Volume 330, number 2, 227–230 FEBS 12973 September 1993

A novel ryanodine sensitive calcium release mechanism in cultured human myometrial smooth-muscle cells

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Received 23 July 1993

In cultured human myometrial cells application of caffeine (1–30 mM) did not result in an elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$). Caffeine was found to reversibly inhibit both spontaneous and agonist-induced repetitive rises in $[Ca^{2+}]_i$ possibly as a consequence of its ability to interfere with the binding of inositol trisphosphate (IP_3) to the receptor on the sarcoplasmic reticulum. Brief applications of ryanodine (1–10 μM) were observed to elevate $[Ca^{2+}]_i$ and repeated exposures to ryanodine could elicit Ca^{2+} transients of similar magnitude. Ryanodine was also observed to mobilise Ca^{2+} in cells bathed in nominally Ca^{2+}-free solution. These observations suggest the presence of a novel type of ryanodine-sensitive Ca^{2+}-induced Ca^{2+} release (R-CICR) system in human myometrial cells.

Ryanodine; Caffeine; Intracellular calcium; Myometrial smooth muscle

1. INTRODUCTION

The mechanisms which lead to a rise in Ca^{2+} in myometrial smooth muscle have been shown to include voltage and receptor operated influx pathways and the mobilisation of Ca^{2+} from intracellular stores [1]. The experiments described in this paper were carried out to identify the nature of the internal Ca^{2+} stores in cultured human myometrial smooth muscle cells using the pharmacological agents caffeine and ryanodine.

In many smooth muscle cell types there appear to be two distinct intracellular stores of Ca^{2+}, which may interact with each other to generate Ca^{2+} signals. One store can be activated by the methyxanthine caffeine to release Ca^{2+} and is sensitive to the plant alkaloid ryanodine. The purified ryanodine receptor from the sarcoplasmic reticulum is a large molecular mass protein which associates as a homotetramer with a large conductance cation channel upon incorporation into planar lipid bilayers [2]. The morphology of the purified ryanodine receptor is identical to the 'feet' structures first seen to span the transverse tubule–sarcoplasmic reticulum junction in striated muscle [3]. Expression studies suggest that the large molecular weight 'foot' protein is the ryanodine receptor and sarcoplasmic reticulum Ca^{2+} release channel [4] and is sensitive to both caffeine and ryanodine. At low concentrations (nanomolar range), ryanodine will promote Ca^{2+} release while at high concentrations (micromolar range) Ca^{2+} release is inhibited [5]. A second intracellular Ca^{2+} store can be activated by inositol trisphosphate (IP_3) [6]. This mechanism is inhibited by caffeine which interferes with the production of IP_3 [7]. Both stores can be activated by Ca^{2+} and consequently operate as Ca^{2+}-induced Ca^{2+} release (CICR) systems. Therefore, these mechanisms may be referred to as the IP_3-sensitive CICR (I-CICR) and the ryanodine-sensitive CICR (R-CICR) systems.

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2. MATERIALS AND METHODS

Human myometrial tissue was obtained from patients with consent from the lower uterine segment from patients undergoing elective caesarean section at term and from hysterectomy (patients under 40 years of age). Ethical approval was granted by Newcastle Area Health Authority. Samples were immediately placed in culture medium 199 (M199; 25 mM HEPES, without Na^+ pyruvate and with 450 mg/l glucose, Gibco Ltd.). Myocytes were prepared by enzymatic dispersion as previously described [8]. Cells were incubated for 1 h at 37°C with the Ca^{2+}-sensitive fluorochrome Fura-2 in M199 containing 5 μM of the acetoxy-methyl ester form of the dye. Cells were placed in a 200 μl bath on the stage of the microscope and solutions were perfused through the bath at 5–8 ml/min. Cells were superfused at 34–37°C with a normal balanced salt solution containing (mM): 140 NaCl; 4.5 KCl; 2.5 $CaCl_2$; 1 $MgCl_2$; 1 NaH_2PO_4; 5 glucose and 10 HEPES buffered to pH 7.4. Balanced salt solutions without Ca^{2+} were supplemented with 200 μM EGTA and used as indicated in the results. Caffeine and oxytocin were obtained from Sigma Chemicals, Dorset, UK. Ryanodine (Lot No. 49626) was obtained from ICN Biochemicals, Cleveland, Ohio.

Fluorescence measurements were carried out using a cooled integrating charged coupled device (CCD) imaging system (Newcastle Photometric Systems, Seaton Burn, Newcastle, UK). During an experiment the light intensity was measured at wavelengths greater than 520 nm using excitation wavelengths of 350/380 nm and from this the wavelength intensity ratio was calculated. This ratio was displayed as a function of time for each experimental area and used as an approximation of $[Ca^{2+}]_i$ [9].

An extracellular calibration was carried out on the system using 10 μM solutions of the penta-potassium salt of the dye in buffers of known Ca^{2+} concentration. This concentration gives fluorescence val-

ues similar to those obtained in dye-loaded cells. Calibration curves can be constructed from the ratio obtained for increasing free Ca^{2+} concentration. However, it must be noted that there are many problems associated with the calibration of Fura-2 and it is generally accepted that this type of calibration cannot be used to estimate absolute ion concentrations in cells loaded with the ester form of the dye [10]. Therefore, all results in this paper will be expressed in terms of fluorescence ratio changes only.

3. RESULTS

In an initial series of experiments cells were exposed to caffeine (1–30 mM) in a balanced salt solution (Fig. 1A). Application of caffeine did not result in an increase in $[Ca^{2+}]_i$. Superfusing a cell with oxytocin (10^{-9} M) led to the activation of a series of transient increases in $[Ca^{2+}]_i$. These agonist-induced oscillations were found to be reversibly inhibited by caffeine (10–30 mM). Fig. 1B shows an example of the inhibition caused by 10 mM caffeine. Approximately 15% of myometrial cells in culture demonstrate spontaneous oscillations in $[Ca^{2+}]_i$ [8]. Fig. 1C illustrates this type of Ca^{2+} activity and shows that these spontaneous oscillations were also inhibited by the addition of 10 mM caffeine to the bathing solution. The absence of any Ca^{2+} mobilising action of caffeine in these cells suggests that the CICR mechanism existing in myometrial cells differs from the caffeine-activated R-CICR systems found in skeletal and cardiac muscle and neural tissue.

If the classical R-CICR system were to operate in these cells then ryanodine, which acts predominantly on the R-CICR system, should have no effect on $[Ca^{2+}]_i$. However, when cells were exposed to ryanodine (1–10 μM), in a normal balanced salt solution for 60–100 seconds, a transient increase in $[Ca^{2+}]_i$ was observed. This effect occurred after a short delay of between 40–50 s and Ca^{2+} returned to resting levels after exposure (Fig. 2A). Several transient responses to ryanodine, all of similar amplitude, could be elicited in the same cell. Fig. 2B shows an example of an experiment to determine the site of action of ryanodine. In cells washed in nominally Ca^{2+}-free solution, ryanodine was still capable of elevating $[Ca^{2+}]_i$. The transient increase in $[Ca^{2+}]_i$ was smaller in amplitude than responses seen in Ca^{2+}-containing solution and consistently exhibited an unresolved double-peak response. Under these conditions the Ca^{2+} must be derived solely from intracellular stores, indicating that ryanodine is capable of modulating the release of stored Ca^{2+}.

4. DISCUSSION

The inhibitory effects of caffeine on agonist-induced generation of intracellular Ca^{2+} oscillations are well documented [11,12]. The mechanism by which caffeine exerts its inhibitory effect is thought to involve the second messenger IP_3. Microinjection of IP_3, in *Xenopus* oocytes, in the presence of caffeine failed to elevate

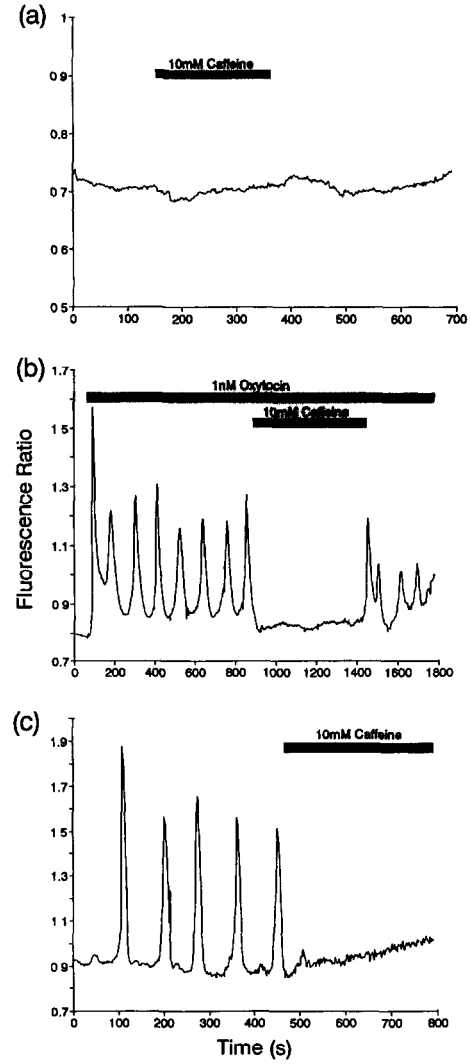

Fig. 1. (a) illustrates the lack of response to 10 mM caffeine on a single cultured human myometrial cell; (b) shows the inhibitory action of 10 mM caffeine on Ca^{2+} oscillations induced by exposing the cells to 10^{-9} M oxytocin; (c) demonstrates the inhibition of spontaneous oscillations in $[Ca^{2+}]_i$ by the application of 10 mM caffeine. Application of caffeine and oxytocin to the bathing solution are indicated by the solid bars. Ordinates denote the ratio of fluorescence intensity measured at 350/380 nm, abscissae show time in seconds. Temperature 34°C.

$[Ca^{2+}]_i$ suggesting that the inhibitory actions of caffeine were either at the level of IP_3 binding to the sarcoplasmic reticulum or at the level of the subsequent release of Ca^{2+} [13]. In human myometrial smooth muscle cells it has been shown that oxytocin will elevate IP_3 [14] and it is suspected that IP_3 can mobilise Ca^{2+} from intracellular stores [6]. Since it is thought that caffeine inhibits the production of IP_3 [7], this mechanism may provide an explanation for the ability of caffeine to block the agonist-induced oscillations in intracellular Ca^{2+} seen in these cells. Spontaneous oscillations in $[Ca^{2+}]_i$ are also inhibited by caffeine which may indicate an involvement of basal levels of IP_3 in their production. These observa-

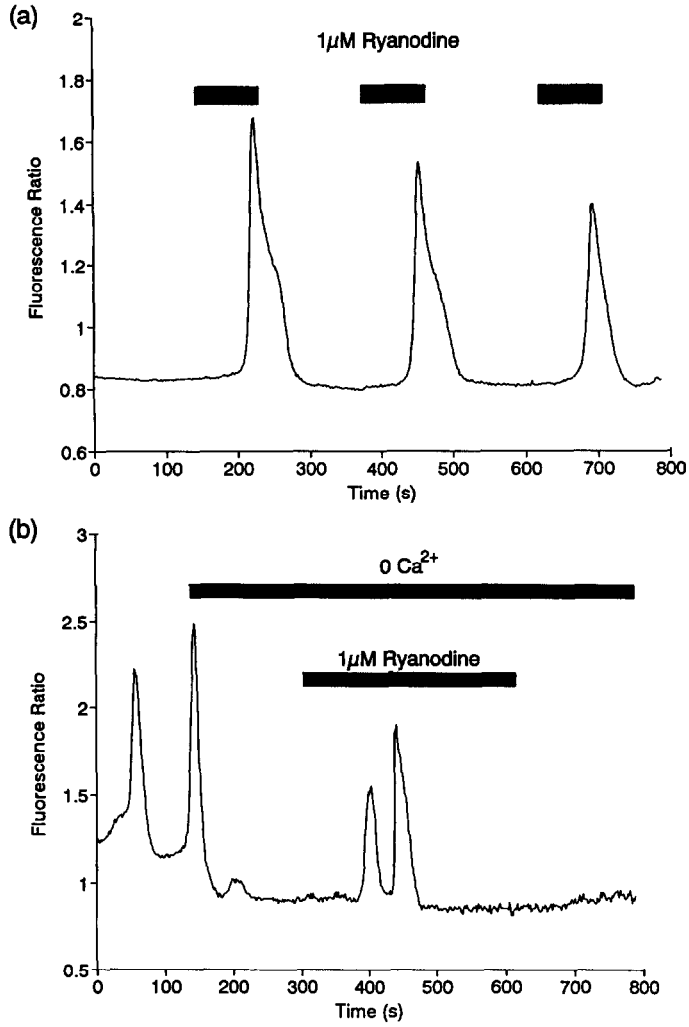

Fig. 2. (a) shows the effect of repeated brief applications of 1 μM ryanodine on $[Ca^{2+}]_i$; (b) illustrates the effect of 1 μM ryanodine on a spontaneous cell bathed in a nominally Ca^{2+}-free solution. Application of ryanodine and nominally Ca^{2+}-free solution are indicated by the solid bars. Ordinates denote the ratio of fluorescence intensity measured at 350/380 nm, abscissae show time in seconds. Temperature 33°C.

tions are consistent with the hypothesis that these cultured human myometrial cells possess a caffeine-sensitive, IP_3-dependent intracellular Ca^{2+} release mechanism (I-CICR). This data also shows that caffeine has no Ca^{2+} mobilising characteristics and indicates that these cells do not possess the classical ryanodine-sensitive intracellular Ca^{2+} store.

At low concentrations (< 10 μM) ryanodine binds to the open Ca^{2+} channel of the Ca^{2+}-induced Ca^{2+} release protein of skeletal and cardiac muscle and maintains the channel in the open state [5]. Ryanodine dissociates from the channels slowly, and consequently Ca^{2+} is lost from the stores despite the activity of Ca^{2+}-ATPase driven pumps. At high concentrations (> 20 μM) ryanodine is capable of inhibiting the Ca^{2+} channels of the release protein, by firstly activating the channel which then slowly closes as it reaches a more stable configuration [15]. The present experiments show that ryanodine can reversibly affect the release of Ca^{2+} from internal stores. Assuming that this effect of ryanodine is not a nonspecific toxic effect, it suggests that there are receptors for ryanodine on the internal membranes of these cells and that activation leads to an opening of Ca^{2+} channels on the sarcoplasmic reticulum resulting in the release of stored Ca^{2+}.

The R-CICR release channels in skeletal and cardiac muscle have been isolated and their structures determined [2,16–18]. Although there is a great deal of similarity between the proteins, specific differences in both structure and function have been noted [19]. This has led to the classification of the proteins as R1-CICR in striated muscle and R2-CICR in cardiac muscle. The structural differences between R1- and R2-CICR are probably related to their different means of activating the Ca^{2+} release mechanisms. For example, the R1-CICR system is linked to the voltage sensor on the T-tubular membrane while the R2-CICR mechanism is not. A third type of ryanodine receptor protein (R3-CICR) has been identified recently in mink lung epithelial cells [20] and in restricted areas of the rabbit brain [21]. Using a cDNA probe for the brain ryanodine receptor it was found that an RNA species of similar size hybridised in many tissues containing smooth muscle, including the uterus [21]. The consequences of the alterations in the structure of the R3-CICR system are at present unclear. However, in the mink lung epithelial cells, this R3-CICR system does not appear to be caffeine-sensitive [20].

In conclusion, it would appear that these cultured human myometrial cells possess an IP_3-dependent intracellular Ca^{2+} release mechanism (I-CICR) and, in addition, may have a second intracellular system which appears to be ryanodine-sensitive. The caffeine sensitivity of this system remains to be established. The role of this second Ca^{2+} storage system in the human myometrium is presently unknown. This novel ryanodine-sensitive system may be similar to the R3-CICR system described in mink lung epithelial cells and involve the protein sequenced from rabbit brain. If this R3-CICR system were to occur in other tissues, care must be exercised in using methylxanthines to diagnose the presence of a R-CICR or eliminate R-CICR from the cellular response, for example in order to investigate IP_3-induced Ca^{2+} release.

Acknowledgements: We gratefully acknowledge the support of the British Heart Foundation and The Physiological Society.

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