Phosphorylation of caldesmon by ERK MAP kinases in smooth muscle

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Hedges, Jason C., Brian C. Oxhorn, Michael Carty, Leonard P. Adam, Ilia A. Yamboliev, and William T. Gerthoffer. Phosphorylation of caldesmon by ERK MAP kinases in smooth muscle. Am J Physiol Cell Physiol 278: C718–C726, 2000.—Phosphorylation of h-caldesmon has been proposed to regulate airway smooth muscle contraction. Both extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein (MAP) kinases phosphorylate h-caldesmon in vitro. To determine whether both enzymes phosphorylate caldesmon in vivo, phosphorylation-site-selective antibodies were used to assay phosphorylation of MAP kinase consensus sites. Stimulation of cultured tracheal smooth muscle cells with ACh or platelet-derived growth factor increased caldesmon phosphorylation at Ser789 by about twofold. Inhibiting ERK MAP kinase activation with 50 µM PD-98059 blocked agonist-induced caldesmon phosphorylation completely. Inhibiting p38 MAP kinases with 25 µM SB-203580 had no effect on ACh-induced caldesmon phosphorylation. Carbachol stimulation increased caldesmon phosphorylation at Ser789 in intact tracheal smooth muscle, which was blocked by the M2 antagonist AF-DX 116 (1 µM). AF-DX 116 inhibited carbachol-induced isometric contraction by 15 ± 1.4%, thus dissociating caldesmon phosphorylation from contraction. Activation of M2 receptors leads to activation of ERK MAP kinases and phosphorylation of caldesmon with little or no functional effect on isometric force. P38 MAP kinases are also activated by muscarinic agonists, but they do not phosphorylate caldesmon in vivo.

Caldesmon is phosphorylated in intact vascular smooth muscle by proline-directed protein kinases, which are presumably members of the mitogen-activated protein (MAP) kinase family (3). Adam et al. (2) identified serine residues phosphorylated in vivo corresponding to Ser759 and Ser789 in human smooth muscle caldesmon (17) (GenBank/Q05682), which accounted for about 70% of the radioactive phosphorous. Both phosphorylated serines are in the carboxy-terminal domain of h-caldesmon known to be functionally important for actin binding, tropomyosin binding, and actomyosin inhibitory activity. This led to the hypothesis that caldesmon phosphorylation by MAP kinase controls or modulates smooth muscle contraction in concert with myosin light chain phosphorylation. Further studies showed that extracellular signal-regulated kinase (ERK) MAP kinases are activated by a variety of contractile agonists in vascular, airway, and intestinal smooth muscles (9, 10, 18). We also know that members of the p38 MAP kinase family are expressed in differentiated smooth muscles and are activated by the same neurotransmitters that activate ERK MAP kinases (14, 19). Both ERK and p38 MAP kinase family members might phosphorylate caldesmon in vivo because these kinases have common substrates in vitro and in vivo (29).

The function of caldesmon and the importance of caldesmon phosphorylation at MAP kinase sites (Ser759 and Ser789) are controversial issues. We reported that phorbol ester activates ERK MAP kinases during sensitization of intact colon smooth muscle (9), and hypothesized that phosphorylation of caldesmon by ERK MAP kinases might contribute to contractile protein sensitization to calcium. Conflicting results were obtained in chemically skinned vascular smooth muscle in which exogenous MAP kinase phosphorylated caldesmon with no increase in contraction (21). Gorenne et al. (11) found that inhibition of MEK, the upstream activator of ERK MAP kinases, had no effect on muscle contraction. The latter results challenge the notion that phosphorylation of caldesmon by ERK MAP kinases is necessary for smooth muscle contraction. Hedges et al. (14) then reported that h-caldesmon is a good substrate for both ERK and p38 MAP kinases in vitro, raising the possibility that caldesmon is a substrate for the p38 MAP kinases in vivo. In the present study, we tested this hypothesis by assaying caldesmon phosphorylation with phosphorylation site-specific polyclonal antibodies. Inhibitors of MEK and p38 MAP kinases were used to define which family of MAP kinases phosphorylate h-caldesmon in tracheal smooth muscle tissue and cultured tracheal smooth muscle cells.

METHODS

Materials. Dual phosphospecific anti-ERK antibodies (9101S) were purchased from New England Biolabs (Beverly, MA). SB-203580 and PD-98059 were purchased from Calbiochem (La Jolla, CA). Anti-rabbit IgG alkaline phosphatase conjugate antibodies were purchased from Promega (Madison, WI).
Alkylation of muscarinic M1 receptors with protection of M2 receptors. M3 muscarinic receptors of trachea smooth muscle strips were alkylated using 4-DAMP mustard. The 2-chloroethylamine moiety was activated to the aziridinium ion in 10 mM phosphate buffer at 37°C for 30 min. After equilibration and setting the muscle at the optimal length for contraction, we elicited a control response to carbachol through the use of a submaximal concentration of 1 µM for 5 min. After three rapid washes with PSS to remove carbachol, tissues were incubated with the respective receptor blockers for 1 h. The following four treatment groups were used: control, 1 µM AF-DX 116, 40 nM 4-DAMP mustard, and a combination of both 1 µM AF-DX 116, and 40 nM 4-DAMP mustard. The control group included 0.1% (vol/vol) DMSO and 4 µM phosphate buffer, which are vehicles for AF-DX 116 and 4-DAMP, respectively. After 60 min, sodium thiosulphate (0.5 mM) was added to the treatment groups to inactivate any remaining unbound activated aziridinium ion. After three rapid washes with PSS, a second response to 1 µM carbachol was elicited. AF-DX 116 (1 µM) was present during the second carbachol stimulation in treatments labeled AF-DX 116 or 4-DAMP mustard plus AF-DX 116.

To determine the effects of selective M1 and M2 receptor blockade on MAP kinase activation and caldesmon phosphorylation, tissue strips were frozen at various times during contraction by immersion in 0.5 mM NaF/acetone chilled to −80°C on powdered dry ice. Frozen muscle strips were allowed to come to room temperature for 30–60 min. The strips were homogenized in 50 µL MAP kinase extraction buffer/mg dry wt. MAP kinase extraction buffer was composed of 2% SDS, 10% glycerol, 5 mM NaF, 0.1 mM leupeptin, 10 mM EGTA, 1 mM EDTA, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF). The protein concentration of the supernatant was determined by the bi-dichroic assay method using BSA as the standard.

Cell culture. Tracheal smooth muscle cells were minced and placed in Ca2+-free Hanks’ solution at 37°C (in mM): 125 NaCl, 5.36 KCl, 15.5 NaHCO3, 0.34 Na2HPO4, 0.44 KH2PO4, 10 glucose, 2.9 sucrose, 10 HEPES, pH 7.4. Minced muscle was digested in Ca2+-free Hanks’ solution for 1–2 h at 4°C with 0.6 mg/ml collagenase ( Worthington, type II, 253 units/mg), 1.6 mg/ml BSA, 1.4 mg/ml trypsin inhibitor (Sigma), 0.25 mg/ml Na2ATP, and 0.07 mg/ml protease (Sigma). Partially digested tissue was warmed to 37°C for 5–10 min and then washed three times in Ca2+-free Hanks’ buffer and cells were recovered in each wash. Dispersed cells were sedimented by centrifugation (100 g, 5 min) and diluted in M199 culture medium (GIBCO) supplemented with 10% newborn calf serum (GIBCO), 0.2 mM l-glutamine, 0.2 units/ml penicillin G, 200 µg/ml streptomycin, and 1 µg/ml amphotericin B. Primary cultures were grown to 80–90% confluence on 75-mm2 culture flasks coated with 15 µg of rat tail collagen. Cells were briefly trypsinized and −105 cells were plated on six-well culture plates and grown to 100% confluence. Cells were grown for 7 days by culturing in Ham’s F-12 medium containing 0.1% newborn calf serum, 0.2 mM l-glutamine, 1 mM nonessential amino acids (GIBCO), 0.1 units/ml penicillin G, 100 µg/ml streptomycin, 6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 µg/ml selenious acid, and 5.35 µg/ml linoleic acid (ITS+; Collaborative Biomedical Products). All biochemical assays were conducted using first-passage cell growth arrested for 7 days according to Halyko et al. (13). Cells cultured in this manner were shown by Western blotting to express h-caldesmon, l-caldesmon, α-calponin, both smooth muscle tropomyosin isoforms, as well as α and γ smooth muscle isoforms of actin (not shown). Expression of these markers of differentiated smooth muscle is consistent with previous studies of similar cultures of airway smooth muscles (13).

Phosphorylation of caldesmon in vitro by ERK2 MAP kinase. Purified porcine stomach h-caldesmon was dephosphorylated by alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) and then phosphorylated in vitro with activated murine GST-p42 MAP kinase in a reaction volume of 60 µl containing 0.2 µM porcine stomach caldesmon, 1.5 µg GST-p42 MAP kinase, 0.25 mM Na2ATP (60 µCi [γ-32P]ATP), 25 mM Tris-HCl, 0.1 mM EGTA, 0.1 mM Na2VO4, 1 mM magnesium acetate, 1 mM dithiothreitol, 0.25 µg/ml leupeptin, and 25 µM AEBSF, pH 7. The reaction was terminated after various times (0–90 min) by removing 7-µl aliquots and diluting them 1:1 with 0.12 M Tris (pH 6.8), 4% SDS, 20% glycerol, and 2 mM dithiothreitol. Phosphorylated caldesmon was isolated by SDS-PAGE (5% acrylamide); proteins were transferred to a nitrocellulose filter, and the filter was incubated with a 1:4,000 dilution of a sheep anti-(h-caldesmon) polyclonal antibody for 1 h. After washing the blot with 0.05% gelatin, 100 mM Tris, 0.05% Tween 20 for 2 h, 32P labeling of caldesmon was imaged with a Bio-Rad model 525 Molecular Imager. Immunodetection of phosphorylated caldesmon was then carried out as described below to correlate antibody labeling with 32P incorporation by ERK MAP kinase.

Caldesmon phosphorylation and ERK MAP kinase threonine/tyrosine phosphorylation. Tracheal smooth muscle cells were grown on six-well plates as described above. Cells were washed with serum-free HAM’s F-12 culture medium, then pretreated with 50 µM PD-98059, 25 µM SB-203580, or 1 µM atropine for 30 min. Cells were then stimulated with ACh (10 µM) or platelet-derived growth factor (PDGF; 10 ng/ml) for 30 min in a CO2 incubator. The reaction was stopped by a wash in ice-cold PBS, and the cells were immediately lysed with 25 mM Tris·HCl, 0.1 mM EGTA, 0.1 mM Na3VO4, 10 mM glucose, 2.9 sucrose, 10 HEPES, pH 7.4. Minced muscle was digested in Ca2+-free Hanks’ solution for 1–2 h at 4°C with 0.6 mg/ml collagenase ( Worthington, type II, 253 units/mg), 1.6 mg/ml BSA, 1.4 mg/ml trypsin inhibitor (Sigma), 0.25 mg/ml Na2ATP, and 0.07 mg/ml protease (Sigma). Partially digested tissue was warmed to 37°C for 5–10 min and then washed three times in Ca2+-free Hanks’ buffer and cells were recovered in each wash. Dispersed cells were sedimented by centrifugation (100 g, 5 min) and diluted in M199 culture medium (GIBCO) supplemented with 10% newborn calf serum (GIBCO), 0.2 mM l-glutamine, 0.2 units/ml penicillin G, 200 µg/ml streptomycin, and 1 µg/ml amphotericin B. Primary cultures were grown to 80–90% confluence on 75-mm2 culture flasks coated with 15 µg of rat tail collagen. Cells were briefly trypsinized and −105 cells were plated on six-well culture plates and grown to 100% confluence. Cells were grown for 7 days by culturing in Ham’s F-12 medium containing 0.1% newborn calf serum, 0.2 mM l-glutamine, 1 mM nonessential amino acids (GIBCO), 0.1 units/ml penicillin G, 100 µg/ml streptomycin, 6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 µg/ml selenious acid, and 5.35 µg/ml linoleic acid (ITS+; Collaborative Biomedical Products). All biochemical assays were conducted using first-passage cell growth arrested for 7 days according to Halyko et al. (13). Cells cultured in this manner were shown by Western blotting to express h-caldesmon, l-caldesmon, α-calponin, both smooth muscle tropomyosin isoforms, as well as α and γ smooth muscle isoforms of actin (not shown). Expression of these markers of differentiated smooth muscle is consistent with previous studies of similar cultures of airway smooth muscles (13).
were determined by the bicinchoninic acid method using BSA as the standard.

Western blotting. To assay caldesmon phosphorylation and threonine-tyrosine phosphorylation of activated MAP kinases, tissue homogenates and cell extracts were resolved by SDS-PAGE. Gels containing 12% acrylamide were used for resolving ERK MAP kinases and gels with 8% acrylamide used for resolving caldesmon. Proteins were transferred to nitrocellulose in 25 mM Tris, 192 mM glycine, 10% methanol buffer with the use of a Genie electrophoretic (24 V, 2 h, 4°C). Blots were blocked at least 1 h with 0.5% gelatin, 100 mM Tris, 150 mM NaCl, 0.05% Tween 20. Phosphospecific ERK (910IS) antibodies were diluted 1:1,000 in 0.1% gelatin, 100 mM Tris, 150 mM NaCl, 0.05% Tween 20. Anti-caldesmon antibodies (described below) were diluted 1:1,000. Immunoreactive bands were visualized with goat anti-rabbit alkaline phosphatase secondary antibody (1:15,000). Secondary antibodies were obtained from Promega Biotec (Madison, WI).

Relative changes in immunoreactive band densities were assayed by loading equal amounts of protein (15 µg) in each lane of SDS-PAGE gels. Blots were labeled, washed, and bands visualized using consistent assay conditions for each set of replicates. The linear range of sample protein concentrations that produced a proportional change in band volume was determined by scanning densitometry. Western blots were scanned with a UMAX Powerlook flatbed scanner and TIF images were analyzed using the Volume Analyze feature of Molecular Analyst software (Bio-Rad, Hercules, CA). Densitometric data were normalized to the unstimulated control cells or tissues, and the linearity of the signal as a function of protein was determined as described previously (14).

Anti-caldesmon antibodies. Three types of polyclonal antibodies were used in the study. First, rabbit polyclonal antiserum was generated against full-length porcine stomach h-caldesmon. Second, affinity-purified antibodies generated against the phosphopeptide, PDGNKSP(PO4)PAPKP, a sequence based on Ser759 of mammalian h-caldesmon (equivalent to Ser702 of gizzard h-caldesmon), were prepared as previously described (8). Third, antibodies were generated against the phosphopeptide, CQSVDKVTSP(PO4)TPKV, a sequence that is analogous to Ser789 of mammalian h-caldesmon (17) and is absent from avian h-caldesmon. Briefly, the phosphopeptide was synthesized, coupled via the cysteine residue to keyhole limpet hemocyanin using sulfo- MBS (Pierce, Rockford, IL), injected into rabbits and, at appropriate times, serum was collected. Antibodies specific for the phosphorylated form of the peptide/protein were purified by a combination of 1) ammonium sulfate precipitation, 2) passage over a column of the corresponding nonphosphorylated peptide, and 3) passage of the flow-through from step 2 over a column of the phosphopeptide.

MAPKAP kinase activity assay. Cells were lysed after stimulation with ACh (10 µM) or PDGF (10 ng/ml) in MAP kinase extraction buffer (see above). Cellular lysate was centrifuged at 10,000 g for 10 min at 4°C. Protein in the clarified supernatant was assayed by the bicinchoninic acid method. Soluble HSP27 kinase activity was assayed in 40-µl kinase reaction containing 25 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 15 mM MgCl2, 1 mM EGTA, 0.1 mM NaF, 1 mM Na3VO4, 4 mM dithiothreitol, cellular lysate (10 µg protein/ml), and 0.15 mg/ml recombinant canine HSP27. Recombinant canine HSP27 (rhHSP27) was expressed and purified as previously described (19). The reaction was started by addition of 10 µCi of 250 µM [γ-32P]ATP. After incubation at 30°C for 30 min the reaction was terminated by diluting 1:4 with concentrated SDS buffer (0.24 M Tris, pH 6.8, 8% SDS, 40% glycerol, 4 mM dithiothreitol). Proteins were resolved by 12% acrylamide SDS-PAGE, and phosphorylated rhHSP27 was visualized and quantitated with a Bio-Rad Model 525 Molecular Imager.

RESULTS

Phosphorylation site-specific caldesmon antibodies. We used antibodies selective for the Ser799 phosphorylation site in h-caldesmon to compare immunoreactivity with 32P labeling catalyzed by activated GST-ERK2 MAP kinase. Figure 1A illustrates the rate of 32P incorporation into caldesmon by ERK2. Figure 1B shows a comparable increase in anti-phosphocaldesmon immunoreactivity detected by the phosphorylation site-specific antibody recognizing phosphorylation of Ser799. The time-course of both signals is very similar; the 32P signal has a slightly lower threshold of detection than the antibody labeling. A control reaction lacking ERK2 MAP kinase resulted in no 32P labeling of caldesmon or immunoreactivity (not shown). Linear regression analysis of 32P labeling intensity and spot volume of the immunoreactive bands showed a strong linear relationship between the two variables (r2 = 0.92). In other preliminary studies using antibodies recognizing phosphorylation of Ser759, activated GST-ERK2 MAP kinase produced minimal phosphorylation of Ser759. We also tested for Ser759 phosphorylation in SDS extracts of cells stimulated with 10 µM ACh or 10 ng/ml PDGF and in tissue strips stimulated with 1 µM carbachol. There was no detectable change in caldesmon phosphorylation at Ser759 in any case (data not shown). Therefore, we focused on using the antibodies selective for Ser799 to investigate MAP kinase activation and caldesmon phosphorylation in cultured cells and intact smooth muscles.

MAP kinase signaling and caldesmon phosphorylation. To determine whether both ERK and p38 MAP kinases phosphorylate caldesmon in vivo, the ERK MAP kinase pathway was blocked by pretreatment with 50 µM PD-98059 to block MEK. The p38 MAP kinase pathway was blocked by pretreatment with 25 µM SB-203580. First-passage tracheal smooth muscle cells were growth arrested 7 days in medium containing 0.1% serum, pretreated 30 min with the MAP kinase pathway antagonists, and then stimulated for 30 min with either ACh (10 µM) or PDGF (10 ng/ml). ACh was chosen because it is the major motor neurotransmitter in parasympathetic target organs. We showed previously that muscarinic agonists activate both ERK and p38 MAP kinases in intact airway and intestinal smooth muscle (9, 10, 19). PDGF was used because it is known to be an effective activator of both ERK and p38 MAP kinase pathways acting via tyrosine kinase receptors. Western blots with equal protein loading for each sample (15 µg/lane) were probed with two anti-caldesmon antibodies, one recognizing caldesmon phosphorylated at Ser759 and the other recognizing caldesmon regardless of the phosphorylation state. Both ACh (Fig. 2A) and PDGF (Fig. 2B) treatments increased caldesmon phosphorylation about twofold, with no change in total caldesmon. The response to ACh was sensitive to atropine (1 µM), verifying that it is...
mediated by muscarinic receptors. The MEK inhibitor, PD-98059 completely blocked caldesmon phosphorylation in both cases. In contrast, ACh-stimulated caldesmon phosphorylation was not significantly affected by the p38 MAP kinase inhibitor, SB-203580.

Two additional experiments were conducted to verify that the protein kinase inhibitors were effective. In the study shown in Fig. 3, dual threonine/tyrosine phosphorylation of ERK1 and ERK2 was assayed using phosphorylation-selective antibodies. Cultured cells were stimulated with ACh or PDGF and total protein extracts assayed for ERK phosphorylation by SDS-PAGE and Western blotting. PD-98059 effectively blocked both ERK1 and ERK2 phosphorylation, thus proving that the inhibitor was effective in preventing agonist-induced activation of MEK and phosphorylation of ERKs. Figure 4 shows that SB-203580 was also effective in blocking the p38 MAP kinase pathway. We assayed p38 MAP kinase activation indirectly by stimulating cells with ACh or PDGF and isolating soluble protein kinases. MAPKAP kinase activity was assayed using recombinant HSP27 in an in vitro kinase assay. MAPKAP kinases are substrates for p38 MAP kinase and are activated to phosphorylate HSP27 in vivo. In the in vitro kinase assay, recombinant HSP27 was phosphorylated by endogenously activated MAPKAP kinases, the substrate separated by SDS-PAGE, and visualized by phosphorimaging. SB-203580 inhibited
MAPKAP kinase activation elicited by both ACh and PDGF. This suggests that the lack of effect on caldesmon phosphorylation was not a result of failure of the drug to inhibit p38 MAP kinases. Therefore, phosphorylation of caldesmon in vivo at Ser789 is catalyzed by ERK MAP kinases and not by p38 MAP kinases.

Coupling of muscarinic receptor subtypes to caldesmon phosphorylation in intact muscle. We showed previously that muscarinic agonists are effective activators of ERK MAP kinases and that muscarinic stimulation increases caldesmon phosphorylation in airway and colonic smooth muscles (9, 10). Most smooth muscles in parasympathetic target organs express M3 and M2 isoforms of muscarinic receptors with a predominance of M2 (80%) over M3 (20%) subtypes. To define which muscarinic receptor subtype is coupled to caldesmon phosphorylation, we used M2- and M3-selective antagonists in intact strips of tracheal smooth muscle. The contribution of M3 receptors was determined with the use of a nitrogen mustard derivative of the M3 antagonist 4-DAMP to selectively alkylate M3 receptors while protecting M2 receptors as in the fourth condition that illustrate the strategy. Figure 5 shows contractile responses of canine tracheal smooth muscle under four conditions that illustrate the strategy. Figure 5A shows contraction in response to carbachol before any treatment. The top trace in Fig. 5B shows contraction after control treatment (DMSO solvent control); force decreased slightly to 95 ± 2% of the initial response to carbachol (Fig. 5C). The second trace shows that blocking only M2 receptors with AF-DX 116 produced a modest but statistically significant inhibition of contraction to 85 ± 1.4% of control (Fig. 5C). The third trace shows that alkylation of M3 receptors with protection of M2 receptors substantially inhibited contraction to 8 ± 1.7% of control. In the fourth trace, the M3 receptors were alkylated while protecting M2 receptors as in the third trace, but AF-DX 116 was also present during the second stimulation with carbachol. Contraction was blocked completely, suggesting that the residual response after M3 alkylation in trace 3 was due to M2 receptors. The data are consistent with several previous studies suggesting M3 receptors mediate the majority of the force response with a minority effect of M2 receptors remaining after selective M3 receptor alkylation (7).

We then used the protocol illustrated in Fig. 5 to determine whether phosphorylation of caldesmon depends on activation of M3 receptors, M2 receptors, or both. Tracheal muscle strips were frozen at 0, 5, and 15 min during the second challenge with carbachol after pretreatment with the muscarinic antagonists AF-DX 116 and 4-DAMP. Caldesmon phosphorylation was assayed by Western blotting with the use of the Ser789 phosphoselective antibodies as in Figs. 1 and 2.
data from densitometric analysis are shown in Fig. 6. Caldesmon phosphorylation increased after 5- and 15-min stimulation with 1 µM carbachol compared with basal levels at 0 min. The M₂ antagonist, AF-DX 116 blocked carbachol-induced caldesmon phosphorylation completely, and the M₃ antagonist produced a partial inhibition. Neither antagonist had any effect on mean phosphocaldesmon band volumes at 0 min (basal values). The results suggest caldesmon phosphorylation in airway smooth muscle depends primarily on M₂ receptor activation.

**DISCUSSION**

The biochemical characteristics of h-caldesmon have led to numerous investigations of hypothesized regulation and functions in smooth muscle contraction. Caldesmon is a component of thin filaments in smooth muscles, which has been shown by isolation of “native thin filaments” by methods designed to preserve associations among proteins existing in vivo (20). There are several functional effects described for caldesmon in vitro, including binding to actin, calmodulin, tropomyosin, and myosin, as well as inhibition of actomyosin ATPase. Current opinion varies as to exactly which biochemical feature of caldesmon is physiologically relevant. The two functions receiving considerable attention are inhibition of actomyosin ATPase via interaction with tropomyosin along the thin filament and cross-linking of actin and myosin filaments. Both functions could have important effects on muscle mechanics, but definitive evidence for either function in vivo is lacking. The ability of caldesmon to cross-link or tether actin to myosin has some interesting implications for muscle contraction that have been studied using the in vitro motility assay. Caldesmon at relatively low concentrations can promote actin filament motility on fully phosphorylated myosin, presumably because a weak tethering effect favors actin binding to myosin (12). Caldesmon also effectively inhibits actin motility, with efficacy that varies with the degree of myosin light chain phosphorylation and the phosphorylation state of caldesmon (16). Caldesmon has little effect on sliding
MAP kinases, and phosphorylation of caldesmon by targets for the MAP kinases. Directed phosphorylation sites that are proposed to be of human caldesmon. These serines are both proline-smooth muscle that correspond to serines 759 and 789 (10). Adam et al. (3) identified two serine residues mon) as well as a significant increase on stimulation (1, 9, 10). Estimates of phosphorylation stoichiometry suggest there is significant antagonism of actin-tropomyosin-activated myosin ATPase (24). These functional and biochemical studies are all consistent with a signal transduction pathway coupling excitation of smooth muscle with activation of MAP kinases and phosphorylation of caldesmon. Several details of this pathway are clarified by the present study. Through the use of site-selective antibodies we find that phosphorylation of Ser759 is minimal in vivo and does not change substantially on muscarinic activation. Phosphorylation of Ser789 increases about 1.5- to 2-fold with muscarinic stimulation (Figs. 2 and 6). Inhibiting the ERK MAP kinase pathway with PD-98059 completely blocks caldesmon phosphorylation, but blocking p38 MAP activity with SB-203580 does not (Fig. 2). This shows clearly that the ERK pathway is necessary in vivo for phosphorylation of caldesmon, at least at the Ser789 MAP kinase site.

We also identified the muscarinic receptor subtype coupled to the pathway leading to caldesmon phosphorylation. M3 muscarinic receptors in smooth muscles are thought to be coupled primarily via Gq11 to phosphoinositide signaling pathways and calcium release mechanisms that support muscle contraction. M2 receptors in smooth muscles couple via Gs to inhibition of adenylate cyclase, activation of nonselective cation channels, and sensitization of the contractile system to calcium (5, 15, 28). Details of signal transduction pathways between receptor activation and effector proteins mediating these diverse effects are lacking. The ERK MAP kinases are known to be activated by several G protein-coupled receptors, including several isoforms of muscarinic receptors (23, 27, 30). We now report that M2 muscarinic receptor stimulation also appears to be critical for caldesmon phosphorylation to occur in vivo (Fig. 6). This represents a novel signaling pathway in smooth muscles, the function of which is uncertain.

One prominent hypothesized function is that caldesmon regulates contraction and is itself regulated by phosphorylation by MAP kinases. Evidence in favor of this hypothesis includes potentiation of Ca2+-induced contraction of permeabilized airway smooth muscle with activated ERK2 MAP kinase (9). Potentiation of contraction by activated ERK2 contrasts with the negative results of Nixon et al. (21) who reported that activated ERK penetrated Triton X-100-permeabilized vascular smooth muscles, phosphorylated caldesmon, but did not potentiate contraction. A functional role for ERK MAP kinases in smooth muscle contraction was also challenged by Gorenne et al. (11), who showed that inhibition of ERK activation with PD-98059 had no effect on isometric contraction of hog carotid artery. These results and previous reports that phosphorylation of the carboxy-terminal domain of caldesmon has only modest or no functional effects in vitro (4, 22) weaken the argument that phosphorylation by MAP kinases is important in regulating contraction. Conflict-

![Fig. 6. Contribution of M2 receptor signaling to caldesmon phosphorylation in tracheal smooth muscle strips. Muscle strips were treated (Inset) as in Fig. 5 to alkylate M2 receptors. One set of strips was exposed to 0.1% DMSO, another set to 1 µM AF-DX 116 alone, and a third set to 4-DAMP mustard (with AF-DX 116 to protect M2 receptors). After these treatments muscles were stimulated with 1 µM carbachol, frozen at times indicated in inset, and caldesmon phosphorylation at Ser789 analyzed by Western blotting. Relative caldesmon phosphorylation was calculated from densitometric analysis of blots by normalizing band volumes to 0-min values. The null hypothesis was that mean relative phosphorylation was equal to 1.0 (*P < 0.05; n = 8; Student's t-test). Velocity when myosin is fully phosphorylated (16). When myosin phosphorylation is less than 0.5 mol P/mole light chain, a level often observed during contraction, caldesmon inhibits sliding velocity more effectively (16), presumably by tethering actin and myosin and imposing a load that retards actin sliding. A modest inhibitory effect of caldesmon on sliding velocity was shown to be sensitive to the phosphorylation state of caldesmon (10). Phosphorylation of caldesmon with ERK1 MAP kinase to 0.7 mol P/mole caldesmon partially antagonized the inhibitory effect of caldesmon on actin sliding velocity. The tethering effect of caldesmon in vivo might be sensitive to the phosphorylation state of caldesmon, however, the issue of whether tethering of actin and myosin occurs in vivo remains controversial. If tethering occurs in vivo it may be regulated in part by phosphorylation of caldesmon by MAP kinases. Phosphorylated caldesmon is proposed to have reduced affinity for actin/tropomyosin, leading to a disinhibition of actomyosin ATPase and contraction. For example, protein kinase C phosphorylation of the carboxy-terminal region of caldesmon reduces the inhibitory effect of caldesmon on actomyosin ATPase activity (26). Phosphorylation by CaM kinase II also abolishes the interaction of caldesmon with myosin (25). These properties of caldesmon suggest that it could regulate smooth muscle contraction in part by a phosphorylation/dephosphorylation mechanism. Caldesmon is in fact phosphorylated in intact smooth muscles in response to diverse contractile stimuli (1, 9, 10). Estimates of phosphorylation stoichiometry suggest there is significant basal phosphorylation (0.4-0.6 mol P/mole caldesmon) as well as a significant increase on stimulation (1, 10). Adam et al. (3) identified two serine residues phosphorylated in caldesmon of intact canine aortic smooth muscle that correspond to serines 759 and 789 of human caldesmon. These serines are both proline-directed phosphorylation sites that are proposed to be targets for the MAP kinases.

Caldesmon is a substrate for the smooth muscle ERK MAP kinases, and phosphorylation of caldesmon by ERK MAP kinase reduces actin binding to a modest extent (4). Mutation of serine 702 to aspartic acid in a 99-amino-acid carboxy-terminal fragment of avian caldesmon prevents phosphorylation by ERK MAP kinase in vitro and reduces the inhibitory effect of the fragment on actin-tropomyosin-activated myosin ATPase (24). These functional and biochemical studies are all consistent with a signal transduction pathway coupling excitation of smooth muscle with activation of MAP kinases and phosphorylation of caldesmon. Several details of this pathway are clarified by the present study. Through the use of site-selective antibodies we find that phosphorylation of Ser759 is minimal in vivo and does not change substantially on muscarinic activation. Phosphorylation of Ser789 increases about 1.5- to 2-fold with muscarinic stimulation (Figs. 2 and 6). Inhibiting the ERK MAP kinase pathway with PD-98059 completely blocks caldesmon phosphorylation, but blocking p38 MAP activity with SB-203580 does not (Fig. 2). This shows clearly that the ERK pathway is necessary in vivo for phosphorylation of caldesmon, at least at the Ser789 MAP kinase site.
ing results may arise from technical differences between laboratories, or they might illustrate important tissue-dependent differences in the role of caldesmon in contraction. Our laboratory has made the unpublished observation that exogenous activated ERK2 MAP kinase does not potentiate contraction of canine colonic smooth muscle, in agreement with the lack of effect on vascular smooth muscle (21). Exogenous MAP kinases added to permeabilized tracheal smooth muscle might phosphorylate targets other than caldesmon in airway smooth muscle, or phosphorylation of caldesmon has different effects in airway smooth muscles compared with gastrointestinal and vascular smooth muscles. This is not unprecedented because substantial differences in correlation of intracellular Ca\(^{2+}\), myosin light chain phosphorylation, and contraction have been described for vascular, airway, and gastrointestinal smooth muscle. Whether phosphorylation by MAP kinases subtly influences dynamic aspects of cross-bridge cycling, dynamic stiffness, or actin filament structure remains to be determined. However, the preponderance of evidence supports the view that caldesmon phosphorylation at Ser789 by ERK MAP kinases has no detectable effect on isometric contraction of smooth muscles.

In summary, this study confirms previous observations that muscarinic receptors are coupled to ERK MAP kinase activation in airway smooth muscle and add the novel observation that M\(_2\) muscarinic receptors appear to be important in eliciting caldesmon phosphorylation in vivo. The pathway leading to caldesmon phosphorylation is clarified by results showing that ERK MAP kinase activation is required for phosphorylation of Ser789. p38 MAP kinases are also activated via muscarinic receptor signaling, but p38 MAP kinases do not phosphorylate caldesmon at Ser789 in vivo.

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