanism in permeabilised human vascular smooth muscle cells

Stephen Lynn, James I. Gillespie*

Department of Physiological Sciences, The Medical School, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK

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Abstract The efflux of $^{45}\text{Ca}^{2+}$ from preloaded intracellular stores of saponin-permeabilised human uterine artery smooth muscle cultured cells was used to study the mechanisms underlying Ca^{2+} release from the sarcoplasmic reticulum (SR). The present paper demonstrates directly a functional Ca^{2+} release mechanism that is dependent on an increase in free Ca^{2+} (100 nM–30 μM) and is completely inhibited by 20 μM Ruthenium red. The amount of Ca^{2+} released at 30 μM free Ca^{2+} was reduced by approximately 50% compared to the release at 10 μM . This Ca^{2+} -induced Ca^{2+} release (CICR) mechanism was not sensitive to caffeine. Exposure of cells to low free Ca^{2+} -containing solutions (10 nM) indicated that a component of the CICR mechanism may be functional at basal free Ca^{2+} levels of 100 nM. Application of ryanodine (0.1–100 μM) induced $^{45}\text{Ca}^{2+}$ efflux from the sarcoplasmic reticulum and this release was also inhibited by 20 μM Ruthenium red.

Key words: CICR; Ryanodine; Saponin; Smooth muscle; Human vascular

1. Introduction

The processes underlying smooth muscle contraction can involve the activation of voltage and receptor operated Ca^{2+} influx pathways and the mobilisation of Ca^{2+} from an intracellular Ca^{2+} store, the sarcoplasmic reticulum (SR). In human uterine artery vascular smooth muscle cells agonists such as histamine can activate repetitive oscillations in intracellular Ca^{2+} in the absence of external Ca^{2+} [1]. These histamine-induced oscillations almost certainly involve the production of inositol 1,4,5-trisphosphate (IP_3) and the mobilisation of Ca^{2+} from an IP_3 -sensitive intracellular store [2].

There appear to be two distinct functional intracellular mechanisms that are involved in Ca^{2+} release, the IP_3 -sensitive and the ryanodine-sensitive (RyR) receptor channel complexes. Physiological studies suggest that different cell types may functionally express one or both of these receptors [3]. The norepinephrine-induced Ca^{2+} release via IP_3 in rat aorta vascular smooth muscle is unaffected by prior depletion of the caffeine-sensitive Ca^{2+} -releasable Ca^{2+} pool and vice versa [4]. This suggests the presence of at least two functionally separate releasable pools [4]. There is also evidence that IP_3 mobilises all of the Ca^{2+} -releasable Ca^{2+} pools in guinea pig intestinal smooth muscle cells, indicating that functional compartmenta-

tion varies among smooth muscle tissues [5]. Caffeine has been the accepted means with which to identify Ca^{2+} -induced Ca^{2+} release (CICR) in many excitable cells, however, both human vascular and myometrial smooth muscle cells do not respond to caffeine with a release of intracellular Ca^{2+} [6]. The IP_3 receptor can be activated by IP_3 and Ca^{2+} , and has been shown to be inhibited by caffeine in some cells [3]. The IP_3 receptor is capable of considerable variability in the sensitivity of IP_3 -induced Ca^{2+} release, which may give rise to the phenomenon of quantal Ca^{2+} release [7]. These variations may depend on receptor types differently expressed from different gene products, alternative splicing or from co-expression of different IP_3 receptor subtypes in a single cell [8,9]. The inhibitory action of caffeine has also been explained by the reduction of IP_3 production in a dose-dependent manner [10]. Parker and Ivorra using *Xenopus* oocytes found that caffeine inhibited responses to IP_3 and this may be explained by an antagonistic effect of binding of IP_3 to its receptor [11].

The RyR has been studied predominantly in cardiac and skeletal muscle and is sensitive to Ca^{2+} , Mg^{2+} and ATP and may be modulated by calmodulin and activated by caffeine [12]. The physiological role of the RyR in smooth muscle is uncertain, however CICR, which is presumed to be the mechanism of RyR-induced Ca^{2+} release in cardiac tissue [13], has been suggested to occur in skinned smooth muscle [14]. Microspectrofluorimetry studies using intact myometrial smooth muscle cells indicate that a ryanodine-sensitive but caffeine-insensitive Ca^{2+} release mechanism may be operational [15]. Therefore, to investigate directly the activation of these complex responses in human uterine artery vascular smooth muscle cells, chemically skinned cultured cells have been used to explore the operation of a ryanodine-sensitive Ca^{2+} -dependent Ca^{2+} release mechanism.

2. Experimental

2.1. Tissue preparation and culture techniques

Sections of uterine artery (1 cm \times 1–2 mm) from the lower uterine segment were taken, with informed consent, from patients undergoing hysterectomy (women under 40 years of age). Ethical approval was obtained from Newcastle Area Health Authority. Myocytes were prepared using a dispase/collagenase digestion [16] and maintained in M199 (Gibco Ltd.) supplemented with 10% foetal calf serum, 1% glutamine and 2% penicillin/streptomycin at 37°C. Cells were grown to confluence in 75 cm² culture flasks before being passaged onto 12 well plastic multiwells for $^{45}\text{Ca}^{2+}$ efflux studies. Cells were not used beyond passage number 6.

2.2. $^{45}\text{Ca}^{2+}$ efflux from saponin-permeabilised cultured cells

Confluent cells, grown in 12-well multiwells, were washed three times

*Corresponding author. Fax: (44) (191) 222 6706.

with a balanced salt solution containing (mM): 135 NaCl, 5.9 KCl, 1.5 CaCl₂, 1.2 MgCl₂, 11.6 HEPES and 11.5 glucose, pH 7.3 to remove the culture medium before being fixed to a mechanical shaker. Cells were permeabilised with 15 µg/ml saponin for 15 min in the following skinning solution (mM): 120 KCl, 10 HEPES, 2 MgCl₂, 1 ATP and 1 EGTA, pH 7.0 at 22°C. The efficiency of skinning was routinely checked using trypan blue exclusion staining. Traces of saponin were removed by washing the cells three times with loading buffer containing (mM): 120 KCl, 10 HEPES, 5 MgCl₂, 5 ATP, 0.44 EGTA and 5 NaN₃, pH 6.89. The mitochondrial Ca²⁺ ATPase inhibitor NaN₃ was used to eliminate mitochondrial loading and remove any contribution of Ca²⁺ released from mitochondrial pools to the overall efflux of ⁴⁵Ca²⁺. Non-mitochondrial intracellular stores were passively loaded to steady state with 10 µCi of ⁴⁵Ca²⁺ per well for 10 min in 0.5 ml of loading buffer. Loading was terminated by washing three times with ice cold loading buffer prior to the start of efflux. The efflux buffer contained (mM): 120 KCl, 10 HEPES, 1 ATP, 3 EGTA and 5 NaN₃. The amount of CaCl₂ and MgCl₂ required to give solutions of known free Ca²⁺ concentration was calculated using the program REACT [17] and basal Ca²⁺ was taken to be 100 nM. Ca²⁺-activated ⁴⁵Ca²⁺ release was induced by additions of 10 nM–30 µM free Ca²⁺ after a steady baseline flux was obtained in 100 nM free Ca²⁺. ⁴⁵Ca²⁺ was monitored at 4 min time intervals and allowed to proceed for 16–20 min. The remaining ⁴⁵Ca²⁺ in the cells at the end of efflux was determined by solubilising the cells with 2% SDS. All experiments were carried out at 25°C as skinned cell preparations tend to deteriorate more rapidly at higher temperatures [18] and the efflux of radiolabelled Ca²⁺ was determined by liquid scintillation counting. The loss of ⁴⁵Ca²⁺ at each four minute time point was calculated from the cpm as a fraction of the total ⁴⁵Ca²⁺ remaining (including the final SDS fraction) within the cells at that time point.

Data are expressed as means ± S.E.M. Where appropriate, data were statistically analysed using a paired *t*-test and *P* values <0.05 were considered significant.

Collagenase, ryanodine and caffeine were obtained from Sigma, UK. Medium 199 and other tissue culture materials were from Gibco Ltd., UK. Dispace was from Boehringer Mannheim. ⁴⁵Ca²⁺ was purchased from ICN Flow Laboratories.

3. Results

3.1. Ca²⁺-activated ⁴⁵Ca²⁺-release in permeabilised cultured cells

The rate of ⁴⁵Ca²⁺ efflux from saponin-permeabilised cultured vascular smooth muscle cells was determined by incubating the cells in a Ca²⁺ solution containing 100 nM free Ca²⁺, which was used as it is considered to be close to the free Ca²⁺ concentration measured in resting intact cells. When the efflux reached steady state the bathing Ca²⁺ was altered from 100 nM to 10 nM, 300 nM, 1 µM and 10 µM and the subsequent ⁴⁵Ca²⁺ release monitored. If a CICR mechanism is present and operational an elevation in the Ca²⁺ concentration of the bathing medium should increase the loss of ⁴⁵Ca²⁺ from the SR. Fig. 1A shows a typical response obtained using this rationale by changing the free Ca²⁺ level. The response appears to be transient with ⁴⁵Ca²⁺ levels at 10 µM free Ca²⁺ returning to baseline levels after approximately 8 min. A decrease in free Ca²⁺ from a resting level of 100 nM to 10 nM resulted in a decrease in the fractional loss of ⁴⁵Ca²⁺, which may demonstrate that Ca²⁺-dependent ⁴⁵Ca²⁺ release may be operating at the basal level of 100 nM free Ca²⁺. Fig. 1B shows the analysis of the data obtained from 6 separate vascular isolations each with duplicate samples as indicated in Fig. 1A. The mean change in fractional loss increases from 10 nM to 10 µM as the bathing free Ca²⁺ is increased. However, at 30 µM free Ca²⁺ the efflux of ⁴⁵Ca²⁺ was significantly decreased compared to 10 µM Ca²⁺. This reduction may indicate an inhibitory action of Ca²⁺ on the CICR channel or an effect of an altered driving force for Ca²⁺ efflux from the SR.

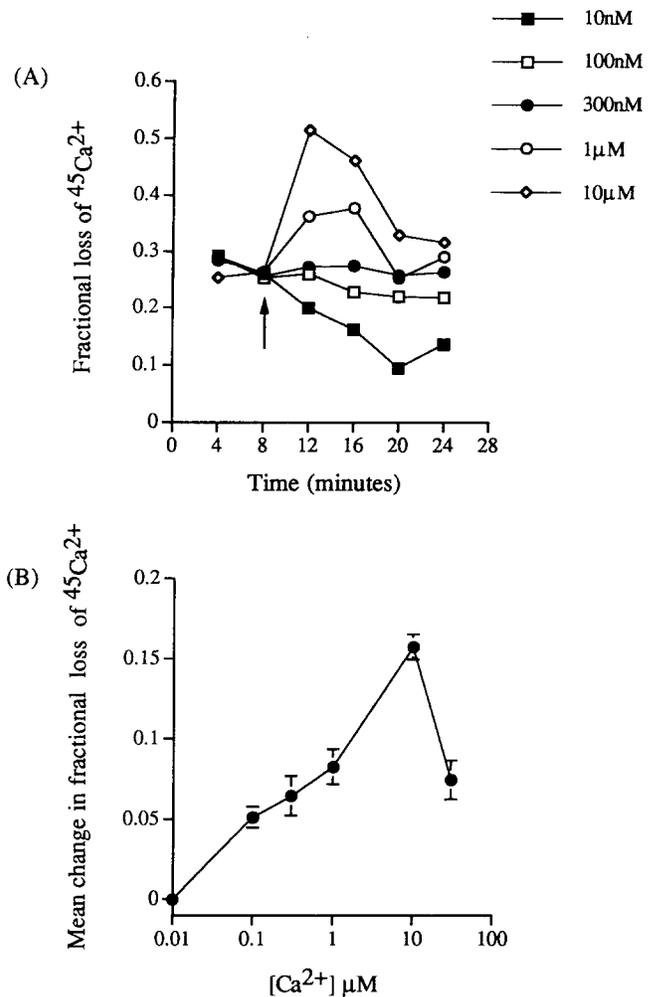


Fig. 1. Ca²⁺-dependent ⁴⁵Ca²⁺ release in saponin-permeabilised cultured human vascular smooth muscle cells. (A) A typical experiment in which the free Ca²⁺ concentration at 8 min (indicated by arrow) is changed from 100 nM (□) to 10 nM (■), 300 nM (●), 1 µM (○) and 10 µM (◇). The cells were exposed to the indicated free Ca²⁺ concentrations until the end of the experiment. (B) Analysis obtained from experiments in A plotted as mean change in fractional loss of ⁴⁵Ca²⁺ (*n* = 6) and the inhibitory effect of 30 µM free Ca²⁺ (*n* = 3). The data in B represents means ± S.E.M.

3.2. Ruthenium red inhibition of Ca²⁺-induced ⁴⁵Ca²⁺ release

Ruthenium red has been used to specifically bind and inhibit Ca²⁺-activated Ca²⁺ release channel complexes from cardiac muscle SR [19]. To demonstrate directly that the ⁴⁵Ca²⁺-dependent Ca²⁺ release response observed in human vascular smooth muscle cells was operating through a CICR channel, cells were pre-incubated with ruthenium red before the step change in bathing free Ca²⁺ concentration. Fig. 2A demonstrates a typical experiment in which pretreatment with 20 µM Ruthenium red completely inhibited the CICR response initiated by 10 µM free Ca²⁺. The analysis of the mean data indicates that Ruthenium red is capable of complete inhibition of the CICR response observed at all free Ca²⁺ concentrations used (Fig. 2B). The application of Ruthenium red was also found to significantly reduce the basal efflux at 100 nM free Ca²⁺ to levels similar to those observed at 10 nM free Ca²⁺ (Fig. 2C). This further supports the suggestion that the Ca²⁺-depend-

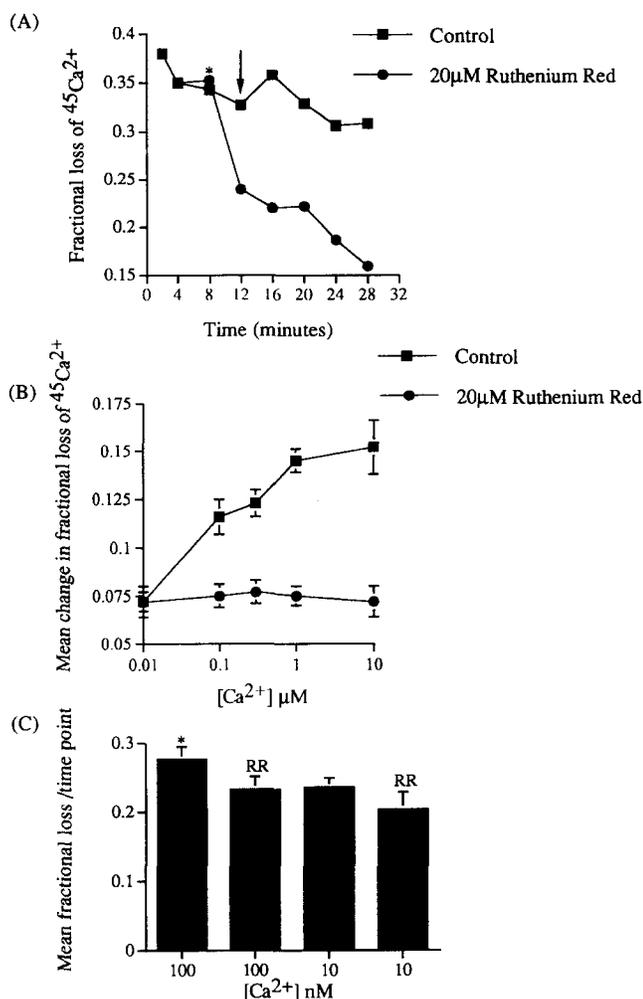


Fig. 2. Effect of Ruthenium red on the Ca^{2+} -dependent $^{45}\text{Ca}^{2+}$ release and basal efflux at 100 nM free Ca^{2+} . (A) A typical experiment in which the Ca^{2+} -dependent $^{45}\text{Ca}^{2+}$ release in response to 10 μM free Ca^{2+} (arrow) is completely inhibited by prior application of 20 μM Ruthenium red (asterisk) until end of the experiment. (B) Analysis of the mean change in fractional loss of $^{45}\text{Ca}^{2+}$ from control Ca^{2+} -dependent $^{45}\text{Ca}^{2+}$ release and Ruthenium red treated permeabilised cells. (C) Bar graph of mean responses over a 20 minute period to illustrate the effect of 20 μM Ruthenium red (RR) on 100 nM and 10 nM free Ca^{2+} basal fluxes (* denotes statistical significant difference where $P < 0.01$). Data represents means \pm S.E.M. for at least four independent experiments, each with duplicate samples.

ent Ca^{2+} release mechanism in human vascular smooth muscle cells may be active at basal levels of cytoplasmic Ca^{2+} .

3.3. Effect of ryanodine and caffeine on $^{45}\text{Ca}^{2+}$ release

Ryanodine is known to have specific effects on the CICR channel of both skeletal and cardiac myocytes and is used to specifically bind and quantitate the RyR in both skeletal and cardiac muscle [20]. At low concentrations ($< 10 \mu\text{M}$) one of its main actions is to induce Ca^{2+} release from SR vesicles incorporated with the RyR protein by locking the channel into a permanently open subconductance state while higher concentrations close the channel [20]. Application of ryanodine (0.1–100 μM) to permeabilised cells exposed to 100 nM free Ca^{2+} induced a progressive dose-dependent release of $^{45}\text{Ca}^{2+}$ (Fig. 3A).

There was no observable inhibition of release at high ryanodine concentrations. The dose-dependent release was not affected by 20 mM caffeine which also had no effect on the basal efflux of $^{45}\text{Ca}^{2+}$ at 100 nM free Ca^{2+} or the Ca^{2+} -dependent $^{45}\text{Ca}^{2+}$ release in response to 10 μM free Ca^{2+} (Fig. 3B,C). The ryanodine-induced Ca^{2+} release was also sensitive to pretreatment with 20 μM Ruthenium red. The presence of Ruthenium red completely blocked the dose-dependent release of $^{45}\text{Ca}^{2+}$ induced by ryanodine (Fig. 3D).

4. Discussion

Both IP_3 receptors and RyR have been isolated from smooth muscle and shown to be localised to the SR, where they are able to function as Ca^{2+} channels enabling the release of Ca^{2+} from the SR [21,22]. The RyR has been shown to be activated through a process of Ca^{2+} -induced Ca^{2+} release (CICR) [14], which occurs following an influx of Ca^{2+} via the action potential and through cyclical SR Ca^{2+} release. This release may induce a positive feedback mechanism by which a localised increase of cytoplasmic Ca^{2+} can propagate as an intracellular Ca^{2+} wave throughout the cell. Caffeine has been used to demonstrate the presence of a RyR channel in many cells [3]. The function of the RyR and the identity of the physiological messenger involved in activation of the receptor in smooth muscle is unknown. The use of caffeine to demonstrate a RyR channel

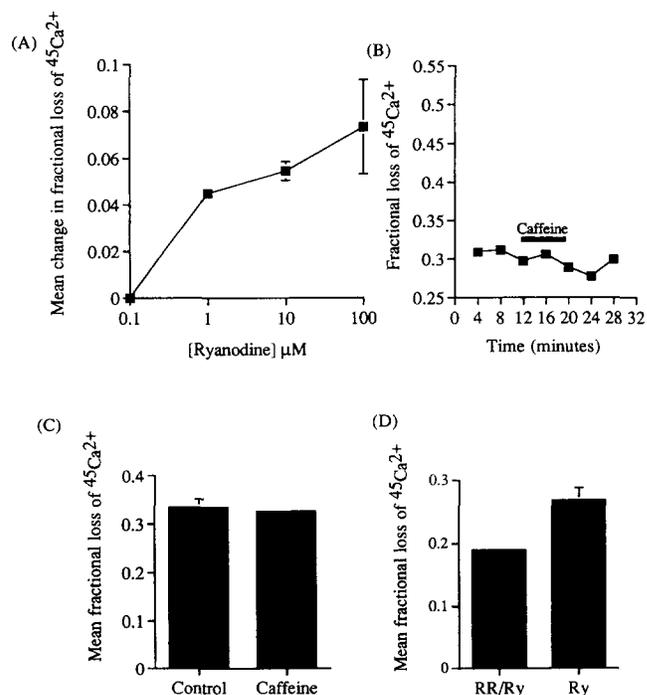


Fig. 3. Effect of ryanodine (0.1–100 μM) and 20 mM caffeine on $^{45}\text{Ca}^{2+}$ release at 100 nM and 10 μM free Ca^{2+} . (A) Mean data of change in fractional loss of $^{45}\text{Ca}^{2+}$ in response to a 4 min exposure to ryanodine at 0.1 μM , 1 μM , 10 μM and 100 μM ($n = 6$). (B) A typical experiment to demonstrate the lack of caffeine (20 mM) sensitivity on basal efflux of permeabilised cells bathed in 100 nM free Ca^{2+} ($n = 5$). (C) Bar graph to demonstrate the lack of effect of a 12 min exposure of 20 mM caffeine on the Ca^{2+} -dependent $^{45}\text{Ca}^{2+}$ release in response to 10 μM free Ca^{2+} ($n = 4$). (D) Bar graph to demonstrate the inhibition of the ryanodine-induced (100 μM) $^{45}\text{Ca}^{2+}$ release by incubation with 20 μM Ruthenium red throughout the experiment ($n = 4$). Data represents means \pm S.E.M.

requires care as the RyR3 isoform expressed in mink lung epithelial cells has been reported to be caffeine-insensitive [23].

Permeabilisation of smooth muscle cells with saponin has allowed filling of the SR with $^{45}\text{Ca}^{2+}$ and precise control of the cytoplasmic environment. The use of a cell culture monolayer is ideal as saponin skinning is able to penetrate evenly into the extracellular space [18]. Skinned cultured smooth muscle cells are suited for measuring $^{45}\text{Ca}^{2+}$ fluxes as it minimises $^{45}\text{Ca}^{2+}$ exchange into the cytoplasm and extracellular space [18]. Chemical skinning with saponin to study Ca^{2+} uptake and release by the SR of smooth muscle cells has the advantage of leaving the SR functionally intact [24]. It is therefore possible to directly stimulate and study a Ca^{2+} -sensitive Ca^{2+} release mechanism by accurately altering the Ca^{2+} content of the bathing solution. The use of permeabilised cells in this study has clearly demonstrated the involvement of free Ca^{2+} levels in the initiation of a CICR response which is sensitive to Ruthenium red in cultured human vascular smooth muscle cells.

It has been shown that Ca^{2+} stores in cultured vascular smooth muscle cells from rat aorta exist as functionally separate entities [25]. In this two-pool model one is sensitive to IP_3 and contains IP_3 receptors and the other is sensitive to caffeine and Ca^{2+} and is associated with a specific RyR [25,26]. These vascular smooth muscle intracellular stores may have both RyR and IP_3 receptors distributed evenly on elements of the SR. Although there is some physiological evidence to support a co-localisation in neurosecretory PC12 cells [27] most studies indicate that these two receptors operate separate Ca^{2+} stores. Studies in the rat vascular smooth muscle cell line A7r5 indicate that the two receptors may operate functionally and spatially distinct Ca^{2+} stores [28]. However, the use of this cell line may require care in the interpretation of results obtained. Missiaen et al. demonstrated a lack of ryanodine and caffeine effect on $^{45}\text{Ca}^{2+}$ fluxes induced by AVP and concluded that CICR was not present [29]. It is possible that these two stores may interact in a co-ordinated manner with each other to generate complex physiological Ca^{2+} signals [3].

The results show that varying the free Ca^{2+} concentration of permeabilised cells from 100 nM to 10 μM increased the $^{45}\text{Ca}^{2+}$ efflux in a dose-dependent manner. The Ca^{2+} concentrations used in the efflux studies to induce CICR are within the range used in other saponin-skinned preparations, such as papillary muscle (1–10 μM) [30], smooth muscle of guinea pig taenia caecum (>3 μM) [31] and rabbit arterial smooth muscle (3–10 μM) [32]. The observation that reducing the free Ca^{2+} concentration to 10 nM reduced the efflux of $^{45}\text{Ca}^{2+}$ below baseline levels is a new observation in these vascular smooth muscle cells and any other cell type investigated. This reduction in the basal efflux may be evidence that a component of the CICR response is operating at basal levels of cytoplasmic Ca^{2+} .

The CICR channel in other smooth muscle cells has been suggested to be inoperative at cytoplasmic (100 nM) levels of Ca^{2+} , in fact, the activation of ryanodine-sensitive CICR in the absence of caffeine required much higher intracellular free Ca^{2+} levels (>1 μM) [14,33]. Increasing the free Ca^{2+} concentration to 30 μM reduced the CICR response to approximately 50% of maximal stimulation with 10 μM free Ca^{2+} in human arterial vascular smooth muscle cells. This biphasic dependence of the efflux rate with respect to Ca^{2+} is similar to the CICR response described in skeletal and cardiac muscle SR [20]. The biphasic relationship of Ca^{2+} release may reflect the operation of more

than one Ca^{2+} -binding site where high Ca^{2+} levels may switch the receptor from a high affinity state that can gate Ca^{2+} to an inactive low affinity state that inhibits release. However, a decrease in the efflux of $^{45}\text{Ca}^{2+}$ at high free Ca^{2+} concentrations (>10 μM), may also reflect a decrease in the Ca^{2+} driving force as the free Ca^{2+} concentration approaches the luminal Ca^{2+} concentration. The contribution of the decrease in driving force to the Ca^{2+} efflux rate requires further investigation.

The RyR Ca^{2+} channel inhibitor Ruthenium red is a polycationic dye that has been reported to inhibit CICR from the SR [34]. This inhibitory action of Ruthenium red was used to investigate the sensitivity and nature of the CICR response in human vascular smooth muscle cells. Application of Ruthenium red (20 μM) to the permeabilised cell preparation prevents CICR-mediated responses at all free Ca^{2+} concentrations used. The basal efflux of $^{45}\text{Ca}^{2+}$ at 100 nM is also sensitive to Ruthenium red which reduces the basal levels similar to those obtained by efflux with 10 nM free Ca^{2+} . This suggests that the minimal $^{45}\text{Ca}^{2+}$ efflux is determined using either of these conditions and so further suggests that a component of CICR is operating at 100 nM or basal cytoplasmic Ca^{2+} levels.

The results also indicate that at 100 nM free Ca^{2+} there is no release of Ca^{2+} following 20 mM caffeine treatment. Similarly, there was no significant enhancement of the CICR response induced in the presence of 20 mM caffeine. This may indicate that the RyR isoform present in these cells is similar to the isoform identified in the mink lung epithelium [23] which is also reported to be caffeine-insensitive. We have previously reported that a ryanodine-sensitive, caffeine-insensitive Ca^{2+} release mechanism is present in intact human myometrial smooth muscle cells [15]. Molecular cloning of RyR-specific transcripts from human myometrial tissue appear to provide evidence for the expression of two isoforms, RyR1 and RyR3 [35]. The myometrial-specific RyR1 isoform may be a tissue-specific transcript derived from the RyR1 gene that lacks the 'foot' region, which is thought to confer caffeine-sensitivity on this receptor [36]. It is possible that human uterine artery smooth muscle in vivo or in the cultured model may possess one or both of these isoforms and so confer the shared property of caffeine insensitivity. This lack of caffeine sensitivity has also been demonstrated using chemically skinned myometrial smooth muscle strips which produced no contractile activity at 0.1–50 mM caffeine [37].

Ryanodine has been shown to stimulate or inhibit Ca^{2+} fluxes in skeletal and cardiac SR depending on the concentration [20]. At concentrations in the range of 0.01–10 μM ryanodine, Ca^{2+} release was stimulated, whereas, at higher concentrations in the range 10–300 μM ryanodine, release was inhibited [20]. This observation suggested that activation and inhibition of SR Ca^{2+} release by ryanodine is mediated by functionally independent sites [38]. In human vascular smooth muscle cells application of 1–100 μM ryanodine induced a progressive dose dependent release of Ca^{2+} from the SR of saponin-permeabilised cells exposed to 100 nM free Ca^{2+} . The maximal release of $^{45}\text{Ca}^{2+}$ by ryanodine is less than the maximal release of $^{45}\text{Ca}^{2+}$ by 10 μM free Ca^{2+} in these cells. This observation may reflect the conformational state of the receptor since ryanodine preferentially binds to the open state of the channel. In the presence of micromolar Ca^{2+} the efflux of $^{45}\text{Ca}^{2+}$ is maximal and this may indicate that the binding and interaction of ryanodine is dependent on the free Ca^{2+} concentration. The ryanodine-induced

$^{45}\text{Ca}^{2+}$ release was sensitive to 20 μM Ruthenium red which inhibited the release of Ca^{2+} at all concentrations of ryanodine used.

It has been shown that millimolar Mg^{2+} can effectively inhibit ryanodine binding to skeletal receptors but has little effect on the binding to cardiac receptors [39]. This Mg^{2+} inhibition of ryanodine binding is due to a direct competition between Ca^{2+} and Mg^{2+} for the Ca^{2+} activation site. In the experimental protocol used to demonstrate the $^{45}\text{Ca}^{2+}$ efflux properties, the efflux solutions used contain 2 mM Mg^{2+} which apparently did not effect the responses demonstrated in this paper. Therefore, the complex responses observed in human vascular smooth muscle cells may be isoform-specific and until a detailed molecular investigation of the RyR isoforms present is complete the exact nature of the caffeine-insensitive ryanodine-sensitivity and Ca^{2+} -dependent Ca^{2+} release mechanism remains unclear.

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