Korean J Physiol Pharmacol
Vol 5: 287–297, August, 2001

G Protein-Coupled Receptor Signaling in Gastrointestinal Smooth Muscle

Uy Dong Sohn, Dong Seok Kim, and Karnam S Murthy[1]

Department of Pharmacology, College of Pharmacy, Chung Ang University, Seoul 156–756, Republic of Korea; [1]Department of Physiology, Box 980551, Medical College of Virginia, Richmond, VA 23298, USA

Contraction of smooth muscle is initiated by an increase in cytosolic Ca^{2+} leading to activation of Ca^{2+}/calmodulin-dependnet myosin light chain (MLC) kinase and phosphorylation of MLC. The types of contraction and signaling mechanisms mediating contraction differ depending on the region. The involvement of these different mechanisms varies depending on the source of Ca^{2+} and the kinetic of Ca^{2+} mobilization. Ca^{2+} mobilizing agonists stimulate different phospholipases (PLC-β, PLD and PLA_2) to generate one or more Ca^{2+} mobilizing messengers (IP_3 and AA), and diacylglycerol (DAG), an activator of protein kinase C (PKC). The relative contributions of PLC-β, PLA_2 and PLD to generate second messengers vary greatly between cells and types of contraction. In smooth muscle cell derived form the circular muscle layer of the intestine, preferential hydrolysis of PIP_2 and generation of IP_3 and IP_3-dependent Ca^{2+} release initiate the contraction. In smooth muscle cells derived from longitudinal muscle layer of the intestine, preferential hydrolysis of PC by PLA_2, generation of AA and AA-mediated Ca^{2+} influx, cADP ribose formation and Ca^{2+}-induced Ca^{2+} release initiate the contraction. Sustained contraction, however, in both cell types is mediated by Ca^{2+}-independent mechanism involving activation of PKC-ε by DAG derived form PLD. A functional linkage between G_{13}, RhoA, ROCK, PKC-ε, CPI-17 and MLC phosphorylation in sustained contraction has been implicated. Contraction of normal esophageal circular muscle (ESO) in response to acetylcholine (ACh) is linked to M_2 muscarinic receptors activating at least three intracellular phospholipases, i.e. phosphatidylcholine-specific phospholipase C (PC-PLC), phospholipase D (PLD) and the high molecular weight (85 kDa) cytosolic phospholipase A_2 ($cPLA_2$) to induce phosphatidylcholine (PC) metabolism, production of diacylglycerol (DAG) and arachidonic acid (AA), resulting in activation of a protein kinase C (PKC)-dependent pathway. In contrast, lower esophageal sphincter (LES) contraction induced by maximally effective doses of ACh is mediated by muscarinic M_3 receptors, linked to pertussis toxin-insensitive GTP-binding proteins of the $G_{q/11}$ type. They activate phospholipase C, which hydrolyzes phosphatidylinositol bisphosphate (PIP_2), producing inositol 1, 4, 5-trisphosphate (IP_3) and DAG. IP_3 causes release of intracellular Ca^{2+} and formation of a Ca^{2+}-calmodulin complex, resulting in activation of myosin light chain kinase and contraction through a calmodulin-dependent pathway.

Key Words: G-protein coupled receptor, Signal transduction, Smooth muscle, Contraction

The focus of this review is on signal transduction by G protein-coupled receptors in gastrointestinal smooth muscle. Most studies of signal transduction in smooth muscle have been done in dispersed smooth muscle cells devoid of neural elements isolated from all regions of the gut including esophagus, lower esophageal sphincter (LES) (Biancani et al, 1997; Sohn et al, 1993, 1994a, 1994b, 1997b), stomach, intestine (Bitar & Makhlouf, 1986; Grider & Murthy, 1995; Makhlouf & Murthy, 1997), gall bladder (Behar et al, 1993), and internal anal sphincter (Chakder et al, 1997). Cell dispersion also uncouples muscle cells from pace maker cells, interstitial cells of Cajal, thereby eliminating phasic (rhythmic) electrical and

Corresponding to: Karnam S Murthy, Department of Physiology, Box 980551, Medical College of Virginia, Richmond, VA 23298, USA. (Tel) 804-828-1853, (Fax) 804-828-2500

contractile activity. Both intact and permeabilized muscle cells were used effectively to characterize the signaling pathways initiated by the action of agonists and the mechanical response was measured by scanning micrometry (Bitar & Makhlouf, 1986). Smooth muscle cells, like other cells have the ability to process and respond to information that is transferred to them by hormones, neurotransmitters and growth factors. Transmitters can be classified pharmacologically in terms of their ability to cause direct contraction (excitatory transmitters) or relaxation (inhibitory transmitters) of smooth muscle cells.

Gastrointestinal smooth muscle cell contains a host of pharmacologically distinct receptors, which are coupled to contraction and relaxation of the cell. Stimulation of these receptors results in activation of effector proteins (e.g. enzymes and ion channels), which mobilize second messengers that cause either contraction or relaxation of smooth muscle (Makhlouf & Murthy, 1997; Sanders, 1998; Harnett et al, 1999). GTP binding proteins (G proteins) transmit information from cell surface receptors to effector proteins (Birnbaumer et al, 1990; Hepler & Gilman, 1992). G protein-coupled receptors, which form a large and functionally diverse superfamily are integral membrane proteins consisting seven transmembrane regions. G proteins are heterotrimers formed of α, β, and γ subunits and are classified into 4 families ($G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$) on the basis of their α subunit. In the basal state, the G protein exists as the heterotrimer with GDP tightly bound to the α subunit. Agonists interact with receptors associated with a GDP-liganded G protein with a higher affinity than with receptors not associated with such a G protein. Activation of receptors by the agonists causes dissociation of GDP in exchange for GTP and release $\beta\gamma$ of subunits as a dimer, which is a functional monomer: both $\beta\gamma$ and GTP-liganded α subunits can transmit signals (Hepler & Gilman, 1992; Clapham & Neer, 1993; Park et al, 1993; Wu et al, 1993). In some cases incoming signals are channeled into different directions (Rozengurt, 1998).

Activation of one G protein gives a bifurcating signal: following G protein activation, the α subunit activates one effector and the $\beta\gamma$ dimmer activates a different effector. Studies in intestinal muscle showed that adenosine A_1 receptors, opioid δ, κ, and μ receptors, somatostain sst_3 receptors, muscarinic m2 receptors and purinergic P_{2Y2} receptors are coupled to distinct pertussis toxin (PTx)-sensitive G proteins (Gi and/or Go): α subunits are coupled to inhibition of adenylyl cyclase and the $\beta\gamma$ subunits are additionally coupled to activation of PLC-β3 isoform (Murthy et al, 1996; Murthy & Makhlouf, 1995a, 1996, 1997, 1998).

In addition many receptors often activate different G proteins to generate multiple intracellular signals. The response to adenosine in intestinal smooth muscel reflects concurrent activation of two receptors (A_1 and A_2 receptors) and three signaling pathways: Ca^{2+} mobilization and inhibition of adenylyl cyclase via A_1 receptors and stimulation of cAMP via A_2 receptors (Makhlouf & Murthy, 1997). Likewise, muscarinic m2 receptors in gastric smooth muscle are coupled to inhibition of adenylyl cyclase Types V/VI via α subunit of Gi_3 and m3 receptors are coupled to activation of adenylyl cyclase V/VI via the $\beta\gamma$ subunit of G_q (Murthy & Makhlouf, 1997). During concurrent activation of both m2 and m3 receptors stimulation of adenylyl cyclase by m3 receptors is masked by the predominant inhibition mediated by the m2 receptors. Thus coexistence of receptor subtypes coupled distinct signaling pathways elicits various patterns of regulation of adenylyl cyclase.

Receptors select G proteins not only on the basis of its α subunit but also on the basis of its $\beta\gamma$ subunit. The intrinsic GTPase activity of the α subunit terminates the activity of the GTP-liganded α subunit and resets the heterotrimeric G protein. The G protein coupled receptor signal is also attenuated by mechanisms that target either receptors via G protein-coupled receptor kinase (GRK)-dependent phosphorylation (Pitcher et al, 1992; Premont et al, 1995; Grady et al, 1997) or G proteins via RGS (regulators of G protein signaling)-dependent stimulation of α-subunit GTPase activity (Lohse et al, 1996; Dohlman & Thorner, 1997; Berman & Gilman, 1998; Hepler, 1999). Phosphorylation of agonist-activated receptors by GRKs uncouples the receptors from the G protein resulting homologous desensitization and stimulation of α subunit GTPase activity terminates G protein activation. Two other mechanisms that target G proteins and limit their ability to transduce signals have been characterized in intestinal smooth muscle: feedback phosphorylation by protein kinase C (PKC) which selectively target Gi_1 and Gi_2, and transient sequestration of $G\alpha$ subunits by caveolin-3 which affects all activated $G\alpha$ subunits (Murthy & Makhlouf, 2000a, 2000b). Both mechanisms result in heterologous desensitization of re-

sponses mediated by different receptors coupled to the same G protein.

In the case of contractile agonists (excitatory transmitters), receptor activation typically results in a hydrolysis of phosphatidylinositols (PI) by one or more phospholipase β isoforms (PLC-β1-β4) and/or phosphatidylcholine (PC) by phospholipase D (PLD) and phospholipase A_2 (PLA_2) (Somlyo & Somlyo, 1994; Makhlouf & Murthy, 1997; Sanders, 1998; Harnett et al, 1999). Hydrolysis of phosphatidylinositol 4,5-bisphosphate results in the generation of second messengers, inositol 1,4,5-trisphosphate (IP_3), which mobilizes intracellular Ca^{2+} and 1,2-diacyglycerol (DAG), which activates PKC (Nishizuka, 1995). Hydrolysis of PC results in the activation of PKC via DAG. Products of PI-specific PLC play a major role in initiation of contraction, whereas the products of PC-specific PLD play a major role in sustained contraction. The mechanism implicated in the initiation of smooth muscle contraction involves increase in intracellular Ca^{2+} leading to the activation of Ca^{2+}/calmodulin-dependent myosin-light chain kinase (MLCK). MLCK phosphorylates the 20-kDa regulatory light chain of myosin (MLC), specifically at Ser,[19] which is an important step in the initiation of smooth muscle contraction (Somlyo & Somlyo, 1994; Stull et al, 1998).

Phosphorylation of the regulatory light chain by MLCK increases actin-activated MgATPase activity leading to muscle contraction. The extent of MLC phosphorylation is determined by the balance between the activities of MLCK and MLC phosphatase. Activation of MLCK is limited since, phosphorylation of MLCK by Ca^{2+}/calmodulin-dependent kinase decrease the Ca^{2+} sensitivity of light phosphorylation (Tansey et al, 1994). The initial Ca^{2+} transient is also rapidly dissipated by active uptake into the stores and extrusion from the cell. Thus, both intracellular Ca^{2+} levels and MLCK activity decline rapidly to near resting levels, whereas MLC phosphorylation and contraction are sustained at lower levels. Because the cytosolic concentrations of Ca^{2+} is not always proportional to the MLC phosphorylation levels several additional Ca^{2+}-independent mechanisms have been proposed for sustained MLC phosphorylation and contraction (Walsh et al, 1994; Horowitz et al, 1996a; Gong et al, 1997; Somlyo & Somlyo, 2000). These Ca^{2+}-independent mechanisms implicated in the sustained contraction involve small monomeric Ras-homology protein (RhoA), Rho-dependent kinase (ROCK) and one or more isoforms of PKC (Khalil & Morgan, 1992; Amano et al, 1996; Horowitz et al, 1996b; Kimura et al, 1996; Gong et al, 1997; Harnett et al, 1999; Lee et al, 1999; Weber et al, 1999; Murthy et al, 2000). Activation of either PKC, via the PKC target protein CPI-17 (17-kDa PKC-dependent inhibitor of protein phosphatase), or ROCK inhibits MLC phosphatase resulting in phosphorylation of MLC and sustained contraction (Masuo et al, 1994; Kimura et al, 1996; Uehata et al, 1997; Li et al, 1998). Activation of ROCK and phosphorylation of CPI-17 were shown to occur in response to agonist stimulation. Contraction can be initiated in the absence of Ca^{2+} when phosphatase inhibitors attenuate MLC phosphatase activity.

In the case of relaxant agonist (inhibitory transmitters), receptor activation typically results in activation of adenylyl cyclase and generation of cAMP. Compelling evidence exists supporting a role for nitric oxide (NO) derived by activation of neuronal NO synthase (nNOS) as inhibitory neurotransmitter (Sander & Ward, 1992; Stark & Szurzewski, 1993). The participation of NO derived from neuronal nitric oxide synthase (nNOS) in enteric neurons, and eNOS in smooth muscle cells is strongly supported by many studies (He & Goyal, 1993; Teng et al, 1998). Nitric oxide interacts with heme moiety of soluble guanylate cyclase and activates the enzyme to generate cGMP (Murad, 1994).

Activation of adenylyl cyclase and NO-dependent soluble guanylate cyclase during nerve-induced relaxation leads to concurrent generation of cAMP and cGMP (Murthy & Makhlouf, 1994; Murthy et al, 1998). Both cAMP and cGMP play important roles in mediating relaxation of smooth muscle via cAMP- and cGMP-dependent protein kinases (PKA and PKG) (Lincoln et al, 1990; Word et al, 1991; Murthy & Makhlouf, 1995d; Francis & Corbin, 1999). The kinases act by (i) inhibiting phospholipase β activity and generation of IP_3; (ii) inhibiting Ca^{2+} release from intracellular stores; (iii) stimulating Ca^{2+} uptake into stores; and (iv) by stimulation of plasmalemmal K^+ channels and inhibiting Ca^{2+} channels. PKA and PKG can also induce relaxation by acting on targets downstream of Ca^{2+} mobilization. Both protein kinases can decrease MLC phosphorylation during the initial phase of contraction by inhibiting Ca^{2+}/calmodulin-dependent activation of MLCK and/or activating MLC phosphatase (Somlyo & Somlyo, 1994). During the sustained phase of contraction, both

kinases decrease MLC phosphorylation by inhibiting the activation of the RhoA. Singular contribution of these mechanisms, however, remains to be established.

Functional diversity of smooth muscle is characterized by different patterns of contractile activity and different signaling pathways depending on the site of origin of the cells. This review addresses much of the information gained to date on the G protein-mediated signaling mechanisms coupled to contraction in muscle cells isolated from esophagus, lower esophageal sphincter and circular and longitudinal muscle layers of intestine. Limitations of space preclude a full justification of other important mechanisms (e.g. ion-channels and receptor-tyrosine kinases) regulating muscle contraction.

Disorders of esophageal motor function and lower esophageal sphincter (LES) competence affect more than one in ten adults over 40 years of age and one in four adults over 60. Knowledge of the mechanisms responsible for esophageal contraction and LES tone may be useful to understand normal function and some of the changes associated with esophageal disease (Dodds et al, 1982; Helm et al, 1983, 1984).

The esophagus is relaxed at rest and contracts with a peristaltic contraction upon swallowing, propelling the food bolus from the pharynx into the stomach, whereas the LES is spontaneously contracted, and relaxes in a timely way when the esophagus contracts, to allow passage of the bolus. The swallow-induced contraction of the esophagus is mediated by the neurotransmitter acetylcholine (ACh). The tonic contraction of the LES is due to specialized myogenic mechanisms, which may be modulated by inhibitory non-adrenergic-non-cholinergic (NANC) and by excitatory cholinergic neural pathways. The present review describes the cellular basis for ACh-induced ESO and LES contraction and spontaneous LES tone and how inflammation induced by acid or by reflux of gastric contents affects the signal transduction mechanisms mediating contraction of these smooth muscles.

A smooth muscle esophagus is present in marsupials, felines and primates. Because of similarities of signal transduction mechanisms with the human esophagus, the cat is a reasonable model for the study of signal transduction in esophageal and LES circular muscle.

Signal transduction mediating muscle contraction in intestine

Smooth muscle of the small intestine consists of an outer longitudinal layer separated from an inner circular muscle layer by the myenteric plexus of the enteric nervous system. The enteric nervous regulates smooth muscle function. Neurons of the myenteric plexus project fibers into the circular and into the longitudinal muscle layer. Considerable progress has been made by Makhlouf's group in characterizing receptors-specific (receptor subtypes) and cell-specific signaling mechanisms in smooth muscle isolated separately from longitudinal and circular muscle layers (Makhlouf & Murthy, 1997; Kuemmerle et al, 1998). Different Ca^{2+} mobilization pathways involving distinct effector enzymes and Ca^{2+} mobilizing second messengers have been characterized to initiate contraction in these two muscle cell types. In both circular and longitudinal muscle cells the contractile agonists, cholecystokinin octapeptide (CCK-8) and acetylcholine (ACh) caused contraction and increase in cytosolic Ca^{2+}. Neither contraction nor increase in cytosolic Ca^{2+} was affected by withdrawal of Ca^{2+} from the medium or addition of Ca^{2+} channel blockers in circular muscle but were abolished in longitudinal muscle cells suggesting that contraction and increase in cytosolic Ca^{2+} were mediated by Ca^{2+} release in circular muscle and Ca^{2+} influx in longitudinal muscle cells (Grider & Makhlouf, 1988; Murthy et al, 1991). In circular muscle cells contractile agonists stimulate preferentially PLC-β1 via the α subunit of pertussis toxin (PTx)-insensitive G protein, Gq resulting in the hydrolysis of PIP_2 and generation of IP_3 and DAG (Murthy & Makhlouf, 1995b). Basal levels of IP_3 were twice as high and the peak increase in IP_3 generation induced by CCK-8 and ACh were 10 times greater in circular muscle cells (Murthy & Makhlouf, 1991). High affinity IP_3 receptors mediating IP_3-dependent Ca^{2+} release are present in circular muscle cells only (Murthy et al, 1991). Consistent with this, initial contraction induced by CCK-8 or ACh in circular muscle cells was abolished by 1) G protein inhibitors (GDPβS, Gα_q antibody), 2) PI-PLC inhibitors (neomycin, U73122 or PLC-β1 antibody), 3) emptying intracellular Ca^{2+} stores with sarcoplasmic Ca^{2+} ATPase inhibitor, thapsigargin, 4) IP_3 receptor antagonist, heparin, and 5) calmodulin antagonists (calmidazolium and W-7). Furthermore, addition of GTPγS or IP_3 to perme-

abilized muscle cells caused Ca^{2+} release and contraction (Murthy et al, 1991; Murthy & Makhlouf, 1995b).

In longitudinal muscle cells, however, contractile agonists stimulated rapidly PLA_2 via the $\beta\gamma$ subunit of PTx-sensitive G protein resulting in the hydrolysis of PC and generation of arachidonic acid (AA) (Murthy et al, 1995). The initial increase in AA coincided with contraction and increase in Ca^{2+}: GDPβs and PTx abolished all three events. AA caused Ca^{2+} influx indirectly by activating Cl^- channels, thereby depolarizing the membrane and opening voltage-sensitive Ca^{2+} channels (Kuemmerle et al, 1998). AA, in the presence of both lipoxygenase and cyclooxygenase inhibitors, caused contraction and increase in Ca^{2+}: the Ca^{2+} channel blocker, nifedipine, abolished both contraction and increase in Ca^{2+} (Murthy et al, 1995a). Ca^{2+} influx in longitudinal muscle acts as a trigger for Ca^{2+}-induced Ca^{2+} release from ryanodine-sensitive channels. Ca^{2+} channels in longitudinal muscle cells possess high-affinity ryanodine receptors and are highly sensitive to Ca^{2+} (Kuemmerle et al, 1994). Cyclic ADP ribose (cADPR) is an endogenous cyclic metabolite of NAD^+ synthesized in cells and tissues expressing ADP-ribosyl cyclase. cADPR enhances the sensitivity of ryanodine-sensitive Ca^{2+} channels. cADPR was shown to bind with high affinity to permeabilized longitudinal cells only and activate ryanodine-sensitive Ca^{2+} channels (Kuemmerle & Makhlouf, 1995). cADPR was synthesized from β-NAD^+ by a membrane-bound ADP-ribosyl cycles in response to contractile agonist suggesting a role for cADP ribose in Ca^{2+}-induced Ca^{2+} release in longitudinal muscle (Kuemmerle & Makhlouf, 1995). Exogenous addition of cADP ribose caused contraction and increase in cytosolic Ca^{2+}: cADPR antagonist, 8-amino-cADP ribose blocked both responses. Ca^{2+} mobilization and contraction in longitudinal muscle cell appears to be independent PI hydrolysis, since PI-PLC inhibitors do not affect contraction and increase in Ca^{2+} (Murthy et al, 1995). Consistent with this initial contraction induced by CCK-8 or ACh in longitudinal muscle cells was abolished by 1) G protein inhibitors (GDPβS and PTx), 2) PLA_2 inhibitors (dimethyleicosadienoic acid (DEDA), $AACOF_3$, 3) Ca^{2+} channel blockers (nifedipine, methoxyverapamil), 4) cADPR antagonist (8-amino-cADPR), and 5) calmodulin antagonists (calmidazolium and W-7) (Makhlouf & Murthy 1997; Kuemmerle et al, 1998).

Contraction induced by agonists in both longitudinal and circular muscle cells consists of a transient initial contraction followed by a prolonged sustained contraction. As noted above, an increase in the cytosolic Ca^{2+} was the major determinant of initial contraction in both cell types. However, sustained contraction in both circular and longitudinal muscle cells, unlike initial contraction, was mediated by Ca^{2+}-independent PKC isoform, PKC-ε. The time course of PKC-ε translocation to membrane coincides with that of sustained contraction (Murthy et al, 2000). Contractile agonist-induced hydrolysis of PC by PLD in intestinal smooth muscle was responsible for sustained formation of DAG and activation of PKC (Murthy & Makhlouf, 1995b). Sustained PLD and PKC activities were abolished by treatment of cells with G protein inhibitor, GDPS but not affected by treatment with PTx suggesting involvement of PTx-insensitive G protein in sustained activation of PLD and PKC (Murthy & Makhlouf, 1995b). Recent studies have provided a link between G protein activation and PKC activation that could lead to inhibition of MLC phosphatase activity and sustained increase in MLC phosphorylation levels (Murthy et al, 2001). Contractile agonists, CCK-8 and ACh, activated G_{13} leading to sequential activation of RhoA, Rho-kinase and PLD. Phosphatidic acid, the primary product of PLD was converted to DAG resulting in the activation of PKC leading to MLC phosphorylation and sustained muscle contraction. MLC phosphatase inhibition occurs via PKC-dependent phosphorylation of CPI-17. This additional mechanism can increase the levels of MLC phosphorylation and induce contraction independently of intracellular Ca^{2+} concentration. Consistent with notion, sustained contraction was inhibited by 1) Gα_{13} antibody or RhoA antibody, 2) RhoA inhibitor, *Clostridium botulinum* C3 exoenzyme, 3) PLD inhibitor (propranolol), 4) PKC inhibitors (calphostin C, bisindolylmaleimide or chelerythrine chloride, and 5) specific PKC-ε antibodies and myristoylated pseudosubstrate inhibitor of PKC-ε (Murthy et al, 2000; Murthy et al, 2001).

Contraction of esophageal circular muscle and LES

Esophageal contraction in response to ACh is mediated by M_2 muscarinic receptors since ACh-induced contraction is selectively inhibited by the M_2 mus-

carinic antagonist methoctramine. We have examined the G-proteins linked to muscarinic and other receptors in the contractile pathway of ESO (Sohn et al, 1993, 1995; Kim et al, 1997) by examining the effect of selective G-protein antibodies on contraction. Our data suggest that M_2 muscarinic receptors are linked mostly to G_{i3} because ACh-induced contraction of permeable cells is inhibited by antibodies against the a-subunit of G_{i3}, but not by antibodies against the a-subunit of G_q, G_o or $G_{i1/2}$.

G-proteins are linked to phospholipases, which generate intracellular second messengers from membrane phospholipids. ACh-induced contraction of ESO is inhibited by selective inhibitors or antibodies of PC-PLC, PLD and $cPLA_2$, suggesting that there is a link between G_{i3} and these phospholipases (Sohn et al, 1993, 1995, 2000).

PLA_2 preferentially hydrolyzes phospholipids containing arachidonic acid (AA) in the sn-2 position (most often phosphatidylcholine), producing AA and lysophospholipid (Dennis, 1994, 1997). PC-PLC hydrolyzes phosphatidylcholine at the sn3 position, producing DAG and phosphocholine (Billah et al, 1989; Qian & Drewes, 1990, 1991). PLD also hydrolyzes phosphatidylcholine at the sn3 position, producing choline and phosphatidic acid (PA). PA may act as a second messenger or may be dephosphorylated to DAG by a phosphatidic acid phosphohydrolase (Billah & Anthes, 1990; Exton, 1990; Qian & Drewes, 1990, 1991; Dennis et al, 1991).

Activation of phospholipases PC-PLC and PLD results in production of DAG (Hillemeier et al, 1996; Cao et al, 1998), and activation of $cPLA_2$ produces AA (Sohn et al, 1994b). In the normal esophagus AA causes little contraction by itself but potentiates contraction induced by the PKC agonist DAG. DAG and AA act synergistically to activate protein kinase C (Sohn et al, 1994b). ACh-induced contraction and activation of phospholipases requires influx of extracellular Ca^{2+} because ACh-induced contraction and DAG production decrease with decreasing extracellular Ca^{2+} levels. However, activation of PKC by DAG is Ca^{2+}-independent because DAG-induced contraction does not significantly change when extracellular or cytosolic Ca^{2+} is reduced to zero (Sohn et al, 1994a). These data suggest that the influx of extracellular Ca^{2+} is required only to activate the phospholipases, and once the second messengers are produced contraction proceeds by the activation of a Ca^{2+}-independent PKC (Sohn et al, 2000; Cao et al, 2001).

PKC isozymes present in ESO and found βII, γ, and ε PKC isozymes (Sohn et al, 1997b). However, when ESO is stimulated by ACh only the Ca^{2+}-independent ε isozyme translocates from the cytosol to the membrane, suggesting that PKC ε is involved in agonist-induced contraction of ESO. This view is supported by the findings that PKC ε antibodies and isozyme-selective pseudosubstrates inhibit ACh-induced contraction of ESO (Sohn et al, 1997b).

In contrast to spontaneous tone, contraction induced by maximally effective doses of the cholinergic neurotransmitter acetylcholine is mediated through muscarinic M_3 receptors, linked to pertussis toxin-insensitive GTP-binding proteins of the $G_{q/11}$ type. They activate phospholipase C, which hydrolyzes PIP_2, producing IP_3 and DAG. IP_3 causes release of intracellular Ca^{2+} and formation of a Ca^{2+}-calmodulin complex, resulting in activation of myosin light chain kinase and contraction through a calmodulin-dependent pathway (Biancani et al, 1994).

Calmodulin and MLCK play a role in ACh-induced LES contraction, whereas the classical MLCK may not be the major kinase responsible for contraction and phosphorylation of MLC in ESO. ESO contraction is PKC-dependent. Caldesmon and/or calponin may play a role in PKC-dependent contraction (Sohn et al, 2001)

The inhibitory role of calmodulin on PKC-induced contraction (unpublished) is relevant in order to understand the switch in signal transduction pathways that occur in an experimental model of acute esophagitis (AE) (Sohn et al, 1997a).

LES tone

The LES circular muscle is a major determinant of LES tone. Although the relative neurogenic contribution may vary with the animal species, a significant component of tone is thought to be myogenic, as it is not affected by neural antagonists, including tetrodotoxin. Functionally, this muscle is specialized, with muscle strips from this region developing higher total and active forces than esophageal strips (Christensen et al, 1973a, 1973b; Biancani et al, 1982). This distinctive contractility may be, at least in part, related to the ability of the LES muscle to handle Ca^{2+} differently than esophageal circular muscle (DeCarle et al, 1977; Biancani et al, 1987). LES muscle main-

tains tension in a Ca^{2+}-free environment for some time after esophageal strips are no longer capable of contraction in response to field stimulation or high concentrations of acetylcholine.

These findings suggest that LES muscle can use Ca^{2+}, released from intracellular storage sites, to maintain tonic contraction, and they are consistent with the histology and biochemistry of these muscles. The LES circular muscle has more abundant endoplasmic reticulum than the esophageal circular muscle (Christensen & Roberts, 1983).

LES tone is associated with spontaneous, low-level activity of PI-PLC, resulting in production of submaximal concentrations of DAG and IP_3, which causes release of Ca^{2+} from stores. The elevated concentrations of IP_3 and DAG, present in LES smooth muscle in the absence of stimulation, decrease when the LES relaxes in response to VIP (Biancani et al, 1992; Hillemeier et al, 1996). Increased IP_3 turnover, resulting in spontaneously elevated IP_3 levels and steady Ca^{2+} release from storage sites, may be responsible for LES tonic contraction. In addition, concurrent activity of a PC-PLC in the LES contributes to the production of additional DAG (Biancani et al, 1994; Hillemeier et al, 1996). IP_3 and DAG, in turn, activate PKC (Sohn et al, 1997a). IP_3 and DAG, produced at submaximal levels, act synergistically, their interaction depends on Ca^{2+} release and is mediated through the Ca^{2+}-sensitive PKC β isozyme (Biancani et al, 1994; Sohn et al, 1997a).

Since G-proteins are linked to phospholipases we examined the G-proteins present in the LES. We find, by Western Blot analysis, that G_q, G_{i3}, and G_{i1-2} are present in LES circular muscle (Sohn et al, 1995) and that these G-proteins are spontaneously active, i.e. bound to GTP, in the absence of exogenously added excitatory neurotransmitters. In unstimulated LES smooth muscle, [^{35}S]GTP γS binding to G_{i3}, $G_{i1/2}$, and G_q antibodies is higher than in ESO smooth muscle, suggesting that these G-protein may be activated. Spontaneous activation of G-proteins may provide the spontaneous activation of the phospholipases required to maintain threshold levels of IP_3 and DAG, which in turn, activate a PKC-dependent tone.

Spontaneous activation of a group I $sPLA_2$ causes production of AA, and AA metabolites such as PGF2 α and thromboxanes, which maintain activation of G-proteins such as G_{i3}, $G_{i1/2}$, and G_q. These G-proteins activate phospholipases such as PI-PLC and PC-PLC which in turn produce DAG and IP_3. DAG and IP_3 synergistically activate PKC. The origin of the $sPLA_2$ remains to be found, however preliminary Western Blot studies using monoclonal antibodies against pancreatic (i.e. group I) human $sPLA_2$ indicate that a pancreatic-like $sPLA_2$ is present in human LES circular smooth muscle.

Contraction induced by $sPLA_2$ is mediated by the same signal transduction pathway that is active in maintenance of LES tone. In control LES, $sPLA_2$-induced contraction is reduced by the same inhibitors that affect LES tone of in vitro circular muscle strips. D609 (PC-PLC inhibitor), U73122 (PI-PLC inhibitor) and chelerythrine (PKC inhibitor) reduces both LES tone and $sPLA_2$-induced contraction (Biancani et al, 1994; Hillemeier et al, 1996) supporting the view that $sPLA_2$ induced contraction, like "spontaneous" LES tone, depends on the activity of PI-PLC, PC-PLC, resulting in contraction through a PKC-dependent pathway.

CONCLUSION

Smooth muscle of the mammalian gut is functionally heterogeneous with its diverse signaling pathways to mediate MLC phosphorylation and contraction. When MLCK was first discovered nearly two decades ago, it was thought that the contractile response is a Ca^{2+}-dependent process and MLC phosphorylation resulted solely from the increase in cytosolic Ca^{2+} and activation of Ca^{2+}/calmodulin- depedent MLCK. The pertinent question that followed much later was obviously how MLC phosphorylation and contraction were sustained in the absence of increase in cytosolic Ca^{2+}. It has become increasingly clear that several other Ca^{2+}-independent mechanism are involved in maintenance of MLC phosphorylation and contraction. In addition studies over the past decade, using single cells and suspensions of muscle cells, have established that smooth muscle cells isolated from different regions of the gut express a unique repertoire of receptors for excitatory and inhibitory transmitters, Ca^{2+} handling mechanisms and signaling pathways to mediate the main function of the smooth muscle, i.e. contraction. These developments have greatly accelerated and modified our understanding of G protein-mediated signal transduction in gastrointestinal smooth muscle.

ACKNOWLEDGEMENTS

Uy Dong Sohn expresses thanks for the financial support by Korean Science and Engineering Foundation (Grant No. 2000-1-21400-001-3).

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