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Multiple effects of ryanodine on intracellular free Ca^{2+} in smooth muscle cells from bovine and porcine coronary artery: modulation of sarcoplasmic reticulum function

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1 The effects of ryanodine and caffeine on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) were studied by use of fura-2 microfluorometry in single smooth muscle cells freshly dispersed from bovine and porcine coronary artery.

2 Bovine and porcine cells demonstrated similar sensitivities to 10 min of exposure to ryanodine in physiological salt solution (PSS), as determined by comparable dose-dependent decreases in the subsequent $[Ca^{2+}]_i$ transient induced by 5 mM caffeine.

3 Ryanodine (10 μM) caused a significant increase in $[Ca^{2+}]_i$ to a plateau level 27 ± 3% and 38 ± 4% above baseline $[Ca^{2+}]_i$ (baseline $[Ca^{2+}]_i$ = $[Ca^{2+}]_i$ at 0 min) in porcine and bovine cells, respectively, when bathed in PSS. In bovine cells the time required to reach ½ the plateau level was only 3 min versus 6 min for porcine cells.

4 The ryanodine-induced plateau increase in $[Ca^{2+}]_i$ was 35 ± 5% above baseline for bovine cells bathed in 0 Ca PSS (PSS including 10 μM EGTA with no added Ca^{2+}), but only 7 ± 3% above baseline in porcine cells during 10 min exposure to 10 μM ryanodine. In bovine cells $[Ca^{2+}]_i$ showed proportional increases when extracellular Ca^{2+} was increased from the normal 2 mM Ca^{2+} PSS to 5 and 10 mM.

5 Cells pretreated with caffeine in 0 Ca PSS, which depleted the caffeine-sensitive sarcoplasmic reticulum Ca^{2+} store, showed no increase in $[Ca^{2+}]_i$ when challenged with 10 μM ryanodine. The ryanodine-associated increase in $[Ca^{2+}]_i$, which was sustained in 0 Ca PSS during the 10 min ryanodine exposure in cells not pretreated with caffeine, suggests that ryanodine releases Ca^{2+} from the sarcoplasmic reticulum, but also inhibits Ca^{2+} efflux.

6 Intracellular free Ba^{2+} ($[Ba^{2+}]_i$) was measured with fura-2 microfluorometry to define further the Ca^{2+} efflux pathway inhibited by ryanodine; specifically, Ba^{2+} is not transported by the Ca^{2+} pump, but will substitute for Ca^{2+} in Na^+-Ca^{2+} exchange. In porcine cells pretreated with caffeine in 0 Ca PSS to deplete the caffeine-sensitive sarcoplasmic reticulum Ca^{2+} store, depolarization with 80 mM K^+ in 2 mM external Ba^{2+} caused a 100 ± 6% increase in fura-2 fluorescence ($[Ba^{2+}]_i$). During the 17.5 min 0 Ca PSS recovery from depolarization, exposure to 10 μM ryanodine inhibited the removal of $[Ba^{2+}]_i$ by 69 ± 3% when compared with control (0 Ca PSS without ryanodine).

7 It was concluded that in bovine and porcine smooth muscle cells: (a) ryanodine (≥ 10 μM) releases Ca^{2+} from the sarcoplasmic reticulum; (b) ryanodine (≥ 10 μM) decreases Ca^{2+} efflux, probably by inhibition of Na^+-Ca^{2+} exchange; (c) the sarcoplasmic reticulum Ca^{2+} store may be larger in bovine than in porcine smooth muscle cells; thus, porcine cells have a relatively greater reliance on Ca^{2+} influx to increase $[Ca^{2+}]_i$.

Keywords: Caffeine; Ca^{2+}; ryanodine; sarcoplasmic reticulum; vascular smooth muscle; Ba^{2+}; Na^+-Ca^{2+} exchange, Ca^{2+} efflux

Introduction

Intracellular free Ca^{2+} ($[Ca^{2+}]_i$ and its regulation are crucial determinants of smooth muscle contractility. The sarcoplasmic reticulum (SR) has been identified as the primary intracellular organelle which modulates or buffers $[Ca^{2+}]_i$ through Ca^{2+} sequestration/storage or agonist-induced Ca^{2+} release (Van Breemen *et al.*, 1988; Van Breeman & Saida, 1989; Wagner-Mann *et al.*, 1991). A thorough understanding of the SR and its roles in vascular smooth muscle function, however, is yet to be attained.

Pharmacological intervention, with agents such as caffeine, is a useful approach to a better understanding of the SR and its functions (Palade *et al.*, 1989). While much information can be acquired regarding the release of Ca^{2+} stored in the SR by use of the SR Ca^{2+} releasing property of caffeine, there are limitations to this use. Reportedly, caffeine interferes with Ca^{2+} influx (Leijten & Van Breemen, 1984) and may increase adenosine 3':5'-cyclic monophosphate (cyclic AMP) and promote SR Ca^{2+} pump activation (Van Breemen & Saida, 1989), thereby potentially confounding the interpretation of $[Ca^{2+}]_i$ regulation by the SR.

Another, seemingly more SR-specific agent is ryanodine, a neutral alkaloid of plant origin (Jenden & Fairhurst, 1969; Sutko *et al.*, 1985). It appears to deplete the SR Ca^{2+} store in smooth muscle, thereby inhibiting or attenuating agonist-induced contraction dependent on Ca^{2+} release (Kanmura *et al.*, 1988; Ashida *et al.*, 1988; Nishimura *et al.*, 1989). The use of ryanodine in smooth muscle experimentation is not without controversy, however, as Ito *et al.* (1986) reported that in guinea-pig aorta, ryanodine inhibited release of intracellular stores of Ca^{2+}. Furthermore, in this laboratory, with the use of ryanodine as a tool for the study of SR function in vascular smooth muscle, species (bovine versus porcine) differences have been noted in response to 10 μM ryanodine (Wagner-Mann & Sturek, 1991; Wagner-Mann *et al.*, 1991). Planar lipid bilayer experiments on SR membranes derived from cardiac and skeletal muscle have focused on examination of ryanodine modulation of single Ca^{2+} release

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channel activity (Palade *et al.*, 1989; Nagasaki & Fleischer, 1989; Chu *et al.*, 1990). These studies, while providing valuable detailed information regarding pharmacology and biophysics of SR Ca^{2+} release channels, do not assess integrated effects of ryanodine in the intact cell. These considerations may profoundly influence the use of ryanodine as a pharmacological tool (Sutko *et al.*, 1985; Sutko & Kenyon, 1990).

The purpose of this study, therefore, was to characterize more fully the actions of ryanodine on $[Ca^{2+}]_i$ regulation in single vascular smooth muscle cells. Specifically we determined whether ryanodine: (1) depletes the SR and/or blocks Ca^{2+} release from the SR; and (2) alters $[Ca^{2+}]_i$ buffering by interfering with Ca^{2+} efflux. Also, we found that differences in responses to ryanodine exist between porcine and bovine coronary smooth muscle. The information gained will increase the value of ryanodine as a pharmacological tool to study SR function in $[Ca^{2+}]_i$ buffering/release in vascular smooth muscle.

Methods

Smooth muscle cell dispersion

Bovine and porcine hearts were obtained fresh from local abattoirs. The left circumflex and right (bovine), or only right (porcine), coronary arteries were dissected from the hearts by sterile techniques less than 30 min following the humane stunning and exsanguination of the animals. The arteries were immediately placed in the following iced medium (in mM): $CaCl_2$ 2, NaCl 135, $MgCl_2$ 1, KH_2PO_4 0.44, Na_2HPO_4 0.34, $NaHCO_3$ 2.6, HEPES 20, glucose 10; 2% horse serum (Hazelton, Lenexa, KS); dilutions (vol:vol) of amino acids (0.02), vitamins (0.01), phenol red (0.002), penicillin/streptomycin (0.01; Gibco, Grand Island, NY, U.S.A.); pH 7.4 with NaOH. Attached cardiac muscle and connective tissue were dissected free by sterile technique in a modification of the above solution, containing low Ca (0.5 mM $CaCl_2$) and no horse serum. Single smooth muscle cells were obtained by enzymatic dispersion techniques described in detail previously (Wagner-Mann *et al.*, 1991; Sturek *et al.*, 1991c). A 1.5 cm long segment was cut longitudinally and pinned lumen side up in a 30 ml jar which contained 2 ml of the dispersion solution (low Ca^{2+} solution also containing 294 uml^{-1} collagenase (CLS II, Worthington), 2 mg ml^{-1} bovine serum albumin (Fraction V, Sigma), 1 mg ml^{-1} soybean trypsin inhibitor (SI, Worthington), and 0.4 mg ml^{-1} DNase I (Type IV, Sigma)). The jar was then placed in a heated (37°C) shaking water bath for 1 h per dispersion fraction. As cells of the first fraction are predominantly endothelial cells, identifiable by their distinct morphology and uptake of acetylated low density lipoprotein (Sturek *et al.*, 1991b), cells from the second or third fraction were used for the experiments. These cells were compared morphologically to cultured cardiac fibroblasts derived from primary biopsy explant cultures. The smooth muscle cells themselves, besides being characteristically oblong in shape, were more readily identified by their contractile responses to agonists such as caffeine (see Results below for more details) (Wagner-Mann *et al.*, 1991; Sturek *et al.*, 1991c).

Microfluorometry

Whole single cells were studied with fura-2 to measure $[Ca^{2+}]_i$ and $[Ba^{2+}]_i$ (Grynkiewicz *et al.*, 1985; Schilling *et al.*, 1989) by microfluorometry methods similar to those described by Thayer *et al.* (1988) and more fully described in other papers from this laboratory (Wagner-Mann *et al.*, 1991; Sturek *et al.*, 1991b,c). Briefly, sample-and-hold analog circuitry amplifiers were used to feed the separate analog signals corresponding to the 340 and 380 nm excitation wavelengths into separate channels of an A-D converter (ADAC Corp., Woburn, MA) and microcomputer equipped with Labtech Acquire (ADAC Corp.) data acquisition system. The fluorescence ratios (F_{340}/F_{380}) were calculated from select fluorescence values, which were transferred into a spreadsheet programme (Quattro, Borland, Inc.). The superfusion chamber, a machined plexiglass block having a glass coverslip bottom, allowed for complete exchange of solutions in 15 s by the solutions flowing in a thin sheet across the cells in the chamber.

Due to the uncertainty of the Ca^{2+}-sensitivity of intracellular fura-2 loaded into the cells by the membrane permeant acetoxymethylester form and the difficulty of *in situ* calibrations on single cells required for accurate absolute $[Ca^{2+}]_i$ determinations, $[Ca^{2+}]_i$ is reported as the ratio of 340 nm to 380 nm fluorescence signals (F_{340}/F_{380}). *In vitro* and 'myoplasmic' calibrations of the fura-2 ratio to $[Ca^{2+}]$ using Ca^{2+}-EGTA buffers are described in detail elsewhere (Wagner-Mann *et al.*, 1991; Sturek *et al.*, 1991c) and briefly presented here. The validity and reliability of the relationship between the fluorescence ratio and $[Ca^{2+}]$ in this laboratory are also described elsewhere (Wagner-Mann *et al.*, 1991) and demonstrated in the present paper (see Results, Figure 2). Briefly, the *in vitro* calibration was generated by adding the fura-2 pentapotassium salt (0.1 mM) to mock intracellular solutions comprised of (in mM) KCl 126, NaCl 10, HEPES 20, $MgCl_2$ H_2O 1 and fura-2 salt 0.1, pH 7.1 with KOH. These solutions were adjusted to 4 different free ionized $[Ca^{2+}]$ with appropriate ratios of EGTA and CaK_2-EGTA (the final [EGTA] was always 10 mM). Using the microfluorometry system described above, fluorescence ratios were measured from 2 µl droplets of the solutions placed on coverslips. For myoplasmic calibrations, smooth muscle cells from bovine and porcine coronary arteries were dispersed separately. For each species, the cells were loaded with 2.5 µM fura-2 acetoxymethyl ester (AM) by incubating them for 15 min at 37°C in a physiological salt solution (PSS, described below), then rinsing the cells for 30 min at 37°C in PSS free of fura-2. The cells were then divided into four fractions for each species, centrifuged, then resuspended in 0.5 ml of calibration solution containing 100, 400, 1000 or 2000 nM free $[Ca^{2+}]$. These fractions were then treated with saponin (100 µg ml^{-1}) to permeabilize selectively the sarcolemma, thereby releasing myoplasmic fura-2 into the solution (van Breemen *et al.*, 1988). Fluorescence ratios were measured from 5 µl droplets of each fraction placed on coverslips. The terms fluorescence ratio and $[Ca^{2+}]_i$ have been used interchangeably throughout the text.

Freshly dispersed smooth muscle cells were loaded with fura-2 according to the protocol described in Wagner-Mann *et al.* (1991). The physiological salt solution (PSS) consisted of (in mM): $CaCl_2$ 2, glucose 10, HEPES 10, KCl 5, $MgCl_2$ 1, NaCl 138, pH 7.4. This solution was used in the experiments as the superfusing fluid and for the direct additions of drugs. Unless otherwise indicated, the cells were constantly superfused with PSS. When the superfusate was changed to e.g. 0 Ca PSS, or ryanodine or caffeine added to the superfusate, the duration of the exposure within a protocol was indicated in the figures by horizontal lines. The Ca^{2+}-free medium (0 Ca PSS) consisted of the PSS solution modified to contain no $CaCl_2$, and to which 2 mM NaCl and 10 µM EGTA were added. All experiments were conducted with cells at room temperature (22–25°C) unless otherwise indicated.

Cells were allowed to equilibrate in PSS for at least 5 min or until the fluorescence ratio (F_{340}/F_{380}) stabilized (baseline). This was followed by a 5 min depolarization with PSS in which 80 mM KCl (80 K) replaced equimolar amounts of NaCl. The depolarization was conducted to verify the viability of each cell by the resulting increase in $[Ca^{2+}]_i$ and contraction observed by video monitoring. Also, the depolarization loaded the SR with Ca^{2+} before the cells were challenged with an agonist. Concentrations of ryanodine ranging from 0.001 µM to 100 µM (Calbiochem, San Diego, CA, U.S.A.) were used. The only concentration of caffeine used

was 5 mM (Sigma, St. Louis, MO, U.S.A.). Cells were randomly assigned to the experimental protocols.

Statistical analysis

Data are expressed as mean ± s.e.mean. When comparing two groups only, Students' *t* test (independent/paired as indicated by the data) was applied. For comparison of multiple groups, analysis of variance with repeated measures was used. When a significant *F* ratio was calculated, an appropriate *post hoc* analysis was applied to compare specific pairs. In all instances, baseline refers to the resting ratio (F_{340}/F_{380}) at the onset of any experimental protocol (0 min), before challenges with any agonist. When expressing any change in $[Ca^{2+}]_i$ over time as the result of a specific agonist's action(s), Δ ratio was calculated by subtracting the ratio value at the onset of the exposure from the highest (or lowest, if appropriate) ratio value attained with that agonist, and % Δ ratio was calculated by dividing Δ ratio by the ratio value at the onset of the exposure to the agonist then multiplying by 100. The significance level for all analyses was $P<0.05$.

Results

Smooth muscle cells from the porcine right coronary artery (RCA) and from the bovine RCA and left circumflex coronary artery (LCFX) were compared regarding baseline $[Ca^{2+}]_i$, and change in $[Ca^{2+}]_i$ in response to 80 mM K^+ (80 K) depolarization and caffeine (5 mM). Table 1 summarizes these data, which indicated that bovine RCA and LCFX were not different and, thus, the data were pooled throughout the remainder of the experiments for comparison with porcine RCA. Representative light micrographs of the cell types that could be seen in the dispersions are pictured in Figure 1. These cell types include a smooth muscle cell (Figure 1a), a cluster of endothelial cells (Figure 1b), a cluster of fibroblasts (Figure 1c), and typical smooth muscle cells compared to adjacent endothelial cells (Figure 1d) or adjacent fibroblasts (Figure 1e). Immunocytochemical methods have been useful for determining the percentage of cells in a population that are smooth muscle versus endothelial or fibroblasts (e.g. Loeb *et al.*, 1985). Unfortunately, the detection of smooth muscle specific actin with antibodies requires dehydration of the cell with methanol and thus, the cell would be useless for fura-2 microfluorometric measurements of $[Ca^{2+}]_i$. Instead, morphological identification (Figure 1), and evidence of contraction (their primary function and the more appropriate and widely used method for identification of smooth muscle cells in freshly dispersed preparations for experiments on single cells) were used. Typically, reports of detailed methods for freshly dispersing smooth muscle cells have relied totally on functional characterization, rather than immunocytochemistry (e.g. Van Dijk & Laird, 1984; DeFeo & Morgan, 1985; Warshaw *et al.*, 1986). The clear advantage of using contraction for smooth muscle cell identification is that every cell studied is identified as a functional smooth muscle cell by simultaneous video monitoring, as shown in our previous papers (Wagner-Mann *et al.*, 1991; Sturek *et al.*, 1991a). This method does not rely on percentages of cells in a population being smooth muscle cells. Similarly, other studies using single cells for voltage-clamp indicate morphological identification of smooth muscle cells (Amédée *et al.*, 1990).

Routinely, in this laboratory, the resting fluorescence ratios in porcine cells are lower than the resting fluorescence ratios in bovine cells (Table 1). From the calibration data depicted in Figure 2 it can be seen that the fura-2 released upon permeabilization of the sarcolemmae of bovine and porcine cells respond comparably to Ca^{2+}. As shown previously (Wagner-Mann *et al.*, 1991; Sturek *et al.*, 1991c) the fura-2 trapped in the myoplasm when loaded via fura-2/AM shows depressed Ca^{2+} sensitivity compared to the *in vitro* fura-2 pentapotassium salt. These data support the contention that differences are the result of species-specific $[Ca^{2+}]_i$ regulation, not differences in fura-2 sensitivity or handling. Temperature controlled studies were conducted in porcine cells to determine whether the action of ryanodine on smooth muscle cells in this experimental setup were significantly affected by temperature. Ryanodine induced a 28.2 ± 4.2% increase in $[Ca^{2+}]_i$ in cells ($n = 9$) maintained at 34°C, a value which was not significantly different from cells maintained at room temperature of 22–25°C (data not shown).

Table 1 Comparison of fura-2 fluorescence ratios (F_{340}/F_{380}) from bovine left circumflex (LCFX) and right coronary artery (RCA) and porcine RCA

	N	*Baseline*	*80 K*	*Caffeine*
Bovine LCFX	34	0.96 ± 0.03	1.44 ± 0.10	2.55 ± 0.21
Bovine RCA	28	0.93 ± 0.03	1.45 ± 0.06	2.28 ± 0.17
Porcine RCA	10	0.75 ± 0.03	1.06 ± 0.04	1.33 ± 0.15

To identifiy the concentration(s) of ryanodine eliciting the greatest changes in $[Ca^{2+}]_i$ and the Ca^{2+} stored in the sarcoplasmic reticulum (SR) in these cells, experiments were conducted in which cells were exposed for 10 min to one of six (seven in porcine) concentrations of ryanodine (0 μM (control), 0.001, 0.01, 0.1, 1.0, 10 μM; plus 100 μM in porcine). The 10 min ryanodine exposure was immediately followed with a 1 min caffeine challenge to assess the effect of ryanodine on the caffeine-sensitive Ca^{2+} store. Figure 3a illustrates the time course of these experiments, for [ryanodine] = 0 (control) and 10 μM (porcine cells). In the porcine cells, only at ryanodine concentrations of 10 μM and 100 μM was there a ryanodine-induced (Figure 3a: Ry Δ ratio) increase in $[Ca^{2+}]_i$ to a plateau level during the 10 min ryanodine exposure, being 27 ± 3% and 25 ± 5%, respectively. In bovine cells during the 10 min ryanodine exposure, only at ryanodine 10 μM did $[Ca^{2+}]_i$ increase significantly, to 38 ± 4% above baseline (Figure 3b). Of particular interest was the subsequent peak caffeine-induced changes in $[Ca^{2+}]_i$. The peak response to caffeine was determined by calculating the % change in the fluorescence ratio from the maximum ryanodine-induced increase in $[Ca^{2+}]_i$ (taken to be the fluorescence ratio at time 19 min) to the peak caffeine-induced increase in $[Ca^{2+}]_i$ (Figure 3a: Caf Δ ratio). The summary of these Caf Δ ratio calculations from these experiments is depicted in Figure 3c. The points graphed represent the average caffeine-induced percentage change in the ratio for each cell at a given concentration of ryanodine. Using the Caf Δ ratio for each cell in these experiments, the caffeine-induced % Δ ratio was calculated, correcting for any gradual shift in ratio (F_{340}/F_{380}) from baseline over the 10 min ± Ry (ryanodine) exposure (see Figure 3a and b). In bovine cells, the caffeine-induced increase in $[Ca^{2+}]_i$ was significantly ($P<0.05$) lower than control for ryanodine concentrations ≥ 1 μM. Typically, porcine cells showed negligible drift. In porcine cells, for ryanodine concentrations ≥ 10 μM, the caffeine-induced increase in $[Ca^{2+}]_i$ was significantly ($P<0.05$) smaller than in cells exposed to ryanodine concentrations < 10 μM. These changes in caffeine-induced increases in $[Ca^{2+}]_i$ are consistent with a ryanodine-induced release of Ca^{2+} from the SR with or without a measurable ryanodine-induced increase in $[Ca^{2+}]_i$ in bovine cells at ryanodine 1 μM.

The above described protocol was also conducted in 0 Ca PSS, in the presence of 10 μM ryanodine or absence of ryanodine (0 μM). These experiments were conducted to rule out an interaction of ryanodine (10 μM) with fura-2 as the cause of the above noted ryanodine-induced increase in the fluorescence ratio. Further, these experiments were designed to identify the contribution of Ca^{2+} release from an internal store, separated from Ca^{2+} influx, to the ryanodine-associated slow increase in $[Ca^{2+}]_i$. Figure 4a depicts the time course of the protocol and summarizes the responses of the

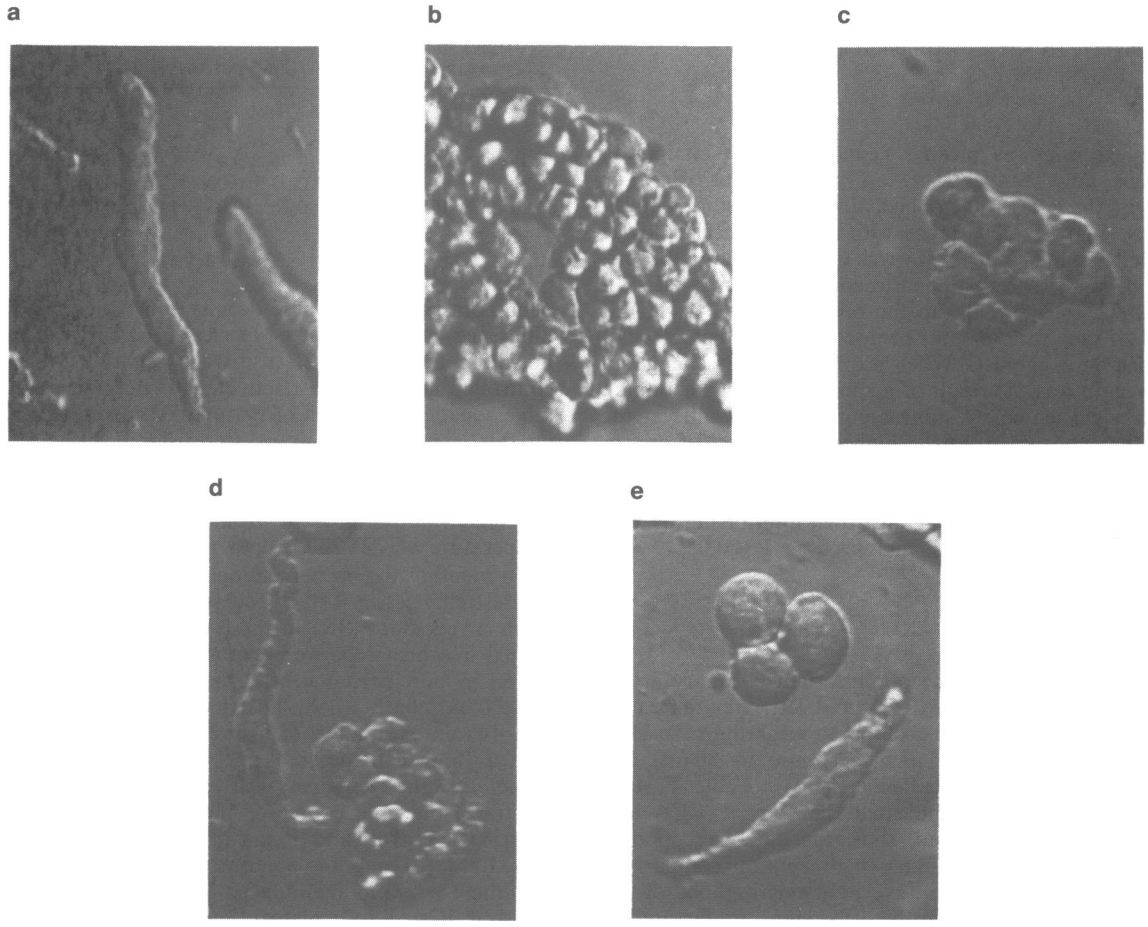


Figure 1 Light micrographs (using Normarski optics) of the cell types seen in the dispersions (calibration bar is 20 μm). (a) In this panel is depicted a typical smooth muscle cell, the predominant cell type of second and third dispersion fractions. (b) Endothelial cells, the predominant cell type of the first dispersion fraction, are typically seen in characteristic grapelike clusters depicted here. (c) The other possible cell type to be found in the dispersion fractions of the coronary arteries is the fibroblast. It typically assumes a rounded configuration, frequently appearing in clusters. These fibroblasts are larger than the endothelial cells. (d) The smooth muscle and endothelial cells seen in close proximity in this panel are readily differentiated morphologically. (e) The smooth muscle cells and fibroblasts seen in close proximity are readily differentiated morphologically.

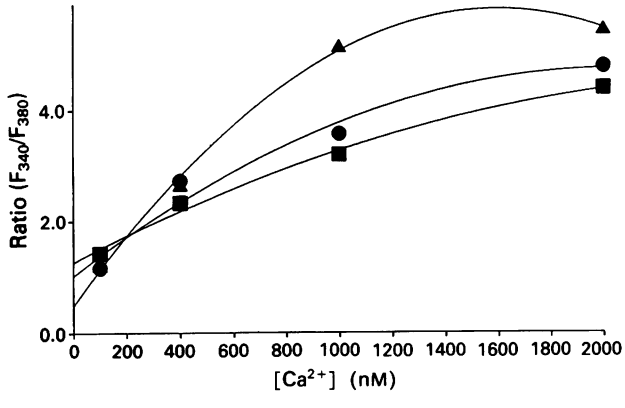


Figure 2 Myoplasmic and *in vitro* calibration curves for bovine and porcine fura-2/AM loaded smooth muscle cells. Data points for the *in vitro* curve (▲) were generated by measuring the fluorescence ratio of specified concentrations of Ca^{2+} (see below) set by Ca^{2+}-EGTA buffers in fura-2 pentapotassium salt (0.1 μM) in mock intracellular solutions (see Methods). For the myoplasmic curves, bovine (●) and porcine (■) smooth muscle cells were enzymatically dispersed then loaded with fura-2/AM. Cells were then divided into 4 approximately equal aliquots (4 for bovine and 4 for porcine), then permeabilized with saponin (100 μg ml^{-1}) and incubated with one of the following four [Ca^{2+}]: 100 nM, 400 nM, 1000 nM, 2000 nM. Ratios were recorded on a minimum of five different 5 μl droplets of the supernate from the saponin-permeabilized cells.

bovine tissue (+ Ry = 10 μM ryanodine, $n = 9$; − Ry = 0 μM, $n = 7$). In bovine cells, 10 μM ryanodine in 0 Ca PSS induced a 35 ± 5% ($n = 9$) increase in $[Ca^{2+}]_i$ above baseline, and $[Ca^{2+}]_i$ increased to 86 ± 5% above baseline with the subsequent caffeine challenge. These are in sharp contrast to the 0 Ca PSS cells exposed to 0 μM ryanodine which showed no change ($n = 7$) in $[Ca^{2+}]_i$ during the 10 min control period in the absence of ryanodine, then responded to the caffeine challenge with a 163 ± 9% increase in $[Ca^{2+}]_i$ above baseline. In porcine cells, the 10 μM ryanodine-induced increase in $[Ca^{2+}]_i$ was only 7 ± 3% ($n = 23$), while the subsequent caffeine-induced $[Ca^{2+}]_i$ increase was 63 ± 7% above baseline. In the corresponding porcine control experiment (ryanodine = 0 μM), there was an 8 ± 3% ($n = 11$) decrease in $[Ca^{2+}]_i$ from baseline during the 10 min 0 Ca PSS exposure and caffeine induced a 94 ± 13% increase in $[Ca^{2+}]_i$ (Figure 4b).

Evidence of ryanodine-associated response differences in bovine and porcine cells were manifest at this point. Specifically, the ryanodine-induced increase in $[Ca^{2+}]_i$ occurred at different rates. In bovine cells, the $[Ca^{2+}]_i$ increased and plateaued within the first 4 min of ryanodine exposure in both 0 Ca PSS and Ca^{2+}-containing PSS. The level of $[Ca^{2+}]_i$ reached was equivalent (Figure 5a). In porcine cells, however, the ryanodine-induced increase in $[Ca^{2+}]_i$ occurred more slowly, only plateauing at the conclusion of the 10 min ryanodine exposure. Furthermore, the ryanodine-induced rate of $[Ca^{2+}]_i$ increase in Ca^{2+}-containing PSS was signi-

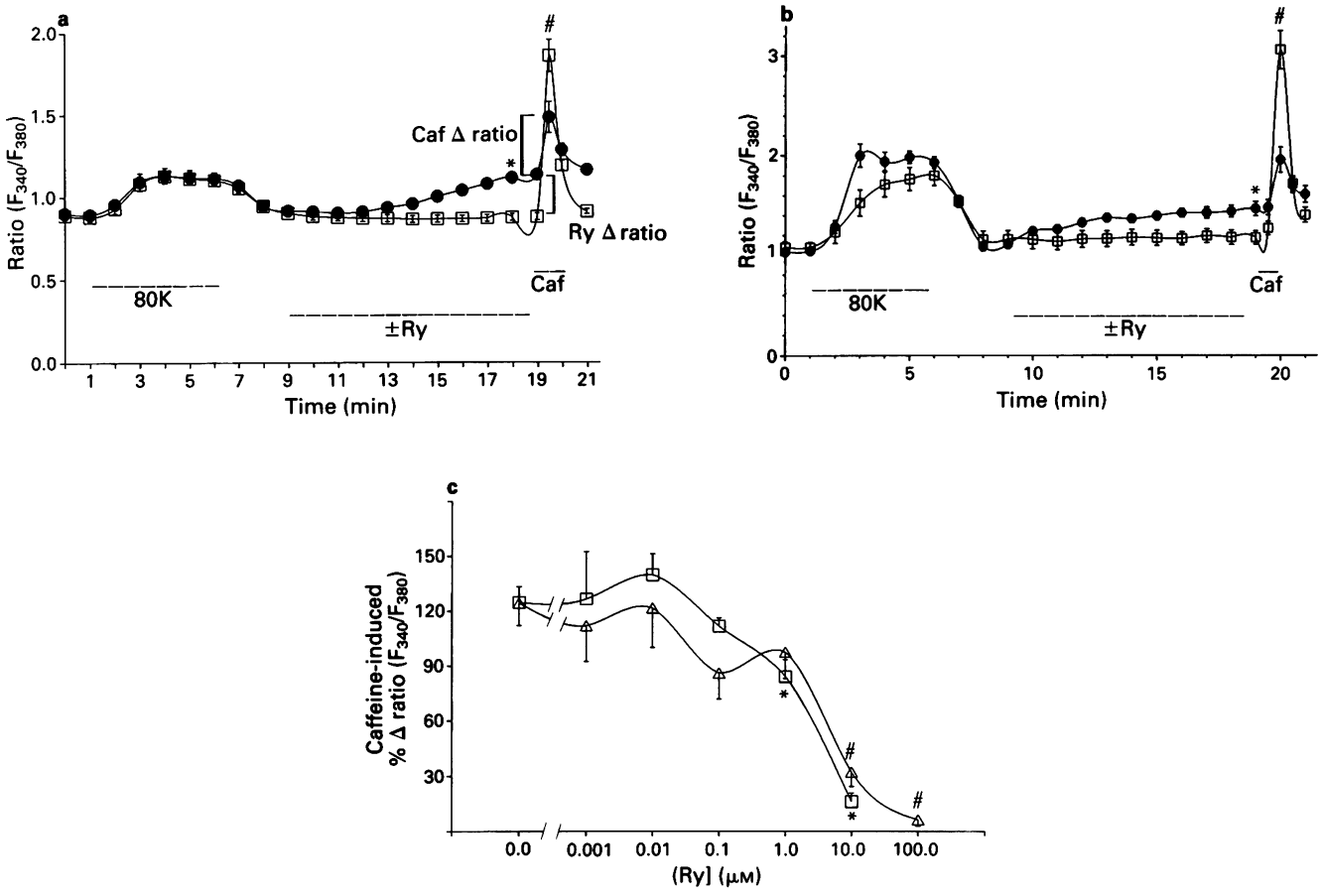
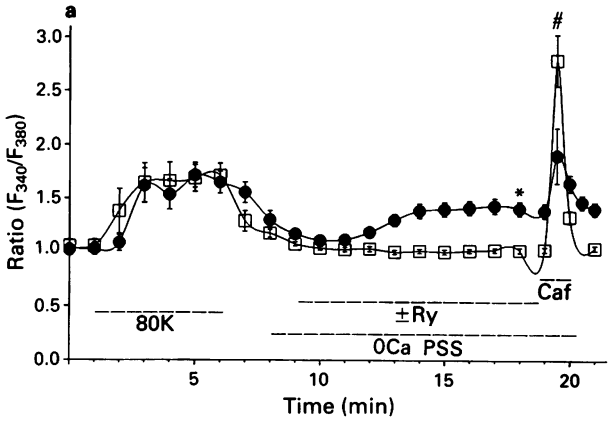


Figure 3 Sustained increase in $[Ca^{2+}]_i$ and inhibition of the caffeine-induced $\Delta[Ca^{2+}]_i$ (Caf Δ ratio = [(peak caffeine-induced ratio (F_{340}/F_{380})) − (ratio (F_{340}/F_{380}) at 19 min)]) by ryanodine. Freshly dispersed smooth muscle cells from porcine ($_p$) and bovine ($_b$) coronary artery were initially depolarized with 80 mM potassium (80 K, duration 5 min as indicated by the horizontal lines and approriate labels) then challenged with various concentrations of ryanodine (see (c) for details). Finally, all cells were exposed to caffeine (Caf; 5 mM) for 1 min. (a) Time course for single dose of ryanodine (+ Ry; 10 μM, $n = 12$; ●) and control (− Ry; 0.0 μM, $n = 29$; □) in porcine smooth muscle (* indicates + Ry fluorescence ratio is significantly greater than the − Ry fluorescence ratio at that data point; # indicates the fluorescence ratios are significantly different at that data point). (b) Time course for single dose of ryanodine (+ Ry; 10 μM, $n = 13$; ●) and control (− Ry; 0.0 μM, $n = 26$; □) in bovine smooth muscle cells (* indicates + Ry fluorescence ratio is significantly greater than the − Ry fluorescence ratio at that data point; # indicates the fluorescence ratios are significantly different at that data point). Contrast the rate of ryanodine-induced $[Ca^{2+}]_i$ increase in bovine versus porcine cells (Figures 3a and 3b). (c) Dose-response curves for ryanodine-induced inhibition of caffeine-induced $\Delta[Ca^{2+}]_i$. Points graphed represent the peak caffeine-induced percentage changes in $[Ca^{2+}]_i$ (% Δ ratio) after 10 min exposure to ryanodine, calculated from Caf Δ ratio (% Δ ratio = (Caf Δ ratio) × (ratio at onset of caffeine challenge)$^{-1}$ × 100) for each cell (see (a); Δ represent porcine data; □ represent bovine data; 0.0 μM (control): $n_b = 26$, $n_p = 29$; 0.001 μM: $n_b = 7$, $n_p = 16$; 0.01 μM: $n_b = 12$, $n_p = 13$; 0.1 μM: $n_b = 8$, $n_p = 6$; 1.0 μM: $n_b = 7$, $n_p = 13$: 10 μM: $n_b = 13$, $n_p = 12$; 100 μM: $n_p = 11$). # Significantly different from control in porcine cells; *significantly different from control in bovine cells.

ficantly ($P < 0.05$) greater than in 0 Ca PSS in porcine cells (Figure 5b).

Next, we identified the effect of releasing Ca^{2+} from the SR with a known agonist (caffeine) on the ryanodine-induced changes in $[Ca^{2+}]_i$ to determine whether the Ca^{2+} stores affected by caffeine and ryanodine overlapped or were in some way functionally linked. To accomplish this, bovine cells were challenged with 1 min of caffeine (5 mM) or 2.5 min caffeine (to more completely release Ca^{2+} stored in the SR) plus ryanodine (10 μM) in 0 Ca PSS to release Ca^{2+} stored in the SR. The exposure to ryanodine (10 μM) in 0 Ca PSS totalled 10 min and finally, cells were exposed for 1 min to caffeine (5 mM) (Figure 6a, open squares) to determine the relative content of the caffeine-sensitive Ca^{2+} store. Even with the initial 1 min caffeine challenge, the total 10 min exposure to ryanodine in 0 Ca PSS induced a 17 ± 2% increase in $[Ca^{2+}]_i$ (relative to baseline). Similar experiments involving an initial 1 min caffeine exposure in 0 Ca PSS and in PSS, but with no ryanodine (control) for 10 min resulted in a 1 ± 2% ($n = 7$) and 12 ± 3% ($n = 9$) increase in $[Ca^{2+}]_i$, respectively after the 10 min control period (data not shown). In cells with the 1 min initial caffeine challenge prior to 10 min of ryanodine exposure in 0 Ca PSS, the second caffeine challenge induced only a 27 ± 4% increase in $[Ca^{2+}]_i$. In contrast, in cells not exposed to ryanodine, the second caffeine challenge induced an 89 ± 7% increase in $[Ca^{2+}]_i$ in PSS, and a 41 ± 4% increase in $[Ca^{2+}]_i$ in 0 Ca PSS (data not shown), thus indicating little depletion of the caffeine-sensitive Ca^{2+} store in the control condition.

In bovine cells in 0 Ca PSS, following the initial 2.5 min caffeine, there was a slight (4 ± 2%) increase in $[Ca^{2+}]_i$ during the remaining 7.5 min of the 10 min exposure to ryanodine in 0 Ca PSS (Figure 6a, closed circles). The second caffeine challenge resulted in only an additional 4 ± 3% increase in $[Ca^{2+}]_i$ to 8 ± 3% above baseline. For cells exposed to caffeine for 2.5 min but not challenged with ryanodine (controls), the $[Ca^{2+}]_i$ dropped 10 ± 2% during the equivalent 7.5 min period in 0 Ca PSS; the second caffeine challenge in this group of cells resulted in a 17 ± 5% increase in $[Ca^{2+}]_i$ (when compared to 1 min before caffeine challenge) to 5 ± 2% above baseline (data not shown). The caffeine-induced change in $[Ca^{2+}]_i$ attained following the 10 min pre-



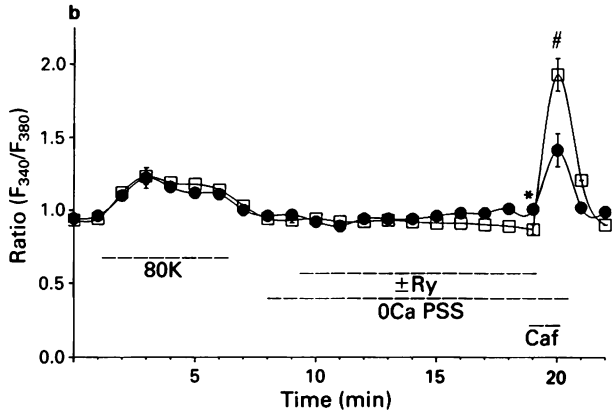
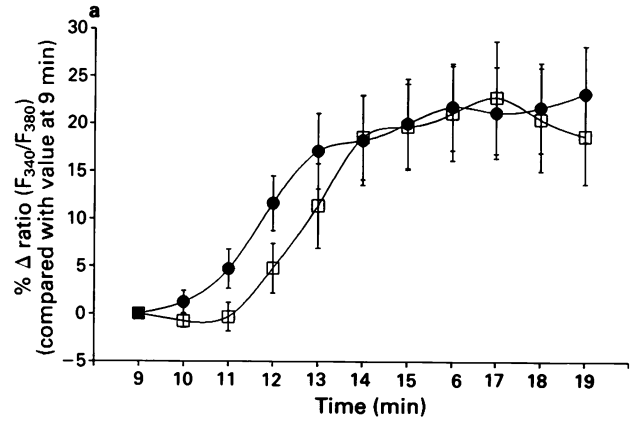


Figure 4 Ryanodine-induced changes in $[Ca^{2+}]_i$ in bovine and porcine cells superfused with 0 Ca PSS. Cells were first depolarized with 80 mM potassium (80 K, duration 5 min, note horizontal line and associated label), then following a 1 min preconditioning in Ca^{2+}-free PSS (0 Ca PSS) cells were either challenged with 10 μM Ry (+ Ry) in 0 Ca PSS or without Ry (− Ry; [ryanodine] = 0.0 μM) in 0 Ca PSS. Finally, cells were exposed to caffeine (Caf; 5 mM) for 1 min. (a) These data were collected from bovine cells (+ Ry: $n = 9$, ●; − Ry: $n = 7$, □; * indicates + Ry fluorescence ratio is significantly greater than the − Ry fluorescence ratio at that data point; # indicates the fluorescence ratios are significantly different at that data point). (b) Ryanodine-induced changes in $[Ca^{2+}]_i$ and subsequent caffeine-induced $[Ca^{2+}]_i$ changes in porcine cells (+ Ry: $n = 23$, ●; − Ry: $n = 11$, □; * indicates + Ry fluorescence ratio is significantly greater than the − Ry fluorescence ratio at that data point; # indicates the fluorescence ratios are significantly different at that data point). Note the difference between the rate of ryanodine-induced changes in $[Ca^{2+}]_i$ increase in bovine compared with porcine cells.



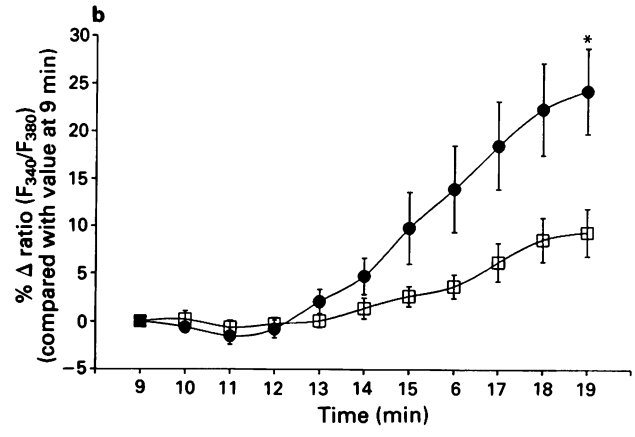


Figure 5 Ryanodine-induced increases in $[Ca^{2+}]_i$ in Ca^{2+}-containing PSS and 0 Ca PSS. Relative rates of ryanodine-induced increases in $[Ca^{2+}]_i$ during the 10 min exposure to 10 μM ryanodine (from Figures 3a, 3b, 4a, and 4b) are shown as % Δ ratio = [ratio (F_{340}/F_{380}) at each of the indicated times (9 min to 19 min)]/[ratio (F_{340}/F_{380}) at 9 min] × 100−100) (Note: this equation is equivalent to that described in Figure 3c). (a) Bovine smooth muscle cells (0 Ca PSS, $n = 9$, □; Ca^{2+}-containing PSS, $n = 13$, ●). Note the equivalent peak Ry-induced increases in the ratio whether in 0 Ca PSS or Ca^{2+}-containing PSS, as well as the overlapping time courses for bovine cells. (b) Porcine smooth muscle cells (0 Ca PSS, $n = 22$, □; Ca^{2+}-containing PSS, $n = 12$, ●). In porcine tissue the peak Ry-induced increase in the ratio is significantly higher (*) in the group of cells superfused with Ca^{2+}-containing PSS than in 0 Ca PSS. Further, the rate of increase in ratio over the 10 min Ry exposure is greater in the Ca^{2+}-containing PSS superfused cells than the 0 Ca PSS superfused cells.

exposure to ryanodine was 59 ± 5% lower than the caffeine-induced change in $[Ca^{2+}]_i$ in the absence of 10 min exposure to ryanodine. The ryanodine-induced changes in $[Ca^{2+}]_i$ were more pronounced in protocols conducted in PSS (Ca^{2+}-containing) than in 0 Ca PSS. Following the initial 2.5 min caffeine plus ryanodine challenge, 10 min total exposure to ryanodine resulted in a 24 ± 3% increase in $[Ca^{2+}]_i$ (Figure 6c), in contrast to 7.5 min of PSS without ryanodine, which resulted in a 6 ± 2% increase in $[Ca^{2+}]_i$ (data not shown). The second caffeine challenge in cells treated with ryanodine resulted in an additional 16 ± 3% increase in $[Ca^{2+}]_i$, whereas the second caffeine challenge in the control cells (without ryanodine treatment) resulted in a 40 ± 5% increase in $[Ca^{2+}]_i$ (data not shown). Figures 6b (0 Ca PSS) and 6c (Ca^{2+}-containing PSS) summarize the effects of variable initial caffeine exposures on the subsequent ryanodine-induced change in fluorescence ratio. Clearly, with prolonged caffeine pretreatment and therefore, release of more stored Ca^{2+} from the SR, the subsequent ryanodine exposure induced a smaller change in $[Ca^{2+}]_i$.

The evidence accumulated thus far supported a ryanodine-induced increase of $[Ca^{2+}]_i$ through release of Ca^{2+} from an internal store. The question of the contribution of influx to the ryanodine-induced elevation in $[Ca^{2+}]_i$ in bovine tissue, while anticipated to be smaller than that of release, still required clarification. To this end Figure 7 is a summary of the results of the experiment evaluating the influence of increasing extracellular $[Ca^{2+}]$ on $[Ca^{2+}]_i$ of cells challenged with ryanodine. The increase in $[Ca^{2+}]_i$ associated with ryanodine rose further as the $[Ca^{2+}]$ increased in the medium bathing the cells. In 0 Ca PSS 10 μM ryanodine (6 min) did not induce a measurable change in $[Ca^{2+}]_i$. With each increase in Ca^{2+} in the bathing medium to 2, 5 and 10 mM Ca^{2+}, while in the continued presence of 10 μM ryanodine (6 min at each $[Ca^{2+}]$), $[Ca^{2+}]_i$ increased to 20 ± 3, 28 ± 4, and 46 ± 4% above baseline, respectively. Subsequent caffeine challenge produced only an additional 7 ± 2% increase in $[Ca^{2+}]_i$ above that attained by ryanodine alone in 10 mM Ca^{2+} PSS.

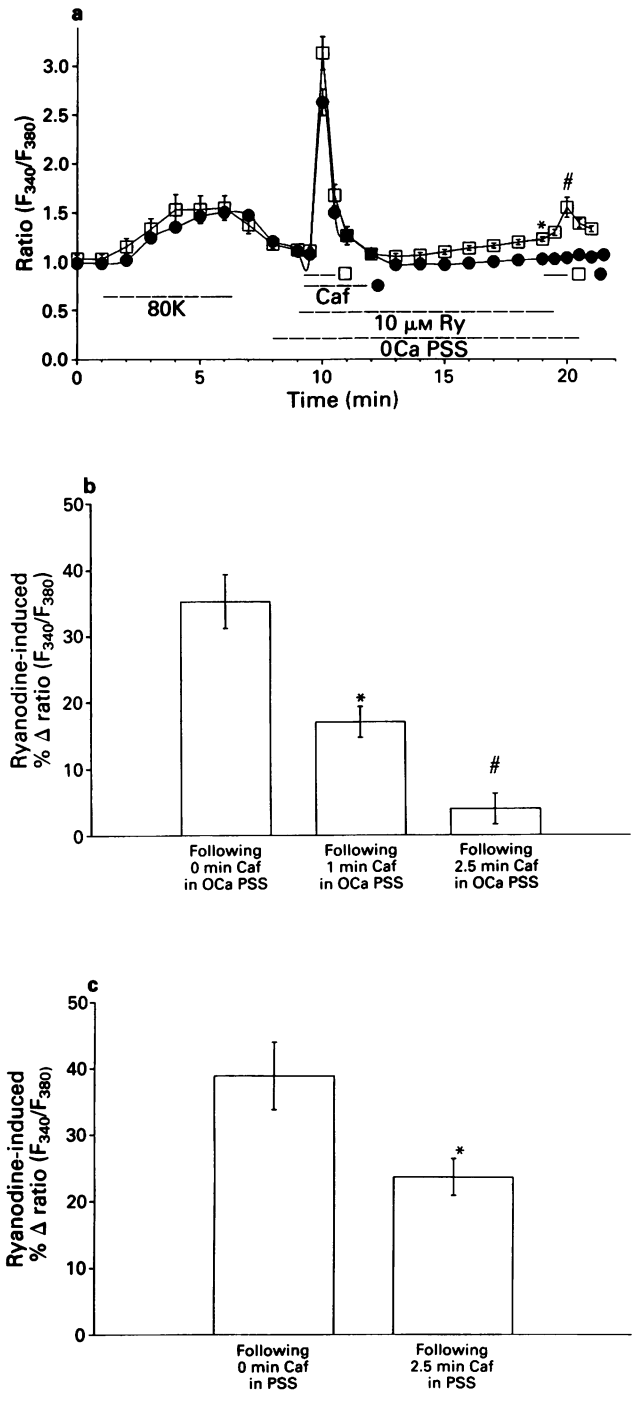


Figure 6 Caffeine-induced Ca^{2+} release from sarcoplasmic reticulum (SR) decreases the subsequent ryanodine-induced $[Ca^{2+}]_i$ increase in bovine coronary artery smooth muscle cells. (a) Time course of the experiments conducted in which 1 min (□) or 2.5 min (●) caffeine (Caf; 5 mM) plus ryanodine (Ry; 10 μM) is followed by 9 or 7.5 min, respectively, ryanodine exposure (10 min total exposure to ryanodine; * indicates + Ry fluorescence ratio is significantly greater than the − Ry fluorescence ratio at that data point; # indicates the fluorescence ratios are significantly different at that data point). As with previously described protocols, cells were first depolarized in 80 mM potassium (80 K) for 5 min. (b) Ryanodine-induced $[Ca^{2+}]_i$ changes in 0 Ca PSS (% Ry Δ ratio = [Ratio (F_{340}/F_{380}) at 19 min]/[Ratio (F_{340}/F_{380}) at 9.5 min] × 100−100) following each of the indicated caffeine exposures (0 min: $n = 9$; 1 min; $n = 9$, * indicates significantly different than the 0 min and 2.5 min caffeine-induced responses; 2.5 min: $n = 30$, # indicates significantly different than the 0 min and 1 min caffeine-induced responses). (c) Ryanodine-induced $[Ca^{2+}]_i$ changes in Ca^{2+}-containing PSS following each of the indicated caffeine exposures (0 min; $n = 13$; 2.5 min: $n = 10$, *significantly different from the 0 min caffeine-induced response; % Ry Δ ratio calculated as indicated in (b) above).

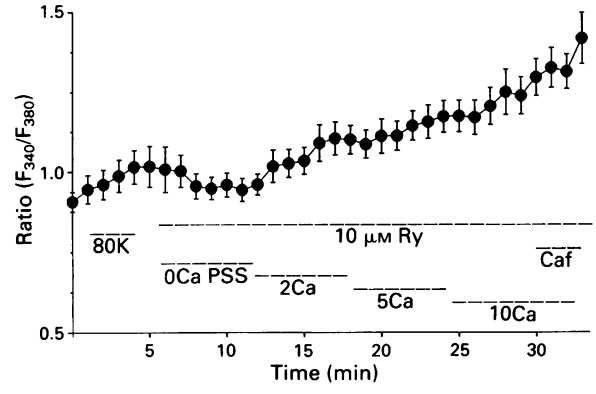


Figure 7 Increases in superfusing $[Ca^{2+}]$ (0, 2, 5 and 10 mM) are associated with increases in ryanodine-induced ([ryanodine] = 10 μM) $[Ca^{2+}]_i$ changes in bovine cells ($n = 16$). It should be noted that while a return to precise baseline ratio values (fluorescence ratio at 0 min) was never achieved following the 80 mM potassium (80 K) exposure, the fluorescence ratio values during the 0 Ca PSS plus 10 μM ryanodine exposure were not significantly greater than baseline. Caffeine (Caf, 5 mM) was without effect.

When ryanodine exposure resulted in elevated $[Ca^{2+}]_i$ (Figures 3–7), the increase in $[Ca^{2+}]_i$ was sustained. This finding implied that $[Ca^{2+}]_i$ removal processes, presumably via the SR and sarcolemmal Ca^{2+} pumps and Na^+-Ca^{2+} exchange (Van Breemen *et al.*, 1986; Slaughter *et al.*, 1989), were not as effective as noted during large increases in $[Ca^{2+}]_i$ evident in response to caffeine without prolonged pretreatment with ryanodine (Figures 3a, 4a, 6a). To test whether these ryanodine-associated sustained elevations of $[Ca^{2+}]_i$ were the result of altered Na^+-Ca^{2+} exchange, the caffeine-sensitive SR Ca^{2+} store was depleted and then the cells were preloaded with barium by depolarization with 80 K (2 Ba, 80 K). The cells were then allowed to remove the intracellular free Ba^{2+} ($[Ba^{2+}]_i$) in 0 Ca PSS in the presence or absence of ryanodine. In cells exposed to ryanodine following the 2 Ba, 80 K challenge, the decrease in $[Ba^{2+}]_i$ was significantly ($P < 0.05$) smaller than the decrease in $[Ba^{2+}]_i$ for cells not exposed to ryanodine (Figure 8).

Discussion

The main findings from these studies on regulation of $[Ca^{2+}]_i$ by ryanodine in bovine and porcine smooth muscle cells are: (1) over the range of dosages examined (0.001 μM to 100 μM), ryanodine effects the release of Ca^{2+} from the caffeine-sensitive internal store; (2) ryanodine (10 μM) also inhibits Ca^{2+}-efflux from these cells; and finally, (3) while the above two effects of ryanodine are detectable in both bovine and porcine smooth muscle cells, there are primary differences between the species.

In these studies 10 μM ryanodine induced an increase in $[Ca^{2+}]_i$, whether the cells were superfused with a Ca^{2+}-containing or Ca^{2+}-free medium. Further, the ryanodine-associated increase in $[Ca^{2+}]_i$ was not different in bovine cells whether the cells were superfused in PSS or 0 Ca PSS. These data support a ryanodine-induced release of Ca^{2+} from an internal store, probably the sarcoplasmic reticulum (SR), over the upper end of the range of dosages studied. While this is in contrast to the findings of Nagasaki & Fleischer (1989), Chu *et al.* (1990), and McGrew *et al.* (1989), who have reported the release of Ca^{2+} in SR vesicles from skeletal muscle at lower concentrations of ryanodine (<μM), and blockade of Ca^{2+} release from the SR at higher concentrations (≥ 10 μM), in smooth muscle ≥ 10 μM concentrations of ryanodine have been shown to induce SR Ca^{2+}-release (Hwang & Van Breemen, 1987; Ashida *et al.*, 1988; Nish-

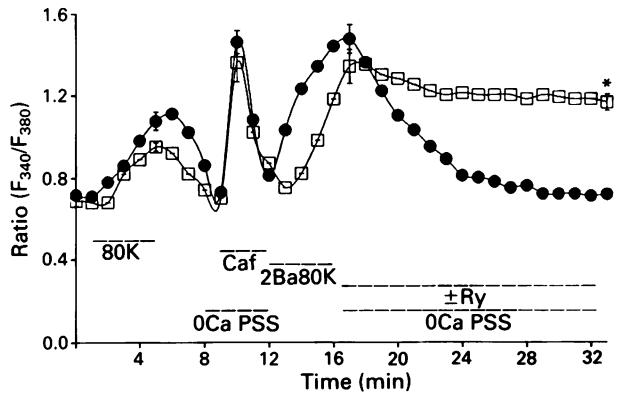


Figure 8 Ryanodine decreases efflux of intracellular free barium ($[Ba^{2+}]_i$ in porcine cells. After depletion of the sarcoplasmic reticulum (SR) with caffeine, Ba^{2+} was substituted for Ca^{2+} (equimolar) during depolarization-induced influx (2 Ba, 80 K). − Ry = control (no ryanodine), $n = 11$, ●; + Ry = 10 μM ryanodine, $n = 14$, □ (s.e. are indicated only at min 4, 9, 16, and 32). Sustained $[Ba^{2+}]_i$ during exposure to 10 μM ryanodine suggests that ryanodine inhibits Na^+-Ca^{2+} exchange. * indicates fluorescence ratios of the two curves at that time (33 min) are significantly different.

imura *et al.*, 1989; Hisayama *et al.*, 1990). The discrepancy between our findings regarding ryanodine-induced Ca^{2+}-release in Ca^{2+}-free solution and those of Kanmura *et al.* (1988), who demonstrated a Ca^{2+}-dependence of the actions of ryanodine might be reconciled by the different species (rabbit versus bovine/porcine), or different smooth muscle source (peripheral versus coronary vasculature). Furthermore, while it is clear that ⩾ 10 μM ryanodine may lock the SR Ca^{2+} release channel in a low subconductance state (Nagasaki & Fleischer, 1989; McGrew *et al.*, 1989), the long duration (> 10 min) exposures to ryanodine in the present studies may result in substantial concentrations of Ca^{2+} being released, thus accumulating as $[Ca^{2+}]_i$ in the intact cell. As indicated by Sutko & Kenyon (1990), the net effect of ryanodine on $[Ca^{2+}]_i$ depends on other $[Ca^{2+}]_i$ removal processes, including the sarcolemmal Ca^{2+} pump and Na^+-Ca^{2+} exchange. Direct bilayer (Ehrlich & Watras, 1988; Nagasaki & Fleischer, 1989; McGrew *et al.*, 1989) or *in situ* (Palade *et al.*, 1989) recordings of single Ca^{2+} release channel activity will be necessary to resolve precise mechanisms of ryanodine action on channels in vascular smooth muscle. The only report of single Ca^{2+} release channel activity in smooth muscle SR membranes did not assess the effects of ryanodine (Ehrlich & Watras, 1988).

Another finding was that both ryanodine and caffeine act on a common internal store of Ca^{2+}. The protocols included in this study were designed to determine both the effects of ryanodine on subsequent caffeine-induced Ca^{2+} release and *vice versa*. The former set of experiments demonstrated that at the middle to upper end of the ryanodine concentrations studied (micromolar), pretreatment with ryanodine resulted in a measurable decrease in the caffeine-induced Ca^{2+} transient. Furthermore, for [ryanodine] ⩾ 10 μM, there was also a sustained increase in $[Ca^{2+}]_i$. In cells pretreated with caffeine the anticipated ryanodine-induced increase in $[Ca^{2+}]_i$ was decreased (1 min caffeine) or abolished (2.5 min caffeine), consistent with a caffeine-induced depletion of the ryanodine-releasable Ca^{2+} store. These data are consistent with other findings regarding the actions of ryanodine on the smooth muscle caffeine-sensitive Ca^{2+} store (Hisayama & Takayanagi, 1988; Kanmura *et al.*, 1988; Ashida *et al.*, 1988).

Differences were detected between bovine and porcine tissue in the ryanodine-induced $[Ca^{2+}]_i$ changes (Figure 4). Compared with the $[Ca^{2+}]_i$ changes in bovine cells, the ryanodine-induced increases in $[Ca^{2+}]_i$ occurred more slowly in porcine cells. Furthermore, the maximum ryanodine-induced $[Ca^{2+}]_i$ increases were greater in bovine than porcine tissue in 0 Ca PSS. Finally, the results were not due to differences in handling of fura-2/AM by the cells as $[Ca^{2+}]$ sensitivity of myoplasmic fura-2 was not different between bovine and porcine cells (Figure 2). Thus, the data collected in this study support the conclusion that the caffeine-sensitive SR Ca^{2+} store in bovine smooth muscle cells may be larger than that in porcine smooth muscle. This would imply a greater dependence on Ca^{2+} influx to increase $[Ca^{2+}]_i$ in porcine cells.

Perhaps the most controversial, and certainly the most challenging, point is the ryanodine-associated sustained elevation in $[Ca^{2+}]_i$. Maintenance of elevated $[Ca^{2+}]_i$ might be explained by relatively enhanced influx over efflux of Ca^{2+} across the sarcolemma or relatively decreased efflux over influx. We have been unable to identify any published data supporting or even suggesting that ryanodine decreases Ca^{2+} influx in any detectable way. In contrast, ryanodine may increase Ca^{2+} influx via decreased inactivation of voltage-gated Ca^{2+} channels in ventricular myoctyes normally resulting from Ca^{2+} release (Balke & Wier, 1991). A ryanodine-induced sustained increase in $[Ca^{2+}]_i$ has been reported in the literature (Hansford & Lakatta, 1987; Nishimura *et al.*, 1989). Results of studies utilizing ^{45}Ca to measure unidirectional Ca^{2+} flux across the sarcolemma have not been consistent. While Kanmura *et al.* (1988) and Hwang & van Breemen (1987) have reported a ryanodine-enhanced ^{45}Ca efflux, Ito *et al.* (1986) found that ryanodine exposure resulted in a decreased agonist-induced ^{45}Ca efflux. These published studies plus the data from this study led to the hypothesis that ryanodine inhibits Ca^{2+} efflux in vascular smooth muscle.

Maintenance of resting $[Ca^{2+}]_i$ in smooth muscle is achieved by the functional Ca^{2+} buffering activity of the SR and Ca^{2+} extrusion via sarcolemmal Ca^{2+}-ATPase activity, and Na^+-Ca^{2+} exchange (Smith *et al.*, 1987; Matlib, 1988; Blaustein, 1988; Slaughter *et al.*, 1989). With elimination of the potential contribution of the SR to Ca^{2+} buffering by caffeine treatment and elimination of extrusion by the sarcolemmal Ca^{2+} pump by using barium (Ba^{2+}) in 0 Ca PSS (Schilling *et al.*, 1989), we were able to evaluate qualitatively the effect of ryanodine on Na^+-Ca^{2+} exchange. Significantly decreased ($P < 0.05$) efflux of $[Ba^{2+}]_i$ from the smooth muscle cells challenged with 10 μM ryanodine versus cells not challenged with ryanodine provided evidence that ryanodine inhibits Ca^{2+} efflux via Na^+-Ca^{2+} exchange. This is of particular interest because 10 μM ryanodine has been used almost exclusively to modulate specifically SR function in intact smooth muscle cells (Hwang & Van Breemen, 1987; Kanmura *et al.*, 1988; Ashida *et al.*, 1988; Hisayama & Takayanagi, 1988; Hisayama *et al.*, 1990). Identification of multiple actions of ryanodine, therefore, is significant.

In conclusion, ryanodine (⩾ 10 μM) releases Ca^{2+} from the SR and decreases Ca^{2+} efflux in bovine and porcine vascular smooth muscle cells, thus causing a sustained increase in $[Ca^{2+}]_i$. Ryanodine is, nonetheless, a valuable tool to aid in the delineation of SR function, as shown by the identification of a greater dependence on Ca^{2+} release from the SR to increase $[Ca^{2+}]_i$ in bovine as compared with porcine smooth muscle.

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