

The modulation and characterisation of the Ca^{2+} -induced Ca^{2+} release mechanism in cultured human myometrial smooth muscle cells

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Abstract The process of Ca^{2+} -induced Ca^{2+} release (CICR) was studied in saponin permeabilised human myometrial smooth muscle cells in which the sarcoplasmic reticulum (SR) was pre-loaded with $^{45}\text{Ca}^{2+}$. A rise in the free Ca^{2+} concentration of the bathing solution from 100 nM to 10 μM increased the rate of $^{45}\text{Ca}^{2+}$ loss, while a reduction to 10 nM decreased the rate of $^{45}\text{Ca}^{2+}$ loss. Ruthenium red (20 μM) lowered the basal rate of $^{45}\text{Ca}^{2+}$ loss and reduced CICR. Caffeine did not activate $^{45}\text{Ca}^{2+}$ release although ryanodine induced $^{45}\text{Ca}^{2+}$ release and CICR was augmented in the presence of caffeine. These data suggest the operation of a Ca^{2+} -activated $^{45}\text{Ca}^{2+}$ release process which is similar in many of its properties to the CICR process described in many cells. The basic properties of the CICR mechanism in myometrium differs in many respects from the CICR process recently described in human vascular smooth muscle.

Key words: Smooth muscle; Ca^{2+} -induced Ca^{2+} release; Human myometrium

1. Introduction

In myometrial smooth muscle, a rise in intracellular Ca^{2+} as a result of a Ca^{2+} influx across the surface membrane, via voltage or receptor-operated ion channels, can activate a further increase in cytoplasmic Ca^{2+} as a result of Ca^{2+} release from intracellular stores [1, (review)]. The total change in cytoplasmic Ca^{2+} is subsequently responsible for activating contraction in smooth muscle. In many cells, the internal mechanisms responsible for these events include an inositol trisphosphate (IP_3)-sensitive receptor/channel complex and a ryanodine-sensitive Ca^{2+} -induced Ca^{2+} release (R-CICR) mechanism [2–5]. Many cell types have been shown to express both of these mechanisms [2], however, the precise role of each system and the possible interaction between the two mechanisms remains unclear.

In cultured myometrial smooth muscle cells, agonists, such as oxytocin, can activate repetitive oscillations in intracellular Ca^{2+} in the absence of external Ca^{2+} [6]. These oscillations are due to the receptor activated production of IP_3 which leads to the release of Ca^{2+} from an IP_3 -sensitive intracellular store [1]. Oxytocin-dependent increases in the production of IP_3 have been reported in intact myometrial smooth muscle cells [7] and recently it has been shown that IP_3 induces a dose-dependent release of $^{45}\text{Ca}^{2+}$ in permeabilised myometrial cells [8]. R-CICR was not thought to be present in the myometrium since caffeine,

a widely used activator of R-CICR, was unable to release Ca^{2+} from an internal store and so did not promote contractions in pregnant rat uterus [9] or mobilise Ca^{2+} from the intracellular stores of single cultured human cells [6]. The effects of caffeine are almost certainly complicated as a result of its known phosphodiesterase activity which leads to an elevation of cAMP and subsequent relaxation of the muscle [9]. Inhibitory actions of caffeine have also been reported on the IP_3 receptor, where caffeine may block the receptor itself [10] or inhibit the production of IP_3 [11]. However, ryanodine was able to mobilise intracellular Ca^{2+} in intact cultured myometrial smooth muscle cells, suggesting that a caffeine-insensitive, ryanodine-sensitive Ca^{2+} release mechanism may be functional in these cells [12].

Three structurally distinct forms of the ryanodine binding Ca^{2+} release channel complex have been identified, RyR1, RyR2 and RyR3 [13]. RyR1 is found predominantly in skeletal muscle and is closely associated with a dihydropyridine (DHP) receptor protein [14]. The RyR1 complex is thought to operate as the voltage sensor of the t-tubular membrane where depolarisation of the t-tubular membrane leads to the release of Ca^{2+} from the sarcoplasmic reticulum (SR). The RyR2 system, found in cardiac muscle, appears to be exclusively activated by cytoplasmic Ca^{2+} derived as a result of Ca^{2+} influx from the external environment. Both RyR1 and RyR2 are known to be modulated by caffeine and calmodulin and are sensitive to Ca^{2+} , Mg^{2+} and ATP [13,15]. A third isoform of the ryanodine receptor/channel complex (RyR3) has been identified in rabbit brain and vascular smooth muscle [16] and in mink lung epithelial cells [17]. The function of RyR3 and its specific sensitivity to Ca^{2+} , ryanodine and other agents are not known. However, it has been reported that the RyR3 isoform in mink lung is not activated by caffeine. The mRNAs encoding for RyR1 and RyR3 have been detected in human myometrial smooth muscle [18] but the details concerning the contribution of these different isoforms to intracellular Ca^{2+} release are not known.

CICR has been described previously in various types of chemically skinned smooth muscle using caffeine [2,19]. Recently, we have reported the presence of a caffeine-insensitive R-CICR mechanism in human vascular smooth muscle cells [20]. The data obtained in the present experiments confirm the presence of a ryanodine-sensitive CICR process in human myometrial smooth muscle cells which demonstrates profound differences in sensitivity to ryanodine, caffeine and ruthenium red compared to the mechanism described in vascular smooth muscle. This paper attempts to highlight these differences and suggests the possible contribution of this Ca^{2+} -mobilising mechanism to the overall Ca^{2+} -signalling processes in human myometrial smooth muscle cells. Part of this work has been published in abstract form [18].

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2. Materials and methods

2.1. Tissue preparation and culture techniques

Myometrial tissue was taken from the lower uterine segment, with informed consent, from patients undergoing either hysterectomy (women under 40 years of age) or elective caesarean section. Ethical approval was obtained from Newcastle Area Health Authority. Myocytes were prepared using a dispase/collagenase digestion [21] and maintained in M199 (Gibco Ltd) supplemented with 10% FCS, 1% glutamine and 2% penicillin/streptomycin. Cells were grown to confluence in culture flasks before being passaged onto plastic 12-well multiwells for $^{45}\text{Ca}^{2+}$ efflux studies. Cells were not used beyond passage number 6. Immunofluorescent staining for α -actin was carried out routinely on cultured cells to check the purity of preparations.

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

Confluent cells, grown in 12-well multiwells, were washed with a balanced salt solution containing (mM): 135 NaCl, 5.9 KCl, 1.5 CaCl_2 , 1.2 MgCl_2 , 11.6 HEPES and 11.5 D-glucose, pH 7.3, to remove the culture medium, before being fixed to a mechanical shaker. Cells were permeabilised at 25°C with 15 $\mu\text{g}/\text{ml}$ saponin for 15 min in the following skinning solution (mM): 120 KCl, 10 HEPES, 2 MgCl_2 , 1 ATP and 1 EGTA, pH 7.0. The efficiency of skinning was routinely checked using trypan blue staining. This permeabilisation technique has been shown to leave the SR functionally intact [19]. It has also been reported that the use of a cultured monolayer of cells in $^{45}\text{Ca}^{2+}$ efflux studies is advantageous since the problem of saponin penetration into tissue strips is overcome, allowing all cells to be skinned simultaneously for the minimum length of time. $^{45}\text{Ca}^{2+}$ exchange into the extracellular space is also minimised under these conditions [22].

Traces of saponin were removed by washing the cells with a loading buffer containing (mM): 120 KCl, 10 HEPES, 5 MgCl_2 , 5 ATP, 0.44 EGTA, 5 NaN_3 and 0.12 CaCl_2 , pH 6.89. The mitochondrial Ca^{2+} ATPase inhibitor NaN_3 was used to eliminate any contribution of Ca^{2+} released from mitochondrial pools to the overall efflux of Ca^{2+} . Non-mitochondrial intracellular stores were passively loaded with 10 μCi of $^{45}\text{Ca}^{2+}$ per well for 15 min in 0.5 ml of the loading buffer. Loading was terminated by washing, at least 3 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 NaN_3 . The amount of CaCl_2 and MgCl_2 required to give solutions of known free Ca^{2+} concentration was calculated using the program REACT [23] and resting Ca^{2+} , in control conditions, was taken to be 100 nM. Unless otherwise stated, paired experiments were carried out where 6 wells per multiwell were used as internal controls. $^{45}\text{Ca}^{2+}$ in the efflux medium was monitored as 2-min time intervals and the remaining $^{45}\text{Ca}^{2+}$ in the cells at the end of efflux was determined by solubilising the cells with 2% SDS. All experiments were carried out at room temperature (25°C) and the efflux of radiolabelled Ca^{2+} was determined by liquid scintillation counting. The loss of $^{45}\text{Ca}^{2+}$ at each time period (i.e. fractional loss) was calculated from the cpm as a fraction of the total $^{45}\text{Ca}^{2+}$ remaining (including the final SDS fraction) within the cells at that time point.

Data are expressed as mean \pm S.E.M. Where appropriate, data were analysed using paired *t* tests and *P* value of < 0.05 was considered significant.

3. Results

3.1. Ca^{2+} -dependent $^{45}\text{Ca}^{2+}$ release in permeabilised cultured cells

Since the physiological stimulus for CICR is, by definition, Ca^{2+} , the effect of changing the free Ca^{2+} concentration in a step wise manner through the range 10 nM to 10 μM was measured on $^{45}\text{Ca}^{2+}$ efflux in permeabilised myometrial smooth muscle cells. The basal level of Ca^{2+} was taken to be 100 nM since this is considered to be close to the resting free Ca^{2+} concentration measured in intact cells. The basal efflux of $^{45}\text{Ca}^{2+}$ reached a steady state within approximately 10 min after which the concentration of Ca^{2+} in the bathing medium was stepped through the range 10 nM to 10 μM Ca^{2+} (Fig. 1A, indicated by bars). The release of $^{45}\text{Ca}^{2+}$ reached a peak after 4 min, declining

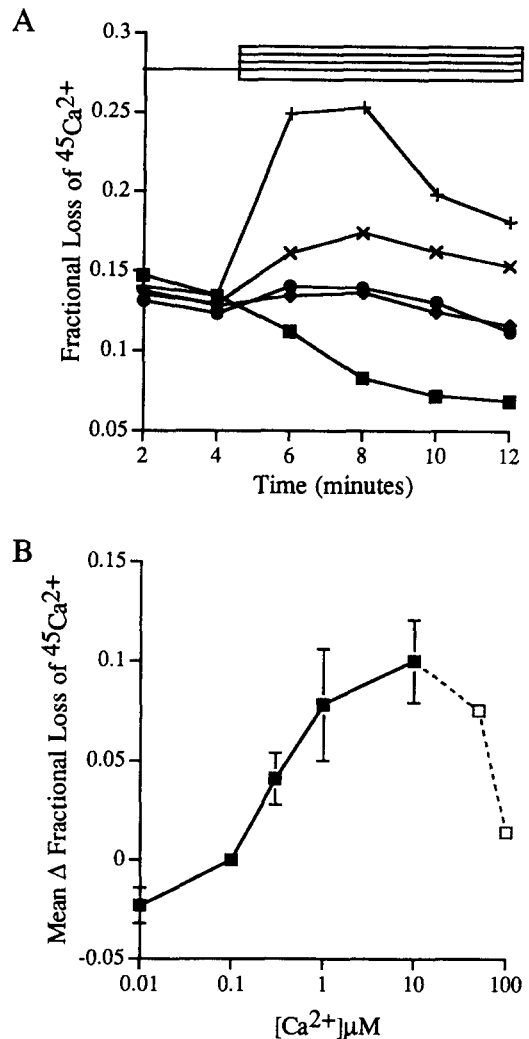


Fig. 1. Demonstration of a Ca^{2+} -dependent $^{45}\text{Ca}^{2+}$ release process in saponin-permeabilised human myometrial smooth muscle cells. A: data from a single experiment (1 of 8) where the concentration of free Ca^{2+} in the bathing solution was changed from 100 nM to 10 nM (■), 100 nM (◆) 300 nM (●), 1 μM (X) and 10 μM (+) at the point indicated by the bar. B: mean data \pm S.E. from 8 experiments as illustrated in Fig. 5A, where results have been plotted to show the amount of $^{45}\text{Ca}^{2+}$ loss at each free Ca^{2+} concentration during the entire period of the step. Data are expressed relative to the loss measured in 100 nM Ca^{2+} . The open symbols (□) and dotted line represents data from a single experiment where the Ca^{2+} concentration range was extended to 50 and 100 μM . Ordinates show fractional loss of $^{45}\text{Ca}^{2+}$ and mean change in $^{45}\text{Ca}^{2+}$ loss, respectively, the abscissae denote time in minutes. Temperature of all experiments, 22–24°C.

slowly over the course of the experiment. Although the responses were not sustained, the efflux at all Ca^{2+} concentrations did not reach baseline values even after 8 min in the respective Ca^{2+} buffers. Incubation of cells in 10 nM Ca^{2+} (■) caused a decrease in the fractional loss of $^{45}\text{Ca}^{2+}$ suggesting that this Ca^{2+} -dependent $^{45}\text{Ca}^{2+}$ release mechanism may be functional at resting levels of Ca^{2+} in the cell. Data analysed from 8 experiments is illustrated in Fig. 1B where the mean change in fractional loss of $^{45}\text{Ca}^{2+}$ released is plotted against the Ca^{2+} concentration of the bathing solution and shows a Ca^{2+} -dependent activation of $^{45}\text{Ca}^{2+}$ loss from the SR in response to the Ca^{2+}

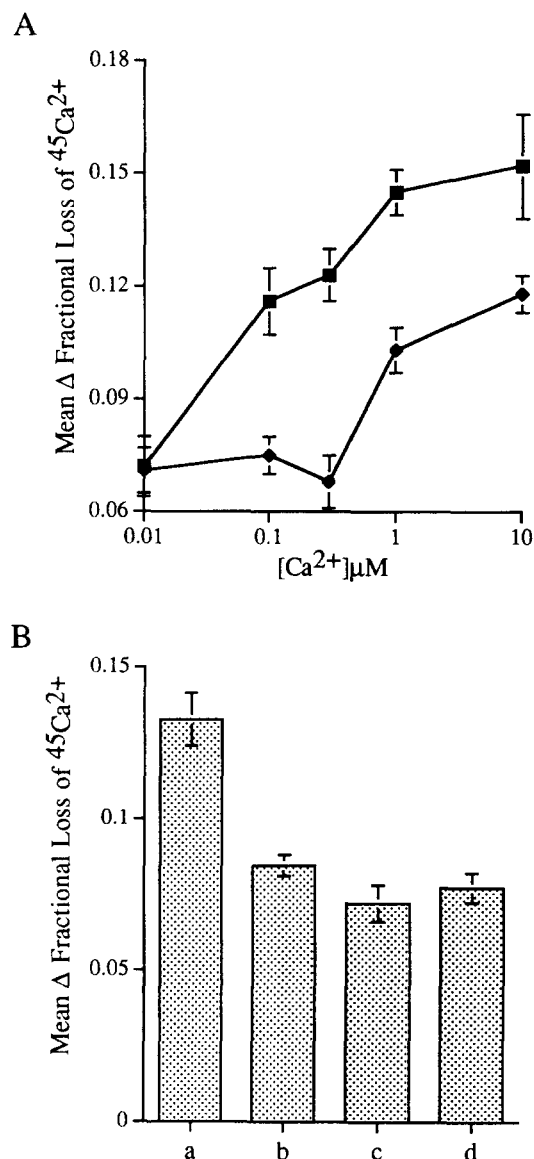


Fig. 2. The effect of ruthenium red on $^{45}\text{Ca}^{2+}$ efflux. A: summarises data from a total of 7 experiments (means \pm S.E.), illustrating the blocking action of ruthenium red on Ca^{2+} -induced $^{45}\text{Ca}^{2+}$ release. (■) shows control CICR data and (◆) shows CICR data in presence of 20 μM ruthenium red. Data was analysed using a Student's paired t test and there is a significant reduction at all Ca^{2+} concentrations, except 10 nM. B: mean data illustrating the efflux of $^{45}\text{Ca}^{2+}$ in (A) 100 nM Ca^{2+} control, (B) 100 nM Ca^{2+} in the presence of ruthenium red, (C) 10 nM Ca^{2+} control and (D) 10 nM Ca^{2+} in the presence of ruthenium red. (B), (C), and (D) are significantly different to (A) where $P < 0.05$. Ordinates show mean change in $^{45}\text{Ca}^{2+}$ loss, the abscissa denotes Ca^{2+} concentration in μM . Temperature of all experiments 22–24°C.

step. Data from a single experiment is shown by the open symbols and dotted line and suggest that, at Ca^{2+} concentrations $> 10 \mu\text{M}$, the Ca^{2+} -induced $^{45}\text{Ca}^{2+}$ release mechanism may decrease. This type of Ca^{2+} step experiment has recently been described in human vascular smooth muscle from the uterine artery [20] where an inhibitory action of Ca^{2+} on the CICR channel has been suggested at Ca^{2+} concentrations $> 10 \mu\text{M}$.

3.2 Inhibition of Ca^{2+} induced $^{45}\text{Ca}^{2+}$ release by ruthenium red

Ruthenium red is routinely used to block the CICR channel

on the SR [24] and Fig. 2 shows data accumulated from 7 paired experiments where cells were incubated in 20 μM ruthenium red for 10 min prior to the Ca^{2+} step. In the concentration range 100–300 nM Ca^{2+} , ruthenium red completely blocked the CICR response, however, there was only 50% inhibition of $^{45}\text{Ca}^{2+}$ release at Ca^{2+} concentrations > 300 nM (Fig. 2A). Data illustrating the effect of ruthenium red on basal $^{45}\text{Ca}^{2+}$ efflux is shown in Fig. 2B where column A and B represents the mean control efflux in 100 nM Ca^{2+} and the effect of ruthenium red on the 100 nM Ca^{2+} efflux, respectively. The results show a significant reduction in basal efflux in the presence of ruthenium red, however, the decrease is not significantly different from the level attained after exposure to 10 nM Ca^{2+} under control conditions (column C) or in 10 nM Ca^{2+} in the presence of ruthenium red (column D). This effect of ruthenium red on basal Ca^{2+} supports the idea that CICR is operating at resting cytoplasmic levels of Ca^{2+} in myometrial cells.

3.3 Modulation of CICR by caffeine

In intact cultured human myometrial cells, caffeine is unable to induce Ca^{2+} release [15]. Similarly, when caffeine is applied as a 2-min pulse in permeabilised cells, there is no change in the fractional loss of $^{45}\text{Ca}^{2+}$ [8]. However, Fig. 3 illustrates data from 5 paired experiments where prolonged incubation of permeabilised cells with 10 mM caffeine, for up to 16 min, caused a small but significant increase in the overall basal efflux of $^{45}\text{Ca}^{2+}$, from a control fractional loss of 0.1052 ± 0.003 to 0.1176 ± 0.003 in the presence of caffeine (Fig. 3A). Fig. 3B illustrates the effect of a prolonged incubation with caffeine on the response of cells to the Ca^{2+} step. There is a small but significant increase in the magnitude of Ca^{2+} -induced $^{45}\text{Ca}^{2+}$ release over the entire range of Ca^{2+} concentrations used. Caffeine appeared to have no effect on the time course of the response (data not shown).

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

We have previously shown that ryanodine can induce a dose-dependent $^{45}\text{Ca}^{2+}$ release from the intracellular stores of permeabilised myometrial cells [8,18]. Fig. 4A shows mean data from 9 experiments illustrating the dose response curve to ryanodine. The curve is complex and demonstrates two bell-shaped curves over the ryanodine concentration range 0.1–100 μM . Fig. 4B demonstrates that this ryanodine-induced $^{45}\text{Ca}^{2+}$ release is sensitive to ruthenium red where column A shows a control response to 1 μM ryanodine and column B illustrates the effect of 20 μM ruthenium red on the 1 μM ryanodine response. Column C shows that heparin (20 $\mu\text{g}/\text{ml}$) does not affect the response to ryanodine.

Caffeine alone does not induce a marked release of Ca^{2+} in intact myometrial cells. However, the present data show that it can modulate the response to ryanodine and Fig. 4C shows a typical experiment (1 of 5 paired experiments) illustrating the effect of caffeine on the ryanodine dose-response curve where caffeine causes an augmentation of the ryanodine induced $^{45}\text{Ca}^{2+}$ release at particularly in the ryanodine concentration range 1–30 μM .

4. Discussion

The present data demonstrate the presence a Ca^{2+} -induced $^{45}\text{Ca}^{2+}$ release mechanism in permeabilised, cultured myomet-

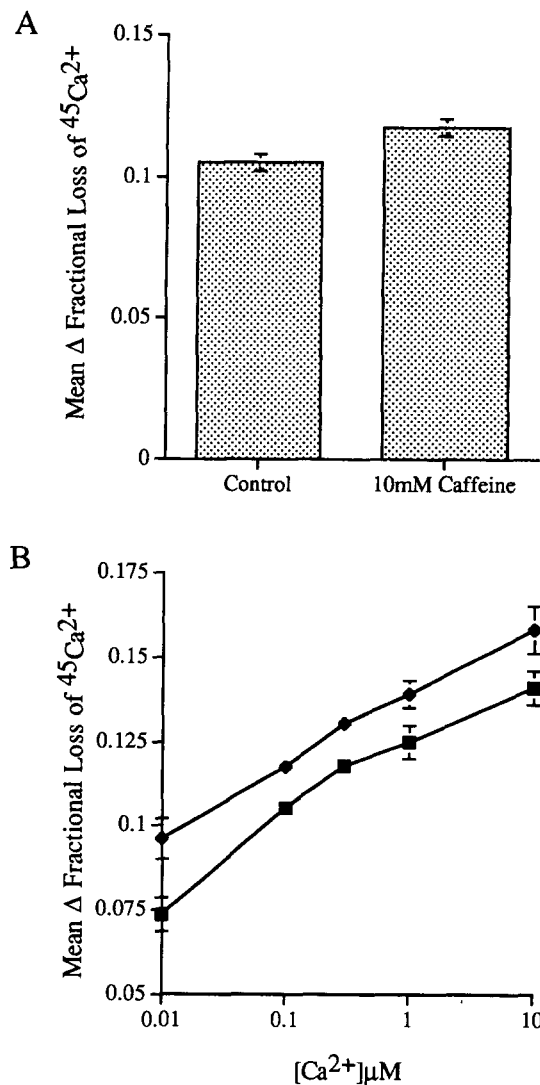


Fig. 3. The effect of caffeine on Ca^{2+} -induced $^{45}\text{Ca}^{2+}$ release. A: a histogram showing mean data from 5 paired experiments illustrating the effect of 10 mM caffeine on the basal flux (100 nM Ca^{2+}) prior to the step. There is a small but significant increase in basal flux in the presence of caffeine where $P < 0.05$. B: mean data \pm S.E. from 5 experiments illustrating the effect of caffeine on CICR over the range 10 nM–10 μM . The increase is significant ($P < 0.05$) at all Ca^{2+} concentrations shown. Ordinates show the mean change in fractional loss of $^{45}\text{Ca}^{2+}$ and the abscissa denotes the concentration of Ca^{2+} in μM . Temperature of all experiments 22–24°C.

rial smooth muscle cells. Using a similar technique of $^{45}\text{Ca}^{2+}$ efflux, CICR has been demonstrated directly in skinned rabbit arterial smooth muscle [25]. The Ca^{2+} concentrations used in this study are similar to those used in other saponin-skinned preparations where Ca^{2+} release from the SR could be facilitated by Ca^{2+} itself when the bathing concentration of Ca^{2+} exceeded 300 nM [20,25,26]. The Ca^{2+} -induced $^{45}\text{Ca}^{2+}$ release mechanism in myometrial cells has many properties of a CICR process mediated by a ryanodine receptor but appears to be different in several respects to the ryanodine-sensitive CICR mechanisms reported in cardiac and skeletal muscle [13,15,27]. In one respect, caffeine has little direct Ca^{2+} -mobilising effect, however, it has been shown to enhance ryanodine-induced

$^{45}\text{Ca}^{2+}$ mobilisation in these cells and it may also modulate R-CICR after prolonged incubation. The lack of a direct stimulatory action of caffeine is in agreement with data reported on pregnant rat uterus [9] and in intact human myometrial cells using optical techniques to monitor intracellular Ca^{2+} [6,20]. The effects of caffeine are almost certainly complicated as a result of its known phosphodiesterase activity which leads to an elevation of cAMP and subsequent relaxation of the muscle [9] as well as its reported effects on IP_3 production and IP_3 binding to its receptor [10,11].

However, the small but significant increase in R-CICR seen after prolonged incubation with caffeine may reflect a differential effect of caffeine on one of the isoforms present in these cells. Two isoforms of the ryanodine receptor/channel complex, RyR1 and RyR3, have been previously identified in myometrial smooth muscle tissue and cultured cells [18]. Additionally, preliminary data using quantitative techniques to assess the relative amounts of each isoform in myometrial tissue indicates that the RyR3 isoform is predominant with RyR1 present only in small amounts (unpublished observations). Since the RyR3 isoform is reported to be caffeine insensitive [17], it is possible that the small effect of caffeine on the CICR response may be due to its effect on RyR1 since, in skeletal muscle, this isoform is known to be caffeine sensitive [15]. However, further studies are required in order to fully characterise the contribution of each isoform to the overall mobilisation of Ca^{2+} in these cells.

Our data show that a reduction in the free Ca^{2+} concentration of the bathing medium from a basal level of 100 nM to 10 nM leads to a reduction in R-CICR suggesting that a component of the R-CICR system appears to be active at Ca^{2+} concentrations similar to those found in the resting cell. This may indicate that R-CICR is involved in the determination of the basal level of Ca^{2+} in the cytoplasm. A similar mechanism has recently been described in human vascular smooth muscle [20] and differs from other smooth muscle types where CICR seems to be inoperative at cytoplasmic levels of Ca^{2+} [3]. At free Ca^{2+} concentrations $>10 \mu\text{M}$, R-CICR is reduced indicating that the process may inactivate at high free Ca^{2+} levels. The biphasic actions of Ca^{2+} suggested from these results are similar to the activation and inactivation of the CICR mechanisms described in skeletal and cardiac tissue [15]. The Ca^{2+} sensitivity of this mechanism suggests that it could operate as a Ca^{2+} -dependent Ca^{2+} leak. If Ca^{2+} in the cytoplasm were to rise, this leak would increase, leading to a further rise in cytoplasmic Ca^{2+} .

The possibility that the Ca^{2+} -induced $^{45}\text{Ca}^{2+}$ release observed in these cells is a direct result of the driving force for Ca^{2+} , i.e., Ca^{2+} – Ca^{2+} exchange across the SR must be considered. However, since the R-CICR response is sensitive to ruthenium red and modulated by other factors such as caffeine this possibility seems unlikely.

Ruthenium red was used in this study to characterise the nature of the R-CICR response since it has been reported to inhibit R-CICR in cardiac muscle [24]. Complete inhibition of the R-CICR response was demonstrated at free Ca^{2+} concentrations between 10 nM and 300 nM but only 50% inhibition was observed between 300 nM and 10 μM Ca^{2+} . In contrast, the R-CICR response in human vascular smooth muscle cells was completely abolished in the presence of ruthenium red over the entire range of free Ca^{2+} concentrations used [20]. These results may indicate that one of the isoforms present in myometrial cells is insensitive to ruthenium red or alternatively, there

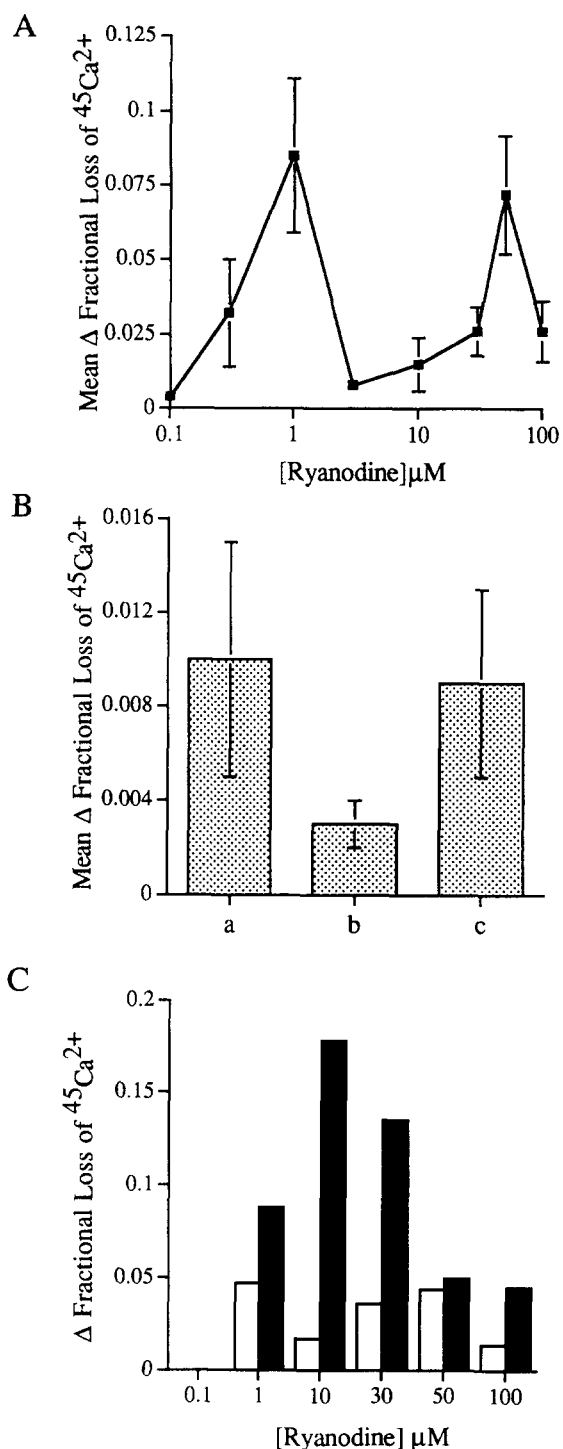


Fig. 4. The effect of ryanodine on $^{45}\text{Ca}^{2+}$ release. A: mean data \pm S.E. from 9 experiments illustrating the dose–response relationship for ryanodine in cultured human myometrial smooth muscle cells. B: a histogram showing mean data \pm S.E. from 4 paired experiments illustrating the effect of ruthenium red and heparin on the ryanodine induced $^{45}\text{Ca}^{2+}$ release from the SR where (A) illustrates the mean control response to 1 μM ryanodine, (B) and (C) ryanodine-induced release in the presence of 20 μM ruthenium red and 20 $\mu\text{g}/\text{ml}$ heparin, respectively. C: a typical experiment (1 of 5) illustrating the effect of 10 mM caffeine on the ryanodine dose–response curve. The open bars show control ryanodine responses and the filled bars show ryanodine responses in the presence of 10 mM caffeine. Ordinates show the mean change in fractional loss of $^{45}\text{Ca}^{2+}$ and abscissa in A and C show the concentration of ryanodine in μM . Temperature of all experiments 22–24°C.

may be other CICR responses which are not mediated through a ryanodine-sensitive channel [28].

In skeletal and cardiac muscle, ryanodine has been used to specifically bind to and quantitate the RyR channel. In these tissues, the dose–response relationship to ryanodine is bell-shaped where the RyR channel is locked into a permanently open state at concentrations $<10 \mu\text{M}$ and at higher concentrations of ryanodine the channel is blocked [15]. Ryanodine has been reported to mobilise Ca^{2+} from internal stores of intact cultured human myometrial smooth muscle cells and this sensitivity led to the suggestion that a ryanodine-sensitive Ca^{2+} release process may be operational [12]. The present data in permeabilised cells demonstrates a complex dose–response relationship to ryanodine over the concentration range 0.1–100 μM where the cells appear to have two bell-shaped responses. This effect is markedly different to the dose–response curve obtained in vascular smooth muscle cells where there is an increase in the ryanodine-induced $^{45}\text{Ca}^{2+}$ release over a similar concentration range [20]. The significance of these differences is unknown at the present time but in myometrial cells the presence of two isoforms of the ryanodine receptor–channel complex may contribute to the complex effects observed with ryanodine. The ryanodine-induced $^{45}\text{Ca}^{2+}$ release was sensitive to ruthenium red, suggesting that these responses are not a non-specific effect of ryanodine but reflect the specific binding of ryanodine to the RyR channel.

It can be concluded from the data reported here that a R-CICR mechanism is operational in cultured human myometrial smooth muscle cells and that the activation and modulation of this process is complex. It would also appear that although this R-CICR process displays some similarity to the R-CICR mechanism described in human vascular smooth muscle cells [20], there are clear differences in the pharmacological modulation by various chemical agents. The physiological significance of these differences and the molecular mechanisms underlying them remain to be determined.

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