Signal-Transduction Pathways that Regulate Visceral Smooth Muscle Function

III. Coupling of muscarinic receptors to signaling kinases and effector proteins in gastrointestinal smooth muscles

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Gerthoffer, William T. Signal-Transduction Pathways that Regulate Visceral Smooth Muscle Function. III. Coupling of muscarinic receptors to signaling kinases and effector proteins in gastrointestinal smooth muscles. Am J Physiol Gastrointest Liver Physiol 288:G849–G853, 2005; doi:10.1152/ajpgi.00530.2004.—Stimulation of muscarinic M3 and M2 receptors on gastrointestinal smooth muscle elicits contraction via activation of G proteins that are coupled to a diverse set of downstream signaling pathways and effector proteins. Many studies suggest a canonical excitation-contraction coupling pathway that includes activation of phospholipases, production of inositol 1,4,5-trisphosphate and diacylglycerol, release of calcium from the sarcoplasmic reticulum, activation of L-type calcium channels, and activation of nonselective cation channels. These events lead to elevated intracellular calcium concentration, which activates myosin light chain kinase to phosphorylate and activate myosin II thus causing contraction. In addition, muscarinic receptors are coupled to signaling pathways that modulate the effect of activator calcium. The Rho/Rho kinase pathway inhibits myosin light chain phosphatase, one of the key steps in sensitization of the contractile proteins to calcium. Phosphatidylinositol 3-kinases and Src family tyrosine kinases are also activated by muscarinic agonists. Src family tyrosine kinases regulate L-type calcium and nonselective cation channels. Src activation also leads to activation of ERK and p38 MAPKs. ERK MAPKs phosphorylate caldesmon, an actin filament binding protein. P38 MAPKs activate phospholipases and MAPKAP kinase 2/3, which phosphorylate HSP27. HSP27 may regulate cross-bridge function, actin filament formation, and actin filament attachment to the cell membrane. In addition to the well-known role of M3 muscarinic receptors to regulate myoplasmic calcium levels, the integrated effect of muscarinic activation probably also includes signaling pathways that modulate phospholipases, cyclic nucleotides, contractile protein function, and cytoskeletal protein function.

heat-shock protein 27; mitogen-activated protein kinase; phosphatidylinositol 3-kinase; mitogen-activated protein kinase activated protein kinase; src

ACETYLCHOLINE ACTIVATES M2 AND M3 muscarinic receptors on gastrointestinal smooth muscle to elicit contraction. M3 receptors are coupled via Gq/11 G proteins to stimulate phospholipase C and phosphatidylinositol (PI) turnover, which triggers release of stored Ca2+ and activates protein kinase C (PKC). The net effect of these proximal biochemical events is a coordinated regulation of K+, Ca2+, and nonselective cation channels, which are ultimately coupled to oscillations in myoplasmic Ca2+ concentration. It is myoplasmic Ca2+ that is the primary intracellular event activating contractile proteins to generate force. Ca2+ activates myosin light chain kinase to phosphorylate the 20-kDa myosin light chains, which activate smooth muscle myosin II to generate force. In colonic smooth muscles, muscarinic stimulation increases phosphorylation of myosin, which correlates with contraction and increased tissue-shortening velocity. Signaling events coupled to the more abundant M2 receptors, which are coupled to Gq or G proteins are not as well defined but have received increased attention in recent years. Inhibition of adenylate cyclase is one of the earliest and best described effects of M2 receptor activation in smooth muscles (23). Newer studies reveal a diverse set of downstream signaling pathways coupled to M2 receptors. These include activation of Src family tyrosine kinases, PI 3-kinases, members of the MAPK superfamily, and PAK1. In some cases, such as Ca2+ activation of myosin light chain kinase and myosin phosphorylation, the effector proteins regulated by muscarinic receptors are well defined, and the functional effects are understood. In other cases, the signaling pathways and effector proteins are less certain. This review will describe M3 coupling to Ca2+ signaling and contraction, and data linking M2 receptors to protein and lipid kinases, contractile proteins, and heat-shock protein (HSP)27.

MUSCARINIC RECEPTOR EXPRESSION

Gastrointestinal smooth muscles typically express both M2 and M3 receptors in varying proportions depending on the region of the gastrointestinal tract (reviewed by Ref. 6). Receptor abundance was established primarily by functional studies with muscarinic antagonists, ligand binding studies, and mRNA levels. Radioligand binding and Northern blot analysis were used in studies of canine colon smooth muscle to establish a majority of muscarinic receptors are M2 receptors (82%) with the remainder being M3 (18%) (24). No evidence of other muscarinic receptor subtypes was reported. This is a common pattern of expression suggested by functional studies in esophagus, ileum, and colon smooth muscles. Functional data from pharmacological studies and knockout mouse models clearly show M3 and M2 receptors are necessary for gastrointestinal smooth muscle contraction, but M4 receptors are not (2). In most gastrointestinal tissues, M2 and M3 receptors are thought to be the only muscarinic receptors that mediate contraction.

EXCITATION-CONTRACTION COUPLING IN GASTROINTESTINAL SMOOTH MUSCLES

Gastrointestinal motility depends on activation and coupling of muscarinic receptors at multiple sites including enteric neurons, interstitial cells of Cajal, and smooth muscle cells. In smooth muscle cells, the canonical excitation-contraction coupling pathway includes activation of phospholipases, production of inositol 1,4,5-trisphosphate (IP3), and diacylglycerol, release of Ca2+ from the sarcoplasmic reticulum, inhibition of potassium channels, activation of L-type Ca2+ channels, and
activation of nonselective cation channels (Fig. 1). These signaling pathways are initiated by coupling of M2 and M3 receptors to downstream effectors through G proteins. M3 receptors couple via G_{11}, and M2 receptors are coupled to G_{i/o} G proteins. Coupling of both M2 and M3 receptors is highly dynamic, being regulated by G protein receptor kinases and regulators of G protein signaling (RGS) proteins. Interesting recent evidence also suggests receptor coupling may depend on disease status. M3 receptors are uncoupled by inflammation of the canine ileum, and M2 signaling is enhanced (17). Shi and Sarna (17) suggested this might underlie reduced motility during inflammation.

Activation of G proteins leads to production of ionic, lipid, and protein second messengers (Fig. 1). Muscarinic regulation of K^+, Ca^{2+}, and nonselective cation channels and corresponding currents has been thoroughly described, and the reader is referred to earlier reviews on this topic. Activation of phospholipases results in production of diacylglycerol and IP_3. Calcium released from the sarcoplasmic reticulum and influx of extracellular Ca^{2+} both occur with the net result being increased global myoplasmic Ca^{2+}. The principle molecular switch that initiates contraction is Ca^{2+} binding to calmodulin. This results in activation of myosin light chain kinase and phosphorylation of the regulatory myosin light chains thus activating myosin II motors. Force is generated, shortening velocity of the muscle cells increases, and the muscularis performs work by moving the contents of the gut.

Muscarinic activation of myosin II in intact smooth muscle can also be modified by several parallel and nonexclusive signaling pathways that result in an apparent “sensitization” or “desensitization” of the contractile elements to Ca^{2+}. Calcium sensitization of gastrointestinal smooth muscle can be induced by activating PKC, by activating the Rho/Rho kinase pathway, and by activating tyrosine kinases (reviewed by Ref. 20). Exactly what these protein kinases do to alter contraction is not clear yet. However, a consensus pathway includes muscarinic receptor stimulation causing activation of Rho A and Rho kinase to inhibit smooth muscle myosin light chain phosphatase. Inhibiting myosin phosphatase keeps myosin II phosphorylated and keeps the muscle contracting. Myosin phosphatase is composed of three subunits: a 37-kDa catalytic subunit (PP1c), a 110-kDa myosin binding subunit (MYPT), and a 20-kDa subunit of unknown function. Phosphatase activity is inhibited by phosphorylation of MYPT1, thereby potentiating the effect of Ca^{2+} to elicit contraction. There is also evidence for a pathway where PKC phosphorylates CPI17, which inhibits myosin phosphatase (14). This mechanism is plausible because muscarinic signaling via G proteins activates PKCs, but the significance of CPI17 phosphorylation may vary with the type of smooth muscle.

Because M2 receptor activation inhibits adenylyl cyclase in gastrointestinal smooth muscles (23), it is important to consider the fact that cAMP and cGMP both desensitize the contractile process. Several mechanisms have been proposed to explain cyclic nucleotide-dependent desensitization: direct binding of cGMP-dependent protein kinase 1a to MYPT1, phosphorylation and inactivation of RhoA, or phosphorylation of MYPT1 at Ser-695, a site that excludes phosphorylation of MYPT1 by Rho-dependent kinases. All three mechanisms would activate myosin phosphatase, promote dephosphorylation of myosin II, and relax smooth muscle. Activation of M2 receptors might contribute to contraction by reducing cAMP, which would favor increased or sustained myosin light chain phosphorylation and activation of myosin II cross bridges. The net effect of muscarinic receptor activation on contraction depends on complex interactions of myoplasmic Ca^{2+} levels, cyclic nucleotide levels, and metabolic coupling to multiple protein kinase cascades that simultaneously regulate myosin light chain kinase and myosin light chain phosphatase (Fig. 1).

**COUPLING OF MUSCARINIC RECEPTORS TO SIGNALING KINASES AND DOWNSTREAM EFFECTORS**

Src-family tyrosine kinases. G protein-coupled receptors activate multiple protein kinase cascades in smooth muscles, and activation of Src-family tyrosine kinases is one of the early postreceptor events (Fig. 2). Src is one member of a large family of tyrosine kinases ubiquitously expressed in many cell types, including smooth muscle. Src family members are membrane-associated nonreceptor protein tyrosine kinases that are activated by neurotransmitters, autacoids, growth factors, and cytokines. In a study of cultured canine colonic myocytes, we found multiple Src family tyrosine kinases are expressed, including Src, Fyn, and Yes (18). Muscarinic stimulation of cultured colon myocytes activated Src but not Fyn. With the use of the muscarinic antagonists 4-DAMP and AF-DX 116, we showed Src activation is mediated through M2 receptors but not via M3 receptors in colonic smooth muscle (18). Once activated, Src family members phosphorylate many substrates and participate in a wide range of cellular functions, including...
contraction of smooth muscles. A role for Src-family tyrosine kinases in contraction was first inferred from studies of inhibitors of tyrosine phosphorylation in intact smooth muscle. Chemical inhibitors of tyrosine kinases such as genistein block Src activation and smooth muscle contraction. In colonic and vascular smooth muscle, Src activates voltage-dependent Ca\(^{2+}\) channels and nonselective cation channels, inactivates Ca\(^{2+}\)-activated K\(^+\) channels, and stimulates phospholipase C activity. Blocking Src activity with PP2, anti-Src antibodies, or Src activated K\(^+\) channels, and contraction via activation of inhibition of adenylate cyclases (AC) and activation of phospholipases (cPLA2). PI 3-kinase, Src, and MAPK-signaling pathways might regulate multiple functions including Ca\(^{2+}\) channels, nonselective cation channels, proliferation, cell migration, and cytoskeletal remodeling.

There are few definitive studies of how muscarinic receptors are linked to Src-family kinases in smooth muscles. One hypothesis is that M2 receptors coupled via G\(_i\) liberate the G\(_{ai}\) and G\(_{ibγ}\) subunits that promote assembly of a signal-transduction complex that includes Src and a PI 3-kinase (Fig. 2). G\(_{ai}\) is implicated by the ability of antibody against G\(_{ai}\) to block methacholine-induced Ca\(^{2+}\) currents in rabbit colon smooth muscle cells (12). G\(_{ibγ}\) subunits are implicated by a recent study in vascular smooth muscle cells in which chemical inhibitors of Src family kinases and anti-G\(_{ibγ}\) antibodies uncoupled M2 receptors and L-type Ca\(^{2+}\) channels (3). This signaling pathway may be quite important in gastrointestinal smooth muscles that express a preponderance of M2 receptors coupled to G\(_{ib}\) G-proteins.

Although it is not entirely clear how nonreceptor tyrosine kinases contribute to smooth muscle contraction, effects on Ca\(^{2+}\), K\(^+\), and nonselective cation channels are probably very important. These channels are most often implicated by the effects of chemical inhibitors such as PP2. Unfortunately, chemical protein kinase inhibitors used to block Src family members are not particularly selective, so it is not clear which member or members of the Src family (Src, Fyn, Yes, Fgr, Lyn, or Lck) are participating in ion-channel regulation. Molecular tools such as blocking antibodies, overexpression of dominant negative constructs, inhibitory peptides, and RNAi are useful alternate approaches that are often quite fruitful. However, the high structural homology and similar substrate profiles of Src family members need to be considered when interpreting results of these approaches. Another emerging issue is the signaling components and effector proteins downstream of Src-family tyrosine kinases in smooth muscles. Some important downstream targets for Src include phospholipase C, PI 3-kinases, and MAPKs.

PI 3-kinases. PI 3-kinases are a family of eight lipid kinases in three structural classes that phosphorylate the 3-hydroxyl of PI to form a variety of phospholipid second messengers. All classes of PI 3-kinases are inhibited by wortmannin and LY294002, which are frequently used to implicate PI 3-kinase in cell functions, including smooth muscle contraction. Class IA isoforms of PI 3-kinase have been linked primarily to activation of receptor tyrosine kinases. The class IB (p110γ) isoform is thought to be activated by ligation of G protein-coupled receptors. The class IB isoform includes a plekstrin homology domain, which is a motif that binds G\(_{ibγ}\) subunits of G proteins. G\(_{ibγ}\) subunits also bind directly to the p110γ isoform independent of the pleckstrin homology domain. The relevance of G\(_{ibγ}\) binding to PI 3-kinase IB remains to be proven in gastrointestinal smooth muscle cells, but there is evidence that PI 3-kinases are components of muscarinic signaling pathways in these cells. In canine colon muscle strips, both class IA and IB PI 3-kinases are expressed based on RT-PCR and Western blot analysis (22). In the same study, acetylcholine was shown to activate a class IB PI 3-kinase but not class IA PI 3-kinase. Wortmannin blocks acetylcholine-induced activation of PI 3-kinase in intact colon muscle tissue (22). Zhou et al. (25) showed that PI 3-kinase is activated selectively via M2 muscarinic receptors in rabbit colon smooth muscle cells. In cultured canine colon muscle cells, serum stimulates both class IA and IB PI 3-kinases and induces cell migration that is blocked by wortmannin (22). In the gastrointestinal tract, cell migration might occur during wound healing or in the pathogenesis of gut tumors. Because cell migration depends on dynamic rearrangement of actin filaments, PI-3 kinases and MAPKs may regulate effector proteins important for actin polymerization and depolymerization or for attachment of actin to force-transducing structures in smooth muscle cells. Whether such an effect contributes to M2 receptor-mediated contraction is unknown, but it seems possible. Mammalian target of rapamycin is another important target for PI 3-kinases that, when activated, favors cell survival in part through regulation of translation of proteins regulating the cell cycle. One effect of muscarinic stimulation of smooth muscle cells may be to promote cell survival and the maintenance of the contractile phenotype, which is necessary to maintain gastrointestinal motility.

MAPKs: ERK. One of the downstream consequences of activation of Src and PI 3-kinases in gastrointestinal smooth muscle cells is stimulation of the ERK and p38 MAPK pathways. Members of all three MAPK families, the ERK, JNK, and the p38 MAPKs are expressed in smooth muscles. ERK MAPKs control proliferation induced by mitogenic agents such as peptide growth factors. MAPKs are also activated via G protein-coupled receptors in gastrointestinal smooth muscles (5, 11). With the use of a receptor protection method, we established that M2 receptors coupled preferentially to activation of ERK in canine colon smooth muscle (5) and that M3 receptors did not appear to signal to the ERK pathway. The failure of M3 receptor activation to stimulate ERK was surprising, because others have reported signaling to ERK from...
other G_q-coupled receptors. Activated ERK transmits signals to a remarkably diverse set of effector proteins. Verified direct substrates for ERKs in smooth muscles include phospholipases, transcription factors, and the actin binding protein caldesmon. A role for ERK activity and caldesmon phosphorylation in contraction was suggested by the fact that both increase following stimulation by acetylcholine and other contractile agonists (8). Phosphorylation of purified caldesmon by purified ERK1 MAPK reversed the inhibitory effect of caldesmon on actin sliding velocity, suggesting that contraction might be regulated by reversing the inhibitory effect of caldesmon on actomyosin ATPase (8). However, this hypothesis is controversial, and there are conflicting negative results in the literature suggesting phosphorylation of caldesmon by ERK MAPK does not affect smooth muscle contraction. Regardless of the controversy over caldesmon phosphorylation, several studies report that the MEK inhibitor PD98059 inhibits ERK activation and blocks contraction of vascular and gastrointestinal smooth muscles. PD98059 blocks spontaneous contraction of lower esophageal sphincter as well as contraction elicited by acetylcholine and phosphatase inhibitors (4). There are also cases where MEK inhibition blocks ERK phosphorylation but does not alter contraction (5, 10). The contributions of ERK and the downstream effector proteins are receptor and cell-type dependent, but the weight of evidence suggests ERK MAPKs target multiple signaling pathways that contribute to smooth muscle contraction. Potential sites of action of ERKs include phospholipase activation (25), regulation of myoplasmic Ca^{2+} levels, activation of myosin light chain kinase, and phosphorylation of caldesmon. Activation of M2 muscarinic receptors on gastrointestinal smooth muscle cells might therefore contribute to contraction, cell migration, and determination of the contractile phenotype by one or more of these biochemical mechanisms (Fig. 2).

MAPK: p38 MAPK. P38 MAPKs are close structural homologs of ERK MAPKs. P38 MAPKs are a family of four isoforms (α, β, γ, δ) that have a minimum consensus phosphorylation motif similar to the ERK MAPKs. Substrates in vivo include transcription factors (ATF2, MEF2C), the RNA binding protein tristetraprolin, and several protein kinases including MAPKAP kinases 2, 3, and 5. P38 MAPKs differ from ERK and JNK MAPKs in that the α- and β-isoforms are sensitive to inhibition by pyridinyl imidazoles SB203580 and SB239063. The α-isoform of p38 MAPK cloned by RT-PCR from canine smooth muscles was found to be expressed in colon, tracheal, and vascular smooth muscles (11). It is phosphorylated by MKK6 and MKK3 at a TGY activation motif in response to motor neurotransmitters acetylcholine and neurokinin A in colon smooth muscle (11). Caldesmon is a good substrate for the kinase in vitro, but caldesmon is not phosphorylated by p38 MAPK in vivo (5). Using 4-DAMP mustard and AFDX 116 (Otenzepad), we showed that activation of p38 MAPK occurred preferentially via M2 but not M3 receptor activation in colon smooth muscle (5).

P38 MAPKs participate in multiple processes in smooth muscle including contraction, cell migration, oxidative stress signaling, and cytokine synthesis. Contraction elicited by some but not all agonists depends on p38 MAPK activity. SB203580 inhibits spontaneous and acetylcholine-induced contraction of esophageal smooth muscle (4). However, Meloche et al. (13) found SB20350 inhibited angiotensin II-induced contraction of rat aorta but not phenylephrine-induced contraction. Therefore, p38 MAPKs mediate signaling events that are not common to all contractile agonists. The mechanisms by which p38 MAPK contributes to contraction are unclear. There is evidence supporting at least three nonexclusive hypotheses: 1) activation of p38 MAPK by some agonists promotes production of reactive oxygen species (13); 2) p38 MAPK phosphorylates and activates phospholipases; or 3) p38 MAPKs phosphorylates and activates MAPKAP kinases 2 and 3, which phosphorylate HSP27. Signaling to phospholipases in colon smooth muscle was shown by Zhou et al. (25), who proposed a pathway composed of M2 receptors, Gβγ13, PI 3-kinase, Gdc42, and Rac1, PAK1, and p38 MAPK, which phosphorylates cPLA2 (Fig. 2). Activation of cPLA2 leads to production of arachidonate, lysophospholipids, and eicosanoids, all of which could promote contraction.

MAPKAP kinase and HSP27. Activation of p38 MAPK and MAPKAP kinases leads to phosphorylation of HSP27 in many cell types. In smooth muscles, MAPKAP kinases are activated to phosphorylate HSP27 by thrombin, serotonin, angiotensin, vasopressin, endothelin, carbachol, ceramide, and cytokines. The functions of HSP27 in smooth muscle are not fully defined, but there is evidence that HSP27 acts as a chaperone or binding partner for Rho and PKC (16); binds to and modulates interaction of actin, myosin, and tropomyosin (19); and regulates actin filament structure (7). This is not surprising given the biochemical properties, the high levels of expression, and cellular localization in smooth muscle. HSP27 is constitutively expressed in smooth muscles at relatively high concentrations (2–8 µg HSP27/mg total protein) (9). HSP27 has been colocalized to contractile proteins in freshly dispersed intestinal smooth muscle cells stimulated with ceramide, and it coprecipitates with actin, tropomyosin, and caldesmon, suggesting some molecular association with contractile proteins (19). HSP27 also associates with integrin-linked actin attachment sites in airway smooth muscle and may be necessary for actin remodeling that occurs during smooth muscle cell migration (9). Bitar and colleagues (1) provided the earliest evidence that HSP27 participates in smooth muscle contraction. They showed bombesin-induced contraction of permeabilized intestinal smooth muscle cells was inhibited by anti-HSP27 antibodies (1) and more recently showed expression of nonphosphorylatable mutant HSP27 blocked acetylcholine-induced contraction (15). Yamboliev et al. (21) subsequently showed anti-HSP27 antibodies also reduced endothelin-1-induced contraction (15). Yamboliev et al. (21) subsequently showed anti-HSP27 antibodies also reduced endothelin-1-induced Ca^{2+}-sensitization of chemically permeabilized canine pulmonary artery muscle. Meloche et al. (13) used SB203580 to block the p38 MAPK/MK2 pathway and block HSP27 phosphorylation in rat aorta cells. Blocking HSP27 phosphorylation substantially inhibited angiotensin II-induced contraction. HSP27 appears to associate with multiple proteins important for signal-transduction (Rho/ROK), cytokoskeletal structure (actin and integrins), and contraction (actomyosin and tropomyosin) (Fig. 2). These proteins are all involved in muscarinic signal transduction in gastrointestinal smooth muscles, but the significance of these various protein-protein interactions in the response of the intact muscle remains to be firmly established. It may be that muscarinic activation of the p38 MAPK/MAPKAP kinase/HSP27 pathway contributes simultaneously to proper protein trafficking of signaling kinases, proper interac-
tion of actin and myosin, and dynamic remodeling of actin filaments.

In summary, muscarinic M3 and M2 receptors are coexpressed in gastrointestinal smooth muscle cells. They are coupled via G_{q11} and G_{i/o} G proteins to multiple protein and lipid kinases and phospholipases. Downstream signaling pathways control myoplasmic Ca^{2+} entry and release as well as the sensitivity of the contractile proteins to activator Ca^{2+}. There is differential signaling through M3- and M2-receptor subtypes. M3 receptors are clearly predominant in controlling cell Ca^{2+} levels. Both receptors regulate phospholipases and the resulting lipid second messengers. Many studies suggest a canonical excitation-contraction coupling pathway that includes activation of phospholipases, production of inositol triphosphate and diacylglycerol, release of Ca^{2+} from the sarcoplasmic reticulum, activation of L-type Ca^{2+} channels, and activation of nonselective cation channels. M2 receptors are prominently coupled to inhibition of adenylate cyclase and activation of PI 3-kinase, Src, ERK, and p38 MAPKs. Although the segregation of M3 and M2 signals is probably not absolute, M2 signaling appears to modulate Ca^{2+} and nonselective cation channels, cross-bridge function, and actin cytoskeleton remodeling. Contraction of intestinal smooth muscle therefore depends on integration of both M3- and M2-signaling networks, with M3 signaling playing a predominant role in excitation-contraction coupling.

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REFERENCES