PKCα and CPI-17 expression and spatial-temporal distribution with activation in pig stomach antrum and fundus

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PKCα AND CPI-17 EXPRESSION AND SPATIAL-TEMPORAL DISTRIBUTION WITH ACTIVATION IN PIG STOMACH ANTRUM AND FUNDUS

by

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ABSTRACT

PKCα AND CPI-17 EXPRESSION AND SPATIAL-TEMPORAL DISTRIBUTION WITH ACTIVATION IN PIG STOMACH ANTRUM AND FUNDUS

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Smooth muscle contraction is a complicated process coordinated by contractile, regulatory and cytoskeletal proteins. The force generation depends on the phosphorylation of Myosin Regulatory Light Chain (MLC_{20}). Myosin Light Chain Kinase (MLCK) and Myosin Light Chain Phosphatase (MLCP) are the two main regulators of the MLC_{20} phosphorylation level. MLCP is further controlled by two known pathways including the G protein coupled receptors (GPCRs)/ phospholipase C (PLC)/ diacylglycerol (DAG)/ protein kinase C (PKC)/ PKC-potentiated inhibitory protein for heterotrimeric myosin light chain phosphatase of 17 kDa (CPI-17) pathway. While messengers involved in this pathway have been proposed, studies on the details of the pathway are still controversial.

This study explored the spatial-temporal regulation and distribution of PKCα and CPI-17 in intact animal tissues. Immunohistochemical results show that the distribution of PKCα in the longitudinal and circular layers of the fundus and antrum under relaxed conditions was predominantly localized at or near the periphery of the smooth muscle cell. Stimulation of the tissues with 1μM phorbol 12,13-dibutyrate (PDBu) for 10 or 30 minutes or 1μM carbachol (CCh) for 3 minutes does not alter the distribution pattern of PKCα. Different from PKCα, CPI-17 appeared to be "uniformly" distributed throughout the smooth muscle cells under relaxed conditions.

Stimulation of the tissues with 1μM PDBu or 1μM CCh for 30 minutes led to a significant distribution shift of CPI-17 from throughout the cytosol to primarily at the cell periphery. Results from double labeling of PKCα and vinculin/talin under relaxed condition or CPI-17 and vinculin/talin under stimulated condition suggested that PKCα and CPI-17 were not associated with the adherens junction. It is likely that PKCα and CPI-17 are localized at the caveolae on the plasma membrane. This study also revealed that the force generated in tonic fundus smooth muscle is much greater than that in phasic antrum tissue upon PDBu stimulation. Immunoblot analyses demonstrated that this difference was not caused by a difference in the expression of PKCα or CPI-17 between these two tissues.

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CHAPTER I

Introduction

Possible mechanisms underlying phasic and tonic smooth muscle contraction

Smooth muscle cells display heterogeneous expression and function of contractile, regulatory and cytoskeletal proteins. Various smooth muscle tissues express different proteins (or protein isoforms), which can modulate their contractile function. Based on their physiological responses, smooth muscles can be divided into tonic and phasic types (Somlyo, 1968). Tonic smooth muscles generate a slow steady isometric contraction that is maintained, while phasic smooth muscles develop a relatively fast transient isometric contraction. In general, visceral organs display primarily phasic contractions, while the vascular system generates predominantly tonic contractions. The tonic vascular smooth muscles such as aorta, femoral, and carotid arteries exhibit a graded force development followed by a well maintained contraction. In contrast to tonic smooth muscles, phasic muscles, such as stomach antrum, bladder, and ileum respond to agonist and generate a very fast but transient contraction. There are exceptions such as the phasic portal vein and vas deferens (both vascular) and the tonic fundus (visceral) smooth muscle. Studies by Himpens et al showed that the time course of $[Ca^{2+}]_i$ is similar in both phasic and tonic muscle types - an initial transient increase to a peak value followed by a drop to approximately 70% of its peak concentration upon high K^+ treatment in tonic smooth muscles and to 60% in phasic smooth muscles (Himpens et al., 1988). Thus, the diversity of contraction pattern between tonic and phasic smooth muscle might

involve factors other than Ca^{2+} modulation. Several hypotheses have been proposed to explain the mechanism(s) responsible for tonic and phasic smooth muscle contractions. These include: 1) myosin heavy chain (MHC) isoforms SMA/B and myosin light chain (MLC) isoforms that are spatially distributed and temporally regulated in smooth muscle tissues. In addition, it has been widely reported that MLC_{17} and MLC_{20} isoforms are differentially expressed in smooth muscles (Cavaillé et al., 1986). It also has been shown that MHC SMA/B isoforms have unique expression pattern in tissues. SMB MHC primarily exists in phasic tissues like stomach antrum and bladder, whereas the SMA MHC isoform is predominantly expressed in tonic smooth muscle tissues, such as stomach fundus, and elastic arteries (Eddiger & Meer, 2007). 2) Smooth muscle cross-bridges called latch bridges could also contribute to the tonic force maintenance. It has been reported that the MgADP has a higher affinity to cross-bridges in tonic smooth muscle than in phasic smooth muscle slowing relaxation. Thus, the MgADP could uniquely affect smooth muscle contractile characters between these two muscle types, especially at low $[Ca^{2+}]_i$ (Khromov et al.,1998; Dillon et al., 1981). 3) Proteins such as caldesmon or calponin, which may play an important role in forming actin-to-myosin cross links are also proposed to affect and regulate force generation (Sutherland & Walsh, 1989). 4) Regulation of actin polymerization/depolymerization has also been reported to be responsible for controlling the force development. Chen et al (Chen et al., 2008) reported that binding of myosin to actin can trigger actin polymerization and enhance the force development in arterial smooth muscle. 5) With the discovery of multiple G-protein coupled receptors (GCPRs) and their downstream regulatory pathways in

smooth muscle contraction (Kitazawa & Somlyo, 1991b; Kubota et al., 1992), these pathways have been reported as additional important regulators of the different contractile responses in smooth muscle tissues (Khalil et al., 1992; Krymsky et al., 2001; Woodsome et al., 2001).

Regulation of Smooth muscle contraction pathways

Regulation of MLCK

It is well accepted that the phosphorylation of MLC_{20} is a critical step leading to smooth muscle contraction (Murthy, 2006). While the steps following MLC_{20} phosphorylation are thought to be the same for smooth muscle contraction under different conditions, two opposing mechanisms are proposed for regulating the phosphorylation level of MLC_{20} (Gong et al.,1992; Ito et al., 2003). A Ca^{2+} related process was reported to be responsible for phosphorylating MLC20 in smooth muscle activation. Identification of the Ca^{2+} binding protein calmodulin (CaM) and multiple other factors showed that the signal transduction is regulated by an interconnected intracellular pathway. The binding of Ca^{2+} /CaM on the catalytic domain of myosin light chain kinase (MLCK) activates its kinase activity and this activity was shown to be responsible for phosphorylating serine 19 on MLC_{20} to cause contraction (Kitazawa et al., 2000). A second pathway for controlling the degree of MLC_{20} phosphorylation is Ca^{2+}- independent and G-protein coupled. The key regulator in this pathway is myosin light chain phosphatase (MLCP) which is responsible for dephosphorylating MLC_{20}. For decades, the importance of MLCP was underestimated because it was assumed that this enzyme is constitutively active.

However, recent work showed that MLCP is regulated in smooth muscle cells and its activity varies under different conditions (Gallagher et al., 1991; Ito et al., 2004). It has been reported that without affecting MLCK, inhibition of MLCP increases MLC_{20} phosphorylation and in turn enhances force production at any given level of Ca^{2+} (Hartshorne et al., 1998). The ratio of activity of MLCK to MLCP is believed to be the critical factor for MLC_{20} phosphorylation and force produced in smooth muscle contraction. The greater the ratio, the greater the force that can be generated.

Regulation of MLCP----RhoA/ROCK pathway

Different from the MLCK regulation mechanism, the pathways controlling MLCP activity may be Ca^{2+}-independent (Fig.1.1). MLCP is a protein with three subunits: a 38-kDa catalytic subunit of type 1 phosphatase (PP1cδ), a 110-kD noncatalytic myosin phosphatase-targeting subunit (MYPT1) and a 20-kDa noncatalytic subunit (M20) of unknown function. Second messengers interacting with different subunits of MLCP are proposed to be a major regulatory mechanism (Hori et al., 1993). Two major signal transduction pathways have been proposed to have the ability of regulating MLCP activity. One pathway is mediated by RhoA activated Rho kinase (ROCK) and this kinase is able to inhibit MLCP through phosphorylation of MYPT1 at Thr696 (Somlyo AP & Somlyo AV, 2003; Wang et al., 2009). Phosphorylated MYPT1 appears to have a reduced affinity for myosin and this decreases the catalytic activity of PP1cδ, which leads to the inhibition of MLCP to dephosphorylate MLC_{20}.

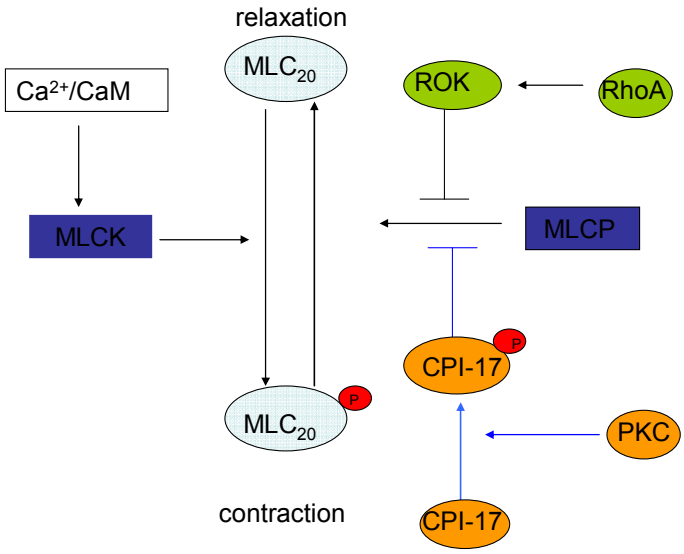


Figure 1.1. Mechanism of smooth muscle contraction. Two major pathways (PKC/CPI-17 pathway and RhoA/ROCK pathway) are involved in regulating the activity of MLCP.

Regulation of MLCP ---- PKC/CPI-17 pathway

Another pathway proposed to be involved in controlling MLCP activity is the Protein Kinase C (PKC)–mediated PKC-potentiated inhibitory protein for heterotrimeric myosin light chain phosphatase of 17 kDa (CPI-17) pathway. CPI-17 is the first PP1cδ inhibitory protein identified in smooth muscle (Velasco et al., 2002). It has been reported that phosphorylation of CPI-17 on Thr-38 increases the phosphatase inhibitory potency more than 1000-fold (Eto et al.,1995). Although several kinases such as RhoA, ROCK and P21-activated protein kinase (PAK) have been shown to have the potential to phosphorylate CPI-17, PKC is believed to be the primary CPI-17 regulating kinase in smooth muscle *in vivo* (Eto et al., 1997; Kitazawa et al., 2003; Koyama et al., 2000).

Studies show that PKC dependent pathways are initiated by agonist binding and a conformation change of G protein coupled receptors (GPCRs) (Somlyo AP & Somlyo AV, 1994). After binding with agonists, altered GPCR have the ability to activate G proteins. G proteins can significantly boost the activity of phospholipase C (PLC), which is in turn utilized by the cell to breakdown phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). As a small soluble protein, IP_3 can move to the sarcoplasmic reticulum (SR) and activate the IP_3 receptors (IP_3R) to release calcium. The released calcium partially contributes to the Ca^{2+}- dependent pathway phosphorylating MLC20. At the same time, DAG remains in the cell membrane. PKC is activated through binding to DAG.

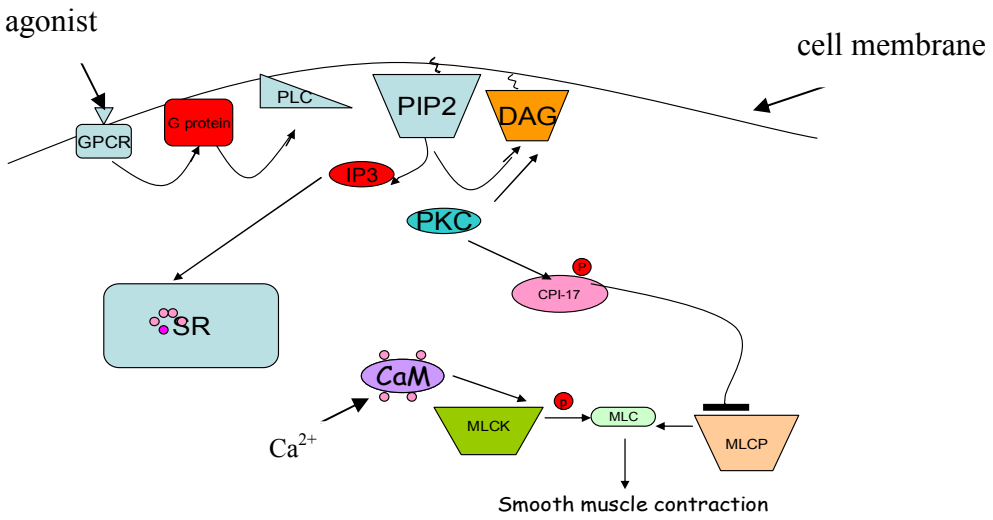


Figure 1.2. PKC/CPI-17 pathway in smooth muscle contraction. With agonists stimulation, GPCR have the ability to activate G proteins. G proteins can significantly boost PLC activity, which are able to breakdown PIP_2 into inositol IP_3 and DAG. IP3 can move to sarcoplasmic reticulum and activate the IP_3 receptor (IP_3R) to open calcium channels. The released calcium partially contributes to the Ca^{2+}-dependent pathway phosphorylating MLC_{20}. At the same time, DAG remains in the cell membrane. PKC is activated through binding to DAG. Activated PKC binds to and phosphorylates CPI-17. Phosphorylated CPI-17 inhibits the function of MLCP and enhances smooth muscle contraction.

Upon phosphorylation by PKC, CPI-17 inhibits the function of MLCP, and enhances smooth muscle contraction (Fig. 1.2).

It is believed that PKC is regulated by its activation status via binding to DAG and spatial distribution within the cell. There are at least 10 PKC isoforms and they can be divided into 3 classes based on their homology: 1) classical PKCs subfamily (cPKCs) including PKCα, βII, and θ, whose activation is Ca^{2+}, phospholipid, and DAG dependent. 2) Novel PKCs (nPKCs) subfamily including PKCδ, ε, η, which require phospholipids and DAG but do not require calcium for activation. 3) Atypical PKC (aPKCs) subfamily including PKCλ, ζ and τ, which do not require DAG or calcium for activation (Reyland, 2009). It is also reported that both the inactive and active PKC isozymes are localized to specific sites because of the finding of several specific anchoring molecules named RICK (receptors for inactivated C-kinase) and RACK (receptors for activated C-kinase) (Mochly-Rosen, 1995; Mochly-Rosen & Gordon, 1998; Mochly-Rosen et al.,1991). In addition, it has been reported that the activation of persistent Ca^{2+} sparklets depend on the PKC binding (Navedo et al., 2006; Navedo et al., 2005). Ca^{2+} sparklets result from activation of small clusters of L-type Ca^{2+} channels (LTCC) to create a continual Ca^{2+} influx. This suggests the particular localization of PKC might be near the membrane where LTCC are located. However, many studies suggest that PKC is diffusely distributed in the cytosol in relaxed conditions and upon stimulation translocates to the membrane (Secrest et al., 1991; Li et al., 2002; Nakamura & Nishizuka, 1994). There are reasons to question these studies of spatial distribution and relative movement of these proteins. No

mechanism has been proposed of how the signal is transferred from the membrane embedded DAG to MLC_{20} which can be located throughout the cytosol. Besides PKC, CPI-17 and MLCP have also been reported to translocate from the cytosol to the membrane upon stimulation (Shin et al., 2002; Sakai et al., 2005). These two molecules are believed to be downstream factors of PKC in this pathway. However, close observation of experiments supporting the translocation of PKC, CPI-17 and MLCP reveals that most of these studies either employed indirect strategies or were done in isolated/cultured cell systems. Isolated cells may not be a reliable system to reflect the *in vivo* physiological situation. It has been reported that *in vivo*, the smooth muscle cells express a contractile phenotype, while isolated/cultured smooth muscle cells differentiate into a synthetic phenotype leading to a loss of contractile capability (Owens, 1995). Because it is well accepted that PKC is activated by newly generated DAG, which is a membrane bound phospholipid, and is a fairly large molecule (~77KD; Reyland, 2009), it might be more reasonable for PKC to stay near the membrane while other smaller messenger molecules translocate to and from the membrane. If this is the case, there must be at least one downstream factor working as a moving messenger to carry the signal from the membrane to the cytosol where MLC_{20} is located. So far, no molecule has been shown to perform this function.

The aims and rationales of this study

To avoid the potential limitations of isolated/cultured cell studies, this study was designed to observe the distribution of PKC and CPI-17 directly in intact tissues in relaxed and agonist stimulated conditions. Pig stomach tissue was selected to be

the model system. Because of its similarities to human, pigs have served as a research model to study physiology and signal transduction for decades. It has been shown that the pig genome and human genome have a high homology in sequence and chromosome structure (Lunney, 2007). Pigs are also a good source for organ transplantation to human (Dekel et al., 2003). Another advantage of using the pig stomach is that it is easily handled due to the relative large size, includes both tonic and phasic SM, and has relatively large smooth muscle cells, allowing for increased resolution with immunostaining.

In this study, phorbol 12, 13-dibutyrate (PDBu) and carbachol (CCh) were used to activate the PKC pathway (Shimamoto et al.,1993). The advantage of using PDBu, an analog of DAG, is that it has the ability to directly initiate the PKC dependent pathway. PDBu can insert into the cell membrane and activate PKCα by binding to its N-terminal region (Kraft & Anderson, 1983). Although PDBu can efficiently activate the PKC pathway, it is not a physiological agonist. Therefore, CCh, a cholinergic agonist, which can stimulate both muscarinic and nicotinic receptors was also used in this study. The purpose of this study was to determine the protein expression levels and distribution of PKCα and CPI-17 under relaxed and stimulated conditions in intact phasic and tonic SM tissues. Li et al. (2002) reported that stimulating cultured smooth muscle cells with PDBu leads to a slow but robust translocation of PKCa from the cytosolic to the plasma membrane by observing with confocal microscopy. These results suggested that the translocation process starts 8 min after PDBu treatment. Sakai reported that CPI-17 begins to translocate to the

membrane fraction after 2 min stimulation with Acetylcholine (Sakai et al., 2005). Based on these studies, the distribution of PKCα and CPI-17 were observed at multiple time points in this study to enhance the likelihood of observing translocation.

Force measurement experiments previously done in our lab show that force generated in rabbit antrum after PDBu treatment is insignificant compared with the response of fundus. This difference suggests diversity in the PKC pathway in the different stomach regions. If PKCα traslocates in a similar pattern in both of these stomach regions, the difference in their force generation is likely to be caused by other steps within this pathway or by other mechanisms. The expression level of CPI-17 is not the same in different types (phasic vs. tonic) of smooth muscle tissue from rabbit, and tonic smooth muscle has more CPI-17 content than phasic smooth muscle. (Woodsome et al., 2001). Thus, this study measured PKC and CPI-17 protein expression in antrum and fundus to determine their potential impact on this pathway and on force generation. Surprisingly, no significant difference in the protein expression level of PKCα and CPI-17 was detected between the fundus and antrum.

Summary

In summary, this study was designed to determine the protein expression and spatial-temporal distribution of PKCα and CPI-17 in phasic stomach antrum and tonic stomach fundus under relaxed and activated conditions at multiple time points. Our results indicate that there is no significant difference in the level of protein expression of PKCα and CPI-17 between pig antrum and fundus. There is also no observable

translocation of PKCα in either relaxed or stimulated conditions in either tissue. PKCα is always located near the cell periphery. On the other hand, CPI-17 was observed to translocate from the cytosol to the plasma membrane upon stimulation (both PDBu and CCh). This translocation is very slow, requiring more than three minutes, and is not observed to return to basal conditions by 30 min of stimulation. Further studies need to be done to determine if and how the CPI-17 that is activated at the plasma membrane can regulate MLCP that is present in the cytosol.

CHAPTER II

Materials and Methods

Organ and tissue handling

Swine tissues (stomachs) were obtained from Hansen Meat Service (Franksville, WI) and put in cold physiological salt solution (PSS (in mM): 140.1 NaCl, 4.7 KCl, 1.2 Na_2HPO_4, 2.0 MOPS (pH 7.4), 0.02 Na_2EDTA, 1.2 $MgSO_4$, 1.6 $CaCl_2$, and 5.6 glucose). Stomachs were cleaned of blood, loose connective tissue, and in some cases, the mucosa, and frozen in isopentane cooled in liquid nitrogen or stored in PSS in the refrigerator for 0-2 days. Some organs were fresh frozen as soon as possible following post-mortem (60-90 minutes). Some organs were incubated in PSS or stimulated with 1.0 μM CCh or PDBu (Sigma) for different time points prior to rapid freezing. Variable incubation times and agonist concentrations were also tested. All tissue was stored frozen until sectioned and immunoreacted. Five to ten μm sections of the frozen tissues were cut on a Leica CM1900 cryostat, picked up on glass slides and stored frozen (0-1 days).

Immunoreactions

Reagents. The antibodies used were obtained from the following sources: PKCα (H-7 and C-20), CPI-17(H-60) from Santa Cruz Biotech, Santa Cruz CA; Vinculin and Talin from Sigma, Saint Louis, Missouri; Cy2 and Cy3 Donkey anti-mouse or rabbit secondary antibodies from Jackson ImmunoResearch, West Grove, PA; Alexa Fluor 594-phalloidin and DAPI from Molecular Probes, Eugene, OR.

Frozen tissue sections picked up on glass slides were fixed with 2% paraformaldehyde for 10 minutes, permeabilized in 0.5% Triton X-100 for 10 minutes and blocked with 5mg/ml BSA for 1 hour prior to incubating with the primary antibody overnight and then the appropriate secondary antibody for one hour at room temperature. After the secondary antibody, the tissues were incubated with DAPI (0.5 μM), phalloidin (10-50 nM) or DAPI/phalloidin as appropriate for staining nuclei and/or filamentous actin. Three washes were used following the primary and secondary incubations, and the counterstaining. Cover glasses were mounted using buffered 75% glycerol with 0.2% n-propyl gallate to minimize fading. All immunoreacting solutions were made in PBS-Tween [(in g/liter: NaCl 8.0, KH_2PO_4 0.2, Na_2HPO_4 1.15, KCl 0.2,), 1% tween-20, pH 7.4] with 0.1% BSA. Negative controls included 0.1%BSA leaving out the primary antibody.

Microscopy. Sections were observed using an Olympus IX70 microscope with epifluorescence illumination. Digital images were taken with a 16bit Princeton Instruments (Princeton, NJ) CCD camera, controlled through a PCI board via IPLab for Windows on a PC (Ver. 3.6, Scanalytics; Fairfax, VA). Images using a 100x (1.3 NA) objective were taken. Emission filters used were 405, 490 and 570 nm. Sections were also viewed using a Nikon confocal microscope (Nikon A1 confocal eclipse ti). The objectives used were 100X(1.4NA) oil lens at 425,488 and 561 nm. Similar results were observed in immunofluorescence distributions using these two different systems.

Image Analysis of protein distribution of single cells in tissues

Profiles of fluorescence intensity were taken for individual cells in transverse tissue sections. Sections were observed at low magnification (10-20x) to avoid areas of apparent artifacts (tissue folding, freeze damage, etc). In an artifact free area the magnification was increased to 40-100x, and pictures were taken at 100x. Three different areas within one section were chosen to take pictures. Z-stack series were taken individually for each of the three color channels used. 1μm thick Z sections were taken, and 10-15 Z sections were taken for each tissue section. Each Z stack series was examined for each section to identify the center z image, and this image was converted to a .bmp file, which was imported to NIS-Elements AR 3.0 (Nikon) on a PC to analyze the data.

Image Analysis of distribution of PKC/CPI-17 of individual Cells in tissues.

Profiles of PKCα and Phalloidin fluorescence intensity were obtained for individual cells in transverse tissue sections. A line was drawn across the center of the image. Ten cells crossed by the line were selected for measurements. Based on our previous protocols (25), the first five pixels (~0.7μm) on either side of the cell where the phalloidin intensity increased sharply from baseline were defined as the periphery of the cell. For cytosolic measurements, a line was placed at least 2 μm away from the cell periphery. Intensity measurements were made using a region of interest (ROI) roughly at the center of the cell (for cytosolic measurements), or along the cell's membrane (for peripheral measurements). For consistency, the ROI is kept constant for the peripheral and cytosolic measurements. Cells in the section with very small

diameters (not able to measure cytosolic ROI at least 2 μm from the periphery) or with nuclei present (visible DAPI staining; spatial limitations and perinuclear organelles could confound the distribution) were excluded from analysis. For PKCα, the ratio of the average intensity of PKCα at the periphery over the total PKCα intensity (sum of PKCα intensity from ROI at periphery and ROI in cytosol) was used. Ten cells per field and three different fields were subjected to measurement for data analysis for each tissue region, i.e. thirty cells were counted and averaged for each sample (n = 1). The final sample size is n=5.The same procedures were applied to measure the intensity of CPI-17, except that only one field of 10 cells were used for each sample (n = 1), with a final sample size of n=3.

Mechanical measurements

Immediately before use, tissue strips were cut and clamped on each end, with the clamps secured between hooks on a stationary metal rod and an isometric force transducer (Harvard Apparatus, Holliston, MA), in PSS bubbled with 95% O_2/5% CO_2 in water-jacketed muscle chambers (Radnoti Glass Technology, Monrovia, CA) at 37^{o}C. The length of each strip was varied by repositioning of the stationary metal rod.

Smooth muscle tissue strips (stomach antrum and fundus) were equilibrated for 1 h and stretched to a passive tension approximating Lo using an abbreviated length-tension curve. To contract tissues, PSS was replaced with K^{+}-PSS (109 mM KCl and 70 mM NaCl substituted for 140 mM NaCl). The muscle strip was activated

repeatedly with stretching of the tissue between each activation, until peak force no longer showed a significant increase over the previous contraction. Chambers were flushed three times with PSS following each tissue activation. At least two successive K^+-PSS contractions were used to get a standard force trace with a 10 minute rest between each contraction before starting the experiment. The tissues were then activated with 1 μM carbachol and relaxed again as for the K^+-PSS contractions. Subsequent contractions all included 1 μM phentolamine and propranolol to block α and β adrenergic receptors, respectively. Following the final 1 μM CCh contraction and wash, the tissues were activated with 1μM PDBu to record their mechanical response.

Analysis of Force Data. Voltage signals from force transducers were digitized by PowerLab 400 or 4SP hardware (ADInstruments, Castle Hill, Australia) visualized on a computer screen (Chart v3.6 or 4.0, ADInstruments) as force (g) at 10 Hz and stored by software command to a hard disk for later analyses. Analyses were performed using the chart software and figures were made with the spreadsheet program Excel 2000 (Microsoft, Redmond, WA).

Gel Electrophoresis and western blotting

Protein expression was analyzed as described previously (Han et al., 2006). Tissues were homogenized in 0.125 M Tris, 2% sodium dodecylsulfate (wt/vol), 20% glycerol, 0.1% bromophenol blue (wt/vol) and 20 mM dithiothreitol. Proteins were resolved on low cross-linking sodium dodecylsulfate gels (Giulian et al., 1983) and

immunoblotting was performed as previously described (Eddinger & Wolf, 1993). Western immunoblots were performed as reported previously (Gaylinn et al., 1989).

Statistics

Statistical comparisons were carried out using MINITAB (Minitab Inc. State College, PA). A one sample t-test was used to test the distribution of PKCα/CPI-17 (peripheral ROI content vs. total ROI content = 0.5 indicating a "uniform" distribution in the SMCs) in antrum and fundus under resting condition. One way ANOVA was performed to test for PKC/CPI-17 distribution differences for the two tissues with different stimulating parameters. Two sample t-tests were used to test the significance of difference for expression level of PKC or CPI-17 in Antrum and Fundus. A value of $P < 0.05$ was considered significant.

CHAPTER III

Results

Swine stomach fundus is a tonic smooth muscle tissue that responds to stimulation with a sustained contraction while the antrum is a phasic smooth muscle tissue that responds to stimulation with a transient contraction (Fig. 3.1). In tissues, these responses are a result of direct stimulation of the smooth muscle tissue as addition of 1 μM propranolol (β agonist blocker) and phentolamine (α agonist blocker), which prohibit neuronal activation via these pathways. Stimulation of smooth muscle with PDBu is used routinely to stimulate PKC and cause smooth muscle contraction via PKC activation. 1 μM PDBU causes a small slow contraction in stomach fundus strips (~40% of K^+ stimulation response) while the antrum shows essentially no response (<5% K^+ stimulation) (Fig. 3.1). The difference between the responses of these two tissues to PDBu stimulation could be due to expression and/or spatial-temporal distribution of the downstream second messengers (PKCα and CPI-17) that are purported to be responsible for MLCP inhibition with PDBu stimulation. To examine this we measured the expression levels and determined the spatial-temporal distribution of PKCα and CPI-17 protein in these tissues.

Figure 3.2 shows western blot results of the expression of CPI-17 and PKCα in the stomach antrum and fundus. Tissues were processed to control for concentration, and the results were calculated based on the intensity of the signal and by normalizing to actin protein expression levels. Both methods indicate that neither

the expression of CPI-17 nor that of PKCα is significantly different between these two smooth muscle tissues. Controls were done using a range of loadings in both antrum and fundus tissues to confirm the range of linearity of loading and that samples used for quantitation were within this range (Fig. 3.3). Because there are no differences in the expression for these two proteins between these two tissues, we proceeded to determine if differences in their spatial-temporal distribution could explain the difference in their responses to agonist stimulation.

Figures 3.4 and 3.5 show immuohistochemical results for the distribution of PKCα in the longitudinal and circular layers of the fundus (Fig. 3.4) and antrum (Fig. 3.5). Under resting conditions in relaxing solution when there is no force generation by the tissue, the PKCα appears to be uniquely distributed near the periphery of the smooth muscle cells (Fig. 3.4 & 3.5 – PSS). Stimulation of the tissues with 1 μM PDBu (10 and 30 minutes) or 1μM CCh (3 minutes) does not alter this primarily peripheral distribution of PKCα. The ratio of the distribution of the PKCα near the plasma membrane relative to that in total was used to quantify possible changes in the distribution of this protein under these different conditions. Figure 3.6 shows the results as the ratio of the PKCα at the cells periphery relative to the total PKCα present in the cell (periperhal/ (peripheral + cytosol), see methods). A ratio of 0.5 would indicate a "uniform" distribution of the protein throughout the cell. The PKCα ratio (peripheral/total) ranged from 0.64 – 0.68 in all the conditions examined. These values are significantly greater than 0.5, indicating that PKC is located primarily at the cells periphery (it is not "uniformly" distributed in the cell)

and that their distribution does not change between the relaxed or stimulated conditions.

Because PKCα is proposed to activate CPI-17, we proceeded to determine the spatial-temporal distribution of CPI-17 in these tissues under these same conditions. Figures 3.7 & 3.8 show immuohistochemical results for the distribution of CPI-17 in the longitudinal and circular layers of the fundus (Fig. 3.7) and antrum (Fig. 3.8). Under resting conditions in relaxing solution when there is no force generation by the tissue, CPI-17 appears to be "uniformly" distributed throughout the smooth muscle cells (Fig. 3.7 & 3.8 – PSS). Stimulation of the tissues with 1 μM PDBu (30 minutes) or 1μM CCh (30 minutes) results in a significant change in this distribution such that the CPI-17 now appears to be primarily at the periphery of the cell in a distribution similar to that observed for PKCα. The ratio of the distribution of the CPI-17 near the plasma membrane relative to total CPI-17 (peripheral + cytosolic) was used to quantify possible changes in the distribution of this protein under these different conditions. Figure 3.9 shows the results as the ratio of the CPI-17 at the cells periphery as a ratio of the CPI-17 total protein. The CPI-17 ratio (peripheral/total) ranged from 0.49– 0.67 in all tissues and conditions examined. The ratio of CPI-17 peripheral/total in relaxed conditions is not significantly different than 0.5 (means 0.49 – 0.5; $P > 0.19$) indicating that the CPI-17 is "uniformly" distributed throughout the smooth muscle cells under this condition. This does not change following 3 minutes of 1 μM CCh stimulation as there is still no significant difference from the relaxed conditions (means = 0.53 – 0.55; $P > 0.05$) with the exception of the fundus

tissues where the CPI-17 peripheral/total ratio is significantly greater ($p < 0.05$) at 3 minutes. With 30 minutes of either 1 μM CCh or 1 μM PDBu stimulation, the CPI-17 distribution becomes significantly greater than 0.5 for all tissue and layers, (means = 0.62 – 0.67, $P < 0.01$), indicating that CPI-17 is now located primarily at the cells periphery (it is no longer "uniformly" distributed in the cell), similar to the distribution of PKCα (Fig. 3.4 & 3.5).

The primarily peripheral distribution of PKCα (under both relaxed and stimulated conditions) and CPI-17 (following 30 minutes stimulation with CCh or PDBu) is not uniform at the cell periphery, but punctuate in distribution (Fig. 3.10). There is also a punctuate distribution of the anatomically and functionally distinct adherens junctions and caveolae at the smooth muscle cell plasmalemma (Eddinger et al., 2007). In order to determine if the punctuate distribution of the PKCα and CPI-17 at the membrane corresponds with the punctuate pattern of the adherens junctions, we did double labeling with two adherens junction associated proteins, vinculin and talin. The double labeling of PKCα with talin/vinculin in relaxed condition and CPI-17 with talin/vinculin under stimulated condition was performed. The results show that PKCα and CPI -17 do not co-localize with either vinculin or talin, suggesting that PKCα and CPI-17 do not associate with the adherens junction complex with the relaxed or activated conditions we examined.

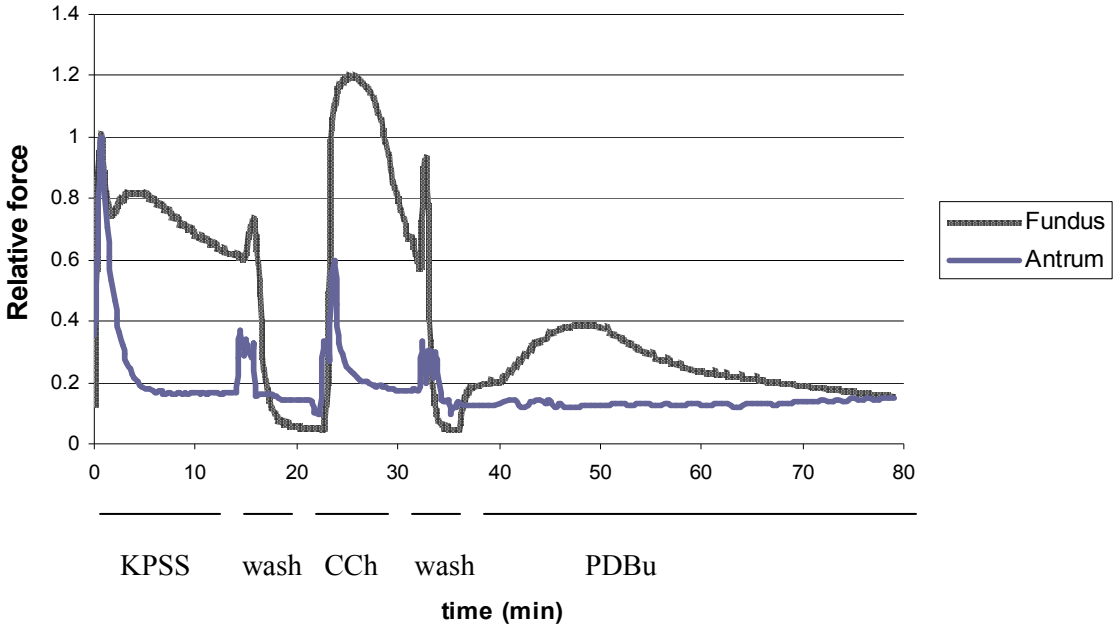


Figure 3.1. Force (normalized to peak force from KPSS stimulation) generated by stimulating with KPSS, CCh, or PDBu in fundus (grey) and antrum (black). Antrum contractile response is not maintained during the stimulation while that in the fundus is. PDBu generated a slow contraction in the fundus that is ~ 40% of its peak KPSS force, but caused little to no contraction in the antrum. Deflections in the traces at the start of each wash are from changing the solution in the chambers.

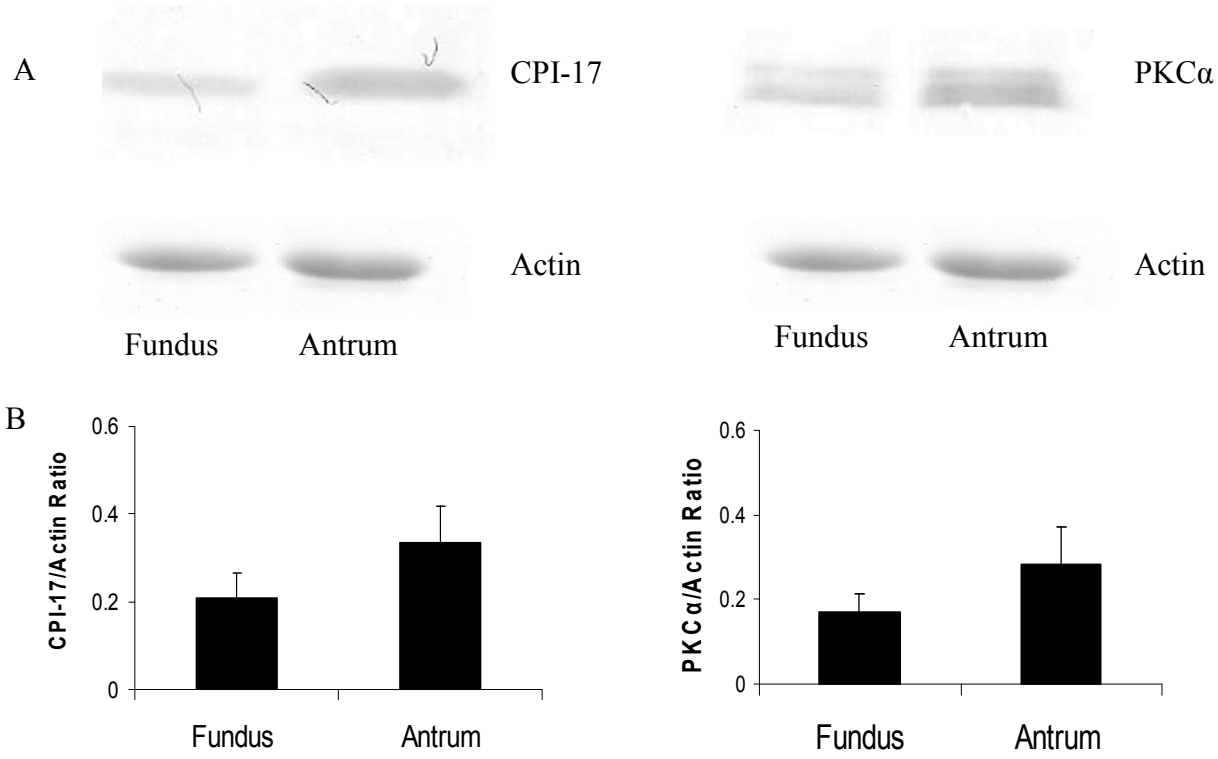


Figure 3.2. Protein expression of CPI-17 and PKCα in fundus and antrum. A: western blot results of CPI-17 (left) and PKC (right) expression in fundus and antrum with actin (coomassie blue stain) expression for each sample shown below. B: Quantitative data of western blot results. Protein expression level of CPI-17 (left) and PKCα in fundus and antrum normalized to actin expression. The expression level of PKCα and CPI-17 were not significantly different between the fundus and antrum.

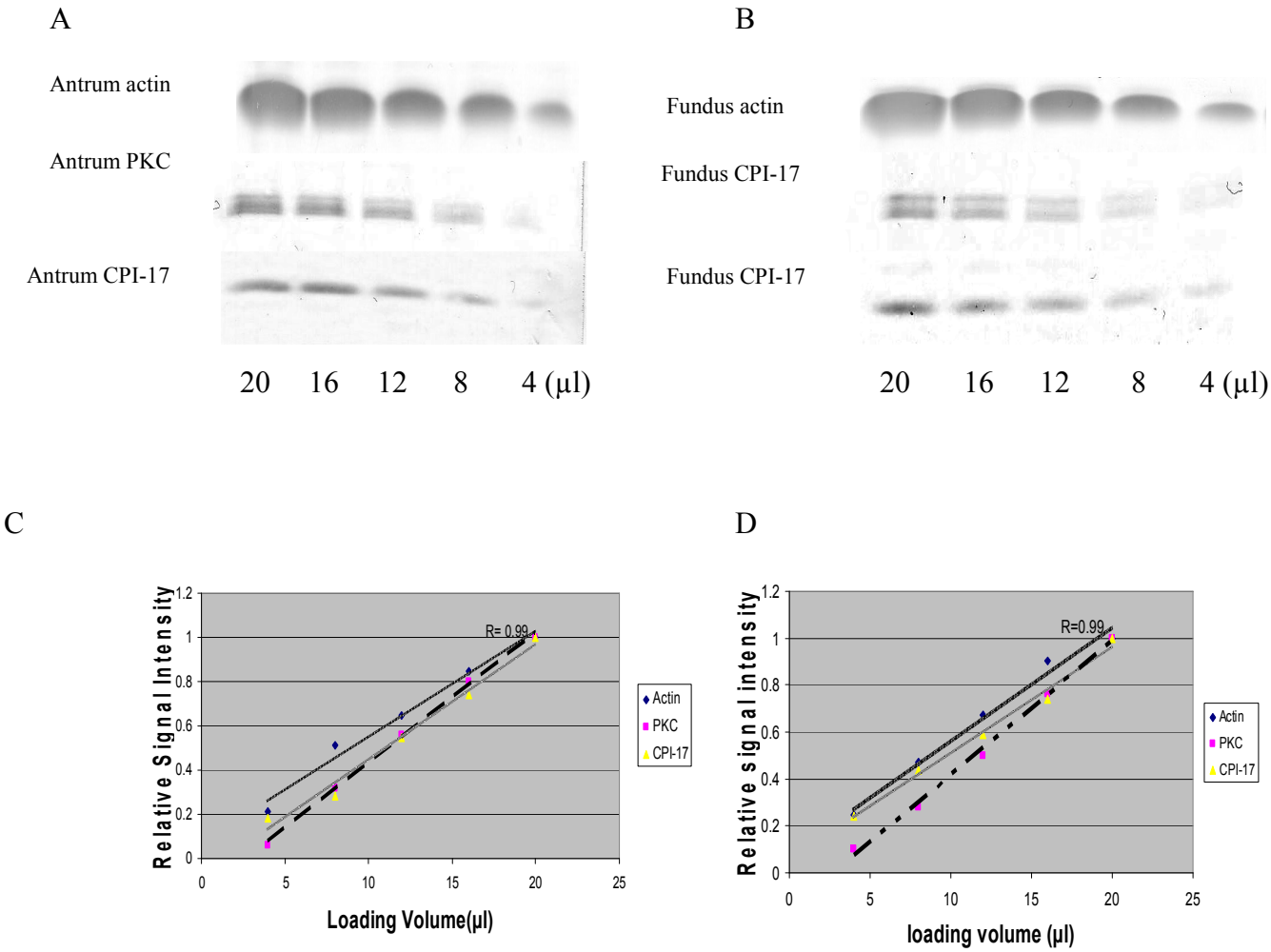


Figure 3.3. Quantitation of PKC and CPI-17 in antrum (A,C) and in fundus (B,D). A and B, loading series from 4µl to 20 µl of swine antrum and fundus extract (50mg tissue:1ml buffer) was subjected to SDS-PAGE. Total Actin was detected by coomassie blue and PKC and CPI-17 were detected by Western blotting. C and D, signal intensity plotted against amount of extract loaded for PKC and CPI-17 Western blots, and Coomassie Blue-stained actin, showing the linear relationship between the amount of sample loaded and the detected signal intensity. (n=3)

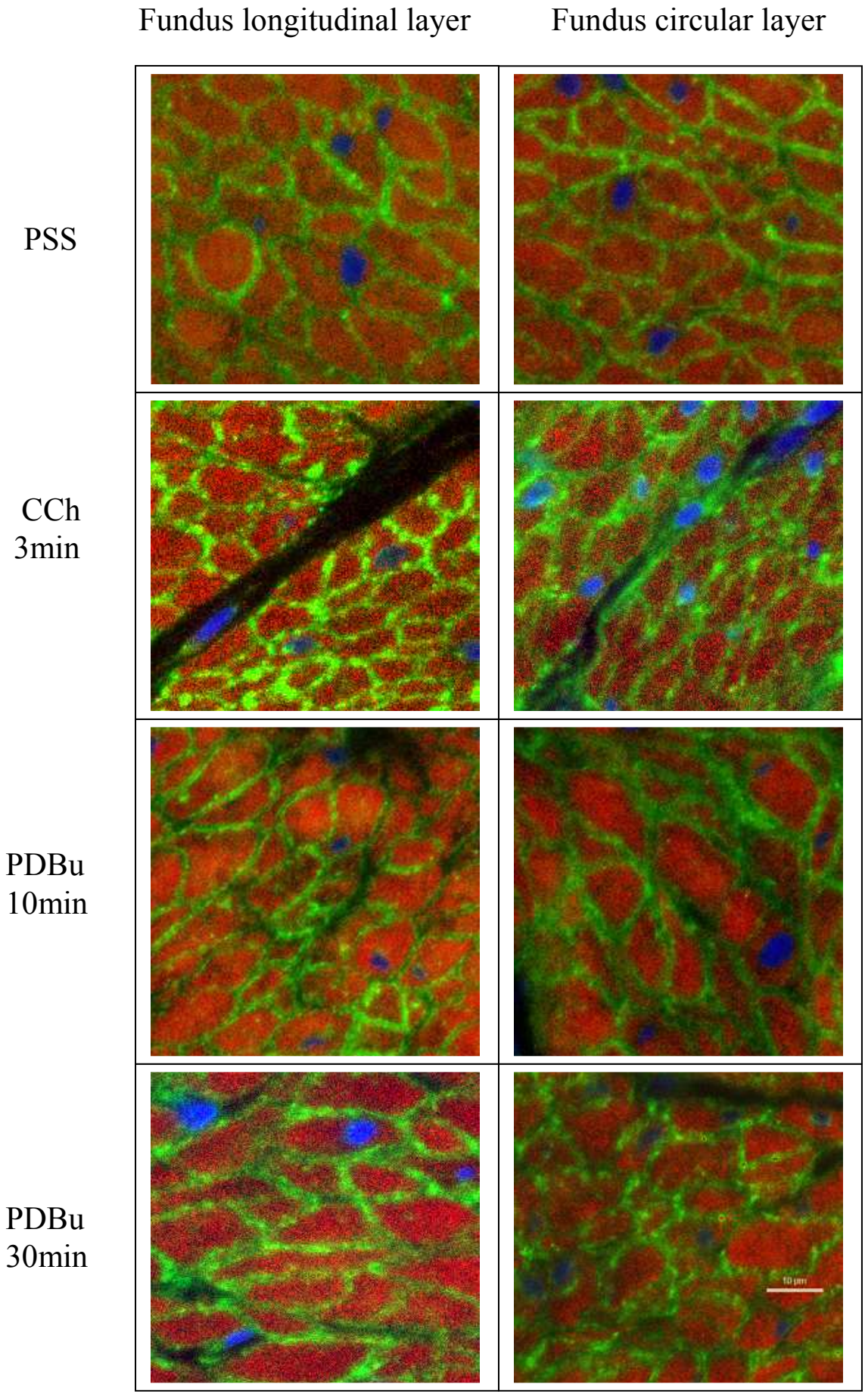

Fundus longitudinal layer
Fundus circular layer
PSS
CCh
3min
PDBu
10min
PDBu
30min

Figure 3.4. Confocal images of PKCα distribution in transverse sections of the longitudinal (left) and circular layer (right) of pig stomach fundus under relaxed (PSS) or stimulated (3 min CCh or 10min & 30 min PDBu) treatments. Tissues were immunoreacted for PKCα (green) and counterstained for phalloidin (red) and DAPI (blue). In all conditions, PKCα is located predominantly at the cell periphery near the plasma membrane. Scale bar - 10μm.

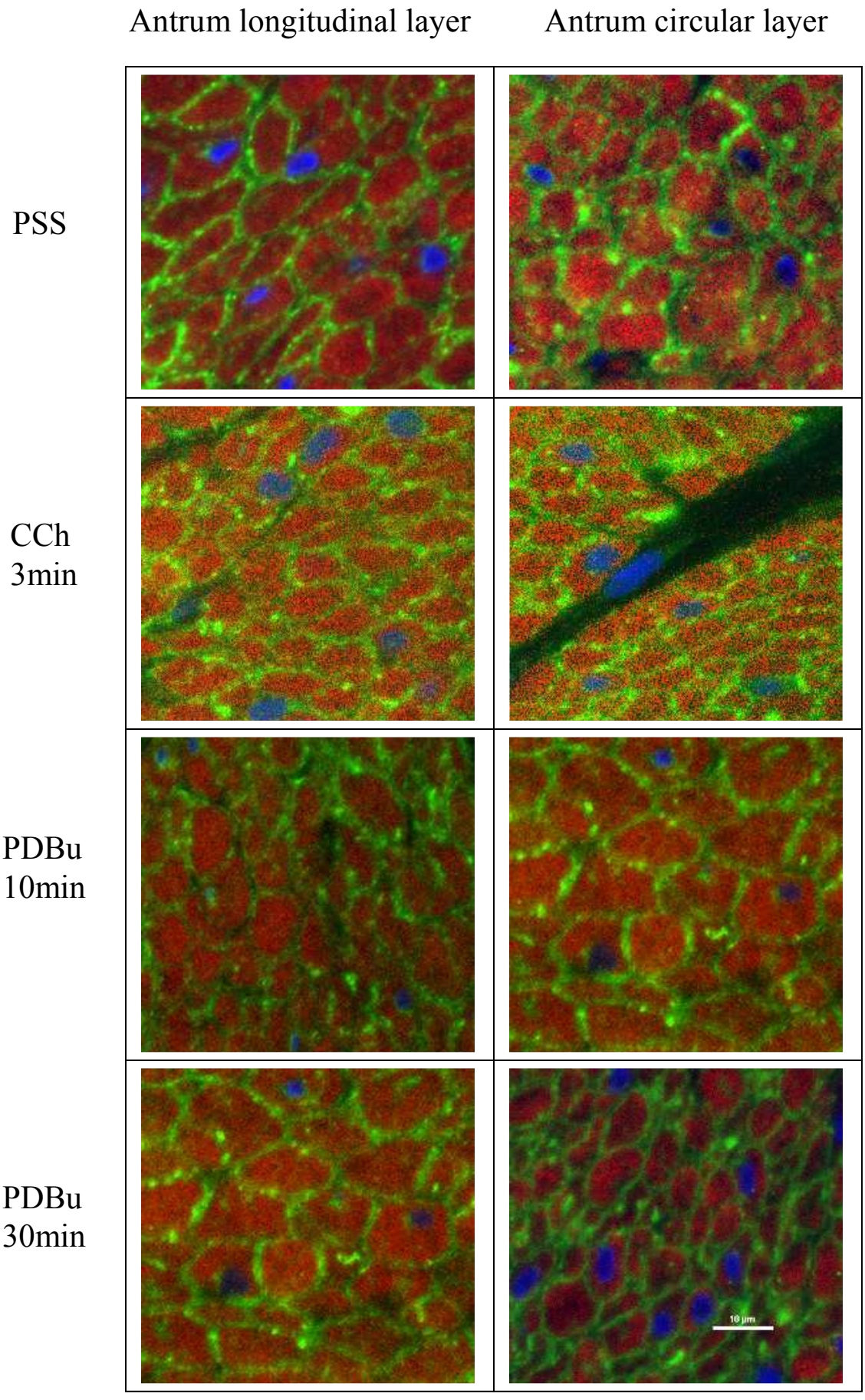

Antrum longitudinal layer
Antrum circular layer
PSS
CCh 3min
PDBu 10min
PDBu 30min
10 µm

Figure 3.5. Confocal images of PKCα distribution in transverse sections of the longitudinal (left) and circular layer (right) of pig stomach antrum under relaxed (PSS) or stimulated (3min CCh or 10min & 30min PDBu) treatments. Tissues were immunoreacted for PKCα (green) and counterstained for phalloidin (red) and DAPI (blue). In all conditions, PKCα is located predominantly at the cell periphery near the plasma membrane. Scale bar - 10μm.

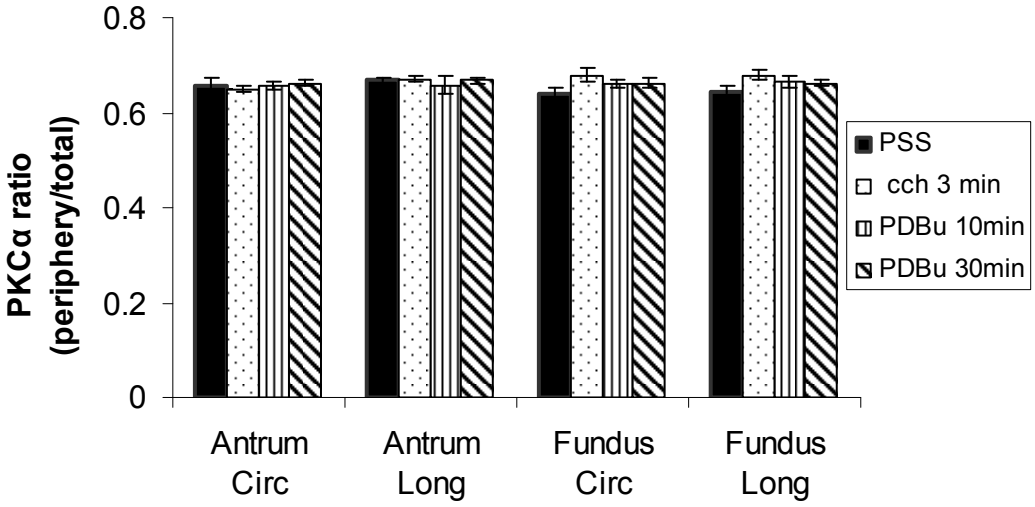


Figure 3.6. Quantitative results of the ratio of the PKCα at the cells periphery to the total PKCα (peripheral + cytosolic) in the circular and longitudinal layers of the antrum and fundus. The ratio in relaxed condition (PSS) in both layers of antrum and fundus is significantly greater than 0.5, indicating a preferential distribution of PKCα near the plasma membrane in the relaxed condition. This ratio does not change significantly with different stimulation treatments, suggesting that PKCα maintains a primarily peripheral distribution in the cell at all times. (n=5)

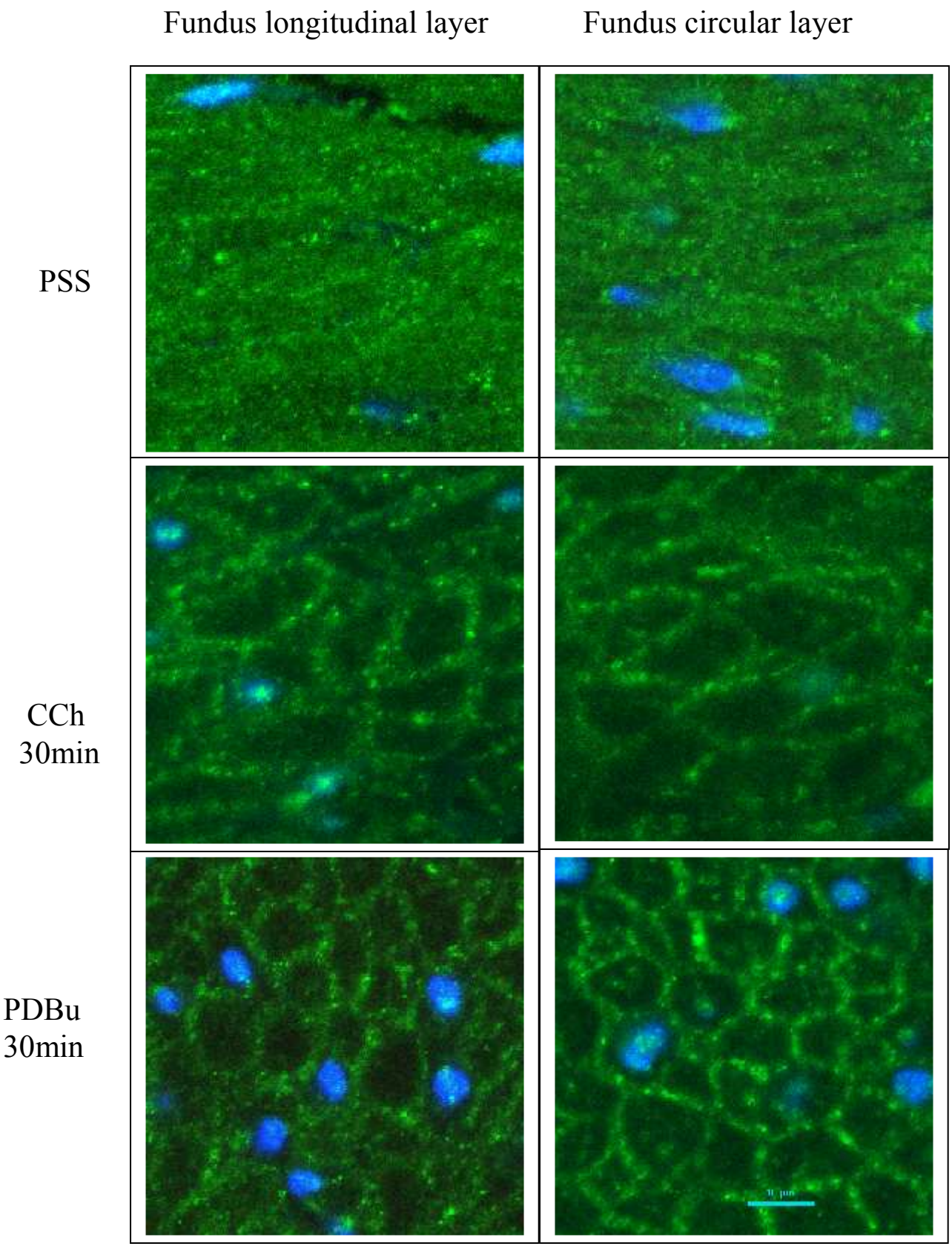

Fundus longitudinal layer
Fundus circular layer
PSS
CCh
30min
PDBu
30min

Figure 3.7. Confocal images of CPI-17 distribution in transverse sections of the longitudinal (left) and circular layer (right) of pig stomach fundus under relaxed conditions (PSS) or stimulated (CCh 30min or PDBu 30min) treatments. Tissues were immunoreacted for PKC (green) and DAPI (blue). In relaxed condition (PSS), CPI-17 appears "uniformly" distributed in the cell, but with either CCh or PDBu stimulation, CPI-17 appears predominantly located at the periphery near the plasma membrane. Scale bar - 10μm.

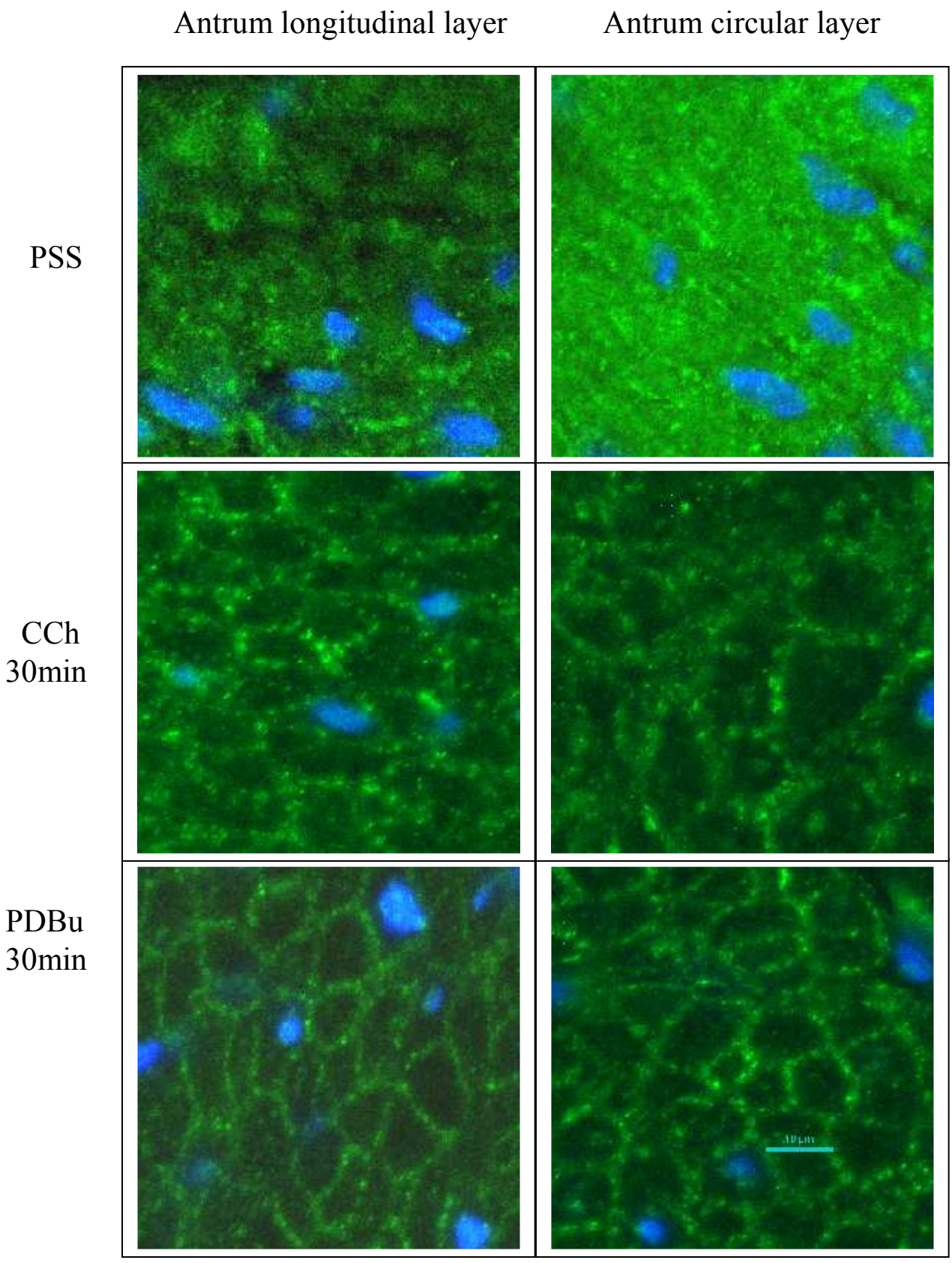

Antrum longitudinal layer
Antrum circular layer
PSS
CCh 30min
PDBu 30min

Figure 3.8. Confocal images of CPI-17 distribution in transverse sections of the longitudinal (left) and circular layer (right) of pig stomach antrum under relaxed conditions (PSS) or stimulated (CCh 30min of PDBu 30min) treatments. Tissues were immunoreacted for PKC (green) and DAPI (blue). In relaxed condition (PSS), CPI-17 appears "uniformly" distributed in the cell, but with either CCh or PDBu stimulation, CPI-17 appears predominantly located at the periphery near the plasma membrane. Scale bar - 10μm.

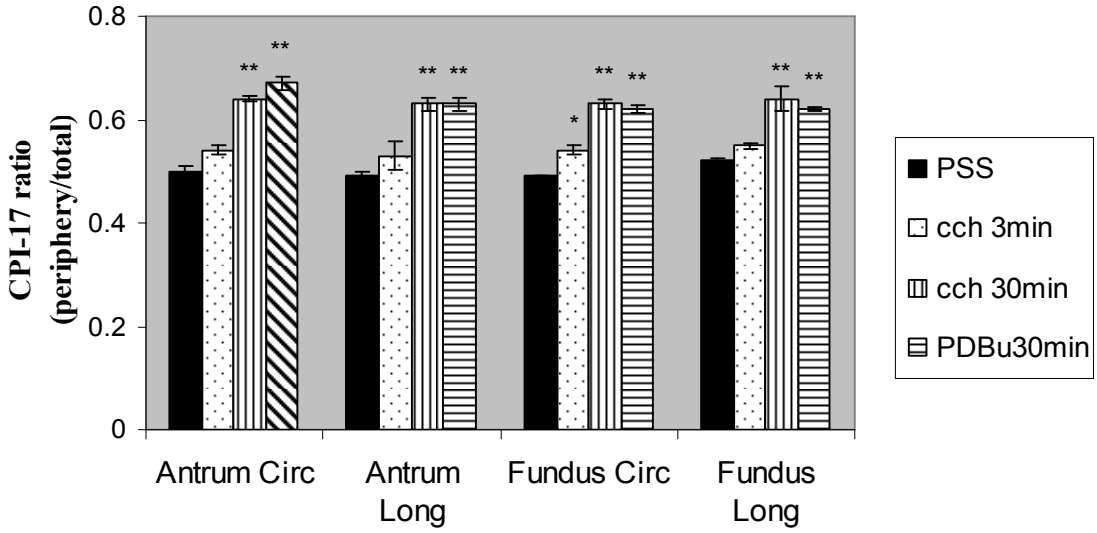
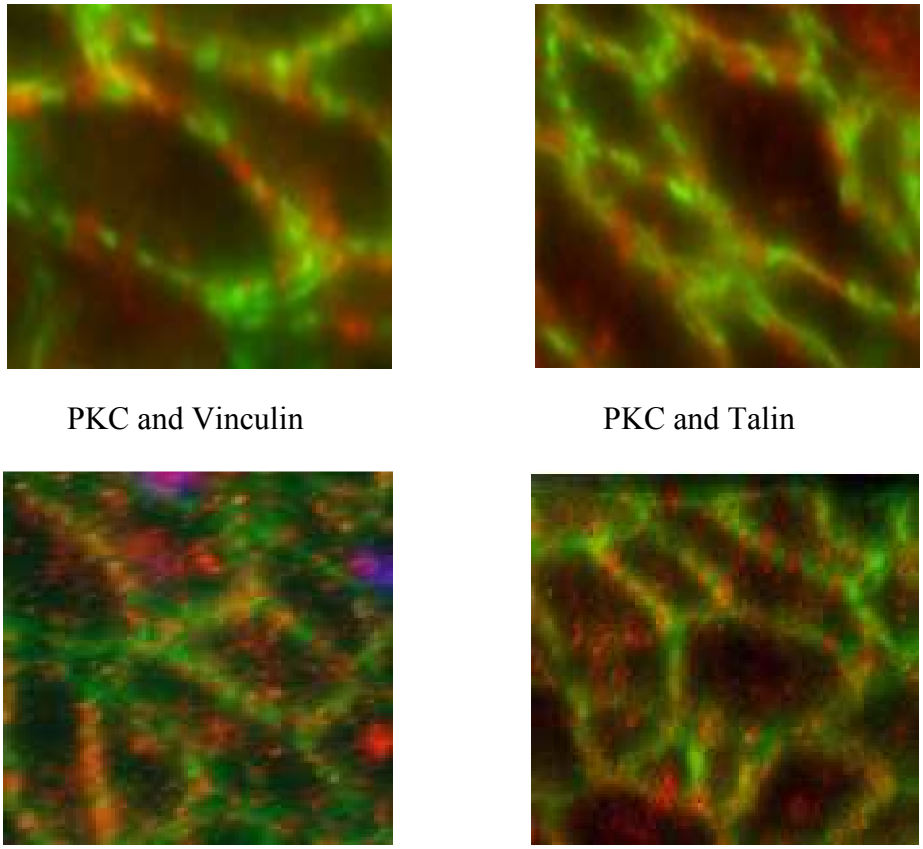


Figure 3.9. Quantitative results of the ratio of the CPI-17 at the cells periphery to the total CPI-17 (peripheral + cytosolic) in the circular and longitudinal layer of the antrum and fundus. The ratio in the relaxed condition (PSS) in both layers of antrum and fundus is not significantly different than 0.5, indicating a uniform distribution of CPI-17 throughout the cells. Three minutes of CCh stimulation does not change the ratio of CPI-17 except in the fundus circular layer. Both CCh (30min) and PDBu (30min) treatments cause a significantly redistribution of CPI-17 to the cell periphery near the plasma membrane (ratios are significantly greater than 0.5). * $p<0.05$, ** $p<0.01$ (n=3)



PKC and Vinculin

PKC and Talin

CPI-17 and Vinculin

CPI-17 and Talin

Figure 3.10. Confocal images of pig Fundus in relaxed condition (top) and with PDBu (30min) stimulation (bottom). Double labeling for PKCα (green) with vinculin (red, upper left) or talin (red, upper right) or CPI-17 (green) with vinculin (red, lower left) or talin (red, lower right). Vinculin and talin are adherens junction associated proteins that appear to have an alternating distribution with PKC and CPI-17, suggesting that these proteins are not localized to the same domains near the plasma membrane. Note that not all of the PKCα or CPI-17 is located near the plasma membrane.

CHAPTER IV

Discussion

Smooth muscle contraction is a complex activity, which involves multiple factors including contractile, cytoskeletal and regulatory proteins. Many questions regarding the molecular pathways participating in the contraction mechanism are still unclear. So far, several regulatory pathways have been reported to control smooth muscle contraction. In all of these pathways, Ca^{2+} is considered to be a primary factor, and elevating $[Ca^{2+}]_i$ will enhance force generation through the activation of MLCK. As a "suppressor", MLCP also plays a significant role in controlling the smooth muscle contraction. Several pathways can regulate MLCP and this regulation plays a key role in determining contractile force in smooth muscle (Ito et al., 2004; Hartshorne et al., 1998; Somlyo AP & Somlyo AV, 2003; Pfitzer G, 2001). In fact, the inhibition of MLCP activity is important for controlling Ca^{2+} sensitization (Kitazawa & Somlyo, 1991b; Somlyo AP & Somlyo AV, 2003).

Studies on PKC distribution

One of the proposed pathways to regulate MLCP is PKC dependent and messengers within the process include GPCR, PLC, DAG, PKC and CPI-17. Numerous publications have reported on the role of this pathway in regulating force production in smooth muscle. However, studies on the spatial distribution and relative movement of PKC and CPI-17 remain controversial. The debate focuses on how the signal is transferred from DAG, which is located primarily at the cell membrane, to myosin (MLC_{20}) which can be detected throughout the cytosol (Parisi & Eddinger,

2002). To explain the signal transduction process, many hypotheses have been developed. One hypothesis suggests that PKC works as a moving messenger to relay the signal from the membrane to the cytosol. PKC, a member of a large family of serine/threonine kinases, was first identified in 1977 by Nishizuka's group (Inoue et al., 1977; Takai et al., 1977). The first study on PKC localization by Kraft suggested that in EL4 mouse thymoma cells, the high level of PKC concentration within the cytosol could be reduced after phorbol ester stimulation (Kraft & Anderson, 1983). Following this study, numerous research groups reported translocation of PKC isoforms during certain cell activities using different techniques. The ability of PKC to move within a cell was further studied in the following three decades (Miyamoto et al., 1995; Nakamura & Nishizuka, 1994; Secrest et al., 1991).

Biochemical analysis of PKC translocation

Much of the evidence supporting the translocation of PKC during smooth muscle cell activation was obtained using biochemical studies. By using cellular fractionation on smooth muscle tissue, Diamond's group explored PKC distribution within the bovine tracheae. They tested the enzymatic activity of generic PKC from membrane and cytosolic fractions to infer the distribution of the protein (Langlands & Diamond, 1992; Langlands & Diamond, 1994). Their results suggest that PKC activity is higher in the cytosolic fraction than the membrane fraction under relaxed conditions while after methacholine stimulation, the situation was reversed. This activity shift was used as evidence for PKC translocation in their study. However, a change of PKC enzymatic activity is not a direct way to determine the distribution

shift of PKC and the two are not necessarily consistent with each other. Agonists could simply increase PKC enzymatic activity in a specific region of the cell without transferring additional PKC molecules from one location to another. To further explore this issue and elucidate possible PKC movement within cells, western blotting was used to study the PKC protein distribution in the bovine tracheae. It has been shown that in relaxed conditions, the concentration of PKCα and PKCβ in the pellet (membrane) fraction is the same as in the supernatant (cytosolic) fraction (the ratio of pellet to supernatant is ~1), while upon stimulation with PDBu, the ratio increased to ~3. This result suggests that administration of PDBu leads to the translocation of PKCα/β from the cytosol to the membrane (Hai & Kim, 2005). In addition, PKC translocates from the cytosol to the membrane in Rat Stomach Fundus upon PDBu treatment with similar methods (Secrest et al., 1991). However, it is possible that the homogenization process used might alter the protein distribution between these fractions, and may not distinguish between membrane fractions (plasmalemmal, nuclear, mitochondrial, SR). Direct observation of the PKC distribution in cells using immunohistiochemical reactions allows direct visualization of protein location and distribution.

Analysis of PKC tanslocation in isolated smooth muscle cells

Immunofluorescence microscopy provides a chance to observe potential PKC movement within cells directly. Freshly isolated smooth muscle cells were used as a model to study translocation of PKC using immunocytochemistry. To obtain isolated single cells, collagenase and elastase were used to digest rat uterine smooth muscle

(Taggart et al., 1999). Isolated single cells were subjected to fixation and immunostaining and then observed with confocal microscopy to determine if the PKC distribution shifts upon different agonist stimulation. The results from this study showed that PKCα underwent a process of relocation from the cytosol to the plasma membrane after either CCh or phorbolester12-deoxyphorbol13-isobutyrate20-acetate (DPBA) incubation. The average ratio of PKC between peripheral to cytosolic was 1.05 with relaxed conditions but increased to 2.09 following CCh stimulation. DPBA activation produced an even bigger shift of PKC distribution and the ratio was 2.77. It also has been reported that in relaxed rat arterial smooth muscle single cells PKCα was uniformly distributed throughout the cytosol, but after incubation with uridine triphosphate (UTP), PKCα moved towards and accumulated adjacent to the plasma membrane (Nelson et al., 2008). Similar changes in isolated rat colon smooth muscle cells after ceramide stimulation have also been shown (Ibitayo et al., 1999). Cultured cells were also used to determine the distribution of PKC with cell activation. Translocation of PKC from the cytosol to cell membrane in response to PDBu in cultured smooth muscle cells has been suggested (Li et al., 2002). At the same time, by using western blotting in isolated single cells, a concentration change of PKC in the particulate fraction of digested rabbit rectosigmoid smooth muscle cells before and after ceramide activation has also been observed (Bitar et al., 2002). However, while their results were statistically significant, the total ratio change was less than 5%. The relevance of such a small shift causes one to question the importance of such a translocation of PKCα with a physiological function. On the other hand, there is also evidence to support a movement of PKC in the opposite direction. Fay's group

reported that in isolated toad stomach single cells, a pool of activated PKC was identified adjacent to the plasma membrane. Upon stimulation with CCh, activated generic PKC was released from the pool and redistributed throughout the cytosol. Further study showed that PKC became associated with contractile filaments after the treatment (Meininger et al., 1999).

Since freshly isolated or cultured smooth muscle cells are easy to obtain and handle, this experimental model is very popular and widely used. However, studies suggest that the phenotype of isolated or cultured smooth muscle cells are not necessarily the same as smooth muscle cells within tissues or organs. When smooth muscle cells are extracted from intact tissue, structure and functionality can be altered compared with that of cells *in vivo* (Owens, 1995). Cultured cells can also develop properties different from when they are associated in their *in vivo* condition. While smooth muscle cells *in vivo* show a contractile phenotype, isolated and cultured smooth muscle cells change with time to a synthetic phenotype. Results obtained from experiments of isolated single cells might be a consequence of alterations of cell structure and protein expression from the cell isolation procedure and/or from culturing. To eliminate potential changes that can occur in isolated cells compared with multicellular preparations, intact tissues were used in this study to determine PKC distribution in smooth muscle cells during relaxation and contraction. Surprisingly, the PKC distribution was not “uniform” throughout the cell in resting conditions and was not changed upon stimulation in intact tissues. PKC was primarily distributed adjacent to membrane, before and after PDBu or CCh treatment (Fig. 3.4

& 3.5). The pattern of PKC distribution was measured at multiple time points and it was always primarily at the cell periphery. Since PKC is a fairly large protein, it seems logical and efficient for the cell to keep it restricted to one place rather than to have it moving within the cell as a means of regulation.

PKC binding proteins in relaxed condition

It is easy to understand the attachment of PKC to cell membrane under stimulation since DAG is generated during the process. Newly produced DAG is membrane bound and it could work as a bridge to link PKC to the cell membrane after PKC is activated. However the accumulation of PKCα at the cell periphery under resting condition observed in this study raises a question about the primarily peripheral distribution of PKCα when DAG is absent. Since there is no evidence to support PKC as a membrane embedded protein, it is reasonable to speculate other membrane associated proteins binding to PKC under resting conditions and preventing it from randomly diffusing throughout the cell. Several proteins have been proposed to have the ability to hold PKC adjacent to cell membrane. The first two candidates, talin and vinculin were identified in the focal contact domain in nonstimulated fibroblasts and in cultured renal cells. It was reported that in cultured cells, PKCα could also be detected in focal contact structures and might bind to talin or vinculin (Dong et al., 1993). In this study, immunohistochemical methods were used to determine the relationship between PKCα and these two candidate proteins under more physiological conditions. Double labeling of PKC with talin or vinculin in intact swine stomach tissues (this study) shows a lack of association between PKC

and talin or vinculin suggesting that PKCα is not associated with the adherens junction. The PKCα appears near the plasmamembrane in an alternate punctuate pattern with either vinculin or talin (Fig. 3.10). Previous studies in our lab and others show that talin and vinculin are distributed alternately with caveolae at the plasma membrane (Eddinger et al., 2007; North et al., 1993; Tanaka et al., 2001). Thus it is possible that PKCα is associated with caveolae as it does not colocalize with talin and vinculin at the adherens junction. It is also possible that it moves from one of these domains to the other during cell activation.

Several other studies suggest PKC could be a resident protein in a membrane structure called caveolae (Smart et al., 1994; Smart et al., 1995). Studies have identified multiple caveolae associated proteins as having the potential to bind to PKC. For instance, a 68kD PKCα binding protein termed serum deprivation response (sdr) was localized in caveolae (Mineo et al., 1998). Taggart et al also reported that PKC*a* has a particular amino acid sequence (522WAYGVLLY528) in the catalytic domain which has the potential of interacting with the scaffolding domain of Caveolin-1, a resident protein in caveolae (Taggart et al., 2000). The results from in vitro assays suggested caveolin-1 has an inhibitory effect on PKC. Sucrose gradient centrifugation also showed Caveolin-1 and PKC were accumulated in the same fraction, which could indicate an interaction between the two proteins (Oka et al., 1997).

Membrane receptors termed RICKs and RACKs were also proposed to be able to interact with inactivated or activated PKC. (Mochly-Rosen, 1995; Mochly-Rosen & Gordon, 1998; Mochly-Rosen et al., 1991). Their function is expected to maintain PKC in distinct locations on or near the cell membrane. One of these scaffolding proteins, AKAP150, has been reported to be able to target PKC to unique plasmalemmal domains where L-type Ca^{2+} channels are located. The contact between PKC and Ca^{2+} channels was suggested to be required for generating constitutive Ca^{2+} influx (Santana & Navedo, 2009). This is consistent with the observation that a population of L-type Ca^{2+} channels is localized to caveolae in ventricular myocytes since PKC was also expected to reside in the same location (Balijepalli et al., 2006). It seems logical to propose that PKC is anchored by caveolae associated protein to the cell membrane where it can be activated by DAG. Our data showing that PKCα does not co-localize with vinculin or talin is consistent with this idea.

Studies on CPI-17 distribution

While many membrane bound proteins were proposed to be able to hold PKC to the cell periphery, no candidate protein has been reported to bind CPI-17, the immediate downstream effecter of PKC, at the plasma membrane under resting conditions. Since PKC is supposed to interact with CPI-17 only when it is activated, CPI-17 is expected to be uniformly distributed throughout the cell in the absence of stimulation. Result from this study confirmed a “uniform” distribution pattern of CPI-17 in stomach SMCs under relaxed condition. Unlike PKC, CPI-17 is only 17 KD and should easily move throughout the cell due to its fairly small size. Thus, it is

reasonable to propose a translocation of CPI-17 from the cytosol to the membrane upon stimulation. Activated PKC could act to sequester free CPI-17 from the cytosol and thus causes a passive translocation of CPI-17 from the cytosol to the membrane. The spatial-temporal regulation on CPI-17 in bronchial smooth muscle of rats under acetylcholine (ACh) stimulation using biochemical methods has been examined (Sakai et al., 2005). Immunoblotting experiments revealed a time-dependent shift of CPI-17 from the cytosol to the membrane upon stimulation. The movement started after 2 minutes of incubation with ACh and lasted for at least 18 minutes. To further study the possible translocation of CPI-17 and eliminate potential concerns introduced by cellular fractionation, this study examined CPI-17 movement in stomach antrum and fundus in intact tissue using Immunofluorescence microscopy. The results suggest that after stimulation with CCh or PDBu, the "uniform" distribution of CPI-17 was altered and the protein moved towards the membrane in a time dependent manner. This is consistent with the observation reported by Sakai et al (Sakai et al., 2005). Our study also revealed a punctuate distribution of CPI-17 at the membrane after stimulation. This suggests CPI-17 specifically accumulates to a particular membrane location. Considering CPI-17 is activated by PKC on the membrane, and PKCα appears to localize with caveolae, it is logical to propose that CPI-17 migrates specifically to caveolae upon stimulation. To eliminate the possibility that CPI-17 was associated with the adherens junction, co-localization between CPI-17 and talin/vinculin was also examined in this study. Double labeling was performed on CPI-17 and talin/vinculin which are resident proteins at the adherens junction. The results show an alternating punctuate distribution between

CPI-17 and talin/vinculin. Based on this observation, it is likely that CPI-17 and PKC colocalize at the caveolae on the cell membrane. While CPI-17 has been observed to translocate from the cytosol to the membrane upon stimulation (Fig. 3.7 & 3.8), there is still 40% CPI-17 remaining in the cytosol (Fig. 3.9). The distribution of CPI-17 was parallel with actin in human pulmonary artery endothelial cell suggesting a co-localization of CPI-17 and actin (Kolosova et al., 2004). Thus it is possible that when CPI-17 is in the cytosol, it associates with actin filaments. Moreover, a certain amount of CPI-17 has been located adjacent to the nucleus. This is consistent with the observation that PKCα can translocate to the perinuclear region and into the nucleus in vascular smooth muscle cells under stimulation (Haller et al., 1998).

Signal transduction between CPI-17 and MLCP

As one of the major regulatory mechanisms of Ca^{2+} sensitization, the PKC/CPI-17 pathway is only effective when it is able to block the downstream MLCP, which in turn lessens the response of the smooth muscle cell to Ca^{2+}. MLCP is reported to be diffusely distributed throughout the cytosol (Shin et al., 2002). If PKC is activated and localized at the membrane and CPI-17 translocates to the membrane to be activated by PKC, then a link between the peripheral CPI-17 and diffusely distributed MLCP needs to be identified. Several possible ways can be proposed to explain the signal transduction between CPI-17 and MLCP. One of them is that CPI-17 is translocated back from membrane to the cytosol after activation and interacts with MLCP in the cytosol. If this is the case, it is a slow process, since

CPI-17 was localized primarily at the cell membrane for at least 30 minutes after CCh/PDBu stimulation (Fig. 3.7& 3.8). A second possible explanation would be that there is another protein involved in this pathway, working as a bridge between CPI-17 and MLCP. However, studies have shown that CPI-17 has the ability to directly inhibit MLCP, suggesting that it is unlikely for an additional messenger molecule to exist (Eto et al., 2004). A third possibility is that MLCP translocates upon stimulation from the cytosol to CPI-17 at the membrane and gets inactivated or is simply kept away from its substrate, MLC_{20} (Shin et al., 2002; Ogut & Brozovich, 2003). However, the fact that permeabilized smooth muscle cells do not seem to lose MLCP, suggests that MLCP is not a freely diffusing molecule in the cytosol. Other soluble proteins such as CPI-17 tend to diffuse out of cells when they are permeabilized (Eto et al., 1995; Eto et al., 1997). Thus, it seems unlikely for MLCP, if it is a bound protein in the cytosol, to diffuse freely towards the cell membrane. A more likely possibility would be that CPI-17 stays at the membrane after activation and inhibits only the MLCP localized near the cell periphery. This is consistent with the observation that $[Ca^{2+}]_i$ is often elevated near the cell membrane region (Jonas & Zelck, 1974; Chen et al, 1991; Poburko et al, 2004). Since the main function of the PKC/CPI-17 pathway is to control Ca^{2+} sensitization of smooth muscle cells, it would be more effective for the regulation to be applied where $[Ca^{2+}]_i$ is high.

Differences between tonic and phasic smooth muscle

This study revealed that the PKC/CPI-17 pathway might not be equally significant for different types of smooth muscle in regulating contraction activity.

While PDBu caused a slow contraction in the fundus which is ~40% of the KPSS induced peak force, little contraction was generated in the antrum with PDBu stimulation. Since fundus and antrum represent two basic types of smooth muscle, tonic and phasic respectively, it seems logical to ascribe the difference in force generation to alternate distinct properties of phasic and tonic muscle. The expression level of CPI-17 in tonic smooth muscle is higher than that in phasic subtypes (Woodsome et al., 2001). This could be used to explain the different ability of tonic muscles to maintain force (CPI-17 inhibits MLCP allowing MLC_{20} phosphorylation to stay high and force to be maintained), but their study was limited to rabbit vascular muscle. This study examined CPI-17 expression in the visceral swine stomach. Surprisingly, no significant difference in the protein expression level of CPI-17 was detected between the fundus and antrum (Fig. 3.2). This result indicates that the difference between tonic and phasic smooth muscle in responding to PDBu treatment cannot be explained by CPI-17 level in all types of smooth muscle tissues. Measurement of PKC expression level with western blotting performed in this study also revealed no significant difference between tonic and phasic smooth muscle (Fig. 3.2). Immunostaining confirmed the similarity between the two muscle types for CPI-17 and PKC expression. Thus, alternative explanations are required. One possibility is that the different responses to PDBu were caused by differences in basal Ca^{2+} level between tonic and phasic smooth muscle. It was shown that tonic tissues have a higher level of $[Ca^{2+}]_i$ than phasic tissues under relaxed conditions (Himpens et al., 1988). It is possible that although PDBu administration could boost Ca^{2+} sensitization in phasic smooth muscle to a similar level as in tonic tissues, the low basal level of

$[Ca^{2+}]_i$ might still not be able to maintain a strong contraction in phasic tissues. The differences of SR distribution in the two types of tissues may be responsible for differences in the $[Ca^{2+}]_i$ in basal conditions (Nixon et al., 1994). A second possibility would be that expression patterns of MLCP are different between tonic and phasic smooth muscle and this difference leads to differences in contraction to PDBu stimulation. This is supported by the observation that phasic smooth muscle contains more MLCP than tonic tissues (Woodsome et al., 2001). The above explanations are not necessarily exclusive to each other and the cause for differences in force generation may be the combination of the two.

Conclusion

As a tissue responsible for the contractility of all hollow organs, smooth muscle is under intensive study since it is important for maintenance of the normal function of many vital systems in organisms. In addition, many diseases are related to defects in the regulation of smooth muscle contraction. Elucidation of the regulatory mechanism for smooth muscle contraction under physiologically relevant conditions is therefore very important. As a major regulatory pathway in smooth muscle contraction, the PKC/CPI-17 signal transduction cascade requires thorough examination in physiologically relevant conditions. This study is the first to directly assess the expression and spatial- temporal regulation of the PKC/CPI-17 pathway in intact animal tissues. Immunostaining was used to determine the distribution patterns of PKC and CPI-17. In contrast with earlier studies, which were performed with isolated/cultured SMCs, this study reports that with or without smooth muscle cell

activation, PKCα is always preferentially localized near the plasma membrane, where it does not co-localize with vinculin or talin and thus is most likely associated with the caveolae (Fig. 4.1). Upon cell excitation, it is likely that the activated PKC acquires the ability to sequester free CPI-17 from the cytosol to the plasma membrane and therefore induce a shift of CPI-17 from the cytosol to the cell membrane. Based on the lack of co-localization of CPI-17 with vinculin/talin, I propose that CPI-17 migrates and attaches to PKCα at the caveolae with stimulation. Membrane associated CPI-17 is reported to be activated by PKC and blocks the activity of MLCP. We were unable to observe CP-17 shift back to the cytosol in a physiologically relevant time frame, and thus propose that it only inhibits MLCP located in close proximity to the cell periphery (Fig. 4.2). This inhibition would lead to an increased Ca^{2+} sensitization and increased force generation in tonic smooth muscle. However, PDBu has little effect on phasic smooth muscle, which is not caused by expression variances of PKC and CPI-17. Differences in response to PDBu between the two tissues may be caused by differences of MLCP expression and/or basal $[Ca^{2+}]_i$ levels.

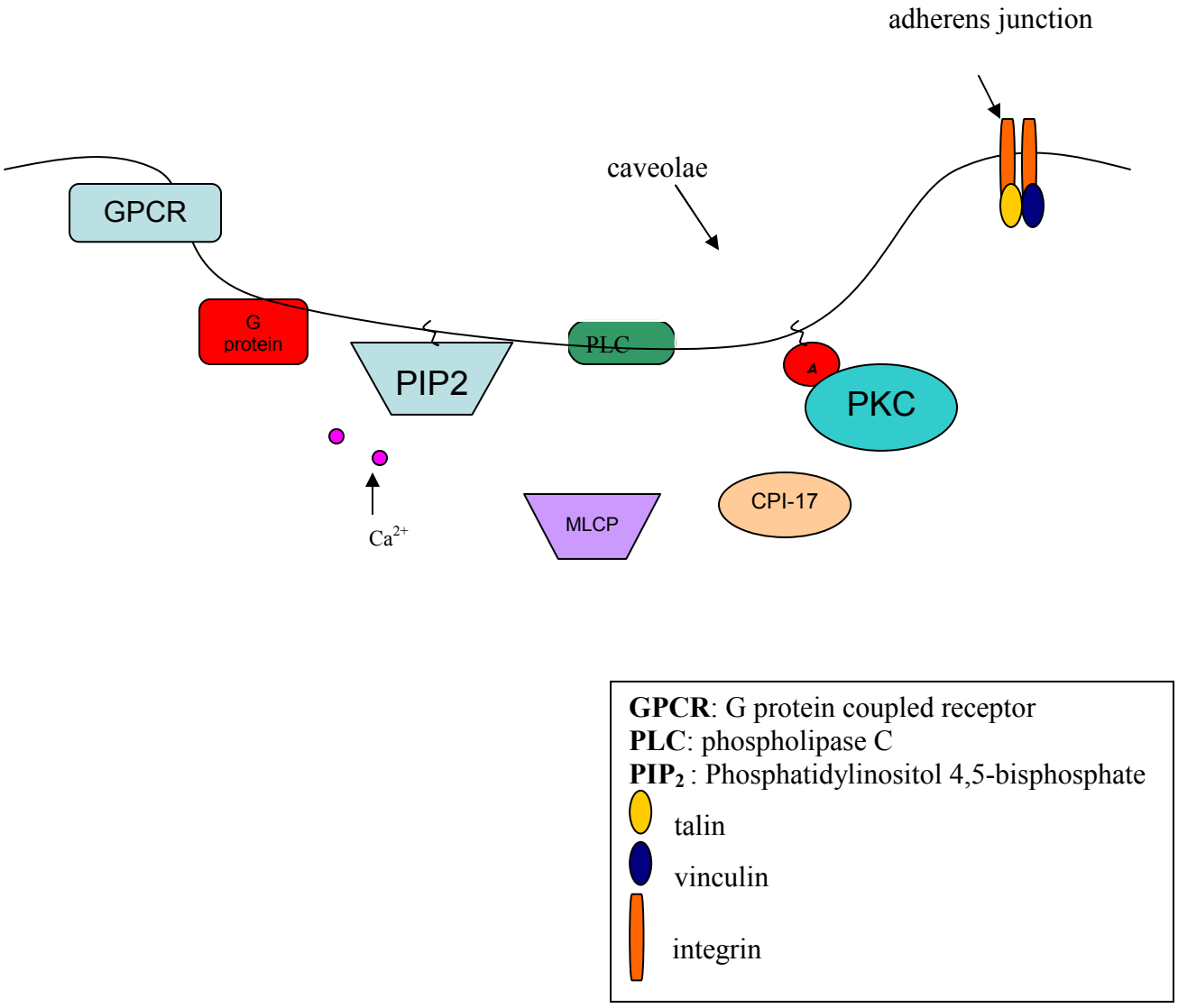


Figure 4.1. A model for PKC/CPI-17 localization under relaxed condition. Without agonist sitmulation, PKCα binds to one or more caveolae resident proteins. In this figure, "A" is used to represent one of such proteins. CPI-17 and MLCP are diffusely distributed in the cytosol under these conditions.

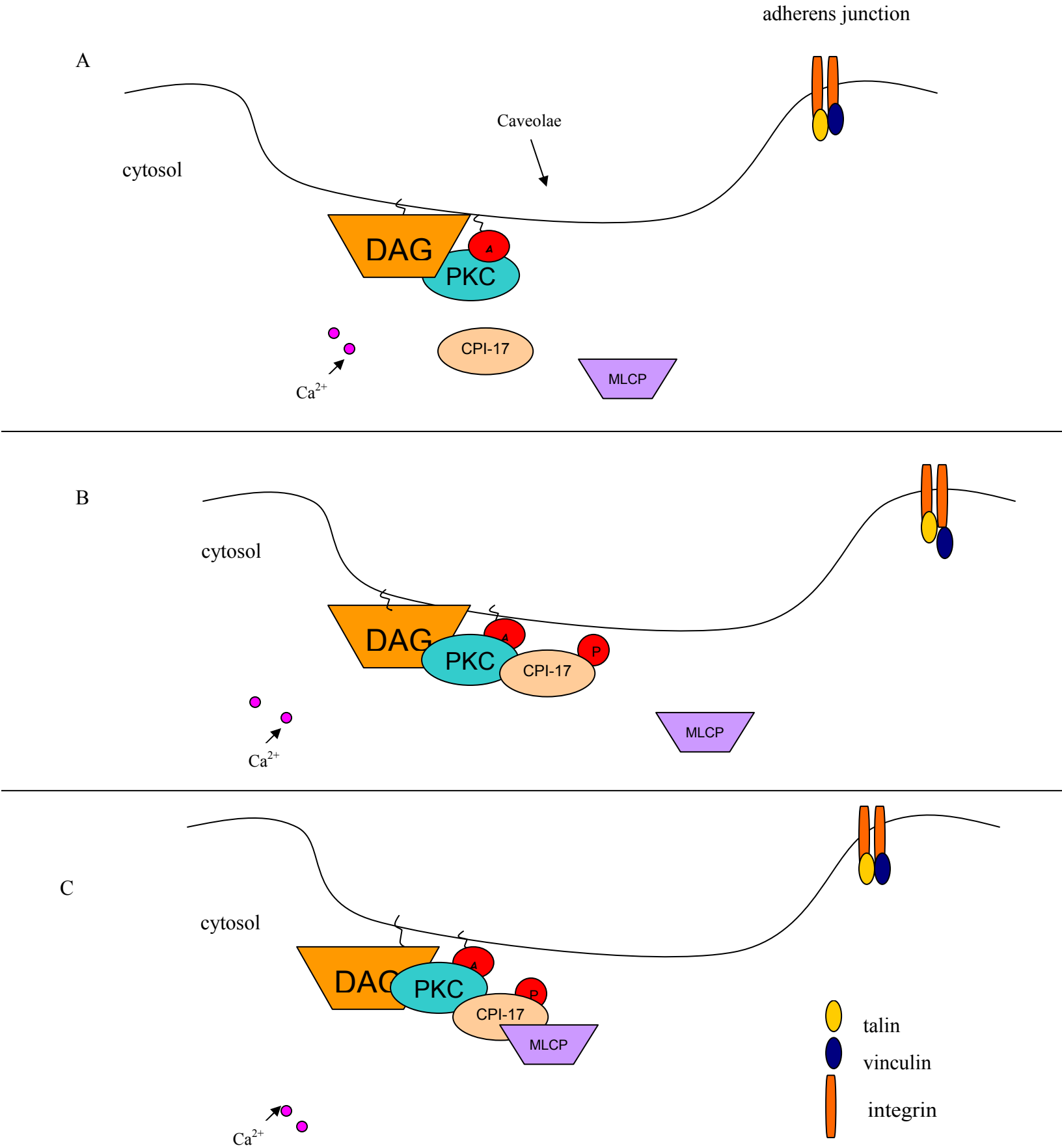

adherens junction
A
Caveolae
cytosol
DAG
A
PKC
CPI-17
MLCP
Ca2+
B
cytosol
DAG
A
PKC
P
CPI-17
MLCP
Ca2+
C
cytosol
DAG
A
PKC
P
CPI-17
MLCP
Ca2+
talin
vinculin
integrin

Figure 4.2. A model for the spatial distribution of PKCα and CPI-17 under stimulated condition. With agonist stimulation, GPCR have the ability to activate G proteins. G proteins can significantly boost PLC activity, which hydrolyses PIP_2 into inostitol IP_3 and DAG. DAG remains in the cell membrane. PKCα is activated through binding to DAG (A). Activated PKC acquires the ability to sequester free CPI-17 from the cytosol and therefore induces a movement of CPI-17 from the cytosol to the cell membrane (B). PKC bounded CPI-17 gets activated by PKCα and further blocks the activity of MLCP located adjacent to cell periphery and enhances smooth muscle contraction by decreasing the cytosolic free MLCP (C).

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