Activation of MAP kinases and phosphorylation of caldesmon in canine colonic smooth muscle

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1. Phosphorylation of caldesmon was assayed in canine colonic circular smooth muscle strips labelled with ^{32}P and stimulated with 10 μM acetylcholine. Caldesmon was isolated by two-dimensional non-equilibrium pH gel electrophoresis. Stimulation with acetylcholine increased caldesmon phosphorylation significantly from a basal level of $0{\cdot}6 \pm 0{\cdot}07$ to $1{\cdot}1 \pm 0{\cdot}15$ mol P_i (mol caldesmon)$^{-1}$ after 2 min.

2. MAP kinase activities were measured in SDS extracts of muscle by a gel reconstitution method using myelin basic protein. Myelin basic protein kinase activities were observed at 38, 44, 50 and 57 kDa by the gel reconstitution method. Endogenous caldesmon kinase activities were also identified by the gel reconstitution method at 38, 44 and 50 kDa. The 38 and 44 kDa kinases comigrated with proteins labelled by anti-ERK1 MAP kinase antibodies on Western blots. Both 38 and 44 kDa MBP kinase activities increased significantly during contractions induced by 10 μM acetylcholine, 0·1 μM neurokinin A and 70 mM potassium.

3. Phorbol dibutyrate (0·1 μM) potentiated activation of MAP kinases and contraction of depolarized muscles while producing a decrease in fura-2 fluorescence ratio. This suggests that protein kinase C activation is coupled to MAP kinase activity in colonic smooth muscle.

4. MAP kinases isolated from muscle homogenates by Mono Q chromatography were assayed using the specific MAP kinase substrate peptide APRTPGGRR. Stimulation of muscles for 2 min with 10 μM acetylcholine activated both ERK1 and ERK2 MAP kinase activities 2-fold.

5. To determine the effects of caldesmon phosphorylation by MAP kinase on the cross-bridge cycle, actin sliding velocity was measured with an *in vitro* motility assay. Unphosphorylated turkey gizzard caldesmon (3 μM) significantly reduced mean sliding velocity. Phosphorylation of caldesmon with sea star ERK1 MAP kinase reversed the inhibitory effect of caldesmon on sliding velocity. The results are consistent with a protein kinase cascade being activated by contractile agonists in gastrointestinal smooth muscle which activates ERK MAP kinases leading to phosphorylation of caldesmon. Phosphorylation of caldesmon *in vivo* may reverse inhibitory influences of caldesmon on cross-bridge cycling.

Gastrointestinal motility is regulated by multiple extracellular messengers including neurotransmitters, autacoids, hormones and drugs. The contractile element of gastrointestinal smooth muscle cells is coupled to extracellular signals by multiple intracellular signal transduction pathways that may vary with the type of stimulant. For example, acetylcholine activates M2 and M3 muscarinic receptors in colonic smooth muscles which are coupled to inhibition of adenylate cyclase, activation of phospholipase C and phosphatidylinositol turnover, release of stored Ca^{2+}, activation of protein kinase C (PKC) and regulation of potassium, calcium and non-selective cation channels (Zhang & Buxton, 1991, 1993; Sanders, 1992; Lee, Bayguinov & Sanders, 1993). Each of these signalling events is ultimately coupled

to changes in myoplasmic Ca^{2+} concentration, which is the primary intracellular event activating contractile proteins to generate force.

Ca^{2+}-dependent phosphorylation of the 20 kDa myosin light chains by myosin light chain kinase is thought to be the primary regulatory event in activation of cross-bridges in mammalian smooth muscles. In colonic smooth muscle, activation of muscarinic receptors by acetylcholine increases phosphorylation of myosin, which presumably increases the number of active cross-bridges and the cycling rate, which results in development of force and increased tissue shortening velocity (Gerthoffer, Murphey, Mangini, Boman & Lattanzio, 1991). However, experiments showing temporal and agonist-dependent dissociation of myosin light chain phosphorylation, force development and tissue-shortening velocity in a variety of smooth muscles suggest there are regulatory mechanisms in addition to myosin light chain phosphorylation (reviewed by Gerthoffer, 1991; Moreland, Pott, Cilea & Moreland, 1991). The identities and specific functions of the putative additional regulatory proteins are not clear, but there has been much effort focused on actin-associated proteins.

Caldesmon is an actin-binding protein abundantly expressed in smooth muscles which inhibits actin-activated myosin ATPase activity *in vitro*. Phosphorylation of caldesmon *in vitro* reverses the inhibition (Ngai & Walsh, 1987) suggesting phosphorylation may be important in regulating contraction. Caldesmon can be phosphorylated *in vitro* by several protein kinases including PKC (Tanaka, Ohta, Kanda, Tanaka, Hidaka & Sobue, 1990), CaM kinase II (Scott-Woo, Sutherland & Walsh, 1990), $p34^{cdc2}$ kinase (Mak, Watson, Litwin & Wang, 1991), the ERK family of mitogen activated protein (MAP) kinases (Childs, Watson, Sanghera, Campbell, Pelech & Mak, 1992) and casein kinase II (Wawrzynow, Collins, Bogatcheva, Vorotnikov & Gusev, 1991). Some important issues raised by biochemical studies are whether phosphorylation of caldesmon occurs *in vivo*, which protein kinase catalyses the phosphorylation, and whether phosphorylation regulates the inhibitory effect of caldesmon on the cross-bridge cycle. Recent studies of vascular smooth muscle show phosphorylation of caldesmon occurs *in vivo* at proline-directed sites indicative of phosphorylation by a member of the $p34^{cdc2}$ or MAP kinase families of proline-directed serine/threonine protein kinases (Adam & Hathaway, 1993).

To address the issue of *in vivo* phosphorylation of caldesmon in gastrointestinal smooth muscle, caldesmon phosphorylation and MAP kinase activities were measured during contraction of circular smooth muscle of canine proximal colon. We find caldesmon is phosphorylated and MAP kinases are activated during contraction induced by acetylcholine, neurokinin A, depolarization or phorbol dibutyrate. Phosphorylation of purified caldesmon by ERK1 MAP kinase reversed the inhibitory effect of caldesmon on actin sliding velocity. The results are consistent with the hypothesis that ERK MAP kinases are activated during colonic smooth muscle contraction to phosphorylate caldesmon. Phosphorylation of caldesmon may contribute to regulation of cross-bridge function by reversing the inhibitory effect of caldesmon on actomyosin ATPase.

METHODS

Tissue preparation, concentration–response methods and fura-2 loading

The circular muscle layer of the proximal colon was isolated from adult mongrel dogs of either sex killed with pentobarbitone sodium (45 mg kg^{-1}, I.V.). Muscle strips (20 × 2 × 0·4 mm) were cut parallel to the long axis of circular muscle fibres. The longitudinal muscle layer and mucosa were removed but the submucosal layer was left attached to the strips of circular muscle. Strips were equilibrated for 30–60 min in Krebs–Ringer bicarbonate (KRB) solution bubbled with 97% O_2–3% CO_2 at 37 °C. Muscle strips to be loaded with fura-2 were incubated in phosphate-free KRB solution containing 10 μM fura-2 acetoxymethyl ester (fura-2 AM) and 0·025% cremophor EL for 3–4 h at 37 °C. Each fura-2-loaded muscle was washed for 30 min with KRB solution at 37 °C and attached to a strain gauge transducer to monitor isometric contraction. Changes in $[Ca^{2+}]_i$ were measured simultaneously with contraction by illuminating muscle strips alternately (60 Hz) at excitation wavelengths of 340 ± 10 nm and 380 ± 10 nm. The intensity of 500 ± 20 nm fluorescence induced by 340 nm excitation (F_{340}) and that induced by 380 nm excitation (F_{380}) was measured using a CAF-102 fluorimeter (Japan Spectroscopic Co., Tokyo). Fluorescence data were expressed as the ratio $R_{340/380}$ calculated by an on-board computer in the fluorimeter.

Tissue $^{32}P_i$ labelling

Adult mongrel dogs of either sex were killed by barbiturate overdose and the circular smooth muscle was isolated from the proximal colon and placed in physiological salt solution (PSS) buffered with 3(*N*-morpholino)propane sulphonic acid (Mops) (Gerthoffer *et al.* 1991). Smooth muscle strips were incubated overnight at 25 °C in oxygenated PSS containing 0·1 mCi ml^{-1} $^{32}P_i$. Tissues strips were warmed to 37 °C in PSS and stimulated three times for 5 min with 70 mM K^+ to produce stable reproducible contractions. They were then stimulated with 10 μM acetylcholine and frozen by immersion in acetone–5% trichloroacetic acid (TCA) cooled on dry ice to −80 °C. After allowing the strips to thaw at room temperature for 30 min, TCA was removed by incubation in acetone at room temperature for an additional 30 min.

Caldesmon phosphorylation

Acetone-treated strips were dried, weighed and bisected for gel purification of caldesmon and monophosphorylated myosin light chains. Myosin light chains were extracted by vigorous shaking in 25 μl urea extraction buffer (mg dry muscle)$^{-1}$ for 90 min. Urea extraction buffer was composed of 8 M urea, 20 mM tris(hydroxymethyl)aminomethane (Tris), 22 mM glycine, 10 mM dithiothreitol, 5 mM EGTA, 2 mM Na_2EDTA, and 5 mM NaF. Caldesmon was extracted from the remaining muscle sample by homogenization (50 μl (mg dry muscle)$^{-1}$) in 9·5 M urea, 5% β-mercaptoethanol, 0·136% Ampholines (pH 3·5–10), 0·012% Ampholines (pH 4–6·5), 0·012% Ampholines (pH 5–8) and 0·5% sodium dodecylsulphate (SDS). Tissue homogenates were then diluted 1 : 1 with 2·5% Triton X-100, 9·5 M urea, 5% β-mercaptoethanol, 0·136% Ampholines (pH 3·5–10), 0·012% Ampholines (pH 4–6·5) and 0·012% Ampholines (pH 5–8). Caldesmon was isolated by non-equilibrium pH gel electrophoresis according to O'Farrell, Goodman & O'Farrell (1977) using a 0·15 × 13 cm tube gel in the pH dimension.

Proteins were separated according to molecular mass using a 0·15 × 14 × 10 cm stacking gel (4·75% acrylamide) and a 0·15 × 14 × 20 cm separating gel (12% acrylamide). SDS–PAGE gels were stained overnight with 0·4% Coomassie Blue R-250, 10% isopropanol, 25% acetic acid. After complete destaining, caldesmon content (moles) was determined by laser densitometry using purified bovine aorta caldesmon run on a separate gel (SDS–PAGE, 8% acrylamide) as the dye-binding standard. Dye binding was linear over a range of 0·1–6 μg bovine caldesmon ($r^2 > 0·95$). An example of a two-dimensional separation is shown in Fig. 1. Caldesmon was identified on Western blots of two-dimensional gels using polyclonal anti-caldesmon antibodies prepared in our laboratory as described below.

Phosphorylation stoichiometry (mol P_i (mol caldesmon)$^{-1}$) was determined by cutting the caldesmon band from the SDS–PAGE gel, incubating at least 2 h in 5 ml Bio Safe II scintillation cocktail (RPI Inc., Mount Prospect, IL, USA) and counting. Monophosphorylated myosin light chains were isolated from the same radiolabelled muscles by glycerol polyacrylamide gel electrophoresis (Hathaway & Haeberle, 1985), incubated in scintillation cocktail and counted. Counts min^{-1} in monophosphorylated myosin light chains were used to estimate specific activity of endogenous [γ-^{32}P]ATP (c.p.m. (mol P_i)$^{-1}$) according to Ludowyke, Peleg, Beaven & Adelstein (1989): mol P_i (mol caldesmon)$^{-1}$ = (c.p.m. of the caldesmon bands/(c.p.m. (mol P_i)$^{-1}$))/mol caldesmon.

MAP kinase assays

A gel reconstitution method was adapted from the methods of Geahlen, Anostario, Low & Harrison (1986) to assay kinase activities in tissue extracts. Muscle strips were frozen by immersion in cold (−80 °C) acetone containing 1 mM NaF and homogenized in 0·1 ml SDS extraction buffer (mg dry weight)$^{-1}$. SDS extraction buffer was composed of 0·1% SDS, 10% glycerol, 5 mM NaF, 1 μM leupeptin, 10 mM EGTA, 1 mM EDTA and 1 mM PMSF. A small sample of the extract was removed for assaying protein concentration by the bicinchonninic acid method using bovine serum albumin dissolved in SDS extraction buffer as the standard. DTT (1 mM) and Bromphenol Blue (0·05%) was added to the remaining extract and 10–25 μg total protein was loaded onto SDS–PAGE mini gels (12% acrylamide) containing 0·5 mg ml^{-1} myelin basic protein (MBP) or, in some experiments, 0·2 mg ml^{-1} bovine aorta caldesmon. Gels were washed in 50 mM Tris (pH 8), 20% isopropanol. Isopropanol was removed by washing in 50 mM Tris, 5 mM β-mercaptoethanol (BME). Proteins were then denatured with 6 M guanidine. Guanidine was removed by washing with 0·04% Tween-40, 50 mM Tris, 5 mM β-mercaptoethanol. After equilibration in 40 mM Hepes (pH 8), 10 mM $MgCl_2$, 2 mM DTT, the kinase reaction was carried out at 22 °C in 10 ml of 40 mM Hepes (pH 8), 10 mM $MgCl_2$, 0·5 mM EGTA, 0·1 mM ATP (5 μCi [γ-^{32}P]ATP ml^{-1}). The kinase reaction was stopped after 2 h. and excess radioactivity removed by washing the gels extensively with 5% trichloracetic acid, 10 mM sodium pyrophosphate. The gels were washed for 4 × 15 min followed by washing overnight with gentle shaking. Extensive washing was critical for reducing the background radiation and obtaining adequate resolution of phosphorylated bands. The gels were then washed for 30 min in 3% glycerol, air dried between sheets of cellophane and subjected to autoradiography of Fuji RX film for 24–36 h at −70 °C using intensifying screens. Each sample was assayed in duplicate on separate gels. Kinase activity was measured by densitometry of the 38 and 44 kDa phosphorylated MBP bands (absorbance units × mm^2) and normalized to total protein loaded in each lane of the gel. Protein content of the homogenates was assayed by the bicinchoninic acid method according to Stoscheck (1990).

MAP kinases were assayed in tissue homogenates and in Mono Q chromatography fractions using a specific peptide substrate (APRTPGGRR), which includes the MAP kinase phosphorylation sequence of myelin basic protein (Erickson *et al.* 1990). Total tissue MAP kinase activity was assayed in muscle strips frozen by immersion in liquid nitrogen, pulverized and homogenized in Tris–EGTA–vanadate extraction buffer, pH 7·5 (mM): 20 Tris, 5 EGTA, 1 Na_3VO_4, 20 β-glycerophosphate, 10 NaF, 1 DTT, 0·1 PMSF; and 1 μg μl^{-1} aprotinin). The extracts were clarified by centrifugation at 100 000 *g* for 10 min at 4 °C. The kinase reaction contained in 40 μl (mM): 25 Mops, 25 β-glycerophosphate, 15 $MgCl_2$, 1 EGTA, 0·1 NaF, 1 Na_3VO_4, 4 DTT, 1 substrate peptide; and 10 μl tissue extract; pH 7·2. The reaction was started by addition of 250 μM ATP (5 μCi [γ-^{32}P]ATP). After incubation at 30 °C for 4 h, two 20 μl samples were spotted on duplicate P81 phosphocellulose filter squares (Whatman Inc.), washed 5 times for 10 min each with ice-cold 0·75% H_3PO_4. After a final 95% ethanol wash for 1 min the filters were dried and counted. MAP kinase substrate peptide was synthesized by Dr Dennis McMaster, University of Calgary, Canada.

Mono Q chromatography

MAP kinase isoforms were separated by Mono Q chromatography of tissue homogenates prepared by freezing muscle strips in liquid nitrogen and homogenizing as described above. Homogenates were clarified by centrifugation at 100 000 *g* for 10 min and then loaded onto a Mono Q HR5 column (Pharmacia) equilibrated with (mM): 20 Tris (pH 7·5), 2 EGTA, 1 Na_3VO_4, 10 β-glycerophosphate, 1 DTT, 0·1 PMSF; and 1 μg ml^{-1} aprotinin. After washing with 25 ml at 1 ml min^{-1}, the column was developed with a 120 ml gradient of 0–0·4 M NaCl. Fractions (1 ml) were collected into tubes containing 50 μg bovine serum albumin as a carrier protein. Ten microlitres of each fraction was used for assaying MAP kinase as described above using the MAP kinase peptide substrate.

Immunoblotting

Proteins were transferred to nitrocellulose by electrophoresis (90 V, 4 h, 15 °C) in 25 mM Tris, 192 mM glycine, 20% methanol transfer buffer. Anti-MAP kinase polyclonal antibody (erk1-CT) was purchased from Upstate Biotechnology Inc. (UBI), Lake Placid, NY, USA. Anti-actin (A-2547) and anti-tropomyosin (T-2780) monoclonal antibodies were purchased from Sigma. Anti-calponin polyclonal antibodies were a gift of Dr M.P. Walsh, University of Calgary, Canada and anti-SM22 antibodies were a gift of Dr L.B. Smillie, University of Alberta, Canada. Anti-caldesmon polyclonal antibodies were prepared by immunizing New Zealand White rabbits with bovine aorta caldesmon. Specific polyclonal antibodies were purified by affinity chromatography with a caldesmon–Sepharose affinity column constructed using bovine aortic caldesmon which was purified according to Clark, Ngai, Sutherland, Groschel-Stewart & Walsh (1986). Proteins on Western blots were visualized using goat anti-rabbit or goat anti-mouse alkaline phosphatase secondary antibodies (Promega, Madison, WI, USA).

In vitro motility assay

Actin sliding velocity was assayed according to Shirinsky, Biryukov, Hettasch & Sellers (1992). Assays were conducted using phosphorylated turkey gizzard myosin, 1 μM turkey gizzard tropomyosin, 20 mM KCl, 5 mM $MgCl_2$, 0·1 mM EGTA, 10 mM Mops (pH 7·35), 0·02% NaN_3, 0·7% methylcellulose, 50 mM dithiothreitol, 0·1 mg ml^{-1} glucose oxidase, 2·5 mg ml^{-1} glucose, 0·02 mg ml^{-1} catalase and 2 mM Na_2ATP. Flow cells were constructed according to Shirinsky *et al.* (1992) by binding monomeric myosin to nitrocellulose-coated glass slides and monitoring movement of rhodamine–phalloidin-labelled actin filaments by enhanced

fluorescence video microscopy. Velocity ± S.D. of each filament calculated from digitized images according to Homsher, Wang & Sellers (1992). Only filaments that moved continuously with a coefficient of variation (mean ± S.D.) of 0·3 or less were included in the data analysis.

Myosin and tropomyosin used in the motility assay were purified from turkey gizzards and actin was prepared from rabbit skeletal muscle (cf. Shirinsky *et al.* 1992). Turkey gizzard caldesmon was purified by a modification of the method of Bretscher (1984). Purified turkey gizzard caldesmon was phosphorylated by purified sea star p44 MAP kinase (UBI). The reaction was carried out for 2 h at 37 °C. The kinase reaction mixture included 0·2 mg ml^{-1} caldesmon, 0·025 mg ml^{-1} sea star MAP kinase (p44), 1 mM ATP, 1 mM $MgCl_2$ and 20 mM Hepes (pH 7·4). Two parallel reactions were conducted – one contained unlabelled ATP and the other contained 100 μCi [γ-^{32}P]ATP ml^{-1}. Phosphorylation stoichiometry of radiolabelled caldesmon was determined by spotting 10 μl samples on P81 filters, washing with trichloroacetic acid and counting. Positive control samples were processed at the same time using myelin basic protein (0·2 mg ml^{-1}) as the substrate.

Solutions and drugs

The normal KRB solution used in this study contained (mM): 137·4 NaCl, 5·9 KCl, 2·5 $CaCl_2$, 1·2 $MgCl_2$, 15·5 $NaHCO_3$, 1·2 Na_2HPO_4 and 11·5 dextrose. The solution had a pH of 7·3–7·4 at 37 °C when bubbled to equilibrium with a 97% O_2–3% CO_2 gas mixture. Mops-buffered PSS contained (mM): 140 NaCl, 4·7 KCl, 1·2 $MgSO_4$, 1·6 $CaCl_2$, 1·2 Na_2HPO_4, 0·02 Na_2EDTA, 2·0 Mops, and 5·6 D-glucose. Fura-2 AM was purchased from Molecular Probes, and dissolved in dimethyl sulphoxide. Cremophor EL, acetylcholine chloride, neurokinin A and phorbol-12,13-dibutyrate (PDBu) were purchased from Sigma. PDBu was dissolved in 95% ethanol. Chemicals used for purification of contractile proteins, assaying caldesmon phosphorylation and assaying myosin light chain phosphorylation were all electrophoresis grade and were obtained from Sigma or Fisher Scientific, Santa Clara, CA, USA. Myelin basic protein, sea star ERK1 MAP kinase, anti-MEK, anti-ras, anti-raf-1, erk1-CT and erk2 antibodies were purchased from UBI.

Statistical methods

Results are presented as means ± S.E.M. Differences between treatment means were evaluated by Student's t test for paired or unpaired data as appropriate. Differences in median actin sliding velocity were tested for using Kruskal–Wallis ANOVA on ranks. A probability of $P < 0·05$ was accepted as a significant difference.

RESULTS

Caldesmon phosphorylation during acetylcholine-induced contraction

To assay phosphorylation of caldesmon during contraction, muscle strips were metabolically labelled with ^{32}P for 16 h prior to stimulation with 10 μM acetylcholine. Unstimulated muscles (basal) and muscles stimulated for 2 min with 10 μM acetylcholine were frozen by immersion in acetone–TCA, and homogenized, and radiolabelled caldesmon isolated by two-dimensional non-equilibrium pH gel electrophoresis (NEPHGE) as shown in Fig. 1. To calculate molar stoichiometry of caldesmon phosphorylation, protein content of the caldesmon band was determined by densitometry prior to cutting out the band for scintillation counting. A dye-binding standard curve was generated by running a dilution series of purified bovine aorta caldesmon on separate SDS–polyacrylamide gels. Coomassie Blue R250 binding was linear from 0·1 to 6 μg of bovine caldesmon ($r^2 > 0·95$). Monophosphorylated myosin light chains (0·2–3 μg) were isolated from the same muscles by glycerol–acrylamide gel electrophoresis (Hathaway & Haeberle, 1985). Protein content and identity of the monophosphorylated 20 kDa light chain bands were determined using purified chicken gizzard 20 kDa myosin light chains. Dye binding was linear from 0·2–10 μg protein ($r^2 > 0·9$). Radioactivity in the monophosphorylated myosin light chains (1 mol ^{32}P (mol protein)$^{-1}$) was used to estimate the specific activity of the λ phosphate of metabolically formed [^{32}P]ATP and the number of moles of ^{32}P incorporated into caldesmon (Ludowyke *et al.* 1989). Caldesmon stoichiometry was then calculated by dividing the number of moles P_i by the number of moles caldesmon determined by densitometry of NEPHGE gels.

Acetylcholine (10 μM) induced an initial rapid, large amplitude transient contraction (Fig. 2*A*) followed by a series of slow phasic contractions that persisted until the agonist was removed. Molar stoichiometry of caldesmon phosphorylation was assayed in unstimulated muscles and after 2 min stimulation with acetylcholine (Fig. 2*B*).

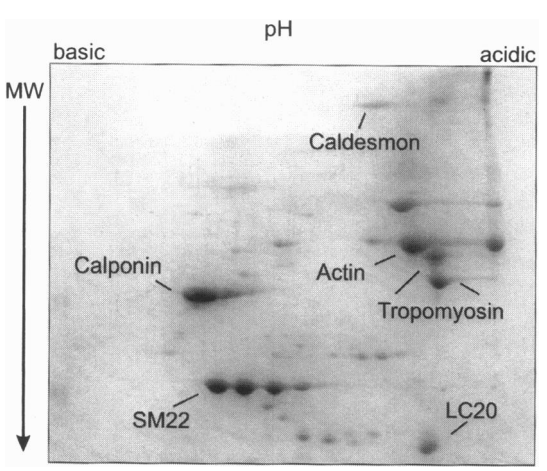

Figure 1. Isolation of caldesmon by two-dimensional non-equilibrium gel electrophoresis

A strip of circular smooth muscle from canine colon was homogenized in 9·5 M urea extraction buffer as described in Methods. Approximately 50 μg of protein was analysed by two-dimensional non-equilibrium gel electrophoresis according to O'Farrell *et al.* (1977). The separator gel from the second dimension (SDS–PAGE, 12% acrylamide) stained with Coomassie Blue R250 is shown. Labelled proteins were identified on separate gels by Western blotting using antibodies specific for calponin, caldesmon, smooth muscle actin, smooth muscle tropomyosin, SM22 and 20 kDa myosin light chains (LC20). MW, molecular weight.

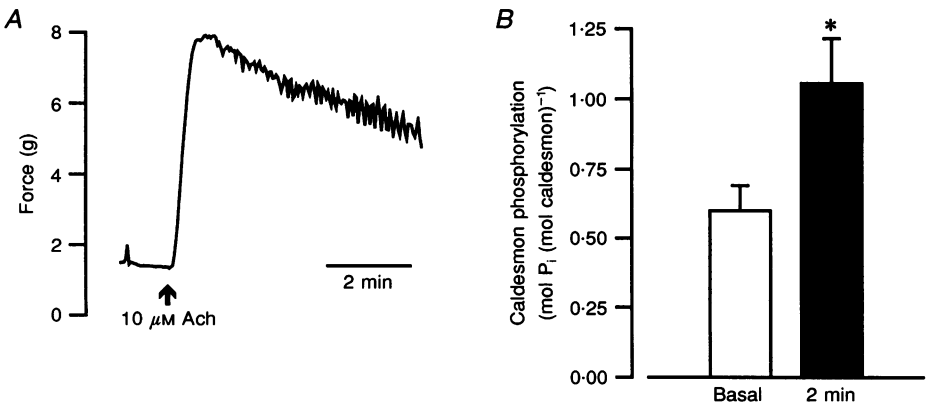

Figure 2. Caldesmon phosphorylation during acetylcholine-induced contraction of colonic smooth muscle

A, representative force trace illustrating a contraction induced by 10 μM acetylcholine (ACh). *B*, the mean molar stoichiometry of caldesmon phosphorylation (± S.E.M.) measured in unstimulated muscles (□) and in muscles stimulated for 2 min with ACh (■). Phosphorylation increased significantly in ACh-stimulated muscles (* $P < 0{\cdot}05$, $n = 5$).

Caldesmon phosphorylation increased significantly after 2 min from basal levels of 0·6 ± 0·07 to 1·1 ± 0·15 mol P_i (mol caldesmon)$^{-1}$.

MAP kinase expression and enzyme activities in colonic smooth muscle

To test the notion that MAP kinases catalyse phosphorylation of caldesmon in intact muscle we used an anti-ERK1 antibody (erk1-CT) to probe Western blots of tissue homogenates. The erk1-CT antibody is a polyclonal peptide antibody designed to recognize the carboxy terminus of ERK1 MAP kinases. It cross-reacts with both ERK1 and ERK2 kinases. Figure 3*A* shows total SDS-extracted proteins resolved on a 12% acrylamide SDS–PAGE gel and stained with Coomassie Blue R250. Figure 3*B* is a Western blot probed with anti-MAP kinase antibody demonstrating immunoreactive proteins with apparent molecular masses of 44 and 38 kDa. The 38 kDa protein also cross-reacted with a specific antibody to ERK2 MAP kinase (erk2), but the 44 kDa protein did not (data not shown). Kinase activities of the 44 and 38 kDa proteins were assayed by a gel reconstitution method in which myelin basic protein (MBP) was incorporated into the gel. Figure 3*C* and *D* are autoradiographs illustrating the MBP kinase activities detected by autoradiography of gels in which proteins were renatured and reacted with [^{32}P]ATP. Figure 3*C* shows an extract of an unstimulated muscle strip, and Fig. 3*D* shows enhanced kinase activity of a muscle stimulated for 5 min with 10 μM acetylcholine. MBP kinase activities were detected at 38 and 44 kDa, which was consistent with antibody labelling of Western blots. MBP kinase activities were also observed at 54 and 57 kDa. These activities did not

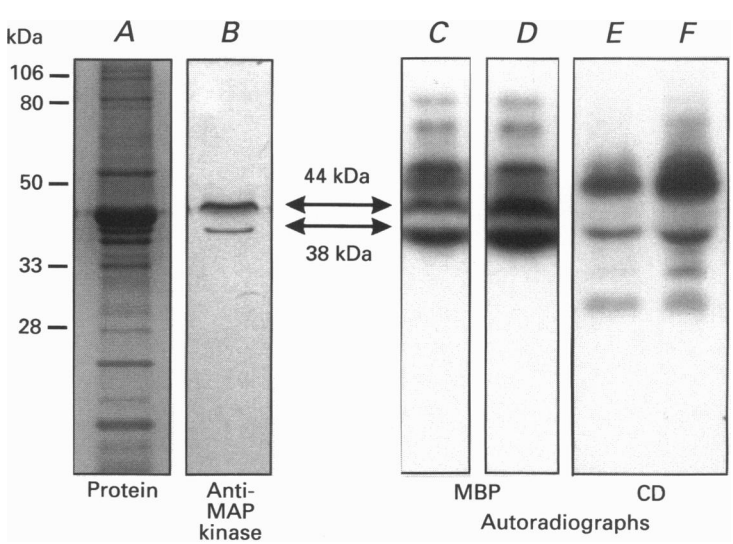

Figure 3. Gel reconstitution assay of MBP kinase activities of colonic smooth muscle

Colonic smooth muscles were homogenized in SDS and proteins resolved by SDS–PAGE (12% acrylamide). *A*, total proteins stained with Coomassie Blue. Molecular mass markers were BioRad-prestained low range markers. *B*, Western blot of SDS homogenate probed with a polyclonal anti-MAP kinase antibody (erk1-CT). *C* and *D*, autoradiographs showing protein kinase activities detected in a 12% SDS-polyacrylamide gel containing 0·5 mg ml^{-1} MBP; *C*, unstimulated muscle; *D*, 10 μM ACh, 5 min. *E* and *F*, autoradiographs of kinase activities assayed using 0·2 mg ml^{-1} bovine aorta caldesmon (CD) incorporated into the gel; *E*, unstimulated muscle; *F*, 10 μM ACh, 5 min. Samples in panels *C*–*E* were exposed to X-ray film and digital imaging under identical conditions.

cross-react with anti-MAP kinase antibodies and are unidentified. Further studies are required to positively identify the higher molecular mass kinase activities, but it is clear that the 38 and 44 kDa MBP kinase activities comigrate with 38 and 44 kDa MAP kinase immunoreactive proteins. Studies described below using a more specific peptide substrate for MAP kinases also support this conclusion.

The aim of adapting the gel reconstitution assay for colonic smooth muscle extracts was to determine whether or not MAP kinase activity increases during contraction. To use the method quantitatively we needed to established the linearity of the assay with respect to enzyme and substrate concentration as well as the degree of background phosphorylation. Background phosphorylation could occur in the absence of added substrate because of autophosphorylation of MAP kinases or phosphorylation of other comigrating endogenous substrates. To assess the degree of background phosphorylation, SDS extracts of the same muscles were run simultaneously on gels with and without MBP and the kinase assay performed as described above. Gels without MBP had no detectable bands at 44 kDa and barely detectable background phosphorylation at 38 kDa comprising no more than 6% of the signal when compared with the gels containing MBP. There was also a linear relationship between total protein loaded and the band volume (absorbance × area) of the 44 and 38 kDa kinases as determined by laser densitometry. Kinase activity was linear over a 4-fold range of protein content (6–24 μg) and over about a 10-fold change in band volume (absorbance × area) on the autoradiograph. In all subsequent studies, the protein loaded on each gel and the exposure time of the autoradiographs (24–36 h) were controlled so all samples fell within the linear ranges of the assay. Adjusting exposure time of the autoradiograph is critical because of the narrow dynamic range of X-ray film, and because overexposure reduces resolution of the individual kinase activities.

Endogenous caldesmon kinase activities in colonic smooth muscle

The gel reconstitution technique was also used to determine whether the 38 and 44 kDa MBP kinase activities could phosphorylate caldesmon. When bovine aorta caldesmon was incorporated into gels instead of MBP, caldesmon kinase activities were detected by autoradiography at approximately 50, 44 and 38 kDa (Fig. 3*E* and *F*). Figure 3*E* shows caldesmon kinase activity of an unstimulated muscle strip and Fig. 3*F* shows caldesmon kinase activity in a tissue stimulated for 2 min with 10 μM acetylcholine. Although the 44 kDa kinase activity is very faint in Fig. 3*E*, longer exposure times revealed a clear band. There were also some faint kinase activities detected at 28 and 32 kDa. The identities of these kinase activities are unknown. The results suggest ERK MAP kinases are expressed in differentiated colonic smooth muscle, and the endogenous 38 and 44 kDa MAP kinases are activated by acetylcholine and can phosphorylate caldesmon. These kinases will be referred to as ERK1 (44 kDa) and ERK2 (38 kDa) kinases according to current conventions for describing MAP kinases.

MAP kinase assay in tissue homogenates

To fortify our conclusion that acetylcholine activates ERK MAP kinases the synthetic peptide APRTPGGRR was used to assay kinase activity of tissue homogenates and in

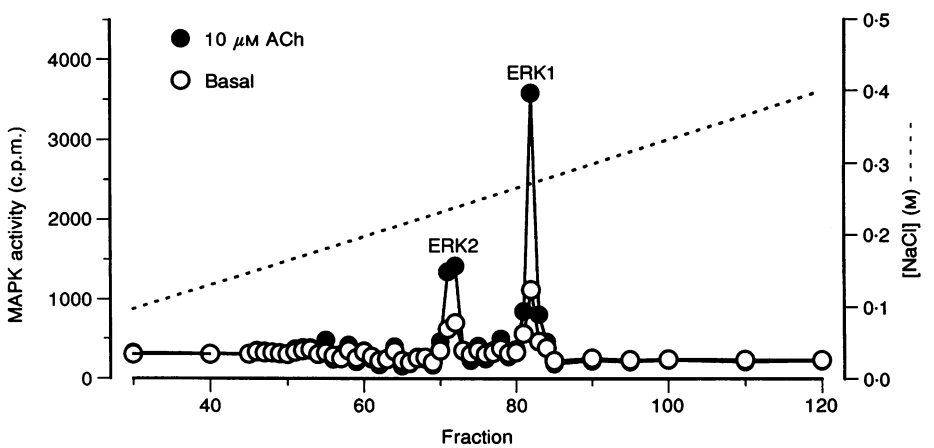

Figure 4. Separation of ERK1 and ERK2 MAP kinases by Mono Q chromatography

Colonic smooth strips (75 mg each) were frozen in liquid nitrogen in the resting state (○, Basal) or after 2 min stimulation with 10 μM acetylcholine (●, 10 μM ACh). Proteins extracted with Tris–EGTA–vanadate extraction buffer were separated by Mono Q chromatography as described in Methods. The column (Mono Q HR5, Pharmacia) was developed with a 120 ml linear NaCl gradient (0–0·4 M) at 1 ml min^{-1}. Fractions (1 ml) eluting between 0·1 and 0·4 M NaCl were assayed for MAP kinase activity using the MAP kinase specific peptide substrate as described in Methods. Kinase peaks were identified by immunoblotting using antibodies against ERK1 and ERK2 (erk1-CT and erk2).

partially purified kinase fractions obtained by Mono Q chromatography. The peptide contains the MAP kinase phosphorylation sequence of myelin basic protein (Erickson *et al.* 1990). Total tissue homogenates were obtained from muscles frozen in liquid nitrogen and kinase activity assayed using P81 filters as described in Methods. Stimulation with 10 μM acetylcholine for 2 min increased total MAP kinase activity in the homogenate from basal activity of $1{\cdot}7 \pm 0{\cdot}39$ to $3{\cdot}7 \pm 1{\cdot}2$ nmol P_i (mg protein)$^{-1}$, which is a 2·1-fold increase over basal activity. This is consistent with the increase in ERK1 and ERK2 MAP kinase activities observed using the gel reconstitution assay (Fig. 3).

Separation of ERK1 and ERK2 MAP kinases by Mono Q chromatography

Mono Q chromatography will resolve ERK1 and ERK2 kinases isolated from crude extracts of mammalian cells, and has been used to demonstrate agonist-induced activation of these kinases in smooth muscle (Adam, Franklin, Raff & Hathaway, 1995). Because assaying kinase activity of a homogenate estimates the sum of all MAP kinase activities it is important to assess activation of the ERK1 and ERK2 isoforms individually. We used Mono Q chromatography to separate the isoforms and the specific peptide substrate to verify activation of each enzyme by acetylcholine. Colonic muscle strips (50 mg) were frozen in the unstimulated state or frozen after 2 min stimulation with 10 μM acetylcholine, homogenized and subjected to Mono Q chromatography. Fractions eluting between 0·1 and 0·4 M NaCl were assayed for MAP kinase activity (Fig. 4). The ERK2 MAP kinase activity eluted at about 0·25 M NaCl and the ERK1 MAP kinase activity eluted at about 0·28 M NaCl. Western blots of column fractions probed with erk1-CT and erk2 antibodies were used to identify the kinases eluting at these salt concentrations (data not shown). Acetylcholine stimulation produced a 1·6-fold activation of the ERK2 MAP kinase and a 2·1-fold increase of ERK1 MAP kinase activity.

Activation of ERK1 and ERK2 MAP kinases by acetylcholine

If ERK MAP kinases catalyse phosphorylation of caldesmon *in vivo*, the activity of the enzymes should increase in response to acetylcholine, which increases phosphorylation of caldesmon. We assayed relative MBP kinase activities of the ERK1 and ERK2 MAP kinases by freezing muscle strips at various times after adding 10 μM acetylcholine (Fig. 5). Band volumes of the ERK1 and ERK2 MAP kinase activities were corrected for protein loaded and normalized to the basal levels determined in unstimulated muscles (0 min). An example of an autoradiograph from a single experiment is shown in Fig. 5*B*. Both MAP kinase

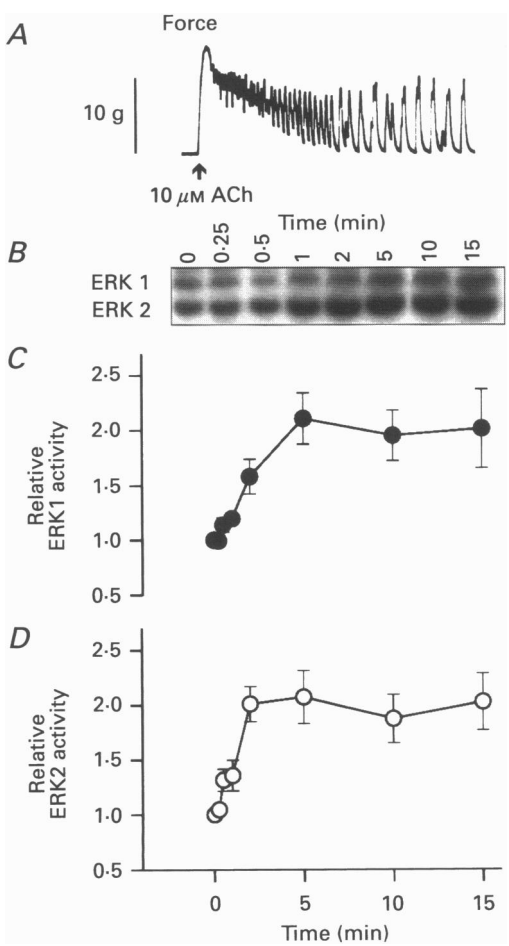

Figure 5. Activation of ERK1 and ERK2 MAP kinases by acetylcholine

A, contraction was induced with 10 μM acetylcholine. A trace from a single representative muscle is shown. *B*, muscle strips were frozen at the indicated times during contraction, and kinase activity was assayed by the gel reconstitution method using myelin basic protein as the substrate. An autoradiograph from a single experiment is shown. Relative kinase activity was determined by densitometry of the 44 and 38 kDa phosphorylated MBP bands which correspond to ERK1 and ERK2 MAP kinases, respectively. Absorbance × mm^2 was divided by total protein in the sample (10–25 μg) and normalized to the kinase activity before stimulation (0 min). Mean kinase activity of the ERK1 (●) and ERK2 (○) bands is shown in *C* and *D*. In both cases kinase activity increased significantly within 30 s ($P < 0{\cdot}05$, *t* test, $n = 10$), reached a maximum level within 5 min and remained activated up to 15 min.

activities increased rapidly compared with basal levels (Fig. 5*C* and *D*). As shown in Fig. 5*B*, there was significant basal kinase activity which on average doubled within 2–5 min of stimulation with acetylcholine and remained at that level for at least 15 min.

After about 5 min the contractile response to a high concentration of acetylcholine (Fig. 4*A*) oscillates nearly in phase with calcium and myosin phosphorylation transients (Gerthoffer *et al.* 1991). These transients are thought to be controlled by co-ordinated changes in cation currents which result in oscillations of $[Ca^{2+}]_i$ that produce transient activation and inactivation of myosin light chain kinase (Gerthoffer *et al.* 1991; Sanders, 1992). To determine whether MAP kinase activities oscillate in a similar manner we assayed kinase activities at the base and the peak of phasic contractions. Sets of four muscle strips from nine animals were placed in isometric muscle baths to measure contraction. An unstimulated strip was frozen to assay basal kinase activity (Fig. 6). The other three strips were stimulated with 10 μM acetylcholine for 15 min. One strip was frozen at the minimum force between contractions, a second strip at the peak of a phasic contraction, and the third after the phasic contraction subsided (Fig. 6, top). There was no significant difference in ERK2 MAP kinase activity during the course of a phasic contraction (Fig. 6, bottom). The results suggest MAP kinase activity does not oscillate significantly during phasic contractions induced by acetylcholine in contrast to other signal transduction events in this muscle.

Activation of MAP kinase by neurokinin A

Neurokinin A is an excitatory agonist in canine colonic smooth muscle that at high concentrations induces a brief tonic contraction followed by phasic contractions (Fig. 7) similar in amplitude and duration to those induced by an equieffective concentration of acetylcholine (cf. Figs 2 and 5). Because neurokinin A may play an important role in enhancing colonic motility we tested the notion that it activates MAP kinase. SDS extracts from unstimulated muscles (basal) and muscles stimulated with 0·1 μM neurokinin A for 30 s or 1 min were assayed for MAP kinase activity by the gel reconstitution method. The ERK2 MAP kinase activity increased rapidly during the initial tonic contraction with kinase activity at 1 min being significantly greater than basal. The ERK2 MAP kinase activity was also measured at the base and peak of phasic contractions to test for oscillation of kinase activity. There was no significant change in ERK2 MAP kinase activity during phasic contractions (data not shown), which is similar to results described above for phasic contractions induced by acetylcholine (Fig. 6). It appears that MAP kinases are activated by at least two excitatory neurotransmitters in colonic smooth muscle, but kinase activity does not oscillate detectably during phasic contractions in contrast with other signal transducers such as cation currents, $[Ca^{2+}]_i$ and myosin light chain phosphorylation.

Activation of MAP kinase by phorbol ester

Protein kinase C has been implicated as an upstream regulator that activates MAP kinases possibly via activation of raf-1 kinase (Kolch *et al.* 1993). We tested for an effect of PKC activation by stimulating potassium-depolarized colonic smooth muscle strips with 0·1 μM phorbol dibutyrate and assaying MAP kinase activity. Depolarization of canine colonic smooth muscle with 70 mM K^+ induced an initial transient contraction followed by significant maintained tone (Fig. 8*A*). In a previous study, we found phorbol dibutyrate potentiated the tonic potassium-induced contraction while causing a decrease in $[Ca^{2+}]_i$ and no change in myosin light chain phosphorylation (Sato, Leposavic,

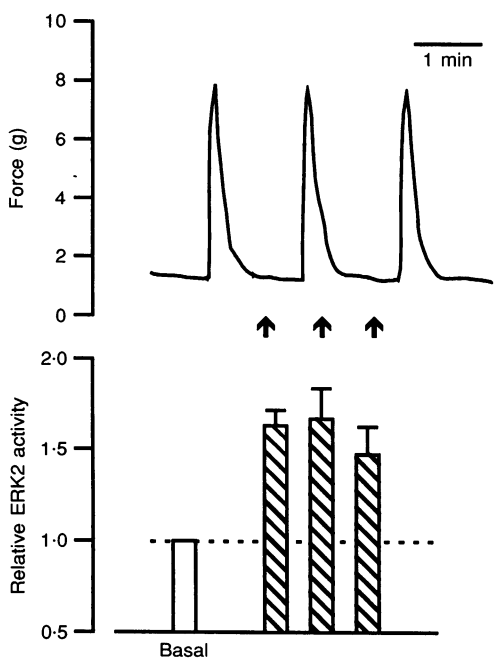

Figure 6. MAP kinase activities during phasic contraction of colonic smooth muscle

The upper panel is an example of slow phasic contractions induced by stimulation with 10 μM acetylcholine for 15 min. MAP kinase activities were assayed during phasic contractions by freezing separate muscle strips from each of 9 animals as indicated by the arrows below the force trace. The ERK1 and ERK2 MAP kinase activities were assayed by the gel reconstitution method with MBP as the substrate. Relative ERK2 MAP kinase activity (lower panel) was determined by densitometry of the 38 kDa phosphorylated band corrected for protein loaded on the gel and normalized to basal kinase activity of unstimulated muscle strips (□ and dashed line). Kinase activity was significantly greater than basal in the presence of acetylcholine (▨), but there was no significant difference in kinase activity between the peak of a phasic contraction and at the minimum force between contractions (Kruskal–Wallis ANOVA, $P = 0{\cdot}4$, $n = 9$).

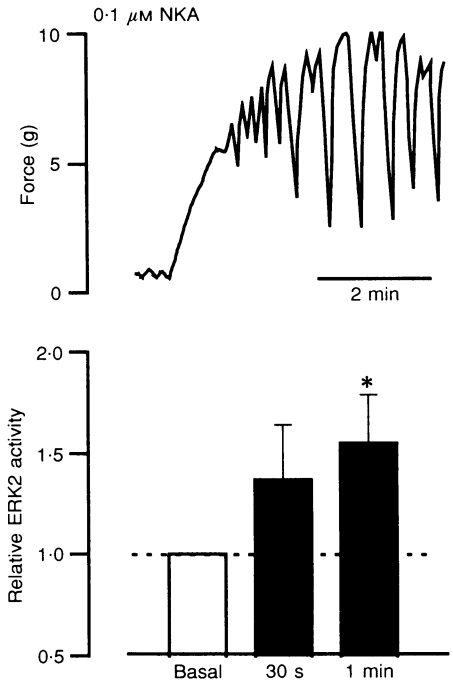

Figure 7. Activation of MAP kinase by neurokinin A

The top panel is a representative contractile response of colonic smooth muscle to stimulation with 0·1 μM neurokinin A (NKA). The bottom panel is mean ERK2 MAP kinase activity assayed by the gel reconstitution assay. Kinase activities were corrected for protein and normalized to basal (unstimulated) activity. Relative kinase activity increased significantly after 1 min stimulation (* $P < 0.05$, Student's t test, $n = 6$).

Publicover, Sanders & Gerthoffer, 1994). This experiment was repeated using a muscle loaded with fura-2 as shown in Fig. 8*B*. When 0·1 μM phorbol dibutyrate was added 5 min after placing the muscle in 70 mM K^+, force increased but the fura-2 fluorescence ratio ($R_{340/380}$) decreased. Potentiation of contraction without a concomitant increase in $[Ca^{2+}]_i$ has been described as sensitization of the contractile system to Ca^{2+}. Because the molecular mechanisms of contractile system sensitization are still undefined we tested the notion that phorbol dibutyrate potentiates contraction, at least in part, by increasing MAP kinase activity. Potassium depolarization alone increased ERK2 MAP kinase activity by 1·3 ± 0·11-fold after 5 min and 1·5 ± 0·15-fold after 10 min (▨ in Fig. 8*C*). Addition of

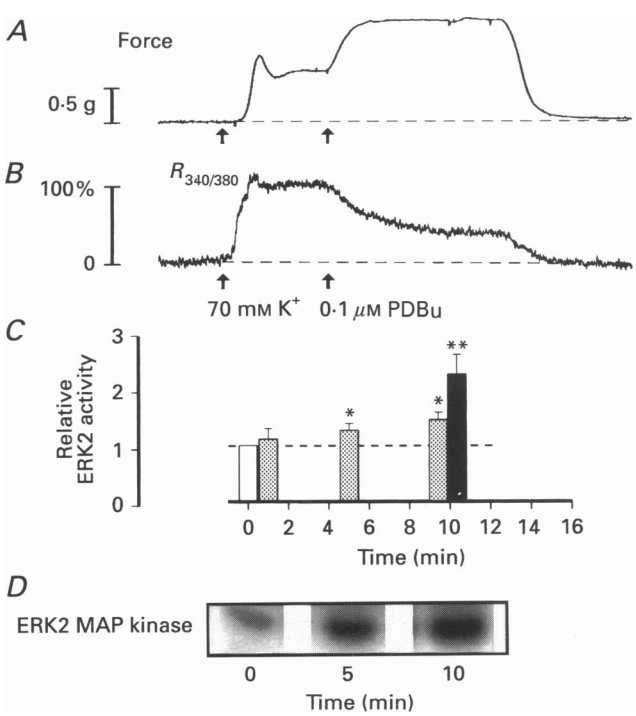

Figure 8. Activation of ERK2 MAP kinase by phorbol dibutyrate

Contraction of colonic smooth muscle was induced by 70 mM K^+ for 5 min followed by addition of 0·1 μM phorbol dibutyrate (PDBu) to produce potentiation of contraction. Force (*A*) and fura-2 fluorescence ratio (*B*, $R_{340}/_{380}$) were measured simultaneously to illustrate the dissociation of force and $[Ca^{2+}]_i$. MAP kinase activity was assayed in muscles treated as in *A* and frozen at the times indicated on the abscissa of *C*. Mean kinase activities were normalized to basal kinase activity of unstimulated muscles (□, *C*, $n = 10$). Kinase activity was measured after 1, 5 and 10 min in 70 mM K^+ (▨, *C*, $n = 10$), and after 5 min exposure to 0·1 μM PDBu (■, *C*, $n = 7$). Potassium depolarization did not significantly increase MAP kinase activity at 1 min, but did increase normalized kinase activity significantly at 5 and 10 min to 1·3 ± 0·1- and 1·5 ± 0·15-fold over basal activity (* $P < 0.05$, Students t test). Addition of 0·1 μM PDBu after 5 min treatment with depolarizing solution increased relative kinase activity further to 2·3 ± 0·35-fold over basal, which was significantly greater than treatment with potassium alone (** $P < 0.05$). Autoradiographs of the ERK2 MAP kinase activity of a single experiment are shown in *D*. The band labelled 10 min is treatment with 70 mM K^+–0·1 μM PDBu.

0·1 μM phorbol dibutyrate after 5 min in potassium depolarizing solution increased ERK2 MAP kinase activity at 10 min to 2·2 ± 0·35-fold over basal activity (■, Fig. 8*C* and the panel labelled 10 min in Fig. 8*D*). The results show that potentiation of contraction by phorbol dibutyrate is associated with increased MAP kinase activity.

Effect of phosphorylation of caldesmon on actin sliding velocity

There are several reports that micromolar concentrations of caldesmon inhibit actin sliding velocity in the *in vitro* motility assay (Okagaki, Higashi-Fujime, Ishikawa, Takano-Ohmuro & Kohama, 1991; Shirinsky *et al.* 1992; Haeberle, Trybus, Hemric & Warshaw, 1992). It is also known that phosphorylation of caldesmon reverses the inhibitory effect of caldesmon on actomyosin ATPase *in vitro* (Ngai & Walsh, 1987). Because caldesmon was phosphorylated and MAP kinase activated during colonic smooth muscle contractions it was important to determine whether phosphorylation of caldesmon by MAP kinase could reverse the inhibition of actin sliding velocity. Using the sliding filament assay of Shirinsky *et al.* (1992) we found that 3 μM caldesmon shifted the frequency distribution toward slower velocities compared with control (Fig. 9). Median velocity decreased from a control value of 0·71 to 0·32 μm s^{-1}. Phosphorylation of caldesmon to 0·7 mol P_i (mol caldesmon)$^{-1}$ with sea star ERK1 MAP kinase partially reversed the inhibitory effect on sliding velocity, shifting the frequency distribution of velocities significantly to the left compared with filaments containing unphosphorylated caldesmon. Median velocity in the presence of phosphorylated caldesmon was 0·54 μm s^{-1}. The results show that when caldesmon is phosphorylated by MAP kinase the inhibitory effect on actomyosin function is reversed. This is consistent with a regulatory role for caldesmon *in vivo* that is controlled by the MAP kinase signal transduction pathway.

DISCUSSION

Excitatory stimuli produce enhanced gastrointestinal motility through electrical excitation of smooth muscle cells of the muscularis. Many studies of smooth muscle contractile proteins indicate that contraction is a result of increased $[Ca^{2+}]_i$ leading to activation of myosin light chain kinase and phosphorylation of the 20 kDa myosin light chains. However, not all experiments are consistent with myosin phosphorylation being the sole regulatory mechanism in smooth muscles, which has prompted the idea that actin-binding proteins including caldesmon exert some regulatory influence. Purified caldesmon binds to actin, tropomyosin and myosin, inhibits actin-activated myosin MgATPase activity, and is a substrate for several protein kinases. Phosphorylation of caldesmon by CaM kinase II disinhibits actomyosin ATPase activity (Ngai & Walsh, 1987), and abolishes the interaction of caldesmon with myosin (Sutherland & Walsh, 1989). PKC phosphorylation of the

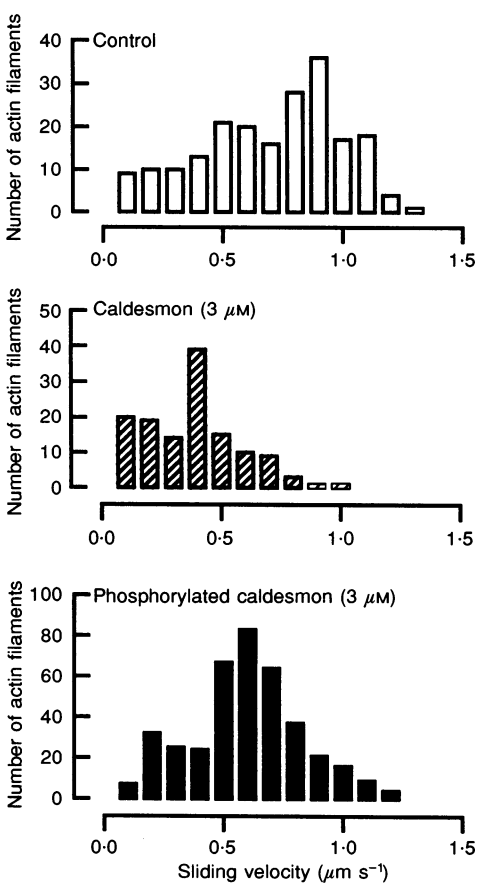

Figure 9. Effects of phosphorylation of caldesmon by MAP kinase on actin sliding velocity

Sliding velocity was assayed using phosphorylated turkey gizzard myosin and rabbit skeletal muscle actin labelled with rhodamine–phalloidin. The top panel shows the frequency distribution of sliding velocities of control actin filaments composed of 2 μM actin and 0·8 μM tropomyosin. The middle panel shows the frequency distribution of velocities in the presence of 3 μM caldesmon, and the bottom panel shows sliding velocity in the presence of caldesmon phosphorylated to 0·7 mol P_i (mol caldesmon)$^{-1}$ with sea star p44 MAP kinase. Median velocities were all significantly different ($P < 0·05$, Kruskal–Wallis ANOVA): Control, 0·71 μm s^{-1}; caldesmon, 0·32 μm s^{-1}; phosphorylated caldesmon, 0·54 μm s^{-1}.

carboxy-terminal region of caldesmon reduces inhibition of actin-activated ATPase activity (Tanaka *et al.* 1990). These results suggested caldesmon regulates smooth muscle contraction and have stimulated further interest in the functional effects of caldesmon phosphorylation *in vivo*.

Caldesmon phosphorylation has been shown by several investigators to occur in intact smooth muscle. Park & Rasmussen (1986) reported that caldesmon is phosphorylated in intact tracheal smooth muscle but the rate and stoichiometry of phosphorylation was not determined. Subsequently, Adam and co-workers demonstrated that caldesmon is phosphorylated during contraction of porcine carotid artery, and that sites phosphorylated in intact fibres are distinct from the sites phosphorylated *in vitro* by PKC or CaM kinase II (Adam, Haeberle & Hathaway, 1989; Adam & Hathaway, 1993). This indicates that phosphorylation is catalysed by a different kinase *in vivo*. Phorbol ester treatment resulted in phosphorylation of two serine residues, one corresponding to S759 and the other corresponding to S789 of human caldesmon (Adam, Gapinski & Hathaway, 1992). Both phosphorylatin sites are immediately adjacent to proline residues meaning phosphorylation is catalysed *in vivo* by a proline-directed kinase such as $p34^{cdc2}$ kinase or a MAP kinase. Both sites are near the carboxy-terminal domain of caldesmon, which interacts with tropomyosin and actin.

In vitro phosphorylation by $p34^{cdc2}$ kinase occurs at five major sites, all located in the carboxy-terminal actin-binding domain of caldesmon: S582, S667, T673, T696 and S702 (Mak, Carpenter, Smillie & Wang, 1991). Phosphorylation of caldesmon by $p34^{cdc2}$ kinase *in vitro* markedly reduces its binding to actin (Mak *et al.* 1991). However, Adam & Hathaway (1993) reported no $p34^{cdc2}$ kinase immunoreactive proteins in intact, differentiated bovine aorta smooth muscle. There was a 34 kDa immunoreactive protein expressed in cultured cells, which is consistent with expression of $p34^{cdc2}$ kinase in proliferating smooth muscle. This suggests MAP kinases are more likely to be the kinases phosphorylating caldesmon in differentiated smooth muscle cells.

In colonic smooth muscle we find two enzyme activities similar to the ERK1 and ERK2 MAP kinases known to be expressed in mammalian and avian smooth muscles (Childs *et al.* 1992; Adam & Hathaway, 1993). Using the gel reconstitution method we detected kinase activities at 38 and 44 kDa that comigrate with anti-MAP kinase immunoreactive proteins (Fig. 3). Higher molecular mass kinase activities were also observed at 50 and 57 kDa, but these enzymes did not cross-react with anti-MAP kinase antibodies. Detection of additional kinase activities in a crude tissue extract is not unexpected because MBP also contains consensus phosphorylation sequences for CaM kinase II and PKC (Erickson *et al.* 1990). However, the assay is conducted in the absence of added calcium, caldmodulin or lipid cofactors which should minimize the activity of these enzymes. There are also higher molecular mass MAP kinases expressed in mammalian cells (rat ERK3 and human ERK3) that do not share the carboxy-terminal epitope recognized by erk1-CT antibody (Gonzalez, Raden, Rigby & Davis, 1992). We may be detecting kinase activity of other canine MAP kinase isoforms that are not recognized by the erk1-CT antibody. An alternative possibility is that constitutively active fragments of catalytic subunits of any number of protein kinases which can phosphorylate MBP are being detected.

MBP kinase activities at 38, 44 and 50 kDa appear to have significant caldesmon kinase activity (Fig. 3*E* and *F*). The 50 kDa caldesmon kinase activity was very prominent, and in two experiments appeared to be activated by 5 min stimulation with 1 μM carbachol (not shown). However, the identity of this kinase is currently unknown. The 38 kDa MBP kinase activity, which appears to have higher caldesmon kinase activity compared with the 44 kDa MBP kinase, is activated rapidly and remains active during acetylcholine-induced contraction. We suggest these MBP kinase activities are canine homologs of ERK1 and ERK2 kinases already described in other mammalian species. This notion is supported by separation of the 38 and 44 kDa kinases by Mono Q chromatography and the ability of both enzymes to phosphorylate a model MAP kinase substrate peptide. The degree of activation of the partially purified enzymes was about 2-fold, which is consistent with the gel reconstitution assay as well as assays of MAP kinase activity of total tissue homogenate.

Both colonic MAP kinase isoforms are activated by physiologically important stimuli including the excitatory neurotransmitters acetylcholine and neurokinin A (Figs 5 and 6). Potassium depolarization also activated the MAP kinases but more slowly than did acetylcholine or neurokinin A (Fig. 8*C*). This shows activation of MAP kinases is not limited to cholinergic stimulation but may be a general response of colonic smooth muscle cells to most or all excitatory stimuli. The most effective stimulus studied was phorbol dibutyrate, producing 2·3-fold activation of ERK2 MAP kinase (Fig. 8*C*). The response to phorbol ester is quite interesting because there was a substantial increase in force while $[Ca^{2+}]_i$ decreased. From our previous study we know under these conditions myosin phosphorylation remains constant (Sato *et al.* 1994). Therefore, additional force is not simply a result of increased $[Ca^{2+}]_i$ activating myosin light chain kinase. Exactly how protein kinase C activation increases force is unclear, but activation of MAP kinase may lead to phosphorylation of caldesmon and production of more force in spite of declining $[Ca^{2+}]_i$. Further studies of caldesmon phosphorylation under the same conditions are required to test this hypothesis.

The results showing activation of MAP kinases by neurotransmitters raise interesting questions about the pathway coupling muscarinic and tachykinin receptors to contraction. Adam *et al.* (1993) showed by immunoblotting the presence

of several components of the ERK MAP kinase signalling pathway including MAP kinase–ERK kinase (MEK), raf-1 kinase and ras. We also find proteins immunoreactive with anti-MEK, anti-raf-1 and anti-ras antibodies in Western blots of canine colonic smooth muscle homogenates (not shown). Therefore, the components of the protein kinase cascade thought to activate ERK MAP kinases in other cells are present in mammalian smooth muscle. This is consistent with a signal transduction pathway coupling excitation of smooth muscle with activation of MAP kinases and phosphorylation of caldesmon.

Although there is substantial evidence favouring caldesmon regulation of the cross-bridge cycle the exact structure and function of caldesmon in smooth muscles remains unclear. It is known to be an elongated molecule which binds actin at the carboxy terminus of caldesmon. Caldesmon also binds to myosin by interaction of the S2 region of myosin and the amino terminus of caldesmon. An important unresolved question is whether caldesmon is bound to actin–tropomyosin filaments along its entire length or whether the carboxy-terminal domain is bound to actin with the amino-terminal domain free to interact with the S2 region of myosin (Mabuchi, Lin & Wang, 1993). Recent electron microscopy studies of cross-linked thin filaments suggest caldesmon extends from thin filaments to thick filaments to form a type of cross-link (Katayama & Ikebe, 1995). This confirms previous binding studies of purified proteins that suggested a cross-linking function of caldesmon (Ikebe & Reardon, 1988; Sutherland & Walsh, 1989). The functions of such cross-links are unclear, but they may contribute to force maintenance as well as acting to slow tissue shortening velocity by imposing a load on the cross-bridges. Our previous study shows shortening velocity slows significantly during acetylcholine-induced contractions of intact colonic smooth muscle (Gerthoffer *et al.* 1991). In the present study caldesmon slowed actin filament sliding velocity and phosphorylation of caldesmon reduced the inhibitory effects on actin sliding velocity (Fig. 9). Caldesmon cross-linking of actin and myosin may be regulated in part by phosphorylation of caldesmon by MAP kinases.

In summary, we find caldesmon is phosphorylated during acetylcholine-induced contraction of canine colonic smooth muscle. There is concomitant activation of several protein kinases, two of which comigrate on SDS–PAGE with proteins immunoreactive with anti-MAP kinase antibodies. Our results are consistent with the notion that caldesmon is phosphorylated by one or more ERK MAP kinases that are activated by excitatory stimuli during colonic smooth muscle contraction. Phosphorylation of caldesmon may regulate cross-bridge cycling by reversing the inhibitory effect of caldesmon on actomyosin ATPase.

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