Physiological and Structural Properties of Saponin-skinned Single Smooth Muscle Cells

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ABSTRACT   The study of the fundamental events underlying the generation and regulation of force in smooth muscle would be greatly facilitated if the permeability of the cell membrane were increased so that the intracellular environment of the contractile apparatus could be manipulated experimentally. To initiate such an analysis, we developed a saponin permeabilization procedure that was used to "skin" isolated smooth muscle cells from the stomach of the toad, *Bufo marinus*. Suspensions of single cells isolated enzymatically were resuspended in high-K^+ rigor solution (0 ATP, 5 mM EGTA) and exposed for 5 min to 25 μg/ml saponin. Virtually all the cells in a suspension were made permeable by this procedure and shortened to less than one-third their initial length when ATP and Ca^{++} were added; they re-extended when free Ca^{++} was removed. Analysis of the protein content of the skinned cells revealed that, although their total protein was reduced by ~30%, they retained most of their myosin and actin. Skinning was accompanied by a rearrangement of actin and myosin filaments within the cells such that a fine fibrillar structure became visible under the light microscope and a tight clustering of acting filaments around myosin filaments was revealed by the electron microscope. Face-on views of saponin-treated cell membranes revealed the presence of 70–80-Å-wide pits or holes. The shortening rate of skinned cells was sensitive to [Ca^{++}] between pCa 7 and pCa 5 and was half-maximal at ~pCa 6.2. Shortening was also dependent on [ATP] but could be increased at low [ATP] by pretreatment with adenosine-5'-O-(3-thiotriphosphate) (ATPγS), which suggests that myosin phosphorylation was more sensitive to low substrate concentrations than was cross-bridge cycling. To determine whether a significant limitation to free diffusion existed in the skinned cells, a computer model of the cell and the unstirred layer surrounding it was developed. Simulations revealed that the membrane, even in skinned cells, could, for short time intervals, significantly inhibit the movement of substances into and out of cells.

INTRODUCTION

In spite of much research effort, a number of fundamental questions about smooth muscle structure and function remain unanswered. It is not well understood, for example, how the contractile elements are arranged in resting muscle,

nor is it completely known how these elements are regulated to bring about contraction or how their organization changes during cell shortening. In recent years, the development of two techniques and their application to the study of smooth muscle have allowed progress to be made in obtaining answers to these questions. It has become possible to increase the membrane permeability of cells in tissue strips so that the concentration of ions in the cell cytoplasm can be controlled and specific molecules can be introduced to alter steps in the pathways involved in the regulation of contraction. It has also become possible to isolate intact cells from whole smooth muscle tissue so that the properties of single cells can be studied in a precisely controlled environment. While the application of these techniques separately has contributed greatly to our understanding of smooth muscle, they have not, with a few exceptions (Small 1974, 1977; Cande et al., 1983; Obara and Yamada, 1984; Caffrey and Magid, 1984), been applied together. There would be several advantages to doing so. Since isolated cells in suspension are all equally exposed to solution, the concentration gradients that exist in whole tissue when solution changes are made would be eliminated. Thus, isolated cells would be more uniformly skinned and, during activation, substrate and product gradients could only exist across cellular dimensions. It should also be possible to introduce molecules into cells to specifically mark or alter various proteins in their cytoskeleton and contractile apparatus. Fluorescent probes could be used, for example, to optically study changes in the organization of contractile proteins during cell shortening in an environment free of contaminating light from surrounding tissue.

Because of these advantages, we sought to develop a skinned single cell preparation that could serve as a reliable, functional model of the intact smooth muscle cell. Previous investigators have examined some of the structural (Small, 1974, 1977), biochemical (Cande et al., 1983), and functional (Obara and Yamada, 1984; Caffrey and Magid, 1984) characteristics of skinned isolated smooth muscle cells. This work, however, was done with a variety of skinning techniques on different preparations, so that it is difficult to integrate the results to obtain a more complete description of the skinned isolated cell. Furthermore, to date, little information is available about the properties of intact single cells in these preparations, so the effects of the skinning process cannot be easily evaluated and, as a consequence, questions remain as to how accurately skinned cells model the intact cell. We were therefore motivated to examine a variety of structural and functional properties of skinned isolated cells from a single system and to compare these properties with those of well-characterized, intact isolated cells from the same system.

The work we present here indicates that the technique we describe can be used to obtain large numbers of uniformly skinned cells that shorten in response to physiological concentrations of Ca^{++} and ATP. Our results also provide evidence supporting the role of myosin phosphorylation in the regulation of smooth muscle contraction and demonstrate the validity of this preparation as a system for the study of the structural organization of smooth muscle and the intracellular processes involved in the regulation of its contractile state. Preliminary accounts of this work have appeared previously (Kargacin and Fay, 1985, 1986).

MATERIALS AND METHODS

Enzymatic Dispersion and Skinning

The procedure for isolating single smooth muscle cells from the stomach of the toad, *Bufo marinus*, has been described by Fay et al. (1982). After isolation, soybean trypsin inhibitor (STI; 5 μg/ml) was added to suspensions of single cells to reduce tryptic activity. Cells were permeabilized as follows: 10-ml aliquots of cells in amphibian physiological saline (APS) containing 2 mM EGTA were centrifuged for 5 min at 250 rpm ($\sim$10–15 g). The APS was aspirated off and replaced with 9 ml of either relaxing or rigor solution (the EGTA was added to the APS to prevent contraction of cells when these solutions were first added). After 5 min of equilibration, 1 ml of rigor or relaxing solution containing saponin was added (final saponin concentration, 5–50 μg/ml) and the cells were immediately centrifuged again at 250 rpm for 5 min. After the saponin-containing supernatant was decanted, the cells were washed in rigor or relaxing solution, centrifuged again, and resuspended in an experimental solution. Suspensions of skinned cells and the solutions used during skinning were kept on ice before use. Centrifugation and experiments were done at room temperature ($\sim$24°C).

Solutions

The rigor solution contained 150 mM K$^+$, 5 mM EGTA, 3 mM Mg^{++}, 6 mM Cl$^-$, 10 mM 3-[N-morpholino]propanesulfonic acid (MOPS), and 135 mM propionate (pH 6.5, ionic strength 0.16). Relaxing solutions contained 1–5 mM ATP, 5 mM EGTA, 10 mM MOPS, 3 mM free Mg^{++}, and 6 mM Cl$^-$, with K-propionate adjusted to maintain ionic strength at $\sim$0.16 and pH 6.5. Contracting solutions had the same composition as relaxing solutions but contained Ca^{++} (added in the form of Ca-propionate). After adding the appropriate amounts of K$^+$ (added as KOH), EGTA, Mg^{++} (part from MgCl$_2$ and part from MgATP), MOPS, MgATP, and Ca-propionate, solutions were titrated to pH 6.5 with concentrated propionic acid. The total [Ca^{++}] and [Mg^{++}] required to obtain the desired free concentrations of these ions were determined with a computer program that used the constants published by Fabiato (1981). In some cases, free Ca^{++} was also determined by potentiometric titration of EGTA with CaCl$_2$ and CdCl$_2$, as described by Moisescu and Thieleczek (1979) with K'_{app} = 5 × 10^5/M. The [Ca^{++}] determined by the two methods agreed to within 2%. Solutions described as having 0.1 mM free Ca^{++} (pCa 4) contained 5 mM EGTA and 4.8 mM Ca-propionate.

To inhibit proteolysis, phenylmethylsulfonyl fluoride (PMSF; 75 mg/liter) and STI (5 μg/ml) were routinely added to solutions. All solutions, unless otherwise stated, contained dithiothreithol (DTT; 5 mM) to prevent −SH oxidation. When used, creatine phosphokinase (CPK; 40 U/ml), phosphocreatine (PC; 4 mM), and calmodulin were added without ionic substitution to solutions. Saponin, CPK (type 1), PC, STI, MgATP, Ca-propionate, and calmodulin were obtained from Sigma Chemical Co., St. Louis, MO, trypan blue from MCB Manufacturing Chemists, Inc., Gibbstown, NJ, adenosine-5'-O-(3-thiotriphosphate) (ATPγS) from Boehringer Mannheim Biochemicals Corp., Indianapolis, IN, and collagenase (CLS II) from Cooper Biomedical Co., Freehold, NJ. Purified calmodulin was a gift of Dr. Michael Walsh, University of Calgary, Alberta, Canada.

Protein Determination

Total cell protein was determined by the Lowry method (Lowry et al., 1951). To eliminate the possibility that DTT interfered with the Lowry assay (Vallejo and Lagunas, 1970), it was either not added to the skinning solutions used in these experiments, or its concentration was only 1 mM. Sodium dodecyl sulfate (SDS) polyacrylamide slab gels (10 or 7% acrylamide) were run according to Laemmli (1970).

To obtain the absorbance map shown in Fig. 3, a gel stained with Coomassie blue was placed over a white-light source and a digitized image of it (transmitted light image) was obtained with a Newvicon camera (model 68, Dage-MTI, Inc., Michigan City, IN). The gel was then removed and an image of the light source alone (total light image) was obtained. An image collected without input to the camera was subtracted pixel by pixel from each of these images to correct for spatial variation in the dark current of the camera. The corrected total light image was then divided pixel by pixel by the corrected transmitted light image and the log of the resultant image was computed to produce an absorbance image of the gel. This image was then plotted as a contour image on a solid view system (model 3400, Lexidata Corp., Billerica, MA).

Assessment of Contraction

Two methods were employed to measure the shortening of skinned cells. The first assessed shortening in populations of cells. Suspensions were rapidly fixed with freshly distilled acrolein (final concentration, 1%) at various times after a putative modulator of contractility was added. Microscopic images of fields containing 10–30 cells were digitized as described by Fay et al. (1986) and cell lengths were determined with a computer algorithm developed to measure the long axis of the cells. This was done for each cell image in a field, and the average length of the cells in three to five fields treated in the same way was computed. The results were plotted either as the average length of cells divided by the average length of cells in rigor solution [$L(ave)/L_i(ave)$] or as percent shortening. In the latter case, the average lengths of cells in fields fixed in rigor solution and after 15 min in 0.1 mM free Ca^{++} plus ATP were determined and used as standards for 0 and 100% shortening, respectively.

In the second method, the length of individual cells was measured at fixed time intervals during shortening from images obtained with a Zeiss inverted microscope and the digital imaging system described by Fay (1986). For observation with the microscope, cells were pipetted into a chamber (~80 μl vol) made from a piece of Parafilm sandwiched between two glass coverslips (Fig. 1). When the input and output ports were clamped to the base, the chamber could be continuously superfused with solutions (62 μl/min) with an infusion/withdrawal pump (Harvard Apparatus Co., Inc., South Natick, MA). At the flow rate used, the cells usually did not wash away with the superfusate if they were allowed to settle to the bottom coverslip before the pump was started. They were nevertheless able to shorten when solution containing Ca^{++} and ATP was injected into the input line of the infusion system and could relax again when Ca^{++} was removed. That this could occur suggests that the cells, if attached to the coverslip, were attached relatively strongly at only one point. Most often, this point appeared to be near the center of the cell since both ends shortened toward the middle.

RESULTS

Permeabilization of Cells

Small (1977) and Cande et al. (1983) have reported that, in the presence of ATP (especially at pH > 6.5), myosin is lost from permeable, isolated smooth muscle cells. We therefore conducted saponin skinning experiments under both relaxing (with ATP) and rigor (without ATP) conditions at pH 6.5. In both cases, fully extended cells able to shorten in response to Ca^{++} (cells skinned in relaxing solution) or Ca^{++} and ATP (cells skinned in rigor solution; see Fig. 8) were obtained. Cells skinned and kept in relaxing solution, however, tended to shorten

spontaneously within 1–2 h of skinning. This may indicate that some proteolysis of the regulatory light chains of myosin or the regulatory subunit of myosin light-chain kinase occurred over time. The shortening may thus be related to the slow shortening observed when cells skinned in rigor solution were exposed to ATP while still in Ca^{++}-free solution (discussed later). In any case, to avoid this problem, cells were skinned and kept in rigor solution and were used as quickly as possible after skinning.
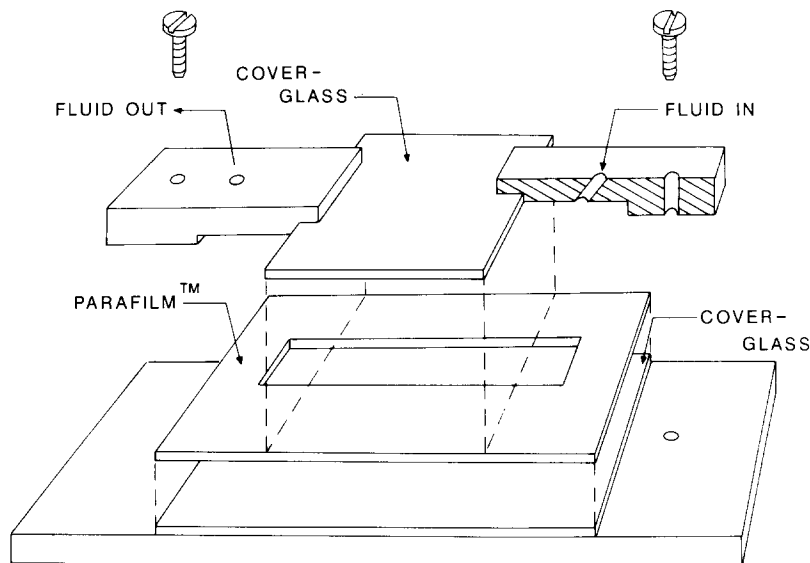
FIGURE 1.   Chamber used for perfusion of skinned single cells. The walls of the chamber were made by the Parafilm layer; the top and bottom were made by the upper and lower coverglasses. The three parts of the chamber were sealed together with petroleum jelly. The input (shown in partial cross section) and output ports were sealed to the upper coverglass and Parafilm with petroleum jelly before they were screwed to the base. When assembled, polyethylene tubing inserted into the ports carried fluid into and out of the chamber. A rectangular opening milled into the base provided the oil immersion objective of an inverted microscope access to the lower coverglass. An oil immersion condenser could be brought down to the upper coverglass to obtain Nomarski or phase-contrast images.

To find the minimum concentration of saponin necessary to permeabilize most of the cells in a population reliably, suspensions of skinned, washed cells were exposed to trypan blue and uptake of the dye was monitored to determine the proportion of permeable cells in a population. Trypan blue (1.2 kD mol wt) is not able to enter intact cells because of its large size, but it is readily taken up by cells with disrupted surface membranes. To eliminate the error that would have been introduced if some dye were taken up by intact smooth muscle cells, cells were counted immediately after they were exposed to the dye. The nuclei of >80% of the cells in suspensions were stained after the cells had been exposed

to 20 μg/ml of saponin for 5 min (Fig. 2). Fewer cells were stained when lower detergent concentrations were used, but higher concentrations did not further increase the percentage of stained cells. These results indicated that a minimum saponin concentration of 25 μg/ml could be used routinely to skin suspensions of cells. This was supported by the finding that after such treatment, virtually all cells could be induced to shorten by Ca^{++} and ATP but not by Ca^{++} alone.

Protein Content of Saponin-treated Cells

To further evaluate the skinning process, the protein content of skinned cells was examined and compared with that of intact cells in the following ways.
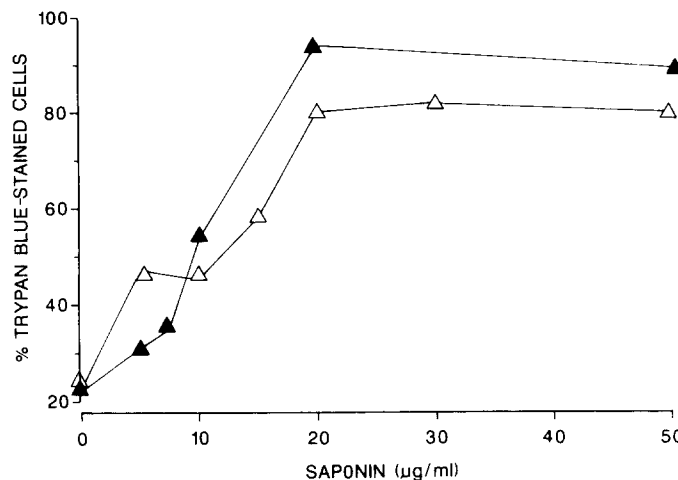

FIGURE 2. Assessment of the extent of saponin skinning. The results of two experiments are shown in which the percentage of cells that were skinned in populations treated with different concentrations of saponin was determined. Within 5 min of the addition of trypan blue, three samples, each containing ~40 cells, were taken from each population and the number of stained and unstained cells per sample was counted to compute the percentages shown. The points at 0 μg/ml saponin were determined from cells that were unskinned but were in rigor solution. Approximately 20% of the control cells kept in APS but centrifuged and resuspended were also stained with the dye.

Aliquots of cells (10 ml) from the same suspension were pipetted into test tubes and either left unskinned in APS or skinned and resuspended in 10 ml of rigor solution. The total protein content of the cells from each aliquot was determined by the Lowry method. To eliminate the possibility that apparent protein loss occurred in the skinned cell suspensions because cells were damaged during centrifugation and resuspension, unskinned cells were spun down and resuspended in APS an equal number of times. To determine protein per cell, the number of cells per unit volume in the skinned and unskinned aliquots was determined with a hemacytometer. When total cell protein was computed, it was found that ~30% (32 ± 3% SD; three experiments) was lost as a result of saponin

treatment. This could have resulted from an overall loss of protein or, as seems more likely, from a more specific loss, through the permeable membrane, of soluble protein and the lower-molecular-weight proteins not attached to the cytoplasmic matrix. To see whether specific proteins were lost, SDS polyacryl-amide gels were run on skinned and unskinned cells. Fig. 3 shows a computer-generated absorbance map (see Methods) of a Coomassie blue–stained 10% SDS gel run to separate the proteins of unskinned cells (lane 1) and those skinned with increasing concentrations of saponin (lanes 3–8). As can be seen, the higher-molecular-weight protein bands were present in all lanes. In particular, filamin, actin, myosin, and the myosin light chains all appeared to be retained by the skinned cells. Only one major protein, as yet unidentified, having a molecular weight of ~26 kD, is absent from the last two lanes (30 and 50 μg/ml saponin).
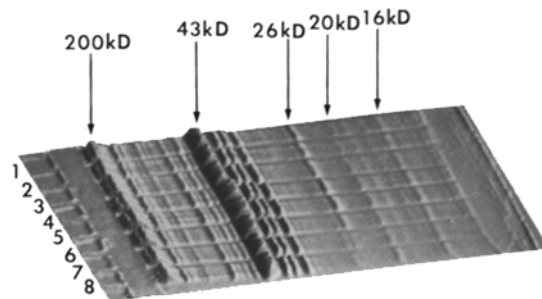

FIGURE 3.   Effect of saponin concentration on the protein content of skinned cells. The absorbance map was generated from a 10% SDS acrylamide gel as described in the Methods. The gel lane at the top of the map contained protein from intact single cells. Lane 2 is from cells in rigor solution but unskinned and the lower lanes show protein from cells treated for 5 min with different concentrations of saponin (lanes 3–8: 5, 10, 15, 20, 30, and 50 μg/ml). The lanes were loaded so that each contained an equal number of cells (determined with a hemacy:ometer). The molecular weights of the bands estimated from molecular-weight standards are indicated at the top of the image. The proteins at 200 and 43 kD are myosin heavy chain and actin, respectively. The highest-molecular-weight protein is probably filamin; the bands at 16 and 20 kD are probably the myosin light chains.

The presence of this protein in cell populations exposed to lower concentrations of saponin (5–15 μg/ml) may have been due to the presence of higher numbers of intact cells in those populations or may reflect less loss from the permeable cells. The protein may be related to a 23-kD protein from chicken gizzard isolated and purified by J. Lees-Miller and L. D. Smillie (personal communication) that is lost during the skinning of gizzard and other smooth muscle (Kerrick, W. G. L., personal communication). The function of this protein is not yet known.

Comparison of the absorbance of actin and myosin bands stained with Coomassie blue on gels from unskinned cells, skinned cells in rigor or relaxing solution, and rigor skinned cells after the addition of ATP failed to reveal any significant change in actin or myosin content or in the ratio of actin to myosin as a result of skinning or of the addition of ATP and Ca^{++} to skinned cells (four

experiments). This result may have been due, in part, to differences in the staining and loading on the different gels.

Structure of Skinned Cells

The results presented above indicate that skinned, isolated cells retain their major structural and contractile proteins. Saponin treatment did alter the appearance of cells, however, which suggests that some structural reorganization may have occurred within them. The most noticeable change that accompanied permeabilization was an increase in cell diameter. As skinning occurred, the cells
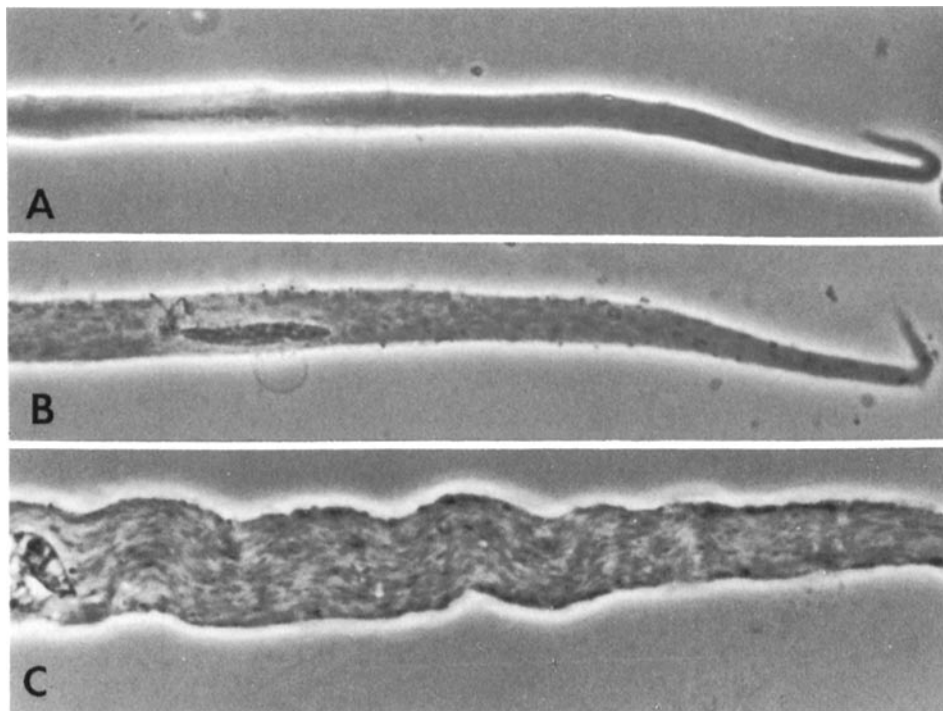

FIGURE 4. (A) Phase contrast image of an intact isolated smooth muscle cell. (B) Phase image of the same cell as in A after saponin treatment. (C) Phase image showing the fine fibrillar structure that became visible in cells after saponin treatment. (A and B) × 1,000. (C) × 2,000.

also straightened and their refractive index decreased. The latter change was made most obvious by the nucleus, which could not be readily seen in intact cells but was clearly visible in skinned cells (Fig. 4, A and B). These changes in gross morphology were accompanied by the appearance of a fine fibrillar structure within the cells that was visible when they were viewed with phase contrast or Normarski optics (Fig. 4C).

The organization of resting intact, isolated cells, viewed with the electron microscope, resembled that observed in other vertebrate smooth muscle (see Cooke and Fay, 1972; Fay and Cooke, 1973; Gabella, 1981). In cross and longitudinal sections of the intact cells (shown in Figs. 5A, 6A, and 7A), bundles
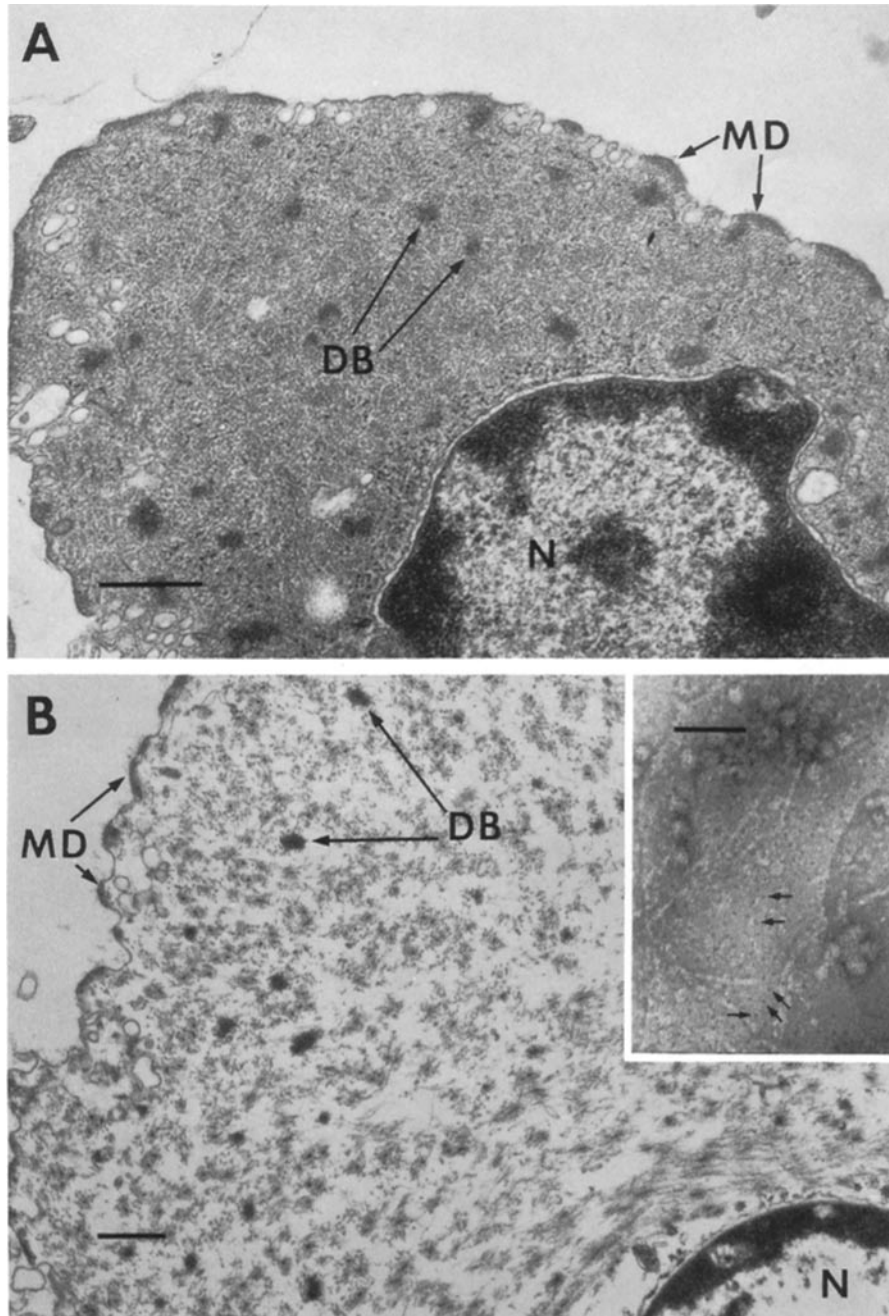
FIGURE 5. (A) Cross section of an intact isolated smooth muscle cell. (B) Cross section of a saponin-skinned isolated cell. The structures indicated are: dense bodies (DB); plasma membrane densities (MD); nucleus (N). (*Inset*) Face-on patch of membrane showing saponin-induced holes (small arrows). (A) × 28,000; bar, 0.5 μm; (B) × 18,000; bar, 0.5 μm; (*inset*) bar, 150 nm.
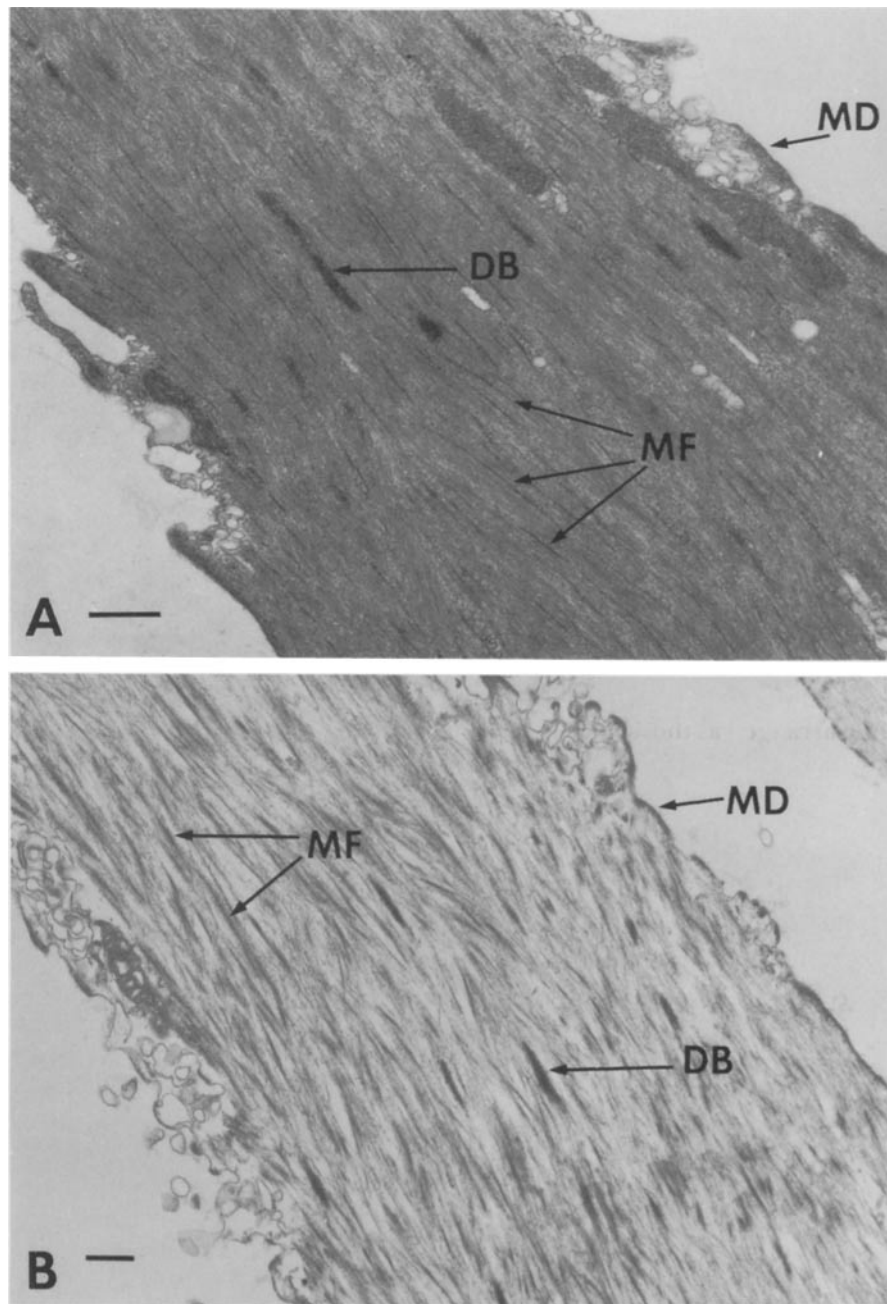
FIGURE 6. Longitudinal sections of an intact (A) and saponin-skinned (B) smooth muscle cell. Indicated structures are: myosin filaments (MF); dense bodies (DB); membrane densities (MD). (A) × 21,000; bar, 0.5 μm; (B) × 13,000; bar, 0.5 μm.

containing hexagonally packed thin (actin) filaments were seen throughout the cytoplasm. Interspaced between these bundles were numerous thick (myosin) filaments and dense bodies. Plasma membrane densities were seen along the cell surface. As is typical of resting smooth muscle, the thick and thin filaments in intact cells were not closely associated with one another except near the periphery of the actin bundles. In contrast, saponin-treated cells fixed in rigor solution were characterized by a more definite actin-myosin association. Rosettes of thin filaments surrounded each thick filament, giving the appearance of a less uniform distribution of actin throughout the cytoplasm (Figs. 6B and 7B). Dense bodies and membrane densities (Figs. 5B and 6B) were still present in the skinned cells. When viewed in cross sections, the plasma membrane appeared to be largely intact. When patches of isolated membrane were seen face on in homogenates of skinned cells, however, numerous 70–80-Å pits or holes (Fig. 5B, inset) were visible in negatively stained preparations. Such structures were similar to those observed in other saponin-treated membranes (Dourmashkin et al., 1962; Bangham and Horne, 1962; Glavert et al., 1962; Seeman et al., 1973). The overall density of material in the skinned cells was less than that in intact cells.

The formation of tight clusters of thick and thin filaments in skinned cells may have been induced by the rigor skinning conditions and was presumably manifest at the light-microscopic level by the appearance of the fine fibrils described above. In cells skinned in relaxing solution, this fibrillar structure was less obvious and the clusters of thick and thin filaments in electron micrographs were not as tightly arranged as those in cells fixed in rigor solution. Actin filaments appeared to be more uniformly distributed throughout the cytoplasm (Fig. 7C). This presumably reflects a weaker link between actin and myosin in the relaxed cell. Cross sections (not shown) of rigor skinned cells pretreated with ATPγS (see below) and Ca^{++} before fixation had a structure more like that of the relaxed cells than the rigor cells, which suggests that some redistribution of the filaments also occurred during myosin phosphorylation.

Shortening of Skinned Cells

Skinned cells shortened in the presence of ATP and Ca^{++} to less than one-third of initial length (L_i) and were able to relax again when Ca^{++} was lowered. Fig. 8 shows two contraction/relaxation cycles of the same cell. As can be seen, the cell shortened to the same extent ($\sim 1/3\ L_i$) and relaxed almost completely to L_i each time. A similar extent of shortening has been observed after activation of intact cells (Fay and Delise, 1973).

As shortening occurs in intact isolated cells, numerous evaginations (blebs) appear on the cell surface. The surface membrane surrounding the blebs appears to be free of plasma membrane densities and the material within them does not contain contractile filaments (Fay and Delise, 1973; Fig. 9A). Skinned cells underwent a similar surface distortion upon shortening, and electron micrographs of skinned, fully contracted cells revealed an ultrastructure similar to that of intact, contracted cells (Fig. 9B). The demarcation between the material within the blebs and the more dense material containing contractile filaments
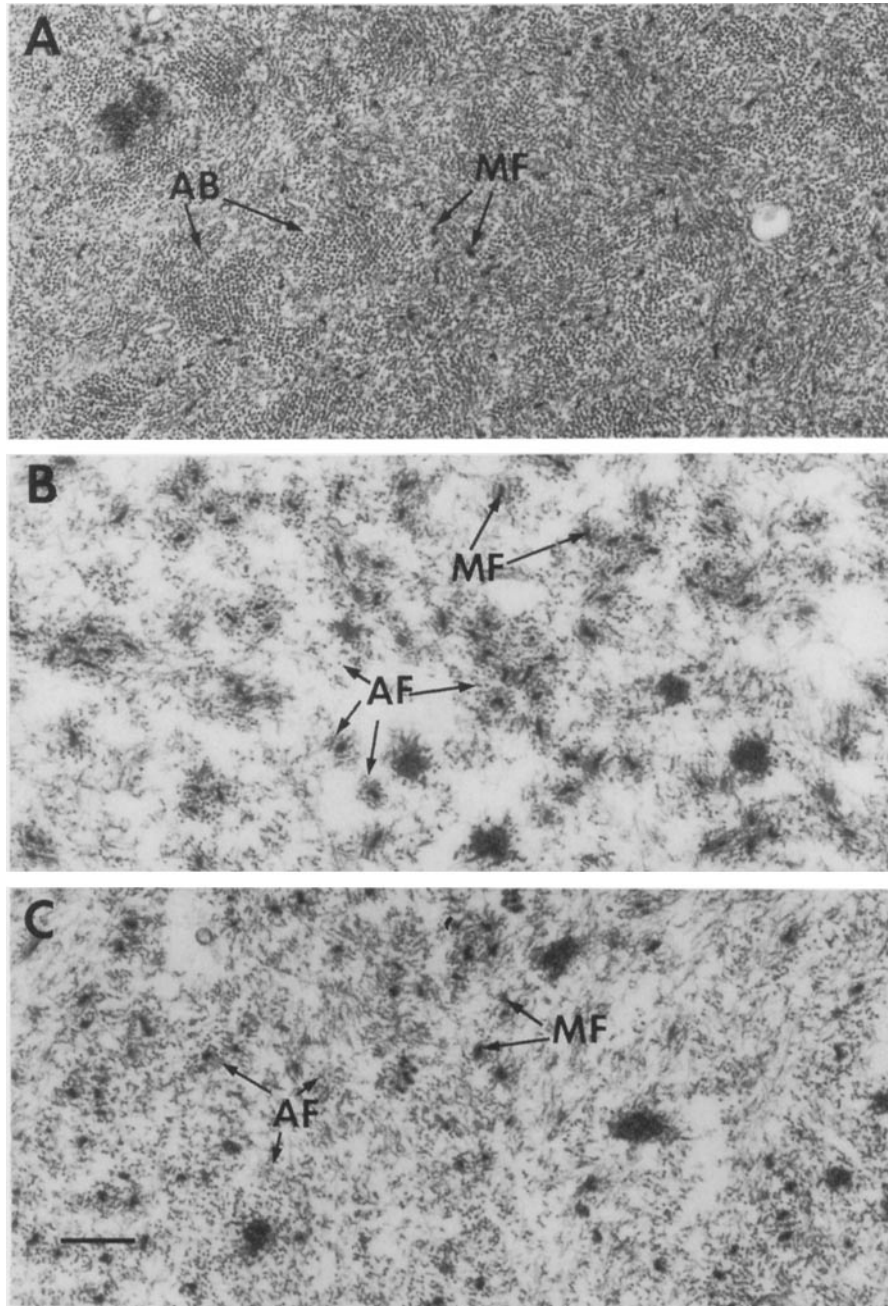
FIGURE 7. Actin and myosin filaments in intact and skinned cells. (A) Intact cell showing myosin filaments (MF) and bundles of actin filaments (AB). (B) Skinned cell in rigor solution: note the clusters of actin filaments (AF) surrounding the myosin filaments. (C) Skinned cell in relaxing solution: note that in this case, the actin filaments are not as tightly clustered around myosin filaments. × 53,000; bar, 0.2 μm.

within the cytoplasm proper was even more prominent in the skinned preparation. In shortened, skinned cells, the surface membrane, particularly that surrounding the blebs, appeared to be disrupted to a greater extent than that of extended cells (compare Figs. 9B and 5B). If a contracted, skinned cell was allowed to relax again, the blebs formed during contraction disappeared as the cell lengthened. When more than one contraction/relaxation cycle was observed, the blebs were seen to form at the same locations along the cell surface during each contraction and became approximately the same size each time.
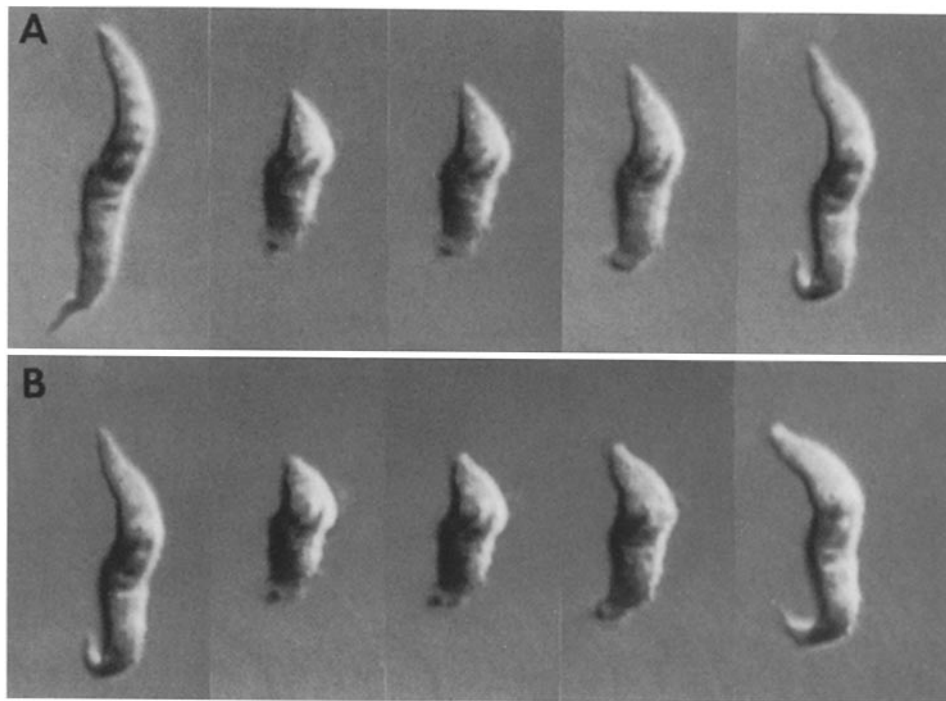

FIGURE 8. Shortening and re-extension of a single skinned cell. Images were chosen during the two contraction/relaxation cycles (A and B) so that the extent of shortening or relaxation of the cell was roughly equal in the top images and the ones immediately below them.

Dependence of Shortening on [ATP]

In 0.1 mM free Ca^{++}, the dependence of shortening on ATP concentration, calmodulin, and the presence of an ATP-regenerating system was examined. Fig. 10 shows the results of a typical experiment. In 1 mM ATP, cells eventually shortened fully (not shown), but they did so at a slower rate than in 5 mM ATP or when the CPK/PC system was included. The initial shortening rate in 5 mM ATP and in 1 mM ATP with CPK/PC was computed for fields of cells from the average length of cells before and 10 s after ATP was added. Because of the slower shortening in 1 mM ATP, fields of cells for these experiments were examined after 20 s or the shortening rate was determined from digitized images of shortening single cells. Values of 0.006 L_i/s and 0.020 L_i/s were obtained for
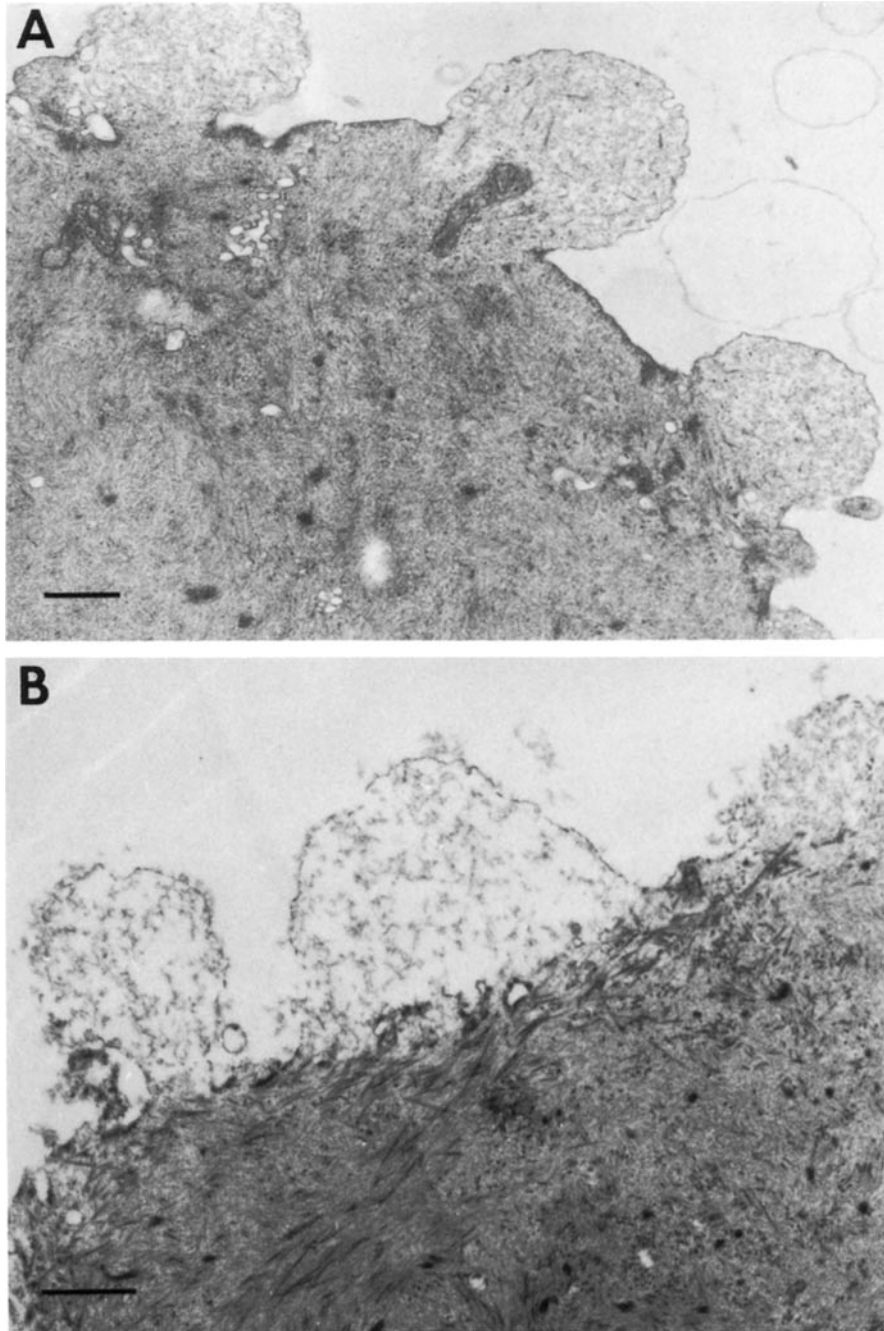
FIGURE 9.   Blebs formed during contraction of intact (A) and skinned (B) single cells. (A) × 21,000; bar, 0.5 μm; (B) × 12,000; bar, 1 μm.

the initial shortening rate in 1 mM ATP without CPK/PC from fields of cells in two experiments. When determined from individual cells, the mean rate was 0.008 ± 0.004 (SD) L_i/s (seven cells). (This value would be an underestimate of the true rate because ATP perfused through the chamber would not arrive at the cell under observation as a rectangular wave front. From monitoring the movement of dye through the chamber, we estimate that, at the flow rate used, if shortening was first detected when extracellular [ATP] was 0.1 mM, it would take ~6 s for extracellular [ATP] to reach 0.5 mM and an additional 9 s for extracellular [ATP] to reach 0.9 mM.) When the regenerating system was included, the shortening rate, determined from fields of cells, increased to 0.042
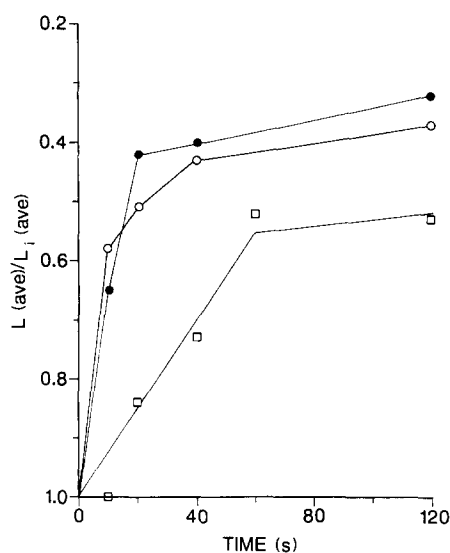

FIGURE 10. Dependence of shortening on [ATP], and an ATP-regenerating system. (□) 1 mM ATP; (●) 5 mM ATP; (○) 1 mM ATP plus CPK/PC. Cells were preincubated in CPK/PC for 5 min. All cells were preincubated in 0.1 mM Ca for 5 min before ATP was added. The line for the cells treated with 1 mM ATP was hand-drawn, with a positive slope at 120 s to indicate that cells in 1 mM ATP eventually shortened to the same extent as those in 5 mM ATP or 1 mM ATP plus CPK/PC.

± 0.004 L_i/s (three experiments). Similar rates (0.035 ± 0.006 L_i/s; five experiments) were found when [ATP] was increased to 5 mM without CPK/PC. No further increase at 5 mM ATP was seen when CPK/PC was included. Preincubation of cells (5–30 min) in calmodulin (2 μM) with or without CPK/PC did not significantly alter the shortening rate in 1 or 5 mM ATP.

In smooth muscle, ATP hydrolysis occurs during light-chain phosphorylation and during cross-bridge cycling. To see whether the shortening rate was limited in 1 mM ATP by cross-bridge cycling, skinned cells were preincubated in Ca^{++} and ATPγS. ATPγS has been shown (Hoar et al., 1979) to activate smooth muscle irreversibly, but, because it cannot be used as a substrate by actomyosin

ATPase, contraction cannot occur unless ATP is also present. Thus, the light-chain phosphorylation step can be effectively bypassed and the dependence of cross-bridge cycling on ATP concentration can be studied independently. Fig. 11 shows that skinned cells shortened very rapidly in 1 mM ATP and 0 Ca^{++} when pretreated with 1 mM ATPγS and 0.1 mM free Ca^{++}, which indicates that light-chain phosphorylation was more affected by low ATP concentrations than was cross-bridge cycling. The average shortening rate of ATPγS-treated cells during the first 5 s after the addition of 1 mM ATP was 0.096 L_i/s (two experiments). To rule out the possibility that this rate reflected some shortening of the cells during the ATPγS incubation, the cells used to determine the initial average cell length (L_i) were also preincubated in ATPγS and Ca^{++} before they
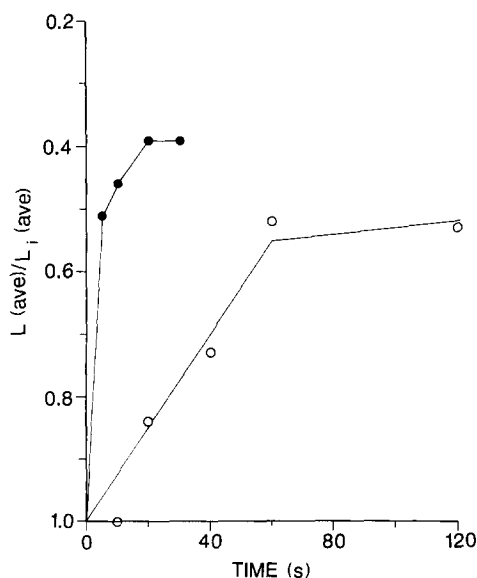

FIGURE 11. Effect of preincubation in ATPγS on the shortening rate of cells. Cells were incubated in ATPγS and 0.1 mM Ca for 30 min before 1 mM ATP was added (●). Control cells were preincubated in 0.1 mM Ca for 5 min before ATP was added (○). Similar results were obtained when the control incubation in Ca was for 30 min. The line for the control points was drawn as in Fig. 10.

were fixed. No shortening was detected during the incubation. In fact, cells could be stored for at least 1 wk in ATPγS and Ca^{++} and still respond to ATP when it was subsequently added.

Dependence of Shortening on Ca^{++}

As another physiological test of the skinned cell preparation and as a first step in the study of the regulation of contraction of smooth muscle, we examined the dependence of shortening on Ca concentration. ATP (5 mM final concentration) was added to suspensions of skinned cells preincubated in solutions with different free Ca^{++} concentrations. After enough time for the cells in the highest Ca^{++} to shorten fully (120 s), acrolein was added to each suspension to fix the cells.

Because skinned cells contracted slowly in the absence of Ca once ATP was added (see Fig. 12, inset), cells in Ca^{++}-free solution with ATP were also fixed after an equal length of time to measure the extent of Ca^{++}-independent shortening. Shortening was dependent on Ca^{++} between pCa 7.5 and pCa 5 (Fig. 12), with half-maximal shortening at pCa ~6.2. Cells at pCa 7.5 did not shorten more than those in Ca^{++}-free solution, which indicates that the 15–20% shortening observed was Ca^{++} independent.

When a single time point is considered, a relationship such as that shown in Fig. 12 could be obtained if cells shortened more slowly at low Ca^{++} or if they shortened to a lesser extent. To distinguish between these possibilities, cells were
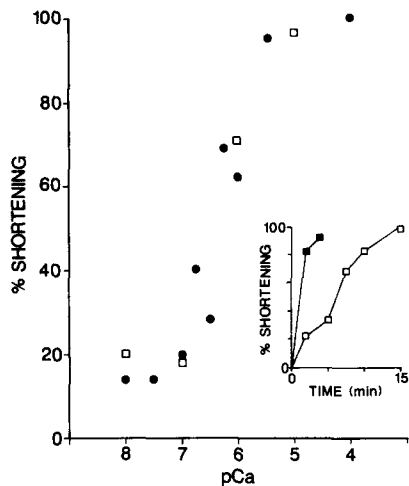

FIGURE 12.   Dependence of shortening on Ca^{++}. Cells were allowed to equilibrate in the different Ca solutions for 5 min at room temperature before 1 mM (□) or 5 mM (●) ATP was added. Cells in 5 mM ATP were fixed with 1% acrolein after 2 min (enough time for those in pCa 4 to shorten fully). The results in 1 mM ATP were obtained from two experiments in which cells were allowed to shorten for 1 and 2.5 min. (*Inset*) Shortening of skinned cells in Ca^{++}-free solution. (□) 0 Ca^{++}, 5 mM EGTA; (■) 0.1 mM Ca^{++}. Contraction was induced with 1 mM ATP.

allowed to shorten for longer periods of time. It was found that cells in lower Ca^{++} eventually shortened to $<1/3$ L_i, which indicates that, even in low Ca^{++}, enough force could be generated for full contraction.

The dependence of shortening velocity on $[Ca^{++}]$ shown in Fig. 12 might be overly steep if the cells in pCa 4 shortened fully in <2 min and those in lower Ca caught up to those in pCa 4. To examine this possibility, cells were fixed at earlier time points. The curves generated at 10, 20, and 40 s were similar to the one shown and had half-maximal shortening near pCa 6.2 (pCa 5.8, pCa 6.3, and pCa 6.5, respectively), which indicates that the relationship in Fig. 12 is representative of the skinned cells under our experimental conditions. Although shortening was slower in 1 mM ATP, the Ca^{++} sensitivity was the same in both 1 and 5 mM ATP (Fig. 12).

DISCUSSION

The work we have presented here indicates that our skinning procedure can be used to reliably produce functional skinned isolated smooth muscle cells. In addition to this, the experiments lay the groundwork for a number of structural, biochemical, and physiological studies that can be undertaken to further probe the contractile process in smooth muscle and its regulation. Such work should be particularly illuminating because it can be done on what is perhaps the most completely characterized system of isolated smooth muscle cells.

The fact that single cells in suspension are all equally exposed to detergent should make this preparation useful for the study of the skinning process itself. We have therefore begun to look at the structural changes that accompany saponin treatment. The swelling of the cells appears to be similar to that seen in other muscle that has been permeabilized (Godt and Maughan, 1977) and is presumably due to osmotic forces and myofilament charge (Collins and Edwards, 1971; Godt and Maughan, 1977; Millman and Nickel, 1980; Magid and Reedy, 1980).

Skinning also appears to be accompanied by a rearrangement of thick and thin filaments in cells and an overall reduction in the density of material in the cytoplasm. The latter may be due in part to the swollen state of the cells but may also reflect the loss of protein that occurs during skinning. Under rigor conditions, a close clustering of actin and myosin filaments similar to that noted by Small (1977) can be seen with the electron microscope. This clustering, which is not obvious in unskinned cells, appears to be due only in part to the rigor state of the muscle since cells to which ATP has been added or those skinned in the presence of ATP still show some clustering of thin filaments around thick filaments. Further experiments are planned to investigate in greater detail the effects of skinning on this filament organization.

The results we have presented indicate that the contractile apparatus of the skinned cells and the mechanisms responsible for regulating contraction and for relaxation remain largely intact. The acrylamide gels show that both myosin and actin are retained by the skinned cells. The structural studies support this and further indicate that the proteins are present in filamentous form. These results are consistent with those of the physiological experiments, which demonstrate that the skinned cells retain their ability to shorten and also indicate that the Ca-regulatory mechanism present in intact smooth muscle is retained after permeabilization. Shortening of the skinned cells is dependent on [Ca^{++}] and ATP, but becomes Ca^{++} independent if the cells are preincubated in ATPγS and Ca^{++}, a procedure believed to cause irreversible phosphorylation of myosin light chains. The latter result indicates that myosin light-chain kinase is retained by the cells since the enzyme is presumably necessary for the thiophosphorylation. Calmodulin also appears to be retained since it is not necessary to add exogenous calmodulin for cell shortening to take place, and shortening is inhibited by the calmodulin blocker trifluoperazine (Kargacin and Fay, 1986). The maximum rate of shortening observed in skinned cells (0.096 L_i/s) is similar to the maximum velocity of shortening of unattached, intact cells (0.13 L_i/s; Fay and Singer,

1977), which suggests that the kinetics of the contractile process have not been altered significantly by the skinning procedure.

A motivation behind the use of skinned preparations has been to obtain information about the physiology of intact cells. It is of great interest, therefore, to compare the Ca sensitivity of skinned cells implied by our experiments with measurements of $[Ca^{++}]$ in relaxed and contracting intact smooth muscle cells. In relaxed single cells, the mean $[Ca^{++}]$ has been estimated with a variety of techniques to be ~140 nM (pCa 6.9; see D. A. Williams et al., 1985). Our results imply that, in the relaxed cell, $[Ca^{++}]$ is just below that necessary for the development of active tension, which suggests that the cell is sensitive to small increases in Ca^{++} above 140 nM. Fig. 12 suggests that full activation of the skinned cells required 2–5 μM Ca^{++}. This could occur if the Ca^{++}-sensitive mechanism saturated above 2–5 μM or if the maximum rate at which cells could shorten was reached. The results with ATPγS show that cells are capable of shortening more rapidly, which makes the latter possibility unlikely and indicates that the regulatory system becomes saturated above these Ca^{++} concentrations. This result leads one to wonder whether saturating levels of Ca^{++} are reached in intact cells during activation. Measurements made with fura-2 indicate that the peak $[Ca^{++}]$ in intact single cells rises to ~1 μM, but not beyond, during maximum activation with K^+ or carbachol (D. A. Williams et al., 1986), which suggests that, in the intact cell, a ceiling on cytoplasmic Ca^{++} may prevent full activation of the contractile machinery. On the other hand, there may be some as yet unknown difference between the intact cells and the saponin-skinned cells, perhaps related to the proteins lost during skinning or to the concentrations of one or more ions. Obara and Yamada (1984) reported that, in skinned single smooth muscle cells from the guinea pig taenia caeci, there is a shortening vs. pCa relationship that is shifted ~0.5 pCa units to the left of ours. They found that this relationship was unaltered by changes in pH and ionic strength but was sensitive to $[Mg^{++}]$ or [MgATP].

There are a number of explanations for the contraction of our cells in the absence of Ca^{++} that are consistent with what is currently known about the light-chain regulatory system in smooth muscle: (*a*) some free Ca^{++} could be present in the rigor solutions; (*b*) light-chain phosphorylation could occur without Ca^{++} if the regulatory site of myosin light-chain kinase were altered by exogenous or endogenous proteases; (*c*) light chains might be phosphorylated if phosphatase activity in the skinned preparations were reduced; (*d*) the structure of the light chain itself might have been altered so that phosphorylation-dependent regulation would be lost. It is also possible that the shortening is similar to that observed by others in high Mg^{++} (Gordon, 1978; Saida and Nonomura, 1978), or it might be related to other regulatory mechanisms.

We have begun to examine Ca-free shortening in the skinned preparation and have eliminated some possibilities but have not yet determined the mechanism responsible for it. Addition of the protease inhibitors PMSF and STI did not prevent Ca^{++}-free shortening, nor did raising EGTA concentrations from 5 to 10 mM. Raising or lowering the free Mg^{++} concentration did appear to alter the rate of contraction in Ca^{++}-free solution, but it also altered shortening rates in

the presence of Ca^{++} in a similar manner. Experiments are currently under way to further study the effect of Mg^{++}, to examine the extent of light-chain phosphorylation in the rigor skinned cells, and to test for the presence of unregulated light-chain kinase.

The finding that the rate at which skinned single cells shortened was less at 1 than at 5 mM ATP might be explained if, at the lower concentration, the cells were not fully activated or if cross-bridge cycling were limited by a lack of substrate. The latter possibility is unlikely since cells pretreated with ATPγS were able to shorten rapidly when 1 mM ATP was subsequently added. It thus seems more likely that the rate of myosin phosphorylation or the extent of phosphorylation was lower in the lower [ATP]. The K_m of myosin light-chain kinase for ATP has been reported to be 0.05–0.1 mM (Adelstein and Klee, 1981; DiSalvo et al., 1981), which indicates that, even at the higher K_m, if 1 mM ATP was present throughout a cell, the rate of light-chain phosphorylation would be 10/11 maximum. However, this rate could be considerably less than maximum if diffusion of ATP into the cells were slow enough to limit the substrate available

TABLE I

Parameters Used in Diffusion Simulations

Parameter	Value
Diffusion coefficient of ATP within the muscle	1.5×10^{-6} cm^2/s*
Diffusion coefficient of ATP in unstirred layer (D_w)	10^{-5} cm^2/s
Thickness of unstirred layer	10 μm (equal to cell diameter)[‡]
ATP consumption rate in relaxed smooth muscle	1 μM/g wet wt/min[§]
ATP consumption rate in contracting smooth muscle	2 μM/g wet wt/min[§]
Estimate of volume of cells per volume of tissue to convert consumption rates to those expected for a single cell	50%[ǁ]
Cell density	1 g/ml

* From Kushmerick and Poldolsky (1969).

[‡] Based on estimates made by J. B. Williams and Kutchai (1985) for the unstirred layer around human red blood cells.

[§] Based on rates tabulated by Paul (1980).

[ǁ] From Cohen and Murphy (1978).

to the kinase. To see whether such a diffusion limitation could exist, we developed a computer model to simulate the movement of ATP into skinned cells. The model, based on the numerical solution described by Crank (1975) for diffusion into a cylinder of infinite length, will be described in a subsequent article. It takes into account diffusion through an unstirred layer surrounding the cell, movement through the cell membrane, consumption of ATP within the cell by the contractile apparatus and other ATPases, and a lower diffusion coefficient for ATP within the cell, which reflects slower diffusion through the cell and/or binding of ATP to proteins. Simulations with the parameters listed in Table I revealed that the ATP available for the kinase could be limited, especially if the cell membrane remained partly intact. To obtain an estimate (see Appendix) of the permeability of the cell membrane, we determined, from electron micrographs of isolated patches of membrane, the density of 80-Å holes (see Fig. 5B) induced by saponin ($\sim$2 $\times$ $10^6/cm^2$). If these holes are water-filled pores, they

would result in a membrane permeability of 1.3×10^{-5} cm/s (>100 times the permeability of the intact cell to K^+; Scheid and Fay, 1980). Under these conditions, the average [ATP] in the cell was predicted to be 0.076 mM 5 s and 0.11 mM 10 s after 1 mM ATP was added outside the unstirred layer and 0.94 mM 5 s and 1.62 mM 10 s after 5 mM ATP was added outside. Thus, after 10 s (assuming a K_m of 0.1 mM), the phosphorylation rate would be ~0.5 maximum when 1 mM ATP was added to the cell suspension and ~0.9 maximum when 5 mM ATP was added (neglecting any initial mixing time). Although we have neglected other factors that might influence the movement of ATP into the cells, these results indicate that, even in skinned cells, the membrane is likely to be a significant barrier to the diffusion of molecules. This suggests that the assumption, often made or implied, that skinning provides free access to the cytoplasm of cells or tissue may be valid for long periods of time but not for short times, especially when consumption or production within the cell of the substance of interest is involved. This conclusion is consistent with the results of Stout and Diecke (1983), who estimated that the permeability of vascular smooth muscle tissue to Ca-EGTA increased by only a factor of 10 upon saponin treatment.

Can the results described above also account for the finding that CPK/PC significantly increased the rate at which cells shortened in 1 mM ATP but not in 5 mM ATP? When the stimulation was run without the term describing consumption of ATP by the cell, at the 10-s time point, intracellular [ATP] was 0.41 mM with 1 mM ATP outside and 2.0 mM with 5 mM ATP outside. Thus, if the regenerating system acted immediately to compensate for any consumption (again assuming a K_m of 0.1 mM), the phosphorylation rate would increase from 0.52 to 0.80 maximum with 1 mM ATP outside but only from 0.94 to 0.95 maximum with 5 mM ATP outside. Considering the inherent inaccuracies in our measurements, CPK/PC could increase the shortening rate in 1 mM ATP to a level indistinguishable from that in 5 mM ATP, but CPK/PC added in the presence of 5 mM ATP would have little measurable effect. We do not feel, however, that this is likely to be the only factor involved. For example, the concentration of ADP or inorganic phosphate within cells might also inhibit the activation process and would be expected to have a significant effect, especially at lower substrate concentrations.

The formation of surface evaginations, or blebs, during contraction of the skinned cells and the ability of the cells to relax again after shortening raise a number of interesting points about the mechanisms involved. The blebs in intact isolated cells are rounded structures (see Fay et al., 1976), which suggests that they may be formed when noncontractile material is squeezed out of the central portion of the cells during shortening and pushes against the cell membrane. If this is the case, the fact that blebs also appear in permeable cells suggests that even though the membrane surrounding them appears to be disrupted (Fig. 9B), the material within the blebs cannot readily leave the cell, perhaps because it is interconnected and thus is part of the cell cytoskeleton. This suggestion is consistent with the observation that the blebs are reincorporated into skinned cells during relaxation and appear again during subsequent contractions.

The ability of skinned cells to re-extend after shortening suggests that the

mechanism responsible for relaxation must be largely retained. It has been suggested (Fay et al., 1976) that relaxation of intact single cells could occur if an ultrafiltrate of the cell cytoplasm was forced out of the cells during contraction and the osmotic gradient formed in this way provided a force for re-extension. The fact that skinned cells also relax requires some modification of this hypothesis. An osmotic gradient could still provide the force needed for re-extension of the cells if water were squeezed out of the filament lattice during shortening. This would in effect concentrate the proteins in the lattice and set up a concentration gradient between it and the extracellular fluid. It is also likely that the charge distribution around the filaments would be altered during contraction so that the restoring force could have an electrical component. It should be possible to test these hypotheses by modification of the ionic and osmotic environment of the skinned cells.

The work done thus far on the skinned cell preparation indicates that it can serve as a reliable model for the investigation of smooth muscle function. Although loss of protein occurs during skinning and there are changes in structural organization, the cells retain their contractile proteins and are able to shorten and re-extend again. They are sensitive to Ca over a physiological range of concentrations and respond in a manner that indicates that their light-chain regulatory system is retained after saponin treatment. We are actively interested in a number of areas of inquiry that have been opened by these experiments and have used the fibrillar structure that becomes apparent in the skinned cells to study the organization of the contractile apparatus during shortening (Cooke et al., 1986).

APPENDIX

The permeability of the skinned cells was estimated from the number of 80-Å holes per unit area of surface membrane in the following way. The flux through each water-filled pore of radius a and length l is

$$\phi = -\frac{\pi a^2 D_w \Delta C}{l}, \tag{1}$$

where D_w is the diffusion coefficient in water. If there are N pores in the membrane, the total flux is

$$\phi_T = N\phi. \tag{2}$$

The total flux can also be estimated if the membrane is thought of as a barrier to free diffusion in which the diffusion coefficient D_1 is determined by the membrane permeability P:

$$D_1 = Pl. \tag{3}$$

Then

$$\phi_T = -\frac{A D_1 \Delta C}{l}, \tag{4}$$

where A is the surface area of the cell. If Eqs. 2–4 are combined,

$$P = \frac{N \pi a^2 D_w}{Al}. \tag{5}$$

N/A was assumed to be $2 \times 10^6/cm^2$ (see Discussion), the pore radius, a, to be 40 Å, membrane thickness, l, to be 75 Å, and the diffusion coefficient of water to be 10^{-5} cm^2/s.

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