A role for focal adhesion kinase in the contraction of gastric fundus smooth muscle evoked by cholinergic neurotransmission

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ABSTRACT

Integrins are trans-membrane receptors that form focal contacts and mediate the attachment of the smooth muscle cell cytoskeleton to the extracellular matrix. Focal adhesion kinase (FAK) regulates the recruitment and assembly of focal adhesion proteins. It has been established that integrin proteins and FAK play important roles in cell adhesion, tension generation, and mechanotransduction. Integrins and FAK are abundant in smooth muscles; however, the function of FAK in gastric smooth muscle cell contractile regulation remains unclear. To better understand the role of FAK in gastric fundus smooth muscle contractile regulation, we performed two major projects: (1) addressing gastric smooth muscles contractile regulation in cholinergic neurotransmission by FAK phosphorylation (Chapter 2) and (2) characterizing the change in Ca\(^{2+}\) sensitization proteins distribution at focal adhesion sites under electric field stimulation induced cholinergic neurotransmission and FAK phosphorylation regulation (Chapter 4). In carrying out the first project, the contractile responses of murine gastric smooth muscles were determined using standard myobath – isometric force transducer techniques and phosphorylated or total proteins determined by automated capillary electrophoresis and immunoblotting by Wes Simple Western. For the second project, we developed smooth muscle tissue \textit{in situ} proximity ligation assay (PLA) for the quantitative PLA analysis of protein-protein interaction and protein phosphorylation (Chapter 3). From these studies, we revealed a novel role of FAK in gastric fundus smooth muscle contractile regulation by cholinergic stimulation. We also demonstrate quantifiable
tissue level PLA that can be extended and applied to studies of protein-protein
interaction, and protein phosphorylation in various tissues and signaling pathways.
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CHAPTER 1: Introduction

1.1 Functional anatomy and physiology of the gastric fundus

1.1.1 Overall goal of stomach function

The stomach is a hollow dilated tube in the gastrointestinal (GI) tract of mouse, human and other animals. This specialized muscular organ carries out complex motor activities to store, mix, propel, and empty the food [1]. The core functions of the stomach are food storage, acid secretion, enzyme secretion and propulsion [2]. These functions of the stomach are critical in churning food into chyme, and passing the chyme into the intestines for food breakdown, absorption, and egestion. Moreover, the stomach is implicated in the metabolic regulation of glucose homeostasis, and satiety-hunger balance [3]. For example, the stomach can sense the glutamate level by the glutamate signaling (Glu) receptors and transmit the signal to the brain through abdominal vagal nerves to control the appetite for food [4]. Obese patients receiving gastric sleeve surgery display altered glucose homeostasis and hormone release, such as glucagon-like peptide 1 (GLP-1) and peptide YY (PPY) [5], [6]. In gastric sleeve surgery, a large portion of the stomach is removed [7]. Most patients survive (around 0.8% mortality rate) and maintain weight loss, but a few patients suffer from complications such as bowel obstruction (2~3%), GI bleeding (~2%) and post-operative infections [7], [8]. The stomach may not be an essential organ for survival, but it has a critical role in the digestive system, chemically and mechanically breaking down ingested food into proteins, lipids, and
carbohydrates for nutrient absorption by the small intestine, as well as metabolic regulation of food intake.

1.1.2 Histology and function of gastric fundus

Anatomically, the stomach consists of the fundus, corpus (body), antrum and pylorus. The gastric fundus by definition is the part of the stomach to the upper left of the lower esophageal sphincter [9]. The gastric fundus wall, like other parts of the gastrointestinal wall, includes 4 tissue layers from outer surface to inside: the serosa, the muscular layer, the submucosa, and the mucosa (Fig.1-1). The mucosa consists of the epithelium, lamina propria and muscularis mucosae. The epithelium protects the gastric wall from the luminal acidic contents and mechanical stress [1]. The lamina propria is the connective tissue beneath the epithelium, containing fibroblasts, lymphocytes, and plasma cells, to provide support by collagen fibrils to the epithelium and nutrition exchange from the capillaries [10]. The muscularis mucosae contains thin layers of smooth muscle fibers, oriented in different ways to support the mucosal surface, contribute to the expulsion of glandular crypts content, mucosal surface organization, and enhancing the contact between luminal contents and the epitheliaums [11]. The submucosa is a thin layer of connective tissue consisting of blood vessels, lymphatic vessels, and nerves. It supports and connects the mucosa to the smooth muscles. The muscularis externa in the fundus is a region of smooth muscle responsible for fundus movement. It consists of an inner circular smooth muscle layer and an outer longitudinal smooth muscle layer. The contraction and relaxation of the circular and longitudinal muscles are responsible for food mixing
and propulsion. The fundus muscle layer motor activity is regulated by enteric neurons, gastric hormones, and the interstitial cells of Cajal (ICC) [12]. Enteric nerves located in the submucosal plexus and the myenteric plexus [13]. The submucosal plexus primarily regulates local blood flow and gastric hormone release while the myenteric plexus in between the circular and longitudinal muscle layers regulates motility. In the gastric fundus, ICCs are coupled with the smooth muscles cells through gap junctions and are present adjacent to the enteric nerves between the circular and longitudinal smooth muscles [14]. The serosa is the outside thin layer of connective tissue surrounding the gastric wall.

![Fig. 1-1. Anatomy of GI wall.](https://en.wikipedia.org/wiki/Gastrointestinal_wall)

From the functional viewpoint, the stomach can be divided into two major parts: the proximal stomach and distal stomach. The proximal stomach is at the orad portion of the stomach, consisting of the fundus and corpus. It primarily stores
the luminal contents and propels the contents to the distal stomach. The human gastric fundus primarily displays tonic contractions and relaxations, with some phasic activity (unpublished observations). The sustained fundus tone helps food propulsion and gastric emptying. The fundus basal tone can be sensed by the “vago-vagal reflex” from the stomach to the brain stem for modulation by the brain. When food enters the stomach, the fundus will reduce its basal tone to lower the stomach pressure and accommodate food. This process is call “the gastric accommodation reflex”, which is mediated by nitric oxide released from nitrergic neurons [15]. Recent studies show that food intake elicits an initial drop in intra-gastric pressure (IGP), followed by the gradual recovery of IGP [16]. The rise in IGP from the nadir correlates with the increased degree of satiation, while the decrease in IGP contributes to the meal volume tolerance [17].

1.1.3 Neural regulation of gastric fundus motility

The enteric nervous system is composed of two major plexuses: (1) the myenteric plexus located between the longitudinal and circular muscle layers, and (2) the sub-mucosal plexus lying in the submucosa. Both plexuses can function on its own or regulated by the sympathetic and parasympathetic fibers connecting to them.

In the myenteric plexus, a linear chain of interconnecting neurons lies between the fundus longitudinal and circular smooth muscles and controls fundus motor activity, primarily the tonic contraction and relaxation. Both excitatory and inhibitory neurotransmitters are present in the fundus myenteric plexus. The primary
excitatory neurotransmitter is acetylcholine, that activates the muscarinic receptors on the ICC-IM, which will transmit the excitatory signal to the smooth muscle cells and result in muscle contraction [18]. Substance P and other tachykinins can also be released together with acetylcholine to stimulate the mucosal immune response [19]. Substance P alone can trigger smooth muscle relaxation through inducing endogenous release of nitric oxide (NO) and/or prostaglandins (PGs) [20]. In addition, in the autonomic nervous system regulation of gastric fundus motility, acetylcholine transmits signal from parasympathetic system to enteric neurons via vagal nerves. The predominant inhibitory neurotransmission in the gastric fundus is nitrergic transmission through the release of nitric oxide [21]. Purinergic transmission by the neural release of β-NAD is also found in the gastric fundus and contributes to inhibition of smooth muscle contraction [22]. Norepinephrine is another inhibitory neurotransmitter secreted by the postganglionic sympathetic nerves. Norepinephrine can be delivered to smooth muscles directly for slight inhibition of gastric fundus motor activity or be delivered to the myenteric neurons under strong sympathetic stimulation and results in great inhibition of fundus motor movements. In general, parasympathetic regulation enhances gastric motor activity and gastric function, whereas the sympathetic regulation generates opposite effects that inhibit digestive activities. More neurotransmitters remained to be discovered and the specific functions to be determined.

In contrast to the myenteric plexus, the submucosal plexus primarily controls the function of the gastric fundus inner wall. The submucosal plexus ganglia spread
through the epithelium and muscularis mucosae. The sensory signals generated from ganglia in the epithelium are transmitted to the submucosal plexus to regulate local secretion and local contraction of the mucosa. 14% of the submucosal plexus neurons are afferent sensory neurons [23]. These sensory nerves can be stimulated by mechanical changes in the gastric wall or specific gastric luminal substances. The afferent sensory system controls gastric movement, blood flow, or secretion in response to the stimulus.

1.2. Cholinergic transmission in gastric fundus smooth muscle

1.2.1 Tonic contraction of fundus smooth muscle

GI smooth muscles exhibit phasic, tonic, or both types of contractions depending upon the specific region. Slow waves are generated and propagated by the myenteric ICC (ICC-MY) to initiate smooth muscle Ca$^{2+}$ influx and motility, and are present in the colon and gastric antrum, where peristaltic movement is critical [24], [25]. Fundus smooth muscles in mice lack ICC-MY, thus displaying no slow waves and lack phasic contraction. [26]. Smooth muscle tonic contraction is regulated by excitatory or inhibitory neurotransmitters from the enteric nerves to the smooth muscle cells. In the murine fundus, smooth muscle tone is specifically elicited by the unitary potentials generated from the intramuscular interstitial cells of Cajal (ICC-IM) [27]. Note that extracellular slow wave recordings from fresh human fundus sample suggest that human gastric fundus may exhibit slow waves, which is different from laboratory animal studies as well as the in vitro studies of
human gastric ICC activity [28]. The measurement standards and the possible experimental artifacts in the studies of slow waves in human gastric pacemaker activity are still under debate [29], [30].

1.2.2 Molecular basis of fundus smooth muscle cholinergic transmission

In gastric fundus smooth muscles, the smooth muscle cells and ICC-IM form the final step in excitation-contraction (E-C) coupling. The enteric motor neurons release neurotransmitters to ICC-IM at the interface between ICC and enteric varicosities. ICC-IM then transmit the signal to smooth muscle cells via gap junctions. Smooth muscle cells also transmit the signal among each other via gap junctions; hence gastric fundus smooth muscle is considered to be a single-unit smooth muscle, in which the smooth muscle cells all behave as one unit in response to neural stimuli. The enteric motor neurons, ICC-IM, and smooth muscle cells form a functional syncytium, which is the general contractile machinery for contraction and relaxation [12].

The major excitatory neurotransmitters in the fundus smooth are acetylcholine and substance P [12]. In cholinergic transmission, acetylcholine is released to synapse-like clefts between the motor neuron varicosities and target ICCs. Acetylcholine binds to muscarinic receptors M3 on the ICC-IM [14]. The activation of M3 receptors will activate the $\text{Ca}^{2+}$-activated $\text{Cl}^{-}$ channels and leads to the depolarization of ICC [31]. The change in membrane potential of the ICC is then transmitted to the electrically coupled smooth muscle cells through the low
resistance gap junctions. Voltage-gated Ca\textsuperscript{2+} channels (L-type) and nonselective cation channels on the smooth muscle cells are activated in response to the depolarized membrane potential, leading to Ca\textsuperscript{2+} influx and G-protein-coupled receptors (GPCR) activation, specifically Gq/G11 subtype in the gastric fundus. Ca\textsuperscript{2+}, inositol 1,4,5-trisphosphate (IP\textsubscript{3}), diacylglycerol (DAG)/ protein kinase C (PKC), and Ras homolog gene family, member A (RhoA)/ Rho-associated kinase (ROCK2) act as second messengers which contribute to the downstream Ca\textsuperscript{2+} sensitization proteins phosphorylation of myosin phosphatase targeting subunit of MLCP (MYPT1) and phosphatase inhibitor protein of 17kDa (CPI-17). Myosin light chain kinase (MLCK) is activated by the increase in [Ca\textsuperscript{2+}], and MLCP inactivated by Ca\textsuperscript{2+} sensitization proteins, which both lead to the increase in myosin light chain phosphorylation and smooth muscle contraction [32], [33]. Along with the increase in [Ca\textsuperscript{2+}], altering CPI-17 phosphorylation is another method that regulates the muscle tone and controls fundus motility (Fig.1-2) [32]. We showed that in gastric fundus smooth muscle cholinergic neurotransmission stimulated by electric field stimulation (EFS), CPI-17 is phosphorylated at T38 and leads to fundus tonic contraction, while no significant change in MYPT1 phosphorylation is observed [18]. When the smooth muscle strip is exposed to carbachol (CCh) in the myobath, the M3 receptors on the ICC and smooth muscle cells and both activated, and contribute to the activation of both MYPT1 and CPI-17 phosphorylation, which results in a greater increase in the smooth muscle tone [18]. The model of EFS inducing endogenous acetylcholine release is more
close to the physiological cholinergic neurotransmission in gastric fundus smooth muscle [32].

**Fig. 1-2. Murine fundus smooth muscle contraction regulated by cholinergic stimulation.** The endogenous acetylcholine (ACh) released upon electric field stimulation. The ACh binds to the M3 muscarinic receptors on the ICC and activates the non-selective cation currents (NSCC) and Ca$^{2+}$-activated Cl$^{-}$ channels (CaCC), which generates excitatory junctional potentials (EJP). The voltage-dependent Ca$^{2+}$ channels (VDCC) on smooth muscle cells are stimulated by the EJP to initiate Ca$^{2+}$ influx and PKC activation of CPI-17 phosphorylation, resulting in the smooth muscle contraction. *Adapted from (Bhetwal et al. 2013)*

1.3. Focal adhesion regulation of smooth muscle contraction

1.3.1 Overview of focal adhesion

Focal adhesions are integrin-based cell to extracellular matrix (ECM) adhesive contacts that tightly link cytoskeletal actin filaments to the ECM [34]. Focal
adhesions mediate cellular responses to the mechanical or biochemical regulatory effects from ECM adhesions [35].

Focal adhesions are large, dynamic multi-protein complexes that form the mechanical links between actin bundles and the ECM at the cell membrane close to the ECM substrate [36]. Focal adhesions can contain up to 160 distinct proteins, most of which are intrinsic components whereas others are transiently localized to the focal adhesion site [34]. The core proteins of focal adhesions are the integrins. Integrins are heterodimeric transmembrane receptors which are formed from one alpha and one beta subunit. Up to 24 integrins are found in vertebrates, with β1-integrins coupled to many different alpha integrins to form multiple heterodimers [37]. In smooth muscles, the expression of alpha 1 to 9, alpha v, beta 1 and beta 3 integrin subunits have been identified [38]. The α5β1 integrins are abundant and widely expressed in arterial and vascular smooth muscles [38]. The αvβ3 integrins are critical in arterial smooth muscle migration [39]. Different forms of integrin receptors bind to different ligands, such as fibronectin, laminin and other proteins with Arg-Gly-Asp (RGD) domains, to trigger integrin activation and corresponding cellular responses, for example, cell surface attachment and cell polarity [40], [41]. The intracellular domains of integrins binds to the cytoskeleton through talin, vinculin, α-actinin, paxillin and other essential adapter proteins at the focal adhesion to form the physical link to the cytoskeleton [42]–[44]. Two non-receptor cytoplasmic tyrosine kinases, focal adhesion kinase (FAK) and c-Src, are key components of focal adhesions to regulate mechanical transduction, actin
remodeling and contraction [45]. Integrins and adaptor proteins are responsible for the focal adhesion structural regulation of the actin polymerization, while FAK, e-Src and other tyrosine kinases at the focal adhesion site belong to the regulation of tyrosine phosphorylation events in focal adhesion. Other major protein components of the focal adhesion site can be categorized into serine/threonine kinases, Rho GTPases, and, lipids based on the integrin adhesome analysis, which summarized the integrin-associated adhesion component proteins [46]. Integrin adhesome network analysis also reveals common scaffolding motifs that allow the regulation of multiple protein binding and recruitment of signaling enzymes and substrates [47]. The identified signaling protein networks and common protein motifs at focal adhesion enable the focal adhesion the feature of switchable and dynamic assembly [48]. This feature plays a crucial role for focal adhesions in cell migration, cell motility and mechanical sensing [49]–[51].

1.3.2 Smooth muscle contractile regulation by integrin proteins

Integrins mediate the regulation of cell shape, stiffness, and motility through the control of actin reorganization and dynamics in non-muscle cells [52]. Integrins transmit the chemical and mechanical signals from the ECM to the intracellular pathways (outside-in). The cytoskeleton events can, in reverse, alter their binding to the integrins and ECM proteins (inside-out) [53]. This crosstalk between the ECM and the cytoplasm dynamically regulate the cytoskeletal organization and enhance the integrin-mediated outside-in signals [54]. In differentiated smooth muscles,
integrins are located at plasma membrane dense plaques, which have long been recognized as the protein complexes that connect smooth muscles to the ECM [55], [56]. The smooth muscle integrin signaling pathways, which are similar to the ones present in non-muscle cells, regulate smooth muscle sensing and response to external mechanical and biochemical stimulus [45].

Integrins function by themselves or interact with other components at the focal adhesion site to regulate the smooth muscle contraction. The cultured airway smooth muscle integrin protein complex elicits increased torsional resistance of integrins to the integrin-bound ferromagnetic beads when the muscle is stimulated by contractile agonists, suggesting actin tension is transmitted to the ECM through integrins [57]. Moreover, in the mechanotransduction of renal autoregulation, integrins can regulate Ca\(^{2+}\) release through ryanodine-sensitive cytoplasmic Ca\(^{2+}\) stores and trigger cytoplasmic Ca\(^{2+}\) waves in renal vascular smooth muscles [58]. In rat arteriole smooth muscle, the binding of RGD peptide to \(\alpha_5\beta_1\) or \(\alpha_v\beta_3\) integrins leads to altered L-type Ca\(^{2+}\) channel and K\(^+\) channel activity, resulting in enhancing the Ca\(^{2+}\) influx and smooth muscle contractile responses for \(\alpha_5\beta_1\) and in reverse for \(\alpha_v\beta_3\) [59]. In addition, adhesion-induced integrin activation can elicit the phosphorylation of FAK, c-Src and other tyrosine kinases, which in turn regulates actin polymerization and cell motility [60] [61].

1.3.3 Smooth muscle contractile regulation by focal adhesion kinase

FAK is a tyrosine kinase that can activate or inactivate various small GTPases in different types of cells [61]. FAK is also a scaffold protein that assembles adaptor proteins, such as talin and paxillin, to connect the cytoskeleton to the ECM.
This dual feature of FAK allows it to regulate actin remodeling, focal adhesion assembly, and turnover [42], [62]. The functional domains of FAK are illustrated in Fig.1-3. The catalytic kinase domain is flanked by the 4.1/JEF and the focal adhesion targeting (FAT) domain. The FAT domain interacts with paxillin, which plays a major role in localizing FAK to the integrin adhesion site [63]. The 4.1/JEF has been shown to facilitate FAK binding to the cytoplasmic tails of β-integrin as well as the receptors for growth factors such as platelet-derived growth factor receptor (PDGFR) and epidermal growth factor receptor (EGFR) [61]. The two proline-rich motifs (PR1 and PR2) are located between the kinase and FAT domain, mediating FAK interaction with Src-homology 3 (SH3) domain such as GTPase regulator associated with FAK (GRAF), which regulates Rho activity and cytoskeleton dynamics [64]. The structure of FAK enables its diverse roles in smooth muscle contractile regulation.

![Fig. 1-3. Major domains, motifs, and tyrosine phosphorylation sites of FAK. Taken from (Hanks et al. 2003)](image)

In smooth muscles, FAK plays critical roles in Ca\(^{2+}\) signaling to regulate smooth muscle contraction. In arteriolar smooth muscle, the platelet-derived growth factor (PDGF) induced Ca\(^{2+}\) influx is partially blocked (43% Ca\(^{2+}\) current) by FAK inhibition with cell dialysis of the anti-FAK [65]. The evidence suggests that FAK
regulates Ca\(^{2+}\) channels in integrin activated pathways. Moreover, FAK was observed to participate in contractile agonist activated smooth muscle contraction. Increasing FAK tyrosine phosphorylation results in downstream activation of Rho GTPase, c-Src and other signaling molecules that affects smooth muscle contraction through participation in Ca\(^{2+}\) signaling and focal adhesion assembly [45]. Vascular smooth muscle cells elicit increased FAK tyrosine phosphorylation under angiotensin stimulation [66]. In tracheal smooth muscle, exogenous acetylcholine-induced muscarinic activation increases tyrosine phosphorylation of FAK [67]. Tracheal smooth muscle with FAK-depletion, by reversible permeabilization and FAK antisense oligonucleotides incubation, shows reduced contractile responses to acetylcholine. However, when tracheal smooth muscle Ca\(^{2+}\) signaling is disrupted by \(\alpha\)-toxin permeabilization, the tissue tension development under increased intracellular Ca\(^{2+}\) is similar between the tissues with or without FAK-depletion [68]. The above evidence suggests that, in tracheal smooth muscle, FAK plays a role in regulating acetylcholine induced Ca\(^{2+}\) influx. also, when tracheal smooth muscle is depleted of Ca\(^{2+}\), FAK tyrosine phosphorylation is still elicited by exogenous acetylcholine [69]. This finding suggests that, in tracheal smooth muscle, acetylcholine activates FAK independent of the Ca\(^{2+}\) influx. A recent study showed that RhoA GTPase recruits FAK to the focal adhesion site, which catalyzes the adhesome assembly to regulate the acetylcholine induced tracheal smooth muscle contraction [70].
Smooth muscle FAK has been implicated in mechanotransduction, which is the translation of mechanical stimuli into biochemical signaling events. FAK tyrosine phosphorylation is increased by cyclical mechanical strain in cultured smooth muscle cells [71]. The length of isometric contraction of tracheal smooth muscle is positively correlated with the FAK tyrosine phosphorylation [45]. This length-sensitive feature of FAK phosphorylation is not affected even when the tracheal smooth muscle is depleted of Ca\(^{2+}\) [67]. Thus, the mechanical sensing of FAK is independent of intracellular Ca\(^{2+}\).

1.4. Proximity ligation assay

1.4.1 The principle of proximity ligation assay

Proximity ligation assay (PLA) is an \textit{in vitro} assay based on traditional immunohistochemistry assays to detect single proteins, protein-protein interactions, and post-translational modifications [72]. This method enables single molecule resolution and generation of quantifiable data for protein analysis with high specificity and sensitivity [73]. The \textit{in situ} PLA, which is the proximity ligation assay carried out specifically in fixed cells or tissue sections, detects transient interactions as well as stable interactions in cells or tissues under different states [74].

The \textit{in situ} PLA is performed after the binding of two primary antibodies produced by different animal species to the target protein or protein-protein interaction complex [72] (Fig.1-4). The species-specific secondary antibodies with attached oligonucleotides, called PLA probes, are then applied to bind to the two
primary antibodies [75]. When the two PLA probes are in close distance (< 40nm), the two probes are hybridized by the added DNA oligonucleotide connectors. The hybridized probes are ligated with T4 DNA ligase to form a circular DNA molecule [74]. Next, the oligonucleotide on one of the PLA probes serves as the primer for the rolling circle reaction (RCA) to amplify the circular DNA molecule, in the presence of Phi29 DNA polymerase and appropriate substrates and buffers [76]. The RCA is performed at 37°C and creates an over 1000-fold amplification of the 100 nt DNA circle. An RCA product is a bundle of DNA oligonucleotides more than 100 kb in length and around 1 μm in diameter [77]. The RCA product is detected by hybridization with fluorescently-labeled complementary oligonucleotides to generate the PLA signal and visualized as a bright spot under the fluorescence microscopes [78]. The PLA signal can be visualized when the microscope objective resolution is below 0.80 μm, namely magnification greater than 20x.
Fig. 1-4. *In situ* proximity ligation assay workflow. (A). Two proximity probe binding to the sample protein complex, bringing the two oligonucleotides in close distance. (B) Connectors hybridizing with the two oligonucleotides and (C) ligated to form a circular DNA. (D) The circular DNA molecule is amplified by rolling circle amplification and the product is hybridized with fluorescently labeled complementary oligonucleotides. The PLA signals can be visualized under the microscope as individual red dots. Two examples are shown as phosphorylated PDGF-β receptor in (E) stimulated and (F) unstimulated human fibroblasts. *Taken from* (Weibrecht et al. 2010)
1.4.2 Applications of proximity ligation assay

PLA is designed to detect proteins, protein-protein interaction and protein post-translational modifications (PTM) [79]. The in situ PLA can be applied to fixed and permeabilized cultured cells and tissue sections [76]. Since the PLA is antibody-based, PTMs such as protein phosphorylation and glycosylation can be probed by one antibody against the core protein, and the other protein against the PTM site [61], [62]. For example, multivalent histone modifications, such as H3K4me3/ H3K9me3 dual methylations, can be visualized by PLA [82]. The PLA technique can also be extended to study RNA-protein interactions or DNA-protein interactions by modifying one of the PLA probes to bind to the target DNA or RNA, while the antibody probe recognizes the particular RNA-binding protein or DNA binding protein [83]–[85]. For PLA signal detection, the enzyme-labeled oligonucleotide can be used to hybridize with the RCA product to generate signals detectable under the bright-field microscope using colorimetric read-outs [86]. PLA has also been combined with flow cytometry, namely to perform PLA to the cell suspension and then examine the PLA signals with a flow cytometer, which enables statistically powerful analysis of protein-protein interactions and PTMs, such as protein phosphorylation [80].

The flexibility, high sensitivity, and high specificity of the PLA approach has wide academic and clinic applications. In addition to protein-protein interactions, PLA can be modified to detect specific DNA-protein or RNA-protein interactions based on different PLA probes using the same downstream procedures.
of probe ligation, RCA, and signal detection [83], [87]. This feature makes PLA an ideal tool for the validation of biomarkers for clinical diagnosis [88], [89]. For example, PLA detection of the association between epidermal growth factor receptor (EGFR) and growth factor receptor-bound protein 2 (GRB2) in patient tumor samples may be an indicator of the patient response to EGFR inhibitor-based therapies [90]. Target recognition by two different primary antibodies instead of one is another outstanding feature of PLA that reduces the cross-reactivity and improves the specificity compared with other antibody-based biochemical assays, such as sandwich ELISA [80]. The excellent sensitivity and specificity of \textit{in situ} PLA are also validated by detection of protein-protein interactions in human and mouse tissue sections with biological controls [91]. In sum, PLA is a powerful and flexible tool for research, diagnostic, and other applications [78], [92].
CHAPTER 2: Regulation of cholinergic transmission induced contraction in gastric fundus smooth muscles by focal adhesion kinase phosphorylation

2.1 Abstract

Smooth muscle contraction involves regulating myosin light chain (LC20) phosphorylation and dephosphorylation by myosin light chain kinase (MYLK) and myosin light chain phosphatase (MLCP). CPI-17 and MYPT1 are crucial for regulating gastrointestinal smooth muscle contraction by inhibiting MLCP. Integrin signaling involves the dynamic recruitment of several proteins, including FAK, to focal adhesions. FAK tyrosine kinase activation is involved in cell adhesion to the extracellular matrix via integrin signaling. FAK participates in linking the force generated by myofilament activation to the extracellular matrix and throughout the smooth muscle tissue. Here, we show that cholinergic stimulation activates FAK in gastric fundus smooth muscles. Electrical field stimulation (EFS) in the presence of L-NAME and MRS2500 contracted gastric fundus smooth muscle strips and increased FAK Y397 phosphorylation (pY397). The FAK inhibitor PF-431396 inhibited the increase in pY397 and inhibited EFS-evoked contractions. PF-431396 inhibited the EFS-induced increase in CPI-17 T38 (pT38) phosphorylation. Atropine inhibited EFS-induced contractions and prevented the EFS-induced increase in pY397. Contractions evoked by high K⁺ increased pY397 and were inhibited by PF-431396. EFS-induced Ca²⁺ influx was unaffected by PF-431396. Nicardipine inhibited the EFS-induced contractions but had no effect on the EFS-induced increase in pY397. PDBu contracted gastric fundus smooth muscle strips and increased FAK Y397 and CPI-17 T38 phosphorylation. Our findings indicate that
FAK is activated by mechanical force or tension, and reveal a novel mechanism for FAK facilitation of gastric fundus smooth muscle contraction by regulating the PKC-dependent phosphorylation of CPI-17.

2.2 Introduction

The trigger for contraction of gastrointestinal (GI) smooth muscles is a rapid increase in intracellular Ca\(^{2+}\) \([\text{Ca}^{2+}]_i\). Membrane depolarization evokes the opening of voltage-dependent (L-type) Ca\(^{2+}\) channels (VDCC), non-selective cation currents (NSCC), and other mechanisms that contribute to the Ca\(^{2+}\) influx and the increase in \([\text{Ca}^{2+}]_i\) [12], [93]. The increase in \([\text{Ca}^{2+}]_i\) activates the calmodulin-dependent MYLK, which phosphorylates LC20 at S19, stimulating myosin ATPase activity to generate cross-bridge cycling and contraction [94], [95]. Termination of the contractile signal leads to a decrease in \([\text{Ca}^{2+}]_i\), and inactivation of MYLK. LC20 is then dephosphorylated by MLCP, leading to relaxation [96]. It is now clear that a key mechanism regulating S19 phosphorylation is inhibition of MLCP by protein kinase C (PKC) mediated phosphorylation of phosphatase inhibitor protein of 17kDa (CPI-17), and/or phosphorylation of myosin phosphatase targeting subunit of MLCP (MYPT1) by Rho-associated kinase 2 (ROCK2) [97]. MYPT1 phosphorylation at T696 (human isoform numbering) inhibits MLCP activity [98], [99]. ROCK2 phosphorylates MYPT1 at T853 and inhibits MLCP in vitro, but phosphorylated T853 does not appear to inhibit MLCP in vivo [93], [100]. However, ROCK2 activity is clearly required for Ca\(^{2+}\) sensitization and augmented contraction [93].
Thus, the level of MYPT1 phosphorylation at T853 (pT853) indicates ROCK2 activity and Ca\(^{2+}\) sensitization in smooth muscles.

In addition to Ca\(^{2+}\) dependent myofilament activation and Ca\(^{2+}\) sensitization by inhibition of MLCP, a number of studies have provided evidence that dynamic changes to the actin cytoskeleton play an important role in smooth muscle contraction [93], [101], [102]. Tyrosine phosphorylation of protein tyrosine kinase 2 beta (Pyk2) and FAK, along with the recruitment of other integrin-associated proteins to focal adhesions occurs during contraction and force development [93], [101], [102]. This remodeling process is thought to facilitate the polymerization of cortical cytoskeletal actin filaments to increase the stability of focal adhesions in the membrane, allowing for the force generated by myofilament activation to be transmitted to the connective tissue of the extracellular matrix [93], [101], [102]. The involvement of FAK and Pyk2 in facilitating smooth muscle contractile responses has previously been studied in various smooth muscle tissues including airway smooth muscle, vascular smooth muscle and prostate smooth muscle [101]–[103]. The Ca\(^{2+}\)-dependent tyrosine kinase Pyk2 has been reported to be activated by membrane depolarization and voltage-dependent Ca\(^{2+}\) influx resulting in RhoA activation, leading to MYPT1 and LC20 phosphorylation and MLCP inhibition [102]. FAK Y397 phosphorylation has been shown to be increased bladder smooth muscle contraction [93], but no studies have addressed the role of FAK in regulating GI smooth muscle contractility.
In gastric fundus smooth muscles smooth muscles, we previously found that EFS-evoked contractions increase CPI-17, but not MYPT1, phosphorylation, and that PKC inhibitors block this increase [18]. Phosphorylation of PKC by tyrosine kinases has been shown to play a role in PKC activation [104]. Due to previous studies implicating a role for FAK or Pyk2 in smooth muscle contraction, in this study we examined FAK activation in gastric fundus smooth muscles by Ca^{2+}-dependent and independent contractile stimuli. We show that FAK Y397 phosphorylation increases in response to EFS-evoked cholinergic neurotransmission, and is inhibited by the FAK inhibitor PF-431396. Pyk2 Y402 phosphorylation is minimal and unchanged during contraction. CPI-17 phosphorylation at T38 and the contractions evoked by EFS or high K^+ were inhibited by PF-431396. Ca^{2+} influx evoked by EFS was not inhibited by PF-431396. Nicardipine inhibited the EFS-evoked contractions but had no effect on the EFS-induced increase in FAK Y397 phosphorylation. We used PDBu to contract gastric smooth muscles Ca^{2+}-independently, and found that PDBu increased FAK Y397 phosphorylation. These findings reveal a novel role for FAK activation in gastric fundus smooth muscle contraction by its involvement in PKC activation and subsequent CPI-17 phosphorylation.
2.3 Materials and Methods

2.3.1 Ethical approval

Male C57BL/6 mice (6- to 8-weeks old, (Charles River Laboratories, Hollister, CA, USA) were maintained and experiments carried out according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Animal protocols were approved by the University of Nevada, Reno, Institutional Animal Care and Use Committee. Mice were housed in a pathogen-free facility with a 12-h light/dark cycle and free access to water and food (Prolab 5P76 Isopro 3000; 5.4% fat by weight). Our methods comply with the animal ethics checklist outlined in the *Journal of Physiology* [105].

2.3.2 Tissue preparation

Mice were anesthetized by isoflurane inhalation and killed by decapitation. The stomachs were removed, pinned to a Sylgard-lined dish and placed in 4°C oxygenated (97%O₂/ 3%CO₂) Krebs-Ringer buffer. The mucosa and sub-mucosa were removed from the gastric fundus by sharp dissection [106]. The smooth muscles were pre-equilibrated for 45 min in 37°C oxygenated Krebs before use.

2.3.3 Mechanical responses

Contractile activity was measured in static myobath [18]. Gastric fundus smooth muscle strips (~10 mm x 10 mm) were attached to a Fort 10 isometric strain gauge (WPI, Sarasota, FL, USA), in parallel with the circular muscles [106]. Each muscle strip was equilibrated in 37°C oxygenated Krebs-Ringer buffer for 1 h, followed by an additional 30 min incubation in 0.3 μM tetrodotoxin prior to KCl
addition, or 100μM L-NAME and 1μM MRS2500 prior to the delivery of square-wave pulses of EFS (0.3 ms duration), 150 V, 30 sec duration (supra-maximal voltage; Grass S48 stimulator) [18]. As indicated, drugs and compounds were added to the tissues in the presence of either 0.3μM tetrodotoxin or 100μM L-NAME and 1μM MRS2500. Muscle responses were recorded and analyzed with Acqknowledge 3.2.7 software (BIOPAC Systems, Goleta, CA, USA). For Wes analysis, the myobath chamber was quickly dropped down and the tissue rapidly immersed into ice cold acetone, 10mM DTT, 10% (w/v) trichloroacetic acid (TCA) for 1 min. During EFS, the stimulation was on while dropping the bath down and submerging the tissue into the ice-cold acetone-DTT-TCA [18]. The tissues were then snap-frozen in liquid N₂, and stored at -80°C [33].

2.3.4 Ca²⁺ imaging

Each gastric fundus muscle preparation was equilibrated by perfusion (1.6 ml/min) with oxygenated Krebs-Ringer buffer for 45 minutes at 37°C. To visualize Ca²⁺ transients, the fundus muscles were incubated in Ca²⁺ PSS containing 10 μM Cal-520 AM (AAT Bio-quest, Sunnyvale CA, USA) and cremophor EL (0.01 %, Sigma) for 15 min at room temperature, followed by de-esterification for 20 min at room temperature. Following incubation, the recording chamber was mounted on the stage of a wide-zoom stereo microscope (SZX16; Olympus Corporation), and visualized and imaged using magnifications from 2x to 10x. The preparations were superfused with dye-free PSS, illuminated at 488 nm using dichroic mirrors and an Olympus light source (Olympus U-LH100HGAPO), and fluorescence emissions at 520 nm were captured through
a barrier filter. Experiments were performed by applying electrical field stimulation (10 Hz for 10 sec) in the presence of 100 μM L-NAME and 1μM MRS2500 before and after adding PF-431396 (300 nM), along with unstimulated, control recordings in the absence and presence of PF-431396. Solutions containing the drugs were perfused into the chamber for 15 min to allow full tissue penetration. Images were captured using a high-speed sCMOS Camera (Andor Neo sCMOS; ANDOR Technology, Belfast, UK). Image sequences were collected at 10-20 fps using Nikon Elements software (Nikon, Tokyo, JP). After acquisition, the video sequences of Ca\(^{2+}\) imaging data were imported into custom software (Volumetry G8d, written by G.W. Hennig) for analysis [107]. The amplitudes of the Ca\(^{2+}\) transients are expressed as the average pixel intensity or (intensity units, iu) from the tissues to generate the traces of calcium activity.

2.3.5 Automated capillary electrophoresis and immunoblotting with Wes Simple Western.

Muscles were washed in ice cold acetone,10 mM DTT for 1 min, 3 times, followed by a 1 min wash in ice cold lysis buffer (mM; 50 Tris HCl pH 8.0, 60 β-glycerophosphate, 100 NaF, 2 EGTA, 25 Na-pyrophosphate, 1 DTT, 0.5% NP-40, 0.2% SDS, and protease inhibitors) [33]. Each muscle tissue was homogenized in 0.3 mL lysis buffer in a Bullet Blender (0.01% anti-foam C, 1 stainless steel bead per tube, speed 6, 5 min), then centrifuged at 16,000 x g, 10 min and 4°C. Supernatants were stored at -80°C. Protein concentrations of the supernatants were determined by the Bradford assay using bovine γ-globulin as the standard. Protein expression and phosphorylation levels were measured and analyzed according to
the Wes User Guide using a Wes Simple Western instrument from ProteinSimple (San Jose, CA, USA). The protein samples were mixed with the fluorescent 5X master mix (ProteinSimple) and then heated at 95°C for 5 min. Boiled samples, biotinylated protein ladder, blocking buffer, primary antibodies, ProteinSimple HRP-conjugated anti-rabbit or anti-mouse secondary antibodies, luminol-peroxide, and wash buffer were loaded into the Wes plate (Wes 12-230 kDa Pre-filled Plates with Split Buffer, Protein Simple). The plates and capillary cartridges were loaded into the Wes instrument, and protein separation, antibody incubation, and imaging were performed using default parameters. Compass software (ProteinSimple) was used to acquire the data, and to generate image reconstruction and chemiluminescence signal intensities. The protein and phosphorylation levels are expressed as the area of the peak chemiluminescence intensity. The following primary antibodies were used: mouse anti-CPI-17 (PPP1R14A) (sc-48406; 1:200), rabbit anti-pT38-CPI-17 (sc-17560; 1:200), rabbit anti-LC20 (sc-15370; 1:400), rabbit anti-pS19-LC20 (sc-12896; 1:200), rabbit anti-MYPT1 (PPP1R12A) (sc-25618; 1:200), rabbit anti-pT696-MYPT1 (sc-17556-R; 1:200), rabbit anti-pT853-MYPT1 (sc-17432-R; 1:200), rabbit anti-Pyk2 (Ptk2b) (sc-9019; 1:200), rabbit anti-pT402-Pyk2 (sc-11767-R; 1:200), (Santa Cruz Biotechnologies, Santa Cruz, CA, USA); and rabbit anti-FAK (#3281; 1:100) and rabbit anti-pY397-FAK (#8556; 1:100) (Cell Signaling Technology, Danvers, MA, USA).

The area of the peak chemiluminescence intensity values of the protein bands were calculated by Compass software. The chemiluminescence intensity values of pT853, pT38, pS19, pY397, and pY402 were divided by the total MYPT1, CPI-17,
LC20, FAK and Pyk2 chemiluminescence intensity values from the same sample, respectively, to obtain the ratio of phosphorylated protein to total protein. The ratios were normalized to 1 for unstimulated muscles (Control, basal levels), and all ratios were subsequently analyzed by non-parametric repeated tests of ANOVA using Prism 7.01 software (Graphpad, San Diego, CA, USA), and are expressed as the mean (SD). Student’s \( t \)-test was used to measure significance and \( P<0.05 \) is considered significant. The digital lane views (bitmaps) of the immunodetected protein bands were generated by Compass software, with each lane corresponding to an individual capillary tube. The immunodetection figures were created from the digitized data using Adobe Photoshop Version: 12.0.3. Contractile responses were compared by measuring the maximum height of each peak (p-p, g), and the area under the curve (AUC) of each peak including the contribution of basal tone (integral, grams \( \times \) seconds) divided by time (sec), per cross-sectional area (cm\(^2\)) of the smooth muscles, using Acknowledge. Average peak responses (mean (SD)) were calculated using Prism, and significance was determined by \( t \)-test using Prism with \( P<0.05 \) considered as significant. Graphs were generated using Prism.

Atropine and tetrodotoxin were obtained from EMD Millipore (Billerica, MA, USA), L-NAME and PF-431396 were obtained from Sigma-Aldrich (St. Louis, MO, USA), MRS2500 and FAK Inhibitor 14 (FI14) were purchased from Tocris Bioscience (Bristol, UK). All other reagents and chemicals purchased were of analytical grade or better. PF-431396 and FI14 were dissolved in dimethyl sulfoxide (DMSO) and then diluted to the indicated final concentrations.
2.4 Results

2.4.1 EFS of cholinergic motor neurons increases FAK Y397 phosphorylation.

As noted previously, FAK and Pyk2 autophosphorylation have both been reported to be involved in the contraction of smooth muscles. To determine whether FAK and Pyk2 are activated in response to cholinergic stimulation in gastric fundus muscles, we measured FAK and Pyk2 phosphorylation during EFS-evoked contractions. As we previously reported with 10Hz stimulation, the peak of contraction occurred at 5 sec (Fig. 2-1A) [18], with an average maximum force generation of $0.14 \pm 0.02$ g (Fig. 2-1B). FAK and Pyk2 phosphorylation were measured from unstimulated muscles, and at the 5sec peak of contraction. (Fig. 2-1C and 2-1E). The basal Y397 phosphorylation level was increased $1.73 \pm 0.35$-fold by EFS (Fig. 2-1D). In contrast to FAK, there was no detectable Pyk2 phosphorylation in control or EFS stimulated fundus muscles (Fig. 2-1E and 2-1F). Thus, we investigated FAK phosphorylation during EFS-induced cholinergic stimulation of gastric fundus smooth muscles.
Fig. 2-1. EFS induces phosphorylation of FAK but not Pyk2 during gastric fundus smooth muscle contraction. A. Representative trace of muscle strips contractions in response to 10Hz EFS in the presence of 100μM L-NAME and 1μM MRS2500. FAK and Pyk2 phosphorylation were measured at the 5 sec time point. B. The mean ± SD of the amplitude of contraction evoked by 10Hz EFS at 5 sec (n=6) ** P<0.01. C. Representative immunoblot of FAK and pY397 from unstimulated and 10Hz 5sec EFS fundus muscles. D. Mean ± SD of the pY397: FAK area under the peak intensity ratios from unstimulated and 10Hz 5sec EFS fundus muscles (n=3) *P<0.05. E. Representative immunoblot of Pyk2 and pY402 from unstimulated and 10Hz 5sec EFS fundus smooth muscles. F. Mean ± SD of the Pyk2 and pY402 area under the peak intensity per μg lysate protein from unstimulated and 10Hz 5sec EFS fundus muscles (n=3).
2.4.2 Effects of FAK inhibitors on basal tone and FAK Y397 phosphorylation.

FAK inhibitor 14 (FI14) and PF-431396 have been used previously to inhibit FAK phosphorylation [108], [109]. We examined the effects of FI14 (1.5μM) and PF-431396 (0.3μM) on basal tone and FAK phosphorylation in murine gastric fundus smooth muscles. PF-431396 had little effect on basal tone (Fig. 2-2A and 2-2B), but reduced FAK Y397 phosphorylation to 24.2 ± 7.9% of the phosphorylation levels in vehicle-treated muscles (Fig. 2-2C and 2-2D). Fundus smooth muscles treated with FI14 (1.5μM) showed a significant increase in basal tone (Fig. 2-1B) (0.19 ± 0.04g), but FAK Y397 phosphorylation was unchanged by FI14 treatment (Fig. 2-2E and 2-2F). Thus, we selected PF-431396 to inhibit FAK phosphorylation for this study.
Fig. 2-2. Effect of FAK inhibitors on basal tone and FAK phosphorylation. A. Representative traces of gastric fundus smooth muscle basal tones without or with PF-431396 or FI14 in the presence of vehicle (0.1% DMSO), or 100μM L-NAME and 1μM MRS2500. B. Mean ± SD of the change in basal tone amplitudes (n=3). C. Representative immunoblot of FAK and pY397 in muscles treated with vehicle, 0.3μM PF-431396, or 1.5μM FI14. Mean ± SD of the pY397:FAK area under the peak intensity ratios from muscles without or with 0.3μM PF-431396 (D) (n=3) or 1.5 μM FI14 (F) (n=3) **P<0.01.
2.4.3 Dose test of PF431396

To investigate the effects of inhibiting Y397 phosphorylation on contraction, we determined the concentration-dependent effects of PF-431396 on FAK Y397 phosphorylation and fundus smooth muscle basal tone. Fundus smooth muscle strips showed little to no change in basal tone when treated with vehicle, 0.1μM, or 0.3μM PF-431396. However, basal tone was reduced by 1μM PF-431396 (0.03 ± 0.01 g reduced tone) and 3μM PF-431396 (0.10 ± 0.01 g reduced tone) (Fig. 2-3A, 2-3B). FAK Y397 auto-phosphorylation was inhibited at each concentration of PF-431396. (Fig. 2-3C and 2-3D). We chose 0.3μM PF-431396 for this study since the inhibition of FAK Y397 phosphorylation was nearly maximal at 0.3μM PF-431396, with no reduction in basal tone.
Fig. 2-3. Dose-response of PF-431396 on basal tone and FAK Y397 phosphorylation. A. Representative traces of gastric fundus smooth muscle basal tones without or with vehicle, 0.1μM, 0.3μM, 1μM or 3μM PF-431396 in the presence of 100μM L-NAME and 1μM MRS2500. B. Mean ± SD of the change in basal tone amplitudes in response to vehicle, 0.1μM, 0.3μM, 1μM or 3μM PF-431396 (*P<0.05, **P<0.01, n=3, each treatment). C. Representative immunoblot of FAK and pY397 in the absence or presence of increasing concentrations of PF-431396. D. Mean ratios ± SD of pY397:FAK in the absence or presence of increasing concentrations of PF-431396 (*P<0.05, n=3, each treatment).

2.4.4 PF-431396 inhibits FAK Y397 phosphorylation and EFS-evoked contraction.

Incubation of gastric fundus smooth muscle strips with 0.3μM PF-431396 markedly inhibited EFS induced contractions (Fig. 2-4A). As measured by the integral of contraction, the contractile responses to 5Hz, 10Hz, and 20Hz EFS were significantly inhibited by 0.3μM PF-431396 (65.1% ± 11.3%, 64.8% ± 11.6%, and 53.9% ± 20.5% inhibition, respectively. The 10Hz 5sec EFS-induced
increase in pY397 was blocked by 0.3μM PF-431396 (Fig. 2-4C and 2-4D). The pY397 levels were increased almost two-fold by EFS (1.73 ± 0.20-fold), but in the presence of PF-431396, the pY397 levels were reduced below the basal, control levels (Fig. 2-4C, 2-4D).

**Fig. 2-4. PF-431396 inhibits EFS-induced contractile responses and FAK Y397 phosphorylation.** Representative traces of the contractile response of gastric fundus smooth muscles to 30 secs of 5Hz, 10Hz or 20Hz EFS without (A) or with (B) 0.3μM PF-431396 in the presence of 100μM L-NAME and 1μM MRS2500 (n=3). C. Representative immunoblot of FAK and pY397 in unstimulated muscles, and muscles contracted with 10Hz 5sec EFS without or with 0.3μM PF-431396, in the presence of 100μM L-NAME and 1μM MRS2500. D. Mean ratios ± SD of pY397:FAK from unstimulated muscles, and muscles contracted with 10Hz 5sec EFS without or with 0.3μM PF-431396 (n=3) *P<0.05, ***P<0.005.
2.4.5 Atropine inhibits EFS-induced FAK Y397 phosphorylation.

To determine that FAK activation and inhibition of FAK activation is occurring in post-junctional cells, we investigated the effects of atropine on the EFS-induced increase in pY397. Atropine completely blocked both the EFS-evoked contractile responses (Fig. 2-5A and 2-5B), and the increase in pY397 evoked by 10Hz 5sec EFS (Fig. 2-5C and 2-5B). In the presence of atropine, the level of FAK Y397 phosphorylation measured at 10Hz 5sec EFS was nearly identical to the basal level of FAK phosphorylation (Fig. 2-5C), indicating that FAK is activated in post-junctional cells by EFS-induced cholinergic neurotransmission.

Fig. 2-5. Atropine blocks EFS-induced contractile responses and the EFS-induced increase in FAK phosphorylation. Representative traces of the contractile response of gastric fundus smooth muscles to 30 sec 5Hz, 10Hz or 20Hz EFS without (A) or with (B) 1μM atropine in the presence of 100μM L-NAME and 1μM MRS2500 (n=3). C. Representative immunoblot of FAK and pY397 from unstimulated and 10Hz 5 sec EFS muscles in the absence or presence of 1μM atropine. D. The mean ± SD of pY397:FAK area under the peak intensity ratios from unstimulated and 10Hz 5sec EFS muscles in the absence or presence of 1μM atropine (n=3) *P<0.05.
2.4.6 PF-431396 inhibits high K⁺-evoked contraction and FAK Y397 phosphorylation.

To further show that FAK activation and inhibition of FAK activation occurs in smooth muscle cells we determined the effects of high K⁺-induced contraction on FAK Y397 phosphorylation. High extracellular K⁺ (33mM) contracted gastric fundus smooth muscle strips, with a maximal force generation of 0.35 ± 0.06g (Fig. 2-6A). No difference in contraction was observed between high K⁺ pre-stimulation and the subsequent high K⁺ stimulation, in which 0.1% DMSO was applied as the vehicle (Fig. 2-6A). Thus, we could compare PF-431396 inhibition of high K⁺ stimulated contraction to the high K⁺ pre-stimulation contraction in the same smooth muscle strip. PF-431396 (0.3μM) inhibited high K⁺-induced contractions by 39.8% ± 7.5% (Fig. 2-6B). High K⁺ also induced a 25 ± 3.2% increase in pY397, which was blocked by 0.3μM PF-431396, further showing that FAK is activated in fundus smooth muscle cells (Fig. 2-6C, 2-6D).
Fig. 2-6. PF-431396 inhibits high K⁺-evoked contractions and FAK Y397 auto-phosphorylation. A. Representative trace of the contractile response of gastric fundus smooth muscles to 33mM KCl without and with vehicle (0.1% DMSO) (n=3). B. Representative trace of the contractile responses to 33mM KCl without and with 0.3μM PF-431396 (n=3). 0.3μM tetrodotoxin was present in the myobath. C. Representative immunoblot of FAK and pY397 from unstimulated and 33mM KCl stimulated muscles in the absence or presence of 0.3μM PF-431396. D. The mean ratios ± SD of pY397: FAK area under the peak intensity ratios from unstimulated muscles and muscles contracted with 33mM KCl in the absence or presence of 0.3μM PF-431396 (n=3, each treatment) *P<0.05, ***P<0.005.

2.4.7 Effect of PF-431396 on EFS-evoked CPI-17, MYPT1 and LC20 phosphorylation.

We previously reported that EFS-evoked cholinergic neurotransmission increases CPI-17 phosphorylation, and is blocked by PKC inhibitors in gastric fundus smooth muscles [18]. As previously noted, phosphorylation of PKC by tyrosine kinases can contribute to PKC activation [104]. Thus, to determine if FAK activation modulates Ca²⁺ sensitization pathways, we examined the effect of
PF-431396 on Ca\(^{2+}\) sensitization protein phosphorylation. As expected, 10Hz 5sec EFS significantly increased pT38 levels by 3.5±0.07-fold, and this increase was blocked by PF-431396 (Fig. 2-7A, 2-7B). The pT853 levels were not significantly increased by 10Hz 5sec EFS, and PF-431396 did not change the pT853 levels, except for a decrease in muscles treated with PF-431396 during EFS, compared to EFS alone (Fig. 2-7C, 2-7D). In addition, as expected, EFS did not increase the pT696 levels, but PF-431396 decreased the pT696 levels below the basal levels by 30±3.1% (Fig. 2-7E, 2-7F). LC20 phosphorylation at S19 was not significantly increased by 10Hz 5sec EFS, and in the presence of PF-431396 during EFS, the pS19 levels were reduced by 64±1% compared to the control levels (Fig. 2-7G, 2-7H).

**Fig. 2-7. Effects of PF-431396 on EFS-evoked CPI-17, MYPT1 and LC20 phosphorylation.** Representative immunoblots of CPI-17 and pT38 (A), MYPT1 and pT853 (C), MYPT1 and pT696 (E), and LC20 and pS19 (G), from unstimulated and 10Hz 5sec EFS gastric fundus smooth muscles in the absence or presence of 0.3μM PF-431396. The mean ± SD of the area under the peak intensity ratios for pT38:CPI-17 (B) (n=3), pT853:MYPT1 (D) (n=3), pT696:MYPT1 (F) (n=3), and pS19:LC20 (H) (n=3), from unstimulated, and 10Hz 5sec EFS muscles in the absence or presence of 0.3μM PF-431396. *P<0.05, **P<0.01.
2.4.8 PF-431396 does not inhibit EFS-evoked Ca\(^{2+}\) influx into smooth muscle cells.

We examined the effect of PF-431396 on EFS-evoked Ca\(^{2+}\) influx because the above findings could also be due to inhibition of Ca\(^{2+}\) influx by tyrosine kinase inhibition, or by off-target inhibition by PF-431396 [110], [111]. Gastric fundus smooth muscle tissues incubated in KRB with 100µM L-NAME and 1µM MRS2500 were loaded with the fluorescent Ca\(^{2+}\) indicator dye Cal-520, and then stimulated with 10Hz for 10sec in the absence or presence of 0.3µM PF-431396. As expected, 10Hz 10 sec EFS evoked a robust Ca\(^{2+}\) influx, as indicated by the increase in fluorescence intensity (Fig. 2-8A, 2-8C, 2-8E). Similar increases in fluorescence intensity were evoked by 10Hz 10 sec EFS in the presence of 0.3µM PF-431396 (Fig. 2-8B, 2-8D, 2-8E), indicating that PF-431396 had no effect on EFS-evoked Ca\(^{2+}\) influx.
Fig. 2-8. PF-431396 does not inhibit EFS-evoked Ca\(^{2+}\) influx into gastric fundus smooth muscle cells. Representative images of the Cal-520 fluorescence evoked by 10Hz 10sec EFS of gastric fundus smooth muscles in the presence of 100μM L-NAME and 1μM MRS2500, in the absence (A) or presence (B) of 0.3μM PF-431396. Representative traces of the fluorescent intensity values evoked by 10Hz 10sec EFS of fundus smooth muscles in the absence (C) or presence (D) of 0.3μM PF-431396. E. The mean ± SD of the area under the curve (AUC) of the Ca\(^{2+}\) intensity units measured from fundus muscle tissues during 10Hz 10sec EFS in the absence or presence of 0.3μM PF-431396 (n=5).
2.4.9 Nicardipine does not inhibit the EFS-evoked increase in FAK pY397.

We examined the effects of nicardipine on the EFS-evoked increase in pY397 to determine whether Ca\(^{2+}\) influx contributes to the increase in FAK pY397. As expected, the EFS-evoked contractions (Fig. 2-9A) were markedly inhibited by 1µM nicardipine (Fig. 2-9B). In contrast, 1µM nicardipine had no effect on the increase in pY397 evoked by 10Hz 5sec EFS (Fig. 2-9C and 2-9E), suggesting that Ca\(^{2+}\) influx is not required for the EFS-evoked increase in FAK pY397.

**Fig. 2-9. Nicardipine does not inhibit the EFS-evoked increase in FAK pY397 phosphorylation.** Representative traces of the contractile response of gastric fundus smooth muscles to 30 sec 5Hz, 10Hz or 20Hz EFS without (A) or with (B) 1µM nicardipine in the presence of 100µM L-NAME and 1µM MRS2500 (n=3). C. Representative immuno-blot of FAK and pY397 from unstimulated and 10Hz 5 sec EFS muscles in the absence or presence of 1µM nicardipine. D. The mean ± SD of pY397:FAK area under the peak intensity ratios from unstimulated and 10Hz 5sec EFS muscles in the absence or presence of 1µM nicardipine (n=3) *P<0.05, ***P<0.005.
2.4.10 **PDBu contracts gastric fundus smooth muscles and increases CPI-17 T38 and FAK Y397 phosphorylation.**

Mechanical stimulation activates FAK in airway smooth muscles [68]. Thus, we examined the effect of PDBu on FAK activation because PDBu contracts smooth muscles in a Ca\(^{2+}\)-independent manner [112]–[116]. Fig. 2-10A shows that 0.5\(\mu\)M PDBu contracted gastric fundus smooth muscle strips. Although the time to achieve maximum force was slower than EFS-evoked contractions, the maximum force generated was similar to the maximum force generated by 20Hz EFS. As expected, PDBu significantly increased the phosphorylation of T38 (Fig. 2-10B), and a 0.5\(\mu\)M PDBu significantly increased Y397 phosphorylation (Fig. 2-10B, 2-10C), supporting the conclusions that mechanical stimulation or tension is sufficient to increase FAK pY397, and that Ca\(^{2+}\) influx is not required for the increase in FAK pY397 evoked by contraction.
Fig. 2-10. PDBu contracts gastric fundus smooth muscles and increases the phosphorylation of FAK Y397 and CPI-17 T38. A. Representative trace of the contractile response of gastric fundus muscles to 0.5 μM PDBu (n=3). 0.3 μM tetrodotoxin was present in the myobath. Representative immunoblots of FAK and pY397 (B) and CPI-17 and pT38 (D) from unstimulated and 0.5 μM PDBu stimulated gastric fundus muscles. The mean ratios ± SD of the pY397:FAK (C) and pT38:CPI-17 (E) area under the peak intensity ratios from unstimulated and 0.5 μM PDBu stimulated gastric fundus muscles. (n=3) *P<0.05, ***P<0.005
2.5 Discussion

In this study, we found that FAK phosphorylation is increased during EFS-evoked cholinergic neurotransmission contraction of gastric fundus smooth muscles. We also found that FAK activation in gastric fundus smooth muscles is \( \text{Ca}^{2+} \) independent, and likely involves mechanosensitive mechanisms. In addition, our findings suggest that FAK expression is higher than Pyk2 expression in gastric fundus muscles, but this could also be due to differences between the antibodies used for FAK and Pyk2 detection. These findings are different from the findings in vascular smooth muscles, in which Pyk2 autophosphorylation is significantly increased during contraction [102], [117], [118]. However, it has been reported that FAK phosphorylation is increased in bladder smooth muscles in response to KCl- or CCh-induced contraction [119]. In tracheal smooth muscles, KCl- or CCh-induced contractions increase FAK phosphorylation, and increases in force, intracellular \( \text{Ca}^{2+} \), and LC20 phosphorylation are inhibited by antisense knock-down of FAK expression [101]. Contractile agonists increase FAK phosphorylation in hyper-plastic human prostate smooth muscle, and the contractile responses are decreased by FAK inhibitors [103].

Although FI14 has been used extensively to inhibit FAK activity, we found that FI14 (1.5\( \mu \)M) had no effect on FAK phosphorylation, and also increased the basal tone of the fundus muscle strips (Fig. 2-1). PF-431396 inhibits FAK and Pyk2 with similar IC\(_{50}\) values [120]. However, since we found that Pyk2 expression appears to be lower than FAK, and Pyk2 phosphorylation was barely detectable and
not increased during contraction (Fig. 2-1), we used PF-431396 to inhibit FAK phosphorylation. Our dose-response studies showed that 0.1µM PF-431396 reduced the basal level of FAK phosphorylation by 90%, with almost complete loss of FAK Y397 phosphorylation at 0.3µM. There was no effect on basal tone by 0.3µM PF-431396, while 1µM and 3µM PF-431396 significantly reduced basal tone (Fig. 2-3). These findings indicate the effect of 0.3µM PF-431396 on contraction is likely due to inhibition of FAK phosphorylation, and not due to off-target effects of the compound. In fact, we found that 0.3µM PF-431396 blocked the EFS-evoked increase in FAK Y397 phosphorylation, reduced Y397 phosphorylation below the basal level, and significantly inhibited EFS induced contractions (Fig. 2-4), suggesting an important role for FAK in gastric fundus smooth muscle contractions.

Gastric smooth muscles are composed of several populations of different cell types, all of which are important for muscle function [12]. Thus, to determine that FAK Y397 phosphorylation is increased in smooth muscle cells in response to cholinergic stimulation, we examined whether the muscarinic receptor blocker atropine would block the increase in FAK Y397 phosphorylation. We found that atropine blocked EFS-evoked contractions, indicating a post-junctional loss of cholinergic signaling (Fig. 2-5). We found that atropine also blocked the increase in FAK Y397 phosphorylation by EFS-evoked cholinergic neurotransmission, indicating that FAK Y397 phosphorylation is increased in post-junctional cells, such as smooth muscle cells. It is interesting that atropine blocked the EFS-evoked increase in FAK Y397 phosphorylation, but did not reduce the level of pY397 below
the basal level (Fig. 2-5C), as PF-431396 did (Fig. 2-5D). These findings suggest that PF-431396 inhibited basal FAK activity, and also inhibited the EFS-evoked increase in FAK activity. In contrast, the finding that atropine did not reduce the level of Y397 below the basal level suggests that basal FAK phosphorylation is maintained by a mechanism independent of muscarinic receptor activity.

In mouse gastric fundus smooth muscles, networks of interstitial cells of Cajal (ICC) and PDGFRα+ cells are also present post-junctionally [14]. FAK expression has been detected in mouse colon ICC and PDGFRα+ cells by transcriptome analysis [121]. To further show that FAK Y397 phosphorylation occurs in smooth muscle cells we investigated the effects of high K+-induced depolarization and contraction on FAK phosphorylation. High extra-cellular K+ contracted gastric fundus smooth muscle strips and increased FAK Y397 phosphorylation, which were both inhibited by 0.3µM PF-431396 (Fig. 2-6), further showing that FAK is activated in fundus smooth muscle cells. These findings provide additional evidence that FAK Y397 phosphorylation and its inhibition occurs in smooth muscle cells, although these findings do not preclude changes in FAK phosphorylation also occurring in other cell types.

Pyk2 is a Ca²⁺-stimulated protein tyrosine kinase, and is activated in caudal artery smooth muscle cells by membrane depolarization and voltage-dependent Ca²⁺ influx, resulting in RhoA activation, leading to MYPT1 and MLC phosphorylation and MLCP inhibition [102]. However, in gastric fundus smooth muscles, the
involvement of RhoA and ROCK2 in MYPT1 phosphorylation by voltage-dependent Ca\(^{2+}\) influx is less likely, based on our findings that EFS-evoked cholinergic contractions do not increase MYPT1 T853 phosphorylation [18]. The phosphorylation of CPI-17 T38 is increased by voltage-dependent Ca\(^{2+}\) influx, and is significantly decreased by PKC inhibitors [18]. The α, β, δ, ε, and ζ PKC isozymes have been identified in GI smooth muscles [122], [123]. PKCδ is a novel PKC isozyme (nPKC) with twin C1 and C2 domains that do not require Ca\(^{2+}\) for activation by DAG [124]. Furthermore, when phosphorylated by Src family kinases at Y311, PKCδ displays lipid-independent kinase activity and can thus potentially phosphorylate target substrates not just on lipid membranes, but also in the cytoplasm, such as CPI-17 [124]. FAK Y397 phosphorylation creates a binding site for the SH2 domain of Src family kinases, resulting in the formation of a transient FAK–Src signaling complex [125]. Because of this possible link between FAK and PKC, we examined whether the regulation of gastric fundus smooth contraction by FAK involves the regulation of PKC activity. CPI-17 T38 phosphorylation was significantly increased by 10Hz 5sec EFS, and PF-431396 blocked this increase. These findings suggest that FAK activity is necessary for PKC activation and CPI-17 T38 phosphorylation by EFS in gastric fundus smooth muscles. Further studies are required to determine whether FAK or a Src family kinase regulated by FAK phosphorylates PKCδ, or other PKC isozymes in murine gastric fundus smooth muscles.
Conflicting reports describe activation or inhibition of L-type Ca$^{2+}$ channel activity in smooth muscles by tyrosine kinases [110], [126], [127]. In addition, it is not uncommon for kinase inhibitors to have off-target effects on ion channels [111]. Thus, we used the fluorescent Ca$^{2+}$ indicator dye Cal-520 to measure EFS-evoked Ca$^{2+}$ influx into gastric fundus smooth muscle cells. The levels and extent of EFS-evoked Ca$^{2+}$ influx into smooth muscles in the absence or presence of PF-431396 were similar, strongly suggesting that the effects of PF-431396 on contraction, FAK Y397 phosphorylation, and CPI-17 T38 phosphorylation, are due to FAK inhibition, and not due to inhibition of EFS-evoked Ca$^{2+}$ influx.

The results of Fig. 2-5 and Fig. 2-8 show that EFS of cholinergic neurons increases FAK phosphorylation and Ca$^{2+}$ influx into smooth muscle cells. Although FAK and Pyk2 share considerable domain structures and sequence homology, the FERM domain of Pyk2, but not the FAK FERM domain, binds to calmodulin in a Ca$^{2+}$-dependent manner [128], [129]. Our finding in Fig. 2-9 showing that the increase in FAK Y397 phosphorylation is nicardipine-insensitive suggests that Ca$^{2+}$ influx is not involved in FAK activation in response to contraction of gastric fundus smooth muscles. The cytoplasmic domain of focal adhesions contains a sizeable array of signaling proteins, which contribute to the mechanosensing function of integrins via associated proteins such as paxillin, vinculin, and FAK [130]. Thus, to further distinguish between the mechanisms of activation of FAK by mechanical stimuli or Ca$^{2+}$ influx, we used PDBu to contract the gastric fundus smooth muscles in a Ca$^{2+}$ independent manner [116]. PDBu contracted the muscle strips and
increased FAK Y397 phosphorylation (Fig. 2-10), providing further evidence that FAK is activated by mechanical stimuli. These findings are similar to the findings that the mechanical strain induced by tracheal smooth muscle contraction activates FAK independent of Ca\textsuperscript{2+} influx [131]. Protein kinase C (PKC) has been implicated in activating FAK in several cell types [132]. However, our finding that FAK inhibition prevented the EFS- induced increase in CPI-17 phosphorylation, suggests that FAK is upstream of PKC, and further suggests that FAK activity is required for PKC activation and CPI-17 phosphorylation. Additional studies are required to determine how cholinergic signaling activates FAK in gastric fundus smooth muscle cells.

In conclusion, my results support a role for FAK in regulating Ca\textsuperscript{2+} sensitization mechanisms during gastric fundus smooth muscle contraction in response to cholinergic motor signaling. FAK Tyr phosphorylation and recruitment to focal adhesions via complex formation with integrins has been shown to occur during smooth muscle contraction [133], [134]. These interactions are thought to facilitate the polymerization of cortical actin filaments to stabilize adhesion complexes in the membrane, allowing the force generated by myofilament activation to be transmitted to the connective tissue of the extracellular matrix [93], [101], [102]. The present work introduces an additional role for FAK in gastric fundus smooth muscle contractile function, by regulating the Ca\textsuperscript{2+} sensitization of myofilament activation.
CHAPTER 3: Quantification and spatial annotation of protein phosphorylation and protein interaction by proximity ligation assays in intact smooth muscle tissues.

3.1 Abstract

Methodologies to study functional protein phosphorylation and protein interaction in intact tissue have the potential to characterize physiological events unbiasedly. Proximity ligation assay (PLA) has wide applications in tissues and cultured or isolated cells [79]. However, PLA assay development and application in physiological studies at tissue level are limited by the lack of solid positive and negative controls and the difficulty of normalization for changes in tissue shape. We demonstrate a set of experimental and computational approaches to improve the credibility of quantitative results in gastric fundus smooth muscles from mouse and human. Based on existing biochemical data, We designed positive and negative controls to ensure experimental rigor. For the consideration of smooth muscle tissue volume change under different physiological state, We normalized the PLA spots to the PLA signal of smooth muscle myosin light chain 20 (LC20). The outcome of the normalization correlates with the morphological the data. We present the steps necessary to clean the noise and quantify PLA signals using Fiji. Our experimental pipelines are tested with key signaling protein phosphorylation and interactions in Ca\textsuperscript{2+} sensitization pathways. In addition, MosaicIA is applied for spatial annotation of the proximity between PLA signals and cellular membrane structures. In conclusion, our tissue-level PLA method enables the unbiased quantification and spatial characterization of protein phosphorylation and
protein-protein interaction in intact smooth muscle tissue, suggesting the potential for PLA applications in other types of intact tissues.

3.2 Introduction

Proteins function in groups and complexes. The protein phosphorylation, protein-protein interaction, and protein complex composition changes in different physiological states in cells and tissues. Proximity ligation assay (PLA) turns out to be a powerful tool to characterize proteins and their interactions in any cell or tissue [77]. Traditional biochemical assays (western blot, co-immunoprecipitation, etc) to study protein phosphorylation and protein-protein interaction are based on protein extraction procedures which may result in loss of proteins [135]. PLA, on the other hand, preserves the intact tissue by fixation. Therefore, PLA is less biased, and maintains cellular structural and spatial information compared to traditional biochemical assays for downstream analysis.

For physiological studies of smooth muscle, cultured cell lines or primary smooth muscle cells are much less favored due to the confounding factors introduced by cell culture conditions compared to smooth muscle cells in the intact tissue. However, the primary quantifiable application of PLA is limited to cultured cells. Tissue section PLA is easy to perform but hard to quantify. PLA signal count can be normalized to the nucleus count in cultured cell PLA applications. On the contrary, in smooth muscle tissue cross-section PLA readout, the nucleus count number in a particular tissue cross-section image does not reflect the actual cell
number in the region of interest. Moreover, the smooth muscle cell structure and volume may change in response to different physiological stimulation [136]. The cell volume change will lead to a change in cell density, which makes it even harder to compare PLA signal intensities for sample tissues under different treatments without proper normalization. In addition, proper PLA positive and negative controls are needed to ensure PLA quality in smooth muscle tissue.

To optimize the PLA application in smooth muscle tissue sections for physiological studies, we demonstrate methods to improve smooth muscle tissue PLA quality control, quantification and spatial analysis. In addition to standard experimental technical controls, we identified a set of PLA pairs for positive and negative controls that correlate with biochemical data from previous studies. We optimized the PLA spot counting method by Fiji [137] to exclude PLA noise. Moreover, we used the LC20 PLA spot count as a novel internal control for PLA signal normalization. In addition, we analyzed the spatial distance between PLA spots and cellular structure with MosaicIA [138]. Thus, our smooth muscle tissue PLA methodology may lead to wider application of PLA in smooth muscle physiology studies.

3.3 Materials and Methods

3.3.1 Tissue preparation

6- to 8- weeks old Male C57BL/6J mice (Charles River Laboratories, Wilmington, MA, USA) were maintained and experiments carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory
Animals. All animal protocols were approved by the University of Nevada, Reno, Institutional Animal Care and Use Committee. Mice were housed in a pathogen-free barrier facility with a 12-h light/dark cycle and free access to water and food (Prolab 5P76 Isopro 3000; 5.4% fat by weight).

3.3.2 Tissue preparation

Mice were anesthetized by isoflurane inhalation and sacrificed by cervical dislocation. Stomachs were dissociated, pinned to a Sylgard-lined dish and immersed in 4°C oxygenated (97%O2/3%CO2) Krebs solution (NaCl, 120.35 mmol/L; KCl, 5.9 mmol/L; NaHCO3, 15.5 mmol/L; NaH2PO4, 1.2 mmol/L; MgCl2, 1.2 mmol/L; CaCl2, 2.5 mmol/L; and glucose, 11.5 mmol/L). The gastric fundus was identified and acquired, followed by removing mucosa and submucosa by sharp dissection [139]. Smooth muscle strips were pre-equilibrated for 45 min in 37°C oxygenated Krebs before use.

3.3.3 Mechanical responses and immunofluorescence

The procedure to measure mechanical responses are the same as described in Chapter 2. Briefly, the gastric fundus smooth muscle strips in static myobath (~10 mm x 10 mm) were hooked onto a Fort 10 isometric strain gauge (WPI, Sarasota, FL, USA), which are in parallel with the circular muscles [33]. A 0.6 gram resting force was initially applied for the force development. Then each muscle strip went through 1 hour equilibration in 37°C oxygenated Krebs to allow establishing a stable level of resting tone. Drugs were applied through the myobath and the mechanical responses
were recorded and analyzed by the Acqknowledge 3.2.7 software (BIOPAC Systems, Santa Barbara, CA, USA). The mechanical responses to neutrally released acetylcholine were measured from muscle strips incubated with 100μM L-Nω-nitro-L-arginine (LNNA) and 1μM MRS2500 for 20 min prior to delivery of electrical field stimulation (EFS) with square wave pulses, 0.3 msec duration, 5-20Hz, 150V and 30 sec durations (supra-maximal voltage; Grass S48 stimulator). The mechanical responses to the bath applied carbachol (CCh) were measured from muscles incubated in the presence of 0.3μM tetrodotoxin. EFS or CCh treated tissues were collected at 5sec and 1min after stimulation, respectively. The myobath chamber was quickly dropped down and the tissue was rapidly immersed into room temperature 4% (w/v) paraformaldehyde (PFA). Then gastric fundus smooth muscle strips were OCT-embedded, and the blocks cut into 10 μm cross sections through the circular smooth muscle layer. For immunofluorescence assays, prepared slides were washed in PBS. After antigen retrieval (antigen retrieval solution, Vector), slides were blocked with PBS containing 0.2% tween-20 and 1% bovine serum albumin for 10 min at room temperature. Then the slides were incubated sequentially overnight at 4°C with the appropriate primary antibody pairs as indicated below. Immunolabeling was performed with Alexa-488 donkey anti-goat IgG and Alexa-594 goat anti-mouse IgG (1:500 in PBS, Cell Signaling Technology, USA). Finally, mounting medium containing DAPI was applied to slides to label the nuclei.

3.3.4 Proximity ligation assay (PLA).

Prepared gastric fundus smooth muscle tissue sections were permeabilized and blocked with PBS containing 0.2% tween-20 and 1% bovine serum albumin for
10 min at room temperature. PLA was performed following the manufacturer’s instructions (Olink Bioscience, Sweden). Tissue sections were incubated with the first primary antibody (1:400 dilution) for 1 hour, at room temperature, washed with PBS and then incubated with the with the second primary antibody (1:400 dilution) for 1 hour at room temperature. Then the slides were incubated with the PLA secondary antibodies at 37°C for 1h, followed by the ligation and amplification reactions (Duolink detection kit red, ex.598/em.634). Finally, mounting medium with DAPI was used.

3.3.5 Confocal microscopy.

The slides were examined using an LSM510 Meta (Zeiss, Jena, Germany) or Fluoview FV1000 confocal microscope (Olympus, Center Valley, PA, USA). Confocal micrographs were digital composites of the Z-series of scans (1 μm optical sections of 10 μm thick sections). Settings were fixed at the beginning of both acquisition and analysis steps and were unchanged. Brightness and contrast were slightly adjusted after merging. Final images were constructed using FV10-ASW 2.1 software (Olympus, USA). In the negative control, primary rabbit anti-Akt, mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and talin (Sigma) antibodies were used. The number of PLA red spots per area was normalized by the number PLA spots of rabbit anti-LC20 (sc-15370; 1:400) and mouse anti-LC20 (sc-28329; 1:400) antibody pair to obtain PLA spot count.
3.3.6 **Statistical analysis.**

Fiji was used to count PLA spots. The red channel was taken out of the raw tiff image, and the threshold adjusted with the MaxEntropy thresholding algorithm [140]. Then the PLA spots were counted by particle analysis with the particle size set between 6 and 40 units. The normalized PLA spots counts were analyzed by non-parametric repeated tests of ANOVA using Prism 6.01 software, and are demonstrated as the average ± SD. *T*-test was used to test the significance and *P* < 0.05 is considered significant. The interaction analysis was performed by MosaicIA [138].

3.3.7 **Drugs.**

Tetrodotoxin was obtained from EMD Millipore, Billerica, MA; L-NNA and carbachol were obtained from Sigma-Aldrich, St. Louis, MO; MRS2500 was purchased from Tocris Bioscience, Minneapolis, MN.

3.4 **Results**

3.4.1 **Data quality for smooth muscle tissue PLA**

To validate the quality of smooth muscle tissue PLA signals, we designed 3 positive controls based on the current experimental evidence and the molecular interaction database IntAct [141]. All the antibodies used for positive controls were validated by immunofluorescence (Fig. 2-1). Actin-myosin interactions are essential for cell movement in muscle cells [142]. The membrane protein talin physically links actin to the integrins [42]. Both myosin and talin directly interact with actin. Thus, we first selected γ-actin vs LC20, and γ-actin vs talin antibody pairs for the positive
PLA controls in murine gastric fundus smooth muscle tissue. We found robust PLA signals for both PLA positive controls (Fig. 3-1B and 3-1C). In other occasions, PLA detects two proteins not directly interacting, but are in close distance (< 40nm) (Fig. 3-1A), which is ideal for studies of protein complexes [76]. To test the use of tissue-PLA for proteins in the same protein complex but not directly interacting, we chose RhoA vs MYPT1. In the IntAct database, RhoA vs MYPT1 is identified as a co-complex interaction via ROCK2, instead of a direct interaction [141]. We detected abundant PLA signals in RhoA vs MYPT1 (Fig. 3-1D), which is consistent with the IntAct database as well as reports that RhoA/ROCK2/MYPT1 forms a signaling complex in smooth muscle [32], [143]. Therefore, the three positive controls we selected are ideal for examining PLA detection of protein-protein direct interaction and co-complex interaction.

We also set up PLA negative controls for smooth muscle PLA. To assess the PLA signal for two proteins with distances over 40 nm, we picked the Akt vs talin PLA pair. No direct interaction or co-complex interaction was found for Akt and talin in the IntAct database [141]. The antibody for the membrane protein talin and intracellular protein Akt both showed robust signal in immunofluorescent assays (Fig. 3-2A). However, the talin vs Akt PLA showed no detectable signal, which agreed with our hypothesis based on the IntAct database information. Moreover, we also set up technical negative controls. Virtually no PLA signal was detected in smooth muscle tissue sections in the absence of one or both primary antibodies (Fig.
3-2B to 3-2E). Thus, the above negative controls are suitable for testing PLA false positive signal in smooth muscle tissue.

Typically, the PLA spots visualized by fluorescence microscopy are expected to be submicrometer-sized [75]. Oversized PLA spots usually occur from oversaturated primary antibody or insufficient washing. Occasionally, we observed PLA signal aggregation when the experiment was performed improperly (Fig. 3-3A). To count the PLA spots, we used Fiji to extract the grey scale image of the red channel and set the threshold of pixels with the MaxEntropy thresholding method which helps the deconvolution of the PLA signals [140], [144]. Based on the PLA methodology, the PLA spot size should be >1 μm in diameter from the 140 min RCA reaction [77]. In consideration of possible variations in PLA signal size, we used the particle analysis to filter out spot sizes below 0.5 μm and above 3 μm (Fig. 3-3A and 3-3B). After filtering the spots, we restored the PLA spot size to a normal distribution compared to a skewed spot size distribution before processing (Fig. 3-3C). Therefore, the PLA spot count is much less biased following these statistical corrections.
Fig. 3-1. Positive controls for smooth muscle tissue PLA. (A) Schematic illustration of PLA positive control. PLA red dots were visualized by confocal microscopy when two antigens (green and red dots) are in close distance (within 40nm). (B) to (D), Immunofluorescent double staining and PLA results. Antibodies were validated with immunofluorescence results for (B) cytoskeleton protein γ-actin (red) and contractile protein MLC20 (green), (C) cytoskeleton protein γ-actin (red) and membrane protein talin (green), and (D) the two cytosolic proteins RhoA (red) and MYPT1 (green). The PLA results of γ-actin vs LC20, γ-actin vs talin, RhoA vs MYPT1 indicate robust PLA signals from these three pairs. Cell nucleus was labeled with DAPI (blue).
**Fig. 3-2. Negative controls for smooth muscle tissue PLA.** (A). Immunofluorescent double labeling of talin (red) and Akt (green) for mouse gastric fundus smooth muscle cross section. Cell nucleus was labeled with DAPI (4’,6-diamidino-2-phenylidole) (blue). (B) to (E) talin vs Akt PLA and other PLA Technical controls show little to no detectable PLA spots. Each negative control is paired with a schematic graph to illustrate the (B) non-interacting proteins control, (C) no talin control, (D) IgG vs Akt control and (E) no primary antibody pair control. Images are representative of three independent experiments. Scale bars, 10 μm. Cell nucleus was labeled with DAPI (blue).
Fig. 3-3. Thresholding and particle analysis of PLA signals. (A) Left figure is the representative image of mouse fundus smooth muscle labeled by α8-integrin vs β1-integrin PLA (red) and DAPI (blue). The right figure is the red channel pulled out by Fiji. The relatively small dots were the PLA signal and larger ones are the noise. (B) The red channel is processed with thresholding by Fiji default parameters (left). Real PLA spots were circled with light blue and distinguished from the noise. Scale bars, 10 μm. (C) The PLA spots size distribution before filtering (left) and after filtering by particle analysis (right). 228 and 162 PLA spots counts were identified before and after filtering.
3.4.2 MLC-rb vs MLC-mo as internal control for PLA spot quantification

To make the PLA signals from protein-protein interactions or protein phosphorylation comparable between excited and non-excited (control) smooth muscles, we measured the contractile response from isometric contractions to exclude changes in cell density due to changes in muscle length, which is a confounding factor in smooth muscle tissue PLA quantification. Smooth muscle isometric contractions generates muscle tension without a change in length or morphology [145]. This ensures similar smooth muscle lengths between different groups under the same tension, whether stimulated or un-stimulated [146]. Because of the smooth muscle cell length-volume relationship, similar smooth muscle length means no significant difference in cell volume and cell density between stimulated and non-stimulated smooth muscles [136]. Therefore, the PLA signal measurements can be carried out by imaging the same volume (same area and z-depth) in the smooth muscle tissue section, as the cell density is equal among the groups. We measured smooth muscle cell areas by using cross-sections of the smooth muscle tissues and immunofluorescent staining of β1-integrin to label the plasma membrane. We found no significant difference in cell volumes for smooth muscles stimulated with EFS or CCh compared to the control groups in contractile experiments with isometric contraction (*unpublished observations*). We also performed PLA of LC20 using primary antibodies against LC20 from different species (rabbit for LC20-rb and mouse for LC20-mo) to quantify the PLA spots from smooth muscle myosin light chain detection, as an indirect measurement of smooth muscle cell density (Fig.
3-5A). Fundus smooth muscle treated with CCh and EFS showed no significant change in the PLA spot count compared to control (Fig. 3-5B, n=5). Therefore, the PLA spot count can be directly compared between different experimental groups in smooth muscle contractile experiments measuring isometric contraction with the same given tension.

Fig. 3-4. Indirect measurement of smooth muscle tissue volume change by PLA spots count of LC20-rb: LC20-mo. (A) Representative images of LC20-rb vs LC20-mo PLA under no treatment (Ctrl), 1μM Carbachol (CCh) in the presence of 0.3μM tetrodotoxin (TTX), and electric field stimulation (EFS) in the presence of 100μM L-NNA and 1μM MRS2500. (B) Average ratios ± SD of LC20-rb vs LC20-mo PLA spots count in Ctrl, CCh, and EFS. (C) Volume change of smooth muscle tissue in CCh and EFS compared to Ctrl.

3.4.3 Detection of LC20 and CPI-17 phosphorylation changes in response to cholinergic stimulation

To assess the efficacy of detecting protein phosphorylation by PLA in smooth muscle tissues, we examined pS19-LC20 and pT38-CPI-17 phosphorylation by cholinergic stimulation from CCh and EFS. Primary antibodies of different species were applied to detect phosphorylated LC20 and CPI-17 only (Fig. 3-5A). Fundus smooth muscle treated with CCh but not EFS showed a significant increase in S19 phosphorylation of 1.80 ± 0.29-fold compared to control (Fig. 3-5B, n=6).
CPI-17 T38 phosphorylation increased CCh 1.66 ± 0.09-fold and 1.32 ± 0.05-fold in response to CCh or EFS, respectively, compared to control. The change in LC20 and CPI-17 phosphorylation levels correlated with our previous western blot data [147].

**Fig. 3-5. Changes in LC20 and CPI-17 phosphorylation in response to physiological stimulation revealed by smooth muscle tissue PLA.** (A) Schematic illustration of protein phosphorylation analysis by phospho-site vs total protein PLA. Note that the primary antibodies to detect the phosphorylation site and the total protein must come from different species. (B and C) Representative PLA images and average ratios ± SD of (B) pS19 vs LC20 and (C) pT38 vs CPI-17 PLA under no treatment (Ctrl), 1μM Carbachol (CCh) in the presence of 0.3μM tetrodotoxin (TTX), and electric field stimulation (EFS) in the presence of 100μM L-NNA and 1μM MRS2500. ***P<0.001, ****P<0.0001, ns P>0.05.

### 3.4.4 Detection of kinase activity at MLC20

We also used tissue PLA to address the question whether a kinase is constitutively phosphorylating its target site as illustrated by the schematic graph (Fig. 3-6A). If a protein is phosphorylated by a kinase constitutively, a certain number of kinase molecules should always be associated with its target protein in stimulated or unstimulated tissue. Moreover, when the kinase is active, the association of the kinase with its target should be increased. We tested this
hypothesis with MLCK and its phosphorylation target pS19-LC20. The pS19 vs MLCK PLA showed increased PLA signals of 4.48 ± 0.20-fold in response to CCh stimulation, and no significant change from EFS compared to the control group. We detected a significant 2.81 ± 0.29-fold increase for CCh but not the EFS groups compared to controls in the LC20 vs MLCK PLA. These PLA results indicate that some MLCK is constitutively associated with LC20, and additional MLCK translocate to LC20 to phosphorylate LC20 in response to cholinergic stimulation by CCh but not EFS.

Fig. 3-6. Characterization of LC20: MLCK and CPI-17: MLC20 PLA. (A) Schematic illustration of phosphorylation site vs total protein PLA. Note that the phosphorylation site and total protein are from two different antigens. (B to E) Representative PLA images and average ratios ± SD of (B) pS19 vs MLCK, (C) LC20 vs MLCK, (D) pT38 vs LC20 and (E) CPI-17 vs LC20 PLA under no treatment (Ctrl), 1μM Carbachol (CCh) in the presence of 0.3μM tetrodotoxin (TTX), and electric field stimulation (EFS) in the presence of 100μM L-NNA and 1μM MRS2500. **P<0.01, ***P<0.001, ****P<0.0001, ns P>0.05.
3.4.5 Analysis of distance distribution and interaction potential between PLA spots and cellular structures.

To study the spatial relationship between the PLA spots and the cellular structure, structural proteins need to be labeled and distinguished from the PLA signals. We performed α8-integrin-mo vs β1-integrin-rb (mouse and rabbit primary antibody) PLA, then counterstained with β1-integrin-gt (goat primary antibody, shown as green the fluorescent signal) to label the cell membrane (Fig. 3-7A and 3-7B). Note that the β1-integrin-rb antibody recognizes the C-terminus of β1-integrin whereas the β1-integrin-gt antibody recognizes the N-terminus. Thus, the β1-integrin-rb antibody in PLA experiments will not interfere the β1-integrin-gt binding to the β1-integrin. The PLA signal was filtered as previously described. Both the PLA signal and the membrane labeling were isolated into separate images by Fiji (Fig. 3-7B). We hypothesize that The PLA signal should colocalize with the β1-integrin-gt labeling, as the β1-integrin-rb antibody binding to β1-integrin contribute to the PLA spots. We used MosaicIA for the interaction analysis because the software is based on Gibbs process which is a statistical model of the point pattern data analysis, suitable for PLA signals [138]. The Mask and combined images of the PLA signal membrane signals were generated from MosaicIA, and the distance distribution between the PLA spots and the membrane signals were predicted by the software (Fig. 3-7C). The strength of the interaction between the PLA spots and membrane signals was estimated by the Hernquist potential. The distance distribution was then fitted with Hernquist potential estimation parameters (Fig. 3-7D). The distance distribution curves indicate the distance between the PLA
signals and the α8-integrin signal is around 20 pixels (2.4 μm). The 1.233 interaction potential calculated by the Hernquist potential interaction analysis indicates there are true interactions between the PLA spots and α8-integrin signals (Fig. 3-7D).

Therefore, the interaction potential prediction is in accord with our hypothesis.
Fig. 3-7. Analysis of distance distribution between PLA spot and cell membrane by MosaicIA. (A) Schematic illustration of α8-integrin-mo vs β1-integrin-rb PLA (red sparks) with Alexa Fluor 488 dye labeling of β1-integrin-gt (green sparks). The Alexa secondary antibody will bind to the β1-integrin that is not binding with PLA secondary antibody. (B) The left figure shows the murine gastric fundus smooth muscle tissue section was labeled with α8-integrin-mo vs β1-integrin-rb PLA (red), β1-integrin-gt (green) and DAPI (blue). PLA signals were filtered (middle figure) and β1-integrin-gt labeling was split out (right figure) by Fiji. (C) Mask and the combined image of filtered PLA signal and β1-integrin-gt were generated by Mosaic Interaction Analysis (MosaicIA) plugin in Fiji. The estimated distance distribution was then generated by MosaicIA. The blue curve is the distribution of nearest neighbor distances (NND) generated by kernel density estimator with suggest Kernel wt(p)=1.398. The red curve is the state density distribution from the kernel density estimator with wt(q). Unit for distance is the pixel. (D) The left figure is the estimated Hernquist potential. The strength of interaction equal to zero means no interaction. The right figure is the distance distribution after fitting. The green curve is the fit p(d) with estimated parameters listed (strength, scale, and residues). The blue curve is the empirical distribution of NND. The red curve is the state density distribution.
3.5 Discussion

In these experiments, we optimized the tissue PLA in smooth muscle tissue sections to obtain quantifiable PLA data and a spatial annotation of the PLA signals. We validated a set of positive and negative controls for direct protein-protein interactions, co-complex interactions, and PLA technical controls. We demonstrated the steps to quantify PLA signal counts and eliminate the noise. We addressed the LC20 PLA labeling as smooth muscle PLA internal controls for the normalization of smooth muscle cell density. Based on the methodology we established, we found increased LC20 and CPI-17 phosphorylation in response to CCh and EFS treatment by pS19 vs LC20 PLA and pT38 vs CPI-17 PLA, respectively. The above data correlates with biochemical data from our previous studies [147]. Additionally, we performed tissue α8-integrin vs β1-integrin PLA with counter-staining of α8-integrin and analyzed the distance distribution and interaction potential between PLA signals and α8-integrin signals.

To validate smooth muscle tissue PLA quality, we recommend performing the following controls: γ-actin vs LC20, γ-actin vs talin, RhoA vs MYPT1 and talin vs Akt PLA for positive controls and negative controls. The immunofluorescence assay should always be performed in advance to ensure the primary antibody quality. For other tissue types, the listed positive controls may not be suitable because myosin expression may be low in other tissue or cell types [148]. Positive controls can be identified from published biochemical data and the IntAct protein-protein interaction database [141]. For the optimal PLA spot count, saturated PLA signal
should be avoided by using proper primary antibody concentrations. Primary antibody titrations may be applied to adjust the PLA signal density. Exact thresholding parameters should be adjusted according to the MaxEntropy algorithm to suit individual PLA images [140]. The 0.5µm to 3µm size filtering helps to filter out outliers/artifacts and make sure the PLA size distribution is close to a normal distribution as shown (Fig. 3-3C). PLA spot quantification with the Fiji steps (Fig. 3-3) removes most of the PLA noise and avoids the traditional time consuming and prone-to-bias quantification process by manual quantification and/or ordinal scoring system quantification [149], [150]. Note that, in order to generate quantifiable PLA data, the primary antibody concentration needs to be optimized to avoid PLA signal saturation. In addition, similar to the notion of using LC20 as the internal control for traditional western blot quantification, we demonstrated that LC20 PLA labeling by LC20-rb vs LC20-mo could serve as an indicator for the relative level of cell density among control and experimental groups in smooth muscle isometric contractile experiments (Fig. 3-4).

With quantifiable PLA data based on the set of controls, noise filtering, automatic quantification and smooth muscle density normalization, we are able to use smooth muscle tissue PLA as a powerful tool to detect changes in protein phosphorylation, protein-protein interactions, and protein complex composition in different physiological and pathophysiological states. We detected changes in protein phosphorylation for CPI-17 and LC20 and demonstrated the relocation of MLCK to LC20 for myosin light chain phosphorylation under cholinergic
stimulation (Fig. 3-5 and Fig. 3-6). The results bolster our previous observations in smooth muscles by biochemical experiments [147], [151]. Therefore, the protein-protein direct interaction or co-complex interaction analysis by PLA could be expanded to help answer more specific physiological questions, and for cross-validation with other biochemical experiments.

To take advantage of confocal microscopy and retrieve more information out of the smooth muscle tissue section PLA, we counterstained the cell structure by immunolabeling and analyzed the spatial relationship between the PLA spots and the cell structure. Few experiments had been performed with antibody counterstaining using tissue-level PLA [152] and no reports have addressed the spatial relationship between PLA spots and the cell structure. We successfully acquired images with α8-integrin-mo vs β1-integrin-rb PLA spots and counterstaining of β1-integrin-gt (Fig. 3-7A). The spatial relationship between sub-cellular structures and PLA spots is important because it reflects compartmentalized physiological events [153] and changes in the micro-environment [149]. We found the MosaicIA (Fiji plugin) software pipeline to be well suited for the analysis of the spatial relationships between the PLA dot signals and the cellular context. The MosaicIA algorithm detects the spatial distance and interaction potential by spatial point pattern analysis taking into account the object pattern and structure, such as the point-like object of PLA signals [154]. Additionally, similar to heart or skeleton muscles, smooth muscle cells are spindle-shaped and stack together in the tissue with possible cell structural change in response to physiological stimulation. To minimize these confounding factors for PLA spot vs membrane structure distance analysis, we prepared cross-sections of circular smooth muscles for imaging. With all these considerations,
we were able to yield meaningful spatial annotations for the PLA spots. The cellular structure labeling is also very flexible that can be applied to any marker protein with a tested primary antibody.

In summary, we overcame several technical hurdles associated with smooth muscle tissue PLA. The smooth muscle tissue PLA quality control and quantification procedures cannot only be directly applied for use in smooth muscle tissues, but also applied to other tissue types with similar methodology to identify suitable positive and negative controls, and internal control for counts normalization. The spatial annotation method also broadens the scope of proximity ligation assays to unveil more knowledge of subcellular compartmentalization and microenvironments, which is critical for physiological cell signaling studies as well as identification of biomarker discovery [149]. We anticipate that smooth muscle tissue PLA will complement current experimental and computational approaches to enable quantifiable and in-depth smooth muscle tissue-level signaling transduction studies.
CHAPTER 4: Focal adhesion kinase regulation of association between β1-integrin and Ca\textsuperscript{2+} sensitization proteins during the contractile response of gastric fundus smooth muscles to cholinergic stimuli.

4.1 Abstract

Contraction and relaxation of smooth muscle involves the regulation of myosin light chain (LC20) phosphorylation by MLCK and MLCP. ROCK2 and MYPT1 are crucial for gastrointestinal smooth muscle contractile regulation via their inhibition of MLCP activity. β1-integrin is a trans-membrane receptor that forms focal contacts and mediates the attachment of smooth muscle cells to the extracellular matrix. Focal adhesion kinase (FAK) regulates the recruitment and assembly of several proteins to focal adhesion. Therefore, β1-integrin and FAK play important roles in cell adhesion, tension generation, and mechanotransduction in smooth muscle cells.

Here we use proximity ligation assays (PLA) to examine the role of FAK in regulating the association of Ca\textsuperscript{2+} sensitization proteins with β1-integrin in response to cholinergic stimulation of gastric fundus smooth muscle. Muscle contraction with neurally released acetylcholine (ACh) by atropine-sensitive electrical field stimulation (EFS) significantly increased the interaction of RhoA/ROCK2/MYPT1, PKC/CPI-17 and S19 phosphorylated LC20 (pS19) with β1-integrin. Furthermore, the FAK inhibitor PF-431396 inhibited the EFS-stimulated increase in p-FAK and FAK association with β1-integrin. PF-431396 also blocked the EFS-induced increase in ROCK2, RhoA, PKC, CPI-17, and pS19 association with β1-integrin. These results indicate that FAK regulates the interaction of Ca\textsuperscript{2+} sensitization and
contractile proteins with β1-integrin in focal contacts in response to cholinergic stimuli, suggesting additional potentially important roles of FAK and β1-integrin in contributing to the contractile responses of fundus smooth muscles to cholinergic stimuli.

4.2 Introduction

In gastrointestinal (GI) smooth muscles, smooth muscle cells are electrically coupled to interstitial cells of Cajal (ICCs) and PDGFRα⁺ cells, forming a contractile unit called the SIP synectium that is involved in motor neurotransmission [14]. Cholinergic neurotransmission in gastric fundus smooth muscles involves a series of synaptic-like signals from enteric motor neurons to the smooth muscle cells mediated by ICCs [12]. Gastric fundus smooth muscle contraction is initiated by an increase in the phosphorylation of LC20 at Ser19 (pS19). Ca²⁺ dependent LC20 phosphorylation is regulated Ca²⁺-calmodulin activation of myosin light chain kinase (MLCK) [32]. Myosin light chain phosphatase (MLCP) dephosphorylates LC20 at pS19, resulting in smooth muscle relaxation [96]. Inhibition of MLCP occurs through two parallel pathways; phosphorylation of phosphatase inhibitor protein of 17 kDa (CPI-17) by protein kinase C (PKC), and MYPT1 phosphorylation by Rho kinase (ROCK), which are key mechanisms regulating pS19 phosphorylation levels [97]. A given increase in Ca²⁺ can yield more pS19 phosphorylation by decreasing MLCP activity via CPI-17 and MYPT1 phosphorylation, and is known as “Ca²⁺ sensitization” [21], [32]. Ca²⁺ sensitization is typically induced by bath-applied agonists or neural release of neurotransmitter [18].
Besides modulating LC20 phosphorylation, emerging evidence indicates smooth muscle contraction may be regulated by cell-to-cell adhesion formation. Integrin proteins, the primary extracellular matrix (ECM) receptors in the focal adhesions, were suggested to transduce environmental signals to activate contractile proteins and rearrange the cytoskeleton proteins [67], [155], [156]. Instead of directly binding to actin filaments, β integrins link to talin, α-actinin, and filamin, which all also bind to actin filaments [157]. Focal adhesion formation is typically accomplished by FAK activation to recruit integrin-associated proteins and assemble the adhesion sites [93], [101], [102]. The regulatory role of FAK in smooth muscle contraction regulation has previously been studied in various smooth muscle tissues including airway smooth muscle, vascular smooth muscle and prostate smooth muscle [60], [65], [68], [103], [156]. In our previous findings detailed in this dissertation, murine gastric fundus smooth muscle contractility under cholinergic stimulation can be regulated by the inhibition FAK phosphorylation at pY397.

4.3 Materials and Methods

4.3.1 Tissue preparation

Mice were anesthetized by isoflurane inhalation and then killed by decapitation. Stomachs were isolated, pinned to a Sylgard-lined dish and immersed in 4°C oxygenated (97%O2/3%CO2) Krebs solution (NaCl, 120.35 mmol/L; KCl, 5.9 mmol/L; NaHCO3, 15.5 mmol/L; NaH2PO4, 1.2 mmol/L; MgCl2, 1.2 mmol/L; CaCl2, 2.5 mmol/L; and glucose, 11.5 mmol/L). The gastric fundus was acquired
and mucosa and submucosa removed by sharp dissection [139]. Smooth muscle strips were pre-equilibrated in 37°C oxygenated Krebs for 45 min prior to use.

4.3.2 Isometric force measurements, immunofluorescence and PLA

The contractile activity measured by static myobath was performed as described in Chapter 2 and Chapter 3. The mechanical responses to neutrally released acetylcholine were measured from muscle strips incubated with 100 μM L-Nω-nitro-L-arginine (LNNA) and 1μM MRS2500 for 20 min prior to delivery of electrical field stimulation (EFS) with square wave pulses, 0.3 msec duration, 5-20Hz, 150V and 30 sec durations (supra-maximal voltage; Grass S48 stimulator). EFS treated in the presence or absence of 0.3 μM PF-431396 tissue was collected at 5sec after stimulation, respectively. The myobath chamber was quickly dropped down and tissue was rapidly immersed into room temperature 4% (w/v) paraformaldehyde (PFA). Then the tissue PLA assays were performed as described in Chapter 3.

4.3.3 Differential centrifuge and western blotting

Muscles were washed for 1 min in ice cold-lysis buffer (mM; 50 Tris-HCl pH 8.0, 60 β-glycerophosphate, 100 NaF, 5 EGTA, 25 Na-pyrophosphate, 1 DTT, with protease inhibitor tablet (Roche, Indianapolis, IA, USA)). Each muscle tissue was homogenized in 0.3 mL lysis buffer in a Bullet Blender (0.01% anti-foam C, 1 stainless steel bead per tube, speed 6, 5 min), and then centrifuged at 1000 x g, 10 min, 4°C. The supernatants from the low-speed centrifugation were taken
centrifuged 16000 x g speed, 10 min, 4°C. The supernatant of this medium speed centrifugation was subjected to 100,000 x g high-speed centrifugation for 1 hour at 4°C to separate cytosolic and membrane proteins. Protein concentrations of the final supernatants and pellets were determined by the Bradford assay. Supernatants and pellets were stored at -80°C.

The supernatants and pellets were analyzed by SDS-PAGE and western blotting with anti-γ-actin, MYPT1, CPI-17, ROCK2, MLC, RhoA antibodies, and phosphorylation levels were determined by Western Blot analyses using anti-pT696-MYPT1, pT853-MYPT1, pT38-CPI-17, pS19-LC20 antibodies (Santa Cruz Biotechnologies, CA, USA). Protein bands were detected using horseradish peroxidase-conjugated secondary antibodies (Millipore-Chemicon, Billerica, MA, USA) and ECL Advantage (GE HealthCare Biosciences, Piscataway, NJ, USA), and visualized with a CCD camera-based detection system equipped with Visionworks software (Epi Chem II, UVP Laboratory Products, Upland, CA, USA). The tiff images were inverted and adjusted to auto levels and resolution with Adobe Photoshop (CS2, V9.0.2, Adobe Systems, San Jose, CA, USA) for densitometry [33].

4.3.4 Statistical analysis

Fiji was used to count PLA spots. The red channel was subtracted from the raw tiff images. The threshold was set using the MaxEntrophy algorithm [140]. Then the PLA spots were filtered and counted by particle analysis with the particle size...
set from 0.5 to 3 μm. The PLA spots counts were analyzed by non-parametric repeated tests of ANOVA using Prism 6.01 software, and are demonstrated as the average ± SD. The $T$-test was used to test the significance and $P < 0.05$ is considered significant. The interaction analysis was performed by MosaicIA [138].

4.3.5 Drugs

Atropine was obtained from EMD Millipore, Billerica, MA; L-NNA and PF-431396 were obtained from Sigma-Aldrich, St. Louis, MO; MRS2500 was purchased from Toeris Bioscience, Minneapolis, MN.

4.4 Results

4.4.1 Distribution of $\text{Ca}^{2+}$ sensitization proteins and contractile proteins.

$\text{Ca}^{2+}$ sensitization proteins have been reported to be important for smooth muscle contraction regulation [32]. To determine the distribution of each $\text{Ca}^{2+}$ sensitization protein, we investigated the distribution of $\text{Ca}^{2+}$ sensitization proteins in the cytosolic protein fraction and membrane protein fraction of murine gastric fundus smooth muscles. After the final high-speed (100,000 x g) centrifugation, membrane proteins were separated from the cytosolic proteins into the pellet, and cytosolic proteins were separated from the membrane proteins into the supernatant. (Fig. 4-1A). To confirm that we successfully separated cytosolic proteins and membrane proteins into the supernatant and pellet fractions, respectively, we investigated the protein expression of the cytosolic marker GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and the plasma membrane protein
marker β1-integrin in both the supernatant and pellet fractions. The 36 kDa GAPDH and 140 kDa β1-integrin were detected uniquely expressed in the supernatant and pellet fractions, respectively (Fig. 4-1B). We then used this method to detect the distribution of Ca\(^{2+}\) sensitization proteins in the cytosolic and membrane fractions. The protein bands for MYPT1, pT853-MYPT1 (pT853), pT696-MYPT1 (pT696), ROCK2, LC20, pS19-LC20, γ-actin were present in both fractions. The protein band for RhoA is barely detectable in the cytosol fraction, while a robust signal is present in the membrane protein fraction. The CPI-17 and pT38-CPI-17 protein bands are almost exclusively present in the cytosolic fraction and barely detectable in the membrane-associated protein fraction (Fig. 4-1C). Gastric fundus smooth muscle tissues were collected in the resting state for the differential centrifugation analysis. The protein distribution results showed the presence of Ca\(^{2+}\) sensitization proteins in the membrane-associated protein fraction of resting gastric fundus smooth muscle. This suggested the potential association between Ca\(^{2+}\) sensitization proteins and membrane proteins in smooth muscle tissue.
Fig. 4-1. Cytosolic protein separated from membrane-associated protein in gastric fundus smooth muscle by differential centrifugation. (A) schematic illustration of differential centrifugation steps to separate cytosolic protein from membrane-associated proteins. (B) Left: representative Western Blots of GAPDH and β1-integrin. GAPDH was detected in the supernatant (s) and β1-integrin detected in the pellet (p) fraction of the final centrifugation product. (C) Representative Western blots of MYPT1, pT853, pT696, ROCK2, MLC, pS19, γ-actin, CPI-17, pT38 and RhoA in the supernatant (s) or pellet (p) fraction of the differential centrifugation product.

4.4.2 PLA spot normalization by myosin.

As demonstrated in Chapter3, the LC20-rb vs LC20-mo PLA can be used to indirectly measure the smooth muscle cell density. We performed the LC20-rb vs
LC20-mo PLA in gastric fundus smooth muscles without stimulation (Ctrl) or stimulated with EFS with and without the presence of PF-431396 (Fig. 4-2A). Fundus smooth muscle treated with EFS in the presence or absence of PF-431396 showed no significant change in the PLA spot count compared to control (Fig. 4-2B, n=5). Therefore, the PLA spot count of Ctrl, EFS, and EFS with PF-431396 groups can be directly compared to each other under isometric contraction.

Fig. 4-2. Indirect measurement of gastric fundus smooth muscle tissue volume change. (A) Representative images of LC20-rb vs LC20-mo PLA under no treatment (ctrl), EFS and EFS with pre-incubation of 0.3μM PF-431396 for 10 min (PF + EFS), all in the presence of 100μM L-NNA and 1μM MRS2500. (B) Average ratios ± SD of LC20-rb vs LC20-mo PLA spots count in Ctrl, EFS, and EFS with PF431396. ns P>0.05, compared to Control.

4.4.3 Effects of cholinergic stimulation and FAK inhibitor PF-431396 on β-1 integrin vs FAK association.

We used PLA to address the association between FAK and β-1 integrin in murine gastric fundus smooth muscle tissue under cholinergic stimulation in the presence or absence of PF-431396. The association of p-FAK and β1-integrin in gastric fundus smooth muscle is significantly increased by 1.60 ± 0.28-fold under cholinergic stimulation by EFS. Little to no detectable PLA signal was found in EFS stimulated smooth muscle with pretreatment of 0.3 μM PF-431396 (Fig. 4-3A, n=6).
The association of p-FAK and β-1 integrin showed a similar pattern to the western blot data we previously reported that FAK phosphorylation is increased by EFS and inhibited by the FAK inhibitor PF-431396 (under Review). The FAK vs β1-integrin PLA signal was increased by 2.15 ± 0.29-fold under EFS compared to control, while PLA spot count under the EFS in the presence of PF-431396 was not significantly different from the control group (Fig. 4-3B, n=6), indicating the relocation of FAK to β1-integrin when FAK pY397 is activated by EFS.

Fig. 4-3. Changed p-FAK and FAK association with β1-integrin in response to cholinergic stimulation and PF431396 inhibition in gastric fundus smooth muscle. (A) and (B) Representative images of (A) β1-integrin vs p-FAK and (B) β1-integrin vs FAK PLA under no treatment (Ctrl), EFS and EFS with pre-incubation of 0.3μM PF-431396 for 10 min (PF + EFS). Average ratios ± SD of (A) β1-integrin vs p-FAK and (B) β1-integrin vs FAK PLA spots count in Ctrl, EFS, and EFS with PF431396. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, ns P>0.05, compared to Ctrl.
4.4.4 Effects of cholinergic stimulation and FAK inhibitor PF-431396 on β1-integrin association with RhoA/ROCK2/MYPT1.

RhoA has been reported to regulate smooth muscle contraction by anchoring cytoskeleton proteins to focal adhesion sites [158]. The Ca^{2+} sensitization signaling between RhoA, ROCK2, and MYPT1 has been identified in smooth muscle tissues [32], [159]. To examine the effect of FAK phosphorylation on the association of β1-integrin with RhoA, ROCK2, and MYPT1, we performed PLA for β1-integrin vs RhoA, ROCK2 and MYPT1. β1-integrin vs RhoA and ROCK2 PLA spots count were significantly increased by 1.93 ± 0.40-fold and 4.59 ± 0.69-fold, respectively, under EFS and inhibited by 0.3 μM PF-431396 (Fig. 4-4A and 4-4B, n=6). The β1-integrin vs MYPT1 PLA signal showed no significant change under EFS with or without PF-431396 (Fig. 4-4C, n=6). Thus, RhoA, and ROCK2 relocate to β1-integrin under EFS. RhoA/ROCK2 redistribution to β1-integrin under EFS is affected by FAK pY397 phosphorylation, while MYPT1 is not. The above evidence indicates the RhoA/ROCK2/MYPT1 Ca^{2+} sensitization pathway might not be involved in FAK regulation of cholinergic stimulation induced smooth muscle contraction.
Fig. 4–4. Changed MYPT1, ROCK2 and RhoA association with β1-integrin in response to cholinergic stimulation and PF431396 inhibition in gastric fundus smooth muscle. (A) and (B) Representative images of (A) β1-integrin vs MYPT1, (B) β1-integrin vs ROCK2 and (C) β1-integrin vs RhoA PLA under no treatment (Ctrl), EFS and EFS with pre-incubation of 0.3μM PF-431396 for 10 min (PF + EFS). Average ratios ± SD of (A) β1-integrin vs MYPT1, (B) β1-integrin vs ROCK2 and (C) β1-integrin vs RhoA PLA spots count in Ctrl, EFS, and EFS with PF431396. * P<0.05, ** P<0.01, **** P<0.0001, ns P>0.05, compared to Ctrl.
4.4.5 Effects of cholinergic stimulation and FAK inhibitor PF-431396 on β1-integrin association with PKC/CPI-17.

We also assessed the relationship between β1-integrin and PKC/CPI-17 pathways proteins by PLA in murine gastric fundus smooth muscles. Active PKC is reported to accumulate near β1-integrin in smooth muscle [160]. CPI-17 is phosphorylated by PKC activation in response to cholinergic stimulation in smooth muscle tissue [18], [161]. We performed PKC and CPI-17 vs β1-integrin PLA under control, EFS and EFS in the presence of PF-431396 (Fig. 4-5). EFS-induced the increased PLA signal for β1-integrin vs PKC and β1-integrin vs CPI-17 PLA by 3.82 ± 0.61-fold and 2.41 ± 0.48-fold, respectively. In the presence of PF-431396, the β1-integrin vs PKC PLA signal is significantly lower by 1.83 ± 0.67-fold than EFS alone (Fig. 4-5A). The β1-integrin vs CPI-17 PLA with PF-431396 inhibited EFS-induced increase of PLA signals completely (Fig. 4-5B). Therefore, FAK phosphorylation regulates PKC/CPI-17 association with β1-integrin under EFS-induced cholinergic stimulation.
Fig. 4-5. Changed PKC and CPI-17 association with β1-integrin in response to cholinergic stimulation and PF431396 inhibition in gastric fundus smooth muscle. (A) and (B) Representative images of (A) β1-integrin vs PKC and (B) β1-integrin vs CPI-17 PLA under no treatment (Ctrl), EFS and EFS with pre-incubation of 0.3μM PF-431396 for 10 min (PF + EFS). Average ratios ± SD of (A) β1-integrin vs PKC and (B) β1-integrin vs CPI-17 PLA spots count in Ctrl, EFS, and EFS with PF431396. ** P<0.01, *** P<0.001, ns P>0.05 compared to Ctrl.

4.4.6 Effects of cholinergic stimulation and FAK inhibitor PF-431396 on β1-integrin association with LC20.

LC20 phosphorylation is downstream of MYPT1 and/or CPI-17 activation in response to cholinergic stimulation [18]. We analyzed LC20 and pS19 association with β1-integrin by PLA (Fig. 4-6). pS19 vs β1-integrin PLA signal was significantly increased under EFS by 2.43 ± 0.57-fold and the increase was completely inhibited in the presence of PF-431396 (Fig. 4-6A, n=6). LC20 vs β1-integrin PLA spot count showed no significant difference under EFS in the presence or absence of PF431396 (Fig. 4-6B, n=6). Therefore, LC20 phosphorylation in β1-integrin region is sensitive to FAK phosphorylation inhibition.
Fig. 4-6. Changed PKC and CPI-17 association with β1-integrin in response to cholinergic stimulation and PF431396 inhibition in gastric fundus smooth muscle. (A) and (B) Representative images of (A) β1-integrin vs PKC and (B) β1-integrin vs CPI-17 PLA under no treatment (Ctrl), EFS and EFS with pre-incubation of 0.3μM PF-431396 for 10 min (PF + EFS). Average ratios ± SD of (A) β1-integrin vs PKC and (B) β1-integrin vs CPI-17 PLA spots count in Ctrl, EFS, and EFS with PF431396. * P<0.05, ** P<0.01, ns P>0.05, compared to Ctrl.

4.5 Discussion

In smooth muscle, integrins transduce chemical and mechanical signals from the cell exterior, across the plasma membrane to intracellular pathways, through proteins assembled at the focal adhesion site [45], [156]. FAK regulates focal adhesion assembly and sensitive to acetylcholine activation of smooth muscle [45]. FAK has been reported to bind to GTPase regulators, GTPase-activating factor (GRAF), suggesting possible cross-talk between FAK and Rho GTPases [162]. Ca\(^{2+}\) sensitization regulation was suggested to be regulated by FAK via multiple Ca\(^{2+}\) influx pathways in different smooth muscle tissues [65], [163]. Our previous data suggest that FAK inhibition in gastric fundus smooth muscle is not inhibiting voltage-gated Ca\(^{2+}\) channels activity induced by EFS. In this study, we address Ca\(^{2+}\)
sensitization protein recruitment to β1-integrin under EFS-induced cholinergic stimulation with or without FAK pY397 phosphorylation inhibition. β1-integrin association with FAK, RhoA/ROCK2/MYPT1, PKC/CPI-17 were increased under EFS. The association of pS19 with β1-integrin is increased while LC20 vs β1-integrin PLA signal showed no change under EFS. RhoA, ROCK2, PKC, CPI-17 and pS19 association with β1-integrin is inhibited by FAK inhibitor PF431396 under EFS. These findings suggest that FAK activation contributes to the attachment of PKC/CPI-17 pathway proteins to focal adhesion site under cholinergic stimulation in murine gastric fundus smooth muscle. The presence of MYPT1 and CPI17 at the focal adhesion site has never been documented in adhesome analysis of any cell type [46]. Together with the recruitment of RhoA/ROCK2 and PKC under EFS in response to FAK phosphorylation, our data suggests Ca\(^{2+}\) sensitization proteins may actively regulate the actin polymerization at the smooth muscle focal adhesion site through the activation of pS19-LC20 phosphorylation.

The Western Blot analysis of total protein and phosphorylation after differential centrifugation demonstrated the co-existence of membrane proteins and most of the Ca\(^{2+}\) sensitization proteins in the membrane fraction from relaxed murine gastric fundus smooth muscle. We found phosphorylation of LC20 at pS19 and MYPT1 at pT853 and pT696 in both the supernatant and pellet fractions. CPI-17 phosphorylation at pT38 was detected only in the supernatant fractions (Fig 4-1C). Thus, differential centrifugation provided initial evidence for the potential correlation between Ca\(^{2+}\) sensitization proteins and membrane proteins. In gastric
fundus smooth muscle, focal adhesions are abundant in the plasma membrane. Therefore, the presence of Ca\(^{2+}\) sensitization proteins in the membrane protein fraction suggests a possible role of Ca\(^{2+}\) sensitization proteins for regulating tension at smooth muscle focal adhesions.

In our previous study, we used western blot and detected significantly increased overall FAK phosphorylation at pY397 under EFS-induced cholinergic stimulation, which was inhibited by the FAK inhibitor PF-431396 in gastric fundus smooth muscle. In our tissue-PLA data, we found significantly increased FAK pY397 phosphorylation at β1-integrin and the pY397 phosphorylation was completely blocked by PF-431396 (Fig 4-3A). We also found the β1-integrin association with FAK, RhoA/ROCK2/MYPT1, and PKC/CPI-17 were significantly increased under EFS (Fig 4-3B, Fig. 4-4 and Fig. 4-5). This suggested the redistribution of FAK and Ca\(^{2+}\) sensitization proteins to β1-integrin under EFS to form a protein complex. Moreover, FAK and Ca\(^{2+}\) sensitization proteins (excluding MYPT1) association with β1-integrin were inhibited by the FAK inhibitor under EFS (Fig. 4-3B, Fig. 4-4 and Fig. 4-5). The above data suggested FAK assembles Ca\(^{2+}\) sensitization proteins when active and the assembly process could be reversed by FAK pY397 phosphorylation inhibition. Interestingly, MYPT1 did not relocate away from β1-integrin when pY397 was inhibited under EFS. Still, a reduced association of ROCK2 with β1-integrin under EFS in the presence of PF-431396 would possibly lead to less activated MYPT1 at the β1-integrin associated protein complex. Thus, tension generation at focal adhesions appears to involve recruitment
of Ca\textsuperscript{2+} sensitization proteins to integrins. Additional PLA experiments of pT853-MYPT1 and pT38-CPI-17 association to the β1-integrin are required to further characterize the Ca\textsuperscript{2+} sensitization protein phosphorylation regulated by FAK activation/inhibition at the focal adhesion site under EFS-induced smooth muscle contraction.
CHAPTER 5: Summary, Limitations, Future directions, and Conclusions

5.1 Summary

The three aims of this dissertation are as follows: (1) To determine the role of FAK phosphorylation in the regulation of gastric fundus smooth muscle contraction in response to cholinergic stimulation. (2) To demonstrate a quantitative tissue proximity ligation assay and its spatial analysis applications in intact smooth muscle tissues and (3) To characterize FAK regulation of Ca\textsuperscript{2+} sensitization proteins association with β1 integrin under cholinergic stimulation in gastric fundus smooth muscle. To achieve the above aims, we first measured FAK Y397 autophosphorylation in the basal state and showed that increased in response to EFS of cholinergic motor neurons. We found that basal and EFS-stimulated FAK Y397 phosphorylation was sensitive to the FAK inhibitor PF-431396 and identified 0.3μM as the suitable dose for around 70% Y397 phosphorylation inhibition without off-target effects on smooth muscle contraction. We found that FAK, but not Pyk2, regulates smooth muscle contraction in murine gastric fundus. We analyzed the phosphorylation of MYPT1 at pT853 and pT696, CPI-17 at pT38 and LC20 at pS19 under conditions, during EFS of cholinergic motor neurons, and EFS in the presence of PF-431396. We found that PF-431396 inhibited pT853, pT696, pT38 and pS19 phosphorylation in response to EFS. For the second aim, we found that the change in LC20 PLA signal is proportional to the change in smooth muscle cell density (cells per image area) between unstimulated smooth muscles and smooth muscles under agonist or EFS-evoked contractions. We developed a novel normalization and PLA
image interpretation method for smooth muscle tissue PLA. In the third aim, we applied the smooth muscle tissue PLA technique to reveal Ca\(^{2+}\) sensitization protein association with β1 integrin under EFS evoked cholinergic transmission with or without the FAK inhibitor PF-431396. We found the association of β1 integrin with Ca\(^{2+}\) sensitization proteins are regulated by FAK phosphorylation at pY397. These findings extend our studies of FAK in gastric fundus smooth muscle Ca\(^{2+}\) sensitization regulation.

FAK is known to regulate cell motility through interactions with GTPases from \textit{in vitro} non-smooth muscle cell studies [61]. Several studies in different smooth muscle tissues show tyrosine kinase regulation of smooth muscle contraction by various mechanisms [102], [103], [164]. No study has addressed FAK regulation of gastric fundus smooth muscle tissue contraction. We showed FAK inhibited the EFS-induced increase in pT38 and pS19 phosphorylation. FAK also reduced pT853 and pT696 phosphorylation in response to EFS-evoked contractions. My results indicate that FAK phosphorylation inhibition blocks Ca\(^{2+}\) sensitization protein activation in response to cholinergic stimulation. This suggests that FAK is involved in the transmission of extracellular cholinergic stimulation to the intracellular Ca\(^{2+}\) sensitization proteins. Moreover, we found atropine inhibited the EFS-induced increase in pY397, indicating FAK Y397 phosphorylation is downstream of ICC activation. Additionally, increased pY397 was detected in high K\(^+\)-contracted smooth muscle and was inhibited by PF-431396. These findings provide evidence that FAK phosphorylation in smooth
muscle cells of gastric fundus smooth muscle tissues is responsible for the smooth muscle contraction regulation since high K$^+$ selectively stimulates smooth muscle cells through membrane depolarization and activation of voltage-dependent Ca$^{2+}$ channels. These results demonstrate the important role of FAK in gastric fundus smooth muscle contraction regulation under cholinergic stimulation.

For the first aim, we used western blot to detect global phosphorylation changes in FAK, Ca$^{2+}$ sensitization proteins, and LC20 in murine gastric fundus smooth muscles. In the second aim, in order to study the localized activation of FAK at focal adhesion sites, we introduced the use of the in situ proximity ligation assay for gastric fundus smooth muscles. The use of in situ proximity ligation assay to study smooth muscle protein-protein association and protein phosphorylation was previously limited to qualitative analysis due to the difficulty of normalization to correct for changes in target location due to changes in cell shape during muscle contraction, and insufficient positive and negative controls [136]. The altered cell volume and cell density of contracted smooth muscles makes it difficult to normalize the confocal images of smooth muscle tissue sections by the counts of nucleus or cell numbers. We identified talin vs γ-actin, γ-actin vs LC20 for direct interaction positive controls, RhoA vs MYPT1 for the co-complex indirect interaction positive control, and Akt vs talin for the non-association negative control. More importantly, we demonstrated LC20 PLA signal as the internal control for PLA signal normalization in consideration of the smooth muscle cell density change during muscle contraction under different physiological stimulation protocols. For accurate quantification, we showed a set
of standard quantification methodologies using Fiji, which overcame the
time-consuming and error-prone manual quantification. Additionally, we
performed counterstaining of the membrane protein after PLA and analyzed the
spatial relationship between PLA signals and the membrane protein signals. We
obtained the spatial distance and interaction potential between the two signals
using the MosaicIA plugin in Fiji. Thus, all these novel smooth muscle tissue PLA
applications bring us much wider PLA applications that can be individualized for
smooth muscle physiology studies. It also enabled my study of localized FAK
function at the focal adhesion site as demonstrated in aim three.

The smooth muscle tissue PLA methodology was applied to the study of
FAK regulation of Ca^{2+} sensitization proteins association with focal adhesion
protein complex due to cholinergic stimulation. Integrins are the mechanical
links between the extracellular matrix (ECM) and cytoskeletal proteins that form
the focal adhesion site [165]. β1 integrin is an essential integrin family member
that pairs with 10 different integrin α subunits in smooth muscles [166]. Thus, we
selected β1 integrin as the marker for focal adhesions. We detected and
increased association of Ca^{2+} sensitization proteins with β1 integrin under
cholinergic stimulation. The increased association of RhoA/ROCK2,
CPI-17/PKC, and pS19 to β1 integrin was blocked by the FAK inhibitor
PF-431396. Therefore, we demonstrated for the first time that the FAK
activation regulates Ca^{2+} sensitization proteins assembly to focal adhesion site.
5.2 Limitations

In the first aim, we characterized the role of FAK in gastric fundus smooth muscle regulation under cholinergic stimulation. The possible limitations of this chapter are as follows. (1) We detected increased FAK pY397 phosphorylation under EFS-induced cholinergic stimulation in smooth muscle tissues. However, FAK is likely expressed in enteric neurons, interstitial cells of Cajal and other cell types in smooth muscle tissues. We could not directly measure FAK Y397 phosphorylation in smooth muscle cells of the muscle tissue. We did high extracellular K+ treatment to selectively activate smooth muscle contraction by voltage-gated Ca2+ channel activation on the smooth muscle cells and showed increased phosphorylation of FAK pY397. This is in contrast to EFS-induced cholinergic stimulation, which activates smooth muscle through excitatory junctional potentials transmitted from ICC-IM to the smooth muscle cells. It would be more informative if we could identify or localize FAK phosphorylation in the smooth muscle cells of the muscle strip. (2) The dose response test for PF-431396 in resting gastric fundus smooth muscles showed that 0.3μM PF-431396 could achieve around 70% inhibition of FAK Y397 phosphorylation while not decreasing basal tone. The Ca2+ imaging data showed that PF-431396 has no effect on the EFS-stimulated Ca2+ influx. Thus PF-431396 is not inhibiting L-type Ca2+ channel activities. But we cannot rule out other, unknown off-target effects of 0.3μM PF-431396 treatment. This study would be further strengthened if we can determine the exact specificity of PF-431396 for FAK inhibition in murine gastric fundus smooth muscles. (3) FAK is recruited to focal adhesions to regulate the signaling between integrins and intracellular proteins [60].
Therefore, analysis of localized phosphorylation is important for future FAK studies. The Western Blot experiments to analyze phosphorylation changes were measuring the global changes in protein phosphorylation, which may not reflect the localized or compartmentalized changes of phosphorylation.

In the second aim, we introduced novel experimental design and analysis methodologies to utilize the proximity ligation assay for smooth muscle tissues. The possible limitations in this chapter are as follows. (1) Based on current knowledge and the molecular interaction database IntAct [141], We identified talin vs γ-actin, γ-actin vs LC20 and γ-actin vs β1 integrin for the tissue PLA positive controls and Akt vs talin for the negative PLA control. We validated these controls for murine gastric fundus smooth muscles. However, these controls might yield different results in different tissues, especially non-smooth muscle tissues. Thus, we suggest do literature research and biochemical assays for the tissue of interest when setting up tissue-PLA in other types of smooth muscles or non-smooth muscle tissues. The positive and negative controls would be valid only when correlated with existing biochemical data from other experiments. (2) Unlike co-immunoprecipitation, Fluorescence Resonance Energy Transfer (FRET) or other techniques, the proximity ligation assay reports a positive signal when the two targets of interest are in a close distance which is less than 40 nm to each other. Therefore, not only proteins that directly interact with each other are counted, but also proteins that are in a multi-protein complex are reported as a positive signal in PLA assays. PLA does not tell the difference between the direct protein-protein interaction and indirect
protein-protein association, without additional experiments such as selective removal of individual proteins from the complex.

In aim three, we demonstrated the FAK regulation of Ca\(^{2+}\) sensitization proteins association with focal adhesions under EFS-induced cholinergic stimulation. The limitations are as follows: (1) Integrins are localized at the dense plaques of smooth muscles (structurally equal to focal adhesion sites of cultured cells) [45]. We used β1 integrin as the marker of focal adhesions. β1 integrin forms various types of integrin receptor heterodimers with at least 10 different α Integrin subunits [167]. In these subunits, α3, α5, α8, and αv are coupled with β1 and bind to fibronectin via its Arg-Gly-Asp (RGD) binding site, indicating that these integrin dimers may share similar cellular function [167]. α5β1 integrins are abundant in vascular smooth muscles and are known to enhance L-type Ca\(^{2+}\) currents [168]. A more accurate interpretation of the functions of the different integrin heterodimers in gastric fundus smooth muscles could be addressed by performing PLA with 3 antibodies to identify which αβ integrin pairs play the most important role in cholinergic transmission regulation in the gastric fundus smooth muscle. The 3PLA technique could be used to detect the proximity between FAK and α5β1 integrins or FAK vs other integrin heterodimers. Although 3PLA assays for proximity ligation assay with 3 probes had been demonstrated [169], the lack of commercially available probes and no reported findings in tissue sections limit its application to our work. (2) To preserve the protein-protein interaction and cellular structure in intact smooth muscle tissue under different physiological conditions, we
used 4% paraformaldehyde (PFA) to fix the tissue. The PFA penetrates the tissue quickly and crosslink the proteins that are interacting with each other. However, the PFA fixative may not retain some of the rapidly-changing protein phosphorylation events because the fixation may not inactive all phosphatase activity in secs. For CPI-17 pT38, FAK pY397 and other protein phosphorylation, we obtained consistent results which correlated with our biochemical data. Thus, the tissue-PLA method for protein phosphorylation analysis could be applied to most of the phosphorylation events if the maintaining the protein phosphorylation status can be achieved without the requirement for specialized treatments.

### 5.3 Future directions

The possible future directions for chapter-2 are as follows; (1) In the second chapter we observed that FAK tyrosine phosphorylation was elicited by EFS-induced cholinergic stimulation. We demonstrated that FAK phosphorylation inhibition is not regulating \( \text{Ca}^{2+} \) influx by \( \text{Ca}^{2+} \) imaging experiments. One possibility for the FAK activation is that FAK sensed the EFS-induced contraction and was then activated to assemble the focal adhesion and amplify the contraction. To test this hypothesis, the smooth muscle can be paralyzed by the actin polymerization blocker cytochalisn D and then stimulated with EFS. We could then conclude that FAK phosphorylation is activated by mechanical force if the FAK phosphorylation not increased under EFS in paralyzed smooth muscle. (2) It might be worthwhile to study FAK expression and phosphorylation levels in different cell types of the murine gastric fundus smooth muscle tissue. Smooth muscle cells, interstitial cells of Cajal,
enteric neuron cells and other cell types could be isolated through cell sorting, followed by Western Blot analysis for FAK expression and pY397 phosphorylation. This would provide a better understanding of FAK distribution and activation in smooth muscle tissues. (3) Cell specific, inducible FAK knockout mice would be helpful to conduct loss-of-function studies in parallel with the FAK inhibitor studies. We could knockout the FAK gene in a cell-type specific manner by the CRISPR/Cas9 system [170]. (4) We showed that FAK phosphorylation inhibition reduced cholinergic transmission induced smooth muscle tonic contraction in murine gastric fundus smooth muscle. However, an effect of FAK phosphorylation on gastric emptying is unknown. Functional studies of gastric emptying during manipulation of FAK expression and/or phosphorylation would help us understand gastric motility modulation by FAK in a broader picture. (5) Since we have human gastric fundus smooth muscle tissue available, we could conduct contractile experiments and perform Wes analysis of Ca\textsuperscript{2+} sensitization protein phosphorylation of human samples in the presence of PF-431396 and compare the similarities and differences between mouse and human gastric fundus smooth muscle. This would improve our understanding of human fundus motility regulation.

The possible future directions for chapter-3 and -4 are as follows; (1) we chose LC20 PLA labeling for smooth muscle tissue PLA internal controls. LC20 PLA labeling would label the myosin light chain proteins in the smooth muscle cells. To confirm the LC20 PLA signals as valid internal controls for PLA, the cell morphology change of isolated gastric fundus smooth muscle cells under EFS and other physiological stimulation could be analyzed by both LC20 PLA along with
geometric volume calculation by microscopy [136]. Thus, the LC20 PLA indirect measurement of smooth muscle cell intracellular density could be correlated with the visualization and image analysis of smooth muscle cell structural changes. (2) The studies in chapter 4 could be further expanded by the spatial annotation of FAK and CPI-17 phosphorylation, which is one of the smooth muscle tissue PLA applications demonstrated in chapter 3. Gastric fundus smooth muscles under different physiological treatments could be labeled with FAK vs pY397 and CPI-17 vs pT38 PLA and then counterstained with β1 integrin. Then we could get the distance distribution of phosphorylated FAK and CPI-17 to β1 integrin under various physiological states, which could also address the hypothesis that Ca\(^{2+}\) sensitization protein assembly to β1 integrin under cholinergic stimulation is regulated by FAK phosphorylation. Additionally, the PKC vs pT38 and PKC vs CPI-17 PLA pairs with the counterstaining of β1 integrin could be performed to illustrate the spatial distribution of PKC in CPI-17 activation. (3) Automatic Fiji analysis pipeline could be established based on the current settings for tissue-PLA analysis workflow. The imaging process power would be much greater with less manual operations on the Fiji software.

5.4 Conclusions

I found FAK and its Y397 autophosphorylation in gastric fundus smooth muscles as a major tyrosine-phosphorylated protein. Furthermore, pY397 phosphorylation increased in response to EFS of cholinergic motor neurons. Inhibition of pY397 phosphorylation results in inhibition of EFS-induced contraction, and inhibition of CPI-17 and LC20 phosphorylation. Atropine blocks
the EFS-induced pY397 phosphorylation. pY397 phosphorylation and smooth muscle contraction evoked by High K$^+$ were inhibited by the FAK inhibitor PF-431396. Together these results indicate that FAK plays an important role in the regulation of gastric fundus smooth muscle contraction in response to cholinergic stimulation.

For the better illustration of FAK functioning at focal adhesions, we developed an improved quantitative PLA approach for smooth muscle tissue sections with novel quality controls and spatial annotation methodology. My tissue PLA method overcomes the difficulty of smooth muscle PLA signal normalization during the tissue density changes, and the morphology changes that occur from different physiological stimulations. We developed a set of experiments for quality controls in addition to the standard PLA technical controls, followed by standardized Fiji imaging analysis. The workflow ensures and validates the PLA signal quality with less biased quantification. Additionally, we introduced PLA with counterstaining of the cell membrane protein to analyze the spatial relationship between PLA signals and the cellular structure. We expect our tissue PLA method will expand the PLA applications to tissue-level physiological studies to help elucidate complex cellular signaling events.

This tissue-PLA method was applied to our study of FAK phosphorylation in smooth muscle contraction regulation. We found FAK regulates the association of Ca$^{2+}$ sensitization proteins to focal adhesions, indicated by PLA analysis of Ca$^{2+}$
sensitization proteins and LC20 association with β1 integrin under EFS with or without FAK inhibitor PF-431396. Taken together with our biochemical and imaging data, we have illustrated the critical role of FAK in Ca^{2+} sensitization pathways regulating murine gastric fundus smooth muscle contraction. This study reveals a novel role of FAK and opens the door for FAK as a new drug target for the gastric motility regulation.
### Supplemental Table 1. Primary antibody list

<table>
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<th>Protein</th>
<th>Vendor</th>
<th>Catalog#</th>
<th>Source</th>
<th>Application Concentration</th>
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<td>CPI-17 (PPP1R14A)</td>
<td>Santa Cruz Biotechnologies, Santa Cruz, CA, USA</td>
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<td>Mouse</td>
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<td>pT38-CPI-17</td>
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<td>sc-17560</td>
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<td>WES (1:200), PLA/IF (1:200)</td>
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<td>pY397-FAK</td>
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