

Ca²⁺ Compartments in Saponin-skinned Cultured Vascular Smooth Muscle Cells

HIROMICHI YAMAMOTO and CORNELIS VAN BREEMEN

From the Department of Pharmacology, University of Miami School of Medicine, Miami, Florida 33101

ABSTRACT A method for saponin skinning of primary cultured rat aortic smooth muscle cells was established. The saponin-treated cells could be stained with trypan blue and incorporated more ⁴⁵Ca²⁺ than the nontreated cells under the same conditions. At low free Ca²⁺ concentration, >85% of ⁴⁵Ca²⁺ uptake into the skinned cells was dependent on the extracellularly supplied MgATP. In the intact cells, both caffeine and norepinephrine increased ⁴⁵Ca²⁺ efflux. In the skinned cells, caffeine increased ⁴⁵Ca²⁺ efflux, whereas norepinephrine did not. The caffeine-releasable ⁴⁵Ca²⁺ uptake fraction in the skinned cells appeared at 3 × 10⁻⁷ M Ca²⁺, increased gradually with the increase in free Ca²⁺ concentration, and reached a plateau at 1 × 10⁻⁵ M Ca²⁺. The ⁴⁵Ca²⁺ uptake fraction, which was significantly suppressed by sodium azide, appeared at 1 × 10⁻⁵ M Ca²⁺ and increased monotonically with increasing free Ca²⁺ concentration. The results suggest that the caffeine-sensitive Ca²⁺ store, presumably the sarcoplasmic reticulum, plays a physiological role by releasing Ca²⁺ in response to norepinephrine or caffeine and by buffering excessive Ca²⁺. The ⁴⁵Ca²⁺ uptake by mitochondria appears too insensitive to be important under physiological conditions.

INTRODUCTION

It is generally accepted that intracellular Ca²⁺ stores play a role during contraction and relaxation of smooth muscle. For instance, norepinephrine can release Ca²⁺ from the caffeine-sensitive Ca²⁺ store in the rabbit aorta and the rabbit mesenteric artery (Itoh et al., 1983; Leijten and van Breemen, 1984; Saida and van Breemen, 1984a). After the contraction induced by high-K⁺ depolarization, the caffeine-sensitive Ca²⁺ store contains more Ca²⁺ than it does in the resting state (Leijten and van Breemen, 1984). In the guinea pig taenia coli, β-adrenergic relaxation is thought to be due to the intracellular Ca²⁺ sequestration (Mueller and van Breemen, 1979).

Morphological identification of the functional Ca²⁺ stores has been a subject of controversy. In earlier electron probe analysis studies, mitochondria had been considered a potential intracellular sink for Ca²⁺ because they could accumulate divalent cations such as Ca²⁺, Ba²⁺, and Sr²⁺ in an energy-dependent manner

Address reprint requests to Dr. Hiromichi Yamamoto, Dept. of Pharmacology, R-189, University of Miami School of Medicine, P.O. Box 016189, Miami, FL 33101.

(Somlyo and Somlyo, 1971; Somlyo et al., 1974). However, later studies showed that, in normal fibers, the mitochondrial Ca^{2+} content was as low as the cytoplasmic Ca^{2+} level and there was no evidence of mitochondrial Ca^{2+} sequestration during the contraction stimulated by high K^+ and supramaximal norepinephrine (Somlyo et al., 1979, 1982). Recently, Bond et al. (1984) have demonstrated that the number of subplasmalemmal regions containing high concentrations of Ca^{2+} , which were identified as part of the sarcoplasmic reticulum (SR), was reduced in the smooth muscles frozen at the peak of contraction by norepinephrine. However, the statistical significance of their data was not shown. This study is the first to prove that norepinephrine releases Ca^{2+} from the "junctional" SR in the smooth muscle.

In subcellular fractionation studies, mitochondrial fractions isolated from the bovine vascular smooth muscle and the guinea pig taenia coli have been shown to have ATP-dependent Ca^{2+} uptake capacity. However, in both cases, they had a low affinity for Ca^{2+} (apparent K_m , 17 and 6 μM) as compared with the microsomal fraction (Vallieres et al., 1975; Raemaekers et al., 1977), which suggested that mitochondria might not regulate the cytoplasmic Ca^{2+} concentration during the normal contraction and relaxation cycle.

Since cultured smooth muscle cells form a cell sheet at the bottom of the dish and have little extracellular material as compared with the smooth muscle tissues, it was expected that the use of this system would greatly improve the resolution of $^{45}\text{Ca}^{2+}$ uptake and efflux experiments and provide a good model for the study of Ca^{2+} accumulation and transport in the smooth muscle cells. The aims of the present study were to establish a method of chemical skinning of cultured smooth muscle cells using saponin and to examine $^{45}\text{Ca}^{2+}$ distribution into the SR and mitochondria *in situ* pharmacologically.

MATERIALS AND METHODS

Cell Culture

Rat aortic smooth muscle cells were cultured by the method of Chamley et al. (1977) with minor modifications (Yamamoto et al., 1983). Briefly, the aorta was excised from male Wistar rats (200–250 g) and the adventitia and the endothelial cells were removed after treatment with collagenase and elastase. The medium thus obtained was dispersed into single cells and cell clumps by a second incubation with collagenase and elastase. These dispersed cells had extremely high viability (>98%), as determined by the trypan blue exclusion test. The cells were inoculated with equal cell density into 14–17 plastic dishes (Falcon Labware, Becton, Dickinson & Co., Cockeysville, MD) of 35 mm diam containing 2 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The medium was changed every 3 d. After 8–10 d, the cells formed a confluent monolayer at the bottom of the dish. These confluent primary cultured cells in a monolayer were found to be optimal for the skinning experiment because the dish had a relatively large number of cells but little extracellular material. The cells cultured for >10 d tended to form a multilayer and were not used for the present study.

The primary cultured cells obtained via this procedure had been previously identified as pure smooth muscle cells by means of electron microscopy and fluorescent staining with antibodies against smooth muscle actin and myosin (Yamamoto et al., 1983).

Cell Skinning

The skinning of the primary cultured smooth muscle cells was performed according to the method described by Saida and van Breemen (1984*b*). After the culture medium was discarded, the cells were successively washed with and incubated for 10–15 min in 1.5 ml of physiological salt solution (PSS). Since the dead cells floated in the culture medium, they were effectively removed by this washing. Since the viable cells stuck to the bottom of the dish, solutions in the dish could be changed rapidly in 4 s using suction and a pipette. There was little mixing of any two solutions. All the experiments in the skinned cells described below were performed at room temperature (25°C) (Saida and van Breemen, 1984*a, b*). The cells were washed five times for 2 min with 1.5 ml of the solution for the skinned cells containing 4 mM EGTA and then incubated with saponin in 1 ml of the same solution for 15 min. The concentration of saponin used was 50 µg/ml in all experiments, except those shown in Fig. 2, where various concentrations were used. After the saponin solution was discarded, the cells were washed five times for 2 min with 1.5 ml of the solution for the skinned cells containing 0.1 mM EGTA (0.1 G solution). All the cells treated with saponin could be stained with trypan blue. The control cells treated with the same solution, but without saponin, could not be stained. The main purpose of using trypan blue was to indicate effective permeabilization of the plasma-membrane. Under the inverted phase contrast microscope, the saponin-treated cells were seen to stick to the bottom of the dish throughout the subsequent experiments.

⁴⁵Ca²⁺ Efflux from Skinned Cells

The skinned cells obtained as above were labeled with ⁴⁵Ca for 20–30 min in 1 ml of 0.1 G solution containing various concentrations of ⁴⁵CaCl₂, which gave the desired free Ca²⁺ concentrations (pCa solution). The cells were then incubated in 1.5 ml of 0.1 G solution (the efflux solution), which was changed every minute for 20 min and trapped in a vial. The cells were harvested by the treatment with 1 ml of PSS containing collagenase (1 mg/ml) and trypsin (0.1 mg/ml). The dishes were rinsed with 1 ml of Ca²⁺-free PSS containing 2 mM EGTA. A 1.5-ml aliquot of the resulting solution containing the harvested cells from each dish was placed into a vial, along with 4.5 ml of scintillation fluid containing Triton X-100, the radioactive content of which was quantified by liquid scintillation counting. The radioactivities of the efflux solution, 20 µl of labeled media, and blanks were also determined. In order to calculate the ⁴⁵Ca²⁺ content in the cells at each time point during the efflux, the amount of ⁴⁵Ca²⁺ in the efflux solution in each vial was back-added to the radioactivity in the cells. The number of cells per dish was counted with a hemocytometer after treatment with collagenase and trypsin in two separate dishes from a group of dishes cultured at the same time. Intact rather than skinned cells were used for the cell count because the latter were digested by the enzymes and could not be used for evaluation of the cell number. By preliminary experiment, it was found that there was little variation (<5% of the mean) in the number of cells among the dishes. Finally, the ⁴⁵Ca²⁺ content and ⁴⁵Ca²⁺ loss per minute were expressed as nanomoles labeled Ca²⁺ per 1 × 10⁶ cells and plotted as a function of the rinsing time. Compartmental analysis of ⁴⁵Ca²⁺ efflux curves was performed by the peeling technique (Casteels and Droogmans, 1975) according to the following equation:

$$C_{(t)} = \sum_{i=1}^n C_i e^{-\lambda_i t},$$

where C_i is the Ca²⁺ content of each compartment, λ_i is the rate constant, and t is the incubation time.

⁴⁵Ca²⁺ Uptake in Skinned Cells

The skinned cells were incubated with ⁴⁵Ca²⁺ for the desired period in 1 ml of pCa solution. To estimate the amount of ⁴⁵Ca²⁺ incorporated into the intracellular organelles, the cells were washed for 7 min with 0.1 G solution, which was changed every minute. After the cells were harvested, the amount of ⁴⁵Ca²⁺ in the cellular suspension was assessed as described for ⁴⁵Ca²⁺ efflux. The number of cells was also counted in the other two separate dishes. The Ca²⁺ content in 1 × 10⁶ skinned cells was calculated and expressed as nanomoles labeled Ca²⁺ per 10⁶ cells.

⁴⁵Ca²⁺ Efflux from Intact Cells

The intact cells were equilibrated with ⁴⁵Ca²⁺ in 1 ml of PSS for 60 min at 37°C. The cells were then incubated in 1.5 ml of Ca²⁺-free PSS containing 2 mM EGTA (the efflux solution) at 37°C, which was changed every minute and trapped in a vial. The radioactivities in the cells and the efflux solution were determined as described for ⁴⁵Ca²⁺ efflux from the skinned cells. The number of cells in the dish was counted. ⁴⁵Ca²⁺ lost per minute per 10⁶ cells was calculated and plotted as a function of the incubation time.

Solutions

The solutions of the following composition in millimolar were used in the present study. PSS: 140 NaCl, 5.0 KCl, 1.0 MgCl₂, 1.5 CaCl₂, 10.0 glucose, 10.0 HEPES, neutralized with NaOH to give pH 7.4 at 37°C. The solution for the skinned cells: 65.0 KCl, 65.0 K-propionate, 5.0 MgCl₂, 3.15 Na₂ATP, 20 Tris-maleate buffer, 4.0 or 0.1 EGTA, pH 6.8 at 25°C (Saida and van Breemen, 1984b). The various free Ca²⁺ concentrations were prepared by adding appropriate amounts of Ca²⁺ to 0.1 G solution (pCa solution). The apparent binding constant of EGTA for Ca²⁺ was found to be 1 × 10⁶ M⁻¹ at pH 6.8 at 25°C (Saida and Nonomura, 1978). The binding constant of ATP for Mg²⁺ was assumed to be 1 × 10⁴ M⁻¹ (Martell and Schwarzenbach, 1956). The ionic strength of pCa solution was kept constant under the various experimental conditions by changing the concentration of KCl.

Chemicals

Caffeine, EGTA, sodium azide, collagenase (type 1), elastase (type 1), and trypsin (type 111) were purchased from Sigma Chemical Co., St. Louis, MO. Saponin was purchased from ICN Pharmaceuticals, Inc., Cleveland, OH. Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Gibco Laboratories, Grand Island, NY.

Statistics

The data collected were expressed as the means ± SD. The difference between the results was compared by paired *t* test; *P* < 0.05 was considered statistically significant.

RESULTS

⁴⁵Ca²⁺ Efflux from Skinned Cells

In order to determine the optimal ⁴⁵Ca²⁺ washout time for the ⁴⁵Ca²⁺ uptake measurement, ⁴⁵Ca²⁺ efflux in the skinned cells was examined. The top curve in Fig. 1A (open circles) shows ⁴⁵Ca²⁺ efflux into 0.1 G solution. The skinned cells were equilibrated with ⁴⁵Ca²⁺ in 1 × 10⁻⁷ M free Ca²⁺ concentration for 30 min and then incubated in 0.1 G solution (the efflux solution) as described in Materials and Methods. The ⁴⁵Ca²⁺ remaining in the cells was plotted as a function of the incubation time in the 0.1 G solution. This curve could be divided into three

components—fast, intermediate, and slow. The parameters for these components are shown in Table I. We attempted to evaluate the contribution of $^{45}\text{Ca}^{2+}$ bound to the extracellular substance and to the dish to the efflux curve. In another dish, the cells were treated for 30 min with a higher concentration of saponin

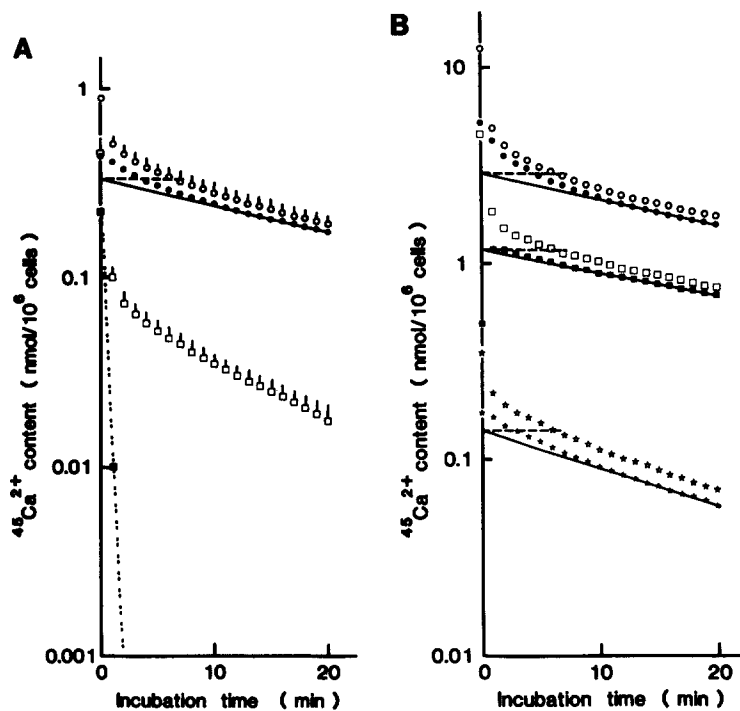


FIGURE 1. $^{45}\text{Ca}^{2+}$ efflux from skinned cells. (A) The saponin-skinned cells (\circ , $n = 5$) were equilibrated with $^{45}\text{Ca}^{2+}$ in 1×10^{-7} M free Ca^{2+} concentration for 30 min and then incubated in the efflux solution for the skinned cells containing 0.1 mM EGTA (0.1 G solution; see Materials and Methods). The efflux solution was changed every minute. The dish (\square , $n = 5$) from which the cells were removed by 30 min treatment with 100 $\mu\text{g}/\text{ml}$ of saponin, followed by 30 min incubation in 0.1 G solution, and the empty dish (\blacksquare , $n = 2$) were treated similarly. The difference of Ca^{2+} content between the skinned cells (\circ) and the dish without the cells (\square) was calculated and plotted (\bullet). Each point gives the mean and standard deviation of the $^{45}\text{Ca}^{2+}$ content. (B) The skinned cells were equilibrated with $^{45}\text{Ca}^{2+}$ in 1×10^{-4} (\circ), 1×10^{-6} (\square), and 3×10^{-8} (\star) M free Ca^{2+} concentration and processed as in A. Corresponding solid symbols (\bullet , \blacksquare , \star) represent the difference in $^{45}\text{Ca}^{2+}$ content between the skinned cells and the dish without the cells (data not shown) at the same free Ca^{2+} concentrations.

(100 $\mu\text{g}/\text{ml}$) than in the standard skinning procedure. The cells were washed five times, incubated in 0.1 G solution for 30 min, and then removed by gentle pipetting. As observed under the phase contrast microscope, no cells remained at the bottom of the dish but most extracellular material did. All the removed cells maintained their original appearance. The dishes thus obtained were processed for $^{45}\text{Ca}^{2+}$ efflux experiments as with the skinned cells. The lower curve

in Fig. 1A (open squares) shows the $^{45}\text{Ca}^{2+}$ efflux in this preparation and has three components (see Table I, B). The initial fast component in this curve has a relatively large Ca^{2+} content and a high rate constant and is similar to the fast component of the $^{45}\text{Ca}^{2+}$ efflux curve in the skinned cells (upper curve in Fig. 1A), which suggests that both components are due mostly to the trace amount of $^{45}\text{Ca}^{2+}$ solution remaining at the surface of the dish and the cell layer. The $^{45}\text{Ca}^{2+}$ efflux from the empty dish showed a similar rapid decay of $^{45}\text{Ca}^{2+}$ (solid squares and double dotted line in Fig. 1A), which supports the above idea. The second and third components of the cell free $^{45}\text{Ca}^{2+}$ efflux curve have significantly smaller Ca^{2+} contents (Table I, B: C_2 and C_3 , $P < 0.005$) and faster rate constants (Table I, B: λ_2 , $P < 0.05$; λ_3 , $P < 0.005$) as compared with those in the skinned cells (Table I, A) and could be attributable to $^{45}\text{Ca}^{2+}$ bound to the extracellular material. The difference in $^{45}\text{Ca}^{2+}$ content between the dish with the skinned cells and that without the cells is plotted in Fig. 1A (middle curve, solid circles).

TABLE I
Ca²⁺ Content and Rate Constant of Each Compartment of the Ca²⁺ Efflux Curves in Fig. 1A

	i	1	2	3
(A) Dish with skinned cells (n = 5)	C_i λ_i	0.391 ± 0.100 2.024 ± 0.771	0.110 ± 0.028 0.299 ± 0.027	0.395 ± 0.058 0.0357 ± 0.0050
(B) Dish without skinned cells (n = 5)	C_i λ_i	0.350 ± 0.081 $3.112 \pm 0.573^\ddagger$	$0.0386 \pm 0.0139^*$ $0.560 \pm 0.257^\ddagger$	$0.0673 \pm 0.0075^*$ $0.0662 \pm 0.0062^*$
(C) Difference curve between A and B (n = 5)	C_i λ_i	0.114 ± 0.034 0.332 ± 0.079	0.338 ± 0.063 0.0331 ± 0.0061	

Values are means \pm standard deviations. n is the number of experiments. * $P < 0.005$; $^\ddagger P < 0.05$. C_i is the Ca^{2+} content (nmol/ 10^6 cells); λ_i is the rate constant (min^{-1}).

This curve is composed of two fractions. Since it is reasonable to assume that $^{45}\text{Ca}^{2+}$ in the cytoplasm of the skinned cells is lost into the efflux solution more rapidly than $^{45}\text{Ca}^{2+}$ stored in the intracellular organelles, it is likely that the first relatively rapid component in the difference curve would reflect $^{45}\text{Ca}^{2+}$ lost from the cytoplasmic space (bound or unbound) and the second slower component (solid line) would be due mostly to $^{45}\text{Ca}^{2+}$ released from the Ca^{2+} stores. Stout and Diecke (1983) proposed a similar interpretation of their $^{45}\text{Ca}^{2+}$ efflux data. We thus conclude that the Ca^{2+} content of the slower component would give us the amount of Ca^{2+} stored in the intracellular organelles. Practically, this value could be estimated by the $^{45}\text{Ca}^{2+}$ content after a 7-min washout in the original $^{45}\text{Ca}^{2+}$ efflux curve of the skinned cells, as shown by the single dashed line in Fig. 1A.

As shown in later experiments (Fig. 4), $^{45}\text{Ca}^{2+}$ uptake in skinned cells was dependent on the free Ca^{2+} concentrations in the range 3×10^{-8} to 1×10^{-4} M free Ca^{2+} . If the $^{45}\text{Ca}^{2+}$ efflux rates from the intracellular organelles are controlled by the Ca^{2+} gradient across the intracellular membranes, it is possible that the skinned cells exposed to the various pCa solutions might give varying $^{45}\text{Ca}^{2+}$ efflux rates such that the 7-min washout method effective in 1×10^{-7} M free Ca^{2+} solution could not be used in the other solutions. Fig. 1B indicates that

this was not the case. In all the solutions with 1×10^{-4} , 1×10^{-6} , and 3×10^{-8} M of free Ca^{2+} , the $^{45}\text{Ca}^{2+}$ content after a 6- or 7-min washout by the efflux solution (0.1 G solution) coincided with the Ca^{2+} content of the slower component of the difference curve. We therefore used a 7-min washout in the Ca^{2+} -free 0.1 G solution in order to assess the organellar Ca^{2+} . However, a significant amount of stored Ca^{2+} might be lost during a 7-min washout. To assess this possibility, we measured the decay of MgATP-dependent stored Ca^{2+} and caffeine-sensitive stored Ca^{2+} from the skinned cells into the washing solution (0.1 G solution). The skinned cells were preloaded with ^{45}Ca labeled at 1×10^{-6} M free Ca^{2+} in the presence or absence of 3 mM MgATP for 20 min and incubated in 0.1 G solution, which was changed every minute. The amount of the MgATP-dependent $^{45}\text{Ca}^{2+}$ at each time point was calculated by subtracting the $^{45}\text{Ca}^{2+}$ content in the skinned cells preloaded in the absence of MgATP from that in the presence of MgATP, and plotted as a function of efflux time assuming that the efflux rates of MgATP-independent $^{45}\text{Ca}^{2+}$ were similar under both conditions. The major exponential component of the Ca^{2+} content decay curve obtained as described above had a half-time of ~ 14 min (data not shown). The decay curve of 25 mM caffeine-sensitive stored Ca^{2+} , which was obtained in a similar manner except that 25 mM caffeine was applied for 1 min at the end of $^{45}\text{Ca}^{2+}$ loading, also had a major component with the same half-time (~ 14 min) as that of MgATP-dependent Ca^{2+} . Thus, $\sim 71\%$ of MgATP-dependent Ca^{2+} and caffeine-sensitive Ca^{2+} was preserved after a 7-min washout.

$^{45}\text{Ca}^{2+}$ Uptake in Skinned Cells

The following experiments were designed to determine the optimal conditions for skinning and the subsequent $^{45}\text{Ca}^{2+}$ uptake. Fig. 2 shows the effect of saponin concentration on $^{45}\text{Ca}^{2+}$ uptake in skinned cells. The cells were treated with various concentrations of saponin for 15 min, incubated with $^{45}\text{Ca}^{2+}$ for 20 min in 1×10^{-6} M free Ca^{2+} concentration, and washed for 7 min with 0.1 G solution. $^{45}\text{Ca}^{2+}$ uptake into the skinned cells was determined as described in Materials and Methods and plotted as a function of the saponin concentration. The $^{45}\text{Ca}^{2+}$ uptake increased abruptly at 40 $\mu\text{g}/\text{ml}$ and reached a maximal level at 50 $\mu\text{g}/\text{ml}$, but did not increase further at higher saponin concentrations, which suggests that the cells were completely skinned at 50 $\mu\text{g}/\text{ml}$. When the incubation time was changed (50 $\mu\text{g}/\text{ml}$ saponin), 15 min incubation with saponin gave a maximal $^{45}\text{Ca}^{2+}$ uptake (data not shown). These results indicated that the optimal condition for complete skinning was 50 $\mu\text{g}/\text{ml}$ saponin for 15 min at room temperature.

The rates of $^{45}\text{Ca}^{2+}$ equilibration of the skinned cells were examined as a function of free Ca^{2+} . As shown in Fig. 3, the skinned cells were fully equilibrated with $^{45}\text{Ca}^{2+}$ in 1×10^{-7} to 1×10^{-4} M free Ca^{2+} concentration at 20 min incubation. In 1×10^{-3} M free Ca^{2+} concentration, a decrease of $^{45}\text{Ca}^{2+}$ uptake was observed after 10 min incubation, which might be due to the deterioration of the function of Ca^{2+} stores, particularly the mitochondria.

$^{45}\text{Ca}^{2+}$ uptake into the saponin-treated skinned cells was compared with that into the nontreated cells under the same experimental conditions (Fig. 4). The skinned cells were incubated in the $^{45}\text{Ca}^{2+}$ -labeled solutions containing various concentrations of free Ca^{2+} for 20 min and $^{45}\text{Ca}^{2+}$ uptake was determined. The

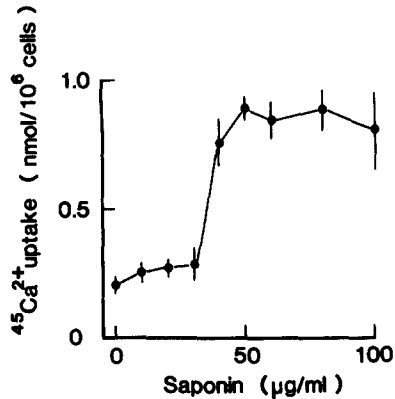


FIGURE 2. Effect of saponin treatment on $^{45}\text{Ca}^{2+}$ uptake. The cells were treated with various concentrations of saponin for 15 min and incubated with $^{45}\text{Ca}^{2+}$ in 1×10^{-6} M free Ca^{2+} concentration for 20 min. The $^{45}\text{Ca}^{2+}$ content was measured after a 7-min washout in 0.1 mM EGTA (0.1 G solution; see Materials and Methods). $n = 3$. Each point represents the mean and standard deviation.

intact cells were treated according to the same protocol except that saponin was not added to the skinning solution. In the range 3×10^{-8} to 1×10^{-4} M of free Ca^{2+} concentration, $^{45}\text{Ca}^{2+}$ uptake into the skinned cells increased with increasing free Ca^{2+} concentration and was 4–10-fold greater than that observed in the nontreated cells. At 1×10^{-3} M free Ca^{2+} concentration, the $^{45}\text{Ca}^{2+}$ uptake decreased in the skinned cells, whereas it reached a maximum in the nontreated cells.

Fig. 5 shows the effect of MgATP on $^{45}\text{Ca}^{2+}$ uptake into skinned cells. The skinned cells were incubated with $^{45}\text{Ca}^{2+}$ at various concentrations of free Ca^{2+} in the absence or presence of 3 mM MgATP. The free Mg^{2+} concentration was

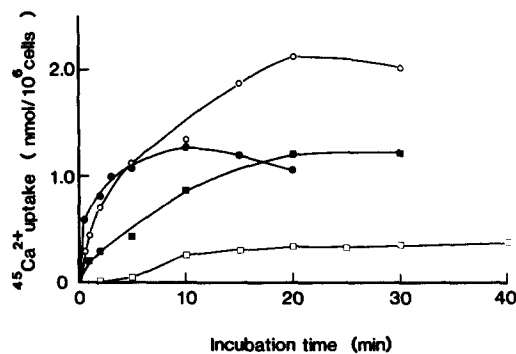


FIGURE 3. Time course of the equilibration of the skinned cells with $^{45}\text{Ca}^{2+}$ in the various concentrations of free Ca^{2+} . The skinned cells were obtained by treatment with $50 \mu\text{g/ml}$ saponin for 15 min and incubated with $^{45}\text{Ca}^{2+}$ in the various concentrations of free Ca^{2+} for various periods. The free Ca^{2+} concentrations were: 1×10^{-7} (\square), 1×10^{-6} (\blacksquare), 1×10^{-4} (\circ), 1×10^{-3} (\bullet) M.

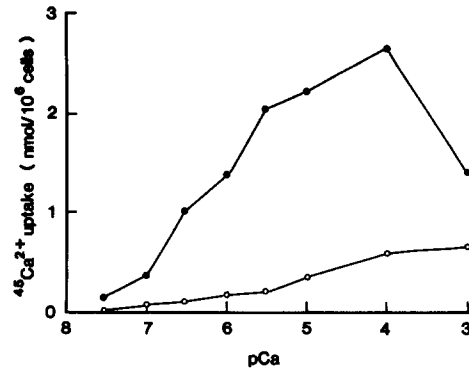


FIGURE 4. Comparison of $^{45}\text{Ca}^{2+}$ uptake in saponin-treated cells and in nontreated cells under the same conditions. The cells were treated with 0 (○) or 50 (●) $\mu\text{g}/\text{ml}$ saponin for 15 min in the presence of 4 mM EGTA and incubated with $^{45}\text{Ca}^{2+}$ for 20 min in the various concentrations of free Ca^{2+} .

kept constant at 2 mM in both cases. 85–92% of the total $^{45}\text{Ca}^{2+}$ uptake was dependent on extracellularly supplied MgATP, which indicates that the bulk of $^{45}\text{Ca}^{2+}$ was taken up by energy-dependent biological processes such as the Ca^{2+} pump in the SR, and that ATP could enter into the cells. The $^{45}\text{Ca}^{2+}$ uptake in the absence of MgATP was probably due to the tightly bound residual $^{45}\text{Ca}^{2+}$ in the cytoplasm.

Effect of Caffeine and Norepinephrine on $^{45}\text{Ca}^{2+}$ Efflux in Intact and Skinned Cells

Itoh et al. (1983) and Saida and van Breemen (1984a) showed that complete skinning abolished the norepinephrine-induced but not the caffeine-induced Ca^{2+} release contraction in the rabbit mesenteric artery preloaded in 1 or 0.5 \times

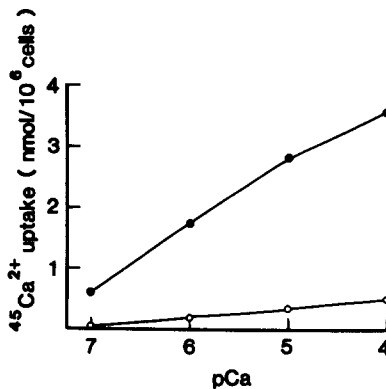


FIGURE 5. Effect of extracellularly supplied MgATP on $^{45}\text{Ca}^{2+}$ uptake by the skinned cells. The skinned cells were incubated with $^{45}\text{Ca}^{2+}$ in pCa solutions containing 0 (○) or 3 (●) mM MgATP. The free Mg^{2+} concentration was kept constant at 2 mM.

10^{-6} M free Ca^{2+} . In order to determine whether the completely skinned cells in the present study had similar properties, we compared the effects of caffeine and norepinephrine on $^{45}\text{Ca}^{2+}$ efflux in intact and skinned cells.

Fig. 6A shows $^{45}\text{Ca}^{2+}$ efflux from the intact cells. The absolute amount of $^{45}\text{Ca}^{2+}$ released per minute was plotted as a function of the time in the efflux

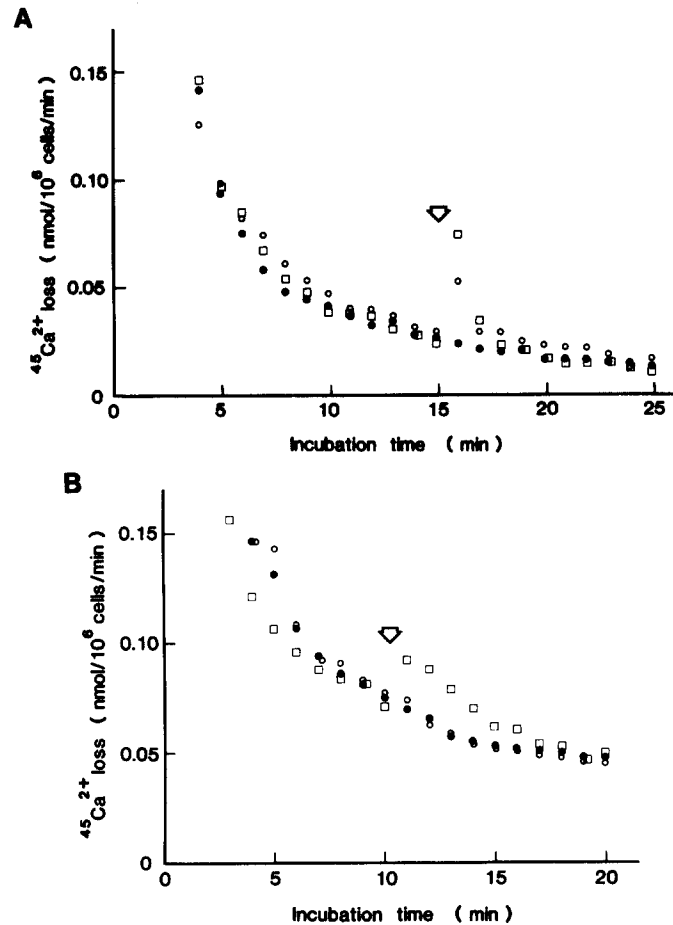


FIGURE 6. Effects of caffeine and norepinephrine on $^{45}\text{Ca}^{2+}$ efflux from intact cells (A) and skinned cells (B). (A) The intact cells were equilibrated with $^{45}\text{Ca}^{2+}$ in PSS for 60 min and then incubated in 2 mM EGTA, Ca^{2+} -free PSS (the efflux solution) at 37°C . The solution was changed every minute. After 15 min, 25 mM caffeine (\square , $n = 3$) or 1×10^{-5} M norepinephrine (\circ , $n = 2$) was added into the efflux solution as indicated by the arrow. \bullet , control ($n = 3$). (B) The skinned cells were equilibrated with $^{45}\text{Ca}^{2+}$ in 1×10^{-6} M free Ca^{2+} concentration for 20 min and then incubated in 0.1 G solution (the efflux solution) at room temperature. After 10 min, 25 mM caffeine (\square , $n = 7$) or 1×10^{-5} M norepinephrine (\circ , $n = 4$) was added to the efflux solution as indicated by the arrow. \bullet , control ($n = 4$). In both graphs, the amount of $^{45}\text{Ca}^{2+}$ lost per minute was calculated and plotted as a function of the time in the efflux solution. Each point represents the mean of the indicated number of observations.

solution. After 25 mM caffeine was added, $^{45}\text{Ca}^{2+}$ efflux increased, presumably because of Ca^{2+} release from the caffeine-sensitive Ca^{2+} store. 1×10^{-5} M norepinephrine produced a smaller increase in $^{45}\text{Ca}^{2+}$ efflux.

Skinned cells were incubated with $^{45}\text{Ca}^{2+}$ in 1×10^{-6} M free Ca^{2+} concentration for 20 min and rinsed in 0.1 G solution (Fig. 6B). Since neither the caffeine-induced nor the norepinephrine-induced increase in $^{45}\text{Ca}^{2+}$ efflux was seen when the skinned cells were loaded in 1×10^{-7} M free Ca^{2+} , the presumed cytosolic free Ca^{2+} concentration in the resting intact cells, we chose 1×10^{-6} M free Ca^{2+} , a nonresting free Ca^{2+} concentration, as a loading condition mimicking the conditions used in the previous studies (Itoh et al., 1983; Saida and van Breemen, 1984a). When 25 mM caffeine or 1×10^{-5} M norepinephrine was applied after 10 min, $^{45}\text{Ca}^{2+}$ efflux increased in response to caffeine but not to norepinephrine,

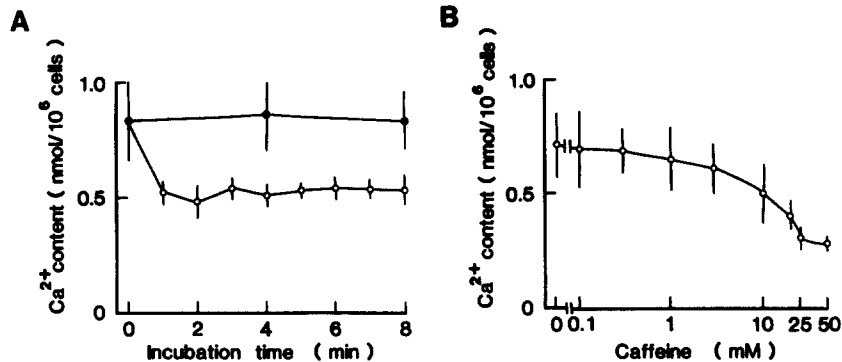


FIGURE 7. Time course (A) and dose-response curve (B) of caffeine-induced Ca^{2+} release from the skinned cells. (A) The skinned cells were equilibrated with $^{45}\text{Ca}^{2+}$ in 1×10^{-6} M free Ca^{2+} concentration for 20 min and incubated in the same solution with (O, $n = 5$) or without (●, $n = 5$) 25 mM caffeine for various periods. (B) The skinned cells were equilibrated with $^{45}\text{Ca}^{2+}$ in 1×10^{-6} M free Ca^{2+} concentration for 20 min and incubated in the same solution containing various concentrations of caffeine for 2 min. Each point represents the mean and standard deviation.

i.e., caffeine could release Ca^{2+} from the Ca^{2+} store in the skinned cells but norepinephrine could not. This agrees with the results obtained in skinned smooth muscle strips and indicates that the caffeine-releasable Ca^{2+} store was at least partially preserved after "optimal skinning."

The rate of $^{45}\text{Ca}^{2+}$ efflux from the skinned cells was apparently greater than that from the intact cells (Fig. 6). Although the loading and efflux conditions were different from each other, it may be that, in intact cells, stored Ca^{2+} exchanges rapidly with cytosolic Ca^{2+} , which in turn exchanges slowly with extracellular Ca^{2+} , as postulated previously by Scheid and Fay (1984).

$^{45}\text{Ca}^{2+}$ Distribution into the Caffeine-sensitive Ca^{2+} Store (SR)

In order to estimate the Ca^{2+} content of the caffeine-sensitive Ca^{2+} store, we used the following protocol. The skinned cells were equilibrated with $^{45}\text{Ca}^{2+}$ at various free Ca^{2+} concentrations for 20 min and then incubated in the same

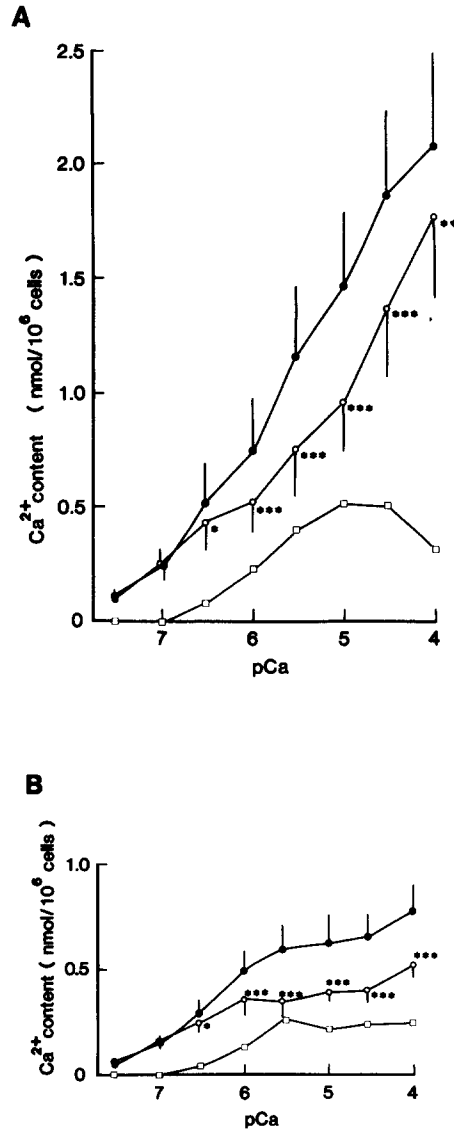


FIGURE 8. Caffeine-releasable Ca^{2+} uptake in skinned cells at various concentrations of free Ca^{2+} . (A) The skinned cells were equilibrated with $^{45}\text{Ca}^{2+}$ at various concentrations of free Ca^{2+} for 20 min and incubated in the same solution with (○, $n = 6-9$) or without (●, $n = 6-9$) 25 mM caffeine for 2 min. (B) The same experiment was done in the presence of 5 mM sodium azide. ○, 25 mM caffeine, $n = 6-7$; ●, control, $n = 6-7$. In both A and B, caffeine significantly reduced the Ca^{2+} content in the range 3×10^{-7} to 1×10^{-4} M of free Ca^{2+} concentration. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$. The caffeine-releasable Ca^{2+} uptake (the difference between the Ca^{2+} content with and without caffeine treatment) was calculated and plotted as a function of free Ca^{2+} concentration (□). Each point and bar represents the mean and standard deviation.

solution with or without caffeine for the desired period. Ca^{2+} content was measured as described in Materials and Methods. Fig. 7 shows the time course (A) and the dose-response curve (B) for the caffeine-induced Ca^{2+} depletion. The Ca^{2+} content was maximally decreased by 25 mM caffeine in 2 min. As demonstrated in Fig. 8A, a 2-min application of 25 mM caffeine could significantly reduce the Ca^{2+} content in the range 3×10^{-7} to 1×10^{-4} M free Ca^{2+} concentration. The amount of caffeine-releasable Ca^{2+} (the difference in the Ca^{2+} content between control and caffeine) increased with the increase in free Ca^{2+} concentration, reached a maximum at 1×10^{-5} M (513 pmol/ 10^6 cells),

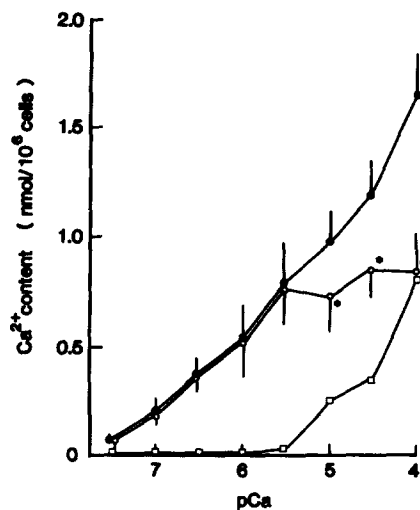


FIGURE 9. Sodium azide-sensitive Ca^{2+} uptake in skinned cells. The skinned cells were first incubated in 0.1 G solution with (○) or without (●) 5 mM sodium azide for 5 min and then equilibrated with $^{45}\text{Ca}^{2+}$ at various concentrations of free Ca^{2+} with (○) or without (●) 5 mM sodium azide. Ca^{2+} uptake was significantly decreased by 5 mM sodium azide in the range 1×10^{-5} to 1×10^{-4} M free Ca^{2+} concentration ($*P < 0.005$). The difference between the Ca^{2+} content in the absence and presence of 5 mM sodium azide was calculated and plotted as a function of free Ca^{2+} concentration (□). Each point represents the mean and standard deviation of four to eight determinations.

and decreased at 1×10^{-4} M. The first caffeine-induced Ca^{2+} release was detected at 3×10^{-7} M and was ~ 80 pmol/ 10^6 cells.

The same experiments in the presence of 5 mM sodium azide produced similar results except that the decrease in caffeine-releasable Ca^{2+} was not observed at 1×10^{-4} M (Fig. 8B).

$^{45}\text{Ca}^{2+}$ Distribution into the Sodium Azide-sensitive Ca^{2+} Store (Mitochondria)

We examined the effect of sodium azide, a known potent inhibitor of mitochondrial Ca^{2+} uptake (Hess and Ford, 1974), on $^{45}\text{Ca}^{2+}$ uptake in the skinned cells (Fig. 9). The skinned cells were pretreated with 0.1 G solution with or without

(control) 5 mM sodium azide for 5 min, incubated in the $^{45}\text{Ca}^{2+}$ -labeled solutions containing various concentrations of free Ca^{2+} with or without 5 mM sodium azide, and then washed with 0.1 G solution in a standard manner. Fig. 9 shows the relationship between the free Ca^{2+} concentration (pCa) and the Ca^{2+} content in the absence and presence of sodium azide. It is clear that the sodium azide-sensitive Ca^{2+} store could accumulate significant amounts of Ca^{2+} when the free Ca^{2+} concentration exceeded 3×10^{-6} M.

DISCUSSION

It is believed that, under appropriate conditions, saponin produces holes in the plasmalemma by forming micellar complexes with cholesterol, while not affecting the intracellular organelles such as the SR and mitochondria, which are thought to have a lower cholesterol content in skeletal and cardiac muscle (Comte et al., 1976; Inamitsu and Ohtsuki, 1984; Lau et al., 1979; Ohtsuki et al., 1978). Although in smooth muscle the cholesterol content of the membranes of the SR and the sarcolemma is not known, the method of saponin skinning has been used successfully in some studies that have illuminated several important aspects of Ca^{2+} mobilization during activation of smooth muscle (Endo et al., 1982; Itoh et al., 1981, 1983; Saida, 1982; Saida and van Breemen, 1984*a, b*). Recent studies showed that $^{45}\text{Ca}^{2+}$ distribution and transport could be studied in the saponin-skinned strips of rat caudal artery (Stout and Diecke, 1983) and rabbit mesenteric artery (Saida and van Breemen, 1984*b*). A few studies (Burgess et al., 1983; Hirata and Koga, 1982) showed that the saponin-skinned isolated cells of other types also could be used to determine the intracellular $^{45}\text{Ca}^{2+}$ distribution. In the present study, we have demonstrated that the saponin-skinned smooth muscle cell in primary culture is a suitable preparation for investigating $^{45}\text{Ca}^{2+}$ distribution and transport in pure smooth muscle cells.

The reasons for studying $^{45}\text{Ca}^{2+}$ exchange in the saponin-skinned smooth muscle cells in culture are twofold. (a) Saponin skinning of intact arterial preparations yields variable results because of the difficulty of saponin penetration into the extracellular space. For this reason, the arterial strip must be as thin as possible (Iino, 1981; Itoh et al., 1981; Saida and Nonomura, 1978). A cell culture monolayer is optimal in this respect because it constitutes the thinnest possible preparation. Saponin can thus interact with all cells simultaneously for the minimally effective time. This is important in "optimal skinning," where the main interest is to preserve the intracellular membranes while permeabilizing the plasmalemma. (b) The monolayer is also perfectly suited for measuring $^{45}\text{Ca}^{2+}$ fluxes because it minimizes $^{45}\text{Ca}^{2+}$ exchange in the extracellular space. The measurement of $^{45}\text{Ca}^{2+}$ uptake into the caffeine-sensitive Ca^{2+} store (SR) is more accurate than measurements from caffeine-induced contractions of skinned arterial preparations because other caffeine effects may alter the relationship between developed force and Ca^{2+} release (Wendt and Stephenson, 1983). In addition, it is clear that caffeine-induced contraction only reflects the Ca^{2+} content of the caffeine-sensitive Ca^{2+} store, presumably SR, which is specialized for Ca^{2+} release and provides no information on other intracellular organelles.

In addition, we note that the experimental model of the skinned cells and

muscle strips have several uncertain factors. (a) Significant amounts of cytoplasmic calcium-binding proteins such as calmodulin, which may regulate Ca^{2+} handling, may be lost after skinning. (b) Significant amounts of modifying factors such as cyclic nucleotides and cyclic nucleotide-dependent protein kinases may be lost. (c) Skinning may abolish the possible function of the plasma membrane as a Ca^{2+} store or a Ca^{2+} -binding site. (d) Saponin may alter the Ca^{2+} storage mechanisms of the intracellular organelles. (e) The local free Ca^{2+} concentrations around the Ca^{2+} stores are not known. Skinning may also abolish possible intracellular Ca^{2+} gradients. These points warrant caution in the interpretation of data obtained from skinned preparations.

Unfortunately, we are unable to measure contractility in these cells, so that the usual criterion for proper skinning, which is a maximal Ca^{2+} -induced contraction equal to or larger than either a maximal high- K^+ -induced contraction or a maximal norepinephrine-induced contraction, cannot be applied. However, we have used the following criteria for "optimal skinning" of the cultured smooth muscle cells. (a) All the cells treated with saponin under the optimal condition were stained internally with trypan blue. (b) $^{45}\text{Ca}^{2+}$ uptake was abruptly increased to a certain sustained level by increasing the saponin concentration to 40 $\mu\text{g}/\text{ml}$ (Fig. 2), which implies that the action of saponin is to make anatomical holes in the plasmalemma rather than to increase Ca^{2+} permeability of the plasmalemma gradually (Ohtsuki et al., 1978). (c) As shown in Fig. 5, >85% of $^{45}\text{Ca}^{2+}$ uptake into the skinned cells was dependent on MgATP supplied from the extracellular solution. (d) Norepinephrine could not cause $^{45}\text{Ca}^{2+}$ release from the saponin-treated cells (Fig. 6B).

Even though the cultured cell sheet is only one layer thick, some time was required to wash off the superficially bound $^{45}\text{Ca}^{2+}$. We estimated this somewhat arbitrarily from the time period necessary for a leveling off of the $^{45}\text{Ca}^{2+}$ washout curve (Fig. 1A) and determined it to be 7 min. Although this is shorter than the 16 min estimated from the study by Stout and Diecke (1983) and the 10 min estimated in an intact cell suspension by Scheid and Fay (1984), it may still be long enough for a significant amount of stored Ca^{2+} to be lost. In the present study, it was found that ~71% of both MgATP-dependent stored Ca^{2+} and caffeine-sensitive stored Ca^{2+} in the skinned cells preloaded with 1×10^{-6} M free Ca^{2+} was preserved after 7 min washing. In other words, the half-time for the loss of both Ca^{2+} fractions was 14 min, which is longer than 6 min for the loss of caffeine-sensitive Ca^{2+} from the arterial SR into the relaxing solution containing 0.5 mM EGTA (Saida and van Breemen, 1984b). Given the above reservations, we were able to make some important conclusions.

In the present study, we have demonstrated that the caffeine-sensitive Ca^{2+} store could accumulate statistically significant amounts of Ca^{2+} when the free Ca^{2+} concentration was equal to or higher than 3×10^{-7} M, and that the amount of Ca^{2+} accumulated increased with the increase in free Ca^{2+} concentration up to 1×10^{-5} M. It has been generally accepted that, in the skinned striated muscle, a higher concentration of caffeine, such as 25 mM, reversibly releases most of the calcium accumulated in the SR but not in mitochondria (Weber and Herz, 1968), and that after a single caffeine treatment, the SR becomes "practically

empty," so that reapplication of caffeine no longer causes any response at all (Endo, 1977). Thus, in the experiments on the skinned striated muscle, caffeine has been used as a tool to estimate the Ca^{2+} content in the SR (Endo, 1977). In the smooth muscle, there is considerable evidence that the intracellularly stored Ca^{2+} , which is responsible for the agonist-induced contraction in the Ca^{2+} -free solution, can be completely released by a high concentration of caffeine (Deth and Casteels, 1977; Endo et al., 1980; Itoh et al., 1983; Leijten and van Breemen, 1984; Saida and van Breemen, 1984a). This caffeine-sensitive Ca^{2+} store in smooth muscle has been considered to be the SR by analogy with the data from skeletal muscle. Thus, it is reasonable to assume that the Ca^{2+} content in the skinned cultured smooth muscle cells, which was reduced by caffeine treatment, reflects, at least partially, the amount of Ca^{2+} accumulated in the SR. Since this Ca^{2+} store increased up to 1×10^{-5} M free Ca^{2+} , we concluded that the caffeine-sensitive Ca^{2+} store, presumably the SR, not only releases Ca^{2+} in response to caffeine and norepinephrine but also acts as a Ca^{2+} sink by buffering excessive Ca^{2+} , which enters from the extracellular space.

The caffeine-sensitive Ca^{2+} store (SR) in the skinned cells could not accumulate a significant amount of Ca^{2+} in 1×10^{-7} M free Ca^{2+} . However, the intact vascular smooth muscle cells in the resting state, in which the cytosolic free Ca^{2+} concentration is believed to be $\sim 1 \times 10^{-7}$ M, contain a significant amount of caffeine-sensitive Ca^{2+} , which is estimated to be $\sim 185 \mu\text{mol/kg}$ cells in the rabbit aorta at 37°C (Leijten and van Breemen, 1984; see also Fig. 6A in the present study). This discrepancy can be explained as follows. We have recently observed (Yamamoto and van Breemen, 1985) that caffeine requires Ca^{2+} on the outside of the SR for its Ca^{2+} -releasing action. In other words, in the skinned cells, in which the free Ca^{2+} concentration is held by the EGTA buffer at 1×10^{-7} M, caffeine may not be able to release Ca^{2+} as effectively as in the intact cells, where the released Ca^{2+} increases the free Ca^{2+} concentration transiently, as indicated by the transient tension development. Therefore, the lack of a caffeine response at 1×10^{-7} M free Ca^{2+} in the skinned cells does not necessarily mean that the SR does not accumulate Ca^{2+} at this concentration. In addition, we have also observed that most of the caffeine-insensitive Ca^{2+} , as well as caffeine-sensitive Ca^{2+} , could be released by 1×10^{-4} M of inositol-1,4,5-trisphosphate (IP_3) (Yamamoto and van Breemen, 1985), which was presumed to release Ca^{2+} from the endoplasmic reticulum in other cell types (Berridge and Irvine, 1984), in the range 3×10^{-8} to 1×10^{-6} M free Ca^{2+} concentration. This IP_3 -releasable Ca^{2+} amounted to $78 \mu\text{mol/liter}$ cells at 1×10^{-7} M free Ca^{2+} and 25°C when the volume of 10^6 cells was assumed to be $2.94 \mu\text{l}$ (Yamamoto, H., and C. van Breemen, unpublished observation). This value is roughly half of that measured in intact cells, which may not be unreasonable considering that reliable absolute values for vascular smooth muscle cytoplasmic free Ca^{2+} have not yet been published. On the other hand, we cannot rule out at present the possibility that saponin skinning may affect the ability of the SR to store Ca^{2+} .

Several investigations have proposed a physiological role for Ca^{2+} -induced Ca^{2+} release (CICR) from the SR in the smooth muscle (Itoh et al., 1981; Saida, 1982; Saida and van Breemen, 1984a, b). Although the protocols in these studies

were different from each other, they all showed that the amount of Ca^{2+} in the SR decreased when the free Ca^{2+} concentration was equal to or higher than 3×10^{-6} M. In the present study, we could not demonstrate a significant decrease in the caffeine-releasable Ca^{2+} content in the range 3×10^{-6} to 1×10^{-5} M free Ca^{2+} concentration. We did, however, observe a decrease in the Ca^{2+} content of the SR at 1×10^{-4} M (Fig. 8A) in the absence but not in the presence of 5 mM sodium azide (Fig. 8B). No conclusions could be derived from these results, since we could not exclude the possibilities that the function of the SR had been impaired by the saponin treatment and the subsequent long incubation in the high Ca^{2+} concentration and that 5 mM sodium azide might inhibit either the caffeine effect or CICR. There are a couple of possible explanations for the discrepancy between the previous studies and the present study. First, we incubated the skinned cells in the labeled pCa solutions for a long time (20 min) to equilibrate them with $^{45}\text{Ca}^{2+}$, whereas in the above studies the skinned smooth muscles were incubated for a relatively short time (30 s to 5 min). Thus, if some kind of inactivation process of CICR had operated during the long incubation, the SR might be able to accumulate Ca^{2+} again after CICR. Fabiato (1983) reported that in skinned cardiac muscle an increase of free Ca^{2+} concentration to a given level induced Ca^{2+} release from the SR when it was applied rapidly, whereas it induced Ca^{2+} loading of the SR when it was applied slowly. Thus, it is possible that the skinned cells show the CICR phenomenon by a short application of the higher triggering Ca^{2+} concentration after loading in the lower Ca^{2+} concentration. Second, we note the possibility that the primary cultured smooth muscle cells, although they retain most of their original characteristics, may be modified to some extent after 10 d cultivation (Thyberg et al., 1983).

Another major finding in the present study is that the sodium azide-sensitive Ca^{2+} store, in contrast to the SR, could not accumulate a significant amount of Ca^{2+} at free Ca^{2+} concentrations of $<1 \times 10^{-5}$ M. It has been reported that sodium azide completely inhibits $^{45}\text{Ca}^{2+}$ uptake by the mitochondrial fraction but does not inhibit that by either the crude microsomal fraction or the plasma membrane-enriched fraction in smooth muscle (Hess and Ford, 1974; Grover et al., 1980). Therefore, we think it is reasonable to assume that the sodium azide-sensitive $^{45}\text{Ca}^{2+}$ uptake fraction reflects mainly mitochondrial $^{45}\text{Ca}^{2+}$ uptake. Thus, we conclude that mitochondria do not play a physiological role in controlling the cytoplasmic Ca^{2+} level. This is in good agreement with the results obtained from electron probe analysis by Somlyo et al. (1982), who reported a significant Ca^{2+} uptake into mitochondria at 1×10^{-5} M free Ca^{2+} concentration, but no detectable Ca^{2+} uptake with this method at 1×10^{-6} M for the skinned rabbit mesenteric vein.

The present study has also provided evidence that a certain fraction of MgATP-dependent $^{45}\text{Ca}^{2+}$ uptake remained in the skinned cells after application of caffeine and sodium azide. This result apparently implies the existence of an alternative Ca^{2+} store that does not overlap with the SR and mitochondria. However, the studies using electron probe analysis showed that neither nuclei nor lysosomes contain Ca^{2+} in excess of the cytoplasmic concentration (Somlyo et al., 1979; James-Kracke et al., 1980). In addition, our recent observation with

IP₃ described above does not support the alternative Ca²⁺ store hypothesis. Taking into consideration the Ca²⁺ requirement of the caffeine action, we conclude that most of the caffeine-insensitive and sodium azide-resistant Ca²⁺ fraction may reflect the remaining Ca²⁺ in the SR. This idea is supported by the observation that Ca²⁺ accumulation into this fraction is dependent on MgATP and is detected at Ca²⁺ concentrations as low as 3×10^{-8} M (Fig. 8), which suggests that it functions as a Ca²⁺-buffering system under physiological conditions.

Fig. 10 schematically illustrates the relative Ca²⁺ contents of the various intracellular Ca²⁺ compartments, which were partially characterized in the present study on saponin-skinned primary cultured aortic smooth muscle cells. We conclude that the caffeine-sensitive Ca²⁺ store (SR) and the caffeine-insensitive, sodium azide-resistant Ca²⁺ fraction ("unidentified" in Fig. 10) play a physiolog-

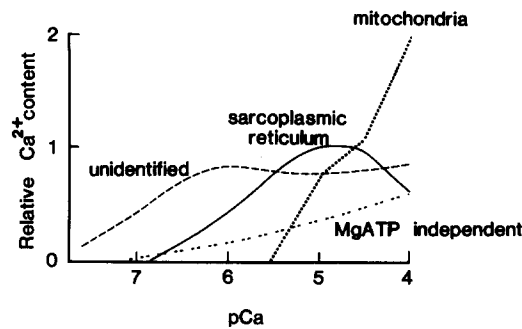


FIGURE 10. An illustration of the various Ca²⁺ fractions, which were partially characterized in this study on saponin-skinned primary cultured aortic smooth muscle cells. The relative Ca²⁺ contents in the various fractions were calculated from the data in Figs. 5, 8A, and 9 and plotted as a function of free Ca²⁺ concentration. "1" corresponds to the Ca²⁺ content in the caffeine-sensitive store (SR) at 1×10^{-5} M free Ca²⁺ concentration. As described in the text, the unidentified Ca²⁺ fraction is believed to be the SR.

ical role in controlling the cytoplasmic Ca²⁺ concentration, whereas the Ca²⁺ uptake by mitochondria appears too insensitive to be important under physiological conditions. However, our results do not exclude the possible contribution of plasmalemma as a functional Ca²⁺ store (Saida and van Breemen, 1984a, b).

This work was supported by National Institutes of Health grant HL29467.

Original version received 31 January 1985 and accepted version received 19 November 1985.

REFERENCES

- Berridge, M. J., and R. F. Irvine. 1984. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature*. 312:315-321.

- Bond, M., T. Kitazawa, A. P. Somlyo, and A. V. Somlyo. 1984. Release and recycling of calcium by the sarcoplasmic reticulum in guinea pig portal vein smooth muscle. *Journal of Physiology*. 355:677-695.
- Burgess, G. M., J. S. McKinney, A. Fabiato, B. A. Leslie, and J. W. Putney, Jr. 1983. Calcium pools in saponin-permeabilized guinea pig hepatocytes. *Journal of Biological Chemistry*. 258:15336-15345.
- Casteels, R., and G. Droogmans. 1975. Compartmental analysis of ion movements. In *Methods in Pharmacology*. Vol. 3: Smooth Muscle. E. E. Daniel and D. M. Paton, editors. Plenum Press, New York. 663-671.
- Chamley, J. H., G. R. Campbell, J. D. McConnell, and U. Groschel-Stewart. 1977. Comparison of vascular smooth muscle cells from adult human, monkey and rabbit in primary culture and in subculture. *Cell and Tissue Research*. 177:503-522.
- Comte, J., B. Maisterrena, and D. C. Gautheron. 1976. Lipid composition and protein profiles of outer and inner membranes from pig heart mitochondria. Comparison with microsomes. *Biochimica et Biophysica Acta*. 419:271-284.
- Deth, R., and R. Casteels. 1977. A study of releasable Ca fractions in smooth muscle cells of the rabbit aorta. *Journal of General Physiology*. 69:401-416.
- Endo, M. 1977. Calcium release from the sarcoplasmic reticulum. *Physiological Reviews*. 57:71-108.
- Endo, M., T. Kitazawa, and S. Yagi. 1980. Different features of responses of the sarcoplasmic reticulum in cardiac and smooth muscles. In *Muscle Contraction: Its Regulatory Mechanism*. S. Ebashi, K. Maruyama, and M. Endo, editors. Japan Science Society Press, Tokyo. 447-463.
- Endo, M., S. Yagi, and M. Iino. 1982. Tension-pCa relation and sarcoplasmic reticulum responses in chemically skinned smooth muscle fibers. *Federation Proceedings*. 41:2245-2250.
- Fabiato, A. 1983. Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. *American Journal of Physiology*. 245:C1-C14.
- Grover, A. K., C. Y. Kwan, R. E. Garfield, J. McLean, J. E. T. Fox, and E. E. Daniel. 1980. Fractionation and Ca uptake studies on membrane of rabbit longitudinal and circular intestinal smooth muscle. *Canadian Journal of Physiology and Pharmacology*. 58:1102-1113.
- Hess, H. L., and G. D. Ford. 1974. Calcium accumulation by subcellular fractions from vascular smooth muscle. *Journal of Molecular and Cellular Cardiology*. 6:275-282.
- Hirata, M., and T. Koga. 1982. ATP-dependent Ca²⁺ accumulation in intracellular membranes of guinea pig macrophages after saponin treatment. *Biochemical and Biophysical Research Communications*. 104:1544-1549.
- Iino, M. 1981. Tension responses of chemically skinned fibre bundles of the guinea-pig taenia caeci under varied ionic environments. *Journal of Physiology*. 320:449-467.
- Inamitsu, T., and I. Ohtsuki. 1984. Characterization of ATP-dependent Ca²⁺ uptake by canine brain microsomes with saponin. *European Journal of Biochemistry*. 145:115-121.
- Itoh, T., H. Kuriyama, and H. Suzuki. 1981. Excitation contraction coupling in smooth muscle cells of guinea-pig mesenteric artery. *Journal of Physiology*. 321:513-535.
- Itoh, T., H. Kuriyama, and H. Suzuki. 1983. Differences and similarities in the noradrenaline- and caffeine-induced mechanical responses in the rabbit mesenteric artery. *Journal of Physiology*. 337:609-629.
- James-Kracke, M. R., B. F. Sloane, H. Shuman, R. Karp, and A. P. Somlyo. 1980. Electron probe analysis of cultured vascular smooth muscle. *Journal of Cellular Physiology*. 103:313-322.

- Lau, Y. H., A. H. Caswell, J.-P. Brunschwig, R. J. Baerwald, and M. Garcia. 1979. Lipid analysis and freeze-fracture studies on isolated transverse tubules and sarcoplasmic reticulum subfractions of skeletal muscle. *Journal of Biological Chemistry*. 254:540-546.
- Leijten, P. A. A., and C. van Breemen. 1984. The effects of caffeine on the noradrenaline sensitive calcium store in rabbit aorta. *Journal of Physiology*. 357:327-339.
- Martell, A. E., and G. Schwarzenbach. 1956. Adenosinphosphate und Triphosphate als Komplexbildner für Calcium und Magnesium. *Helvetica Chimica Acta*. 39:653-666.
- Mueller, E., and C. van Breemen. 1979. Roles of intracellular Ca^{2+} sequestration in β -adrenergic relaxation of a smooth muscle. *Nature*. 281:682-683.
- Ohtsuki, I., R. M. Marzi, G. E. Palade, and J. D. Jamieson. 1978. Entry of macromolecular tracers into cells fixed with low concentrations of aldehydes. *Biologie Cellulaire*. 31:119-125.
- Raemaekers, L., F. Wuytack, and R. Casteels. 1977. Comparison of the Ca accumulation by different subcellular fractions of smooth muscle. In *Excitation Contraction Coupling in Smooth Muscle*. R. Casteels, T. Godfraind, and J. C. Ruegg, editors. Elsevier/North-Holland Biomedical Press, Amsterdam. 233-238.
- Saida, K. 1982. Intracellular Ca^{2+} release in skinned smooth muscle. *Journal of General Physiology*. 80:191-202.
- Saida, K., and Y. Nonomura. 1978. Characteristics of Ca^{2+} - and Mg^{2+} -induced tension development in chemically skinned smooth muscle fibers. *Journal of General Physiology*. 72:1-14.
- Saida, K., and C. van Breemen. 1984a. Characteristics of the norepinephrine-sensitive Ca store in vascular smooth muscle. *Blood Vessels*. 21:43-52.
- Saida, K., and C. van Breemen. 1984b. Cyclic AMP modulation of adrenoceptor-mediated arterial smooth muscle contraction. *Journal of General Physiology*. 84:307-318.
- Scheid, C. R., and F. S. Fay. 1984. Transmembrane ^{45}Ca fluxes in isolated smooth muscle cells: basal Ca^{2+} fluxes. *American Journal of Physiology*. 246:C422-C430.
- Somlyo, A. V., and A. P. Somlyo. 1971. Strontium accumulation by sarcoplasmic reticulum and mitochondria in vascular smooth muscle. *Science*. 174:955-958.
- Somlyo, A. P., A. V. Somlyo, C. E. Devine, P. D. Peters, and T. A. Hall. 1974. Electron microscopy and electron probe analysis of a mitochondrial cation accumulation in smooth muscle. *Journal of Cell Biology*. 61:723-742.
- Somlyo, A. P., A. V. Somlyo, and H. Shuman. 1979. Electron probe analysis of vascular smooth muscle composition of mitochondria, and nuclei and cytoplasm. *Journal of Cell Biology*. 81:316-335.
- Somlyo, A. P., A. V. Somlyo, H. Shuman, and M. Endo. 1982. Calcium and monovalent ions in smooth muscle. *Federation Proceedings*. 41:2883-2890.
- Stout, M. A., and F. P. J. Diecke. 1983. ^{45}Ca distribution and transport in saponin skinned vascular smooth muscle. *Journal of Pharmacological and Experimental Therapeutics*. 225:102-111.
- Thyberg, J., L. Palmberg, J. Nilsson, T. Ksiazek, and M. Sjolund. 1983. Phenotype modulation in primary cultures of arterial and smooth muscle cells. *Differentiation*. 25:156-167.
- Vallieres, J., A. Scarpa, and A. P. Somlyo. 1975. Subcellular fractions of smooth muscle. Isolation, substrate utilization and Ca^{2+} transport by main pulmonary artery and mesenteric vein mitochondria. *Archives of Biochemistry and Biophysics*. 170:659-669.
- Weber, A., and R. Herz. 1968. The relationship between caffeine contracture of intact muscle and the effect of caffeine on reticulum. *Journal of General Physiology*. 52:750-759.
- Wendt, I. R., and D. G. Stephenson. 1983. Effects of caffeine on Ca-activated force production in skinned cardiac and skeletal muscle fibres of the rat. *Pflügers Archiv European Journal of Physiology*. 398:210-216.

- Yamamoto, H., H. Kanaide, and M. Nakamura. 1983. Metabolism of glycosaminoglycans of cultured rat aortic smooth muscle cells altered during subculture. *British Journal of Experimental Pathology*. 64:156-165.
- Yamamoto, H., and C. van Breemen. 1985. Inositol-1,4,5-trisphosphate releases calcium from skinned cultured smooth muscle cells. *Biochemical and Biophysical Research Communications*. 130:270-274.