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SARCOPLASMIC RETICULUM BUFFERING OF MYOPLASMIC CALCIUM IN BOVINE CORONARY ARTERY SMOOTH MUSCLE

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SUMMARY

1. We tested the hypothesis that the sarcoplasmic reticulum (SR) buffers (attenuates) the increase in averaged myoplasmic free [Ca^{2+}] (Ca_{im}) resulting from Ca^{2+} influx.

2. Fura-2 measurements of Ca_{im} were obtained in single smooth muscle cells freshly dispersed from bovine coronary artery.

3. Caffeine (5×10^{-3} M) elicited a transient increase in Ca_{im} and depleted the SR Ca^{2+} store. In the continued presence of caffeine or 10^{-5} M-ryanodine SR buffering of Ca_{im} was inhibited. Subsequent exposure to high extracellular [K^+] (> 30 mM, equimolar Na^+ removal) elicited a 2-fold more rapid and 2-fold greater peak increase in Ca_{im} than high K^+ elicited when SR buffering of Ca_{im} was normal. The augmented increase in Ca_{im} was inhibited 35% by 10^{-5} M-diltiazem, 65% by 2×10^{-4} M-$LaCl_3$, and 87% in Ca^{2+}-free external solution.

4. When Ca_{im} buffering capacity was increased by partially depleting the SR with a transient (1 min) exposure to caffeine, subsequent exposure to 80 mM-K^+ solution increased Ca_{im} almost 2-fold more slowly than 80 mM-K^+ before depletion of Ca^{2+} from the SR. However, the influxing Ca^{2+} was sequestered by the SR and refilled it, as evident by the subsequent caffeine-induced Ca_{im} transient being identical to the first. Increasing extracellular [K^+] (thus, increasing depolarization and Na^+ removal) caused proportional increases in Ca_{im} and the subsequent caffeine-induced Ca_{im} transients were proportionally larger, indicating a graded filling of the SR by Ca^{2+} influx.

5. Diltiazem (10^{-5} M) inhibited the refilling of the SR achieved by 80 mM-K^+, by 26%. Refilling was inhibited 76% by 80 mM-K^+, Ca^{2+}-free solution, indicating the fraction of refilling dependent on influx of Ca^{2+} through voltage-gated Ca^{2+} channels, leak channels, and other influx pathways. Mild depolarization with 35 mM-K^+ (no Na^+ removal) often caused no increase in Ca_{im}, but influx through voltage-gated Ca^{2+} channels occurred because the SR Ca^{2+} store was refilled. Also, 10^{-5} M-diltiazem or 10^{-6} M-TA3090 inhibited the refilling to levels attributable only to leak influx of Ca^{2+}.

6. All data support our hypothesis that the SR significantly attenuates the amount of Ca^{2+} influx that accumulates to increase Ca_{im}. The main implications are: (a) vasoactive agents that cause external Ca^{2+}-dependent increases in Ca_{im} may inhibit SR buffering of Ca_{im}, rather than increasing Ca^{2+} influx across the

sarcolemma; (b) classical voltage-gated Ca^{2+} channel antagonists may also act by inhibiting both localized increases in Ca^{2+} in a restricted subsarcolemmal compartment and refilling of the SR, while not affecting Ca_{im}.

INTRODUCTION

Maintenance of low myoplasmic free Ca^{2+} concentration (Ca_{im}) in smooth muscle cells of the coronary artery is essential for minimizing vascular tone and, thereby, insuring adequate blood flow to the myocardium. This task is especially demanding during elevated influx of Ca^{2+} resulting from depolarization by elevated external K^+ (Sumimoto & Kuriyama, 1986; Bradley & Morgan, 1987; Kanaide, Kobayashi, Nishimura, Hasegawa, Shogakiuchi, Matsumoto & Nakamura, 1988; Rembold & Murphy, 1988; Nishimura, Khalil & van Breeman, 1989), removal of external Na^+ (Blaustein, 1988), or by vasoconstrictor agonists (Bradley & Morgan, 1987; Kanaide *et al.* 1988; DeFeo & Morgan, 1989; Rembold, 1989). About a decade ago van Breemen (1977) and Casteels & Droogmans (1981) proposed that a fraction of the Ca^{2+} that enters the cell during depolarization is sequestered by the sarcoplasmic reticulum (SR) before it accumulates as Ca_{im} in the bulk myoplasm for activation of the Ca^{2+}–calmodulin complex and contraction. These data suggest a protective, Ca_{im} buffering action of the SR, rather than its classical role of releasing Ca^{2+} to initiate contraction (van Breemen, Saida, Yamamoto, Hwang & Twort, 1988; van Breemen & Saida, 1989).

Indirect evidence for this function of the SR was the finding that K^+ depolarization-induced contraction of vascular smooth muscle was reduced when the SR Ca^{2+} content was minimal and, hence, the Ca_{im} buffering capacity of the SR was near maximal (Casteels & Droogmans, 1981; van Breemen *et al.* 1988; Hisayama & Takayanagi, 1988; van Breemen & Saida, 1989). Conversely, when the SR was prevented from sequestering Ca^{2+} by a noradrenaline-induced maintained increase in SR permeability, the Ca^{2+} influx threshold for initiation of contraction was lower (van Breemen *et al.* 1988). Van Breemen and co-workers have refined their hypothesis, which is referred to as the 'superficial buffer barrier' hypothesis (van Breemen *et al.* 1988; van Breemen & Saida, 1989). In addition, necessary morphological support for the buffer barrier hypothesis (Casteels & Droogmans, 1981; van Breemen *et al.* 1988; van Breemen & Saida, 1989) is the finding that a portion of the SR in vascular smooth muscle cells of the coronary artery is located less than 50 nm from the sarcolemma (Devine, Somlyo & Somlyo, 1972; Forbes, 1982). Accordingly, the buffer barrier hypothesis predicts a localized gradient of free Ca^{2+} in the subsarcolemmal region of the cell (van Breemen *et al.* 1988). Similarly, a critical component of the 'Ca^{2+} cycling' hypothesis of Rasmussen (Rasmussen & Barrett, 1984) is a subsarcolemmal Ca^{2+} gradient. Putney (Putney, 1986) recently proposed a 'capacitative' model for ligand-gated Ca^{2+} influx in secretory cells whereby Ca^{2+} influx is enhanced when the endoplasmic reticulum has first been emptied and maintained in a hyperpermeable state during activation by secretagogues. This model differs significantly, however, from the above hypotheses, because the capacitative model predicts a normal or lower $[Ca^{2+}]$ in the subplasmalemmal region (Putney, 1986).

The most fundamental and novel aspect which may be common to these three

hypotheses is that accumulation of free Ca^{2+} in the bulk cytosol resulting from Ca^{2+} influx is tightly regulated by intracellular organelles, not just plasmalemmal regulators (Casteels & Droogmans, 1981; Putney, 1986; van Breemen *et al.* 1988). Despite the insights of these three hypotheses, however, there are few direct measurements of Ca_{im} in single vascular smooth muscle cells during alterations in SR buffering of Ca_{im}. Our specific hypothesis was that the SR buffers (attenuates) the increase in Ca_{im} normally resulting from Ca^{2+} influx. We increased Ca^{2+} influx by high-K^+, low-Na^+ solutions. The purpose of these studies was to test several major predictions of the SR Ca_{im} buffering hypothesis in smooth muscle cells: (1) the increased Ca_{im} normally resulting from Ca^{2+} influx is augmented during inhibition of Ca_{im} buffering by the SR; (2) when the SR is depleted of Ca^{2+} it has a greater Ca_{im} buffering capacity, thus it will require longer for increased Ca^{2+} influx to increase Ca_{im}; (3) Ca^{2+} influx refills the SR Ca^{2+} store and; (4) loading of the SR Ca^{2+} store is proportional to Ca^{2+} influx.

The four predictions of the hypothesis were confirmed, thus providing strong evidence for a major role of the SR in buffering of Ca_{im}, which further predicts physiological localization of free Ca^{2+} in the subsarcolemmal region. The companion paper addresses the prediction of Ca^{2+} localization as a result of SR Ca_{im} buffering (Stehno-Bittel & Sturek, 1991). The results in the present manuscript necessitate novel interpretations for mechanisms by which vasoactive agents regulate Ca^{2+} in vascular smooth muscle. Preliminary reports of these data have appeared (Sturek, Kunda & Bowman, 1989; Kunda, Bowman & Sturek, 1989).

METHODS

Fresh dispersion of smooth muscle cells from the coronary artery

Single cells from the bovine left circumflex and the proximal 1/3 of the left anterior descending coronary artery were enzymatically dispersed (Van Dijk & Laird, 1984; Wagner-Mann, Bowman & Sturek, 1991). At a local abbatoir cows were killed by exsanguination according to US Department of Agriculture guidelines. The arteries were cut open longitudinally while still on the heart and then removed and placed in a container with ice-cold dispersion 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. The artery was cleaned of connective tissue and fat in a solution similar to the above, but containing only 0·5 mM-Ca^{2+} and no horse serum. The artery was cut into segments and then pinned with the lumen facing upward in a 30 ml bottle. The 0·5 mM-Ca^{2+} solution was removed and 2 ml of enzyme solution which contained the following components in 0·5 mM-Ca^{2+} solution was added: 294 U ml^{-1} collagenase (CLS II, Worthington, Freehold, NJ, USA), 2 mg ml^{-1} bovine serum albumin (Fraction V, Sigma Chemical Co., St Louis, MO, USA), 1 mg ml^{-1} soybean trypsin inhibitor (Worthington), 0·4 mg ml^{-1} DNAase I (Type IV, Sigma). The vessels were then placed in a shaking water bath at 37 °C for 60 min. The supernatant at this step contained mostly endothelial cells, easily identified morphologically; few smooth muscle cells were seen. The supernatant was removed and 2 ml enzyme solution again added and the vessel placed in the water bath for 60 min. The next fraction usually contained a substantial number of relaxed smooth muscle cells. This dispersion procedure was repeated several times as desired and the cells were suspended in physiological saline solution (PSS, see *Solutions and drugs for microfluorometry* below). Smooth muscle cells remained relaxed and contracted when exposed to vasoconstrictors for up to 6–8 h after the dispersion. It is emphasized that these experiments were conducted on relaxed, freshly isolated cells, verified as healthy, normally functioning smooth muscle cells by their ability to contract in response to > 30 mM-extracellular K^+, caffeine, or ryanodine. A video system was used to verify contraction during experiments (see below).

Microfluorometry

The fluorescent indicator, Fura-2, was used for measurement of Ca_{im} in single cells based on methods previously reported in detail (Thayer, Sturek & Miller, 1988). The microfluorometry system has been further refined with the following main modifications: (1) an excitation filter wheel, (2) an analog fluorescence signal processor, (3) simultaneous video monitoring, and (4) a computer system based on the 80286 microprocessor. The cell superfusion chamber consisted of a plexiglass block machined to accommodate a thin glass cover-slip as a bottom. A droplet of cell suspension was placed in the perfusion chamber and a thin sheet of buffer flowed across the cells in the experimental chamber; solutions in the chamber could be completely exchanged within 15 s. The superfusion chamber was mounted on a Nikon Diaphot inverted microscope (Nikon Inc., Garden City, NY, USA) and cells were localized by observation in the microscope or video system. The epifluorescence microscope was mounted on a vibration isolation table within a light-tight Faraday cage with the Xe light source isolated electrically, as well as mechanically, outside the cage. To excite the Fura-2, light from a 150 W Xe arc lamp was passed via a liquid light guide (Oriel Corp., Stratford, CT, USA) as a collimated beam through a circular interference filter wheel (Omega Optical, Brattleboro, VT, USA) that had two pie-shaped sections that each comprised 144 deg of the circle. One of the filters provided 340 nm (10 nm half-bandwidth) and the other provided 380 nm (10 nm half-bandwidth) illumination. Two opaque sections each comprising 36 deg of the circle separated the sections providing light transmission. Excitation in our previous report was provided by the diffraction grating of a spectrophotometer (Thayer *et al.* 1988). This excitation filter wheel was mounted on an adjustable speed servo motor (B & B Electric, Indianapolis, IN, USA) that rotated the wheel, thereby allowing the changing of the excitation wavelengths at rotational periods of 20, 50, 100, or 200 ms. Immediately after the interference filter wheel a collimating beam probe was placed to focus the light onto the end of another liquid light guide, which directed light to the epifluorescence illuminator of the microscope. The light was reflected by a dichroic mirror (Nikon, DM 400) through a 40 × phase contrast oil immersion objective with a numerical aperture of 1·3. The fluorescence emission was selected for wavelength with a 480 nm barrier filter and recordings were defined spatially to only one cell with an adjustable rectangular aperture (Ealing Electro-Optics, Holliston, MA, USA).

During typical microfluorometry the only light illuminating the cell is that required for excitation of the chromophore, i.e. alternating 340 and 380 nm light for Fura-2 excitation. Also, all emitted fluorescence is passed to the photomultiplier for quantitation, leaving no possibility for simultaneous observation of the cell. This problem was overcome by inserting onto the video port of the Nikon Diaphot a binocular head (Science Instrument Shop, University of Missouri) containing a dichroic mirror (Omega Optical) that reflected the Fura-2 fluorescence (peak of the emission is about 510 nm) to the photomultiplier. Simultaneously, additional illumination of the cell was provided by placing a 600 nm centre wavelength (10 nm half-bandwidth) interference filter in the filter holder of the Diaphot microscope. Only this narrow bandwidth light was then allowed to pass from the halogen lamp of the Diaphot through the dichroic mirror to a Panasonic WV-D5010 CCD camera, thus permitting observation of the cell on a video monitor during Fura-2 microfluorometry. A 510 nm centre wavelength (60 nm half-bandwidth) interference filter was placed immediately in front of the photomultiplier to further insure no interference from the 600 nm illumination; indeed, background fluorescence was the same with or without simultaneous 600 nm illumination.

The fluorescence emission was amplified with a photomultiplier tube (No. 9893A/350, Thorn EMI Gencom, Inc., Plainview, NY, USA) that was selected for low background counts and high sensitivity at 510 nm, the peak of the Fura-2 emission spectrum. The amplified emission signal was then analysed by a discriminator (APED-II, Thorn EMI Gencom). The resulting pulses were then fed into a circuit based on a monostable multivibrator (74123) previously described (Thayer *et al.* 1988), which generates pulses of user-selectable widths of 1, 10, or 100 μs by means of external capacitors. The pulses were then essentially integrated by passing the signal through on 8-pole low-pass Bessel filter (Frequency Devices, Haverhill, MA, USA) set at 500 Hz. Gain of the photomultiplier tube output instrument chain was proportional to the width of the pulse from the monostable multivibrator, since the analog output from the low-pass filter integrates a larger area under wider pulses.

A reflective optocoupler monitored the position of the interference filters in the wheel and transmitted this information to the analog fluorescence signal processor, based on sample-and-hold

circuitry, which demodulated the raw, oscillating fluorescence signal from the photomultiplier into two separate analog voltages corresponding to signals resulting from excitation at 340 and 380 nm. The separated signals were then fed into separate channels of an analog-to-digital converter. The Labmaster analog-to-digital converter (Scientific Solutions, Inc., Solon, OH, USA) was used with the microcomputer equipped with the pCLAMP data acquisition system (Axon Instruments, Foster City, CA, USA) and the ADAC analog-to-digital converter was used with Labtech Acquire. The sample interval in each channel for analog-to-digital conversion of fluorescence signals was 2 s for the pCLAMP system and 5 s for the Labtech Acquire system; often the output of the optical processor was low-pass filtered at 0·2 Hz for these data acquisition.

[Ca^{2+}] calibration of Fura-2 fluorescence ratio

The method of Grynkiewicz, Poenie & Tsien (1985) is to use the equation $[Ca^{2+}]_i = K_D(\max_{380}/\min_{380})(R-R_{min})/(R_{max}-R)$, where K_D is the dissociation constant for Fura-2, $\min_{380}$ and $\max_{380}$ are the minimum and maximum fluorescence at 380 nm, R is the measured ratio (the independent variable, 340/380 fluorescence intensity), and R_{max} and R_{min} are the maximum and minimum ratios of (340 fluorescence)/(380 fluorescence) which would be obtained at saturating amounts of Ca^{2+} and at 0 Ca^{2+}, respectively. In theory, we could obtain ratios in only saturating and 0 Ca^{2+} and use these directly; however, in these smooth muscle cells this technique was subject to several errors. First, relative background fluorescence due to excitation at 380 nm sometimes increased greatly at saturating Ca^{2+} concentrations because the fluorescence emission from Fura-2 became very small. This consequently made the error of the measurement greater in these cases. Second, it was difficult to obtain maximum ratio measurements in a single cell loaded with Fura-2 AM without lysing the cell and losing all the Fura-2 from the myoplasm. Third, and most importantly, the Grynkiewicz *et al.* (1985) equation assumes that the pentapotassium salt form of Fura-2 is the only Fura-2 species. Scanlon, Williams & Fay (1987) have found other Ca^{2+}-insensitive forms of Fura-2, presumably partially de-esterified forms, that result in decreased Ca^{2+} sensitivity of Fura-2 in intact cells loaded with Fura-2 AM. For these reasons (and others discussed below) a calibration curve for the Fura-2 salt was determined in the range of Ca^{2+} concentration likely to be encountered in the experiments and over the working range of the dye, which is 50 nM to about 2000 nM. This procedure validated the use of ratio fluorescence F_{340}/F_{380}) as an estimate of myoplasmic [Ca^{2+}]. Twelve different solutions of varying [Ca^{2+}] were used with Fura-2 pentapotassium salt in a mock intracellular solution containing (mM): 126 KCl, 10 NaCl, 20 HEPES, 1 $MgCl_2.2H_2O$, 0·1 Fura-2 salt, pH adjusted to 7·1 with KOH. Free Ca^{2+} concentrations ranging from 0 to 2000 nM were achieved by using appropriate ratios of H_2K_2-EGTA and CaK_2-EGTA calculated using the apparent stability constant of $3{\cdot}969 \times 10^6$ (temperature 22 °C, pH 7·1) (Fabiato & Fabiato, 1979); the final EGTA concentration was always 10 mM. The data were fitted by a second-order polynomial with a non-linear least-squares fit computer program available on Sigmaplot graphics program (Jandel Scientific, Corte Madera, CA, USA). These *in vitro* calibration constants for Fura-2 fluorescence signals were obtained using the mock intracellular solution indicated above and the two microfluorometry systems (system 1 and system 2; see Fig. 1) used here, thus accounting for the unique optical arrangement, etc. of the systems. The same type of cover-slip (25 mm diameter, 0·17 mm thickness; Fisher Scientific) used as the bottom of the superfusion chamber (see Thayer *et al.* (1988) for an illustration) was used in these calibrations. A diamond pencil was used to make a light etch on the top of the cover-slip upon which the investigator focused for proper optical alignment. A 2 μl droplet of the final calibration solution was placed on the cover-slip and fluorescence ratio recorded.

'Myoplasmic' calibration of Fura-2 fluorescence ratios was also conducted in order to estimate whether Fura-2 AM was properly hydrolysed to the pentapotassium salt (Scanlon *et al.* 1987; Peeters, Hlady, Bridge & Barry, 1987) and whether the myoplasmic calibration for the salt was similar to the *in vitro* measurements (Peeters *et al.* 1987). One suspension of $5–10 \times 10^6$ cells was loaded with Fura-2 by incubating with $2{\cdot}5 \times 10^{-6}$ M of the permeant ester form, Fura-2 AM, for 15 min at 37 °C in physiological saline solution plus 0·2% bovine serum albumin. Another suspension of cells was treated similarly, being subjected to the same incubation and centrifugation steps, except that no Fura-2 AM was added. Following incubation the cells were centrifuged to form a pellet, the Fura-2 AM-containing physiological solution removed, and the two separate fractions rinsed at 37 °C for 30 min with 2 ml dispersion media containing 2% horse serum with no added Fura-2 AM. It was found that rinsing with serum-containing medium more effectively removed the Fura-2 AM, most likely due to the presence of esterases in the serum. The two separate

suspensions of cells were then each divided further into three fractions. After another centrifugation the cell pellets (volume of approximately 25 μl) were resuspended in 500 μl of final Fura-2 [Ca^{2+}] calibration solution containing either 100, 400, or 1000 nM free [Ca^{2+}], but excluding the Fura-2 pentapotassium salt, and then allowed to rinse for 15 min. This procedure strongly buffered the external Ca^{2+} to the desired free [Ca^{2+}]. The cells were centrifuged, the 500 μl of calibration solution removed, and 25 μl of the same calibration solutions containing 100 μg saponin ml^{-1} added to the cell pellet. For the three cell fractions that had not been loaded with Fura-2 AM, 40 μM of Fura-2 pentapotassium salt was also included in the saponin solution added to the cell pellet. Saponin has been shown to effectively remove the sarcolemma from cells without affecting the intracellular organelles (van Breemen *et al.* 1988). In the case of Fura-2 AM-loaded cells the myoplasmic Fura-2 was released from the cells into the suspension in a manner very similar to that done in cuvette measurements in cell suspensions (Grynkiewicz *et al.* 1985). Buffering the [Ca^{2+}] to known levels thus permitted determination of the Ca^{2+} sensitivity of the myoplasmic Fura-2 that the cells have processed (presumably hydrolysed) from the Fura-2 AM which entered the cells during the loading period. Addition of Fura-2 pentapotassium salt in different cell pellets was a control for instrument calibration, possible turbidity of the suspension because of membrane lysis, interaction of Fura-2 with myofilaments (Baylor & Hollingworth, 1988), etc. Droplets (2 μl) of each of the lysed cell fractions were removed and the ratio fluorescence determined with the microfluorometry system 1. The data for all the calibration analyses were fitted to a second-order polynomial. The somewhat depressed Ca^{2+} sensitivity of myoplasmic Fura-2 shown in Fig. 1 led us to express the data as ratios. Obtaining this myoplasmic calibration curve from cell suspensions gave a reasonable estimate of the Ca^{2+} sensitivity of the myoplasmic Fura-2 in cells which had been loaded by Fura-2 AM. None the less, accurate generalization to measurements on only a single cell in the larger population ($5–10 \times 10^6$ cells) was not considered most prudent (see Results, Fig. 1).

Solutions and drugs for microfluorometry

The physiological saline solution was composed of (mM): 2 $CaCl_2$, 138 NaCl, 1 $MgCl_2$, 5 KCl, 10 HEPES, 10 glucose, pH adjusted to 7·4 with NaOH. Depolarization of the cells by elevating extracellular [K^+] to 30 or 80 mM was accomplished by equimolar replacement of NaCl with KCl and the solutions referred to as 30 K or 80 K, respectively. Also, mild depolarization without the effects of extracellular Na^+ removal involved adding 25 mM-KCl to increase the total extracellular KCl to 30 mM and adjusting the pH with 5 mM-KOH, rather than NaOH, thus yielding 35 mM-final extracellular [K^+] (solution termed +35 K). The added osmolarity from the KCl was without adverse effects (Magliola & Jones, 1987). Calcium-free solution was made by omitting $CaCl_2$ and adding 10^{-5} M-EGTA to the appropriate buffer. Caffeine (Sigma Chemical Co.) was used at a final concentration of 5×10^{-3} M. Ryanodine (Calbiochem, La Jolla, CA, USA) was made as a stock solution of 10^{-2} M in 100% ethanol and diluted into the appropriate buffer for use at the final concentration of 10^{-5} M. Diltiazem (a kind gift of Dr Ronald Gaddis, Marion Laboratories, Kansas City, MO, USA) was made as a stock solution of 10^{-2} M in distilled water and diluted into the appropriate buffer for use at the final concentration of 10^{-5} M. The period of solution changes are indicated in all figures by horizontal lines labelled with the appropriate solution name. All experiments were conducted at room temperature (22–25 °C).

Statistics

Group data are expressed as means ± S.E.M. unless otherwise indicated. Student's t test for independent groups and paired t tests were used where appropriate when comparing only two groups. Analysis of variance was used to compare multiple groups. If a significant F ratio was found, a *post hoc* analysis was used to compare specific pairs of groups.

RESULTS

The curves fitted to the open circles and open triangles in Fig. 1 demonstrate the relationship between fluorescence ratio (F_{340}/F_{380}) and [Ca^{2+}] in mock intracellular solutions for the two microfluorometry systems. The data are very similar to other reports (Poenie, Alderton, Steinhardt & Tsien, 1986; Thayer *et al.* 1988) using very

different optics. These standard curves are appropriate to use for determination of maximum and minimum fluorescence for insertion into the equation of Grynkiewicz *et al.* (1985; Fabiato & Fabiato, 1979) or for using simple regression prediction to derive Ca_{im} directly from the curve (Thayer *et al.* 1988) if an essential prerequisite is

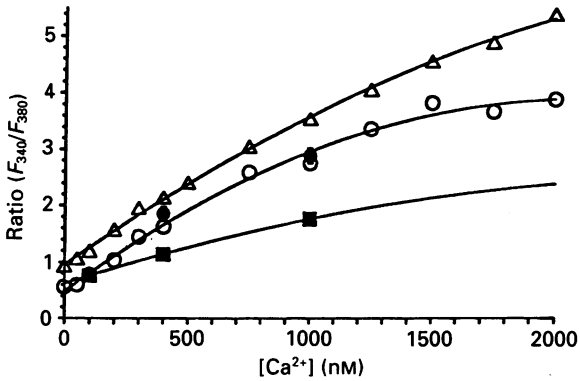

Fig. 1. Calibration of Fura-2 fluorescence ratios with ionized $[Ca^{2+}]$. Ratios were obtained as described in Methods. ○, values for *in vitro* calibration solutions on microfluorometry system 1, with which data for Figs 2–5 were obtained ($n = 3$ replicate measurements per point); polynomial fit to $[Ca^{2+}] = -11{\cdot}68 + 51{\cdot}789\,R + 106{\cdot}78\,R^2$, where R = ratio. △, values for *in vitro* calibration solutions in microfluorometry system 2, with which data for Figs 6 and 7 were obtained ($n = 3$ replicate measurements per point); polynomial fit to $[Ca^{2+}] = -205{\cdot}07 + 192{\cdot}93\,R + 40{\cdot}891\,R^2$. ●, 'myoplasmic' ratios obtained in permeabilized cell suspensions when Fura-2 pentapotassium salt was added exogenously ($X \pm$ S.E.M., $n = 5$); polynomial fit to $[Ca^{2+}] = 85{\cdot}854 - 95{\cdot}304\,R + 143{\cdot}30\,R^2$. ■, 'myoplasmic' ratios obtained in suspensions of permeabilized cells which were first loaded normally with Fura-2 AM (See Methods) ($X \pm$ S.E.M., $n = 5$; S.E.M. values are not visible at some points because of low variability); polynomial fit to $[Ca^{2+}] = -345{\cdot}55 + 450{\cdot}76\,R + 178{\cdot}27\,R^2$.

met. The prerequisite is that the Fura-2 has the same Ca^{2+} sensitivity in the myoplasm as it does in mock intracellular solutions *in vitro*. We attempted saponin permeabilization of the sarcolemma in single cells during constant superfusion to obtain the maximum ratio but were not successful because of rapid loss of the myoplasmic Fura-2 from the cells. Also, $20–100 \times 10^6$ M-ionomycin only transiently elevated the fluorescence ratio and, thus, could not be used as a method to 'clamp' Ca_{im} to the $[Ca^{2+}]$ of the extracellular solution as reported for Ca^{2+} ionophores by others (Williams *et al.* 1985; Peeters *et al.* 1987; Pallotta, Helper, Oglesby & Harden, 1987).

In the companion paper (Stehno-Bittel & Sturek, 1992) the maximum fluorescence ratio in cells loaded with Fura-2 pentapotassium salt via the patch pipette was similar to the *in vitro* ratio in saturating Ca^{2+}. Furthermore, when Ca^{2+} was 'clamped' at several concentrations in cells by inclusion of Ca^{2+}-EGTA buffered solutions in the pipette the ratios were also in close agreement with the *in vitro* ratio (Fig. 1). These data indicate that Fura-2 pentapotassium salt behaves similarly *in vitro* and '*in situ*'. The maximum ratio was also determined in cells loaded with

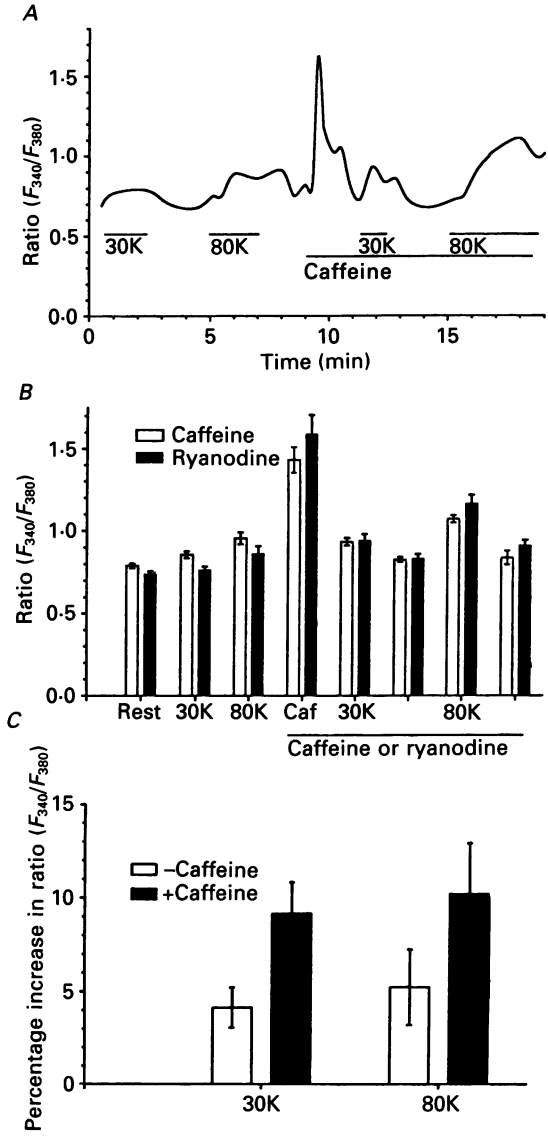

Fig. 2. Increased Ca_{im} resulting from Ca^{2+} influx during exposure to high extracellular $[K^+]$ was augmented during inhibition of SR buffering of Ca_{im}. *A*, representative recording from a single cell. The cell was exposed to 30 and 80 mM-K^+ (30 K and 80 K, respectively) for the durations indicated by the horizontal line. The cell was then exposed to caffeine (5×10^{-3} M) for the remaining 10 min of the protocol and 30 K or 80 K were again applied to the cell simultaneously with the caffeine for the durations indicated. For the ryanodine experiments caffeine and ryanodine were added simultaneously for minutes 10–12; thereafter, only ryanodine was present for the remainder of the experiment. Ryanodine (10^{-5} M) had similar effects to those of caffeine. *B*, group data, $n = 11$–19 cells in each caffeine group, except in the last group where there are only three cells; $n = 10$ cells in each ryanodine group. *C*, rate of increase in Ca_{im} expressed as percentage increase in the fluorescence ratio in the first minute of exposure to 30 K or 80 K before exposure to caffeine or during continued exposure to caffeine ($n = 14$–17 cells per group).

Fura-2 AM by piercing the cell with a patch pipette when the cell was bathed in physiological saline solution and rapidly monitoring the fluorescence before all the Fura-2 escaped from the cell (Stehno-Bittel & Sturek, 1992). This ratio was consistent with the maximum ratio predicted from the myoplasmic calibration of Fura-2 AM from cell suspensions (■, Fig. 1). The filled circles in Fig. 1 indicate that the pentapotassium salt of Fura-2 added exogenously to the $[Ca^{2+}]$-clamped cell lysate does not bind any organelles and undergoes no quenching by myoplasmic contents, and that saponin alone had no endogenous fluorescence or Fura-2 fluorescence quenching properties in the cell lysate. In contrast, when cells were loaded with Fura-2 AM by the normal loading procedure (see Methods) and then allowed to cleave the molecule with endogenous esterases, the Fura-2 released by saponin permeabilization of the sarcolemma showed a reduced apparent Ca^{2+} sensitivity (■, Fig. 1). At low Ca^{2+} concentrations the differences between the pentapotassium salt and myoplasmic Fura-2 from cells loaded with Fura-2 AM were not easily detected, but at 1000 nM $[Ca^{2+}]$ the fluorescence ratio noted in lysate of Fura-2 AM loaded cells was only 60% of that seen when Fura-2 pentapotassium salt was added to saponin permeabilized cells. Note that all the myoplasmic calibrations were conducted on one microfluorometry system, system 1. The *in vitro* Ca^{2+} calibration of a separate system (system 2) used for collection of a portion of these data (Fig. 6) is shown (△) to indicate a relatively parallel upward shift from the *in vitro* relationship obtained using system 1; thus, regardless of the differences in fluorescence ratios, the data in Figs 2–5 are comparable to Fig. 6.

Prediction 1: inhibition of Ca^{2+}_{im} buffering by the SR increases Ca_{im}

If the SR Ca_{im} buffering hypothesis is true then the increased Ca_{im} normally resulting from Ca^{2+} influx should be augmented during inhibition of Ca_{im} buffering by the SR; specifically, both the rate and maximum amplitude of the increase in Ca_{im} should be enhanced. The experiment shown in Fig. 2*A* is the general format for conduct of most of the experiments described in this report. Each cell was first monitored for 3–4 min during superfusion with physiological saline solution (PSS) prior to beginning each protocol to be certain that Ca_{im} was stable. The cell was then exposed to 30 K solution (equimolar Na^+ removal) for the 2 min period indicated by the horizontal line, which caused a 15% increase in the Fura-2 fluorescence ratio. The 2 min exposure to 80 mM-K^+ (equimolar Na^+ removal) caused a 33% increase in the Fura-2 fluorescence ratio above resting value, which would correspond to approximately 200 nM-Ca_{im} (> 100% increase over resting Ca_{im}) when the myoplasmic calibration curve in Fig. 1 (■) was used for conversion of the ratio. Although we have quantified our data in ratio fluorescence the terms fluorescence and Ca_{im} are used interchangeably. In these protocols in Figs 2–5 the cell was exposed to the next vasoactive agent only after the Ca_{im} returned to resting level in physiological solution. Caffeine (5×10^{-3} M) caused a large increase in Ca_{im} that subsequently decayed to resting level in the continued presence of caffeine; in this case, the peak increase in Ca_{im} would have been about 1000 nM when the myoplasmic calibration curve in Fig. 1 (■) was used for conversion of the ratio. Because the cell was continuously exposed to caffeine for the remaining ten minutes of the protocol, this procedure functionally inhibited Ca_{im} buffering by the SR. Caffeine opens the SR

Ca^{2+} release channel and, although the SR Ca^{2+} ATPase remains functional, the SR is maintained in a hyperpermeable state and unable to sequester Ca_{im} (Palade, 1987). Subsequent exposure to 30 K and 80 K in the presence of caffeine caused 21 and 38% increases in Ca_{im} above resting, respectively. This corresponds to a 110 and 65%

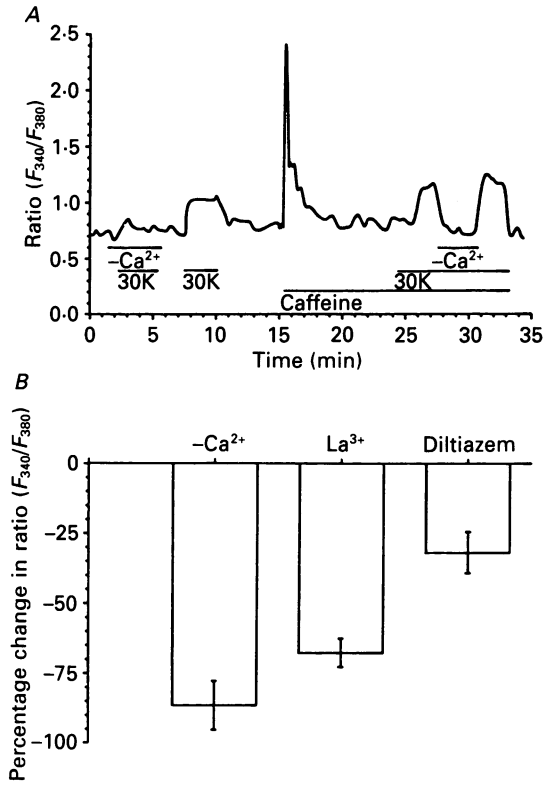

Fig. 3. Extracellular Ca^{2+} dependence of the augmented increase in Ca_{im} resulting from exposure to high extracellular $[K^+]$ during inhibition of SR buffering of Ca_{im}. *A*, representative recording from a single cell. The cell was exposed to Ca^{2+}-free physiological buffer ($-Ca^{2+}$, 10^{-5} M-added EGTA) to remove extracellular Ca^{2+} and then Ca^{2+}-free 30 mM-K^+ (30 K) solution for the duration indicated by the horizontal line. Normal PSS containing 2 mM-Ca^{2+} superfused the cell prior to a second exposure to 30 K. The cell was then superfused with caffeine (5×10^{-3} M) for the next seventeen minutes of the protocol and 30 K and Ca^{2+}-free ($-Ca^{2+}$) extracellular solutions were again applied to the cell simultaneously with the caffeine for the durations indicated. *B*, summary of group data for inhibition of the high K^+-induced increase in Ca_{im} in the presence of caffeine by Ca^{2+}-free solution ($-Ca^{2+}$), lanthanum (La^{3+}, 2×10^{-4} M), and diltiazem (10^{-5} M). The percentage inhibition was expressed as a negative percentage change in the fluorescence ratio. Ca^{2+}-free solution inhibited the 30 K-induced increase by 87% ($n = 6$). La^{3+} ($n = 5$) and diltiazem ($n = 5$) inhibited the 80 K-induced increase by 65 and 35%, respectively.

increase over that elicited by 30 K or 80 K alone ($P < 0{\cdot}05$). Ryanodine (10^{-5} M), a more specific opener of the SR Ca^{2+} release channel (Imagawa, Smith, Coronado & Campbell, 1987), also had effects on SR Ca_{im} buffering that were similar to those of caffeine, but ryanodine caused an irreversible increase in the baseline level of Ca_{im}

(note the last value in Fig. 2*B*). Although the effects of ryanodine appear larger than those of caffeine in Fig. 2*B*, upon subtraction of the elevated baseline Ca_{im} (data not shown) the responses to ryanodine were similar to those of caffeine. As a measure of the rate of increase of Ca_{im} we used the percentage increase in the fluorescence ratio in the first minute of exposure to 30 K or 80 K, which resulted in 121% ($P < 0.05$) and 97% greater rates of increase in Ca_{im}, respectively, when buffering by the SR was inhibited in the presence of caffeine (Fig. 2*C*). The relatively large variability is probably associated with cell-to-cell variability and alterations in solution exchange in the superfusion chamber.

The 30 K-induced increase in Ca_{im} during inhibition of SR Ca_{im} buffering by caffeine was dependent on external Ca^{2+} (Fig. 3*A*). The protocol was similar to Fig. 2*A*, except that: (1) the cell was exposed to 30 K, not 80 K, and (2) in Fig. 3*A* caffeine was not applied until minute 15 because this cell required longer to achieve a steady baseline Ca_{im} level. In the continued presence of caffeine and 30 K there was a 26% greater increase in Ca_{im} than when the cell was exposed to 30 K alone. Furthermore, Ca^{2+}-free 30 K (10^{-5} M-added EGTA) solution abolished the increase. Almost identical results were found when comparing 80 K to Ca^{2+}-free 80 K solution ($n = 7$ cells, results not shown). To define the Ca^{2+} influx pathway more specifically, two other pharmacological agents were used. Lanthanum is an inorganic blocker of voltage-gated Ca^{2+} channels at $> 5 \times 10^{-5}$ M (Bean *et al.* 1986) and inhibitor of Na^{+}–Ca^{2+} exchange at concentrations greater than $1–2 \times 10^{-4}$ M (Miura & Kimura, 1989), and diltiazem is a very specific inhibitor of voltage-gated Ca^{2+} channels at a concentration of 10^{-5} M (Godfraind, Miller & Wibo, 1986). The group data are shown in Fig. 3*B*, where the results are expressed as a negative percentage change in the fluorescence ratio (or percentage inhibition of the high K^{+}-induced increase). Calcium-free 30 K solution decreased the ratio 87%, lanthanum (2×10^{-4} M) decreased the ratio by 65%, and diltiazem (10^{-5} M) decreased the ratio by only 35%. The ratio was decreased 44% by 5×10^{-5} M-lanthanum ($n = 5$). Inhibition by all three agents was rapidly reversible.

Prediction 2: increased Ca_{im} buffering capacity of the SR decreases Ca_{im}

The basic rationale for these experiments was that when the Ca_{im} buffering capacity is increased the SR will more avidly sequester influxing Ca^{2+} and, thus, it will require longer for Ca^{2+} influx to cause a net increase in Ca_{im}. Increasing the Ca_{im} buffering capacity was accomplished by a transient, 2 min exposure of the cell to caffeine to cause partial depletion of the SR Ca^{2+} store (Kanaide *et al.* 1988). Cells as in Fig. 4*A* were exposed to 80 K for 3 min and then Ca_{im} allowed to return to baseline before exposing the cells to caffeine (5×10^{-3} M) for 2 min. It required 90% longer (5.5 ± 0.2 min, $P < 0.05$) for subsequent exposure to 80 K to cause Ca_{im} to reach the peak level obtained during the first exposure to 80 K; the percentage increase in Ca_{im} at 3 min of the second 80 K exposure was only 27%, compared to an increase of 47% in the first exposure to 80 K. The third exposure to 80 K after partial depletion of the SR similarly required 148% longer (7.2 ± 1.0 min) to comparably increase Ca_{im}.

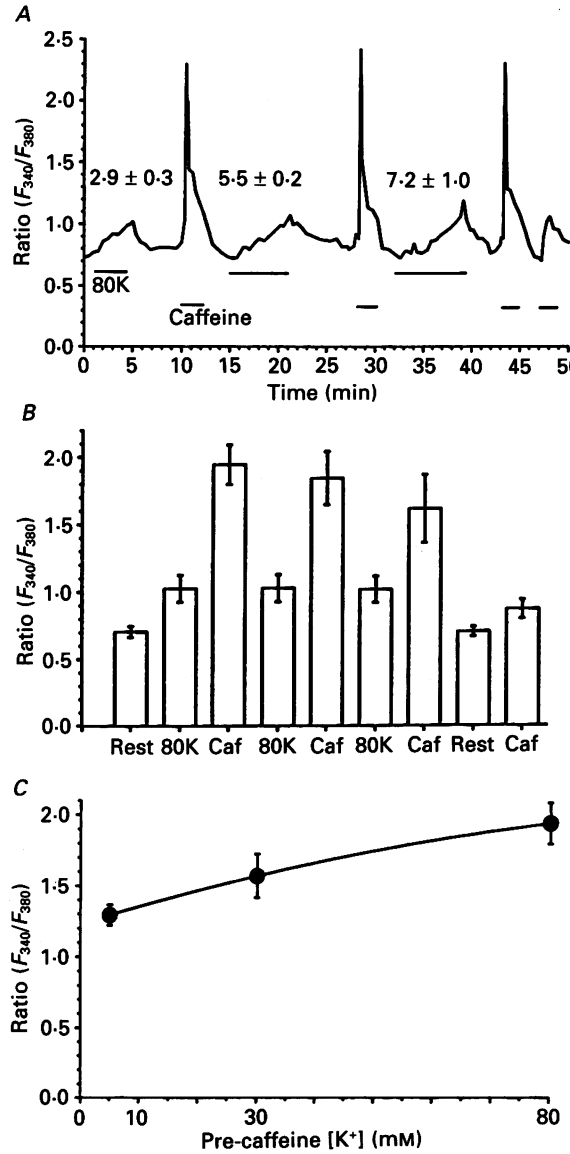

Fig. 4. Partial depletion of the SR Ca^{2+} store increased the time required for Ca^{2+} influx during exposure to high extracellular [K^+] (80 K) to increase Ca_{im} and refilled the SR Ca^{2+} store. *A*, representative recording from a single cell. The $X \pm$ S.E.M., ($n = 5$ cells) of the time for the 80 K-induced increase in Ca_{im} to reach the level in the first 2 min exposure to 80 K is shown above the 80 K-induced increase in Ca_{im}. The 2·9 ± 0·3 min indicated above the first exposure to 80 K was the time at which Ca_{im} peaked as a result of the 3 min exposure. *B*, group data for peak Ca_{im} responses, $n = 5$ cells/group. *C*, group data for caffeine-induced Ca_{im} transient (fluorescence ratio) as a function of 3 min exposure to 5, 30, or 80 mM-extracellular [K^+] prior to caffeine exposure. $n = 7$, 7 and 5 cells for 5, 30 and 80 mM-[K^+]. Significant correlation of 0·99 of least-squares fit to data by equation: ratio $= 1{\cdot}2298 + (1{\cdot}2745 \times 10^{-2} K) - (4{\cdot}7901 \times 10^{-5} K^2)$, where $K = [K^+]$.

Prediction 3: the SR is refilled by Ca^{2+} influx

Data in Fig. 4*B* are group data obtained from the protocol of Fig. 4*A*. The identical caffeine-induced peak increase in Ca_{im} indicates that the Ca^{2+} influx that occurred during exposure to 80 K was sufficient to refill the partially depleted SR Ca^{2+} store. The peak caffeine-induced increases in Ca_{im} were 185, 170 and 163% above the resting fluorescence ratio in the first, second, and third caffeine challenges, respectively. A final exposure to caffeine only 2 min after the third exposure to caffeine, with no intervening depolarization, elicited a modest 26% increase in the Ca_{im}.

Prediction 4: filling of the SR Ca^{2+} store is proportional to Ca^{2+} influx

A corollary to prediction 3 is that filling of the SR Ca^{2+} store noted in Fig. 4*A* and *B* should be proportional to Ca^{2+} influx. The first 15 min of the protocol of Fig. 4*A* was replicated, except that the cells were exposed to 30 K or 80 K for the 3 min of PSS (normal 5 mM-extracellular $[K^+]$) for 10 min preceding exposure to caffeine. The resulting peak of the caffeine-induced Ca_{im} transient represents the amount of Ca^{2+} in the caffeine-sensitive SR store (Stehno-Bittel, Laughlin & Sturek, 1990). The caffeine-induced Ca_{im} peak was proportionally larger when cells were exposed to 30 K and 80 K, as shown by a significant least-squares fit to a second-order polynomial (correlation coefficient, R, equals 0·99) in Fig. 4*C*. Another test of prediction 4 was to decrease Ca^{2+} influx pharmacologically with the specific inhibitor of voltage-gated Ca^{2+} channels, diltiazem, and then determine whether there was a subsequent parallel inhibition of the caffeine-induced release of Ca^{2+} from the SR. The protocol was similar to Fig. 4, except that cells were exposed to 80 K solution which contained diltiazem (10^{-5} M) and after the 80 K solution was washed out diltiazem remained present for 1 min. Again, as in Figs 2–4, each cell was allowed to recover for variable times until Ca_{im} reached resting levels (Fig. 5*A*). The cell was superfused with diltiazem-free solution for at least 1–2 min prior to exposure to caffeine. Diltiazem inhibited the 80 K-induced increase in Ca_{im} by 34% (Fig. 5*B*; and see also Fig. 3*B*) and the peak increase above resting Ca_{im} of the subsequent (second) caffeine-induced Ca_{im} transient was inhibited by 26% (Fig. 5). Diltiazem had no direct effects on caffeine-induced Ca^{2+} release, as evident by lack of inhibition of the Ca_{im} transient when cells were exposed to diltiazem for 1 min in normal PSS solution prior to caffeine exposure ($n = 3$, data not shown). The third exposure to 80 K was in Ca^{2+}-free solution, thus enabling the assessment of the degree of SR refilling that was the result mainly of resequestration of Ca_{im} by the SR. Similar to Fig. 3, Ca^{2+}-free 80 K solution did not increase Ca_{im} (Fig. 5). The peak of the Ca_{im} transient in the subsequent (third) exposure to caffeine was decreased 76% from the first caffeine-induced Ca_{im} transient, thus indicating that 76% of the refilling was due to external Ca^{2+}. Accordingly, diltiazem inhibited 34% of the refilling attributable to external Ca^{2+}, exactly parallelling the 34% inhibition of the 80 K-induced increase in Ca_{im}.

The data of Figs 3 and 5 clearly indicated that almost 90% of the 30 K- and 80 K-induced increase in Ca_{im} was dependent on extracellular Ca^{2+} and that diltiazem only blocked about 35% of the Ca^{2+} influx, while lanthanum blocked 65%

of the high-K^+-induced increases in Ca_{im}. Accordingly, we postulated that removal of extracellular Na^+ may be responsible for the majority (perhaps > 50–65 %) of the Ca^{2+} influx. In contrast, diltiazem (10^{-4} M) completely abolished the 80 K-induced increase in Ca_{im} in cells from porcine left circumflex artery (data not shown). Because

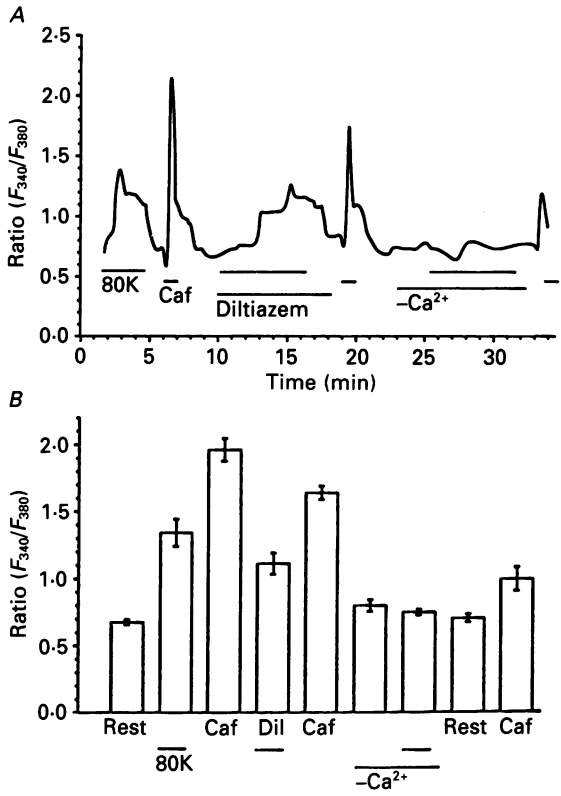

Fig. 5. Inhibition of Ca^{2+} influx during exposure to 80 mM-K^+ (80 K) inhibited refilling of the SR. *A*, representative recording from a single cell. The protocol was similar to that in Fig. 4, except that diltiazem (Dil, 10^{-5} M) was present during the second exposure to 80 K and the third exposure to 80 K was in Ca^{2+}-free external solution. *B*, group data, $n = 4$ cells per group. The second and third caffeine-induced Ca_{im} transients were significantly different than the first ($P < 0{\cdot}05$).

external Na^+-dependent Ca^{2+} influx may be involved in the 80 K-induced increase in Ca_{im} in bovine cells, we sought to eliminate any effects of extracellular Na^+ removal by adding the appropriate concentration of K^+ to the PSS, while not removing extracellular Na^+. In these experiments the time of solution exposure was kept constant for each cell and, thus, group data are plotted in Fig. 6 over the entire protocol at 1·0 min intervals. The first exposure to +35 K was 5 min duration and elicited less than a 5–10 % increase in the fluorescence ratio in all three protocols (Fig. 6*A–C*). Data in Fig. 6*A* clearly indicate that the SR Ca^{2+} was completely refilled with a 7 min exposure to +35 K following partial depletion of the SR by 1 min exposure to caffeine as shown by the peak of the second caffeine-induced Ca_{im} transient not being different than the first. A 5 min second exposure to +35 K only

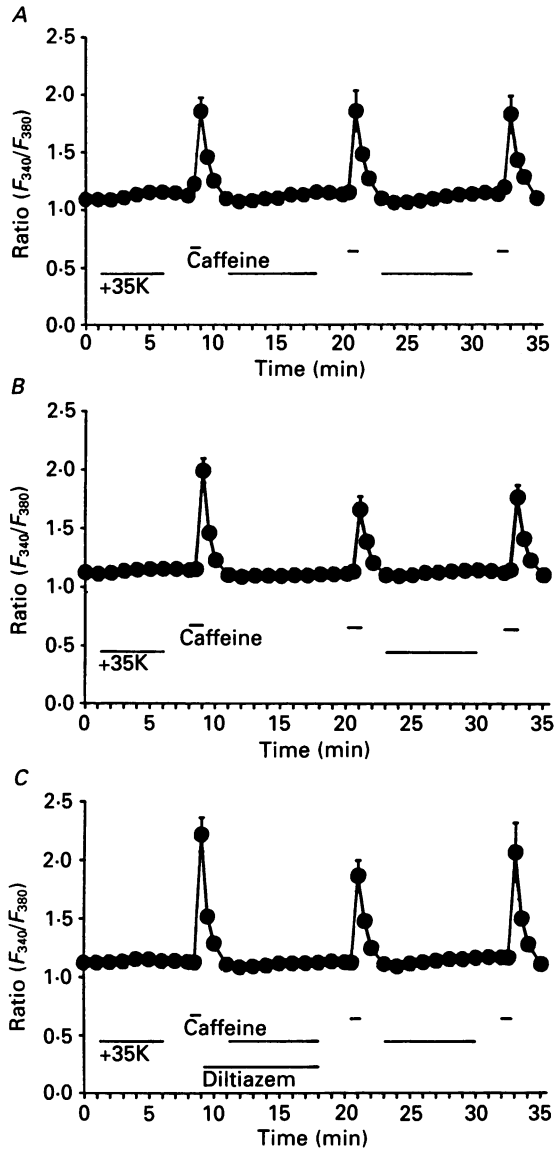

Fig. 6. Effect of inhibition of Ca^{2+} influx during mild depolarization with 35 mM-K^+ (no Na^+ removal) on refilling of the SR Ca^{2+} store. All cells in each group were exposed to each of the solutions for the exact minutes indicated by the horizontal lines. *A*, complete refilling of the SR Ca^{2+} store by +35 K. Cells were depolarized with 35 mM-K^+, but no Na^+ was removed (+35 K) for 5 min indicated by the horizontal line, followed by 2 min recovery in PSS, and then exposure to caffeine (5×10^{-3} M). The treatments were repeated twice, except that the cells were exposed to +35 K for 7 min ($n = 8$ cells). *B*, partial refilling of the SR Ca^{2+} store by recovery in PSS. The protocol was similar to *A*, except that the cells were allowed to recover in PSS for 7 min after the first exposure to caffeine. The peak of the second caffeine-induced Ca_{im} transient was less than the first ($P < 0{\cdot}05$; $n = 11$ cells). *C*, partial inhibition of refilling of the SR Ca^{2+} store by diltiazem (10^{-5} M) during exposure to +35 K for 7 min. The peak of the second caffeine-induced Ca_{im} transient was less than the first ($P < 0{\cdot}05$; $n = 5$ cells).

refilled the SR by 61% ($n = 6$ cells, data not shown), thus seven full minutes of Ca^{2+} was essential for complete refilling. Given the small amount of Ca^{2+} influx that was likely with exposure to +35 K in Fig. 6*A*, exposure to only PSS for the entire 10 min intervening period between the first and second exposures to caffeine was necessary

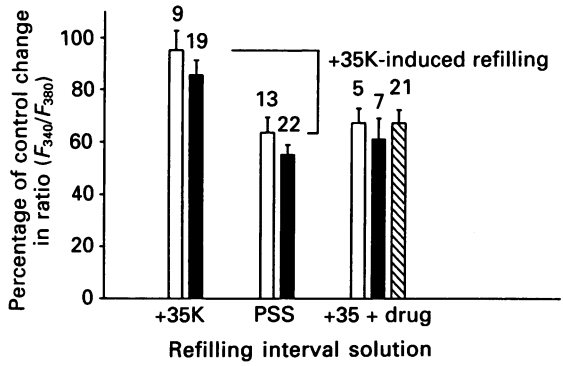

Fig. 7. Summary of effects of Ca^{2+} antagonists on refilling of the sarcoplasmic reticulum. Data were derived from protocol of Fig. 6. The peak ratio induced by the first exposure to 5×10^{-3} M-caffeine as in Fig. 6 was the control measurement and all data were plotted as percentage of control change in ratio induced by the second exposure to caffeine after the refilling interval as in Fig. 6. The depolarization-induced (+35 K-induced) refilling of the SR is noted by the difference in +35 K *vs.* PSS refilling conditions and is bracketed and labelled. Data for diltiazem (open bars, 10^{-5} M) experiments were summarized from Fig. 6 and represent the second peak caffeine-induced increase in *A* (+35 K), (PSS), and *C* (+35 K + drug). All TA3090 experiments were conducted at about the same time, but 1–2 months after the diltiazem experiments. Therefore data were compiled for the +35 K- and PSS-refilling conditions (filled bars) for the 10^{-7} M (filled bar) and 10^{-6} M (crosshatched bar) TA3090 concentration in the '+35 K + drug' column. The number of cells per group is indicated above bars.

to ascertain any refilling due simply to net leak influx of Ca^{2+}, which is estimated to be 5×10^{-6} mol kg^{-1} min^{-1} in bovine coronary artery (Ratz & Flaim, 1984, 1985) and may be sufficient over a long period to refill the SR. Partial refilling was evident over the total 11 min recovery in PSS, as the second caffeine-induced Ca_{im} transient was decreased 39% from the first (Fig. 6*B*). This is in contrast to the 76% decrease in the peak of the final Ca_{im} transient in Fig. 5 after recovery of only 2 min in PSS following exposure to caffeine. The presence of diltiazem (10^{-5} M) for 2 min preceding and during 7 min exposure of cells to +35 K decreased the subsequent caffeine-induced Ca_{im} transient by 34% (Fig. 6*C*). Refilling of the SR Ca^{2+} was possible after a third 7 min depolarization with +35 K in the absence of diltiazem.

Figure 7 summarizes the data on inhibition of SR refilling by diltiazem and its analogue, TA3090. The first caffeine-induced peak change in ratio using the protocol of Fig. 6 was the control. The second caffeine-induced Ca_{im} transient was plotted as a function of the solution superfusing the cells during the refilling interval. Similarly to Fig. 6*A*, when +35 K superfused the cells the resulting Ca^{2+} influx largely refilled the SR, as shown by the caffeine-induced percentage of control change in ratio being 85–95% (Fig. 7). Data on SR Ca^{2+} refilling induced by +35 K and PSS are shown for both diltiazem and TA3090 because these data were collected between 1 and 2 months apart and, thus, reproducibility of refilling by +35 K and PSS solutions

needed to be documented. The caffeine-induced increase in Ca_{im} after exposure to PSS during the refilling interval as in Fig. 6*B* clearly indicated refilling to about 60% of the control level (Fig. 7). Note that cells used to document refilling associated with exposure to PSS for the TA3090 condition (PSS, filled bars, Fig. 7) showed a non-significant trend toward less refilling in PSS when compared to the diltiazem condition (PSS, open bars, Fig. 7). The difference between the percentage of control change in ratio for the +35 K compared to the PSS refilling interval solutions was the +35 K-induced (depolarization-induced) refilling of the SR Ca^{2+} store. In the presence of either 10^{-5} M-diltiazem or TA3090 (10^{-7} or 10^{-6} M) the peak caffeine-induced Ca_{im} transient was decreased to about 60% of the control, which represents the same amount of SR Ca^{2+} refilling that occurred with exposure to PSS alone. A full dose–response curve was not determined for either drug because we were interested primarily in complete inhibition of depolarization-induced Ca^{2+} influx. Diltiazem was clearly less potent than TA3090 as shown from the concentrations used here. Collectively, the data in the three protocols of Fig. 6 that are summarized in Fig. 7 indicated that diltiazem and TA3090 inhibited virtually 100% of the depolarization-induced (+35 K-induced) refilling of the SR Ca^{2+} store.

DISCUSSION

The main conclusion of these studies is that the SR buffers, i.e. attenuates, the increase in Ca_{im} resulting from Ca^{2+} influx. Our findings support general concepts of several other major hypotheses of cellular Ca^{2+} regulation in that our data show important interactions between intracellular Ca^{2+} stores and the plasmalemma and suggest subcellular Ca^{2+} localization. The major hypotheses include the superficial buffer barrier hypothesis of van Breemen and co-workers (van Breemen *et al.* 1988; van Breemen & Saida, 1989), the Ca^{2+} cycling hypothesis of Rasmussen (Rasmussen & Barrett, 1984), and the capacitative model of Putney (1988). We propose, similarly to these investigators, a model of free Ca^{2+} regulation in cells involving two major Ca^{2+} compartments (Fig. 8): (1) free Ca^{2+} in the subsarcolemmal region (Ca_{is}) and (2) free Ca^{2+} concentration averaged over the bulk myoplasm (Ca_{im}). The SR may attenuate the increase in Ca_{im} resulting from Ca^{2+} influx through channels or Na^+–Ca^{2+} exchange (Fig. 8). We have provided evidence for Ca_{im} buffering due to influx through voltage-gated Ca^{2+} channels, external Na^+-dependent pathways, and leak pathways. The data necessitate novel interpretations of cellular mechanisms for drug and vasoconstrictor actions on smooth muscle cells of the coronary artery.

The SR Ca_{im} buffering hypothesis specifically tested in this report has been supported by confirming four major predictions of the hypothesis. First, the increased Ca_{im} normally resulting from Ca^{2+} influx was augmented during inhibition of SR buffering of Ca_{im} (Fig. 2). This inhibition of Ca_{im} buffering by the SR was achieved by caffeine and also, ryanodine, an agent with high selectivity for the SR Ca^{2+} release channel (Imagawa *et al.* 1987). Second, when the SR was depleted of Ca^{2+} it had a greater Ca_{im} buffering capacity and, thus, it required longer for Ca^{2+} influx to increase Ca_{im} (Fig. 4). Kanaide *et al.* (1988) and van Breemen *et al.* (1988) indicate that multiple caffeine challenges in Ca^{2+}-free solution are necessary for full depletion of the SR Ca^{2+} and that a single 2 min exposure to caffeine may only reduce the Ca^{2+}

store by 50% (Kanaide *et al.* 1988). We exposed cells to caffeine for only 2 min in physiological buffer containing 2 mM-Ca^{2+} to partially deplete the SR Ca^{2+} store (Fig. 4). In this regard, our results showing an almost twofold longer time for high-K^+ (80 mM-K^+) solution to increase Ca_{im} to the level achieved by 80 mM-K^+ before

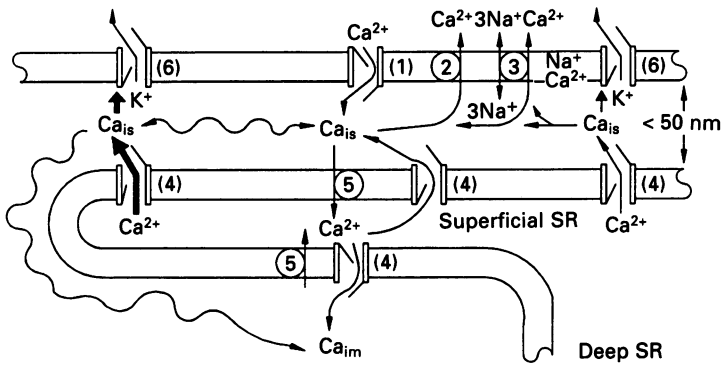

Fig. 8. Model of Ca^{2+} regulation in vascular smooth muscle cells whereby Ca^{2+} buffering by the SR may result in two major free Ca^{2+} compartments: Ca_{is} and Ca_{im}. The SR buffers the increase in Ca_{im} resulting from Ca^{2+} influx through voltage-gated Ca^{2+} channels, ligand-gated channels, or leak pathways (all three pathways indicated as (1) for simplicity) or reverse mode Na^+–Ca^{2+} exchange or other external Na^+-dependent mechanisms (3). The sarcolemmal (2) and SR (5) Ca^{2+} ATPases may also be involved in active Ca^{2+} buffering. The SR is sufficiently close (< 50 nm) from the sarcolemma such that release of Ca^{2+} via SR Ca^{2+} release channels (4) results in activation of Ca^{2+}-dependent K^+ channels (6). Bolus, very localized release of Ca^{2+} from the SR results in spontaneous transient outward K^+ currents (indicated by broad arrow at left of figure). See text for other details.

partial depletion of the SR are particularly striking and are more support for the physiological relevance of SR Ca_{im} buffering. Third, Ca^{2+} influx refilled the SR Ca^{2+} store (Figs 4 and 6). Fourth, filling of the SR Ca^{2+} store was proportional to Ca^{2+} influx (Fig. 4*C*). Collectively, these data support four major predictions of the SR Ca_{im} buffering hypothesis and suggest that a localized Ca^{2+} gradient exists between the SR and the sarcolemma, as addressed in the companion paper regarding Ca^{2+} release from the SR (Stehno-Bittel & Sturek, 1992).

Involvement of Ca_{im} buffering by the SR in the actions of vasoactive agents

The most important general implication of the Ca_{im} buffering function of the SR is that novel interpretations for cellular mechanisms of vasoactive agents on vascular smooth muscle cells are necessary.

Implications of SR Ca_{im} buffering in the action of vasoconstrictors

A common interpretation of the cellular mechanism of vasoactive agents is that a vasoconstrictor agonist is judged to have a sarcolemmal site of action if the agonist causes an increase in Ca_{im} which is: (1) completely abolished in Ca^{2+}-free solution and; (2) blocked by specific voltage-gated Ca^{2+} channel antagonists, such as diltiazem. We propose that the site and mechanism of action of the vasoconstrictor

may be the inhibition of Ca_{im} buffering by the SR, which would result in an increased Ca_{im}, without any change in Ca^{2+} influx across the sarcolemma (Fig. 2). Evidence suggests that this is one mechanism of action of endothelin (Wagner-Mann, Bowman & Sturek, 1990, 1991); another mechanism includes endothelin potentiation of voltage-gated Ca^{2+} channels (Inoue, Oike, Nakao, Kitamura & Kitayama, 1990). Thus, a vasoconstrictor may have the SR as its site of action, while removal of extracellular Ca^{2+}, etc. may yield results which are inappropriately interpreted as indicating a sarcolemmal effect. Although we used both caffeine and ryanodine, a toxin specific for the Ca^{2+} release channel of the SR (Imagawa *et al.* 1987), thapsigargin is proposed to be a specific inhibitor of the endoplasmic reticulum Ca^{2+} ATPase in secretory cells (Thastrup, Cullen, Drobak, Hanley & Dawson, 1990) and studies are now underway in our laboratory using thapsigargin as another test of the Ca_{im} buffering hypothesis in smooth muscle.

Role of SR Ca_{im} buffering in the action of Ca^{2+} channel antagonists

Our data reveal only a maximal 35% inhibition of the 80 K-induced increase in Ca_{im} by diltiazem (Figs 3 and 5). In contrast, Kanaide *et al.* 1988) recently reported complete block of the 100 mM-K^+-induced (equimolar Na^+ removal) increase in Ca_{im} in rat aortic smooth muscle cells by diltiazem. Similar to our methods, Kanaide *et al.* depolarized the cells with high K^+ and removed extracellular Na^+ equimolar to the added K^+. However, the cells in that report were subcultured, not freshly dispersed. Their data could be reconciled with ours if the cultured aortic cell preparation lacked extracellular Na^+-dependent Ca^{2+} transport systems, while retaining voltage-gated Ca^{2+} channels as the primary Ca^{2+} influx pathway. Furthermore, consistent with our data are those of DeFeo & Morgan (1989) showing that in intact ferret aorta 10^{-7} M-nifedipine caused only a 50% decrease in the aequorin light signal induced by 24 mM-K^+ (equimolar Na^+ removal). Given the high potency block of Ca^{2+} channels in depolarized cells (Bean, Sturek, Puga & Hermsmeyer, 1986; Hermsmeyer, Sturek & Rusch, 1988), complete inhibition of the 24 mM-K^+-induced increase in Ca_{im} should have occurred unless other Ca^{2+} transporters were involved (DeFeo & Morgan, 1989). In other words, maybe a portion of the increase in Ca_{im} was the result of extracellular Na^+-dependent Ca^{2+} influx.

Ratz & Flaim (1985) reported that 10^{-6} M-diltiazem blocked 83% of the 80 K-induced (equimolar Na^+ removal) contraction of bovine coronary artery; however, no determination of effects on $^{45}Ca^{2+}$ uptake or Ca_{im} was conducted. Direct voltage-clamp studies show complete block of Ca^{2+} current in cells by 10^{-5} M-diltiazem (Godfraind *et al.* 1986). Data of Ratz & Flaim could be reconciled with ours if they used mainly segments from the left anterior descending artery, because we have noted differences in regulation of Ca_{im} in cells of the left anterior descending and left circumflex arteries (C. C. Wagner-Mann & M. Sturek, unpublished observations). Surprisingly, we also found that 2×10^{-4} M-$LaCl_3$ was required for the maximal (65%) block of 80 K-induced Ca_{im} increase in our studies (Fig. 3). Also, 5×10^{-5} M-La^{3+} inhibited the Ca_{im} increase by 44%, despite complete block of voltage-gated Ca^{2+} channel currents in whole-cell voltage clamp (Bean *et al.* 1986). However, this degree of inhibition by 5×10^{-5} M-La^{3+} is entirely consistent with the small component of the 80 K-induced increase in Ca_{im} that is likely to result from Ca^{2+}

influx through voltage-gated Ca^{2+} channels (Figs 3 and 5) in the bovine cells studied here. Other Ca^{2+} transport mechanisms, i.e. Na^+–Ca^{2+} exchange, may be inhibited by 2×10^{-4} M-La^{3+} (Miura & Kimura, 1989).

The strongest support for the above interpretation of the modest inhibition of high K^+-induced increases in Ca_{im} is the effect of diltiazem on refilling of the SR by exposure to +35 K (no Na^+ removal). Figures 6 and 7 show complete block of the +35 K-induced component of SR refilling. The caffeine-induced Ca_{im} transient obtained after exposure to +35 K and 10^{-5} M-diltiazem (Fig. 6*C*) was not different to the caffeine-induced Ca_{im} transient obtained after the cells were only exposed to normal PSS (Fig. 6*B*). Partial refilling of the SR is likely to have occurred simply by the leak of Ca^{2+} into the cells during the 11 min interval between the first and second exposures to caffeine (Fig. 6). The results were also confirmed using the more potent analogue of diltiazem, TA3090 (Fig. 7). Note that in Fig. 5 the peak of the final caffeine-induced Ca_{im} transient was only 20% of the caffeine-induced Ca_{im} peak occurring only 2 min prior. The possibility of refilling by Ca^{2+} leak seems plausible, since the basal rate of Ca^{2+} entry in bovine coronary arteries is over 5×10^{-6} mol kg^{-1} min^{-1} (Ratz & Flaim, 1984, 1985). It is intriguing to consider whether voltage-gated Ca^{2+} channel antagonists affect refilling of the SR Ca^{2+} store that is mobilized by vasoconstrictors such as noradrenaline or endothelin, given the large functional overlap of these intracellular Ca^{2+} stores (van Breemen *et al.* 1988; van Breemen & Saida, 1989; Wagner-Mann *et al.* 1991; Wagner-Mann & Sturek, 1991).

Another implication of the SR Ca_{im} buffering hypothesis is that Ca^{2+} channel antagonists have indirect effects on release of Ca^{2+} from the SR, despite the well-known finding that their site of action is the voltage-gated Ca^{2+} channel (Bean *et al.* 1986; Hermsmeyer *et al.* 1988). This implication was clearly shown in Figs 6 and 7, in which diltiazem caused a 34% reduction in the caffeine-induced Ca_{im} transient by its inhibition of SR refilling. Diltiazem inhibited Ca^{2+} release from the SR *indirectly* by inhibiting SR refilling (Figs 6 and 7). Furthermore, TA3090, a more potent analogue of diltiazem showed similar effects (Fig. 7). These data indicate that the cellular mechanism of clinically used Ca^{2+} antagonists must be extended to include effects on Ca^{2+} release from the SR. This finding at least partly reconciles two seemingly inconsistent bodies of data: (1) *almost all* humoral vasoconstrictors implicated in coronary vasospasm release Ca^{2+} from the SR (Godfraind *et al.* 1986) and; (2) clinically effective Ca^{2+} channel antagonists show little binding or direct action on SR vesicles (Godfraind *et al.* 1986), while having high affinity binding to and inhibition of sarcolemmal voltage-gated Ca^{2+} channels (Bean *et al.* 1986, 1987; Godfraind *et al.* 1986; Hermsmeyer *et al.* 1988). Therefore, in addition to a direct action on voltage-gated Ca^{2+} channels to inhibit constriction of coronary arteries that have been depolarized by localized release of K^+ from ischaemic areas of the myocardium (Hill & Gettes, 1980), diltiazem and other Ca^{2+} entry blockers may indirectly inhibit release of Ca^{2+} from the SR by inhibiting refilling of the SR Ca^{2+} store. Our data showing that diltiazem did not affect the caffeine-induced Ca_{im} transient if applied during caffeine application are consistent with other data showing resistance of noradrenaline-induced contractions to inhibition by D600 and nicardipine (Casteels & Droogmans, 1981). Most importantly, our data extend those findings to indirect effects of diltiazem and TA3090 on Ca^{2+} release by a primary

effect on refilling the SR Ca^{2+} store. The effects of +35 K may be especially relevant, given the closer approximation to *in vivo* conditions of myocardial ischaemia in which extracellular K^+ increases to 20–30 mM (Hill & Gettes, 1980).

Implication that SR Ca_{im} buffering results in Ca^{2+} localization

Despite several excellent discussions of theoretical calculations supporting the localization of free Ca^{2+} in neurons and other cell types (e.g. Smith & Augustine, 1988), and electron probe data on localization of total Ca^{2+} (bound and ionized) (Devine *et al.* 1972), there are few critical tests of the Ca^{2+} localization hypothesis in smooth muscle. We cannot provide direct spatial imaging of Ca^{2+} with our optical methods of fluorescence recording from whole smooth muscle cells using photon counting. Digital imaging of Fura-2 fluorescence in smooth muscle cells has provided critical evidence for localization to large organelles, such as the nucleus (Williams, Fogarty, Tsien & Fay, 1985). Unfortunately, however, the resolution of $[Ca^{2+}]$ in the space of less than 50 nm between the sarcolemma and superficial SR (Fig. 8) is also not possible with the conventional digital imaging techniques using epifluorescence microscopy that are presently available (Williams *et al.* 1985; Goldman, Wier & Blaustein, 1989). None the less, our demonstration that the SR is refilled with Ca^{2+} with no increase in Ca_{im} strongly implies Ca^{2+} localization in a restricted cellular compartment, i.e. Ca_{is}, or that the influxing Ca^{2+} entered the SR via a direct route involving a low-resistance pathway, rather than sequestration by the SR Ca^{2+} pump (Fig. 8) (Putney, 1986). Evidence for a direct pathway is provided by the finding that manganese influx into cells from a rat aortic cell line (A7r5) occurs without involvement of the Ca^{2+} pump when the SR has been depleted (Missiaen, Declerck, Droogmans, Plessers, De Smedt, Raeymakers & Casteels, 1990). At the present time our data cannot differentiate between these mechanisms. Using aequorin to estimate Ca_{im}, Rembold (1989) has found the aequorin light signal to increase during Ca^{2+} influx after depletion of the SR, while there was no change in myosin phosphorylation in swine carotid artery. The data would also suggest Ca^{2+} localization, since aequorin is very sensitive to localized changes in Ca^{2+}, i.e. Ca_{is}, while the measurement of myosin phosphorylation would be more representative of changes in Ca_{im}.

Rigorous quantitative evaluation of Ca^{2+}-activated K^+ current as an estimate of Ca_{is} during simultaneous voltage clamp and Ca_{im} measurement will be necessary to resolve spatially and temporally the nature of the restricted Ca_{is} compartment. The feasibility of using Ca^{2+}-activated K^+ currents to indicate changes in Ca_{is} in the absence of any change in Ca_{im} is indicated in several reports of spontaneous transient outward currents in smooth muscle due to localized release of Ca^{2+} from the SR, which activates Ca^{2+}-dependent K^+ channels without causing contraction of the cell (Benham & Bolton, 1986; Ohya, Kitamura & Kuriyama, 1987; Hume & Leblanc, 1989; Desilets, Driska & Baumgarten, 1989; Ganitkevich & Isenberg, 1990; Stehno-Bittel & Sturek, 1992). These data strongly indicate a tight association of the SR and sarcolemma in smooth muscle cells. This relationship is depicted schematically in Fig. 8 and explored in greater detail in another report (Stehno-Bittel & Sturek, 1992). Similarly, an association between Na^+ influx and activation of reverse mode Na^+–Ca^{2+} exchange in cardiac myocytes has been recently reported (Leblanc & Hume, 1990), which can best be explained by localization of Na^+ in a 'fuzzy space'

analogous to the subsarcolemmal region we have proposed (Lederer, Niggli & Hadley, 1990). In conclusion, our data provide a step toward explaining important interactions of the SR with Ca^{2+} influx through the sarcolemma, which has relevance to several interrelated hypotheses of Ca_{im} regulation (Rasmussen & Barrett, 1984; Putney, 1986; van Breemen *et al.* 1988; van Breemen & Saida, 1989; Lederer *et al.* 1990). The companion paper (Stehno-Bittel & Sturek, 1992) provides data indicating another important interaction of the SR with the sarcolemma and prediction of the SR Ca_{im} buffering hypothesis; namely, the SR releases Ca^{2+} toward the sarcolemma, resulting in Ca_{is} localization.

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