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Structural and biochemical analysis of skinned smooth muscle preparations

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Summary

This paper describes a biochemical and immunocytochemical analysis of smooth muscle strips that were chemically skinned and subjected to contraction and relaxation cycles according to procedures commonly employed in current skinned smooth muscle work. The fate of four major proteins, myosin, filamin, caldesmon and actin, was followed with respect to the proportionate loss of these proteins to the bathing medium as well as to their structural redistribution within the cells in the muscle strips. Large losses (of the order of 50%) of both myosin and filamin occurred at the skinning step, using either Triton X-100 or Saponin as the detergent; losses of actin were up to 30% with Triton X-100 and around 15% with Saponin. Losses of caldesmon were difficult to assess due to the rapid degradation of this protein in the bathing medium. Subsequent cycles of contraction and relaxation resulted in accumulated loss, notably of myosin and filamin, so that after the third contraction as little as 20% and 40% respectively of the original complement of these proteins remained in the muscle strips. These changes in protein composition were accompanied by a drastic redistribution of the proteins in the muscle cells. Most marked were the changes seen with myosin, significant amounts of this protein being already found in the connective tissue space after the first relaxation. These findings point to the need for a careful reappraisal of the conditions currently used in skinned smooth muscle research.

Introduction

Chemically skinned smooth muscles, commonly prepared via the use of detergents (Endo *et al.*, 1977; Gordon, 1978) or glycerol (Filo *et al.*, 1965), have received widespread acceptance as models for the study of smooth muscle function (see review by Meisheri *et al.*, 1985). Skinned muscles respond mechanically to externally applied ATP and the contractile process can, potentially, be analysed in detail by the infiltration of probes applied via the external bathing medium. This experimental system has already been used to advantage to show that the salient features of activation of the actin–myosin interaction in smooth muscle as deduced from biochemical studies (see review by Small & Sobieszek, 1980; Marston, 1982) apply also in intact tissue (Meisheri *et al.*, 1985).

A notable characteristic of skinned smooth muscles is, however, their relatively poor force production, ranging down to 20% as compared to intact muscle prior to skinning (Endo *et al.*, 1977; Gordon, 1978; Saida & Nonomura, 1978; Peterson, 1980, 1982; Spedding, 1981). For example, in intact guinea pig taenia coli, maximal force development has been reported to be 178 mN mm^{-2} (Aberg & Axelsson, 1965), 200 mN mm^{-2} (Lowy & Mulvany, 1973) and 416 mN mm^{-2} (Gabella, 1976). This compares to forces of 28.5–60.6 mN mm^{-2} in Saponin-skinned preparations (Iino, 1981) and to 67–100 mN mm^{-2} in muscles skinned with Triton X-100 (Arner, 1982). Moreover the capacity of skinned smooth muscles to perform repeated cycles of contraction and relaxation is highly variable.

A comparison of the procedures normally used for skinning, storage and for the contraction and relaxation of skinned smooth muscles (Schneider *et al.*, 1981; Sparrow *et al.*, 1981, 1984; Meisheri *et al.*, 1985) with those used for the isolation of smooth muscle contractile proteins is revealing. In particular protocols commonly employed for storage and relaxation of skinned muscles are not far removed from those used to extract myosin and/or thin filaments from tissue homogenates (Sobieszek & Bremel, 1975; Sobieszek & Small, 1976). The question may then be asked to what extent the skinned muscle system reflects the true composition and structural organization of the intact tissue? In this study we show that current conditions employed in skinned smooth muscle work are far from ideal in that they lead to the gross extraction and redistribution of major proteins of the contractile machinery.

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These results point to the need for a careful reappraisal of methods employed in such studies.

Methods

Skinned fibre preparation

Guinea pigs were sacrificed by cervical dislocation. The taenia coli were dissected carefully into strips 3–5 cm long and subjected to a skinning procedure, essentially as described by Gordon (1978), as follows:

(1) Preincubation (10 min) in one of solutions 1–4 (Table 1) at 2–4° C (on ice in the cold room).
(2) Skinning by addition to the same solution of either 0.5% Triton X-100 for 4 h or 50 μg ml^{-1} Saponin for 12 h, both on ice.
(3) The fibres were then rinsed for 10 min in their respective bathing solutions and stored either at 2–4° C in the same solution or at −20° C in a solution (Sparrow *et al.*, 1981; Paul *et al.*, 1983) containing 50% glycerol and (in mM): imidazole 20, EGTA 4, $MgCl_2$ 10, ATP 7.5, NaN_3 1, DTE 0.5 at pH 6.7.

Preparation and conditions for isometric force measurements

Fibres were first bathed at 22° C in a relaxing solution containing (in mM): imidazole 20, EGTA 4, ATP 7.5, $MgCl_2$ 10, NaN_3 1, DTE 2, phosphate 6, calmodulin 10^{-3}, and an ATP-regenerating system consisting of 10 mM phosphocreatine and 10 U ml^{-1} creatine phosphokinase at pH 6.7.

The contraction solution had a composition identical to the relaxing solution with the exception that EGTA was replaced by Ca–EGTA to give an estimated pCa of 4.5 (Portzehl *et al.*, 1964).

Thin taenia coli strips (100–300 μm thick) needed for the measurements were teased out of the skinned fibres and attached vertically with a cellulose based glue between an adjustable glass rod mounted on a micrometer drive and another rod extending from an AME 801 force transducer. Tissues were bathed in the appropriate solutions in 1.0 ml chambers and the solutions exchanged (in less than 5 s) via raising and lowering the transducer–fibre assembly. Fresh solutions were used at each step.

Immunocytochemistry: tissue sections and antibodies

At the end of the experiments the fibres were fixed in a mixture of 3% paraformaldehyde and 0.1% glutaraldehyde (Polaron, EM grade) at room temperature for 1 h in solution 4 and embedded in polyvinyl alcohol for subsequent immunocytochemistry as described elsewhere (Small *et al.*, 1986).

Antibodies against smooth muscle actin, myosin light chain (mol. wt. 20 000), light meromyosin (LMM), filamin, caldesmon and tropomyosin were raised in collaboration with Dr J. de Mey (Janssen Pharmaceutica, Belgium) and purified according to previous reports (Fürst *et al.*, 1986; Small *et al.*, 1986).

Light microscopy

Light microscopy was carried out using a Zeiss photomicroscope II equipped with epifluorescence optics.

Electrophoresis and immunoblotting of muscle strips and their extracts

The various solutions used for skinning, contraction and relaxation were collected and dialysed for 1 h against water. The samples were lyophylized, redissolved in 50 μl water and 10 μl of SDS–sample buffer (17.5% SDS, 7% β-mercaptoethanol, 437.5 mM Tris–HCl, pH 6.8) and, after boiling for 2 min, 10 μl of 50% glycerol containing 0.075% bromphenol blue added. The muscle strips were dissolved in 100 μl 8 M urea (containing 0.5 mM EGTA, 40 mM imidazole, pH 7.0) with vigorous mixing; 25 μl of SDS–sample buffer was then added and the mixture warmed to 60° C for 5 min prior to addition of 20 μl of the glycerol–bromphenol blue cocktail. SDS–PAGE was carried out using the minislab system of Matsudaira & Burgess (1978) and the Laemmli buffer system (Laemmli, 1970). Proteins were transferred electrophoretically (Towbin *et al.*, 1979) to nitrocellulose sheets (Schleicher & Schüll, West Germany) and the blots processed for immunogold silver staining as described (Moeremans *et al.*, 1984). For quantitation purposes, photographic negative reversals of the immunoblots were scanned on a Joyce-Loebel scanning densitometer (Chromoscan II). Appropriate corrections were made for different gel loadings and for the volume of the original muscle strips as estimated from the length and diameter measured under a dissecting microscope after dissection (for further details see legend to Fig. 3). For the purpose of estimates of the relative losses of each protein we assumed that the transfer efficiency during blotting was independent of sample band size and that the final signal after immunolabelling and enhancement was linearly related to protein content. Calibration tests with purified filamin at gel loadings ranging from 0.5 μg to 6 μg showed a linear relationship between sample loading and the area of the peaks obtained by densitometry. To minimize error, samples to be compared were run on the same gel and thus blotted and immunolabelled under identical conditions.

Results

Tension measurements

A typical trace of isometric force development by a skinned smooth muscle fibre from the guinea pig taenia coli is shown in Fig. 1. Cycling from relaxation to contraction was achieved by changes in the free Ca^{2+} concentration. Whatever the skinning conditions (skinning buffer 1–4, Triton or Saponin) the maximal force development was essentially similar for all muscle strips listed and ranged from 30 to 95 mN mm^{-2}. This applied to muscles stored at −20° C in the glycerol storage solution as normally employed in skinned muscle studies. For muscles stored at 4° C in relaxation buffer alone, force development was much reduced, by around ten-fold compared with parallel strips stored in glycerol. These latter muscles were excluded from immunocytochemical studies.

Samples of muscle strips for electrophoresis and immunocytochemistry were taken at the points

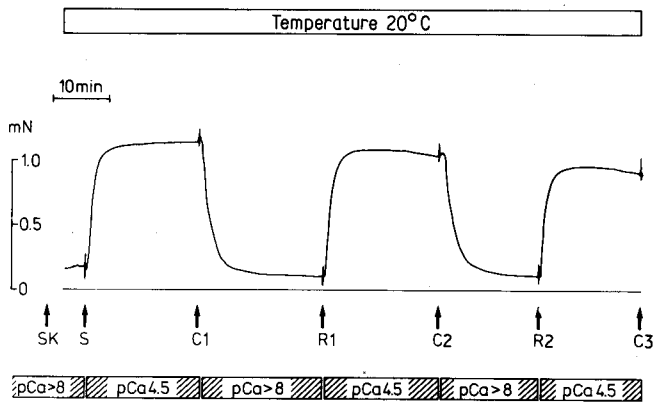

Fig. 1. Isometric contraction–relaxation cycles of a skinned smooth muscle strip (skinned in solution 1, Triton X-100) from guinea pig taenia coli. Contraction–relaxation cycles were induced by changes in the external free calcium concentration (see Methods for additional conditions). Samples for biochemical and immunocytochemical analysis were taken at the points indicated. SK, skinning step; S, equilibration prior to first contraction; C1, end of contraction 1; R1, end of relaxation 1; C2, end of contraction 2; R2, end of relaxation 2; C3, end of contraction 3.

indicated on the typical tracing shown in Fig. 1. Samples of the solutions bathing the muscles were taken at all the points indicated on Fig. 1 as well as from the additional washes made before and after the skinning step.

Loss of proteins to the external medium

From direct inspection of the SDS–polyacrylamide gels it was clear that significant quantities of protein were extracted at the different steps of handling of the muscle strips. This was demonstrated even more dramatically on immunoblots of the electrophoresis gels (Fig. 2a, b). The data shown correspond to skinning with solution 1 with either Triton (Fig. 2a) or Saponin (Fig. 2b): the use of different skinning conditions (Table 1) had little effect on the degree and the pattern of extraction. For illustrative purposes these blots were labelled with a mixture of monospecific polyclonal antibodies against filamin, light meromyosin (LMM), caldesmon and actin. The antibody concentrations were adjusted to give qualitatively similar relative intensities as for the different proteins after Coomassie Blue staining of the original gels. Although the relative proportions of the different proteins for each preparation could not readily be estimated in this way the relative losses of each individual protein from one step to the next were apparent (see Fig. 2). Fig. 2a shows the losses accompanying the most commonly employed procedure in skinned muscle studies, utilizing solution 1 and Triton X-100 for skinning (Gordon, 1978; Sparrow *et al.*, 1981). Most marked are the losses of myosin from the muscle strips as evidenced from this protein's presence, not only in the skinning solutions but in all bathing, contraction and relaxation solutions (Fig. 2). Note that all the washing and bathing solutions originate from the same muscle strip, finally dissolved for electrophoresis after the third contraction (C3, lane 16). The losses of myosin, as quantitated on separate blots with myosin antibody alone and with appropriate corrections for muscle size and sample loading, are presented in Fig. 3a. The shaded bars correspond to the muscle strips taken at the different steps indicated and the open bars to the intervening bathing solutions (for further details see legend to Fig. 3). The corresponding data for skinning with Saponin are shown in Figs 2b and 3b. As can be seen the losses of myosin are considerable, amounting to more than 50% at the skinning step and 5 to 12% of the total at each subsequent contraction or relaxation. After the third contraction only around 20% of the total myosin remained in the muscle strips.

The quantitations of the amounts of filamin and actin retained in the muscle strips are shown in Fig. 4. As for myosin, marginally less protein was lost after skinning with Saponin but the losses, particularly for filamin, were considerable, amounting to more than 50%. Filamin showed a degradation product in the skinned muscles and supernatants at a position just below the myosin heavy chain and this was also included for the purpose of quantitation of this protein. Of the different components assayed caldesmon appeared the least extracted (see Fig. 2). However, quantitation of caldesmon was complicated by the appearance of several breakdown products around M_r 80 000 and below. Absolute values for the amounts retained in the muscle are therefore not yet available.

Redistribution of contractile proteins

In recent studies the relative distributions of contractile and cytoskeletal proteins within the intact smooth

Table 1. Composition of solutions (in mM) used for skinning. Skinning was achieved by adding either 0.5% Triton X-100 or 50 μg ml^{-1} saponin.

	Solution			
	1	*2*	*3*	*4*
EGTA	5	5	5	2
Imidazole	20	–	20	
KCl	50	50	50	Ca-Mg free
Sucrose	150	150	150	Hanks
DTE	0.5	0.5	0.5	
$MgCl_2$	5	5	–	2
MES	–	20	–	10
pH	7.4	6.1	7.4	6.1

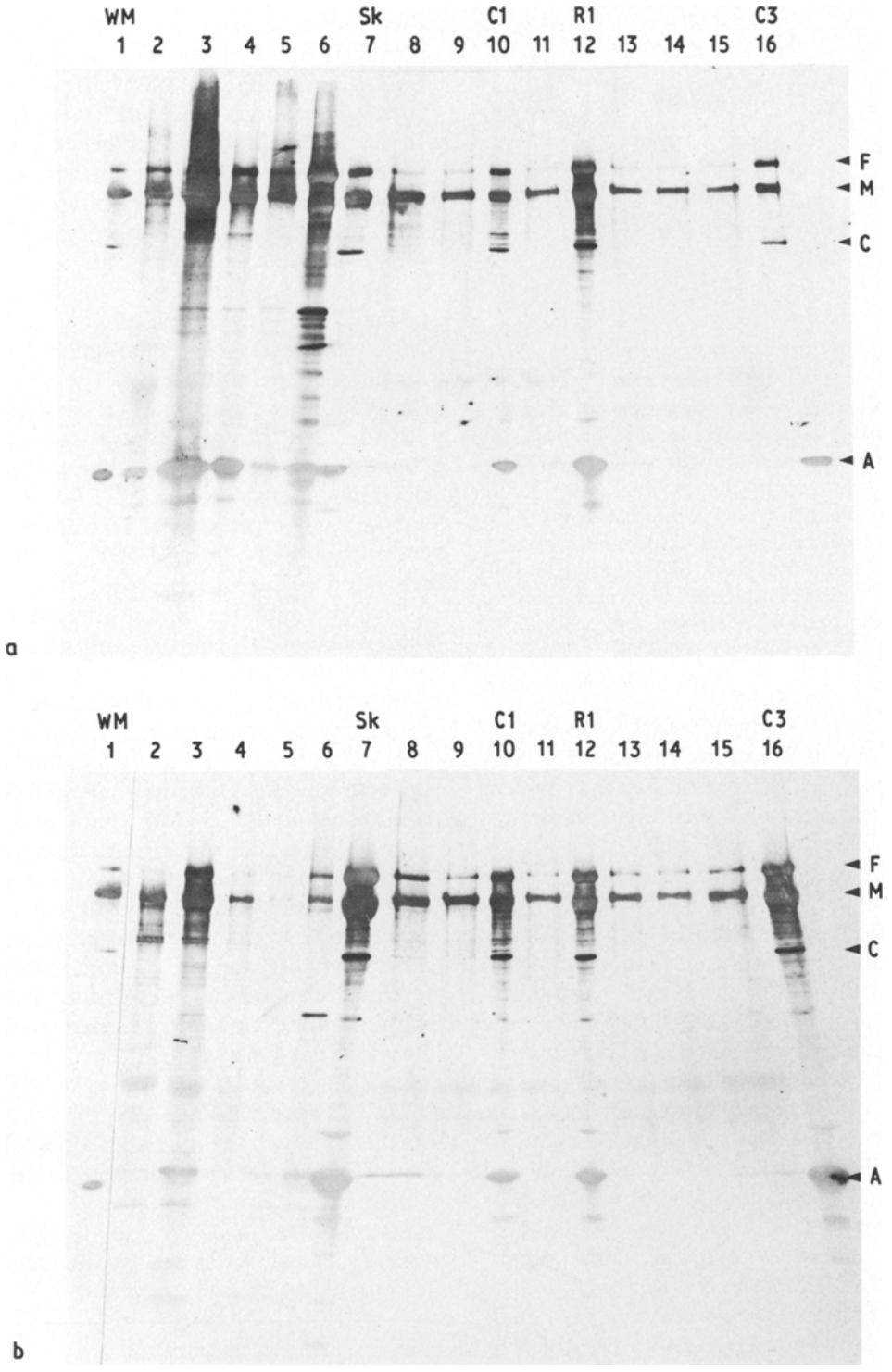

Fig. 2. Immunoblots of the SDS electrophoresis gels of the skinned muscle preparations and their bathing solutions taken at the different points of the handling scheme shown in Fig. 1. (a) and (b) differ in the use at the skinning step (solution 1) of either Triton X-100 or Saponin, respectively; the corresponding samples are otherwise identical. The blots were labelled with a mixture of monospecific polyclonal antibodies against filamin (F), light meromyosin (M), caldesmon (C) and actin (A). The lanes marked with letters correspond to the muscle strips taken at the stages indicated in Fig. 1 (WM in lane 1 is a control, whole muscle at low loading to indicate position of bands). The lanes between these correspond to the samples of the sequential bathing solutions, as follows: 2, whole muscle rinse; 3, skinning solution; 4, wash after skinning; 5, glycerol containing storage solution taken after storage at −20° C; 6, alternative 4° C storage solution; 8, 9, 11, 13, 14, 15 bathing solutions taken at points indicated in Fig. 1, respectively S, C1, R1, C2, R2 and C3. Note the presence of protein, in particular myosin, in all bathing solutions. The relative amounts of protein released at the different steps cannot be directly appreciated on these blots since appropriate corrections are necessary for specimen loadings (see text and Figs 3 and 4). It may be noted, however, that the washing and bathing solutions on the gel all originate from the same muscle strip: C3, lane 16.

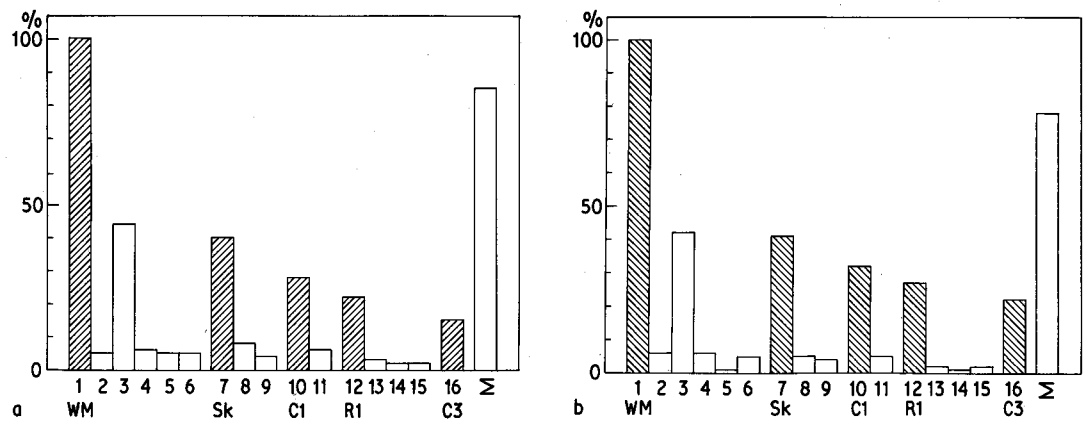

Fig. 3. Loss of myosin from skinned muscle strips as quantitated from scans of immunoblots like Fig. 2 but labelled with anti-myosin alone (not shown). Bars indicate percentage of myosin present in the bathing solutions (open bars) and muscle strips (hatched bars) at the different steps given in Fig. 1, and further detailed in Fig. 2. The data for the bathing solutions correspond to the average values for two muscle strips that were taken through the entire handling scheme from skinning to the end of the third contraction. Correction for the original sample volumes and gel loadings gave the loss at each step relative to the total, summed value (including final muscle strips) taken as 100%. The values for the amount of myosin left in the muscle strips taken at earlier steps (Sk, C1 and R1) were estimated in a similar way by comparison of the blot for a given strip with parallel blots of its own, summed extracts taken up to that step. Σ corresponds to the summed, total losses of myosin from the muscle strips. (a) For muscles skinned with Triton X-100. (b) For muscles skinned with Saponin.

muscle cell have been mapped by immunocytochemical methods (Fürst *et al.*, 1986; Small *et al.*, 1986). Using the same methods we have followed the changes in protein distribution caused by the skinning of smooth muscle and by the subsequent contraction and relaxation cycles already described. Five proteins have been monitored: actin, myosin, tropomyosin, filamin and caldesmon.

In accordance with the immunoblotting data significant changes in protein distributions in the muscle strips were observed. This applied in particular to the strips that had been additionally taken through one or more contraction–relaxation cycles. Typical examples of the protein patterns obtained are shown in Figs 5 and 6, corresponding to muscles skinned using Triton X-100 or Saponin, respectively (both solution 1).

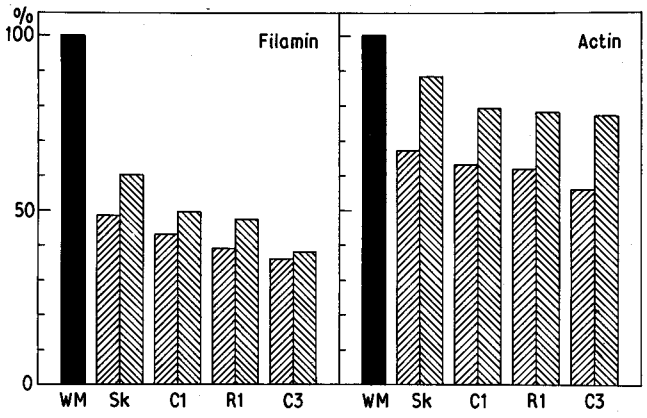

Fig. 4. The relative losses of filamin and actin from the skinned muscles as quantitated by immunoblotting with the appropriate antibodies. Solid bars indicate starting, whole muscle and the hatched bars the skinned muscle strips taken at the given stages in the handling procedure. The bars on the left of each pair correspond to skinning with Triton and on the right to skinning with Saponin. Each value is the average of at least two strips and was obtained in the same manner as described for myosin (see text and legend to Fig. 3).

The distribution of actin remained notably homogeneous through all the steps and the labelling pattern of this protein provided an immediate overview of any induced changes in cell shape and diameter. As can be seen in the figures the cycling of smooth muscle strips through relaxation and contraction induced heterogeneous changes in cell size even within the same fibre.

In contrast to actin the other four proteins assayed showed significant changes in their distributions, the changes in the localization of myosin being perhaps the most dramatic. Thus while the skinning step itself did not significantly modify the myosin pattern (compare Fig. 6, intact muscle with Fig. 5, Triton–Sk) subsequently imposed contraction–relaxation cycles led to a major displacement of myosin from the intracellular to the extracellular space (see myosin blocks, Figs 5 and 6). For muscles skinned in Triton X-100 such major redistributions were seen even after the first contraction cycle (Fig. 5: myosin, relaxation 1). The changes in the redistribution of caldesmon tended to follow those shown by myosin although this protein appeared to be retained far more tenaciously within the cells; this was evidenced by a lesser difference in staining between the intracellular and extracellular compartments (compare caldesmon and myosin blocks; relaxation 1, Fig. 5).

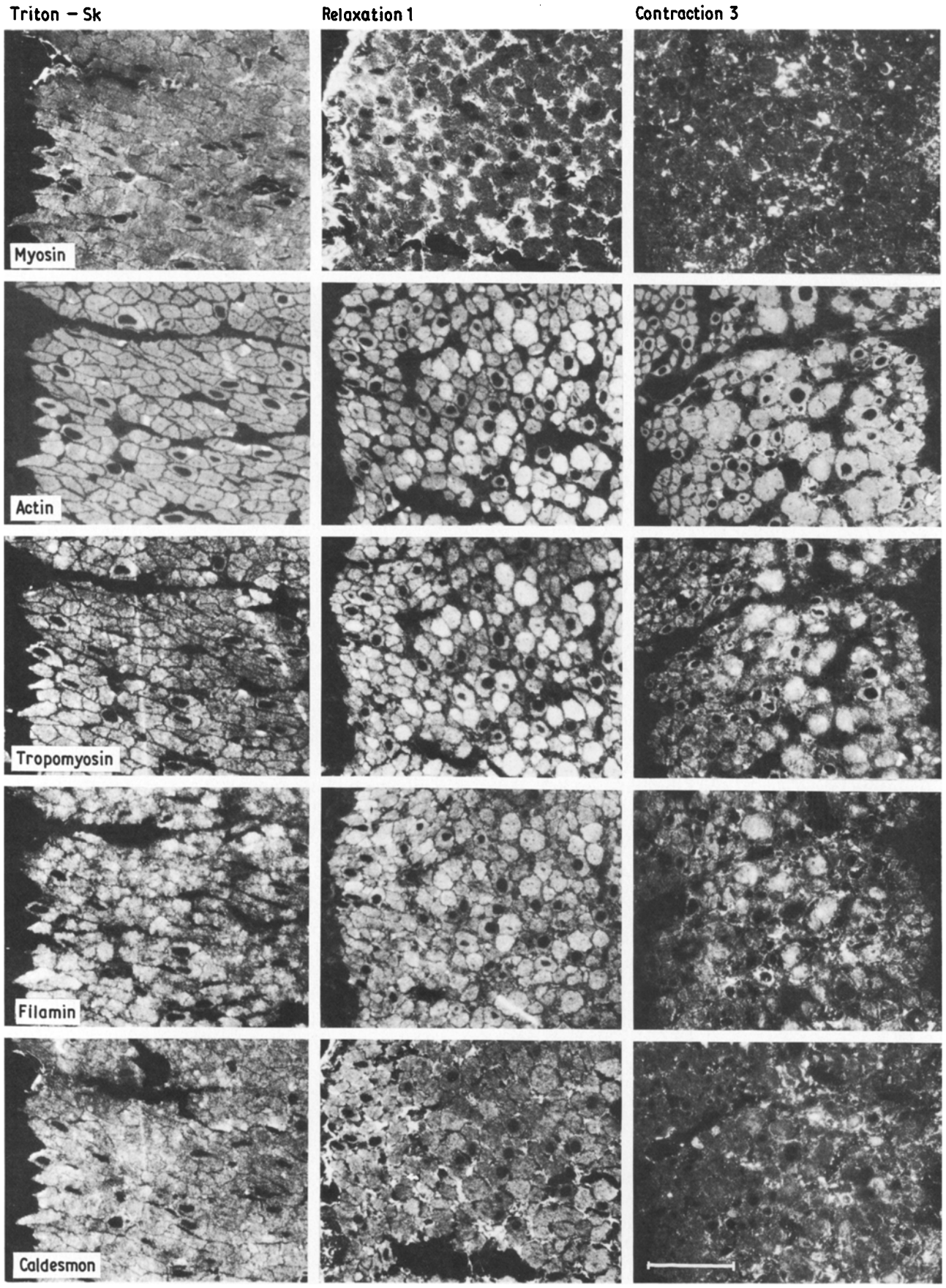

Fig. 5. Immunofluorescence analysis of ultrathin sections of muscle strips (skinned with Triton X-100; solution 1) taken at the three steps: Sk, (skinned muscle), relaxation 1 and contraction 3. Pseudo serial sections from each muscle are shown labelled with antibodies against the five proteins indicated (myosin, labelled with anti-20 000 myosin light chain). Note that apart from actin, all proteins suffer a drastic rearrangement as a result of passage through the different handling steps. For further details see text. Scale bar, 20 μm.

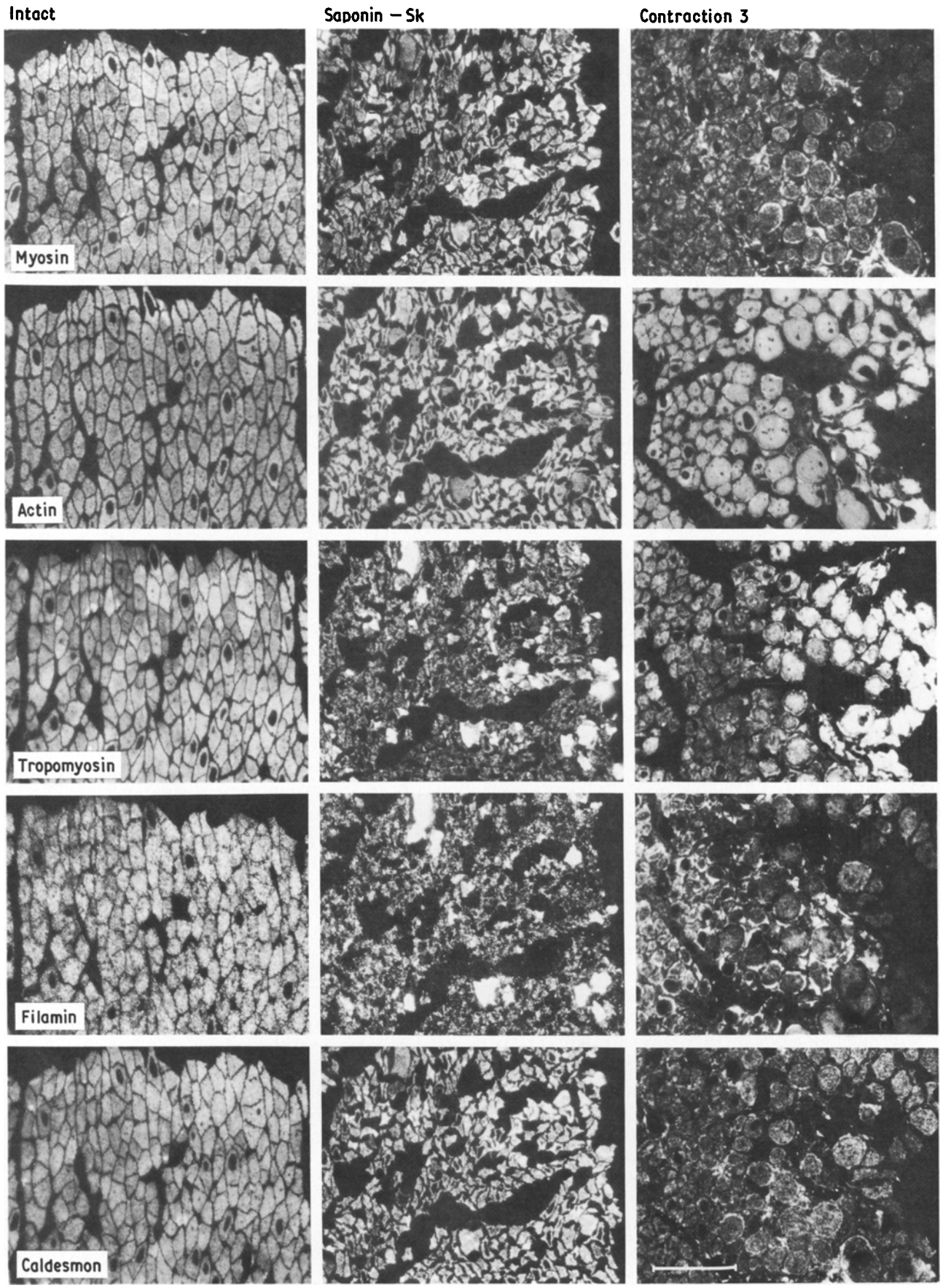

Fig. 6. Distribution of the contractile and structural proteins in intact muscle (intact) compared to the distributions in a muscle skinned with Saponin (Sk) and additionally passaged through three contraction cycles (contraction 3). Otherwise as for Fig. 5. Scale bar, 20 μm.

It was somewhat surprising to find that tropomyosin showed distinct changes in its distribution and did not remain homogeneously associated with actin (Figs 5 and 6). This was first evident even after the skinning step, the pattern of tropomyosin being then punctate as compared to homogeneous in the intact muscle (not clear at the magnification shown). In subsequent cycles tropomyosin became heterogeneously distributed within the cell population and some label was also detectable in the extracellular space. The redistribution of filamin followed a similar but not identical course to that shown by tropomyosin. For this protein considerably more label was detected in the extracellular space in cycled muscle strips (Figs 5 and 6, contraction 3).

As shown in Fig. 6 skinning with Saponin produced additional osmotic shrinkage of the cells, presumably because of this detergent's slower action in dissolving the cell membranes.

Discussion

The extensive studies on skinned smooth muscles carried out over the last ten years have been adequately reviewed by Meisheri *et al.* (1985). Notably, and in concert with the biochemical data (e.g. Marston, 1982), these studies have confirmed the central role played by myosin phosphorylation in the activation of contraction, and the corresponding requirement for calmodulin and myosin light chain kinase to effect this phosphorylation; likewise data on the requirement of a phosphatase for the relaxation of tension, predicted by the biochemical work, is now emerging (e.g. Haeberle *et al.*, 1985b). Potentially, skinned smooth muscles offer the possibility of investigating additional and apparently more complex processes that modulate the contractile state, in particular those responsible for the maintenance of tension at low energy cost. However, the results of studies on skinned muscle can only be viewed with confidence once it is shown that the act of demembranation and subsequent handling does not grossly disturb the composition and organization of the contractile apparatus.

Hitherto, the effect of detergent-skinning on smooth muscle has been evaluated almost exclusively on the basis of subsequent tension development, and, as indicated in the Introduction, the tensions developed by smooth muscle strips are reportedly much lower, with one exception (Gordon, 1978), than for intact tissue. In a more recent study, Haeberle and coworkers (Haeberle *et al.*, 1985a) did present ultrastructural data on their skinned smooth muscles, but by necessity at the level of single cells without an overview of the whole tissue. In no case has any systematic control been performed on the effect that the skinning, storage and handling solutions have on the integrity of the contractile machinery. We aimed in this work to rectify this situation.

Using the conditions of skinning developed by Gordon (1978) and the storage and handling media commonly employed in skinned muscle studies (e.g. Sparrow *et al.*, 1981) we observed both high losses of contractile and structural proteins and drastic structural rearrangement of the remaining proteins within the muscle strips. Most significant was the effect on myosin, as was to be expected from the use of ATP at around neutral pH in the storage, relaxation and contraction solutions (Laszt & Hamoir, 1961; Sobieszek & Bremel, 1975; Sobieszek & Small, 1976). However the largest losses of myosin were observed at the skinning step, in the absence of exogenous ATP. Subsequent studies, now in progress (Cross & Small, unpublished observations) suggest that these losses may be attributed in the main to the presence of endogenous ATP in the tissue. To what degree myosin actually exists in soluble form *in vivo* is a question of very topical interest that is currently unsettled.

Both filamin (Wang, 1977) and caldesmon (Sobue *et al.*, 1981) are major actin-binding proteins in smooth muscle that together match in amount the myosin content (Wang, 1977; Bretscher, 1984). It is to be expected that these proteins play significant roles in the modulation of the contractile process (see Marston & Smith, 1985; Small *et al.*, 1986). As shown by the present studies both proteins are lost to different extents in smooth muscle strips and suffer significant rearrangement during contraction and relaxation cycles. Likewise, both proteins are also susceptible to proteolysis and efforts to minimize proteolytic activity have been taken only rarely in skinned muscle studies (Haeberle *et al.*, 1985a). Since proteolysis must inevitably have a significant effect on the function of these proteins more attention to this problem is warranted and worthwhile (Cross & Small, unpublished observations).

In conclusion, the methods described here provide a means of controlling the integrity of skinned smooth muscle preparations. It is to be hoped that this type of approach will stimulate a more critical evaluation of conditions used for the preparation and handling of skinned smooth muscle strips.

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