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SPONTANEOUS SARCOPLASMIC RETICULUM CALCIUM RELEASE AND EXTRUSION FROM BOVINE, NOT PORCINE, CORONARY ARTERY SMOOTH MUSCLE

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SUMMARY

1. We tested the hypothesis that the Ca^{2+}-loaded sarcoplasmic reticulum (SR) of coronary artery smooth muscle spontaneously releases Ca^{2+} preferentially toward the sarcolemma to be extruded from the cell without increasing the average free myoplasmic [Ca^{2+}] (Ca_{im}) concentration.

2. The SR of bovine cells was Ca^{2+}-loaded by depolarization-induced Ca^{2+} influx. Release (unloading) of Ca^{2+} from the SR during recovery from depolarization was determined by Fura-2 microfluorometry of Ca_{im}. The SR Ca^{2+} unloading was maximal following a long (14 min) recovery from depolarization, as shown by the 66% decrease in the peak caffeine-induced Ca_{im} transient compared to the Ca_{im} transient after a short (2 min) recovery. No increase in Ca_{im} occurred during the long recovery. No unloading of the SR Ca^{2+} store was noted in porcine cells.

3. Approximately 80% of the outward K^+ current in bovine and porcine cells was sensitive to subsarcolemmal Ca^{2+} (Ca_{is}) concentrations. Whole-cell voltage clamp using pipette solutions with Ca^{2+} concentrations clamped between 0 and 1000 nM with Ca^{2+}-EGTA or Ca^{2+}-BAPTA buffers showed increasing K^+ currents (normalized for cell membrane surface area) as a function of both membrane potential and Ca_{is}. Clamping of Ca_{im} and Ca_{is} was verified by the lack of changes in K^+ current and Fura-2 ratio in response to Ca^{2+} influx, Ca^{2+}-free external solution, or caffeine-induced Ca^{2+} release. At +30 to +50 mV the K^+ current amplitude showed a similar sensitivity to Ca^{2+} as Fura-2. These data indicate that in this experimental preparation Ca^{2+}-activated K^+ current is a valid estimate of Ca_{is}.

4. Simultaneous Ca_{im} and Ca_{is} measurements in bovine cells which were not Ca^{2+}-clamped (2×10^{-4} M-EGTA pipette solution) showed that during the long recovery period the K^+ current (reflecting Ca_{is}) increased 55%, while Ca_{im} did not change.

5. In quiescent bovine cells the Ca_{is} was higher than Ca_{im}, while the higher resting Ca_{is} gradient was not apparent in porcine cells.

6. The Ca_{is} concentration was directly related to the amount of Ca^{2+} in the SR in bovine, but not porcine cells. Depletion of the SR in bovine cells by caffeine resulted in a 58% decrease in K^+ current compared to the resting K^+ current.

7. Caffeine-induced Ca^{2+} release caused an increase in Ca_{is} which preceded the increase in Ca_{im} by approximately 2 s. Such a long delay between the change in Ca_{is}

and Ca_{im} is > 100-fold slower than the delay predicted by simple diffusion of Ca^{2+} through the cytosol, thus indicating preferential release of Ca^{2+} toward the sarcolemma and significant Ca_{im} buffering.

8. Spontaneous Ca^{2+} efflux (as measured by extracellular Fura-2) was proportional to the initial amount of Ca^{2+} loaded into the SR of bovine, but not porcine cells. When the SR of bovine cells was Ca^{2+}-loaded (following depolarization in high Ca^{2+}) spontaneous Ca^{2+} efflux was greatest. When the SR was Ca^{2+}-unloaded (following caffeine exposure) the spontaneous Ca^{2+} efflux from the cell was greatly attenuated.

9. The results support the hypothesis that the SR spontaneously releases Ca^{2+} to be extruded from the cell. The species variation in the results may provide insight into the role of the SR as a Ca^{2+} buffer barrier, which is sufficiently powerful to give rise to a restricted subsarcolemmal Ca^{2+} compartment in the resting cell.

INTRODUCTION

The handling of Ca^{2+} by vascular smooth muscle is important in the control of blood pressure and blood flow. Recently, three distinct models have been proposed to describe the regulation of intracellular Ca^{2+} by the endoplasmic/sarcoplasmic reticulum (see companion paper (Sturek, Kunda & Hu, 1992) for details). The extent of control that the sarcoplasmic reticulum (SR) might have in regulating intracellular Ca^{2+} in vascular smooth muscle is an area of much discussion (Allen & Seidel, 1986). Sturek *et al.* (Sturek *et al.* 1992) demonstrated that the SR in vascular smooth muscle attenuates the increase in myoplasmic free Ca^{2+} (Ca_{im}) resulting from Ca^{2+} influx. This being the case, when the SR is Ca^{2+}-loaded it can no longer sequester the continuing influx of Ca^{2+} into the cell. Thus, it has lost its Ca^{2+} buffering capabilities, unless the SR has a mechanism to release a portion of its Ca^{2+} store in the absence of agonist stimulation.

Van Breemen & Saida (1989) proposed a mechanism for Ca^{2+} buffering in resting smooth muscle cells in which Ca^{2+} was released by the SR preferentially toward the sarcolemma to be extruded from the cell. They further proposed in their model that Ca^{2+} buffering gives rise to a Ca^{2+} gradient near the sarcolemma with subsarcolemmal free Ca^{2+} (Ca_{is}) concentrations being higher than Ca_{im} concentrations (van Breemen & Saida, 1989). Results which support this model are that: (1) the rate of loss of ^{45}Ca from the SR increases after exposure to high K^+ (Deth & Casteels, 1977; Leijten & van Breemen, 1986); (2) ryanodine releases Ca^{2+} from the SR of smooth muscle but it does not cause contraction (Nishimura, Khalil & van Breemen, 1989), and; (3) coronary artery smooth muscle spontaneously loses Ca^{2+} from the SR without increasing Ca_{im} (Stehno-Bittel, Laughlin & Sturek, 1990, 1991).

Direct measurement of Ca^{2+} movement from the SR toward the sarcolemma is difficult due to the complex morphology of smooth muscle cells (Somlyo, Somlyo, Shuman, Sloane & Scarpa, 1978; Allen & Seidel, 1986). The SR is not continuous with the sarcolemma in vascular smooth muscle (Allen & Seidel, 1986), but may be located within 50–100 nm from the sarcolemma (Devine, Somlyo & Somlyo 1972). The membrane arrangement is highly irregular and appears to be species dependent (Allen & Seidel, 1986). Measuring Ca_{im} concentrations in such a small area is nearly impossible; however, Ca^{2+}-activated K^+ currents enable monitoring of changes in Ca_{is} concentrations. Several reports indicate that vascular smooth muscle cells have

an abundance of these channels which are sensitive to Ca_{is} concentrations (Benham, Bolton, Lang & Takewake, 1986; Ohya, Kitamura & Kuriyama, 1987; Sadoshima, Akaike, Tomoike, Kanaide & Nakamura, 1988; Desilets, Driska & Baumgarten, 1989; Hume & Leblanc, 1989; Wilde & Lee, 1989). Work with Ca^{2+}-activated K^+ channels in skeletal muscle indicates that the average open time of single channels is directly related to Ca_{is} concentrations (Barrett, Magleby & Pallotta, 1982; Pallotta, Helper, Oglesby & Harden, 1987). We show that if several controlled experimental conditions are used, Ca^{2+}-activated K^+ currents estimate relative changes in Ca_{is} in intact cells.

The purpose of this study was to test the hypothesis that Ca^{2+}-loaded SR releases Ca^{2+} spontaneously toward the sarcolemma to be extruded from the cell. This hypothesis led to four testable predictions: (1) the SR unloads Ca^{2+} spontaneously without increasing Ca_{im} concentrations, (2) Ca^{2+} released spontaneously from the SR is extruded from the cell, (3) Ca^{2+} concentrations are higher in the subsarcolemmal region (Ca_{is}) than in the myoplasmic region (Ca_{im}) during spontaneous SR unloading of Ca^{2+}, and (4) Ca_{is} concentrations are directly related to the amount of Ca^{2+} in the SR. All four predictions were confirmed in bovine, but not in porcine cells. Since the two species appear to handle Ca^{2+} differently we compare and contrast results obtained from the two cell types to identify pathways and subsequently determine the physiological significance of spontaneous SR Ca^{2+} release. The results stress the importance of choosing appropriate models when studying SR Ca^{2+} regulation and imply that in bovine vascular smooth muscle the SR plays a more active role in regulating intracellular Ca^{2+} under resting conditions and conditions of spontaneous SR Ca^{2+} unloading.

METHODS

Dispersion of cells from the coronary artery

Single smooth muscle cells were obtained from the bovine and porcine right and left circumflex and the left anterior descending coronary arteries as described in the accompanying paper (Sturek *et al.* 1992*a*). Briefly, the arteries were removed from the heart and placed in a media consisting of (mM): 2 $CaCl_2$, 135 NaCl, 1 $MgCl_2$, 5 KCl, 0·44 KH_2PO_4, 0·34 Na_2HPO_4, 2·6 $NaHCO_3$, 20 HEPES, 10 glucose; plus dilutions of (vol:vol) 0·02 amino acids, 0·01 vitamins, 0·002 Phenol Red, 0·01 penicillin/streptomycin (GIBCO, Grand Island, NY, USA), 2% horse serum (Hazelton, Lenexa, KA, USA), pH adjusted to 7·4 with NaOH. Arteries were cleaned of connective tissue and cut into segments and pinned in 30 ml bottles with the lumen facing up. Two millilitres of enzyme solution in 0·5 mM-Ca^{2+} containing 294 U ml^{-1} collagenase (Worthington), 2 mg ml^{-1} bovine serum albumin (Sigma), 1 mg ml^{-1} soybean trypsin inhibitor (Sigma), 0·4 mg ml^{-1} DNAase (Sigma, Chemical Co.) were applied to the tissues. The arteries bathed in the enzyme solution for 60 min in a shaking bath at 37 °C. Every hour the enzyme solution was removed and a new fraction added. The second or third fractions of enzyme solution predominantly contained smooth muscle cells and were used for subsequent studies.

Fura-2 microfluorometry

Fura-2, a fluorescent dye, was used to measure intracellular free Ca^{2+} concentrations in the bulk myoplasm (Ca_{im}) (Grynkiewicz, Poenie & Tsien, 1985). The microfluorometry system, based on methods described by Thayer, Sturek & Miller (1988), has been refined and is described in detail in the companion paper (Sturek *et al.* 1992). Intracellular Fura-2 results are expressed as fluorescence ratios for reasons presented by Sturek *et al.* (1992), but the terms ratio and Ca_{im} are used interchangeably throughout the text. Drops of cells in suspension were placed in a chamber on an inverted microscope (Nikon Diaphot) and the cells quickly attached to the cover-slip at the base of the chamber. Cells were superfused with physiological salt solution (PSS) containing (mM):

2 $CaCl_2$, 138 NaCl, 1 $MgCl_2$, 5 KCl, 10 HEPES, 10 glucose, pH adjusted with NaOH to 7·4. Caffeine (5 mM; Sigma Chemical Co., St Louis, MO, USA), glyburide (10^{-6} M; gift from A. Jones), and 9-anthracenecarboxylic acid (Aldrich Chemical Co., Milwaukee, WI, USA) were added to PSS for application to cells. Depolarization with high external K^+ was accomplished with equimolar replacement of 80 mM of Na^+ with K^+ and the solution referred to as 80K. The period of solution changes are indicated in all figures by horizontal lines labelled with the appropriate solution names. All experiments were performed at room temperature (22–25 °C).

The SR of coronary artery smooth muscle cells from both pigs (Stehno-Bittel *et al.* 1990) and cows (Sturek *et al.* 1992) can be loaded with Ca^{2+} to a reproducible level by depolarizing the cells with high external K^+ for 3–5 min. Depolarization allows Ca^{2+} to enter the cell and subsequently be taken up by the SR (Sturek *et al.* 1992). Alternatively, the same intracellular Ca^{2+} store can be nearly emptied by exposure to caffeine (5×10^{-3} M). Caffeine releases Ca^{2+} from the same intracellular pool in rabbit aorta that is sensitive to noradrenaline, histamine, angiotensin, vasopressin, and prostaglandins (for review see van Breemen & Saida, 1989). Porcine coronary artery smooth muscle showed no greater release of Ca^{2+} from the SR with 25 mM-caffeine than with 5 mM-caffeine (Stehno-Bittel *et al.* 1991). A 2–3 min caffeine application (5×10^{-3} M) abolished subsequent measurable Ca^{2+} release by endothelin (Wagner-Mann & Sturek, 1991); ryanodine, and noradrenaline (L. Stehno-Bittel, unpublished observations). Therefore, we use depolarization (holding potential of −40 mV in a patch clamp mode or by applying 80 mM-K^+ when the membrane potential is not voltage clamped) as a tool to load the SR Ca^{2+} store, and caffeine as a tool to measure the relative amount of Ca^{2+} released from the SR.

Net Ca^{2+} efflux using extracellular Fura-2 free acid

Prior to initiating experiments all glassware (including pipette tips) was thoroughly rinsed in 0·5 mM-EGTA (Fluka Chem. Co, Ronkonkoma, NY, USA) consisting of (mM): 140 NaCl, 1 $MgCl_2$, 5 KCl, 10 HEPES, 0·5 K-EGTA, 10 glucose, pH adjusted to 7·4 with NaOH to remove any contaminating Ca^{2+}. Glassware received a second rinse in deionized water to remove any remaining EGTA and was subsequently air dried. Cells were dispersed as described above; however, fractions of enzyme solution with smooth muscle cells were combined to form a suspension of cells containing an average of 2×10^6 cells. We found this cell number to be optimal for net Ca^{2+} efflux studies. The cells were centrifuged at 3200 *g* for 5 min and the supernate removed and discarded. The cells were resuspended in 5 ml of pretreatment solutions, 80 mM-K^+ or 5 mM-caffeine (described above), and placed in a shaking bath for 5 min. Both pretreatment solutions contained 2 mM-Ca^{2+}. The suspensions were centrifuged again and the supernate removed. The cell pellets were placed in 0·5 mM-EGTA solution to remove excess external Ca^{2+}, spun again, and finally resuspended in the Fura-2 solution, in which 0·5 mM of membrane impermeant Fura-2 replaced EGTA as the external Ca^{2+} buffer. The cell suspensions (total of 150×10^{-6} l) were transferred to a chamber made of a cloning ring attached to a glass cover-slip with Sylgard and, therefore, the suspensions were not superfused during the data collection. The chamber was placed on the microscope and Fura-2 fluorescence ratio collected continuously as previously described (Sturek *et al.* 1992).

Following the experiments the number of cells per suspension was determined. Cells were washed from the chamber using the physiological buffer and placed on a haemocytometer (Sigma Chemical Co.) for counting. Cells on five individual grids of the haemocytometer were counted and the mean taken as an approximation of the cellular concentration per suspension (Freshney, 1987).

In control experiments changes in the extracellular Fura-2 emission signal were measured during Triton X-100 application to the suspension. Triton X-100 (gift from Calvin Hale) was added directly to the cell suspension while in the chamber. Triton X-100 (0·02 and 2·0%) was made as a 10% stock in a Ca^{2+}-free solution made similar to the EGTA solution described above without the 0·5 mM-EGTA.

Whole-cell voltage-clamp and microfluorometry

The whole-cell configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) was used to record ionic currents from single cells. The configuration, including dialysis of the cells with the contents of the pipette, thus permitted control of the intracellular ionic environment and injection of Fura-2 pentapotassium salt. A fire-polished micropipette (4–7 MΩ) was sealed (seal resistance greater than 1 GΩ) against the cell membrane by applying suction following initial contact. After obtaining a gigaohm seal one edge of the measurement aperture was

moved (using a knurled finger screw which did not mechanically disturb the seal) to mask off the pipette tip from the measurement area of the photomultiplier tube. Care was taken to minimize background fluorescence by the following procedures. First, a small area (about 5 μm diameter) near the cell–pipette seal was outside the light-sensitive (photodetection area). Second, the rectangular aperture was closed 5 μm beyond the pipette tip. Third, the 5–8 MΩ pipettes resulted in only a little 'spray' of Fura-2 to the outside of the cell. Fourth, the focal plane of the microscope was on the cell not the pipette. The pipette was at a 60 deg angle to the cover-slip of the chamber and, therefore, was largely out of the focal plane. Fifth, the illumination aperture on the epifluorescence attachment was adjusted to be only 90 μm diameter of the field, thus excluding the pipette from direct excitation.

After the gigaseal was formed the cell membrane at the pipette tip was ruptured by suction to allow simultaneous whole-cell voltage-clamp and Fura-2 measurements. The most common pipette solution used was termed KCl and was composed of (mM): 135 KCl, 10 NaCl, 1 $MgCl_2$, 2 MgATP, 0·5 Tris-GTP, 0·2 EGTA, 0·1 Fura-2 pentapotassium salt, pH adjusted to 7·1 with KOH. To identify the portion of the outward current carried by K^+, intracellular KCl was replaced with CsCl in appropriate solutions. The external solutions were identical to those used for Fura-2 experiments (described above).

For cells with desired internal Ca^{2+} concentrations pipette solutions were made consisting of (mM): 126 KCl, 10 NaCl, 20 HEPES, 1 $MgCl_2$, 0·1 Fura-2, pH adjusted to 7·1 with KOH. Free Ca^{2+} concentrations ranging from 0 to 1000 nM were achieved by adding appropriate amounts of H_2K_2-EGTA and CaK_2-EGTA calculated using the apparent stability constant of $3{\cdot}969 \times 10^6$ (Fabiato & Fabiato, 1979; Thayer *et al.* 1988). The total EGTA concentration was always 10 mM. BAPTA (Calbiochem Corp., San Diego, CA, USA) was also used as a Ca^{2+} buffer in pipette solutions. The Ca^{2+} concentration *vs.* Fura-2 fluorescence calibrations were fitted with a non-linear least-squares fit routine available on the Sigmaplot graphics program (Jandel Scientific, Corte Madera, CA, USA).

Ionic currents were amplified by a List EPC-7 patch-clamp amplifier with a headstage having switchable feedback resistors of 0·5 and 50 GΩ. Capacitance transients were measured for each cell during 10 ms pulses from a holding potential of −80 mV to a test potential of −70 mV. Capacity currents were filtered at a low-pass cut-off frequency of 8·4 kHz and digitized at 25 μs intervals. The K^+ currents were then filtered by an 8-pole low-pass filter at a cut-off frequency of 400 Hz, digitized at 600 μs intervals, and stored and analysed on computer using AxoBASIC software (Axon Instruments, Foster City, CA, USA).

Statistics

Group data are expressed as means ± S.E.M. unless otherwise indicated. Paired *t* tests were used when comparing only two groups. However, most frequently multiple groups were compared and analysis of variance was utilized. Significance was defined as $P < 0{\cdot}05$ for all results.

RESULTS

Prediction 1: the SR spontaneously unloads Ca^{2+}

The protocol to measure the unloading of Ca^{2+} from the SR is illustrated in Fig. 1 and basically involves loading the SR with Ca^{2+} by depolarization-induced influx and determining the relative Ca^{2+} content of the SR after varying recovery periods. One group of bovine cells (Fig. 1*A*, ●) was superfused with physiological buffer until minute 9 of the protocol when they were exposed to high external K^+ (80K) which depolarizes the cells allowing Ca^{2+} influx, and, thereby, loading the SR with Ca^{2+} (Sturek *et al.* 1992*a*). The ratio increased from 0·76 ± 0·02 to 0·84 ± 0·03 during the depolarization which corresponds to Ca_{im} concentrations of 93·5 ± 5·1 and 109·7 ± 7·2 nM-Ca^{2+}, respectively using Fura-2 *in vitro* calibration curves similar to that shown in Sturek *et al.* (Sturek *et al.* 1992). Following a 3 min exposure to 80K the cells were returned to physiological buffer for 2 min followed by exposure to

caffeine (5 mM), which induces a rapid release of Ca^{2+} from the SR. We have previously termed the time between depolarization and caffeine application the 'recovery period' (Stehno-Bittel *et al.* 1990). Therefore, this protocol (●) resulted in a short, 2 min recovery.

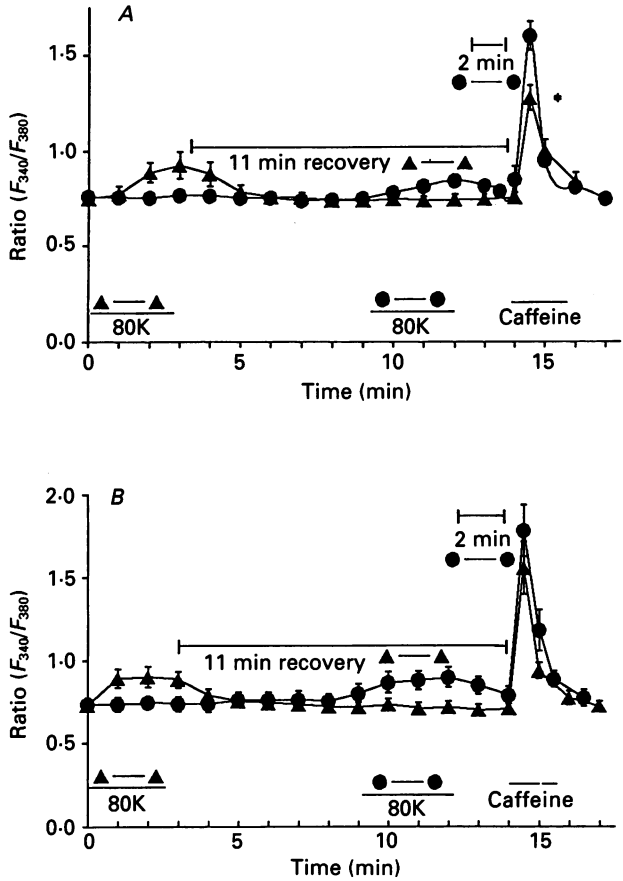

Fig. 1. The caffeine-sensitive SR Ca^{2+} store decreases with long recovery times. Changes in Ca_{im} were measured with Fura-2. A, one group of bovine cells (●, $n = 15$) was superfused with physiological buffer until minute 9 when exposed to 80 mM-K^+ (80K) for 3 min as indicated by the horizontal line. There was a 2 min recovery period and then at minute 14 caffeine (5 mM) was applied. The second group of bovine cells (▲, $n = 17$) were exposed to 80K for 3 min at minute 0 and then an 11 min recovery period in physiological buffer prior to caffeine exposure. Asterisk indicates significant ($P < 0{\cdot}05$) decrease in the response. *B*, porcine cells were subjected to the same two protocols described above involving short 2 min recovery (●, $n = 7$) and long, 11 min recovery from depolarization (▲, $n = 8$). The cells caffeine-induced increases in Ca_{im} did not differ with the two protocols.

The second group of bovine cells were exposed to 80K from minutes 0–3 and the Fura-2 ratio (indicating Ca_{im}) increased from $0{\cdot}75 \pm 0{\cdot}01$ to $0{\cdot}83 \pm 0{\cdot}07$. A long, 11 min recovery in physiological buffer followed until the caffeine exposure at minute 14. We predicted that as the time between depolarization and caffeine application

(recovery time) increased more Ca^{2+} would be allowed to spontaneously leave the Ca^{2+}-loaded SR, thus the caffeine-induced Ca_{im} peak would be attenuated. The results of bovine cells confirm this prediction as the caffeine-induced Ca_{im} peak was significantly less (31 % less) following an 11 min recovery as compared to a 2 min recovery. The mean caffeine-induced increases in the Fura-2 ratios were $1{\cdot}60 \pm 0{\cdot}08$ and $1{\cdot}28 \pm 0{\cdot}07$ for cells with a 2 and 11 min recovery, respectively. Using a myoplasmic calibration curve such as that shown in Sturek *et al.* (Sturek *et al.* 1992) the resting Ca_{im} concentration of these cells was approximately 100 nM and the caffeine-induced Ca_{im} peaks were 1000 and 700 nM-Ca^{2+} following 2 and 11 min recovery periods, respectively. During the 11 min recovery period (minute 3–14) the Ca_{im} concentrations remained steady at resting values. We assume the 31 % difference in the caffeine-induced Ca_{im} peak was due to unloading of Ca^{2+} from the SR during the recovery period and that the Ca^{2+} unloaded from the SR did not accumulate significantly in the bulk myoplasm since the Fura-2 ratio did not increase during the recovery period (Fig. 1*A*).

In contrast, porcine coronary artery cells failed to show a significant decline in the relative SR Ca^{2+} store with an 11 min recovery. Figure 1*B* illustrates the same two protocols shown in Fig. 1*A* except using porcine cells. The caffeine-induced Ca_{im} peaks were not different following either a 2 or 11 min recovery period (ratios of $1{\cdot}78 \pm 0{\cdot}16$ and $1{\cdot}66 \pm 0{\cdot}16$ following 2 and 11 min recovery periods, respectively). These Fura-2 ratios correspond to Ca_{im} concentrations of approximately 1200 and 1000 nM-Ca^{2+} using myoplasmic calibration curves (Sturek *et al.* 1992).

In order to characterize the time dependence of the spontaneous unloading of Ca^{2+} from the SR we repeated the protocols described in Fig. 1, except that the length of the recovery time was varied. In Fig. 2 the caffeine-induced Ca_{im} peaks are plotted as an increase in Ca_{im} above resting Ca_{im} levels. The relative amount of Ca^{2+} in the SR in porcine cells (hatched bars) that was released by caffeine did not differ statistically with changes in recovery times. In contrast, the amount of Ca^{2+} in the SR of bovine cells (open bars) released by caffeine was attenuated with long recovery periods. To our surprise, the caffeine-induced Ca_{im} peaks did not differ statistically following a 2 or 7 min recovery in bovine cells (percentage increase of $218{\cdot}4 \pm 18{\cdot}6$, and $263{\cdot}7 \pm 54{\cdot}9$ for 2 and 7 min, respectively). Following 11 min of recovery the increase in Ca_{im} was $171{\cdot}1 \pm 16{\cdot}8$ % and following 14 min of recovery only $80{\cdot}8 \pm 9{\cdot}8$ % in bovine cells. In all experiments the Ca_{im} returned to resting levels and remained steady during the recovery periods. Based on the results shown in Fig. 2, we further predicted that, without stimulation (depolarization-induced Ca^{2+} influx) to load the SR with Ca^{2+}, the SR Ca^{2+} store would be depleted further as evidenced by a small caffeine-induced Ca_{im} peak relative to the peak following a 14 min recovery. Therefore, cells were superfused with physiological buffer until minute 14 when they were exposed to caffeine. The mean caffeine-induced Ca_{im} peak measured during this protocol is shown in Fig. 2 (control). The SR Ca^{2+} store of bovine cells was not depleted partially, as we had predicted; instead, the store was not different from the amount of Ca^{2+} following a 2 or 7 min recovery (control cells = $255{\cdot}5 \pm 4{\cdot}24$ % increase). Consistent with the Ca^{2+} in the SR following 2, 7, 11 or 14 min recovery periods, there was no difference in the relative amount of Ca^{2+} released by the SR of porcine cells.

Prediction 2: Ca^{2+}released spontaneously from the SR is extruded from the cell

Ca^{2+} efflux studies have shown that *agonist-induced* Ca^{2+} release from the SR of vascular smooth muscle cells increases Ca^{2+} efflux to the extracellular space (Leijten & van Breemen, 1984). However, there is little published evidence demonstrating

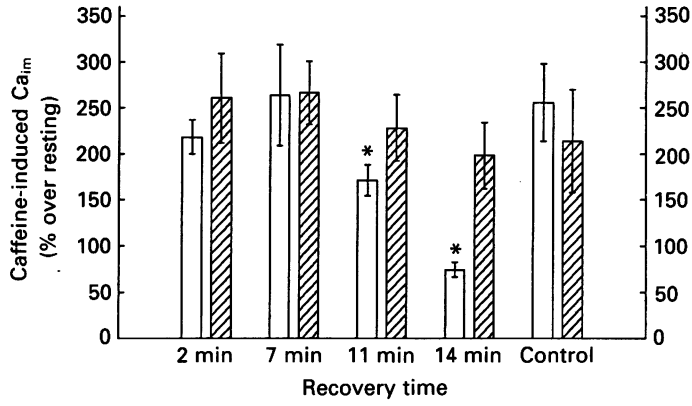

Fig. 2. Dependence on recovery time of unloading of Ca^{2+} from the SR Ca^{2+} store of bovine, but not porcine cells. The figure summarizes data collected from ninety-three cells with six to seventeen cells per value. The caffeine-induced Ca_{im} peaks at minute 14 in Fig. 1 are plotted as a percentage over resting Ca_{im} concentrations *versus* different recovery periods. The control value indicates the caffeine-induced Ca_{im} increase at minute 14 without prior intervention. The SR Ca^{2+} store of bovine cells (open bars) was significantly less (*) than other values following 11 or 14 min recovery periods, while porcine cells (hatched bars) showed no change.

that in the absence of agonist stimulation, Ca^{2+} efflux from the cell is correlated with the amount of Ca^{2+} in the SR store. We have designed experiments using membrane impermeant Fura-2 free acid extracellularly to monitor changes in free extracellular Ca^{2+} as an index of net Ca^{2+} efflux from the cell. To our knowledge this experimental design has not been used with intact cells, but has been used successfully with skeletal muscle membrane vesicles (Kargacin, Scheid & Honeyman, 1988) and saponin-permeabilized cells (Meyer, Holowka & Stryer, 1988).

Control experiments determined that extracellular Fura-2 was sufficiently sensitive to monitor changes in extracellular Ca^{2+}. Triton X-100 (0·02%) added to the cell suspension increased the Fura-2 ratio 123% ($n = 3$ suspensions) and 2·0% Triton X-100 increased the ratio 247% ($n = 5$ suspensions). To ensure that the measured fluorescence reflected Ca^{2+} accurately for the entire suspension the following experiments were conducted ($n = 6$ suspensions). Cells were exposed to Triton X-100 (2·0%) resulting in an increase in extracellular Ca^{2+}. After the Ca^{2+} concentration had stabilized, the chamber was removed from the microscope stage, mixed vigorously and replaced on the stage for Fura-2 emission measurements. In all experiments mixing did not alter statistically the Fura-2 ratio and, therefore we conclude that solutions in the chamber were homogeneous. Control experiments indicate that the fluorescence signal from the Fura-2 solution without cells present in the chamber was constant over the 18 min time course of net Ca^{2+} efflux

experiments. The addition of caffeine, 80K, or Triton X-100 (all in Ca^{2+}-free solutions) to Fura-2 solution without cells failed to alter the Fura-2 emission ratio ($n = 2$). These results support the use of extracellular Fura-2 as a method for monitoring accurately net Ca^{2+} efflux from suspensions of intact cells.

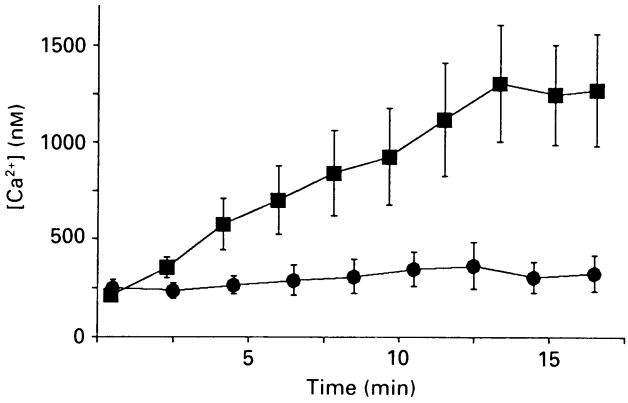

Fig. 3. Bovine cells illustrate spontaneous loss of Ca^{2+} from the cell when the SR is loaded with Ca^{2+}. Suspensions of cells were exposed to 80K (■, $n = 4$ suspensions) or caffeine (●, $n = 4$ suspensions) for 5 min followed by rinsing. Changes in extracellular Ca^{2+} were monitored with extracellular Fura-2 free acid and converted to changes in extracellular Ca^{2+} concentrations in nM. The values at min 0 and 18 are given in Table 1.

Bovine coronary smooth muscle cells were dispersed and placed in suspension to be exposed for 5 min to either 80K (to load the SR Ca^{2+} store) or 5 mM-caffeine (to unload the SR Ca^{2+} store). The cells were rinsed in 0·5 mM-EGTA solution to remove excess extracellular Ca^{2+} and finally suspensions pretreated with either 80K or caffeine were placed in 0·2 mM-Fura-2 solution in which external Fura-2 was the major Ca^{2+} buffer present. As shown in Fig. 3 when cells were pretreated with 80K (■) the Fura-2 ratio increased significantly, indicating an increase in extracellular Ca^{2+}. Cells that were pretreated with caffeine (●) showed little increase in the Fura-2 ratio during the same time course. The results indicate that the initial Ca^{2+} content of the SR in bovine smooth muscle cells may be correlated with net Ca^{2+} efflux from the cell during SR Ca^{2+} unloading, thus providing support for the second prediction. In contrast to the results obtained using bovine cells, pretreatment with 80K or caffeine did not affect the extracellular Fura-2 signal when using porcine cells.

The Fura-2 ratio values obtained during the net Ca^{2+} efflux experiments (Fig. 3) were converted to free extracellular Ca^{2+} concentrations. Typical *in vitro* conversion curves are shown in Fig. 4. The mean free extracellular Ca^{2+} concentrations (nM) are shown in Table 1 at minute 0 (initial Ca^{2+}) and minute 18 (final Ca^{2+}) of the experiments with the calculated change in free extracellular Ca^{2+} shown (ΔCa^{2+}). Free external Ca^{2+} concentrations were converted to total Ca^{2+} concentrations using methods of Kargacin *et al.* (1988) which allows for the calculation of total Ca^{2+} with known or measured parameters.

$$Ca_{total} = Ca^{2+}_{free} + \text{Fura-2}_{total} - (K_D \times \text{Fura-2}_{total})/(K_D + Ca^{2+}_{free}),$$

where K_D is the dissociation constant. The change in external Ca_{total} concentration

was converted to a change in the number of external Ca^{2+} ions (knowing the total volume of the suspension) and finally divided by the number of cells in the suspension, resulting in the net number of Ca^{2+} ions released per cell. Changes in external Ca^{2+} concentrations were monitored over time; therefore, changes in the number of external Ca^{2+} ions were divided by the number of minutes, resulting in an

TABLE 1. Ca^{2+} efflux in bovine and porcine cells

	Bovine		Porcine	
Treatment	Caffeine	80K	Caffeine	80K
Initial Ca^{2+} (nM)	250 ± 55	214 ± 24	230 ± 62	225 ± 48
Final Ca^{2+} (nM)	307 ± 78	1269 ± 290	344 ± 73	328 ± 60
ΔCa^{2+} (nM)	60 ± 30	1002 ± 282	113 ± 39	104 ± 52
Ca^{2+} ions min^{-1} $cell^{-1}$	$4{\cdot}58 \times 10^7$	$2{\cdot}42 \times 10^{8}$*	$7{\cdot}66 \times 10^7$	$7{\cdot}03 \times 10^7$

Ca^{2+} efflux was greatest in bovine cells pretreated with 80K. Initial Fura-2 ratio values obtained from bovine (Fig. 3) and porcine cell suspensions were converted into free extracellular Ca^{2+} concentrations (nM) using the polynomial $110\,R^2 + 63\,R - 24 = Ca^{2+}_{free}$ where R = ratio. The change in Ca^{2+} was converted to total Ca^{2+} and normalized for time and cell number (Ca^{2+} ions min^{-1} $cell^{-1}$).

average number of Ca^{2+} ions released per minute from each cell. The results of these calculations are shown in the last row of Table 1 (Ca^{2+} ions min^{-1} $cell^{-1}$). The number of Ca^{2+} ions released per minute from bovine cells differed statistically from the results of porcine cells only when the bovine cells were pretreated with 80K to load the SR Ca^{2+} store. Bovine cells pretreated with 80K released 5·25 times more Ca^{2+} ions min^{-1} $cell^{-1}$ than bovine cells pretreated with caffeine. The number of Ca^{2+} ions released from porcine cells was not altered by the pretreatment.

Prediction 3: subsarcolemmal Ca^{2+} concentrations (Ca_{is}) are higher than Ca^{2+} in the bulk myoplasm (Ca_{im})

Whole-cell recordings allow cells to be dialysed with the membrane-impermeant Fura-2 free acid. To ensure that the Fura-2 free acid emission ratio was not altered by cytoplasmic components, solutions with known concentrations of Ca^{2+} (using EGTA or BAPTA) were placed on a cover-slip (Fig. 4, *in vitro*, ●; see (Sturek *et al.* 1992) for details of *in vitro* measurements) or were injected into cells using patch-clamp micropipettes (intracellular, ■). The Fura-2 emission ratio was measured in triplicate with both techniques and the results are plotted in Fig. 4. Clearly, the Fura-2 signal was not altered whether the Fura-2 free acid was intracellular or *in vitro*.

It has been proposed that Ca^{2+}-activated K^+ currents provide a qualitative index of Ca_{is} localization (Benham *et al.* 1986*b*; Pallotta *et al.* 1987; Desilets *et al.* 1989). Ca^{2+}-activated K^+ currents provide a major portion of the total whole-cell K^+ current in most types of vascular smooth muscle (Desilets *et al.* 1989). To identify the Ca^{2+} sensitivity of the whole-cell outward current, Ca^{2+}-buffered solutions were injected into bovine and porcine cells via patch-clamp pipettes and the resulting Fura-2 emission ratio and whole-cell current amplitude (at a test potential of +30 mV) were measured.

To verify that intracellular Ca^{2+} (including Ca_{is}) was fully buffered, we applied caffeine (5 mM) to the cell. In all cells with buffered Ca^{2+} solutions caffeine failed to raise Ca_{im} as measured by Fura-2 and it failed to change Ca_{is} as measured by the whole-cell recording ($n = 51$ cells). In contrast, when bovine and porcine cells were

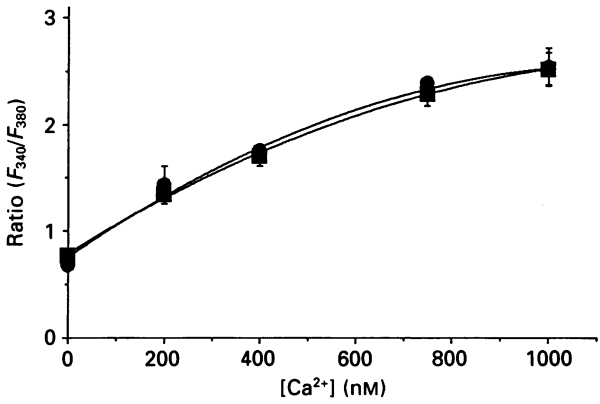

Fig. 4. Calibration of extracellular Fura-2 ratio (●, $n = 3$ replicates per point) and intracellular Fura-2 ratio (■, $n = 3$ cells per point) with [Ca^{2+}]. Fura-2 free acid (in solution with Ca^{2+} concentrations clamped by Ca^{2+}-EGTA buffers) was placed on a coverslip on the microscope used to collect data (*in vitro*) or injected into a cell via a micropipette (intracellular) and the corresponding fluorescence ratio (F_{340}/F_{380}) values measured. *In vitro* and intracellular data were fitted by the second-order polynomial equations: $-441{\cdot}1\,R^2 + 507{\cdot}6\,R + 5{\cdot}2 = Ca^{2+}_{free}$ and $-475{\cdot}6\,R^2 + 547{\cdot}6\,R + 5{\cdot}6 = Ca^{2+}_{free}$, respectively, where R = ratio. See Methods and the companion paper (Sturek *et al.* 1992) for details of Ca^{2+}-EGTA buffer solutions and *in vitro* calibrations.

dialysed with intracellular solutions with low Ca^{2+} buffering, both Ca_{im} and Ca_{is} increased dramatically in response to caffeine ($n = 142$ cells, see Fig. 10 for examples). Therefore, we conclude that intracellular Ca^{2+}, even in the subsarcolemmal region, was fully buffered. The voltage-clamp template is shown in Fig. 5*A*. The holding potential (V_h) was −80 mV and every 5 s the cells were depolarized to a test potential (V_t, in these experiments +30 mV) for 323 ms; thus the cells were depolarized only 6·5% of the time. Increases in Ca_{im} were not noted in these cells during brief depolarizations (less than 5 s) because the cells have a small Ca^{2+} current of less than 10–20 pA under physiological conditions with 2 mM-Ca^{2+} (M. Sturek, unpublished observation). At least 30 s of depolarization was required to increase Ca_{im} in these cells (see change in Fura-2 ratio in Fig. 1). While depolarization of 3–5 min increased Ca_{im} in cells without buffered Ca^{2+}, depolarization did not increase Ca_{im} in cells infused with Ca^{2+}-clamped solutions, thus providing further evidence that cells were Ca^{2+} clamped. The voltage template for long depolarizations is shown in Fig. 5*A* (dashed line). The protocol is similar to that already described except that the holding potential was −40 mV. In Ca^{2+}-clamped porcine cells, when the holding potential was −40 mV the amplitude of the I_K at +30 mV was reduced by 56% ($n = 3$ cells) due to inactivation of the channels. If, however, the membrane potential was returned to −80 mV (conditioning pulse, CP) for a duration as short as 45 ms prior to the test potential, the amplitude of the I_K did not decrease during

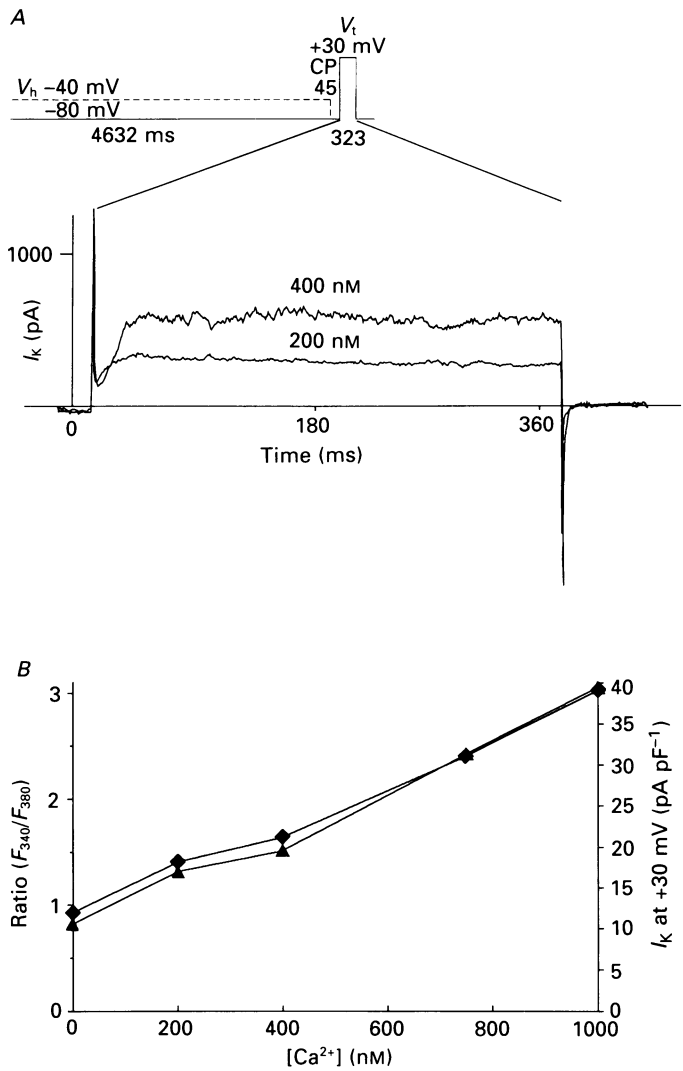

Fig. 5. Changes in the Fura-2 ratio (▲) and the I_K (◆) in response to clamped Ca^{2+} concentrations in bovine cells. Five bovine cells were dialysed with intracellular solution containing Fura-2 and clamped Ca^{2+} concentrations using EGTA. *A*, the voltage-clamp template (insert) shows the membrane potential held at −80 mV (V_h) and depolarized to a test potential (V_t) of +30 mV at 5 s intervals. The voltage-clamp protocol for long-duration depolarization to holding potential of −40 mV is also shown (dashed line, see text for discussion of this protocol). Two raw whole-cell recordings taken during the test potential of +30 mV from a holding potential of −80 mV are shown. The upper recording was obtained from a cell with 400 nM clamped intracellular Ca^{2+} while the lower tracing was obtained from a cell with 200 nM intracellular Ca^{2+}. *B*, the Fura-2 ratio and the current amplitude were measured during the plateau of the outward K^+ current, which was maintained for the last 200 ms of the 323 ms test potential, and plotted.

a 3–5 min depolarization. Therefore, in order to avoid inactivation of the channels during long depolarization, we returned the membrane potential to −80 mV for a 45 ms conditioning potential (CP) prior to the test potential (Fig. 5*A*).

During the test pulse the amplitude of the whole-cell recording was measured. Figure 5*A* shows raw tracings of whole-cell currents from porcine cells during experiments with 400 and 200 nM-intracellular Ca^{2+} (current amplitude of 567 and 308 pA with 400 and 200 nM-Ca^{2+}, respectively). The holding potential for these tracings was −80 mV. The whole-cell current was normalized for differences in plasma membrane surface area by dividing the amplitude of the current by the capacitance of the cell (pA pF^{-1}). The current amplitude/capacitance (pA pF^{-1}) from bovine cells is plotted in Fig. 5*B* (◆) along with the Fura-2 ratio (▲) at clamped Ca^{2+} concentrations of 0, 200, 400, 750, and 1000 nM. The figure illustrates the relationship between changes in the current and the Fura-2 ratio with increasing Ca^{2+} concentrations in bovine cells. A similar relationship was noted in porcine cells (Fig. 5*A*, note the increase in amplitude of the raw tracings from porcine cells in 400 *vs.* 200 nM-Ca^{2+}). At each Ca^{2+} concentration the changes in the Fura-2 ratio and current were parallel. This graph can be used to convert currents to Ca_{is} concentrations and the Fura-2 ratio to Ca_{im} concentrations.

The outward current shown in Fig. 5 was carried predominantly by K^+ as replacement of K^+ with Cs^+ in the micropipette greatly attenuated the current in both bovine and porcine cells to $3{\cdot}4 \pm 0{\cdot}1$ pA pF^{-1} at a test potential of +30 mV ($n = 7$ cells). This is 4 times less than the mean current amplitude when K^+ was present. The whole-cell current amplitude in Cs^+ reversed at −8 mV and the current–voltage relationship was linear, suggesting primarily leakage current. These results did not differ in cells from bovine or porcine tissue. Ca^{2+}-sensitive Cl^- channels have been reported in smooth muscle (Byrne & Large, 1988). To ensure that the current monitored was K^+, cells were superfused with 10 mM-9-anthracenecarboxylic acid (9-AC), a Ca^{2+}-sensitive Cl^- channel blocker (Welsh, 1986). Addition of 9-AC to the bath did not alter the amplitude of the whole-cell current at test potentials of −60 to +50 mV in Ca^{2+}-clamped cells ($n = 2$ cells). Aspartate was used as an anion replacement for Cl^- in cells ($n = 23$ cells) since aspartate will not pass through Cl^- channels. Replacement of Cl^- with aspartate did not alter the amplitude of the current. Therefore, we conclude that the majority of the current consists of the outward movement of K^+ ions. Furthermore, the current did not appear to be ATP-sensitive as the addition of glyburide (10^{-6} M) to the external solution failed to reduce the current amplitude in twenty-five of twenty-six cells. In only one bovine cell did glyburide reduce the current amplitude (reversible 24% decrease in amplitude) following a 2 min exposure and that occurred 1 h into the whole-cell recording, suggesting that after such a long whole-cell recording the cell may have been partially ATP-depleted. Currents in cells with clamped intracellular Ca^{2+} concentrations did not diminish when exposed to glyburide ($n = 5$ cells). Finally, cells with Ca^{2+}-clamped intracellular solutions were exposed to Ca^{2+}-free and 5 mM-Ca^{2+} external concentration solutions ($n = 5$ cells). Neither affected the amplitude of the Ca^{2+}-activated K^+ current (I_K).

The I_K was sensitive to membrane potential. We identified the voltage sensitivity by completing current *vs.* voltage relationships while intracellular Ca^{2+} concentrations were clamped (as described above). With a holding potential of −80 mV test potentials were applied starting at −60 mV and increasing by 10 mV increments to +50 mV. As the intracellular Ca^{2+} concentration increased and the membrane potential became more positive the whole-cell current amplitude also increased

(Fig. 6). The solutions used for the data obtained in Fig. 6 were made with EGTA as the Ca^{2+} buffer; however, Ca^{2+}-free solutions were also made with BAPTA as the Ca^{2+} buffer. The mean amplitude of the whole-cell current did not differ whether EGTA or BAPTA was used. Mean current amplitudes in BAPTA at +30, 40 and 50 mV

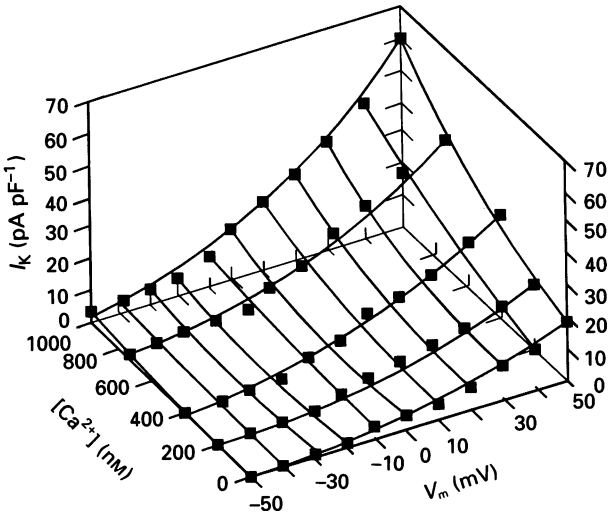

Fig. 6. Whole-cell I_K is sensitive to Ca_{is} and membrane potential (V_m). Bovine cells (2–3 cells per value) were dialysed with Ca^{2+}-clamped intracellular solutions. Current–voltage relationships from a holding potential of −80 mV by depolarizing the cells every 5 s to test potentials ranging from −60 to 50 mV in 10 mV increments. The mean I_K amplitude (pA pF^{-1}) is plotted *versus* the test potential and intracellular Ca^{2+} concentration. Standard error bars are not included for clarity.

were 10·8, 15·4 and 18·3 pA pF^{-1}, respectively, which were all similar to values obtained in Ca^{2+}-EGTA solutions (see Fig. 6). Using EGTA-buffered solutions the I_K amplitude (measured in pA pF^{-1}) at +30 mV of bovine cells (Fig. 6) and porcine cells was 2·5 times greater in 750 nM-Ca^{2+} *vs.* Ca^{2+}-free internal solutions. The Ca^{2+} sensitivity of the I_K did not differ in bovine or porcine cells; however, the variability in the current amplitude increased at 1000 nM clamped internal Ca^{2+}. Barrett *et al.* (1982) found that the Ca^{2+} sensitivity of the I_K increased at positive membrane potentials in skeletal muscle. We found a similar increase in the sensitivity as the slope of the line (I_K amplitude *vs.* Ca^{2+} concentration) increased at more depolarized membrane potentials.

With the whole-cell current calibrated to estimate Ca_{is} concentrations, we compared bovine cells with similar Fura-2 ratios in which intracellular Ca^{2+} was allowed to fluctuate freely *vs.* bovine cells in which intracellular Ca^{2+} concentrations were clamped at 400 nM (Fura-2 ratio of 1·67±0·02 for both groups). The current–voltage relationship was again compared with a holding potential of −80 mV and test potentials from −60 to +50 mV. Full Ca^{2+} clamp of cells was verified in all cells by the absence of changes in I_K or ratio in response to caffeine-induced Ca^{2+} release. In bovine cells (Fig. 7*A*) the I_K was significantly larger in cells where Ca^{2+} was allowed to fluctuate (○, unclamped) than cells with clamped intracellular Ca^{2+} (●, clamped) even though the Fura-2 signal indicated that Ca_{im} in

cells from both groups was less than 40 nM different. At +30 mV the I_K amplitude in cells with unclamped Ca^{2+} was 64·3±15·3 pA pF^{-1} *versus* 25·9±9·7 pA pF^{-1} for Ca^{2+}-clamped cells. Using Figs 5 and 6 to convert the I_K to Ca_{is} the cells with unclamped intracellular Ca^{2+} had a mean Ca_{is} concentration of approximately

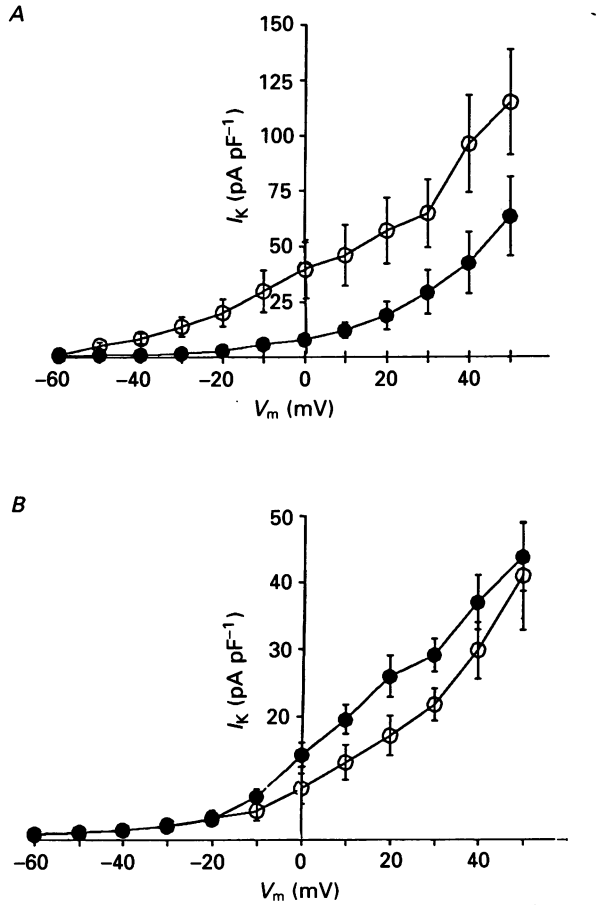

Fig. 7. Ca_{is} gradient exists in bovine, but not porcine cells. I_K in cells with similar intracellular Ca^{2+} concentrations were compared. *A*, bovine cells were dialysed with Ca^{2+}-clamped intracellular solutions of 400 nM-Ca^{2+} (●, $n = 3$) and the amplitude of the whole-cell current at different test potentials was compared to cells with unclamped intracellular Ca^{2+} solutions (○, $n = 5$ cells). Bovine cells with unclamped Ca^{2+} had a significantly greater whole-cell current even though the Fura-2 ratio (1·67±0·02) indicated Ca_{im} in all cells was similar. *B*, porcine cells with the same intracellular Ca^{2+} concentration (400 nM-Ca^{2+}, ratio of 1·64±0·02) were compared with Ca^{2+}-clamped (●, $n = 3$ cells) and unclamped (○, $n = 4$ cells) intracellular solutions. The amplitude of the I_K did not differ significantly except at membrane potentials of 0 to +30 mV.

1800 nM (obtained by extrapolation of the I_K *vs.* Ca^{2+} concentration in Fig. 5) while Ca_{im} measured 400 nM.

Porcine cells (Fig. 7*B*) showed little difference in the I_K whether intracellular Ca^{2+} was clamped or unclamped. For porcine cells the Fura-2 ratio which corresponded with 400 nM-Ca^{2+} was 1·64±0·02. Only at a membrane potential of 0 to +30 mV was

there a significant difference between the two groups; however, I_K was actually lower when Ca^{2+} was allowed to fluctuate freely (○) than when clamped (●). At a test potential of +30 mV the I_K values were 20·3±2·4 and 27·7±2·5 pA pF^{-1} for unclamped and clamped cells, respectively. Therefore, there does not appear to be a significant Ca_{is} gradient in porcine smooth muscle cells under resting conditions.

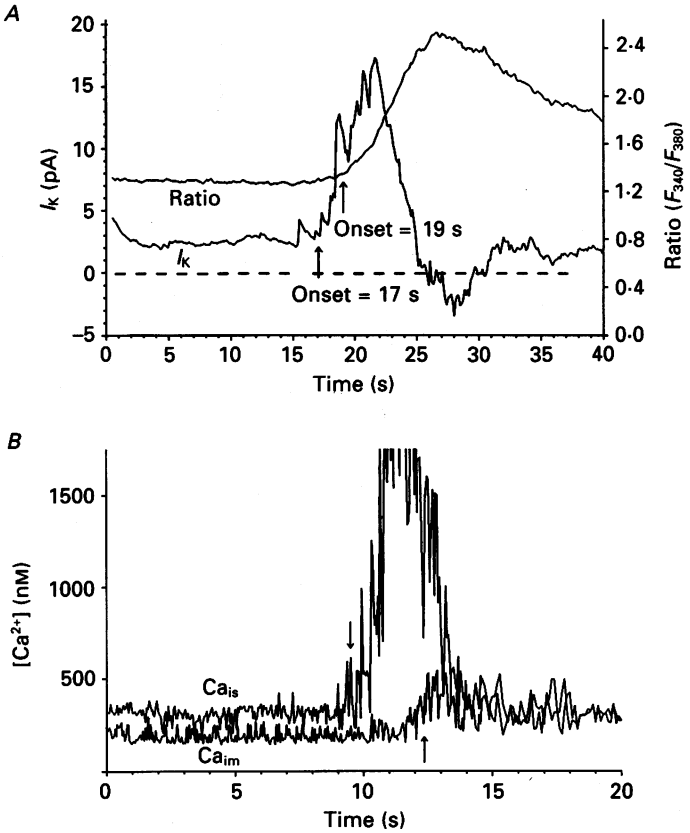

Fig. 8. Caffeine-induced increases in the I_K were noted prior to changes in the Fura-2 ratio. *A*, the Fura-2 ratio and the current were monitored simultaneously in a typical bovine cell. Caffeine was applied at time 0 with a constant holding potential of −40 mV. The onset of the increase in the I_K preceded the increase in the ratio by 2 s. *B*, Fura-2 ratio and I_K from a porcine cell during exposure to caffeine were converted to Ca_{im} and Ca_{is}, respectively. The onset of the increase in Ca_{is} occurred at 9·7 s while the onset of the increase in Ca_{im} was at 12·4 s. Both the I_K and Fura-2 ratio were low-pass filtered at 100 Hz.

Prediction 4: Ca_{is} is directly related to the amount of Ca^{2+} in the SR

The first indication that Ca^{2+} might preferentially be released from the SR toward the sarcolemma was based on agonist-induced Ca^{2+} release. Rather than focusing on differences in Ca^{2+} concentrations between Ca_{im} and Ca_{is}, we focused on a temporal dissociation between changes in Ca_{im} and Ca_{is} that was large enough to rule out simple diffusion of Ca^{2+}. Figure 8*A* shows the Fura-2 ratio and K^+ current obtained from a bovine cell which was held at a constant membrane potential of −40 mV

during a caffeine exposure. The increase in outward current (Ca_{is}) preceded the increase in the ratio (Ca_{im}) by 2 s. The methods used for this study enable resolution of the Fura-2 ratio every 20 ms, thus measurements are not limited by instrumentation. The onset in the increase in the ratio and current values was defined as a 10% increase over the mean value during the first 2 s in five consecutive values. The inward current that followed at 25 s was of uncertain ionic specifity but was most likely due to Ca^{2+}-activated non-selective cation channels (Sturek, Thayer & Miller, 1988; Sturek, Stehno-Bittel & Obye, 1991) and Cl^- channels (Byrne & Large, 1988) that are activated at negative membrane potentials and high Ca^{2+} concentrations. The outward current was again verified as being carried by K^+ because the current was blocked by inclusion of Cs^+ in the pipette ($n = 39$ cells) leaving the inward current unmasked. In seven of nine bovine cells (78%) studied with this protocol the caffeine-induced increase in the I_K preceded the increase in Ca_{im} by a mean value of $1{\cdot}4 \pm 0{\cdot}2$ s. In the other two bovine cells the two events coincided.

The same protocol was completed using porcine cells with holding potentials of either -20 or -40 mV. The I_K increased prior to the change in the Fura-2 ratio in three of four cells. In the fourth cell the ratio and I_K increased together. The holding potentials (-20 or -40 mV) altered the amplitude of the I_K response, but did not affect the time delay in the Fura-2 ratio. The change in I_K preceded the change in the Fura-2 ratio by a mean of $2{\cdot}3 \pm 0{\cdot}3$ s. The Fura-2 ratio values were converted to Ca_{im} using the myoplasmic calibration curve for Fura-2 pentapotassium salt shown in Fig. 1 of the companion paper (Sturek *et al.* 1992). The amplitude of I_K was converted to Ca_{is} using a graph similar to Fig. 6 except with results from porcine cells. Figure 8*B* shows a typical response from a porcine cell while caffeine was applied during a holding potential of -20 mV. The I_K (Ca_{is}) increased at 9·7 s into the recording while the Fura-2 ratio (Ca_{im}) increased 2·7 s later. The results are consistent with those shown in Fig. 8*B* showing little or no subsarcolemmal Ca^{2+} gradient in resting porcine cells. The initial Ca^{2+} concentrations were 169 nM and 251 nM for myoplasmic (Ca_{im}) and subsarcolemmal (Ca_{is}) compartments, respectively. Given the calculations that were performed to obtain these values, the figure indicates little difference in the Ca_{is} and Ca_{im} concentrations in a resting porcine cell. However, there was a large difference in the peak caffeine-induced increases in Ca^{2+} in the two compartments. The peak increase in Ca_{is} has not been included in Fig. 8*B* in order to clarify the time difference in the two Ca^{2+} compartments. The peak caffeine-induced increase in Ca_{is} was extrapolated to be 4368 nM at 12 s, while the peak increase in Ca_{im} was 472 nM-Ca^{2+}. One minute following the caffeine-induced Ca^{2+} peak, the Fura-2 ratio (Ca_{im}) returned to resting levels. We include Ca^{2+} calculations shown in Fig. 8*B*, not to indicate absolute concentrations of Ca_{is} and Ca_{im}, rather to illustrate the vast differences between the compartments in both time and magnitude of responses.

Recordings of I_K were obtained during protocols similar to that shown in Fig. 1 with varying recovery times. The amplitude of the I_K was compared at rest (1 min following access to the whole-cell or when the I_K was steady), 2 min after depolarization to a holding potential of -40 mV (Post-depolarization), and 2 min after the removal of caffeine (Post-caffeine). The time points, 2 min after depolarization and 2 min after caffeine, were chosen because extensive work with

Fura-2 indicated that the Ca_{im} concentration normally returned to resting levels within 2 min (see Fig. 1*A* and *B* for examples). Figure 9*A* illustrates typical recordings of I_K from a bovine cell. At rest the holding potential was maintained at −80 mV (rest) and the test potential was +30 mV (see inset for template). The mean

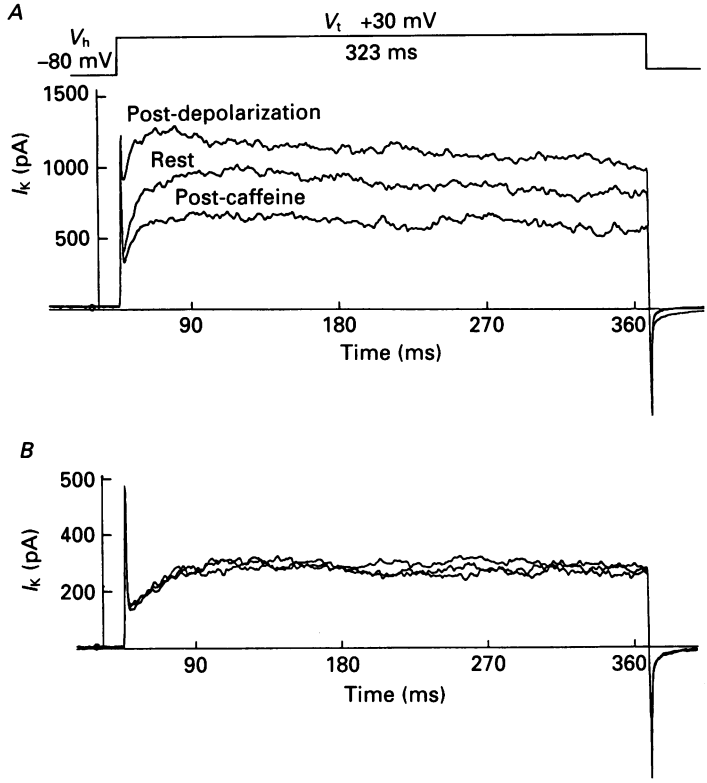

Fig. 9. The amplitude of the I_K was altered by the amount of Ca^{2+} in the SR. *A*, the voltage-clamp protocol during depolarization is shown in the inset (full protocol is shown in Fig. 5*A*). Raw whole-cell recordings from a typical bovine cell are shown during the 323 ms test potential to +30 mV. The current amplitude 2 min following depolarization was significantly greater than the current at rest, and 2 min following the removal of caffeine the current amplitude was decreased. At each time point the Fura-2 ratio did not differ statistically. *B*, whole-cell recordings from a typical porcine cell show no difference in the amplitude of the current whether the cell was at rest, 2 min following depolarization, or 2 min following caffeine.

current amplitude, representing the mean of points between about 180 and 360 ms of the test pulse, under resting conditions was 883 pA. The cell was then depolarized for 3 min, mimicking protocols shown in Fig. 1. The depolarization pulse pattern is shown in Fig. 5*A*. The holding potential was −40 mV with a test potential every 5 s to +30 mV.

Following the 3 min depolarization the holding potential was returned to −80 mV and the test potential +30 mV. Two minutes following the depolarization (Fig. 9*A*, Post-depolarization) the I_K increased 23% to 1145 pA. After a 7 min recovery period the cell was exposed to caffeine and 2 min after the removal of caffeine (Post-caffeine)

the amplitude of the I_K was 31% less (614 pA) than the resting current amplitude. At each of these time points the Fura-2 ratio did not differ statistically, therefore the change in the Ca^{2+}-sensitive I_K cannot be attributed to changes in Ca_{im}.

The amplitude of the I_K from porcine cells, in sharp contrast, did not differ from resting conditions, 2 min following depolarization, or 2 min following caffeine as is shown in Fig. 9*B*. In fact, the three currents are indistinguishable even though the protocols were identical to those used in obtaining Fig. 9*A*. The porcine whole-cell current is smaller than the bovine which can be accounted for by a difference in cell size. The capacitance was 23·7 for bovine cells and 12·1 pF for porcine cells, thus explaining the higher resting whole-cell I_K in the bovine cell.

To show the full time course of the changes in the whole-cell current the Fura-2 ratio (▲) and the I_K (●) were measured simultaneously, are shown in Fig. 10. Data from a typical bovine cell (Fig. 10*A*) show that depolarization to a holding potential of −40 mV increased the I_K only slightly ($V_t = +30$ mV). The I_K increased from a resting value of 203 to 290 pA at minute 3. Depolarization caused a significant increase in the Fura-2 ratio (ratio of 1·18 and 1·66 at minutes 0 and 3, respectively). Following a return to a holding potential of −80 mV the Fura-2 ratio returned to baseline (ratio = 1·17) while the current amplitude increased (453 pA) and remained double its resting level throughout the 11 min recovery. Caffeine was applied at minute 15 and both current (1553 pA) and the Fura-2 ratio (2·22) increased. The sampling interval during this protocol did not allow an accurate measurement of any possible time discrepancy between the onset of the rise in the Fura-2 ratio and the K^+ current. Finally, following the 2 min caffeine application the Fura-2 ratio returned to baseline levels (1·25), while the I_K fell slightly below its resting level (180 pA).

The results shown in Fig. 10*B* were obtained from a porcine cell with a 47% larger surface area than the bovine cell shown in Fig. 10*A* (thus explaining the larger resting current amplitude). When the I_K of the two cells was normalized for capacitance the resting current was 17·1 and 14·2 pA pF^{-1} in the porcine and bovine cells shown in Fig. 10, respectively. The protocol used was identical to that shown in Fig. 10*A*. Unlike the bovine cell, the current did not increase during the depolarization (amplitude of 508 and 466 pA at minutes 0 and 3, respectively) nor did it increase following the depolarization (473 pA); rather, the current was very steady until minute 15 when the cell was exposed to caffeine (3847 pA). The Fura-2 ratio, however, did change in a manner similar to the bovine cell during the depolarization (ratio of 1·25 and 1·98 at minutes 0 and 3, respectively). Porcine and bovine cells both illustrated a caffeine-induced increase in the ratio and current, thus indicating the viability of the porcine cells and an agonist-releasable Ca^{2+} store. Following the caffeine exposure the ratio and current returned to resting levels (ratio = 1·23, $I_K = 481$).

Group data from protocols similar to those illustrated in Fig. 10 are shown in Fig. 11. To illustrate the difference in the results obtained from bovine and porcine cells the current was normalized for cell size by dividing the I_K by the capacitance. In bovine cells the current increased 42% following depolarization (from resting value of $38·8 \pm 6·8$ to $66·5 \pm 10·4$ pA pF^{-1} post-depolarization), while the I_K from porcine cells did not change ($23·4 \pm 3·2$ and $22·11 \pm 2·9$ pA pF^{-1} at rest and post-depolar-

ization, respectively). The caffeine-induced increase in I_K is shown for both porcine and bovine and did not differ statistically (97·4±11·8 and 95·8±23·8 pA pF^{-1} for bovine and porcine cells, respectively). Two minutes following the removal of caffeine the bovine I_K was reduced by 17·5% from its resting value to 19·3±2·7 pA pF^{-1}

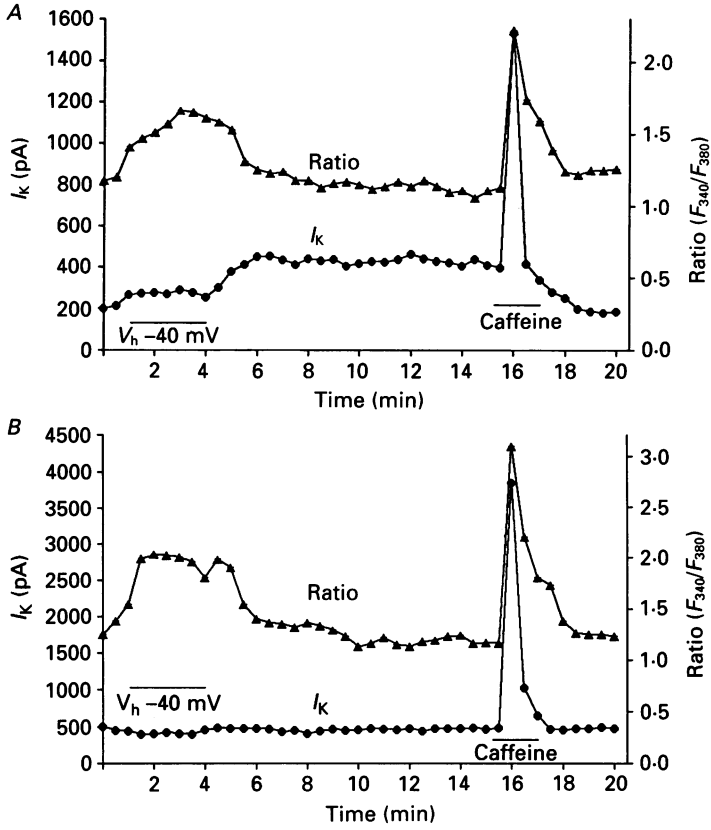

Fig. 10. Simultaneous monitoring of Fura-2 ratios and I_K illustrates the time course of the changes. *A*, Fura-2 ratios and I_K were measured as the bovine cell was depolarized to a holding potential of −40 mV (V_h) for 3 min (see Fig. 5*A* for protocol). During that time the current increased approximately 30% while the Fura-2 ratio rose to 1·65. Following depolarization the Fura-2 ratio returned to resting values while the I_K amplitude increased from 290 to 453 pA and remained high until caffeine was applied at minute 15. Caffeine induced a dramatic increase in both the ratio and current which was followed by a return of the ratio to resting values while the I_K amplitude fell below the initial levels. *B*, the same protocol was completed on porcine cells and the results show that the Fura-2 ratio changed in a manner similar to that seen in bovine cells. In contrast the I_K amplitude did not increase following the depolarization but remained steady until the caffeine application.

while the porcine current returned to levels that did not differ from resting (27·2±4·1 pA pF^{-1}). Thus, there is a correlation between the amount of Ca^{2+} in the SR and the Ca_{is} concentration in bovine, but not porcine, smooth muscle cells.

During experiments shown in Fig. 10 large spontaneous transient outward currents (STOCs) were occasionally noted. These fluctuations have previously been

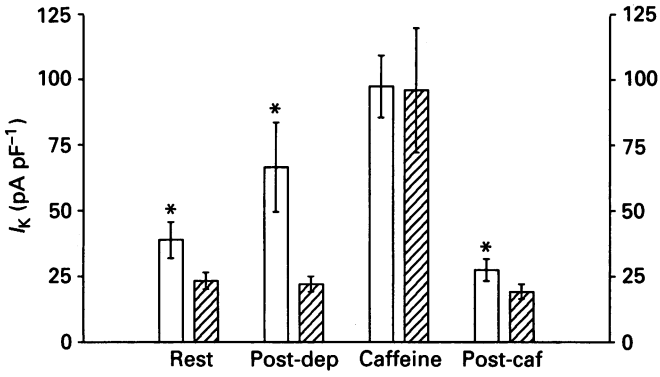

Fig. 11. Group data compiled from protocols similar to that shown in Fig. 10 illustrate the change in the I_K amplitude of bovine ($n = 12$ cells), but not porcine ($n = 10$ cells), cells with alterations in the SR Ca^{2+} store. Following depolarization (Post-dep, when the SR is Ca^{2+}-loaded) the I_K amplitude in bovine cells increases and following caffeine (Post-caf, when the SR is unloaded of Ca^{2+}) the I_K amplitude decreases. In contrast, porcine cells showed no change in the I_K except in response to caffeine. Significant differences in the mean I_K between resting values, and following depolarization and caffeine are indicated by asterisks.

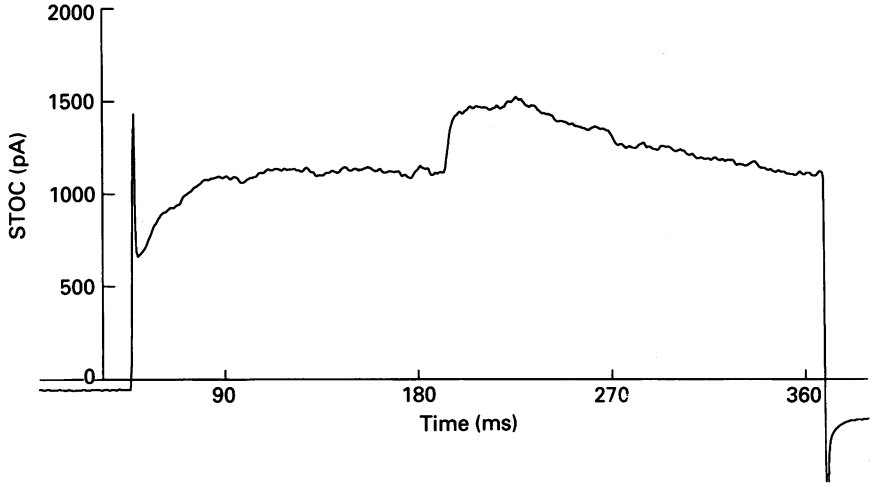

Fig. 12. Typical example of a STOC during a depolarization to a test potential of +30 mV ($V_h = -80$ mV).

described in smooth muscle cells and results indicate that STOCs reflect changes in I_K resulting from localized Ca^{2+} release from the SR (Benham & Bolton, 1986; Desilets *et al.* 1989; Hume & Leblanc, 1989). We defined a STOC as a minimum of a 20% increase in the amplitude of the steady-state I_K. A typical STOC from a porcine cell is shown in Fig. 12. The I_K shown in Fig. 12 increased from a steady-state level of 1103 to 1518 pA during the STOC (38% increase). A dramatic difference was noted in the occurrence of STOCs between bovine and porcine cells. Only seven of eighty-nine (8%) bovine cells had STOCs while porcine cells (using the same

protocol) had STOCs in twenty-six of seventy-two (36%). STOCs were most commonly noted in bovine cells when the SR was Ca^{2+}-loaded (immediately following depolarization). Of the bovine cells that demonstrated STOCs, the frequency of STOCs was 47% greater following depolarization than prior to depolarization (1st *vs.* 6th minute of protocol in Fig. 10). When STOCs were noted in porcine cells depolarization did not affect the frequency. However, the application of caffeine completely abolished the occurrence of STOCs in both cell types which is consistent with previous reports (Desilets *et al.* 1989). STOCs did not appear in cells with intracellular Ca^{2+} clamped at any concentration (1000 nM-Ca^{2+} to Ca^{2+}-free).

DISCUSSION

The main findings of this study are that: (1) the SR unloads Ca^{2+} from its caffeine-sensitive store without increases in Ca_{im}, (2) Ca^{2+} released spontaneously from the SR is extruded from the cell, (3) Ca^{2+} concentrations are higher in the subsarcolemmal region (Ca_{is}) than in the myoplasmic region (Ca_{im}) during spontaneous SR unloading of Ca^{2+}, and (4) Ca_{is} concentrations are directly related to the amount of Ca^{2+} in the SR. Taken together with the results discussed in the companion paper, these findings strongly support the role of the SR in vascular smooth muscle as a Ca^{2+} barrier so that Ca^{2+} fluctuations near the sarcolemma do not affect Ca_{im}. Thus, the SR would inhibit Ca_{im} concentrations from rising in the absence of stimulation, perhaps promoting relaxation.

Prediction 1: the SR spontaneously unloads Ca^{2+}

We predicted, based on the superficial buffer barrier hypothesis (van Breemen & Saida, 1989) that, when filled with Ca^{2+}, the SR releases Ca^{2+} preferentially to be extruded from the cell. Therefore, measuring the amount of Ca^{2+} in the SR store at varying times after a Ca^{2+} loading period should demonstrate a loss of Ca^{2+} from the SR. Previously we demonstrated a time-dependent loss of Ca^{2+} from the caffeine-sensitive store in coronary artery smooth muscle from exercise-trained miniature pigs, but not in sedentary pigs (Stehno-Bittel *et al.* 1990, 1991). Furthermore, we found spontaneous SR Ca^{2+} release to be sensitive to ryanodine (blocked by high concentrations of ryanodine and enhanced by low concentrations), suggesting that spontaneous SR Ca^{2+} release is via ryanodine-sensitive SR Ca^{2+} release channels (Stehno-Bittel *et al.* 1991). In this study we used the same initial protocols to determine whether a time-dependent spontaneous release of Ca^{2+} was present in bovine and porcine (not miniature swine) coronary artery smooth muscle cells. Consistent with our previous work which found no spontaneous SR Ca^{2+} release in control miniature pigs, we found that smooth muscle cells from local farm pigs did not illustrate a loss in the caffeine-sensitive SR Ca^{2+} store with varying recovery times. In contrast, bovine cells did show spontaneous SR Ca^{2+} unloading (Fig. 2), which increased in magnitude with longer recovery periods. It is interesting that no depletion of the SR Ca^{2+} store was noted following a 7 min recovery (Fig. 2). The results shown in Figs 10*A* and 11 suggest that the SR is already releasing Ca^{2+} toward the sarcolemma during the first 7 min of the recovery period as the Ca^{2+}-activated K^+ current is elevated in bovine cells. However, each value shown in Fig. 2

is a measurement of the net change in the amount of Ca^{2+} in the SR. Sturek *et al.* (1992) have shown that during depolarization the SR sequesters Ca^{2+} that is entering the cell via voltage-gated Ca^{2+} channels, thus filling its store. Perhaps the depolarization-induced Ca^{2+} uptake is still accelerated 2 and 7 min following the depolarization; therefore, no net change in the store is measured until minute 11. An additional surprise in the results was that the caffeine-induced Ca_{im} transient in cells that had not been previously Ca^{2+}-loaded (Fig. 2, Control) was as large as cells receiving a 2 min recovery. The results stress the time dependence of SR Ca^{2+} unloading (time between depolarization and caffeine application). Perhaps SR Ca^{2+} unloading is initiated by depolarization and/or some event dependent upon depolarization. At present we lack a full explanation for these findings, and experiments are currently underway to characterize better the time dependence of Ca^{2+}-unloading in bovine cells.

Prediction 2: Ca^{2+} released spontaneously from the SR is extruded from the cell

Results summarized in Table 1 show the correlation between the pretreatment with caffeine or 80K and the change in extracellular Ca^{2+} in bovine, but not porcine cells. When the free extracellular Ca^{2+} concentrations were converted to changes in the average number of Ca^{2+} ions released per minute the only value that was different was for bovine cells which had been pretreated with 80K. Care must be taken in using this technique as extracellular Ca^{2+} concentrations must be greater than 100 nM or Mg^{2+}-Fura-2 binding becomes significant (Kargacin *et al.* 1988). Further, Ca^{2+} contamination may be a factor, thus repeated rinsing of glassware with EGTA is necessary. The greatest degree of error is introduced in the counting of cells in the suspension (Freshney, 1987). We took several precautions to ensure that the number of cells on the haemocytometer grids were representative of the entire cell suspension, but errors will still be made. We conclude that quantitative measurements of Ca^{2+} stores using this protocol are, at best, estimates; however, comparisons between caffeine and 80K pretreatments are valid since both treatments were administered under the same conditions.

It is most important to note that this technique measures Ca^{2+} changes similar to values obtained with other techniques. The application of caffeine and subsequent increase in ^{45}Ca in Ca^{2+}-free external solutions were used to estimate the concentrations of Ca^{2+} in rabbit aortic smooth muscle SR (Leijten & van Breemen, 1984). The investigators found that following prolonged Ca^{2+} loading in an 80 mM-K^+ solution the rate of loss of Ca^{2+} from the SR was increased. They calculated that the SR Ca^{2+} concentration was 5·3 mM and following the depolarization the Ca^{2+} concentration increased to 8·5 mM (Leijten & van Breemen, 1984). Iino (1989) calculated the SR Ca^{2+} concentration in guinea-pig taenia to be as high as 15 mM with a gradual decrease in the Ca^{2+} store (half-time of 6 min) which roughly averages 1·25 mM of Ca^{2+} lost from the SR per minute. If we consider the Ca^{2+} increase in the extracellular solution that is due to pretreatment (difference in extracellular Ca^{2+} measured from cells pretreated with 80K *vs.* caffeine) as the change in extracellular Ca^{2+} due to SR Ca^{2+} release and extrusion then we calculate a rate of loss of the SR Ca^{2+} of 1·59 mM min^{-1}. This calculation assumes that the average volume for the cell is 10^{-12} l (estimate calculated from smooth muscle cell dimensions

given by Wagner-Mann, Bowman & Sturek, 1991) and that the SR occupies 5% of that volume (Allen & Seidel, 1986). This calculation agrees generally with Iino's data (Iino, 1989) whereas Leijten & van Breemen's data (Leijten & van Breemen, 1984) indicate an average rate of Ca^{2+} loss from the SR of 130 μM min^{-1} following depolarization. Methods employed by Leijten & van Breemen (1984) examined, more specifically, the loss of Ca^{2+} from the SR, while the technique used in this paper measures loss of all Ca^{2+} from the cells. This may explain the difference in our results along with the many assumptions which had to be made to calculate these estimates.

Prediction 3: subsarcolemmal Ca^{2+} concentrations (Ca_{is}) are higher than Ca^{2+} in the bulk myoplasm (Ca_{im})

It is difficult to measure accurately Ca^{2+} concentrations confined within the 50 nm distance between the sarcolemma and superficial SR. Although confocal microscopy may provide high spatial resolution, a less expensive procedure involves monitoring changes in Ca^{2+} concentrations directly under the sarcolemma via Ca^{2+}-activated K^+ currents. Whole-cell recordings have shown that current through Ca^{2+}-activated K^+ channels increases as Ca_{is} rises. We have shown that these channels in both bovine and porcine smooth muscle cells are sensitive to Ca_{is} and membrane potential (Fig. 6). In this regard, our findings are particularly important, since simultaneous voltage clamp and Ca_{im} measurement is the only method currently available to resolve the dynamic nature of the Ca_{is} compartment. Several considerations must be addressed first before the use of I_K as an estimate of Ca_{is}.

First, Ca^{2+}-sensitive I_K should be the dominant ionic current of the preparation. Our results determined that the current obtained in our preparations was carried predominantly by K^+ (Cs^+ in the pipette virtually abolished the outward current with the remaining current primarily leakage). The dominant current of the cells was sensitive to intracellular Ca^{2+} concentrations as the amplitude of the current at +50 mV was decreased by greater than 80% when intracellular Ca^{2+} was clamped at 0 (comparison of current amplitude in Fig. 6, 0 Ca^{2+} and Fig. 7*A*, unclamped). Second, intracellular Fura-2 free acid should have the same Ca^{2+} sensitivity as *in vitro* mock intracellular Ca^{2+}-clamped solutions. The results of Fig. 4 clearly show that the Fura-2 ratio was not altered by cytosolic factors. Third, in order to convert the I_K to Ca_{is}, experiments must be performed in which both Ca_{is} and Ca_{im} are Ca^{2+}-clamped. In the Ca^{2+}-clamped experiments performed the kinetics of the Ca^{2+} buffer (EGTA or BAPTA) had to be sufficiently fast to buffer the most rapid Ca^{2+} transients under experimental conditions. We have shown that in Ca^{2+}-clamped cells neither the Fura-2 ratio nor I_K changed under several experimental conditions including increased Ca^{2+} influx during depolarization, Ca^{2+}-free extracellular solutions, or caffeine-induced Ca^{2+} release, which causes the largest and most rapid increase in Ca_{im} that can be elicited in these cells. Additionally, STOCs were abolished in cells that were Ca^{2+}-clamped, suggesting an absence of localized Ca_{is}. Fourth, I_K should be normalized for sarcolemmal surface area (in pF) to account for variations in absolute I_K as a function of the number of channels. Although we noted differences in the amplitudes of I_K in Ca^{2+}-clamped cells, when the amplitudes were divided by each cell's capacitance the amplitude of the I_K at each Ca^{2+} concentration was not different in porcine *vs.* bovine cells. Fifth, there should be no direct modulation of Ca^{2+}-activated K^+ channels by experimental agents used in these experiments. The I_K

should be a result of changes in Ca_{is} of membrane potential only. In support of this requirement, we found no modulation of the I_K in Ca^{2+}-clamped cells when caffeine or glyburide were applied. Sixth, with all of the above conditions satisfied, a membrane potential should be selected with which to 'assay Ca_{is}'. Most of the voltage-clamped protocols reported here used a test potential of +30 mV. At this membrane potential the K^+ channels are sufficiently sensitive to Ca^{2+} (Fig. 6) and the cells appear to tolerate a brief depolarization to +30 mV well.

There are weaknesses in using the Ca^{2+}-sensitive I_K to estimate Ca_{is}. The major weakness is that measuring the whole-cell I_K assumes that the K^+ channels and Ca_{is} are evenly distributed along the sarcolemma. In fact, there may be localization of the K^+ channels and/or localization of Ca_{is} gradients. Therefore, the calculated Ca_{is} concentration in the unclamped bovine cell (Fig. 7*A*) was only approximately 1800 nM. The high Ca_{is} gradient may exist only in localized areas (perhaps only between the superficial SR and the sarcolemma). There is evidence for localization of Ca_{is} along the sarcolemma as Bond, Shuman, Somlyo & Somlyo (1984) used electron probes to locate 'spots' of total subsarcolemmal Ca^{2+}. They found regional differences in the subsarcolemmal Ca^{2+} concentrations in relaxed guinea-pig portal vein varying from $0{\cdot}0 \pm 3{\cdot}5$ to $11{\cdot}9 \pm 3{\cdot}5$ mmol (kg dry weight)$^{-1}$ only 75 nm from each other along the subsarcolemma region (37 nm beneath the sarcolemma). Figure 7*A* indicates that Ca_{is} was approximately 1800 nM while Ca_{im} was 400 nM in bovine cells. If we assume that the difference between the Ca_{is} and Ca_{im} concentrations is due to Ca^{2+} in the region between the superficial SR and the sarcolemma (approximately 2% of the sarcolemma is adjacent to superficial SR) then the Ca_{is} concentration must be as high as 70 μM in those regions to account for the increase in I_K alone. It seems unlikely that localized Ca_{is} concentrations would reach 70 μM in a resting cell, suggesting that a Ca_{is} gradient may exist that is not completely due to Ca^{2+} between the sarcolemma and the superficial SR. However, the results shown in Fig. 11 indicate there is a correlation between the amount of Ca^{2+} in the SR and the Ca_{is}. Even taking into account localization of Ca_{is} along the sarcolemma, the data presented in this paper support the hypothesis of a higher Ca_{is} concentration than Ca_{im} concentrations in resting bovine cells (Fig. 7*A*).

Previously published electrophysiological studies have provided evidence to support the existence of a Ca^{2+} gradient underneath the sarcolemma. It has been found that Ca^{2+}-sensitive K^+ channels of smooth muscle may show spontaneous activation while no contractile activity is stimulated (Benham *et al.* 1986*b*). Furthermore, a Ca_{is} gradient attributed to SR Ca^{2+} uptake has been suggested by Rembold (Rembold, 1989). Finally, prominent pyroantimonate precipitate containing Ca^{2+} has been shown to localize along the inner surface of the plasma membrane of relaxed fibres of guinea-pig taenia coli (Sugi & Daimon, 1977) and rabbit cerebral artery (Nakayama, Suzuki & Sugi, 1986). These reports are also consistent with our findings that a subsarcolemmal Ca^{2+} gradient exists in coronary smooth muscle in which Ca_{is} is higher than Ca_{im}.

Prediction 4: Ca_{is} is directly related to the amount of Ca^{2+} in the SR

If the SR Ca^{2+} buffering hypothesis is true then the increase in Ca_{is} should precede an increase in Ca_{im}. The temporal dissociation shown in Fig. 8 leaves little doubt. The release of Ca^{2+} from the SR in response to 5 mM-caffeine elicited an increase in K^+

current that preceded the increase in Ca_{im} by often greater than 2 s (Fig. 8). These data offer strong support for the close functional association between the SR and the sarcolemma in coronary artery smooth muscle. The temporal dissociation of 2 s noted here is more than an order of magnitude greater than the temporal dissociation recently reported by Foskett, Gunter-Smith, Melvin & Turner (1989) for membrane hyperpolarization and free Ca^{2+} transients caused by acetylcholine-induced Ca^{2+} release in parotic acinar cells. The temporary dissociations we found are more than 100-fold slower than predicted by simple diffusion through cytosolic material (Hille, 1989). These results (Fig. 8) are unique to this paper as this is the only figure in which there was no difference in the response between bovine and porcine (both showed approximately a 2 s delay in the change in Ca_{im}). The results indicate that agonist-induced SR Ca^{2+} release may not differ in porcine and bovine cells and that the species-related differences are greatest when measuring spontaneous SR Ca^{2+} release. There does not appear to be as great a time delay between changes in I_K and Ca_{im} during Ca^{2+}-loading (Fig. 10*A*, depolarization to V_h of -40 mV). The sampling interval used was not as frequent in Fig. 10*A* as compared to Fig. 8, therefore, a delay in the increase in Ca_{im} may have occurred at the beginning of the depolarization which was not measured. Alternatively, there may exist Ca^{2+} influx pathways by which Ca^{2+} may enter intracellular organelles (SR) without increasing Ca_{is}. Cultured smooth muscle cells demonstrate a pathway of Ca^{2+} entry which loads the SR but is not dependent on the SR Ca^{2+} pump (Missiaen, Declerk, Droogmans, Plessers, De Smedt, Raeymakers & Casteels, 1990).

It is highly unlikely that Ca_{im} buffering by soluble Ca^{2+} buffering proteins is significant enough to cause a 2 s delay in the change in Fura-2 during the caffeine exposure, because in regions of less than 200 nM, Ca^{2+} buffering factors have been shown to have little affect on the intracellular diffusion rate of Ca^{2+} (Simon & Llinás, 1985; Smith & Augustine, 1988). Simon & Llinás (1985) mathematically modelled the steady-state Ca^{2+} concentration immediately under the membrane of active sites of neurons. They found that mobile Ca^{2+} buffers had little effect on the steady-state distribution of Ca^{2+}, while non-mobile buffers had no effect (Simon & Llinás, 1985).

An alternative explanation for the results shown in Fig. 8 is that the K_D of Ca^{2+} binding to Fura-2 may be larger than the K_D for Ca^{2+} binding to the K^+ channels, therefore explaining the delay in any change in the Fura-2 ratio relative to a change in the I_K. This is unlikely because Fig. 6 shows a lower Ca^{2+} sensitivity of I_K at the membrane potentials of -20 and -40 mV which were used in Fig. 8. In a whole-cell recording it is impossible to reach an intracellular Ca^{2+} concentration that would saturate all the sarcolemmal Ca^{2+}-activated K^+ channels, therefore it is impossible to determine a K_D for Ca^{2+} binding to the channel using the whole-cell I_K. We have clamped intracellular Ca^{2+} up to 1500 nM-Ca^{2+} and have seen no indication of the current amplitude saturating. At higher intracellular Ca^{2+} concentrations the whole-cell recordings are not stable. A future project in this laboratory is to calibrate the probability of opening of single Ca^{2+}-activated K^+ channels in excised patches to clamped Ca^{2+} concentrations; therefore a K_D for the channels could be determined. Recently, Giangiacomo, Garcia-Calvo, Garcia & McManus (1991) extracted the Ca^{2+}-activated K^+ channel from bovine aortic smooth muscle and reconstituted it into lipid bilayers. They found the channel had a probability of opening of 0·5 at a Ca^{2+}

concentration of approximately 4 μM. Although reconstitution into a lipid membrane may have altered the Ca^{2+} sensitivity of the channel the results still suggest that the affinity of the channel to Ca^{2+} is much lower than that of Ca^{2+} binding to Fura-2 which has a K_D of 224 nM-Ca^{2+} (Grynkiewicz *et al.* 1985). Therefore, a higher Ca^{2+} binding affinity of Fura-2 *vs.* Ca^{2+}-activated K^+ channels cannot explain the delay in the Fura-2 ratio relative to the change in the I_K.

When correlating the amount of Ca^{2+} in the SR with Ca_{is} concentrations we made two important assumptions: (1) that Ca^{2+} entering the cell during depolarization to -40 mV fills the SR with Ca^{2+}, and (2) caffeine (5 mM) empties the SR Ca^{2+} store. Both of these assumptions have been repeatedly supported by work in our laboratory and in independent laboratories which we have already summarized in the Methods section of this paper. The finding that the SR Ca^{2+} store is directly proportional to the Ca_{is} concentration and the increase in Ca^{2+} efflux measured when the SR was filled with Ca^{2+} strongly support the release of Ca^{2+} from the SR which is extruded by the cell.

Species variation

The differences found when duplicating the same protocols using bovine or porcine coronary artery smooth muscle cells lend some insight into the physiological significance of the role of the SR as a Ca^{2+} buffer barrier. On the surface, one might conclude that the buffer barrier role is purely species specific (found in bovine smooth muscle, but not in porcine). However, our previous work suggests that spontaneous SR Ca^{2+} release may be altered with a long-term physiological stimulus. Matched groups of miniature pigs were chronically exercise-trained or pen-confined (sedentary). Although spontaneous SR Ca^{2+} unloading was not noted in cells from sedentary pigs it was noted in cells from exercise-trained pigs (64% depletion of the SR Ca^{2+} store following an 11 min recovery period) (Stehno-Bittel *et al.* 1990, 1991). It is important to note that a net loss of SR Ca^{2+} does not occur in miniature pigs (Stehno-Bittel *et al.* 1990, 1991) or farm pigs (Fig. 2), yet SR Ca^{2+} release does occur in porcine cells, as noted by STOCs (Fig. 12, this paper) and (Stehno-Bittel *et al.* 1991). In farm pigs 36% of the cells studied showed STOCs, compared to only 8% of the bovine cells. In addition, 73% of the cells from sedentary miniature pigs had STOCs, compared to only 3% of the cells from exercise-trained pigs (Stehno-Bittel *et al.* 1991). We interpret these findings within the model depicted in Fig. 8 of Sturek *et al.* (1992) and propose that the Ca^{2+} released in a bolus amount during a STOC is not extruded from smooth muscle cells of the porcine coronary artery (miniature or farm pigs). Support for this is provided by studies on arterial smooth muscle cells in which STOCs were not dependent on extracellular Ca^{2+} leading the authors to conclude that the Ca^{2+} initiating the STOCs was released from an intracellular store and preferentially resequestered by the store rather than lost from the cell (Benham & Bolton, 1986*a*). Requestration of the Ca^{2+} by the SR of porcine cells would explain the constant amount of SR Ca^{2+} with varying recovery periods (Fig. 2) and why Ca^{2+} efflux from the cell did not increase when the SR was Ca^{2+}-loaded. In contrast, we propose that bovine cells (or porcine exercise trained) has a mechanism of spontaneous SR Ca^{2+} release which allows extrusion of Ca^{2+} from the cell (see Fig. 8 of Sturek *et al.* 1992). This model is consistent with the following findings: (1)

depleted SR Ca^{2+} store with increasing recovery times, (2) increased Ca^{2+} efflux from cells with Ca^{2+}-loaded SR, and (3) higher Ca_{is} concentrations when the SR is Ca^{2+} loaded. Therefore, we propose that the action of the SR as a buffer barrier is not only species related, but may be modulated by physiological stimuli; thus, it may have relevance in the health and disease of the coronary arteries.

The results of this study, taken together with the results discussed in the companion paper, strongly suggest that the SR of coronary artery smooth muscle acts as a buffer barrier to protect the bulk myoplasm (most likely calmodulin and the myofilaments) from fluctuations in Ca^{2+} that may occur within the subsarcolemmal region.

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