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Signal Transduction in Esophageal and LES Circular Muscle Contraction

Karen M. Harnett, Weibiao Cao, Nayoung Kim, Uy Dong Sohn, Harlan Rich, Jose Behar, and Piero Biancani[a]

Department of Medicine, Rhode Island Hospital and Brown University, Providence, Rhode Island

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^{++} and formation of a Ca^{++}-calmodulin complex, resulting in activation of myosin light chain kinase and contraction through a calmodulin-dependent pathway.

Signal transduction pathways responsible for maintenance of LES tone are quite distinct from those activated during contraction in response to maximally effective doses of agonists (e.g., ACh). Resting LES tone is associated with activity of a low molecular weight (~14 kDa) pancreatic-like (group I) secreted phospholipase A_2 ($sPLA_2$) and production of arachidonic acid (AA), which is metabolized to prostaglandins and thromboxanes. These AA metabolites act on receptors linked to G-proteins to induce activation of PI- and PC-specific phospholipases, and production of second messengers. Resting LES tone is associated with submaximal PI hydrolysis resulting in submaximal levels of inositol trisphosphate (IP_3)-induced Ca^{++} release, and interaction with DAG to activate PKC.

In an animal model of acute esophagitis, acid-induced inflammation alters the contractile pathway of ESO and LES. In LES circular muscle, after induction of experimental esophagitis, basal levels of PI hydrolysis are substantially reduced and intracellular Ca^{++} stores are functionally damaged,

[a]*To whom all correspondence should be addressed:* Piero Biancani, Ph.D., Rhode Island Hospital, 593 Eddy Street, 5SWP, Providence, RI 02903. Tel.:401-444-5629; Fax: 401-444-5890; E-mail: piero_biancani@brown.edu.

[b]*Abbreviations:* AA, arachidonic acid; AE, acute experimental esophagitis; ACh, acetylcholine; CE, chronic experimental esophagitis; $cPLA_2$, cytosolic phospholipase A_2; DAG, diacylglycerol; ESO, esophagus/esophageal; GERD, gastro-esophageal reflux disease; IL, interleukin; IP_3, inositol 1-4-5 triphosphate; LES, lower esophageal sphincter; LT, leukotriene; NANC, non-adrenergic non-cholinergic; NDGA, nordihydro-guaiaretic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PC-PLC, phosphatidylcholine-specific phospholipase C; PG, prostaglandin; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; PIP_2, phosphatidylinositol bisphosphate; PKC, protein kinase C; PLD, phospholipase D; $sPLA_2$, secreted phospholipase A_2; TX, thromboxane.

resulting in a reduction of resting tone. The reduction in intracellular Ca^{++} release causes a switch in the signal transduction pathway mediating contraction in response to ACh. In the normal LES, ACh causes release of Ca^{++} from intracellular stores and activation of a calmodulin-dependent pathway. After esophagitis, ACh-induced contraction depends on influx of extracellular Ca^{++}, which is insufficient to activate calmodulin, and contraction is mediated by a PKC-dependent pathway. These changes are reproduced in normal LES cells by thapsigargin-induced depletion of Ca^{++} stores, suggesting that the amount of Ca^{++} available for release from intracellular stores defines the signal transduction pathway activated by a maximally effective dose of ACh.

INTRODUCTION

Disorders of esophageal motor function and lower esophageal sphincter (LES)[b] 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.

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. We have used the cat to study esophageal and lower esophageal sphincter function and have occasionally obtained normal esophageal/LES muscle specimens from human organ donors. We observed that 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.

We have examined normal animals and two experimental models of esophagitis. An acute model of experimental esophagitis (AE) was obtained by esophageal perfusion with 0.1 N HCl for 45 min on three successive days, with experiments carried out on the fourth day [1-5].

More chronic models of experimental esophagitis (CE) were obtained by performing a myotomy of the LES circular muscle and allowing esophageal inflammation to develop over the course of six months (six-month CE) or 12 to 14 months (one-year CE) [6-9]. In the 6-month CE model we find that inflammation-induced changes in mucosal histology and in contractile mechanisms are similar to the acute model and reversible by treatment with acid suppressants [8]. Histologic and mechanical changes were more pronounced in the acute model, supporting this as a reasonable model for the study of inflammation-induced disturbances of esophageal and LES motor function.

ACh-induced contraction of ESO and LES circular muscle depends on distinct intracellular pathways beginning with the muscarinic receptors acted upon by the neurotransmitter, extending to the G-proteins, phospholipases, second messen-

gers, and effector mechanisms mediating muscle contraction. We will review separately the signal transduction pathways responsible for contraction of ESO and LES and for maintenance of LES tone in normal animals and in the two experimental models of esophagitis.

CONTRACTION OF NORMAL ESOPHAGEAL CIRCULAR MUSCLE

Esophageal contraction in response to ACh is mediated by M_2 muscarinic receptors since ACh-induced contraction is selectively inhibited by the M_2 muscarinic antagonist methoctramine. We have examined the G-proteins linked to muscarinic and other receptors in the contractile pathway of ESO [10-12] 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 α-subunit of G_{i3}, but not by antibodies against the α-subunit of G_q, G_o or $G_{i1/2}$ (Figure 1).

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 phosphatidylcholine-specific phospholipase C (PC-PLC), PLD, and $cPLA_2$ suggesting, a link between G_{i3} and these phospholipases [10, 11].

PLA_2 preferentially hydrolyzes phospholipids containing arachidonic acid (AA) in the sn-2 position (most often phosphatidylcholine), producing AA and lysophospholipid [13, 14]. PC-PLC hydrolyzes phosphatidylcholine at the sn3 position, producing diacylglycerol (DAG) and phosphocholine [15-17]. 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 [16-20].

Activation of phospholipases PC-PLC and PLD results in production of DAG [21, 22], and activation of $cPLA_2$ produces AA [23]. 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 [23]. ACh-induced contraction and activation of phospholipase requires influx of extracellular Ca^{++} because ACh-induced contraction and DAG production decrease with decreasing extracellular Ca^{++} levels. However, activation of PKC by DAG is Ca^{++}-independent because DAG-induced contraction does not significantly change when extracellular or cytosolic Ca^{++} is reduced to zero [24]. These data suggest that the influx of extracellular Ca^{++} is required only to activate the phospholipases, and once the second messengers are produced contraction proceeds by the activation of a Ca^{++}-independent PKC [22].

We have examined the PKC isozymes present in ESO and found βII, γ, and ε PKC isozymes [25]. However, when ESO is stimulated by ACh only the Ca^{++}-independent ε isozyme translocates from the cytosol to the membrane, suggesting that PKCε is involved in agonist-induced contraction of ESO. This view is supported by the findings that PKCε antibodies and isozyme-selective pseudosubstrates inhibit ACh-induced contraction of ESO [25].

To conclude, in normal ESO ACh-induced contraction is mediated by activation of M_2 muscarinic receptors coupled to G_{i3} type G-proteins. G-protein activation of PC-PLC, PLD and $cPLA_2$ phospholipases results in phosphatidylcholine hydrolysis and production of the second messengers DAG and AA. DAG and AA synergistically activate a Ca^{++}-independent PKCε and produce a PKC-dependent contraction.

ESOPHAGEAL CONTRACTION IN MODELS OF ESOPHAGITIS

Acute esophagitis.

In vivo esophageal contraction in response to swallowing and *in vitro* response to electrical stimulation are antagonized by atropine, suggesting the involvement of cholinergic excitatory neurons. After induction of acute experimental esophagitis by repeated acid perfusion, contraction in response to KCl and to ACh, which act directly on the muscle, is not affected [1, 2]. However, the *in vivo* response to swallowing and the *in vitro* response to electrical, i.e., neural stimulation, are significantly reduced. These data suggest that after acid perfusion esophageal muscle is still capable of contraction, but that the cholinergic neural mechanisms responsible for release of excitatory neurotransmitters may be affected.

Intestinal inflammation in man [26] and animals [27] has been reported to result in changes in motility caused by alterations in enteric nerve and muscle. Pro-inflammatory cytokines present in inflammatory sites, such as interleukin-1β (IL1β), have been shown to alter muscle contractility by suppressing the release of neurotransmitters ACh and norepinephrine [28, 29].

We examined the effect of pro-inflammatory cytokines on normal esophageal smooth muscle function. We find that in

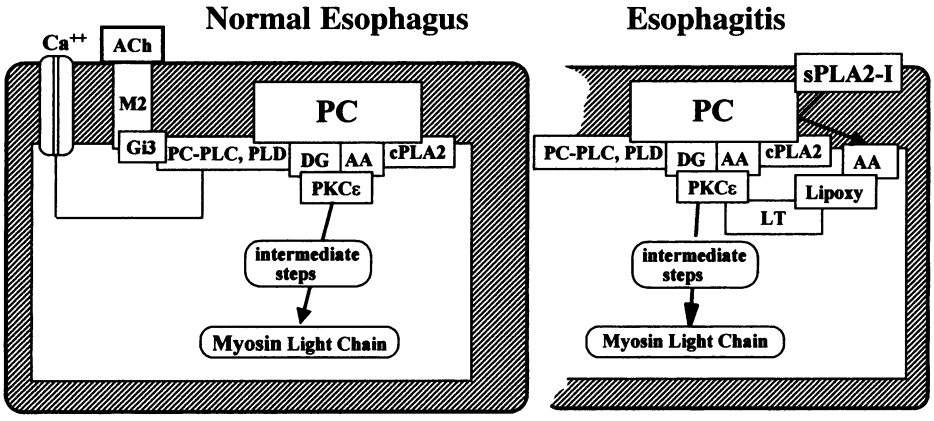

Figure 1. In the normal esophagus, ACh-induced contraction is mediated by M_2 muscarinic receptors linked to a pertussis toxin-sensitive GTP-binding protein of the G_{i3} type and results in activation of at least three phospholipases acting on membrane phospholipids [10]. Phosphatidylcholine-specific phospholipase C (PC-PLC) and phospholipase D (PLD) hydrolyze phosphatidylcholine (PC) to produce diacylglycerol (DAG). Cytosolic phospholipase A_2 ($cPLA_2$) is also activated, resulting in production of arachidonic acid (AA) [23]. Activation of these phospholipases requires the presence of Ca^{++} [22, 24], which is provided by the influx of extracellular Ca^{++} through voltage-dependent channels [50], possibly augmented by release of Ca^{++} from stores. Once DAG is produced, the Ca^{++}-independent protein kinase Cε (PKCε) is activated, and contraction proceeds through a calmodulin-independent pathway [24]. AA, produced by PLA2, which is also Ca^{++}-dependent, potentiates the DAG-induced activation of PKC [23]. Acute esophagitis (AE) modifies ACh signaling in the esophageal circular smooth muscle: a group I secreted PLA_2 ($sPLA_2$) produces AA, which is metabolized by lipoxygenase (lipoxy) to leukotrienes (LT), which contribute to activation of PKC [3].

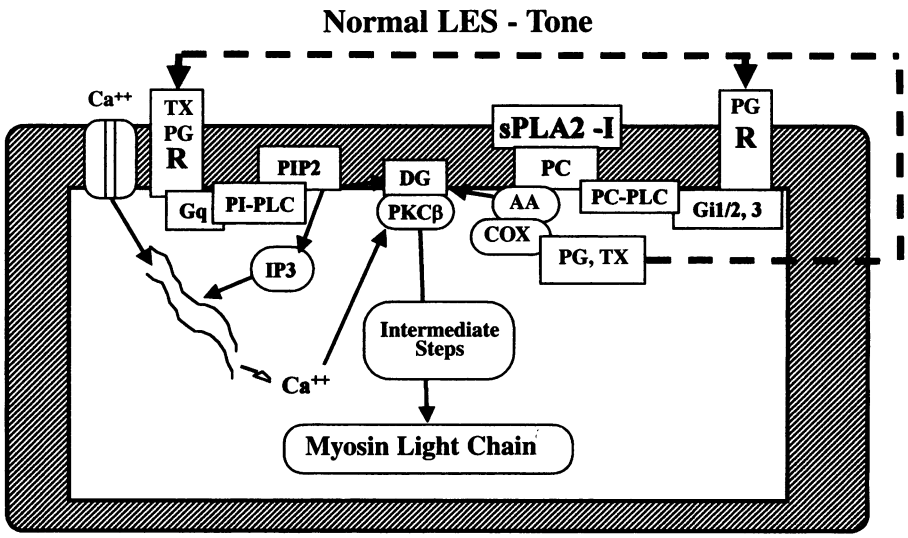

Figure 2. Resting LES tone is associated with spontaneous activity of phosphatidylinositol-specific phospholipase C (PI-PLC) [53] and phosphatidylcholine-specific phospholipase C (PC-PLC) [21] resulting in production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) [21] and activation of a Ca^{++}-sensitive protein kinase Cβ (PKCβ) [25]. A low molecular weight (14 kDA) secreted, pancreatic-like (group I) phospholipase A_2 ($sPLA_2$) may play a role resting LES tone by producing arachidonic acid (AA), which is then metabolized to prostaglandins (PG) and thromboxanes (TX). PG and TX are membrane permeable and bind to specific G-protein-linked receptors, to cause activation of PI-PLC and PC-PLC, producing second messengers and contraction [57].

normal esophageal muscle IL-1β and IL-6 cause a significant reduction in contraction in response to electrical stimulation but has no effect on ACh-induced contraction. In contrast, IL-8 and tissue necrosis factor α (TNFα) do not effect either the response to ACh or the response to electrical stimulation, but inhibit both responses at higher concentrations [30]. These results suggest that IL-1β and IL-6, but not IL-8 or TNFα inhibit the release of excitatory neurotransmitter in response to electrical stimulation without affecting the ability of the muscle to contract in response to ACh, and, thus, mimic the changes observed in our model of acute experimental esophagitis. The data also suggest that ACh release from the *in vitro* esophageal strips in response to electrical stimulation may be reduced after induction of acute experimental esophagitis and that this reduction may be caused by inflammatory cytokines such as IL-1β and IL-6.

In addition, induction of AE modifies ACh-induced signaling (Figure 1). In normal esophageal muscle a high molecular weight (85 kDa) (group IV) cytosolic phospholipase A_2 ($cPLA_2$) participates in acetylcholine-induced contraction of esophageal circular smooth muscle [23]. Since PLA_2, arachidonic acid, and its metabolites are involved in inflammatory responses, we examined their role in esophageal smooth muscle cells (ESO) isolated by enzymatic digestion from the circular layer of normal and esophagitis animals.

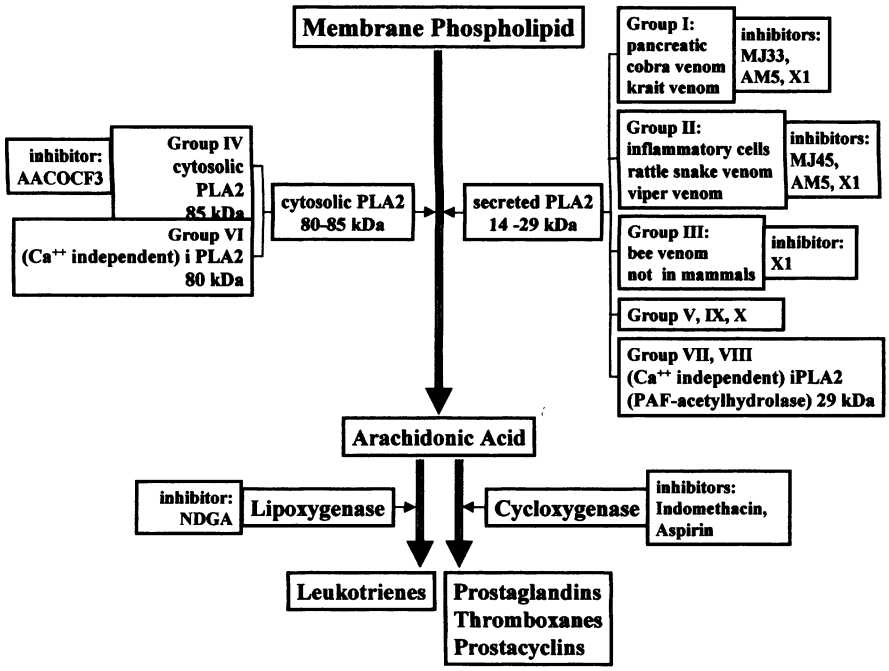

Figure 3. Membrane phospholipids are acted upon by phospholipases A_2, a family of enzymes that catalyze the hydrolysis of glycerolphospholipids at the sn-2 position, producing free fatty acids and lysophospholipids [13, 14, 58]. PLA_2s are divided into two major classes, intracellular or cytosolic (high molecular weight, 80-85 kDa) $cPLA_2$, and secretory or secreted (low molecular weight, ~14 kDa) $cPLA_2$. The intracellular $cPLA_2$s include the 85 kDa calcium-sensitive $cPLA_2$, and the 80 kDa calcium-insensitive $cPLA_2$. The $sPLA_2$ s are divided into different Groups (i.e., I, II, III, V, VII, VIII, IX) according to their molecular structure and the localization of their disulfide bridges [13, 14, 58, 59, 80]. Mammalian PLA_2 enzymes produce arachidonic acid (AA), an important regulator of specific cellular processes, and precursor to biologically active lipids including prostaglandins, leukotrienes, thromboxanes and prostacyclins.

After induction of experimental esophagitis, AA is produced by the same $cPLA_2$ and, in addition, a second pathway is activated in response to ACh (Figure 1). ACh causes activation of a low molecular weight (14 kDa) group I-secreted PLA_2 ($sPLA_2$). After induction of AE, ACh-induced contraction is significantly inhibited by the $sPLA_2$ antagonists AM5 and MJ33. The lipoxygenase inhibitor nordihydro-guaiaretic acid (NDGA) and the leukotriene D_4 (LTD_4) antagonist ICI 198,615 inhibits ACh-induced contraction of esophagitis ESO, suggesting that the AA produced by this $sPLA_2$ may be metabolized by lipoxygenase to leukotrienes (LT). Peptido-leukotriene (LTC_4, LTD_4, LTE_4) content is higher level in esophagitis than in normal circular esophageal muscle, and increases in response to ACh in esophagitis but not in normal esophageal muscle [3].

The same changes are observed in the one-year CE model [6]. These data suggest that in inflammation-free controls AA is produced by $cPLA_2$ and not by $sPLA_2$ and is metabolized by cycloxygenase, and not by lipoxygenase. In AE and CE, activation of $sPLA_2$ causes additional production of AA that is metabolized by lipoxygenase to produce leukotrienes, which contribute to ACh-induced contraction.

Production of IL-1β by inflammatory or target cells may explain some of these changes in esophageal circular muscle. IL-1β has been shown to cause activation of secreted PLA_2 and of 5-lipoxygenase in several experimental preparations [31-39], and subsequent production if IL-6 [40-46].

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 [47-49]. This distinctive contractility may be, at least in part, related to the ability of the LES muscle to handle Ca^{++} differently than esophageal circular muscle [50, 51]. LES muscle maintains tension in a Ca^{++}-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^{++}, 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 [52].

We have reported that LES tone is associated with spontaneous, low-level activity of phospholipase C, resulting in production of submaximal concentrations of DAG and inositol trisphosphate (IP_3), which causes release of Ca^{++} from stores (Figure 2). 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 [2, 21]. Increased IP_3 turnover, resulting in spontaneously elevated IP_3 levels and steady Ca^{++} 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 [21, 53]. IP_3 and DAG, in turn, activate PKC [25]. IP3 and DAG, produced at submaximal levels, act synergistically; their interaction depends on Ca^{++} release and is mediated through the Ca^{++}-sensitive PKCβ isozyme [25, 53].

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 [11] 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, $[^{35}S]GTP\gamma S$ 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.

Evidence from our laboratory suggests that production of AA by a low molecular weight (14 kDa) group I-secreted PLA_2 [54-55] may contribute to maintenance of LES tone by producing AA metabolites, such as prostaglandin-$F_{2\alpha}$ or thromboxanes, which maintain activation of G-proteins [56-57] (Figure 2).

Phospholipases A2 are a growing family of enzymes that catalyze the

hydrolysis of glycerolphospholipids at the sn-2 position, producing free fatty acids and lysophospholipids (Figure 3) [13, 14, 58]. PLA_2s are divided into two major classes, intracellular or cytosolic (high molecular weight, 80-85 kDa) group IV $cPLA_2$, and secretory or secreted (low molecular weight, ~14 kDa) $sPLA_2$.

The $sPLA_2$s are divided into different groups (i.e., I, II, III, V, VII, VIII, IX) according to their molecular structure and the localization of their disulfide bridges [59]. Many sPLA2 s function extracellularly, but some have also been localized within mitochondria [60, 61].

LES tone may be mediated by the activity of a group I (secreted) $sPLA_2$ because: 1) unstimulated LES circular smooth muscle has higher AA levels than ESO and spontaneously releases more AA than ESO smooth muscle; 2) MJ33, a selective inhibitor of group I $sPLA_2$, significantly reduces AA content and spontaneous tone of LES circular muscle strips, whereas the group II $sPLA_2$ antagonist MJ45 and the $cPLA_2$ inhibitor AACOCF3 has no effect on LES tone; 3) cobra venom (group I) $sPLA_2$, but not rattlesnake (group II) or bee venom (group III) $sPLA_2$, causes dose-dependent contraction of LES strips [56].

These data suggest that AA production, through group I $sPLA_2$, participates in maintenance of LES tone. It is possible that the selectivity of the group I $sPLA_2$ in LES muscle may be conferred by the specific interaction of $sPLA_2$ with cell surface receptors. Specific membrane receptors for neuronal (N)-type and muscle (M)-type $sPLA_2$s, have been identified with snake venom $sPLA_2$ [62-67]. One of these $sPLA_2$ receptors, the 180 kDa muscle M-type, has been cloned in rabbit [66] and man [68] and has been shown to have very high affinity for mammalian $sPLA_2$. Receptor binding of $sPLA_2$ is thought to mediate some of the physiological effects of mammalian $sPLA_2$, including vascular smooth muscle contraction, cell proliferation, and internalization of $sPLA_2$ [69-71]. For example, antigen stimulation results in the selective binding of group I $sPLA_2$ and release of AA from bone marrow mast cells, which have been shown to contain the mRNA for the group I PLA_2 receptor [72].

The AA produced by $sPLA_2$ in the LES is metabolized to prostaglandins, such as $PGF_{2\alpha}$ and thromboxanes which, in turn contribute to maintaining tone because the cycloxygenase inhibitors indomethacin and aspirin, and not the lipoxygenase inhibitor NDGA, dose-dependently reduce LES tone. We find that $PGF_{2\alpha}$ content is significantly higher in LES than in ESO and that PGF2α dose-dependently contracts LES strips and single cells. Thromboxanes A2 and B2 may also be involved in LES tone, since thromboxane B2 dose-dependently contracts LES strips and the thromboxane A2 antagonist SQ29548 dose-dependently reduces LES tone [56, 57]. Whether other products of AA metabolism are present and play a role in maintenance of LES tone remains to be determined.

The AA metabolites $PGF_{2\alpha}$ and thromboxanes A_2/B_2 may maintain tone by binding to their respective receptors which are coupled to G-proteins. $PGF_{2\alpha}$ and the thromboxane A_2 analog U46619 significantly increases the $[^{35}S]GTP\gamma S$ binding of G_{i3}, and G_q in solubilized LES circular muscle membranes. In addition, $[^{35}S]GTP\gamma S$ binding in LES circular smooth muscle is significantly reduced by indomethacin, suggesting that G-proteins are activated by cycloxygenase-dependent production of AA metabolites [57]. These data suggest the following hypothesis:

Spontaneous activation of a group I $sPLA_2$ causes production of AA, and AA metabolites such as $PGF_{2\alpha}$ 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 phosphatidylinositol-specific phospholipase C (PI-PLC) and PC-PLC, which, in

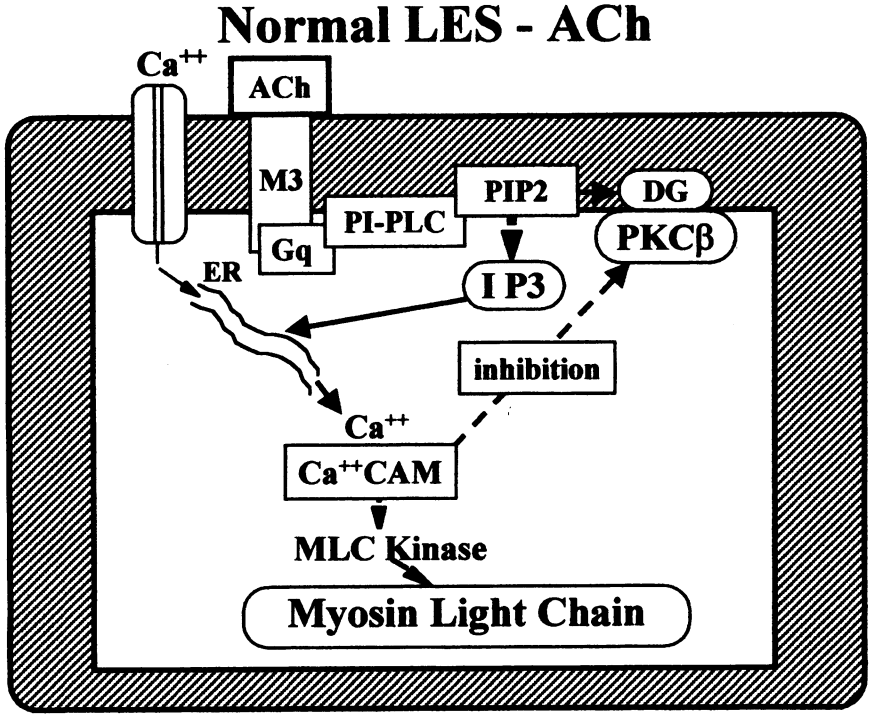

Figure 4. Contraction of LES cells by a maximally effective dose of ACh is mediated by activation of phosphatidylinositol-specific phospholipase C (PI-PLC), and production of inositol 1,4,5-trisphosphate (IP_3) [10] and diacylglycerol (DAG). IP3 causes release of Ca^{++} from stores at a concentration sufficient to cause activation of calmodulin (CAM) [53]. Ca^{++}-CAM causes activation of myosin light chain kinase (MLC kinase) and inhibition of protein kinase C (PKC), inducing a contraction that is entirely calmodulin-dependent [81]. Ca^{++}-CAM-induced inhibition of PKC masks the presence of other factors that would otherwise contribute to activation of PKC.

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.

Since LES tone may be maintained by the activity of a secreted PLA_2, we have used $sPLA_2$-induced contraction as a possible model of tone. We find that 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 [21, 53], 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.

ACH-INDUCED LES CONTRACTION IN NORMAL ANIMALS

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^{++} and formation of a Ca^{++}-calmodulin complex, resulting in activation of myosin light chain kinase and contraction through a calmodulin-dependent pathway (Figure 4) [53].

Thus, unlike LES tone, which is associated with spontaneous, submaximal phospholipase C activity and activation of a PKCβ-dependent pathway, maximal cholinergic stimulation activates a calmodulin-dependent pathway. The mechanisms responsible for the switch from a PKC-dependent to a calmodulin-dependent pathway are not entirely clear. They may result from the different Ca^{++} requirements of calmodulin and PKC. Lower Ca^{++} levels are required for PKC activation than for calmodulin activation [4, 5, 53]. For instance, relatively low (180 nmol/L) cytosolic Ca^{++} levels can support contraction induced by the PKC agonist DAG, but contraction induced by calmodulin requires Ca^{++} levels approaching 1 μmol/L [73-78]. In addition, when Ca^{++} levels are sufficiently elevated to activate calmodulin, calmodulin may inhibit PKC. The mechanism of calmodulin-induced inhibition of PKC activity has not been extensively investigated. Kruger et al. [76] examined tryptic fragments of calmodulin and found that two PKC inhibitory sequences were localized to the first and third Ca^{++} binding domains of calmodulin, and that calmodulin-induced PKC inhibition was not affected by calmodulin antagonists. Thus it is possible that, at Ca^{++} levels insufficient to activate calmodulin, contraction will be PKC-dependent. In contrast, at Ca^{++} levels sufficient to fully activate calmodulin, the contraction will be calmodulin-dependent, and PKC activity will be inhibited [79].

The inhibitory role of calmodulin on PKC-induced contraction is relevant in order to understand the switch in signal transduction pathways that occurs in an experimental model of acute esophagitis (AE) [5].

ACH-INDUCED LES CONTRACTION IN ACUTE AND CHRONIC MODELS OF ESOPHAGITIS

Acute esophagitis

Repeated perfusion of the esophageal lumen with 0.1 N hydrochloric acid for three to four days causes a reduction in resting *in vivo* LES pressure, in spontaneous *in vitro* tone, in levels of IP_3, and in releasable intracellular Ca^{++} stores [1, 2, 4].

We have discussed how contraction of normal LES smooth muscle in response to a maximally effective dose of ACh activates M3 muscarinic receptors, which are coupled to Gq/11 type G-proteins, linked to PI-PLC. Activation of PI-PLC produces DAG and IP3, which causes release of Ca^{++} from intracellular stores, activation of calmodulin and contraction by a calmodulin-dependent pathway. AE causes a shift in the intracellular pathway mediating the response to a maximally effective dose of ACh from a calmodulin-dependent to a PKC-dependent pathway (Figure 5) [5]. After AE, contraction induced by a maximally effective dose of ACh is mediated through M_2 muscarinic receptors, linked to G_{i3}-type G proteins, which activate phosphatidylcholine-dependent phospholipase C and phospholipase D to produce DAG. This ACh-induced contraction depends on influx of extracellular Ca^{++} which is insufficient to

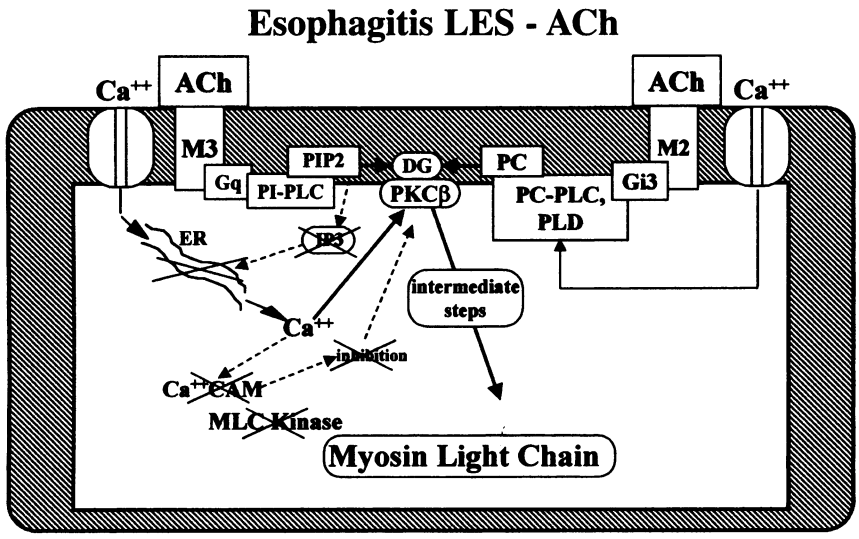

Figure 5. Acute esophagitis (AE) modifies ACh signaling in LES circular smooth muscle. After induction of AE, intracellular Ca^{++} stores are functionally damaged or depleted [4], basal and ACh-induced phosphatidylinositol bisphosphate (PIP_2) hydrolysis are substantially reduced [2, 5], and the resulting reduction in IP_3 and intracellular Ca^{++} release is insufficient to activate calmodulin (CAM), and inhibit PKC. The signal transduction pathway mediating contraction in response to ACh, thus switches to a PKC-dependent pathway, activated by M2 muscarinic receptors linked to G_{i3}, PC-PLC and PLD resulting in hydrolysis of phosphatidylcholine (PC), production of DAG and activation of PKCβ [5]. Influx of extracellular Ca^{++} is required to activate PC-PLC and PLD. These changes are reproduced in normal cells by thapsigargin-induced depletion of Ca^{++} stores.

activate calmodulin, resulting in a PKC-dependent contraction [2, 4, 8].

These changes in the functional signal transduction pathway are mimicked in normal LES muscle by acute depletion of intracellular Ca^{++} stores by thapsigargin [5]. They are, therefore, related to impaired release of Ca^{++} from intracellular stores, which arises both from impaired production of IP_3 [2] and from depletion of releasable Ca^{++} stores [4] subsequent to induction of AE. Because release of Ca^{++} from intracellular stores is reduced in AE, the available Ca^{++}, which arises mostly from Ca^{++} influx, may be insufficient to activate calmodulin, and, thus, a PKC-dependent pathway is "unmasked" that would otherwise be suppressed by calmodulin activation. A reduction in Ca^{++} release by inflammation, secondary to AE, may be the central event, from which all other observed changes follow, and may also explain the reduction in resting tone associated with AE.

Chronic esophagitis

The significance of the subchronic changes in cat acute esophagitis to the understanding of esophagitis in humans, where gastroesophageal reflux disease (GERD) is likely to develop over a longer time period, remains to be established. However, in CE, we find similar but less accentuated, endoscopic, histologic and functional changes, up to four to six months after surgery [8]. The magnitude

of the changes may be related to the degree of damage caused by repeated acid perfusion, as the degree of damage in the acute model (45-min acid perfusion x 3 days) is greater than the damage induced by spontaneous reflux in the chronic model. In addition, in the chronic model, the suppression of HCl secretion, either after the onset of mild chronic esophagitis, or at the time of myotomy, reverses or prevents the changes in smooth muscle signal transduction, presumably by inhibiting or preventing the injury caused by reflux [8].

LES TONE IN ACUTE ESOPHAGITIS

Acute esophagitis causes a decrease of *in vivo* and *in vitro* resting LES tone. The decrease in tone may be explained by the same mechanisms that affect the signal transduction pathway activated by ACh.

In normal LES, resting tone depends on activity of PI-PLC, resulting in equimolar formation of IP_3 and DAG. Additional DAG is produced by activity of PC PLC. IP_3-induced Ca^{++} release potentiates DAG-induced activation of the Ca^{++} sensitive PKCβ, responsible for maintenance of tone. Since AE reduces IP3 formation by PI-PLC the associated DAG production is also decreased, resulting in a decrease of total DAG. In addition, AE causes depletion of Ca^{++} stores, thus Ca^{++} release and DAG formation are substantially decreased, resulting in reduced activation of PKCβ and reduced LES tone.

CONCLUSION

We conclude that in esophageal circular muscle ACh-induced contraction, is mediated by M_2 muscarinic receptors, linked to G_{i3}, PC-PLC, PLD, and $cPLA_2$, resulting in production of DAG and AA and activation of the Ca^{++}-insensitive PKCε. In this contractile pathway Ca^{++} is required for activation of the phospholipases and production of the second messengers DAG and AA, as PKCε is Ca^{++}-independent and DAG causes contraction of esophageal muscle cells even in the absence of Ca^{++}.

Inflammation causes activation of a second PLA_2, which is a group I $sPLA_2$. This PLA_2 causes additional production of AA, which is metabolized to leukotrienes, resulting in increased levels of LTs in the basal state and in response to ACh. LT formation in response to ACh contributes to contraction of esophageal muscle, which remains of normal amplitude, when directly exposed to ACh. Inflammation causes a reduction in contraction in response to neural stimulation, most likely due to reduced neurotransmitter release secondary to inflammation. These results are mimicked by exposing normal esophageal muscle strips to the pro-inflammatory cytokines IL-1β and IL-6.

In normal LES smooth muscle cells, ACh-induced contraction is mediated by M_3 receptors linked to $G_{q/11}$ and PI-PLC, causing formation of IP_3, release of Ca^{++} from stores and activation of calmodulin. This results in inhibition of PKC and activation of a calmodulin-dependent contractile pathway. In AE, releasable Ca^{++} stores and IP_3 formation are reduced, resulting in reduced Ca^{++} release in response to ACh and dependence on Ca^{++} influx for contraction. The reduced Ca^{++} release prevents activation of calmodulin and prevents the calmodulin-induced inhibition of a PKC-dependent pathway, which is not activated in the normal LES. Thus AE causes a switch in contractile pathways, from a calmodulin-dependent to a PKC-dependent contraction. This switch is mimicked by thapsigargin-induced depletion of Ca^{++} stores in normal LES muscle.

The reduction in Ca^{++} release may also account for the reduction of *in vivo* and *in vitro* LES resting tone associated with AE [1, 2, 49].

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