Phosphorylation of caldesmon by mitogen-activated protein kinase with no effect on Ca^{2+} sensitivity in rabbit smooth muscle

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1. Recombinant, activated mitogen-activated protein kinase (3·3 μM; $p42^{mapk}$) phosphorylated caldesmon in phasic (rabbit portal vein) and tonic (rabbit femoral artery) smooth muscle strips permeabilized with Triton X-100.

2. Phosphorylation of caldesmon by $p42^{mapk}$ neither induced contraction of relaxed smooth muscle nor affected the Ca^{2+} sensitivity of submaximally contracted permeabilized phasic or tonic smooth muscle.

Ever since the discovery of caldesmon, a thin filament-associated, calmodulin-binding protein, numerous attempts have been made to determine whether it has a role in modulating contraction, regulated primarily by myosin light chain phosphorylation, in smooth muscle (Sobue & Sellers, 1991; Walsh, 1991). The fact that caldesmon can be phosphorylated at multiple sites by several kinases *in vitro* (reviewed in Pato, Sutherland, Winder & Walsh, 1993) and the report that phosphorylation by calmodulin-dependent kinase II reverses the *in vitro* inhibition of actomyosin ATPase by caldesmon (Walsh, 1991) raised the possibility that this putative contractile regulatory function is, in turn, regulated by a caldesmon kinase. Indeed, early studies showed that phosphorylation of caldesmon in intact smooth muscles is stimulated by a variety of excitatory agents (Park & Rasmussen, 1986). The search for the identity of the endogenous caldesmon kinases recently led to the presumptive identification of mitogen-activated protein (MAP) kinase, based on the finding that the sites of caldesmon phosphorylated *in situ* are identical to those phosphorylated *in vitro* by MAP kinase, but not by kinase C or other kinases (Adam, Gapinski & Hathaway, 1992; Adam & Hathaway, 1993; Childs & Mak, 1993). However, the direct effect of phosphorylation of caldesmon by MAP kinase *in situ* has not been previously explored, and the purpose of the present study was to determine whether a purified, recombinant MAP kinase ($p42^{mapk}$) could phosphorylate caldesmon *in situ* and/or affect the Ca^{2+} sensitivity of smooth muscle contraction.

METHODS

Adult male rabbits were killed by an overdose of halothane and exsanguinated, following a protocol approved by the University of Virginia Animal Research Committee. Portal vein and femoral artery were dissected and placed in Hepes-buffered normal Krebs solution. Connective tissue was removed and small strips (150–200 μm wide, 3 mm long) were cut. Strips for measurement of Ca^{2+} sensitivity as well as for caldesmon phosphorylation were mounted on a 'bubble' plate and attached to a force transducer (AE801, AME, Horton, Norway) for permeabilization. After determining the contractile responses to 143 mM K^+ and to 0·1 mM phenylephrine, the strips were incubated in normal relaxing solution (Ca^{2+} free, 1 mM EGTA) for 5 min and permeabilized for 20 min at 22–24 °C with 0·1% Triton X-100 in an EGTA-free solution, G0, containing: 7·2 mM magnesium methanesulphonate, 77·1 mM potassium methanesulphonate, 30 mM Pipes, 10 mM creatine phosphate, 5·16 mM Na_2ATP, 0·1 μM calmodulin and 10 μM A23187 added to release stored calcium. The Ca^{2+} concentration, measured by Ca^{2+} electrode, was 1 μM. Details of the solutions used for permeabilized strips have been previously described (Kobayashi, Kitazawa, Somlyo & Somlyo, 1989). Unless otherwise noted, all solutions contained 1 μM calmodulin to (partially) compensate for leakage of endogenous calmodulin from permeabilized cells.

To determine if caldesmon was phosphorylated by recombinant $p42^{mapk}$ *in vivo*, permeabilized strips were incubated for 1 h at 22–24 °C in a relaxing solution containing 10 mM EGTA and 0·5 mM ATP with 0·14 mg ml^{-1} phosphorylated $p42^{mapk}$ and 1·5 mCi of [^{32}P]ATP (6000 Ci $mmol^{-1}$; Dupont NEN, Boston, MA, USA). Control strips were treated identically, except no $p42^{mapk}$ was added to the incubation solution. Treated strips were placed in a homogenizing buffer (1% SDS, 10% glycerol and 20 mM dithio-

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threitol) and homogenized using a small hand-held glass homogenizer. Samples were then boiled for 2 min and placed on ice, before subjecting them to two-dimensional electrophoresis. Briefly, 50 μl of homogenate was loaded to an isoelectric focusing tube gel with 5% ampholytes, pH range 5·0–7·0 (caldesmon pI 5·7–6·0), and run overnight. The tube gel was then placed on an SDS–polyacrylamide slab gel (10%), and the second dimension was run. Slab gels were transferred overnight onto polyvinylidene difluoride ('Immobilon') membranes (Millipore), and the membranes were exposed to X-ray film (Fuji) overnight. To determine the position of caldesmon on the two-dimensional gels, membranes were incubated with a rabbit polyclonal antibody raised against chicken gizzard caldesmon, followed by a secondary anti-rabbit antibody coupled to HRP. Stained proteins were visualized by enhanced chemiluminescence (ECL) (Amersham Life Science, Arlington Heights, IL, USA).

Recombinant $p42^{mapk}$ (from the rat sequence) was purified as previously described with minor modifications (Haystead, Dent, Wu, Haystead & Sturgill, 1992). The purified $p42^{mapk}$ was activated by phosphorylating it with constitutively active recombinant MAP kinase kinase (MEK) in the presence of ATP. The activity of the phosphorylated $p42^{mapk}$ ($0·18 \pm 0·09$ μmol min^{-1} mg^{-1}) was determined by its ability to phosphorylate myelin basic protein (Haystead *et al.* 1992). This activity is comparable to that (0·49 μmol min^{-1} mg^{-1}) of seastar $p44^{mapk}$ and about 360-fold higher than the activity of $p42^{mapk}$ isolated from gizzard (Childs & Mak, 1993). The $p42^{mapk}$-containing solutions used in this study also contained the recombinant MAP kinase kinase (MEK) in a molar ratio of at least 1:1 MEK: $p42^{mapk}$ to maintain the level of $p42^{mapk}$ phosphorylation.

To determine the effect of phosphorylated recombinant $p42^{mapk}$ on the contractile responses in rabbit portal vein and femoral artery, experiments were carried out on Triton X-100-permeabilized strips (150 μm width, 3 mm length) mounted in a bubble chamber as described above. All solutions contained 1 μM calmodulin. The experimental strips of either rabbit portal vein or femoral artery were incubated in relaxing solution, G10, containing: 10 mM EGTA, 4·5 mM ATP and 0·14 mg ml^{-1} of phosphorylated $p42^{mapk}$ for 1 h at 22–24 °C, and then exposed sequentially to the following calcium-containing solutions buffered with 10 mM EGTA to pCa 6·5, pCa 6·3 and pCa6·0. Each of these solutions also contained 0·14 mg ml^{-1} of phosphorylated $p42^{mapk}$ with MEK, as described above. A maximal contraction was obtained using a pCa 4·5 solution with 5 μM calmodulin. Control strips were treated identically, but had no $p42^{mapk}$ added.

In order to estimate the concentration of endogenous $p42^{mapk}$ in smooth muscle, purified MAP kinase standards of known protein concentration (0·85, 8·5 and 85 ng) were run on 10% SDS–PAGE with homogenized rabbit portal vein strips of known dimensions. The gel was tranferred onto a polyvinylidene difluoride membrane overnight and incubated with an anti-MAP kinase polyclonal antibody followed by a secondary anti-rabbit antibody. Stained proteins were visualized by ECL. Blots were subjected to densitometry for quantitation. The MAP kinase concentration in portal vein smooth muscle was estimated on the basis of 56% cellular volume of the strips (calculated using the dimensions of the strips and 44% extracellular space measured by morphometry of electron micrographs).

To assess the endogenous MAP kinase concentration after Triton X-100 permeabilization in portal vein and to examine the entry of the exogenously added $p42^{mapk}$, strips were permeabilized with Triton X-100 and incubated with or without $p42^{mapk}$ (0·14 mg ml^{-1}) under the same conditions as described above. Strips were washed and homogenized in SDS loading buffer. Samples were run on 10% SDS–PAGE, transferred onto membranes and immunoblotting was carried out using the anti-MAP kinase antibody as described.

Caldesmon content was measured by electrophoresis of homogenates of intact and Triton X-100-permeabilized muscle, as described above. Transferred gels of homogenates and calmodulin standards were probed with a rabbit caldesmon antibody made to purified chicken caldesmon and kindly provided by Dr Michael Walsh.

RESULTS

Incubation of phosphorylated $p42^{mapk}$ with Triton X-100-permeabilized portal vein and femoral artery strips for 1 h at room temperature caused phosphorylation of several proteins, compared with controls, as observed by autoradiography of two-dimensional gel electrophoretograms. In portal vein samples, one spot appeared on control autoradiographs (labelled 'a'; Fig. 1*B*) and it was also present in the $p42^{mapk}$-treated muscle (Fig. 1*A*). In the autoradiographs of the $p42^{mapk}$-treated portal vein samples (Fig. 1*A*) seven major spots could be observed (in addition to spot 'a'). When the membranes of these autoradiographs were subjected to immunoblotting with anti-caldesmon antibody, one spot was observed (Fig. 1*C*). The approximate molecular weight of this protein, as determined by molecular weight markers, was 140 kDa (corresponding to the migration of high molecular weight caldesmon on SDS gels). When the immunoblot was aligned with autoradiographs, the 140 kDa spot matched exactly with a spot on the $p42^{mapk}$-treated autoradiographs (Fig. 1*A* compared with Fig. 1*C*). Comparison of immunoblots of unpermeabilized with Triton X-100-permeabilized strips probed with the anti-caldesmon antibody showed that caldesmon was not extracted during permeabilization (data not shown). In autoradiographs of $p42^{mapk}$-treated femoral artery samples (Fig. 1*D*) several proteins were also phosphorylated compared with controls (Fig. 1*E*; 'b' denotes protein phosphorylated under control conditions). In $p42^{mapk}$-treated femoral artery samples, more proteins appeared to be phosphorylated compared with portal vein, and the background was higher, giving a lower resolution. This was the result of longer exposure of the autoradiograph, which was necessary because of the lower caldesmon content of tonic, compared with phasic, smooth muscle (Haeberle, Hathaway & Smith, 1992). Phosphorylated caldesmon was identified by immunoblotting the membrane (Fig. 1*D*) with the anti-caldesmon antibody (shown in Fig. 1*F*), and run at the expected molecular weight of approximately 140 kDa. The migration of caldesmon by isoelectric focusing, although in a similar position, was less diffuse in femoral artery than in portal vein (Fig. 1*C*).

Phosphorylated $p42^{mapk}$, under conditions identical to those that produced phosphorylation of caldesmon, as demonstrated above, did not cause tension development of permeabilized, relaxed strips of rabbit portal vein or femoral artery over the 1 h incubation period. Subsequently, the Ca^{2+} sensitivity of contraction was examined in both $p42^{mapk}$-treated and control strips. In both, pCa 6·5, pCa 6·3 and pCa 6·0 solutions caused successive increments in force, with pCa 6·0 inducing close to maximal force (maximum force was achieved by the addition of pCa 4·5 with 5 μM calmodulin; Fig. 2*A* and *B*). The Ca^{2+} sensitivity and maximum force developed by $p42^{mapk}$-treated strips of both the phasic and tonic muscles were unchanged, compared with control strips, as shown in Table 1. Estimates of the rate of rise in force, obtained by comparing half-times, were also similar in treated and untreated strips

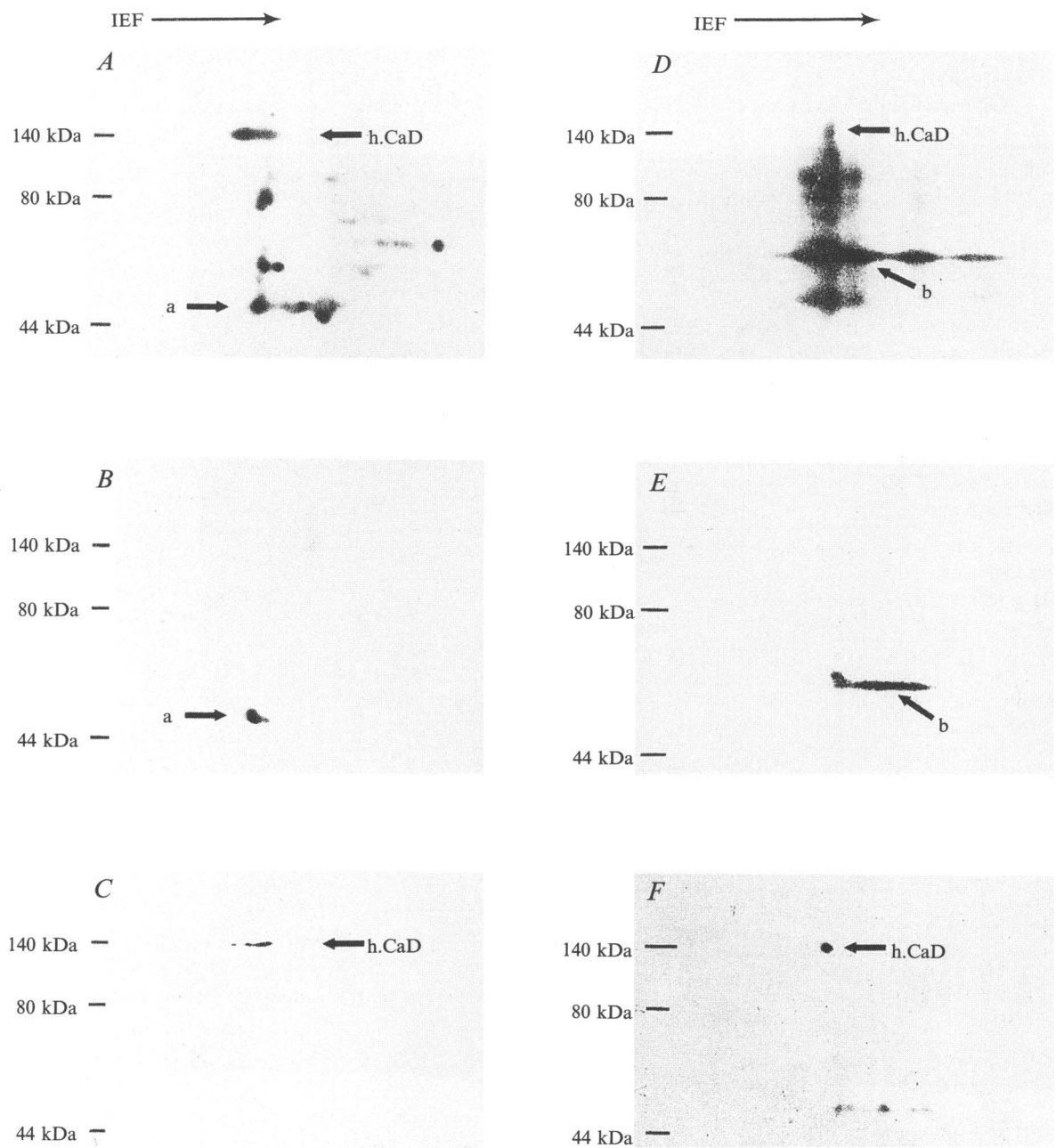

Figure 1. Two-dimensional gel electrophoresis of rabbit portal vein and femoral artery

Autoradiographs showing phosphorylated proteins in $p42^{mapk}$-treated permeabilized strips (*A*, *B* and *C*, portal vein; *D*, *E* and *F*, femoral artery) and control permeabilized strips (*B* and *E*). *C*, immunoblot of *A* using an antibody raised against caldesmon. *F*, immunoblot of *D* using an antibody raised against caldesmon. IEF, isoelectric focusing; h.CaD, high molecular weight caldesmon. Spots labelled 'a' and 'b' denote phosphorylated proteins visible in both control and $p42^{mapk}$-treated samples.

Table 1. Tension responses to calcium-containing solutions in permeabilized rabbit portal vein and femoral artery in the presence and absence of $p42^{mapk}$

	pCa 6·5	pCa 6·3	pCa 6·0	pCa 4·5 + 5 μM CaM
Portal vein				
$p42^{mapk}$	0·07 ± 0·01	0·34 ± 0·05	0·46 ± 0·10	0·49 ± 0·13
Control	0·07 ± 0·02	0·36 ± 0·03	0·43 ± 0·05	0·50 ± 0·02
Femoral artery				
$p42^{mapk}$	0·49 ± 0·05	0·75 ± 0·10	0·82 ± 0·10	0·86 ± 0·12
Control	0·43 ± 0·10	0·77 ± 0·13	0·85 ± 0·15	0·89 ± 0·19

Responses are normalized to the contraction in 1 μM Ca^{2+} solution (see Methods) after 20 min permeabilization but before addition of $p42^{mapk}$; $n = 3$. CaM, calmodulin.

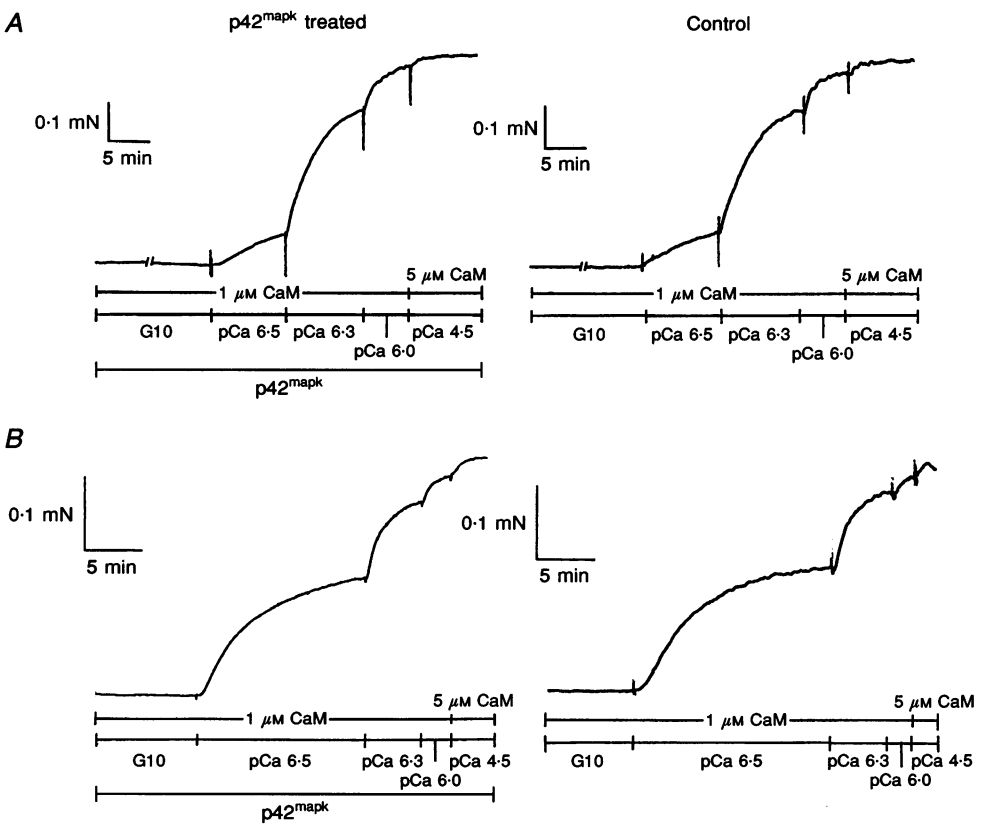

Figure 2. Calcium sensitivity of permeabilized smooth muscle strips in which caldesmon was phosphorylated by $p42^{mapk}$

A, force development by permeabilized rabbit portal vein strips in response to increasing Ca^{2+} concentrations after 1 h incubation with $p42^{mapk}$ and by permeabilized control strips (no $p42^{mapk}$). *B*, force development by permeabilized rabbit femoral artery strips in response to increasing Ca^{2+} concentrations after 1 h incubation with $p42^{mapk}$ and force development by permeabilized control strips (no $p42^{mapk}$). CaM, calmodulin. As previously reported (Kitazawa, Gaylinn, Denney & Somlyo, 1991) the Ca^{2+} sensitivity of tonic smooth muscle (femoral artery) is greater than that of phasic muscle (portal vein).

(femoral: pCa 6·5, 227 ± 31 s (treated) *versus* portal 198 ± 18 (untreated): pCa 6·5, 156 ± 28 s (treated) *versus* 144 ± 13 (untreated), $n = 3$). The maximum force response to 1 μM Ca^{2+}, reached by portal vein strips following the 1 h incubation (Table 1, last column), was approximately 50% of the initial response obtained immediately after permeabilization. This is due to 'run down' of the strips, typically seen in smooth muscle permeabilized by the method used to permit extensive penetration of high molecular weight proteins (Iizuka, Ikebe, Somlyo & Somlyo, 1994).

The concentration of endogenous MAP kinase was estimated by comparing immunoblots of smooth muscle with those of known MAP kinase standards. These blots are shown in Fig. 3*A*. In the tissue samples both the $p44^{mapk}$ and $p42^{mapk}$ were identified. Rabbit portal vein samples contained 5 ng of $p42^{mapk}$, compared with $p42^{mapk}$ standards and measured by densitometry. Adjusting this for the total cell volume in these samples (0·041 μl) yields an endogenous $p42^{mapk}$ concentration of approximately 0·12 mg ml^{-1} (2·8 μM).

Triton X-100-permeabilized portal vein strips incubated for 1 h with 0·14 mg ml^{-1} of purified $p42^{mapk}$ revealed a strong single band on immunoblots (Fig. 3*B*) probed with the anti-MAP kinase antibody. Permeabilized strips incubated (for 1 h) without exogenous $p42^{mapk}$ showed no evidence of MAP kinase on immunoblots, suggesting that the endogenous MAP kinase had been depleted.

DISCUSSION

Our results show that, although $p42^{mapk}$ can phosphorylate caldesmon *in situ*, this phosphorylation has no detectable effect on either the Ca^{2+} sensitivity or the maximum amplitude of contraction in smooth muscle.

The access of the exogenous $p42^{mapk}$ to cellular phosphorylatable sites was indicated by the phosphorylation of several proteins (Fig. 1), including caldesmon. This phosphorylation is not due to activation of endogenous MAP kinase, as by the end of the 1 h incubation period the permeabilized strips did not retain detectable concentrations of this enzyme (Fig. 3). This was expected,

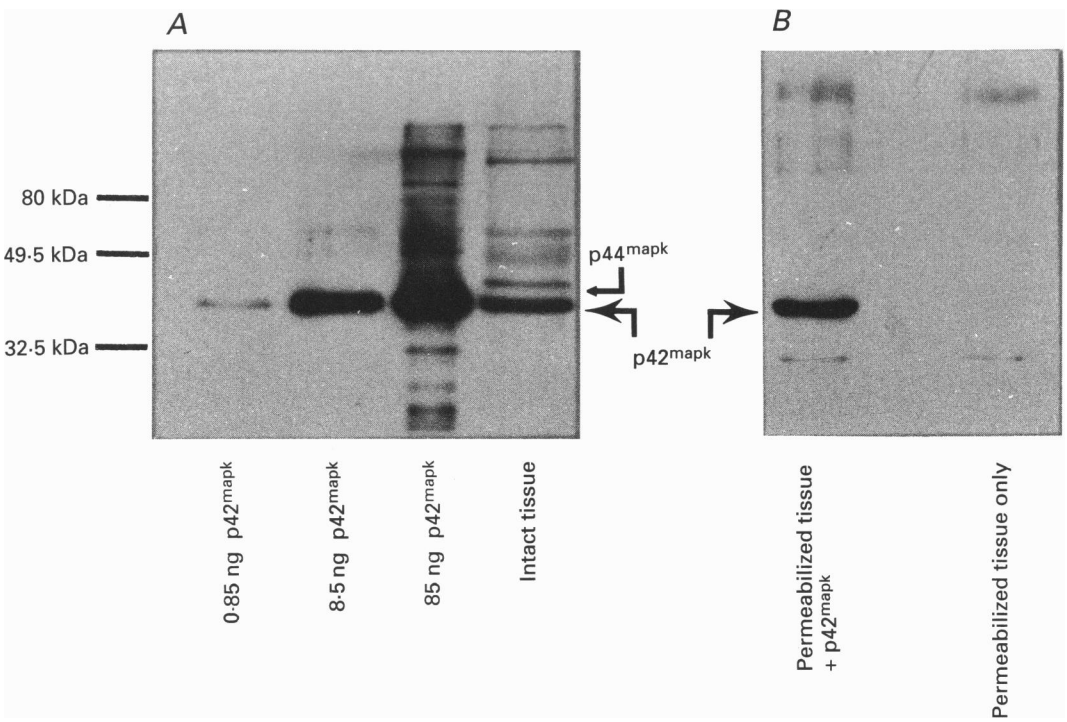

Figure 3. Immunoblots of purified $p42^{mapk}$ and of rabbit portal vein probed with anti-MAP kinase antibody

A, known amounts of purified $p42^{mapk}$ (lanes 1,2, 3) and homogenate of intact tissue (lane 4) were loaded in order to estimate the endogenous concentration of $p42^{mapk}$ (see Results). *B*, homogenate of rabbit portal vein permeabilized with Triton X-100 and incubated with (lane 1) or without (lane 2) $p42^{mapk}$. Note the presence of $p42^{mapk}$ in the intact muscle, and its absence (loss) from the permeabilized preparation, to which no exogenous enzyme had been added.

because permeabilization of smooth muscle by the method employed permits extensive transmembrane passage of even much higher molecular weight (150 kDa) proteins (Iizuka *et al.* 1994). Our findings are in agreement with those of Adam & Hathaway (1993), who found that the MAP kinase sites of caldesmon were phosphorylated in intact carotid arteries stimulated with high K^+ or phorbol ester from resting levels of 0·45 mol PO_4 (mol caldesmon)$^{-1}$ to 0·96 (KCl stimulation) and 1·1 mol PO_4 (mol caldesmon)$^{-1}$ (phorbol ester stimulation) (Adam *et al.* 1989). It is likely that caldesmon was as available to the highly active exogenous $p42^{mapk}$ as to the, probably less active (Childs & Mak, 1993), endogenous enzyme, However, the stoichiometry of caldesmon phosphorylation could not be accurately determined in our small, permeabilized samples; therefore, we cannot exclude the possibility that MAP kinase phosphorylated only a fraction of the caldesmon pool in the smooth muscle (see Note added in proof).

From immunoblots, we estimated the total $p42^{mapk}$ concentration in portal vein to be in the low micromolar range. Although this may be an overestimate, compared with the yield of $p42^{mapk}$ isolated from (gizzard) smooth muscle (Childs & Mak, 1993), it demonstrates that the lack of effect of exogenous $p42^{mapk}$ on contractility is highly unlikely to be the result of less than physiological $p42^{mapk}$ activity in our experiments (exogenously added, phosphorylated $p42^{mapk}$ was 3·3 μM).

The fact that phosphorylation of caldesmon by $p42^{mapk}$ had no effect on the parameters of contractility measured, and in particular, Ca^{2+} sensitivity, argues against a contractile regulatory role of caldesmon phosphorylation. This conclusion is consistent with *in vitro* studies that showed that phosphorylation (C-terminal) of caldesmon had little or no effect on its binding to actin, calmodulin or tropomyosin or on its effect on actomyosin ATPase (Childs, Watson, Sanghera, Campbell, Pelech & Mak, 1992; Pinter & Marston, 1992), although other studies have shown some effects on the actomyosin ATPase. The faster time course of relaxation than that of caldesmon dephosphorylation in arterial smooth muscle (Adam *et al.* 1989) also argues against a regulatory function of caldesmon phosphorylation. Therefore, we consider it unlikely that caldesmon plays a contractile regulatory role as the downstream target of a MAP kinase cascade.

Our results do not directly address the general question of whether caldesmon, independently of its state of phosphorylation on the MAPK sites, modulates contractility in smooth muscle. Some of the different experiments supporting this hypothesis are difficult to reconcile, because peptide fragments and synthetic polypeptide sequences of the C-terminal actin-binding region of caldesmon have been reported to have both inhibitory (Pfitzer, Zeugner, Troschka & Chalovich, 1993) and potentiating (Katsuyama, Wang & Morgan, 1992) effects on force, albeit in different smooth muscles. Exogenous caldesmon also inhibits force development in striated muscle that does not contain endogenous h-caldesmon (Brenner, Yu & Chalovich, 1991), hence the specificity and physiological significance of its effects in smooth muscle remain to be established. Furthermore, for caldesmon to regulate contraction, it would have to have a 'switch' that moves it preferentially into an 'on' or 'off' position. Caldesmon is neither a Ca^{2+}-binding protein nor does its phosphorylation affect contraction (present study), thus excluding the two major possible switching mechanisms. It is also unlikely that interaction with calmodulin regulates caldesmon by removing its inhibitory effect (*in vitro*; Walsh, 1991) during tonic contractions, after the decline in $[Ca^{2+}]_i$ and myosin light chain phosphorylation ('latch'), because of the very high concentrations of Ca^{2+}–calmodulin required for this mechanism (Smith, Pritchard & Marston, 1987). Furthermore, given the low affinity of Ca^{2+}–calmodulin for, and its rapid off-rate (13·5 s^{-1}) from caldesmon (Kasturi, Vasulka & Johnson, 1993), compared with that for myosin light chain kinase (MLCK), the dissociation of any calmodulin–caldesmon complex would precede the dissocation and decline in the activity of the $4Ca^{2+}$–calmodulin–MLCK complex. Of course, it is possible that, regardless of its state of phosphorylation or association with calmodulin, caldesmon has a stabilizing effect on thin filaments, favouring either the relaxed or activated state.

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Note added in proof

We obtained an estimate of the approximate level of caldesmon phosphorylation by comparing the extent of autoradiographically detected phosphorylation by $p42^{mapk}$ in portal vein strips with the levels reached, under identical conditions (in 1 h), in purified gizzard caldesmon. The respective values were normalized to the concentrations of caldesmon determined from immunoblots. Assuming that the purified caldesmon was stoichiometrically (1 mol mol^{-1} in 1 h; Adam & Hathaway, 1993) phosphorylated, we find that exogenous $p42^{mapk}$ phosphorylated approximately 69% of the caldesmon in the portal vein strips. Given a resting level of 0·45 mol PO_4 (mol caldesmon)$^{-1}$, (Adam *et al.* 1989), this would imply near-stoichiometric phosphorylation of caldesmon in our study.

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