Coupling of M2 muscarinic receptors to ERK MAP kinases and caldesmon phosphorylation in colonic smooth muscle

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Coupling of M2 muscarinic receptors to ERK MAP kinases and caldesmon phosphorylation in colonic smooth muscle. Am. J. Physiol. Gastrointest. Liver Physiol. 278: G429–G437, 2000.—Coupling of M2 and M3 muscarinic receptors to activation of mitogen-activated protein (MAP) kinases and phosphorylation of caldesmon was studied in canine colonic smooth muscle strips in which M3 receptors were selectively inactivated by N,N-dimethyl-4-piperidinyl diphenylacetate (4-DAMP) mustard (40 nM). ACh elicited activation of extracellular signal-regulated kinase (ERK) 1, ERK2, and p38 MAP kinases in control muscles and increased phosphorylation of caldesmon (Ser789), a putative downstream target of MAP kinases. Alkylation of M2 receptors with 4-DAMP had only a modest inhibitory effect on ERK activation, p38 MAP kinase activation, and caldesmon phosphorylation. Subsequent treatment with 1 µM AF-DX 116 completely prevented activation of ERK and p38 MAP kinase and prevented caldesmon phosphorylation. Caldesmon phosphorylation was blocked by the MAP kinase/ERK kinase inhibitor PD-98059 but not by the p38 MAP kinase inhibitor SB-203580. These results indicate that colonic smooth muscle M2 receptors are coupled to ERK and p38 MAP kinases. Activation of ERK, but not p38 MAP kinases, results in phosphorylation of caldesmon in vivo, which is a novel function for M2 receptor activation in smooth muscle.

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N,N-dimethyl-4-piperidinyl diphenylacetate mustard; AF-DX 116; p38 mitogen-activated protein kinase; PD-98059; SB-203580.

Muscarinic receptors are comprised of five different families of receptors (M1–M5) that are expressed in different tissues. M2 and M3 muscarinic receptors are both expressed in different proportions in mammalian smooth muscle (2). In the colon of the dog, radioligand binding data suggest that circular smooth muscle cells predominantly express M2 (80%) over M3 (20%) subtypes, which is a pattern similar to that in many other visceral smooth muscles. Although muscarinic receptors certainly respond to vagal excitation and stimulate smooth muscle contraction, the details of the signaling networks activated by these two receptors are not completely understood. M3 muscarinic receptors are known to couple via Gi/11 to phosphatidylinositol signaling pathways, resulting in release of calcium from the sarcoplasmic reticulum. In many smooth muscles including colonic smooth muscle, this appears to be the major excitatory pathway that produces muscle contraction (13, 15). M2 receptors in smooth muscle couple via Gi to several effector proteins that might also contribute to, or modulate, the primary response to calcium release via M1 signaling. M3 activation is known to inhibit adenylate cyclase, which might potentiate contraction indirectly by reducing cAMP levels. M2 receptors also activate nonselective cation channels (1, 17), which could depolarize the cell membrane, causing a net increase in cell calcium via L-type calcium channels. Cell calcium might also be increased directly by calcium entering via nonselective cation channels. Activation of M2 receptors in permeabilized airway smooth muscle sensitizes the contractile system to calcium (9). The signaling pathway or pathways mediating M2 effects on ion channels and the contractile machinery are undefined but probably involve one or more protein kinase signaling cascades. There are several signaling cascades that might be coupled to M2 receptors in smooth muscles including Rho-activated kinases, p21-activated protein kinases, and the mitogen-activated protein kinases (MAP kinases) (5, 14, 16).

Autonomic neurotransmitters activate multiple protein kinase cascades in smooth muscles, including several members of the MAP kinase family (5, 8, 10). However, details of the pathways coupling muscarinic receptors to downstream protein kinases are ambiguous. We (4, 5, 8) have suggested that caldesmon is one of the downstream effectors of the MAP kinase pathways in smooth muscle. Caldesmon is an actin-binding protein expressed in smooth muscle that appears to be a substrate for extracellular signal-regulated kinase (ERK) MAP kinase. Caldesmon inhibits actin-activated myosin ATPase in vitro, and phosphorylation of caldesmon in vitro reverses the inhibition. These findings suggest that caldesmon phosphorylation might regulate contraction. In addition, phosphorylation of purified caldesmon by purified ERK1 MAP kinase reverses the inhibitory effect of caldesmon on actin sliding velocity in an in vitro motility assay (5). It has been proposed that phosphorylation of caldesmon may contribute to the regulation of contraction by reversing the inhibitory effect of caldesmon on actinomyosin ATPase; however, this hypothesis is controversial.

In addition to the uncertainty about the function of caldesmon phosphorylation, it is not clear how M2 and
M₃ muscarinic receptors are coupled to this event. To address this question, we monitored contraction, ERK and p38 MAP kinase activation, and caldesmon phosphorylation in response to ACh in colonic smooth muscle strips. To investigate differential coupling of M₂ and M₃ muscarinic receptors to these events, we inactivated M₃ receptors with N,N-dimethyl-4-piperidinyl diphenylacetate (4-DAMP) mustard and blocked both M₂ and M₃ muscarinic receptors with combined treatment with activated 4-DAMP mustard and AF-DX 116. We found that M₁ receptors are primarily responsible for smooth muscle contraction in canine colon. We also show that activation of p38 MAP kinases, ERK MAP kinases, and caldesmon phosphorylation are selectively coupled to M₂ muscarinic receptors.

**METHODS**

Materials. 4-DAMP mustard was purchased from Research Biochemicals International (Natick, MA). Dual phospho-ERK (Thr202/Tyr204, no. 9101S) and dual phospho-p38 MAP kinase (Thr180/Tyr182, no. 9211S) antibodies were purchased from New England Biolabs (Beverly, MA). Rabbit polyclonal antibodies generated against full-length porcine stomach h-caldesmon and affinity-purified antibodies generated against the phosphopeptide CQSVDKVTS(PO₄)PTKV, a sequence that is analogous to Ser⁸⁵⁹ of mammalian h-caldesmon, were prepared by L. Adam (Bristol Myers Squibb, Princeton, NJ). SB-203580 and PD-98059 were purchased from Calbiochem, (La Jolla, CA). Anti-rabbit IgG alkaline phosphatase conjugated antibodies were purchased from Promega (Madison, WI). Other analytical reagents were purchased from Sigma Chemical (St. Louis, MO).

Qualitative RT-PCR. Total RNA was extracted from canine colonic circular smooth muscle with TRIzol reagent (1 ml/100 mg wet muscle; Gibco BRL). First-strand cDNA synthesis was performed at 42°C from 2 µg of RNA using 250 ng of random hexamers, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol (DTT), 0.125 mM dATP, dCTP, dGTP, and dTTP, and 1 µM dXTP, and 1 unit SuperScript II reverse transcriptase (Gibco BRL). Twenty units of RNaseH (Promega, Madison, WI) was added at 37°C for 20 min. Muscarinic receptors were amplified using PCR in a thermal cycler (GeneAmp PCR System 2400, Perkin-Elmer). The reaction mixture contained 60 mM Tris-HCl (pH 8.5), 15 mM NH₄SO₄, 1.5 mM MgCl₂, 0.25 mM dATP, dCTP, dGTP, and dTTP, 10% DMSO, 0.2 ng/ml of each primer, template cDNA, and 2.5 units of Thermus aquaticus polymerase (Taq). Amplification took place at 94°C for 1 min, 57°C for 1 min, and 72°C for 1.5 min using the following oligonucleotides obtained from the human sequence of the M₂ muscarinic receptor (X15264): 5'-CGGACCACAAACATG- GCAGTTA-3' (sense, nt 403-424) and 5'-TGGTATGGGC- CCAAATGTATG-3' (antisense, nt 1211-1190). Oligonucleotides for the M₂ muscarinic receptor were also obtained from the human sequence (U29589): 5'-ACGGTGGACATGGAG- GCAGTTT-3' (sense, nt 1237-1259) and 5'-TGCCACACGC- AGCACATTTGAG-3' (antisense, nt 1685-1663). Oligonucleotides were purchased from Bio-Synthesis (Lewisville, TX). Reaction products (15 µl) were electrophoresed through a 1% agarose-Tris-acetate EDTA gel and visualized with ethidium bromide.

Relative RT-PCR. Semi-quantitative relative PCR was performed using QuantumRna 18S ribosomal RNA internal standards (Ambion) according to the manufacturer’s protocol. Total RNA isolation, cDNA synthesis, and PCR amplification took place as described in Qualitative RT-PCR, with the following exceptions. The linear range of PCR amplification (period in which amplification efficiency remains constant over a number of cycles) for the M₂ and M₃ muscarinic receptors was determined and quantified by ethidium bromide staining of agarose gels followed by densitometry. Multiplex PCR took place within the linear range for M₂ and M₃ muscarinic receptors with an optimized ratio of 18S primers used as an endogenous standard along with 18S competitors. These competitors attenuate 18S amplification without affecting the performance of the muscarinic receptor PCR targets in the reaction. PCR products were quantified by ethidium bromide staining and analyzed by densitometry. Results were normalized to the amount of 18S ribosomal RNA present in each sample from five separate colonic smooth muscle RNA preparations.

Mechanical studies with muscarinic antagonists 4-DAMP mustard and AF-DX 116. Adult mongrel dogs of either sex were killed by barbiturate overdose. The colon was promptly removed and placed in cold physiological saline solution (PSS) composed of (in mM) 2 MOPS, pH 7.4, 140 NaCl, 4.7 KCl, 1.2 MgSO₄, 2.5 CaCl₂, 1.2 Na₂HPO₄, 0.02 EDTA, and 5.6 d-glucose. The circular smooth muscle layer of the colon was dissected free of longitudinal smooth muscle and mucosa. Colonic muscle strips - 10 mm long and 2 mm wide and in parallel to the long axis of the circular muscle fibers and included the full thickness of the circular muscle layer. Canine colonic smooth muscle strips were mounted between stainless clamps with one end attached to an isometric force transducer (Grass FT03). Strips were equilibrated at least 60 min at 37°C in oxygenated PSS plus 10 µM L-nitroarginine to block nitric oxide synthetase. Muscle strips were stimulated three times for 5 min with 0.6 µM ACh to produce stable, reproducible contractions. Strips were then stimulated with 10 µM ACh to obtain a maximum control response. Phasic contractile responses were quantitated by digitizing force traces using Sigma Scan (Jandel Scientific, San Rafael, CA). Areas under each curve (g x s) were calculated with correction for basal force, which was -5% of the maximum force. The response to the initial test dose of ACh (10 µM for 5 min) was defined as 100% and subsequent responses of each strip to ACh (10 µM for 5 min) were expressed as percent of the initial maximum control response. The M₂ muscarinic receptors were then alkylated using 4-DAMP mustard with simultaneous protection of M₃ receptors with AF-DX 116 (3). 4-DAMP mustard was activated in 10 mM phosphate buffer at 37°C for 30 min, and then test strips were incubated for 1 h with 1 µM AF-DX 116 plus 40 mM 4-DAMP mustard. Unbound active 4-DAMP mustard was inactivated by 0.5 mM Na₂S₂O₃ for 15 min in the continued presence of 1 µM AF-DX 116. Selective inactivation of the M₃ receptors can be achieved by protecting M₃ muscarinic receptors with the competitive antagonist AF-DX 116 at a concentration of 1 µM, according to Ehliert and Griffin (3). Including an M₂ antagonist is necessary because activated 4-DAMP mustard is only modestly selective for M₂ receptors [dissociation constant (Kd) = 7.2 nM for M₂ receptors and Kd = 43 nM for M₃ receptors]. When M₂ receptors were occupied by AF-DX 116 during the dialytic period, Ehliert and Griffin (3) reported 85% alkylation and 90–95% functional inactivation of M₃ receptors with 9% alkylation and minimal functional inactivation of M₂ receptors.

Two general protocols were used, one for assessing effects of muscarinic antagonists on contraction and one for assessing effects on MAP kinase activation and caldesmon phosphorylation. To define the contribution of M₂ receptors in contraction, three repeated responses to 10 µM ACh were obtained from each strip: a control response, a response after alkyla-
tion of M3 receptors in the continued presence of AF-DX 116, and a third response after washout of AF-DX 116. The rationale was to alkylate M3 receptors while protecting M2 receptors and then test the response to ACh with and without the equilibrium-competitive M2 antagonist present, thus defining the magnitude of the M2-mediated contraction.

For MAP kinase and caldesmon phosphorylation assays, four muscle strips were cut from each colon, equilibrated as described above, and then stimulated twice with 10 µM ACh, once to elicit a control contraction and a second response after treatment with a muscarinic antagonist. DMSO control tissues were exposed to 0.1% (vol/vol) DMSO and 0.1% DMSO and 4 µM phosphate buffer during the 60 min “alkylation” reaction. A second strip was treated for 60 min with 1 µM AF-DX 116 only. The third and fourth strips were treated 60 min with 40 nM 4-DAMP mustard plus 1 µM AF-DX 116. After 60 min, sodium thiocyanate (0.5 mM) was added to all strips for 10 min to inactivate any remaining unbound activated aziridinium ion. After three rapid washes with PSS, a second response to 10 µM ACh was elicited.

MAP kinase and caldesmon phosphorylation immunoblotting assays. To determine the effects of selective M2 and M3 receptor blockade on p38 and ERK MAP kinase activation and caldesmon phosphorylation, tissue strips were frozen at 0, 5, and 15 min during the second 10 µM ACh-induced 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 of MAP kinase extraction buffer/mg dry weight. MAP kinase extraction buffer was composed of 2% SDS, 10% glycerol, 5 mM NaF, 0.1 mM leupeptin, 10 mM EGTA, 1 mM EDTA, and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF). Tissue homogenates were clarified by centrifugation at 10,000 g at 4°C. Proteins (15 µg protein/lane) were resolved by SDS-PAGE (10% acrylamide) and transferred to nitrocellulose membranes. The nitrocellulose membranes were blocked 2–14 h with 0.5% gelatin, 100 mM Tris (pH 7.5), 150 mM NaCl, and 0.05% (vol/vol) Tween-20. ERK MAP kinase was detected by anti-phospho-ERK primary antibody (diluted 1:1,000) followed by labeling with goat-anti-rabbit alkaline phosphatase secondary antibody (diluted 1:15,000). Phosphorylated caldesmon was immunodetected by anti-phosphocaldesmon primary antibodies (diluted 1:1,000) and goat-anti-rabbit alkaline phosphatase secondary antibody (diluted 1:15,000). Affinity-purified anti-phosphocaldesmon antibodies were generated against the phosphopeptide CQSVDKVTS(PO4)PTKV, a sequence that is analogous to Ser789 of mammalian h-caldesmon.

Blots were scanned with a UMAX Powerlook flatbed scanner, and TIFF images were analyzed using the Volume Analyze feature of Molecular Analyst software (version 1.4; BioRad, Hercules, CA). Densitometric data were normalized to the nonstimulated control tissues, and the linearity of the signal as a function of protein was determined as described previously (12).

Statistical methods. Results are presented as means ± SE of three to seven experiments for each experimental data point. Protein kinase activity and caldesmon phosphorylation were normalized to basal levels (defined as 1.0) to give a relative activity, and statistically significant activation was tested for using Student’s t-test. The null hypothesis was rejected if relative activity or relative phosphorylation was > 1.0.

RESULTS

Expression of M2 and M3 receptors in canine colonic smooth muscle. M2 and M3 muscarinic receptor expression in canine colonic smooth muscle was determined by qualitative RT-PCR (Fig. 1A). PCR products of the predicted sizes of 810 bp for the M2 receptor and 450 bp for the M3 receptor were observed. Semiquantitative RT-PCR was then performed to determine the relative ratio of M2 to M3 expression in canine colonic smooth muscle (Fig. 1B). Multiplex PCR reactions were performed using M2 or M3 receptor primers along with the 18S primers and competimers described above. These reactions resulted in amplification of either the predicted M2 or M3 PCR product along with a 324-bp 18S ribosomal RNA product. Figure 1C depicts a graphic summary of M2 and M3 muscarinic receptor expression after normalization of the muscarinic receptor expression to 18S ribosomal RNA amplification from five separate canine colonic smooth muscle preparations. In

![Fig. 1. M2 and M3 muscarinic receptor expression in canine colonic smooth muscle. A: qualitative RT-PCR demonstrating amplification of 810-bp M2 receptor PCR product and 410-bp M3 receptor PCR product. B: representative semiquantitative RT-PCR experiment depicting M2 and M3 receptor amplification in presence of endogenous 18S RNA as an internal control for variables in cDNA synthesis. C: graphic summary of M2 and M3 receptor expression normalized to 18S RNA amplification from 5 separate canine colonic smooth muscle preparations.](http://ajpgi.physiology.org/)
M3 receptors are primarily responsible for canine colonic smooth muscle contraction. 4-DAMP mustard is an irreversible alkylating agent that is relatively selective for M3 receptors, and AF-DX 116 is an equilibrium competitive antagonist relatively selective for M2 receptors. The use of both 4-DAMP mustard and AF-DX 116 allows for simultaneous inactivation of M3 receptors and preservation of M2 receptors (3). A previous study using this strategy showed M3 receptors to be predominant for eliciting contraction of guinea pig colonic smooth muscle (13), with a minor residual response being mediated by M2 receptors. We conducted studies of canine colonic smooth muscle strips to determine the minimum incubation time required for 4-DAMP mustard to inactivate M3 receptors. It was found that 1-h incubation with 40 nM 4-DAMP mustard and 1 µM AF-DX 116 was sufficient to maximize the inhibition of ACh-induced contraction.

This optimized method was then used to define the relative contribution of M2 and M3 receptors to isometric contraction (Fig. 2) and to verify the effectiveness of receptor blockade for further studies of the relative contribution of M2 and M3 receptors to MAP kinase activation and caldesmon phosphorylation. A control response to a maximum concentration of ACh (10 µM) was elicited (Fig. 2A), followed by alkylation of M3 receptors. Excess activated mustard was quenched with Na2S2O3 and a second response to 10 µM ACh was elicited in the continued presence of 1 µM AF-DX 116. Under these conditions, both M2 and M3 receptors were blocked and there was minimal residual contraction averaging 7 (± 4)% of the control response (Fig. 2B). When AF-DX 116 was washed from the bath and the third response to 10 µM ACh elicited, there was a small increase in phasic contractions to 15 (± 6)% of the control response (Fig. 2B). When muscle strips were treated with 1 µM AF-DX 116 alone without prior treatment with 4-DAMP, mustard contraction was inhibited to 57 ± 16% of control (data not shown). M2 receptor activation alone, after alkylation of M3 receptors, appears to mediate only modest colonic smooth muscle contractions. M3 receptor activation is necessary for maximum contractile response, and there are probably important interactions between the two receptors that determine the net isometric force developed in response to ACh (cf. Refs. 17 and 19).

ERK MAP kinase activation is coupled to M2 receptors in colonic smooth muscle. To determine whether ERK MAP kinase phosphorylation is differentially coupled to M2 and M3 muscarinic receptors, tissue strips were stimulated with ACh (10 µM) and frozen at various times during contraction for assaying dual phosphorylation of ERK1 and ERK2. Contraction was recorded throughout the experiment to confirm muscle viability and to verify efficacy of 4-DAMP mustard and AF-DX 116. The protocol and representative contractile response are illustrated in Fig. 3. Strips were then frozen at 0, 5, and 15 min during the second response to ACh for assaying dual phosphorylation of ERK MAP kinase. One set of three muscle strips was treated 60 min with DMSO as a solvent control (Fig. 3A). Two other sets of three strips were exposed to 40 nM activated 4-DAMP mustard plus 1 µM AF-DX 116 to alkylate M3 receptors and protect M2 receptors (Fig. 3, B and C). One set of muscles was stimulated a second time with 10 µM ACh alone (Fig. 3B) to activate residual M2 receptors. The third set of strips was stimulated with ACh in the presence of 1 µM AF-DX to block M2 receptors (Fig. 3C).

ERK1 and ERK2 MAP kinase activation was assayed by Western blotting using phospho-p44/42 MAP kinase antibodies to monitor dual phosphorylation of Thr202/Tyr204 at the regulatory TEY sequence (Fig. 4A). In muscles treated only with DMSO, ERK1 and ERK2 phosphorylation significantly increased above basal activity by three- to fivefold after 5- and 15-min stimulation with ACh (Fig. 4, B and C). Stimulation with ACh after alkylation of M3 receptors with 4-DAMP increased ERK phosphorylation above basal levels by two- and
fourfold at 5 and 15 min, respectively (Fig. 4, B and C). 
M₃ receptor blockade had only a modest inhibitory effect at 5 min but did not affect phosphorylation at 15 min. In contrast, combined treatment with 4-DAMP and AF-DX 116 completely blocked ERK phosphorylation (Fig. 4, B and C). We suggest that M₂ receptors are prominently coupled to ERK activation and that full activation probably involves the combined action of M₂ and M₃ receptors.

Caldesmon phosphorylation is coupled to M₂ receptors in colonic smooth muscle. We have shown previously (5) that caldesmon is phosphorylated in colonic smooth muscle stimulated with ACh and that ERK MAP kinases are activated under identical conditions. This is consistent with the ERK MAP kinases catalyzing caldesmon phosphorylation in vivo. However, we also reported that p38 MAP kinase from airway smooth muscle could phosphorylate caldesmon in vitro and that p38 MAP kinases are activated by muscarinic stimulation of intact tracheal smooth muscle (8, 12). These results prompted us to suggest that both ERK and p38 MAP kinases might phosphorylate caldesmon in vivo. To test this notion in colonic smooth muscle, we first determined which muscarinic receptors are coupled to activation of p38 MAP kinase, whether p38 MAP kinase or ERK MAP kinase catalyzes caldesmon phosphorylation in vivo, and which muscarinic receptors are coupled to phosphorylation of caldesmon.

We assessed relative activation of p38 MAP kinases by measuring dual Thr¹ eighty-Tyr¹ eighty-two phosphorylation of p38 MAP kinase phosphorylation by Western blotting using the protocol illustrated in Fig. 3. p38 MAP kinase phosphorylation was measured using the same tissue homogenates in which ERK MAP kinase phosphorylation was measured in Fig. 4. Relative p38 MAP kinase phosphorylation increased in response to ACh in a similar fashion in control and 4-DAMP-treated muscles (Fig. 5). The response was completely blocked by 1 µM AF-DX 116 after M₃ receptor alkylation. We also found that p38 MAP kinase phosphorylation was significantly inhibited by 1 µM AF-DX 116 alone before alkylation of M₃ receptors (data not shown). Relative p38 MAP kinase phosphorylation compared with basal activity was 0.82 ± 0.05 after 5-min stimulation and 1.47 ± 0.10 after 15-min stimulation. This compares to relative p38 phosphorylation of 1.7 ± 0.19 at 5 min and 2.2 ± 0.26 at 15 min in control tissues stimulated with ACh (Fig. 5B). The results suggest that p38 MAP kinases, like the ERK MAP kinases, are prominently coupled to M₂ receptors in colon smooth muscle.
To establish which MAP kinase family phosphorylates caldesmon in vivo we used PD-98509 and SB-203580 to block activation of ERK and p38 MAP kinases, respectively. We have shown previously (7, 12) that both compounds are effective in canine smooth muscle tissues and cells in blocking the target pathways, with no detectable crossover of effects on the ERK and p38 MAP kinase pathways. One set of three muscle strips was exposed for 30 min to 0.1% DMSO (Fig. 6); two other sets of strips were treated for 30 min with 25 µM SB-203580 to block p38 MAP kinase activity or treated with 50 µM PD-98059, which inhibits ERK activation (7), completely blocked phosphorylation of caldesmon at Ser789 (Fig. 6).

The results in Figs. 4 and 5 suggest that both ERK and p38 MAP kinases are coupled primarily to M₂ receptors in colon smooth muscle. Data from Fig. 6 show that ERK MAP kinases, but not p38 MAP kinases, phosphorylate caldesmon in vivo at Ser789. If this is correct, then caldesmon phosphorylation should also be differentially coupled to M₂ and M₃ muscarinic receptors. To test this hypothesis, the homogenates used to generate data in Figs. 4 and 5 were assayed for phosphorylation of caldesmon at Ser789 by Western blotting (Fig. 7). Phosphorylated caldesmon was assayed in tissues frozen at 0, 5, and 15 min of ACh stimulation after treatment with the respective antagonists.
M2 receptors are coupled to nonselective cation channels (19). These ionic and biochemical events ultimately result in coordinated regulation of potassium, calcium, and nonselective cation channels, all of which are coupled to oscillations in myoplasmic Ca\(^{2+}\) concentration, and activation of the contractile proteins. Myoplasmic Ca\(^{2+}\) is the primary intracellular trigger that causes contractile proteins to generate force. In addition, other signal transduction pathways can modify Ca\(^{2+}\) sensitivity or cytoskeletal structure in smooth muscle, and recent work suggests activation of M2 receptors enhances Ca\(^{2+}\) sensitivity of permeabilized airway smooth muscle (9).

Previous studies have demonstrated some tissue variability in the role of M2 receptors in the contractile response of smooth muscles to excitatory agonists. In guinea pig trachea, M2 receptors dominate the contractile response after a majority of M3 receptors have been inactivated by selective alkylation with 4-DAMP mustard. However, it has also been reported that in guinea pig esophagus and colon, M2 receptors do not play a dominant role in the overall contractile response (15). In the present study we also used 4-DAMP mustard, a muscarinic antagonist that preferentially binds irreversibly to M3 receptors, to study the relative contribution of M2 and M3 muscarinic receptors to canine smooth muscle contraction, activation of MAP kinase pathways, and phosphorylation of caldesmon.

The protocol developed by Ehler and Griffin (3) for preferential inactivation of M3 receptors depends on kinetic differences between the binding of 4-DAMP mustard and AF-DX 116 to M2 receptors. The rate constants for alkylation of M2 and M3 receptors by 4-DAMP mustard are approximately the same (k = 0.1 min\(^{-1}\)). In contrast, the affinity of 4-DAMP mustard for M3 receptors (K\(_d\) = 7.2 nM) is ~6.3-fold greater than that for the M2 receptors (K\(_d\) = 43 nM). The specificity of 4-DAMP mustard for alkylating M3 receptors can be enhanced by simultaneous exposure to AF-DX 116, an equilibrium-competitive antagonist with modest M2 selectivity. In the presence of micromolar concentrations of AF-DX 116, Ehler and Griffin (3) reported that 85% of M3 receptors were alkylated whereas 7.8% of M2 receptors were alkylated. We stimulated colon smooth muscle strips before and after M3 alkylation with protection of M2 receptors and found that blocking M3 receptors reduced ACh-induced contraction to 15% of the control response (Figs. 2 and 3). About one-half of the residual response was blocked by AF-DX 116, suggesting a primary role of M3 receptors (85% of maximum) in mediating contraction with a minor role played by M2 receptors (~7% of maximum). The remaining ACh-induced contraction might be an indirect nictinic effect on enteric neurons to release neurotransmitters other than ACh. These data probably can be explained by the notion that M2 receptors cause direct contraction through Ca\(^{2+}\) mobilization. M2 receptors might elicit contraction indirectly by inhibiting cAMP increase, by activation of nonselective cation channels, by enhancing sensitivity of the contractile proteins to Ca\(^{2+}\), or by some combination of these events.

MAP kinase pathways were proposed by us (5) to be one of the pathways that contribute to Ca\(^{2+}\) sensitization in colonic smooth muscle via phosphorylation of caldesmon. Phosphorylation was hypothesized to relieve the inhibition of actomyosin ATPase by caldesmon and thus enhance force development. Further studies by us and others show that G protein-coupled receptors are linked to activation of MAP kinases and to phosphorylation of caldesmon in vivo. In the present study we show that both ERK and p38 MAP kinases are activated by ACh in colonic smooth muscle (Figs. 4 and 5) and that activation of both MAP kinases appears to be primarily coupled to M2 receptors. Previously, we (8)
demonstrated that caldesmon is a good substrate for both enzymes, and in this study we find that blocking activation of ERK MAP kinases with PD-98509 prevents phosphorylation of caldesmon. In contrast, blocking the p38 MAP kinase pathway with SB-203580 had no effect on caldesmon phosphorylation (Fig. 6). Caldesmon phosphorylation, like activation of ERKs, was unaffected by alkylation of M3 receptor but completely blocked by AF-DX 116. We interpret this to mean that ACh activates M3 receptors to trigger a signal transduction cascade including ERK MAP kinases, which phosphorylate caldesmon at Ser789. We find no evidence for any change in phosphorylation of caldesmon at Ser759 using phosphorylation site-selective polyclonal antibodies developed by L. Adam (data not shown).

The functional significance of caldesmon phosphorylation at MAP kinase sites (Ser759 and Ser789) are controversial issues for which there is conflicting evidence. Evidence in support of functional significance of caldesmon phosphorylation includes reversal of the inhibitory effect of caldesmon on actin sliding velocity (5) and potentiation of contraction of chemically permeabilized smooth muscle by active ERK MAP kinase (4). However, Gorenne et al. (6) reported no effect of inhibiting the ERK MAP kinases on isometric contraction of vascular smooth muscle, and Krymsky et al. (11) reported little biochemical effect of caldesmon phosphorylation by ERKs on actomyosin ATPase and actin sliding velocity (11). Krymsky et al. (11) suggested that phosphorylation of caldesmon by ERKs is not the principal regulatory event in vivo and that there is another undefined caldesmon kinase activity in smooth muscle. Previously, we (5) reported a prominent 50-kDa caldesmon kinase activity detected by an in-gel kinase assay of colon smooth muscle extracts that did not cross-react with anti-caldesmon antibodies. However, in that study we clearly showed that ERK MAP kinases are activated by ACh, and in this study we show that ACh acting via M2 receptors induces phosphorylation of caldesmon at Ser789 (Fig. 7), which is blocked by the MEK inhibitor PD-98509 (Fig. 6). Therefore, if there are caldesmon kinase activities in vivo that are not ERK MAP kinases, they must be phosphorylating additional sites or the unidentified kinase activity is downstream of MEK.

In summary, we suggest that M3 muscarinic receptors are primarily responsible for colonic smooth muscle contraction, although M2 receptors contribute somewhat and activation of both receptors is required for maximum force development. We also present evidence suggesting that ACh activates ERK MAP kinases that phosphorylate caldesmon in vivo through activation of M2 muscarinic receptors and not via M1 receptors. This is a novel function for M2 receptors in smooth muscle, which to date have been linked primarily to decreased adenylate cyclase activity and activation of nonselective cation channels in smooth muscles. Although we show that Ser789 in caldesmon is a target for the ERK MAP kinases in colonic smooth muscle, the functional significance of that phosphorylation event for isometric force development appears to be minimal. It seems likely that other known substrates for the MAP kinases, such as phospholipases and transcription factors, are also phosphorylated via M2 receptor-mediated pathways in colonic smooth muscle, but the functional significance of these signaling events remain to be defined.

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