ABSTRACT

CHILCOAT, CLAYTON DOUGLAS. Protein Kinase A Regulates β2 Integrin Avidity Activation and Subsequent Neutrophil Activation via Modulation of Myosin Light Chain Kinase. (Under the direction of Wayne A. Tompkins and Samuel L. Jones)

β2 integrins are adhesion molecules on the surface of neutrophils. Avidity activation of β2 integrins includes transportation of pre-formed integrins to the cell surface and a conformational change in the integrin to a high-binding state. Upon binding ligand, β2 integrins initiate a signaling cascade that results in activation of the neutrophil to a pro-inflammatory state, and the inhibition of this signal can prevent further activation of the neutrophil. cAMP and it effector protein kinase A (PKA) exert a generally inhibitory effect upon neutrophil activation. PKA has been shown to inactivate myosin light chain kinase (MLCK). Myosin light chain (MLC) phosphorylation is crucial for actin-myosin complex formation, which is required for stability and contraction of the actin cytoskeleton in neutrophils as well as β2 integrin-dependent adhesion. We hypothesize that the inhibitory effect of PKA upon neutrophils is due to inhibition of β2 integrin avidity activation resulting in the subsequent inhibition of neutrophil activation. Furthermore we hypothesize that the effect of PKA upon β2 integrin avidity activation is mediated through PKA's effect upon MLCK. We demonstrate that inhibition of PKA induces β2 integrin-dependent adhesion and that augmentation of cAMP prevented β2 integrin-dependent adhesion and subsequent respiratory burst activity. Further, we demonstrate via flow cytometric detection of antibodies directed against β2 integrins that pharmacologic inhibition of PKA activity results in overall increased β2 integrin expression on the neutrophil surface, as well as increased expression of the activated form of the integrin. This upregulation and activation of β2 integrins due to inhibition of PKA is abolished by pharmacologic MLCK inhibition. Inhibition of MLCK also blocked β2 integrin-dependent neutrophil adhesion achieved by inhibition of PKA, as well as neutrophil migration along towards a PKA inhibitor. These findings demonstrate that PKA regulation of β2 integrin affinity activation and subsequent neutrophil activation is via an MLCK-dependent pathway.

Protein Kinase A Regulates β2 Integrin Avidity Activation and Subsequent Neutrophil Activation via Modulation of Myosin Light Chain Kinase

by

Clayton D. Chilcoat

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Approved by:

_____	_____
Dr. Scott M. Laster	Dr. Edward A. Havell
_____	_____
Dr. Wayne A. Tompkins Chair of Advisory Committee	Dr. Samuel L. Jones Co-Chair of Advisory Committee

BIOGRAPHY

Clayton D. Chilcoat was born on September 10, 1969 in Silver Spring, Maryland. He attended Virginia Polytechnic Institute and State University where obtained a Bachelor of Science degree in Biochemistry in 1991 and a Doctorate of Veterinary Medicine in 1996. He then moved on to Colorado State University where he completed an internship in equine medicine in 1997, followed by a residency in large animal internal medicine at Purdue University which he completed in 2000. Upon completion of his residency, Clayton began his immunology training at North Carolina State University with Dr. Samuel L. Jones, where he studied the role of β2 integrins in the regulation of neutrophil activation. Upon completion of his doctoral training, Clayton will pursue post-doctoral research at North Carolina State University with Dr. Wayne Tompkins studying the role of regulatory T lymphocytes in feline immunodeficiency virus infection as a model of human immunodeficiency virus infections.

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INTRODUCTION

The innate immune system plays a crucial role in the defense of the host against infectious agents. The primary cell type of the innate immune system is the neutrophil or "polymorphonuclear cell" (PMN), which circulate through the bloodstream in a quiescent, monitoring state. When the PMN detects a pro-inflammatory signal, it leaves the circulation and begins its journey to the site of inflammation within the tissue. When the PMN arrives at the site of inflammation it becomes activated to an aggressive, pro-inflammatory phenotype. In this state, the activated PMN can engulf invading organisms via phagocytosis and can release toxic substances such as proteases and reactive oxygen species to destroy the pathogens still free in the affected tissue. Given the destructive potential of PMN, strict regulation of the activation of these cells is required to prevent unnecessary tissue destruction as well as a means to resolve inflammation once the pathogenic threat has been eliminated. One form of regulation acts through the adhesion molecules known as integrins.

Integrins are heterodimeric glycoprotein cell surface adhesion molecules found on a myriad of cell types and they play a key role in the attachment of the cell to other cells and to the extracellular matrix. The most common integrins on the surface of PMN belong to the β2 class of integrins, and they exist in either a state of low or high affinity for ligand. While the PMN is in its quiescent state in the circulation, the β2 integrins are in the low affinity state. If the PMN encounters a pro-inflammatory signal, the β2 integrins will cluster upon the cell surface and become activated to a high affinity state which allows subsequent binding to their specific ligands. This up-regulation of integrins

is referred to as “avidity activation”. This avidity activation provides a greatly increased integrin-ligand interaction which is crucial for the extravasation and migration of PMN to the inflamed tissue. The intracellular biochemical signals which result from the reception of the pro-inflammatory mediators and lead to avidity activation are called “inside-out signals”.

β2 integrins mediate activation of PMN to their terminal, pro-inflammatory state. When β2 integrins bind ligand, conformational changes within the integrin result in the generation of a signaling cascade, and this “outside-in signal” leads to the transformation of resting PMN to a more aggressive phenotype. The critical role of the integrin upon the regulation of PMN can be seen in clinical patients afflicted with leukocyte adhesion molecule deficiency (LAD). This genetic condition results in a lack of β2 integrins which prevents the activation of PMN, and therefore these individuals cannot mount an appropriate innate immune response. β2 integrins have been demonstrated to be key regulators of PMN activation, however the mechanisms regulating integrin activation are still not completely known.

Cyclic AMP (cAMP) is a common “second messenger” molecule that has been shown to have an overall anti-inflammatory affect in PMN, including suppression of adhesion, phagocytosis and respiratory burst secondary to several different pro-inflammatory activators of PMN. A common effector of cAMP is protein kinase A (cAMP-dependent protein kinase, PKA). Like cAMP, PKA is known to prevent the inflammatory activity of PMN. The significant effects of β2 integrins, cAMP and PKA on the regulation of PMN activation raises the following question, are these various molecules working in concert in the PMN? The link between cAMP and PKA is well established; however

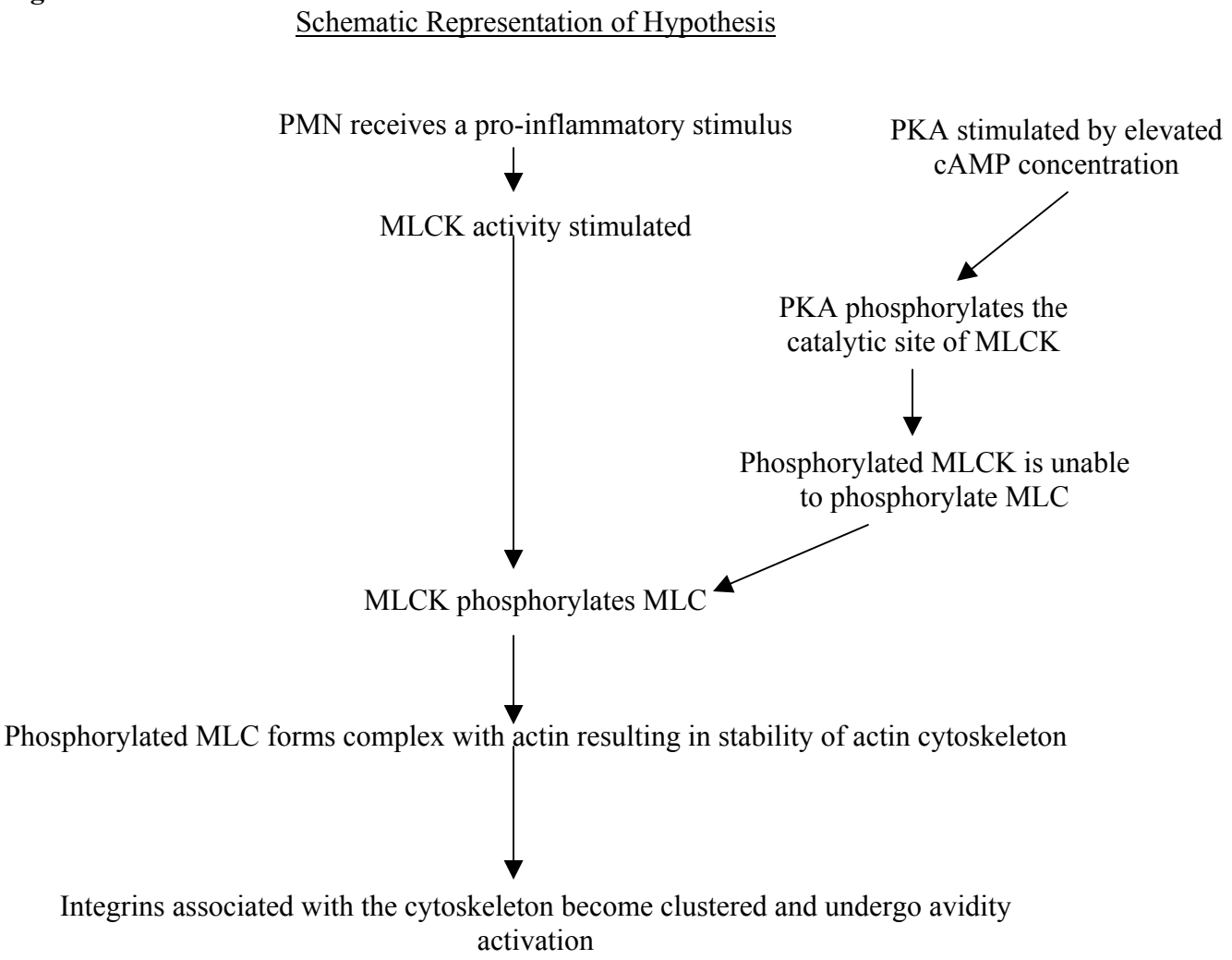
cAMP can act independently of PKA through modulation of intracellular calcium and the Epac class of signaling molecules. A possible connection between cAMP/PKA and the integrins exist within the actin cytoskeleton. The actin cytoskeleton plays a pivotal role in the clustering and anchoring of integrins which are required for adhesion and the generation of outside-in signals. The contraction of the cytoskeleton also generates the motility of the PMN as it migrates out of the vasculature and through the tissue. Disruption of the actin cytoskeleton has been shown to prevent integrin-mediated adhesion. cAMP can block polymerization of the actin cytoskeleton through upregulation of PKA activity. PKA can phosphorylate, and thereby inactivate, myosin light chain kinase (MLCK); an enzyme required for the phosphorylation of myosin light chain (MLC). Phosphorylation of MLC is required for the formation of the actin-myosin complex which leads to cytoskeletal stability. Given this information, I have hypothesized that **PKA inhibits β2 integrin affinity and avidity, and subsequent PMN activation, via inhibition of MLC phosphorylation by MLCK** (see figure 1). In this dissertation, I have set out to prove this hypothesis through the following specific aims:

Specific Aim 1. Determine if PKA regulates β2 integrin avidity activation.

Specific Aim 2. Determine if PKA regulates β2 integrin-dependent PMN adhesion, migration and respiratory burst activity.

Specific Aim 3. Determine if MLC phosphorylation via MLCK is required for PKA regulation of β2 integrin avidity activation and subsequent β2 integrin-dependent PMN adhesion and migration.

Figure 1


Schematic Representation of Hypothesis
PMN receives a pro-inflammatory stimulus
PKA stimulated by elevated cAMP concentration
MLCK activity stimulated
PKA phosphorylates the catalytic site of MLCK
Phosphorylated MLCK is unable to phosphorylate MLC
MLCK phosphorylates MLC
Phosphorylated MLC forms complex with actin resulting in stability of actin cytoskeleton
Integrins associated with the cytoskeleton become clustered and undergo avidity activation

CHAPTER 1

Regulation of the Inflammatory Response of Neutrophils:

The Role of β2 Integrin Activation

The tissues of the body sustain damage from sterile trauma and invasion of foreign organisms on a continual basis. The disruption of the offended tissue from these insults can range from undetectable to complete loss of tissue function, resulting in the incapacitation or death of the affected individual. To halt ongoing damage to the affected tissue and to prevent the spread of infectious organisms to unaffected tissues, the body has developed a system to address this pathology, namely the immune system. While the adaptive immune system is generally the focus of most classical immunologists, it is the innate immune system which first responds to the initial pathology generated in the affected tissues. In fact it is the response of the innate immune system, through its antigen presentation and initiation of biochemical signals, which leads to an adaptive immune response. The innate immune system deals with insult to tissues by several mechanisms, including phagocytosis of foreign material, the release of toxic substances to kill invading organisms and the generation of biochemical signals to both alter the local environment by recruiting and activating various types of cells to aid in the response. The innate immune response can also lead to regional and global alterations within the body, such as changes in regional blood flow, metabolism and body temperature to assist in the defeat of invading pathogens. These affects of the innate immune system are referred to as inflammation. While appropriate inflammation is critical for the body's survival in the face of injury and infection, it is intuitive that the inflammatory response must be tightly controlled to prevent inappropriate inflammation in the absence or after the resolution of an insult to the tissues. In many disease processes it is the inflammatory response itself and not the initial insult that results in the generation of tissue damage and clinical disease.

Initial activation of neutrophils in circulation:

The recruitment of leukocytes, the primary effector cells of inflammation, from the blood stream is one of the earliest steps in the inflammatory response and has been determined to be one of the initial sites of regulation of inflammation. Neutrophils or "polymorphonuclear leukocytes" (PMN), the predominant effector cell of the innate immune system, leave circulation and migrate to the site of infection or injury in the peripheral tissues. PMN in the vasculature respond to biochemical signals generated in the insulted tissue by actively penetrating the vascular wall and proceeding into the extravascular space. This activation can occur in response to several different types of molecules, including chemokines which signal through the G-protein coupled receptors as well as the 'classical chemoattractants", which include complement factor 5a, leukotriene B4 (LTB4), platelet-activating factor (PAF) and tumor necrosis factor-α (TNFα) **(**Rollins 1997**,** Baggiolini 2001**,** Bokoch 1995**,** Sanchez-Madrid and del Pozo 1999**).** PMN can also be activated to undergo extravasation by microbial products such as formylated peptides and antigen-antibody complexes. When PMN reach the affected tissue, they undergo further activation which leads to the phagocytosis and destruction of invading pathogens and damaged tissue. The extracellular destructive capabilities of PMN are propagated through the release of proteolytic proteins and the generation of reactive oxygen species, which occurs in the terminally activated PMN. These inflammatory chemicals generated by the PMN are toxic not only to the invading organisms but also cause damage to host tissues.

PMN are generated in the bone marrow and upon maturation move into the vasculature where they travel through the bloodstream in a quiescent, monitoring state.

As PMN enter the vasculature of insulted tissue, they encounter activating signals which leads to adherence to the vascular endothelium. This interaction between PMN and the vascular endothelium is mediated through a series of interactions between cell surface adhesion molecules on both the PMN and the vascular wall **(**Carlos and Harlan 1994**).** The initial arrest of PMN in the circulation is caused by low-affinity interactions with the endothelium which causes rolling of the PMN along the endothelium. This initial loose interaction is mediated by the selectin family of adhesion molecules **(**Lawrence and Springer 1991**,** Ley and Tedder 1995**).** The selectins are characterized by their ability to bind carbohydrate moieties on the surface of other cells and include L-selectin (CD62L) which is constitutively expressed on leukocytes, and P-selectin (CD62P) and E-selectin (CD62E) which reside on the endothelium **(**Vestweber and Blanks 1999**).** Expression of the selectins upon the surface of the vascular endothelium is upregulated in response to pro-inflammatory mediators such as histamine, thrombin and other cytokines **(**Ley *et al* 1995**,** Jung and Ley 1999**).** This capture of the PMN by the vascular endothelium of inflamed tissue allows retardation of the PMN's progress through the circulation, which in turn improves the ability of the PMN to detect pro-inflammatory signals and become activated to undergo a more substantial adhesion and subsequent evacuation of the vasculature **(**Lindbom *et al* 1992**).** This firm adhesion and the subsequent migration to the site of insult are mediated by a group of cellular adhesion molecules known as integrins.

Integrins and avidity activation:

Integrins bind to several different ligands to assist the PMN in its inflammatory response, and the ability of the integrins to bind to ligand is regulated to prevent

inappropriate adhesion. Not only is the ability of integrins to bind ligand under cellular regulation, but the act of integrin binding to ligand generates intracellular signals within the PMN which lead to further activation of the PMN to a pro-inflammatory state. Genetic abnormalities that cause loss of integrin binding function, referred to as leukocyte adhesion deficiency, result in an abnormal accumulation of PMN in circulation and the inability to mount an affective inflammatory response in insulted tissue.

Integrins are heterodimeric glycoprotein adhesion molecules comprised of α and β subunits. Integrins have the ability to regulate the level of affinity for their ligands through a process known as "inside–out signaling". This "inside-out" signal is mediated by the intracellular signals generated by other cell surface or intracellular receptors upon binding of their appropriate ligands. These signals act upon the cytoplasmic domains of the integrin and alter the integrins affinity for ligand. Integrins can also generate intracellular signals; upon binding ligand, the integrin undergoes a conformational change that results in the generation of an intracellular signal. This integrin-generated signal is referred to as an 'outside–in signal" (Carman and Springer 2003, Ley 2002, Travis *et al* 2003). Integrins are commonly classified by their β subunit; β2 integrins are the most common class on PMN. The most prevalent integrin on PMN is αMβ2 (CD11b/CD18, Mac-1, CR3), while the most prevalent integrin on lymphocytes, not to mention the best characterized, is αLβ2 (CD11a/CD18, LFA-1). αMβ2 has several ligands, including complement, LPS and the membrane protein ICAM-1. When a PMN traveling through the circulation receives a pro-inflammatory signal, such as endogenous mediators (TNF-α, IL-8, immune complexes, etc.) or microbial products (LPS, fMLP, etc.), its integrins will undergo a transformation that will improve their ability to bind

ligand. The PMN may then adhere to the vessel wall and proceed to emigrate out of the circulation. Thus regulation of the integrins results in regulation of the PMN.

Migrating leukocytes undergo a myriad of adhesive events, some characterized by a tight adherence to substrate and others by a loose tethering. This variability in intensity of the cell's interaction with its environment is crucial for proper function; the PMN needs to be slowed in circulation by a loose tethering to decrease transit time through possibly inflamed vasculature, while a strong adhesion is required to cause arrest of the PMN to allow extravasation. "Avidity" is the term used to describe the strength of an adhesive event between a cell and its substrate. Intuitively, since the cellular adhesion is due to multiple adhesion molecules with varying ligand binding strengths, avidity is a function of both the affinity of each individual adhesion molecule for its ligand and the total number of bonds between cellular adhesion molecules and ligands, referred to as "valency" (Shimaoka *et al* 2002). Because the surface of the PMN is not homogeneous, but is in fact characterized by the grouping of cell surface molecules (including adhesion molecules), and because only a portion of the PMN can interact with the substrate due to simple geographic constraints, the valency of an adhesive event is a function of the juxtaposition of the cell and the substrate, the total number of adhesion molecules and ligands available and the mobility of adhesion molecules to the site of adhesion. Many researchers have historically believed that alteration of integrin affinity for substrate has had little to no role in the modulation of cellular avidity, suggesting that cellular avidity is regulated predominately through the cell's valency, especially through the clustering of adhesion molecules at the site of binding to substrate. To support this hypothesis, data has been presented suggesting that upregulation of integrin affinity is not required to generate

integrin-mediated cellular adhesion (Bazzoni and Hemler 1998). Current dogma in the cellular biology community holds that while regulation of integrin-mediated adhesion is surely affected by alterations of integrin valency, there is also a crucial role for modulation of integrin affinity in avidity regulation (Shimaoka *et al* 2002). To support this, the three-dimensional structure of both free and ligand-bound integrins has been determined, and it has been shown that integrins can undergo changes in conformation which result in significant modulation of integrin affinity for substrate (Carman and Springer 2003).

As previously stated, integrins are adhesion molecules that play critical roles in several steps in the transition from the quiescent PMN circulating through the vasculature to the terminally activated, pro-inflammatory PMN at the site of insulted tissue. To date, 25 different integrin heterodimers have been detected in vertebrates created by the combination of one of 19 different α-subunits with one of 8 different β-subunits (Humphries 2000, Hynes 1992, Shimaoka *et al* 2002). The diversity generated by the different combinations of integrin subunits results in adhesion molecules that have various affinities for different ligands, allowing for differentiation of cellular adhesion to a substrate based upon the integrins presented on the cell's surface to the extracellular environment. The integrin heterodimers also differ in their response to inside-out signals, their ability to generate outside-in signals and the type of signals generated, and their attachment to the cytoskeleton, creating further diversity which allows for different cellular responses to integrin-ligand interactions. The α and β subunits are both classified as type I transmembrane glycoprotiens, and the α subunit is comprised by a minimum of 940 amino acids while the β subunit contains at least 640 amino acids (Shimaoka *et al*

2002). Examination of the extracellular portions of integrins via electron microscopy reveals that the integrin heterodimer binds its respective ligand through a globular headpiece, and this headpiece is situated at the end of two long stalks which attach the ligand binding site to the transmembrane portion of the molecule (Du *et al* 1993, Takagi *et al* 2001, Weisel *et al* 1992). Structural analysis of integrins in both low and high affinity states has shown that conformational changes occur between these two states (Xiong *et al* 2001, Beglova *et al* 2002). While the integrin is in a low affinity state, the extracellular stalks are folded in a manner that protects the ligand binding site from the environment. Upon activation of the integrin, the stalks unfold into a straightened conformation which presents the ligand binding site to the environment. Along with this presentation of the ligand binding site, conformational changes occur within the integrin headpiece resulting in increased affinity for ligand (Beglova *et al* 2002, Shimaoka *et al* 2002).

The α subunit:

The integrin α subunit is a transmembrane protein with an intracytoplasmic C terminus and an extracellular N terminus. All α subunit integrin molecules include a β-propeller domain and a stalk region in their extracellular portion, and about half of the α subunits identified thus far contain a region referred to as the I domain. The I domain consists of appoximately 200 amino acids and it is the I domain that contains the ligand binding site for α subunits that contain this domain; α subunits that lack the I domain have their ligand binding site in their β-propeller domain (Diamond *et al* 1993, Michishita *et al* 1993, Humphries 2000). Many integrins are dependent upon divalent cations to bind ligand affectively, and the I domain contains a divalent cation binding site

referred to as the metal ion-dependent adhesion site (MIDAS), and the MIDAS interacts with these cations to generate binding of integrins to negatively charged amino acids in ligands(Shimaoka *et al* 2002). The I domain has also been shown to contain a region which binds dinucleotides, similar to the dinucleotide-binding regions of both small G proteins and the trimeric G protein α subunit (Lee *et al* 1995).

The β-propeller domain includes the N terminus of the α subunit and modeling of this region suggests a seven-bladed β propeller structure, where each of the seven sub-regions contains approximately 60 amino acids in β sheets (Springer 1997). The I domain, in the α subunits that posess an I domain, resides between the proposed β sheets 2 and 3 of the β-propeller (Springer 1997). As previously mentioned, the β-propeller domain contains the ligand binding site for the α subunits lacking an I domain. The ligand-binding role of the β-propeller in I domain-containing α subunits is variable; both αM and αL contain I domains, and the β-propeller is involved in ligand binding in αM (Yalamanchili *et al* 2000) while the β-propeller is not involved in αL ligand binding (Shimaoka *et al* 2001, Lu *et al* 2001). This β-propeller model proposed by Springer has been confirmed by both epitope mapping (Oxvig and Springer 1998) and crystal structure analysis of the integrin αVβ3 (Xiong *et al* 2001). To address the role of the β-propeller in ligand binding, mutagenesis studies were performed which demonstrated that the ligand-binding capability of the β-propeller is focused in a region loceated on the top and side of the structure (Kamata *et al* 2001).

The majority of the extracellular portion of the integrin α subunit consists of the stalk region which is C terminal to the β-propeller domain. This domain spans from the globular head of the α subunit to the transmembrane portion, and consists of three

domains of two-layered β sandwiches (Xiong *et al* 2001, Lu *et al* 1998). Both the α and β subunits (except β4) contain only minimal intracytoplasmic domains, which results in the need for adaptor proteins for interaction and anchoring to the cytoskeleton (de Pereda *et al* 1999).

The β subunit:

As with the α subunit, the integrin β subunit is comprised of three distinct extracellular domains with an extracellular N terminus and a cytoplasmic C terminus. Starting at the N terminus, the first domain shares sequence homology with both plexins and semaphorins, and thus has been named the plexins, semaphorins and integrins (PSI) domain (Bork *et al* 1999). The PSI domain is of particular interest because it is cysteine rich and can interact via a disulfide bond with the cysteine rich stalk at the C terminal portion of the extracellular domain (Calvete *et al* 1991). This disulfide bond forces the integrin into a conformation which maintains the integrin in a low affinity state, demonstrating a regulatory role for the PSI domain (Zang and Springer 2001).

Immediately C terminal to the PSI domain is a region which exhibits some homology in both sequence and structure to the I domain of the α subunit and is known as the I-like domain (Lee *et al* 1995, Ponting *et al* 2000). Ligand binding at the I-like domain has been demonstrated in integrins lacking an I domain in the α subunit, and the I-like domain may play a role in regulation of ligand binding in integrins containing an I domain (Shimaoka *et al* 2002). The I-like domain has been shown to interact with the β-propeller of the α subunit (Huang *et al* 1997, Huang and Springer 1997, Puzon-McLaughlin *et al* 2000, Zang *et al* 2000). This interaction between the subunits is crucial for integrin function; point mutations within the I-like domain have been detected

resulting in the loss of integrin binding ability which in turn results in leukocyte adhesion deficiency syndromes (Bilsland and Springer 1994).

Like the integrin α subunit, the β subunit also has a stalk region at the C terminal end of the extracellular domain. As previously mentioned, the β subunit stalk is cysteine rich and forms a disulfide bond with the PSI domain to maintain the integrin in a low affinity state (Shimaoka *et al* 2002). There is strong evidence that the β subunit stalk region plays a role in modulating integrin affinity since many of the integrin-activating antibodies, as well as many of the antibodies specific for the high affinity state of integrins, are directed against epitopes located within the β stalk region (Humphries 2000, Lu *et al* 2001).

Integrin affinity activation:

Modulation of integrin affinity is a major regulator of cellular avidity, and alterations in this affinity are controlled by alterations in the conformation of the integrin. To generate an integrin-ligand complex, the ligand binding site of the integrin must be available to the ligand. Integrins are maintained in a low affinity state by protecting the binding site from the ligand, and this is created by folding the α and β subunit stalk which places the ligand binding headpiece near the cell membrane, thus sterically preventing the exposure of the binding site to the ligand (Xiong *et al* 2001, Lu *et al* 2001, Beglova *et al* 2002, Takagi *et al* 2002). Opening of the stalks into an extended conformation not only presents the integrin headpiece to ligand for binding, but freeing the integrin also allows for further conformational changes that are required for both increasing integrin affinity and for the generation of outside-in signals subsequent to integrin ligand interaction (Beglova *et al* 2002, Takagi *et al* 2002, Takagi *et al* 2001). Examination of the closed,

low affinity conformation has elucidated sites within the integrin which are protected in this conformation. When ectopic glycosylation sites were experimentally introduced into these sites in the β subunit, the integrin was no longer able to achieve the folded conformation, and these altered integrins expressed constitutive high affinity for ligand, confirming the importance of this conformational shift in affinity regulation (Kashiwagi *et al* 1999, Luo *et al* 2003).

While it is intuitive that conversion of the integrin stalks from a folded, closed conformation into an open conformation will allow for better presentation of the ligand binding site and therefore increased integrin affinity, further comparison of the conformational differences between high and low affinity states has demonstrated that conformational shifts occur within domains of the subunits which result in increased ligand affinity (Takagi *et al* 2001, Takagi *et al* 2002, Takagi *et al* 2003). Examination of the I domain, which is responsible for ligand binding in the α subunit, has shown that this domain can exist in two different conformations, called "open" and "closed" (Shimaoka *et al* 2002, Emsley *et al* 2000, Lee *et al* 1995, Shimaoka *et al* 2003). The open conformation of the I domain is created by a downward shift in an α helix and also demonstrates coordination of the MIDAS, which is reqiured for binding negatively charged residues on the ligand (Emsley *et al* 2000, Lee *et al* 1995, Shimaoka *et al* 2003). To confirm the role of these changes in affinity regulation, mutations have been introduced into the integrins that hold the I domain in either the open or closed conformation. Integrins with a constitutively open I domain show constitutive high integrin affinity, and conversely mutants with constitutively closed I domains have low affinity for ligand (Shimaoka *et al* 2000, Shimaoka *et al* 2001, Shimaoka *et al* 2002,

Shimaoka *et al* 2003, Lu *et al* 2001a, Lu *et al* 2001b, Xiong *et al* 2000, Vorup-Jensen *et al* 2003). Further experiments were performed to lock the affected α helix in an open state by the addition of disulfide bonds, and the findings confirmed that the open conformation of the I domain is associated with high integrin affinity (Shimaoka *et al* 2001, Shimaoka *et al* 2002, Shimaoka *et al* 2003, Lu *et al* 2001a, Lu *et al* 2001b). Because the integrin is both acted upon by inside-out signals and generates outside-in signals, it is frequently difficult to assess if the alteration of the integrin conformation is due to affinity activation or ligand binding. Through the use of x-ray crystallography and antibodies recognizing the open conformation of the I domain, both in the absence of ligand, it has been determined that the open conformation of the I domain can be detected, thus demonstrating that this conformational shift is a part of integrin affinity activation and not a part of the conformational changes due to ligand binding (Shimaoka *et al* 2003, Oxvig *et al* 1999, Ma *et al* 2002).

The above demonstrates that alterations in integrin conformation result in upregulation of affinity for ligand, and it is believed that these conformational changes are created by separation of the α and β subunits. When in a low affinity state, the proximal portions of the integrin stalks and the transmembrane portions of the subunits are in close proximity (Xiong *et al* 2001, Takagi *et al* 2002, Adair and Yeager 2002). Experimental mutation of the cytoplasmic domains of integrins leads to both separation of the subunits and creation of a constitutive high affinity state (Travis *et al* 2003, Vinogradova *et al* 2002). Conversely, if the dissociation of the subunits is prevented, the integrin is maintained in a low affinity state (Travis *et al* 2003). The physiological role of this dissociation has been shown by demonstrating that G-protein coupled receptor signaling, which results in

upregulation of integrin affinity, also leads to separation of the integrin subunits in a manner similar to that seen in the mutational analysis studies (Kim *et al* 2003).

Integrin clustering:

While it is becoming readily apparent that activation of integrins to a high affinity state plays a significant role in the upregulation of cellular avidity, the role of integrin valency can not be overlooked. It is well established that integrins can cluster on the cell surface resulting in localized areas of adhesion (van Kooyk *et al* 1994, Hogg *et al* 2002). In fact, treatment of T lymphocytes with either the PKC agonist phorbol myristate acetate (PMA) or stimulation through the T cell receptor (TCR) will lead to clustering of integrins upon the cell surface but will not activate the integrins to a high affinity state (Kucik *et al* 1996, Stewart *et al* 1996). Further examination of this effect showed that integrin-dependent adhesion could be stimulated in T cells by PMA or TCR stimulation, and these findings lead researchers to the conclusion that affinity activation did not play a role in cellular avidity (Stewart *et al* 1998). As demonstrated above, it now apparent that regulation of both affinity and valency affect integrin avidity. The clustering of integrins required for focal adhesions requires the release of the integrin from its cytoskeletal restraint, and this is achieved through the activity of the protease calpain (Stewart *et al* 1998, Hogg *et al* 2002). Indeed, inhibition of calpain results in the inhibition of integrin clustering and the subsequent integrin-mediated adhesion. Unsurprisingly, many different signaling pathways can stimulate integrin clustering including SLAP 130, Rap-1 and the Rac-1 GEF protein, Vav-1 (Peterson *et al* 2001, Griffiths *et al* 2001, Sebzda *et al* 2002, Krawczyk *et al* 2002, Hogg *et al* 2002).

Lipid rafts in the cell membrane are sites of presentation of many different membrane bound proteins, and it appears that integrins utilize lipid rafts as well. Several integrins have been detected within the lipid rafts and it has been demonstrated that activation of cells results in translocation and clustering of the integrins in the rafts (Krauss and Altevogt 1999, Green *et al* 1999, Skubitz *et al* 2000, Claas *et al* 2001, Leitinger and Hogg 2002). In fact, αLβ2 mediated adhesion in T cells appears to be lipid raft dependent (Hogg *et al* 2002). The mechanism targeting the integrins to the lipid rafts remains unclear. Many lipid raft-associated proteins are palmitoylated to initiate transport to the rafts, but this modification has not been identified in any integrin subunits (Cheruki *et al* 2001). More likely, given the minimal intracellular domains of both subunits, integrins are drawn to the lipid raft through interactions with other proteins. Integrin associated protein and members of the transmembrane 4 superfamily, both known raft associated proteins, are also known to interact with integrins and may play a role in integrin targeting (Green *et al* 1999, Claas *et al* 2001, Shibagaki *et al* 1999, Skubitz *et al* 2000).

Integrin-generated signals:

It is important to remember that integrins not only respond to intracellular signals initiated by transmembrane receptors, but they are themselves receptors capable of generating signals which propel PMN to a pro-inflammatory phenotype. To illustrate this point, while still able to receive pro-inflammatory signals, β2 integrin deficient PMN are unable to generate oxygen radicals (Scharffetter-Kochanek *et al* 1998). It has also been shown in β2 deficient mice and through the use of blocking antibodies that β2 integrins retard PMN rolling along the endothelium of the vasculature in the presence of TNF-α

(Kunkel *et al* 2000, Dunne *et al* 2002, Jung *et al* 1998). Degranulation and respiratory burst activity can both be generated in PMN through cross-linking β2 integrins and the subsequent signaling through nonreceptor tyrosine kinases (Dib 2000). The small G protein RhoA can also be activated subsequent to integrin ligand binding, which causes reorganization of the actin cytoskeleton (Dib *et al* 2001). The urokinase receptor CD87 is known to modulate integrin-ligand interactions, and when CD87 is acting upon β2 integrins changes in PMN effector functions can be detected (Sitrin *et al* 1996, Petty *et al* 1997). Ligation of β2 integrins can lead to the upregulation of β1 integrins on the surface of PMN; in fact, β2 integrin generated signals are required for the expression of both α4β1 and α2β1 on the surface of PMN (Poon *et al* 2001, Werr *et al* 2000). These findings are physiologically reasonable since β2 engagement is required for arrest of the PMN on the vascular endothelium, while β1 integrins are required for the subsequent migration through the tissue. The signals generated by β2 integrins can also lead to apoptosis through activation of either tyrosine kinases and c-Jun N-terminal kinase (JNK) (Walzog *et al* 1997, Avdi *et al* 2001). The binding of ligand by β2 integrins in PMN can also lead to the release of pro-inflammatory mediators such as IL-1β, IL-8 and heparin binding protein (Walzog *et al* 1999, Gautam *et al* 2000, Ley 2001).

The role of integrins in the regulation of neutrophils:

Integrin-mediated adhesion is required for both PMN extravasation and activation of the PMN's terminal inflammatory phenotype (Brown and Lindberg 1996, Berton *et al* 1996). The requirement for β2 dependent adhesion for the subsequent generation of the activated phenotype of PMN has been demonstrated both *in vivo* by the characterization

of β2 leukocyte adhesion deficient (LAD) patients (Anderson *et al* 1986, Anderson *et al* 1984, Nathan *et al* 1989, Nathan and Sanchez 1990, Graham *et al* 1993, Gresham *et al* 1991, Shappell *et al* 1990) and experimentally *ex vivo* (Berton *et al* 1996). To prevent inappropriate and overzealous inflammation, PMN are regulated by β2 integrins at two distinct stages corresponding with the aforementioned inside-out and outside-in signaling. While in circulation, PMN maintain their β2 integrins in a low affinity state until a pro-inflammatory signal is detected, which generates an inside-out signal resulting in upregulation of β2 expression and affinity for ligand and integrin clustering (Brown and Lindberg 1996, Diamond and Springer 1994, van Kooyk and Figdor 2000). Several different pro-inflammatory stimuli, including chemokines such as IL-8, cytokines such as TNF-α, LTB4 and PAF, immune particles such as compliment and antigen-antibody complexes, signaling through selectin clustering and pathogen derived molecules such as lipopolysaccharides (LPS) and formylated peptides can all initiate integrin activation through their respective signaling pathways (van Kooyk and Figdor 2000, Jones *et al* 1998, Jones *et al* 2001, Ninomiya *et al* 1994).

Since the combination of inside-out signals generated by inflammatory mediators and outside-in signals generated by integrin-ligand binding is required for PMN activation, it has been difficult to determine which signaling pathways correspond to these distinct events. It has been determined that phosphatidylinositol-3 kinase (PI3K) is involved in the β2 integrin activation witnessed in PMN secondary to Fcγ receptor stimulation (Jones *et al* 1998). Chemokine stimulation of lymphocytes leads to PI3K dependent clustering of the β2 integrin LFA-1, reiterating the role for this kinase in β2 integrin activation (Constatin *et al* 2000). While PI3K is required for β2 integrin activation in PMN to some

pro-inflammatory stimuli, some inflammatory mediators including formylated peptides and complement fragments activate β2 integrins in a PI3K independent manner (Jones *et al* 1998). It has been recently demonstrated that TNF-α induced activation of β2 integrins in PMN relies on both the Src family of tyrosine kinases as well as the MAP kinase p38 (Bouaouina *et al* 2004). However, our laboratory has demonstrated that while LTB4 and PAF both result in activation of p38 and the subsequent PMN polarization and migration are p38-dependent, the upregulation of β2 integrin expression seen with these mediators is p38-indipendent (Jones *et al* unpublished data). The small GTPases have also been demonstrated as having a role in β2 integrin activation, with H-Ras acting through a PI3K pathway (Weber 2002) and Rap1 acting through a PI3K independent pathway (Kinashi and Katagiri 2004).

Integrin regulation in neutrophils:

Regardless of the initial stimulus, integrin activation and the subsequent phagocyte activation of PMN must be tightly regulated. If integrin activation were to occur in the absence of a pro-inflammatory signal, the integrin could engage ligand resulting in further activation of the PMN and an unnecessary inflammatory response. Inflammation tends to be self-propagating and the ability to down-regulate the inflammatory response is crucial for the normal resolution of inflammation. A major regulator of the inflammatory response of PMN is the second messenger cAMP and its effector PKA. Increased PKA activity has been shown to inhibit integrin-dependent adhesion, migration and respiratory burst activity in PMN (Laudanna *et al* 1997, Bloemen *et al* 1997, Derian *et al* 1995, Nielson *et al* 1992, Chilcoat *et al* 2002), and PKA has been shown to inhibit β2 integrin

upregulation and activation (Jones 2002) as well as upregulation of the β1 integrin VLA-4 (Sulivan *et al* 2004). cAMP upregulation of PKA has also been demonstrated to inhibit 5-lipoxygenase translocation and subsequent leukotriene biosynthesis (Flamand *et al* 2002) and to inhibit TNF-α induced apoptosis (Krakstad *et al* 2004). The anti-inflammatory effect of cAMP can also act through the regulation of intracellular calcium concentrations, which can prevent the release of elastases and reactive oxygen species (Tintinger *et al* 2001, Theron *et al* 2002).

While cAMP has been shown to play an anti-inflammatory role in PMN, it has recently been demonstrated that cAMP also plays a crucial role in expression of an inflammatory phenotype in PMN. A newly characterized group of molecules called Epac are exchange factors for the small GTPases Rap1 and Rap2 and these Epacs are activated by binding cAMP (Rangarajan *et al* 2003). Rap1 is capable of initiating β2 integrin activation and subsequent integrin-dependent adhesion in PMN (Rangarajan *et al* 2003, Bos *et al* 2003). At first glance, it appears that the activity of PKA and Rap1, both of which are stimulated by cAMP, are in direct opposition. In fact, the PMN can utilize these opposing effects to its advantage by discreet localization of PKA and Rap1 geographically within the cell. PKA is localized within the cell by A-kinase anchoring proteins (AKAPs), which can associate PKA with the membrane and cytoskeleton (Edwards and Scott 2000, Howe 2004). It has been shown that inhibition of AKAP activity and the subsequent release of PKA from its geographical restraints results in integrin-dependent adhesion in PMN, suggesting that the inhibitory effect of PKA on integrin activation does indeed have a spatial component (Jones 2002). Further study has demonstrated that while exposure of PMN to a gradient of the PKA inhibitor KT5720

results in F-actin reorganization, cell polarization and integrin-mediated migration, global exposure of PMN to KT5720 does not generate a polarized phenotype in the PMN (Jones unpublished data). It should be noted that the integrin-mediated adhesion generated by AKAP inhibition does not result in affinity activation of the β2 integrins (Jones 2002), but is more likely due to the loss of PKA-mediated inhibition of cytoskeletal reorganization. These findings support the model of integrin-dependent migration in PMN that suggests that integrin activity is required at the forward moving, "leading edge" of the PMN to provide traction for locomotion, while integrin activity must be down regulated at the receding "lagging edge" to allow cellular detachment for forward movement. The opposing roles of Epac/Rap and PKA upon integrin activation in the face of cAMP stimulation further support this model for PMN migration.

Given the inhibitory effect of PKA upon both integrin activation and cytoskeletal reorganization leading to cell polarization, as well as the finding that loss of PKA localization results in integrin-mediated adhesion independent of affinity activation, it is reasonable to hypothesize that PKA may inhibit integrin activation and subsequent PMN activation through inhibition of cytoskeletal reorganization. The actin cytoskeleton plays a critical role in PMN adhesion and migration as it clusters integrins generating increased valency and spatial association with integrin activating signals, as well as the polarity required for direction movement. The cytoskeleton also provides stability to the PMN while the cell interacts with its environment, and the contraction of the actin cytoskeleton generates the locomotion required for cellular migration. An attractive target for PKA inhibition of the cytoskeleton with subsequent inhibition of integrin activation is the formation of the actin-myosin complex, since myosin light chain kinase (MLCK), a

known substrate of PKA, is critical for actin-myosin complex formation (Howe 2004, Smith *et al* 2003). MLCK phosphorylates myosin light chain (MLC) at thr18ser19 which allows activates myosin ATPase activity which is required for actin-myosin complex formation as well as myosin motor activity (Bresnick 1999). MLCK must bind calmodulin to act upon MLC, and PKA is capable of inhibiting MLCK activity by phosphorylating MLCK in its calmodulin binding site, with the overall result of PKA-mediated inhibition of actin-myosin complex formation (Howe 2004, Smith *et al* 2003). In a series of experiments utilizing T lymphocytes to examine the role of MLCK upon β2 integrin-mediated migration, MLCK was found to co-localize with the actin cytoskeleton in the leading edge of the cell, and MLCK activity was found to be required for migration (Smith *et al* 2003). Data will be presented in this dissertation that confirms that PKA does inhibit MLCK activity in PMN and that the regulation of β2 integrin activation and subsequent phagocyte activation in PMN by PKA is via a MLCK-dependent pathway.

Inflammation is a common occurrence in the body with PMN defending the tissues from invading organisms on a daily basis. While crucial for survival, the response of PMN to inflammatory signals also generates significant tissue damage which can be profoundly devastating to the wellbeing of the host. The current anti-inflammatory therapeutic modalities, while effective, have a myriad of deleterious effects which make their administration a balancing act between the alleviation of inflammation and the creation new pathology. Further study into the regulation of inflammation is critical for our understanding of the disease processes involved and will no doubt lead to novel anti-inflammatory therapies that will result in less damage to the host without sacrificing the PMN's ability to defend against infection.

REFERENCES

Adair B, Yeager M. 2002. Three-dimensional model of the human platelet integrin αIIbβ3 based on electron cryomicroscopy and X-ray crystallography. *Proc Natl Acad Sci USA* 99:14059–14064

Anderson D, Schmalstieg F, Arnaout M, Kohl S, Dickey W, Abramson J, Springer T, Boxer L, Hollers J, Smith C. 1984. Abnormalities of polymorphonuclear leukocyte function associated with a heritable deficiency of high molecular weight surface glycoproteins (GP138): common relationship to diminished cell adherence. *J. Clin. Invest.* 74: 536-551

Anderson D, Miller L, Schmalstieg F, Rothelein R, Springer T. 1986. Contributions of the Mac-1 glycoprotein family to adherence-dependent granulocyte functions: structure-function assessments employing subunit- specific monoclonal antibodies. *J. Immunol.* 137: 15-27

Alonso J, Essafi M, Xiong J, Stehle T, Arnaout M. 2002. Does the integrin αA domain act as a ligand for its βA domain?. *Curr Biol* 12:R340–R342

Avdi NJ, *et al.* 2001. Tumor necrosis factor-α activation of the c-Jun N-terminal kinase pathway in human neutrophils. Integrin involvement in a pathway leading from cytoplasmic tyrosine kinases apoptosis. *J Biol Chem* 276: 2189-2199

Baggiolini M. 2001. Chemokines in pathology and medicine. *J. Intern. Med.* 250:91–104

Bazzoni G, Hemler ME. 1998. Are changes in integrin affinity and conformation overemphasized? *Trends. Biochem. Sci.* 23:30-34

Beglova N, Blacklow S, Takagi J, Springer T. 2002. Cysteine-rich module structure reveals a fulcrum for integrin rearrangement upon activation. *Nat Struct Biol* 9:282–287

Berton G, Yan S, Fumagalli L, Lowell C. 1996. Neutrophil activation by adhesion: mechanisms and pathophysiological implications. *Int J Clin Lab Res* 26:160

Bilsland CAG, Springer TA. 1994. Cloning and expression of the chicken CD18 cDNA. *J. Leukoc. Biol.* 55:501-6

Bloemen P, van den Tweel M, Henricks P, Engels F, Kester M, van de Loo P, Blomjous, F, Nijkamp F. 1997. Increased cAMP levels in stimulated neutrophils inhibit their adhesion to human bronchial epithelial cells. *Am. J. Physiol.* 272:L580-L587

Bokoch GM. 1999. Chemoattractant signaling and leukocyte activation. *Blood*. 86:1649-1660

Bork P, Doerks T, Springer TA, Snel B. 1999. Domains in plexins: links to integrins and transcription factors. *Trends Biochem. Sci.* 24:261-63

Bos JL, de Bruyn K, Enserink J, Kuiperij B, Rangarajan S, Rehmann H, Riedl J, de Rooij J, van Mansfeld F, Zwartkruis F. 2003. The role of Rap1 in integrin-mediated cell adhesion. *Biochem Soc Trans*. 31:83-6

Bouaouina M, Blouin E, Halbwachs-Mecarelli L, Lesavre P, Rieu P. 2004. TNF-Induced β2 Integrin Activation Involves Src Kinases and a Redox-Regulated Activation of p38 MAPK. *J Immunol*. 173: 1313-1320

Bresnick A. 1999. Molecular mechanisms of nonmuscle myosin-II regulation. *Curr.Opin. Cell Biol.* 11, 26-33

Brown E, Lindberg F. 1996. Leucocyte adhesion molecules in host defence against infection. *Ann Med.* 28:201

Calvete JJ, Henschen A, González-Rodríguez J. 1991. Assignment of disulphide bonds in human platelet GPIIIa. A disulphide pattern for the β-subunits of the integrin family. *Biochem. J.* 274:63-71

Carlos TM, Harlan JM. 1994. Leukocyte-endothelial adhesion molecules. *Blood.* 84:2068–2101

Carman C, Springer T. 2003. Integrin avidity regulation: are changes in affinity and conformation underemphasized? *Curr.Op. Cell Biol.* 15:547-556

Cherukuri A, Dykstra M, Pierce SK. 2001. Floating the raft hypothesis: lipid rafts play a role in immune cell activation. *Immunity* 14: 657-660.

Chilcoat C, Rowlingson K, Jones S. 2002. The effects of cAMP modulation upon the adhesion and respiratory burst activity of immune complex-stimulated equine neutrophils. *Vet Immunol Immunopath.* 88: 65-77

Claas C, Stipp CS, Hemler ME. 2001. Evaluation of prototype transmembrane 4 superfamily protein complexes and their relation to lipid rafts. *J Biol Chem* 276: 7974-7984

Constantin G, Majeed M, Giagulli C, Piccio L, Kim J, Butcher E, Laudanna C. 2000. Chemokines Trigger Immediate β2 Integrin Affinity and Mobility Changes Differential Regulation and Roles in Lymphocyte Arrest under Flow. *Immunity*. 13: 759-769

de Pereda JM, Wiche G, Liddington RC. 1999. Crystal structure of a tandem pair of fibronectin type III domains from the cytoplasmic tail of integrin α6β4. *EMBO J.* 18:4087-95

Derian C, Santulli R, Rao P, Solomon H, Barrett J. 1995. Inhibition of chemotactic peptide-induced neutrophil adhesion to vascular endothelium by cAMP modulators. *J. Immunol.* 154:308-317

Diamond M, Springer T. 1994. The dynamic regulation of integrin adhesiveness. *Curr Biol* 4:506

Diamond MS, Garcia-Aguilar J, Bickford JK, Corbi AL, Springer TA. 1993. The I domain is a major recognition site on the leukocyte integrin Mac-1 (CD11b/CD18) for four distinct adhesion ligands. *J. Cell Biol.* 120:1031-43

Dib K. 2000. β2 integrin signaling in leukocytes. *Frontiers Bioscience* 5: D438-D451

Dib K, Melander F, Andersson T. 2001. Role of p190RhoGAP in β2 integrin regulation of RhoA in human neutrophils. *J Immunol* 166: 6311-6322

Du X, Gu M, Weisel JW, Nagaswami C, Bennett JS, *et al*. 1993. Long range propagation of conformational changes in integrin αIIbβ3. *J. Biol. Chem.* 268:23087-92

Dunne JL, Ballantyne CM, Beaudet AL, Ley K. 2002. Regulation of leukocyte rolling velocity by LFA-1 and Mac-1. *Blood* 99: 336-341

Edwards A, Scott J. 2000. A-kinase anchoring proteins: protein kinase A and beyond. *Curr. Opin. Cell Biol.* 12:217-221

Emsley J, Knight C, Farndale R, Barnes M, Liddington R. 2000. Structural basis of collagen recognition by integrin α2β1. *Cell* 101:47–56

Feldhaus MJ, Weyrich AS, Zimmerman GA, McIntyre TM. 2002. Ceramide generation *in situ* alters leukocyte cytoskeletal organization and β2-integrin function and causes complete degranulation. *J Biol Chem*277:4285-4293.

Flamand N, Surette ME, Picard S, Bourgoin S, Borgeat P. 2002. Cyclic AMP-mediated inhibition of 5-lipoxygenase translocation and leukotriene biosynthesis in human neutrophils. *Mol Pharmacol*. 62:250-6

Gautam N, Herwald H, Hedqvist P, Lindbom L. 2000. Signaling via β2 integrins triggers neutrophil-dependent alteration in endothelial barrier function. *J Exp Med* 191:1829-1839

Graham I, Lefkowith J, Anderson D, Brown E. 1993. Immune complex-stimulated neutrophil LTB4 production is dependent on beta 2 integrins. *J. Cell Biol.* 120: 1509-1517

Green JM, *et al.* 1999. Role of cholesterol in formation and function of a signaling complex involving αVβ3, integrin-associated protein (CD47), and heterotrimeric G proteins. *J Cell Biol* 146: 673-682

Gresham H, Graham I, Anderson D, Brown E. 1991. Leukocyte adhesion-deficient neutrophils fail to amplify phagocytic function in response to stimulation. Evidence for CD11b/CD18-dependent and -independent mechanisms of phagocytosis. *J. Clin. Invest.* 88: 588-597

Griffiths EK, *et al.* 2001. Positive regulation of T cell activation and integrin adhesion by the adapter Fyb/Slap. *Science* 293: 2260-2263

Hogg N, Stewart M, Scarth S, Newton R, Shaw J, Law S, Klein N. 1999. A novel leukocyte adhesion deficiency caused by expressed but nonfunctional β2 integrins Mac-1 and LFA-1. *J Clin Invest* 103:97–106

Howe A. 2004. Regulation of actin-based cell migration by cAMP/PKA. *Biochimica et Biophysica Acta Mol Cell Res.* 1692:159-174

Huang C, Lu C, Springer TA. 1997. Folding of the conserved domain but not of flanking regions in the integrin β2 subunit requires association with the α subunit. *Proc. Natl. Acad. Sci. USA* 94:3156-61

Huang C, Springer TA. 1997. Folding of the β-propeller domain of the integrin αL subunit is independent of the I domain and dependent on the β2 subunit. *Proc. Natl. Acad. Sci. USA* 94:3162-67

Humphries MJ. 2000. Integrin structure. *Biochem. Soc. Trans.* 28:311-39

Huth J, Olejniczak E, Mendoza R, Liang H, Harris E, Lupher M, Wilson A, Fesik S, Staunton D. 2000. NMR and mutagenesis evidence for an I domain allosteric site that regulates lymphocyte function-associated antigen 1 ligand binding. *Proc Natl Acad Sci USA* 97:5231–5236

Hynes RO. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69:11-25

Jones S, Knaus U, Bokoch G, Brown E. 1998. Two signaling mechanisms for the activation of αMβ2 avidity in polymorphonuclear neutrophils. *J Biol Chem* 273:10556

Jones S., Sharief Y, Chilcoat C. 2001. Signaling mechanism for equine neutrophil activation by immune complexes. *Vet. Immunol. Immunopathol*. 82: 87-100

Jones S. 2002. Protein kinase A regulates ß2 integrin avidity in neutrophils. *J Leuk Biol.* 71:1042-1048

Jung U, Ley K. 1999. Mice lacking two or all three selectins demonstrate overlapping and distinct functions for each selectin. *J. Immunol.* 162:6755–6762

Jung U, Norman KE, Ramos CL, Scharffetter-Kochanek K, Beaudet AL, Ley K. 1998. Transit time of leukocytes rolling through venules controls cytokine-induced inflammatory cell recruitment *in vivo*. *J Clin Invest* 102: 1526-1533

Kamata T, Tieu KK, Springer TA, Takada Y. 2001. Amino acid residues in the αIIb subunit that are critical for ligand binding to integrin αIIbβ3 are clustered in the β-propeller model. *J. Biol. Chem.* 276:44274-83

Kashiwagi H, Tomiyama Y, Tadokoro S, Honda S, Shiraga M, Mizutani H, Handa M, Kim M, Carman C, Springer T. 2003. Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. *Science* 301:1720-5

Kinashi T, Katagiri K. 2004. Regulation of lymphocyte adhesion and migration by the small GTPase Rap1 and its effector molecule, RAPL. *Imm Letters* . 93: 1-5

Krakstad C, Christensen A, Døskeland S. 2004. cAMP protects neutrophils against TNF-α-induced apoptosis by activation of cAMP-dependent protein kinase, independently of exchange protein directly activated by cAMP (Epac). *J Leuk Biol.* 76:641-647

Krauss K, Altevogt P. 1999. Integrin leukocyte function-associated antigen-1-mediated cell binding can be activated by clustering of membrane rafts. *J Biol Chem* 274: 36921-36927

Krawczyk C, *et al.* 2002. Vav1 controls integrin clustering and MHC/peptide-specific cell adhesion to antigen-presenting cells. *Immunity* 16: 331-343

Kucik DF, Dustin ML, Miller JM, Brown EJ. 1996. Adhesion-activating phorbol ester increases the mobility of leukocyte integrin LFA-1 in cultured lymphocytes. *J Clin Invest* 97: 2139-2144

Kunkel EJ, Dunne JL, Ley K. 2000. Leukocyte arrest during cytokine-dependent inflammation *in vivo*. *J Immunol* 164: 3301-3308

Kurata Y, Matsuzawa Y, Shattil S. 1999. A mutation in the extracellular cysteine-rich repeat region of the β3 subunit activates integrins αIIbβ3 and αVβ3. *Blood* 93:2559–2568

Laudanna C, Campbell J, Butcher E. 1997. Elevation of intracellular cAMP inhibits RhoA activation and integrin-dependent leukocyte adhesion induced by chemoattractants. *J. Biol. Chem.* 272:24141-24144

Lawrence MB, Springer TA. 1991. Leukocytes roll on a selectin at physiologic flow rates: Distinction from and prerequisite for adhesion through integrins. *Cell.* 65:859–873

Lee J, Bankston L, Arnaout M, Liddington R. 1995. Two conformations of the integrin A-domain (I-domain): a pathway for activation? *Structure* 3:1333–1340

Lee J-O, Rieu P, Arnaout MA, Liddington R. 1995. Crystal structure of the A domain from the αsubunit of integrin CR3 (CD11b/CD18). *Cell* 80:631-38

Leitinger B, Hogg N. 2002. The involvement of lipid rafts in the regulation of integrin function. *J Cell Sci*;115: 963-972

Ley K, Bullard DC, Arbones ML, Bosse R, Vestweber D, Tedder TF, Beaudet AL. 1995. Sequential contribution of L- and P-selectin to leukocyte rolling *in vivo*. *J. Exp. Med*. 181:669–675

Ley K. 2001. Plugging the leaks. *Nature Med* 7:1105-1106

Ley K. 2002. Integration of inflammatory signals by rolling neutrophils. *Imm. Rev.* 186:8

Ley K, Tedder TF 1995. Leukocyte interactions with vascular endothelium. New insights into selectin mediated attachment and rolling. *J. Immunol.* 155:525–528

Lindbom L, Xie X, Raud J, Hedqvist P. 1992. Chemoattractant-induced firm adhesion of leukocytes to vascular endothelium *in vivo* is critically dependent on initial leukocyte rolling. *Acta Physiol. Scand.* 146:415–421

Lu C, Shimaoka M, Ferzly M, Oxvig C, Takagi J, Springer T. 2001. An isolated, surface-expressed I domain of the integrin αLβ2 is sufficient for strong adhesive function when locked in the open conformation with a disulfide. *Proc Natl Acad Sci USA* 98:2387–2392

Lu C, Shimaoka M, Zang Q, Takagi J, Springer T. 2001. Locking in alternate conformations of the integrin αLβ2 I domain with disulfide bonds reveals functional relationships among integrin domains. *Proc Natl Acad Sci USA* 98:2393–2398

Lu C, Ferzly M, Takagi J, Springer TA. 2001. Epitope mapping of antibodies to the C-terminal region of the integrin β2 subunit reveals regions that become exposed upon receptor activation. *J. Immunol.* 166:5629-37

Lu C, Oxvig C, Springer TA. 1998. The structure of the β-propeller domain and C-terminal region of the integrin αM subunit. *J. Biol. Chem.* 273:15138-47

Lu C, Takagi J, Springer T. 2001. Association of the membrane-proximal regions of the α and β subunit cytoplasmic domains constrains an integrin in the inactive state. *J Biol Chem* 276:14642–14648

Luo B, Springer T, Takagi J. 2003. Stabilizing the open conformation of the integrin headpiece with a glycan wedge increases affinity for ligand. *Proc Natl Acad Sci USA* 100:2403–2408

Ma Q, Shimaoka M, Lu C, Jing H, Carman C, Springer T. 2002. Activation induced conformational changes in the I domain region of LFA-1. *J Biol Chem* 277:10638–10641

Michishita M, Videm V, Arnaout MA. 1993. A novel divalent cation-binding site in the A domain of the β2 integrin CR3 (CD11b/CD18) is essential for ligand binding. *Cell* 72:857-67

Nathan C, Srimal S, Farber C, Sanchez E, Kabbash L, Asch A, Gailit J, Wright S. D. 1989. Cytokine-induced respiratory burst of human neutrophils: dependence on extracellular matrix proteins and CD11/CD18 integrins. *J. Cell Biol.* 109: 1341-1349

Nathan C, Sanchez E. 1990. Tumor necrosis factor and CD11/CD18 (β2) integrins act synergistically to lower cAMP in human neutrophils. *J. Cell Biol.* 111: 2171-2181

Nielson C, Bayer C, Hodson S, Hadjokas N. 1992. Regulation of the respiratory burst by cyclic 3',5'-AMP, an association with inhibition of arachidonic acid release. *J. Immunol.* 149: 4036-4040

Ninomiya N, Hazeki K, Fukui Y, Seya T, Okada T, Hazeki O, Ui M. 1994. Involvement of phosphatidylinositol 3-kinase in Fc gamma receptor signaling. *J. Biol. Chem.* 269: 22732-22737

Oxvig C, Lu C, Springer T. 1999. Conformational changes in tertiary structure near the ligand binding site of an integrin I domain. *Proc Natl Acad Sci USA* 96:2215–2220

Oxvig C, Springer TA. 1998. Experimental support for a β-propeller domain in integrin α-subunits and a calcium binding site on its lower surface. *Proc. Natl. Acad. Sci. USA* 95:4870-75

Peterson EJ, *et al.* 2001. Coupling of the TCR to integrin activation by Slap-130/Fyb. *Science* 293: 2263-2265

Petty HR, Kindzelskii AL, Adachi Y, Todd RF. 1997. Ectodomain interactions of leukocyte integrins and pro- inflammatory GPI-linked membrane proteins. *J Pharm Biomed Anal* 15: 1405-1416

Ponting CP, Schultz J, Copley RR, Andrade MA, Bork P. 2000. Evolution of domain families. *Adv. Protein Chem.* 54:185-244

Poon BY, Ward CA, Cooper CB, Giles WR, Burns AR, Kubes P. 2001. 4-integrin mediates neutrophil-induced free radical injury to cardiac myocytes. *J Cell Biol* 152: 857-866

Puzon-McLaughlin W, Kamata T, Takada Y. 2000. Multiple discontinuous ligand-mimetic antibody binding sites define a ligand binding pocket in integrin IIb 3. *J. Biol. Chem.* 275:7795-802

Rangarajan S, Enserink J, Kuiperij H, de Rooij J, Price L, Schwede F, Bos J. 2003. Cyclic AMP induces integrin-mediated cell adhesion through Epac and Rap1 upon stimulation of the β2-adrenergic receptor. *J Cell Biol.* 160: 487-493

Rollins BJ. 1997. Chemokines. *Blood* 90:909–928

Sanchez-Madrid F, del Pozo MA. 1999. Leukocyte polarization in cell migration and immune interactions. *EMBO J.* 18:501–511

Scharffetter-Kochanek K, *et al.* 1998. Spontaneous skin ulceration and defective T cell function in CD18 null mice. *J Exp Med* 188: 119-131

Sebzda E, Bracke M, Tugal T, Hogg N, Cantrell DA. 2002. Rap1A positively regulates T cells via integrin activation rather than inhibiting lymphocyte signaling. *Nat Immunol* 3: 251-258

Shappell S, Toman C, Anderson D, Taylor A, Entman M, Smith C. 1990. Mac-1 (CD11b/CD18) mediates adherence-dependent hydrogen peroxide production by human and canine neutrophils. *J. Immunol.* 144: 2702-2711

Shibagaki N, Hanada K, Yamashita H, Shimada S, Hamada H. 1999. Overexpression of CD82 on human T cells enhances LFA-1/ICAM-1-mediated cell-cell adhesion: functional association between CD82 and LFA-1 in T cell activation. *Eur J Immunol* 29: 4081-4091

Shimaoka M, Salas A, Yang W, Weitz-Schmidt G, Springer T. 2003. Small molecule integrin antagonists that bind to the β2 subunit I-like domain and activate signals in one direction and block them in another. *Immunity* 19:391-402

Shimaoka M, Xiao T, Liu J, Yang Y, Dong Y, Jun C, McCormack A, Zhang R, Joachimiak A, Takagi J *et al.* 2003. Structures of the αL I domain and its complex with ICAM-1 reveal a shape-shifting pathway for integrin regulation. *Cell* 112:99–111

Shimaoka M, Shifman J, Jing H, Takagi J, Mayo S, Springer T. 2000. Computational design of an integrin I domain stabilized in the open, high affinity conformation. *Nat Struct Biol* 7:674–678

Shimaoka M, Lu C, Salas A, Xiao T, Takagi J, Springer T. 2002. Stabilizing the integrin αM inserted domain in alternative conformations with a range of engineered disulfide bonds. *Proc Natl Acad Sci USA* 99:16737–16741

Shimaoka M, Takagi J, Springer T. 2002. Conformational regulation of integrin structure and function. *Annu Rev Biophys Biomol Struct* 31:485–516

Shimaoka M, Lu C, Palframan R, von Andrian UH, Takagi J, Springer TA. 2001. Reversibly locking a protein fold in an active conformation with a disulfide bond: integrin αL I domains with high affinity and antagonist activity *in vivo*. *Proc. Natl. Acad. Sci. USA* 98:6009-14

Sitrin RG, Todd RF III, Petty HR, *et al.* 1996. The urokinase receptor (CD87) facilitates CD11b/CD18-mediated adhesion of human monocytes. *J Clin Invest* 97: 1942-1951

Skubitz KM, Campbell KD, Skubitz AP. 2000. CD63 associates with CD11/CD18 in large detergent-resistant complexes after translocation to the cell surface in human neutrophils. *FEBS Lett* 469: 52-56

Smith A, Bracke M, Leitinger B, Porter J, Hogg N. 2003. LFA-1-induced T cell migration on ICAM-1 involves regulation of MLCK-mediated attachment and ROCK-dependent detachment. *J Cell Sci.* 116:3123-3133

Springer TA. 1997. Folding of the N-terminal, ligand-binding region of integrin α-subunits into a β-propeller domain. *Proc. Natl. Acad. Sci. USA* 94:65-72

Springer TA, Jing H, Takagi J. 2000. A novel Ca^{2+}-binding β-hairpin loop better resembles integrin sequence motifs than the EF-hand. *Cell* 102:275-77

Stewart MP, Cabanas C, Hogg N. 1996. T cell adhesion to intercellular adhesion molecule-1 (ICAM-1) is controlled by cell spreading and the activation of integrin LFA-1. *J Immunol* 156: 1810-1817

Stewart MP, McDowall A, Hogg N. 1998. LFA-1-mediated adhesion is regulated by cytoskeletal restraint and by a $Ca2^{+}$-dependent protease, calpain. *J Cell Biol* 140: 699-707

Sullivan G, Lee D, Ross W, DiVietro J,. Lappas C, Lawrence M, Linden J. 2004. Activation of A2A adenosine receptors inhibits expression of α4/β1 integrin (very late antigen-4) on stimulated human neutrophils. *J Leuk Biol.* 75:127-134

Takagi J, Strokovich K, Springer T, Walz T. 2003. Structure of integrin α5β1 in complex with fibronectin. *EMBO J* 22:4607-15

Takagi J, Petre B, Walz T, Springer T. 2002. Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. *Cell* 110:599–611.

Takagi J, Beglova N, Yalamanchili P, Blacklow SC, Springer TA. 2001. Definition of EGF-like, closely interacting modules that bear activation epitopes in integrin β subunits. *Proc. Natl. Acad. Sci. USA* 98:11175-80

Takagi J, Erickson HP, Springer TA. 2001. C-terminal opening mimics "inside-out" activation of integrin α5β1. *Nat. Struct. Biol.* 8:412-16

Tan S, Walters SE, Mathew EC, Robinson MK, Drbal K, *et al*. 2001. Defining the repeating elements in the cysteine-rich region (CRR) of the CD18 integrin β2 subunit. *FEBS Lett.* 505:27-30

Theron AJ, Steel HC, Tintinger GR, Anderson R. 2002. Endogenous adenosine regulates neutrophil pro-inflammatory activities by cyclic AMP-dependent accelerated clearance of cytosolic calcium. *Inflamm Res*. 51:594-602

Tintinger GR, Theron AJ, Anderson R, Ker JA. 2001. The anti-inflammatory interactions of epinephrine with human neutrophils *in vitro* are achieved by cyclic AMP-mediated accelerated resequestration of cytosolic calcium. *Biochem Pharmacol*. 61:1319-28

Travis M, Humphries J, Humphries M. 2003. An unraveling tale of how integrins are activated from within. *Trends Pharmacol Sci* 24:192–197

van Kooyk Y, Figdor C. 2000. Avidity regulation of integrins: the driving force in leukocyte adhesion. *Curr Opin Cell Biol.* 12:542

van Kooyk Y, Weder P, Heije K, Figdor CG. 1994. Extracellular $Ca2^{+}$ modulates leukocyte function-associated antigen-1 cell surface distribution on T lymphocytes and consequently affects cell adhesion. *J Cell Biol* 124: 1061-1070

Vestweber D, Blanks JE. 1999. Mechanisms that regulate the function of the selectins and their ligands. *Physiol Rev.* 79:181–213

Vinogradova O, Velyvis A, Velyviene A, Hu B, Haas T, Plow E, Qin J. 2002. A structural mechanism of integrin αIIbβ3 "inside-out" activation as regulated by its cytoplasmic face. *Cell* 110:587–597

Vorup-Jensen T, Shimaoka M, Ostermeier C, Hommel U, Springer T. 2003. Structure and allosteric regulation of the αXβ2 integrin I domain. *Proc Natl Acad Sci USA* 100: 1873–1878

Walzog B, Weinmann P, Jeblonski F, Scharffetter-Kochanek K, Bommert K, Gaehtgens P. 1999. A role for β2 integrins (CD11/CD18) in the regulation of cytokine gene expression of polymorphonuclear neutrophils during the inflammatory response. *FASEB J* 13: 1855-1865

Walzog B, Jeblonski F, Zakrzewicz A, Gaehtgens P. 1997. 2 integrins (CD11/CD18) promote apoptosis of human neutrophils. *FASEB J* 11: 1177-1186

Weber C. 2002. Novel mechanistic concepts for the control of leukocyte transmigration: specialization of integrins, chemokines, and junctional molecules. *J Mol Med.* 81: 4-19

Weisel JW, Nagaswami C, Vilaire G, Bennett JS. 1992. Examination of the platelet membrane glycoprotein IIb-IIIa complex and its interaction with fibrinogen and other ligands by electron microscopy. *J. Biol. Chem.* 267:16637-43

Werr J, Eriksson EE, Hedqvist P, Lindbom L. 2000. Engagement of 2 integrins induces surface expression of 1 integrin receptors in human neutrophils. *J Leukoc Biol* 68: 553-560

Xiong J, Li R, Essafi M, Stehle T, Arnaout M. 2000. An isoleucine-based allosteric switch controls affinity and shape shifting in integrin CD11b A-domain. *J Biol Chem* 275: 38762–38767

Xiong J-P, Stehle T, Diefenbach B, Zhang R, Dunker R, *et al*. 2001. Crystal structure of the extracellular segment of integrin αVβ3. *Science* 294:339-45

Yalamanchili P, Lu C, Oxvig C, Springer TA. 2000. Folding and function of I domain deleted Mac-1 and LFA-1. *J. Biol. Chem.* 275:21877-82

Yuan Q, Jiang W-M, Leung E, Hollander D, Watson JD, Krissansen GW. 1992. Molecular cloning of the mouse integrin β_7 subunit. *J. Biol. Chem.* 267:7352-58

Zang Q, Lu C, Huang C, Takagi J, Springer TA. 2000. The top of the I-like domain of the integrin LFA-1 βsubunit contacts the αsubunit β-propeller domain near β-sheet 3. *J. Biol. Chem.* 275:22202-12

Zang Q, Springer TA. 2001. Amino acid residues in the PSI domain and cysteine-rich repeats of the integrin β2 subunit that restrain activation of the integrin αXβ2. *J. Biol. Chem.* 276:6922-29

CHAPTER 2

The Effects of cAMP Modulation upon the Adhesion and Respiratory Burst Activity of Immune Complex-Stimulated Equine Neutrophils

Abstract

Toxic products such as reactive oxygen intermediates released by activated neutrophils (PMN) have an important role in the pathophysiology of diseases associated with the deposition of immune complexes (IC) in tissues. IC-induced activation of PMN requires adhesion mediated by integrin adhesion receptors. Of the integrins expressed on PMN, the β2 family has been found to be of particular importance for activation of PMN by IC. β2 integrin ligand binding must be activated to enable adhesion to IC. Both activating and inhibitory signals regulate β2 integrin ligand avidity and adhesion. The second messenger cAMP has been demonstrated to inhibit the activation of PMN in response to a variety of stimuli. The purpose of this study is to test the hypothesis that cAMP-dependent signals inhibit β2 integrin dependent adhesion of equine PMN to immobilized IC and subsequent adhesion-dependent activation of respiratory burst activity. Treatment of equine PMN with β2 adrenergic agonists isoproterenol or clenbuterol, which trigger an increase in intracellular cAMP concentration, inhibited adhesion of equine PMN to IC in a dose dependent manner. Similarly, inhibition of cAMP hydrolysis by the non-specific phosphodiesterase inhibitor pentoxifylline and the phosphodiesterase 4-specific inhibitor rolipram inhibited adhesion of equine PMN to IC. Elevation of intracellular cAMP levels with pentoxifylline, clenbuterol and rolipram also inhibited IC-induced activation of respiratory burst activity in equine PMN. Importantly, co-treatment of equine PMN with rolipram and either β2 adrenergic agonist synergistically inhibited both the adhesion of equine PMN to IC as well as the subsequent respiratory burst activity.

Introduction

Phagocytes are a critical component of the innate immune system due to their role in protection against local infection and systemic dissemination of pathogens. The most abundant of these cells are the neutrophils, also known as polymorphonuclear neutrophils (PMN), which circulate throughout the bloodstream and tissues to provide a rapid response to any infectious organisms that they may encounter. PMN use many lethal weapons in their response such as phagocytosis of invading organisms, generation of a variety of microbicidal products including reactive oxygen intermediates, and production of inflammatory mediators which potentiate the host response to infection. The transition of PMN from a quiescent, monitoring phenotype to an aggressive pro-inflammatory state is referred to as phagocyte activation. Cellular adhesion to specific substrates is an essential step in the activation of PMN. This activating adhesion occurs via adhesion molecules called integrins (Jones *et al* 2001, Jones *et al* 1998, Tang *et al* 1997, Graham *et al* 1993).

Classified by their β sub-unit, integrins are heterodimeric glycoproteins that, when activated to their high affinity state, bind to cell surface or extracellular matrix ligands. Integrins also contain a cytoplasmic tail that is capable of activating intracellular signaling pathways and assembling a critical cytoskeletal structure called a podosome when integrins are bound to ligand. While PMN express β1, β2 and β3 integrins on their cell surface, it is the β2 integrin (CD18, LeuCAM) and specifically αMβ2 (Mac-1, CD11b/CD18) that are the most abundant and functionally relevant (Jones *et al* 1998, Tang *et al* 1997, Nagahata *et al* 1994, Graham *et al* 1993, Gresham *et al* 1991). It has

been previously demonstrated that blocking β2 integrin function with specific Mab prevents activation of PMN by many pro-inflammatory activators (Brown 1997).

Integrins bind poorly to their ligands when the PMN is quiescent. When the cell is exposed to inflammatory stimuli such as chemokines, cytokines, bacterial products, complement fragments or immune complexes (IC), the integrins undergo avidity activation which mobilizes the integrins to the site of activation and transforms them to a high affinity state (Jones *et al* 2001, Ninomiya *et al* 1994). In this state, the integrins bind to their ligands, which in turn augments activation of the PMN.

Immune complexes, a potent stimulant of inflammation, are generated when multiple soluble antibodies bind to their specific antigen. This binding results in the clustering of the Fc portion of the antibodies, rendering them available for binding to multiple Fcγ receptors (FcγR) on the PMN in a 1:1 stoichiometry (Kato *et al* 2000). Binding of IC to FcγR results in FcγR clustering on the surface of the PMN, which in turn initiates a signaling cascade that leads to the activation of β2 integrins and adhesion (Kato *et al* 2000, Jones *et al* 1998). This type of "inside-out" signaling leading to activation of integrins is common to many inflammatory stimuli (Brown 1997).

It has been previously shown in humans that cAMP can inhibit adhesion and adhesion-dependent activation of PMN through its effects on the protein kinase A (PKA) pathway (Bloemen *et al* 1997, Derian *et al* 1995, Condino-Neto *et al* 1991, Nielson *et al* 1990). Both phosphodiesterase (PDE) inhibitors and β2 adrenergic agonists have been shown to increase intracellular cAMP concentrations by preventing the degradation of cAMP and increasing cAMP synthesis, respectively. Our data show that treatment of PMN with either PDE inhibitors or β2 adrenergic agonists has a dose-dependent,

inhibitory effect on both IC-induced adhesion and subsequent respiratory burst activity. Moreover, inhibition of PKA activity is sufficient to activate adhesion to β2 integrin substrate. These data suggest that cAMP and PKA have a role in the inside-out signaling mechanism regulating β2 integrin activation in response to stimulation with IC.

Materials and methods

Reagents - Powdered phosphate buffered saline (PBS) and Hank's balanced salt solution (HBSS) were purchased from Gibco BRL, Grand Island, NY. Ficoll-Paque PLUS was obtained from Amersham Pharmacia Biotech, Piscataway, NJ. Calcein was purchased from Molecular Probes, Eugene, OR. 8-Br-cAMP, pentoxifylline, rolipram and KT5720 were purchased from Alexis Corporation, San Diego, CA. Bovine fetal calf serum (FCS) and bovine serum albumin (BSA) were obtained from HyClone, Logan UT. Human serum albumin (HSA) was purchased from Bayer, Elkhart, IN. Rabbit anti-BSA IgG, phorbol myristate acetate (PMA), scopoletin, horseradish peroxidase (HRP), clenbuterol and isoproterenol were purchased from Sigma, St Louis, MO.

Neutrophil isolation – Heparinized whole blood was collected from healthy, adult horses via jugular veinipuncture. The blood was then allowed to settle for 60 minutes, after which time the leukocyte rich plasma was collected and layered onto 5mL of Ficoll-Paque PLUS in 15mL conical tubes. The plasma was centrifuged @ 2000RPM for 20 minutes at room temperature. The remaining pellet was re-suspended in Hank's balanced salt solution containing 20 mM HEPES and 8.9 mM sodium bicarbonate without calcium

or magnesium (HBSS), yielding PMN that were greater than 98% pure and greater than 99% viable (data not shown).

Well coating with adhesion substrates – 96-well microtiter plates (Immulon 2HB, Dynex Technologies, Inc.) were coated with 50μL of either 100 ug/ml BSA or 5% FCS for 2 hours followed by 150μL 1% HSA in 0.1M glycine. IC immobilized to the microtiter plate plastic were generated by the addition of 50μL of either 1:50 or the indicated dilution of rabbit anti-BSA IgG (Sigma, St Louis MO) in PBS to wells previously coated with BSA. Rabbit anti-BSA IgG was used due to the lack of a commercially available source for equine anti-BSA IgG. Immobilized IC generated with rabbit IgG have been previously demonstrated to induce equine PMN adhesion in a β2 integrin dependent manner (Jones *et al* 2001).

Adhesion assay – PMN adhesion was quantified as previously described (Jones *et al* 2001). PMN were suspended in HBSS at a concentration of $1X10^7$ cells/mL. PMN were then incubated with 2μg/mL calcein at room temperature for 30 minutes. Following incubation with calcein, the cells were washed once with HBSS. The PMN were then re-suspended in HBSS containing 1mM Ca^{2+} and 1mM Mg^{2+} (HBSS++) at a concentration of $2X10^6$ cells/mL and treated with vehicle control or various cAMP modulators at the indicated concentration for 20 minutes at 37^0C. 50μL of the PMN solution was then added to the appropriate wells of a substrate-coated microtiter plate and incubated at room temperature for 10 minutes to allow the PMN to settle to the bottom of the wells. In some wells 70ng/mL PMA was added as a stimulant. The microtiter plate was then

incubated for 30 minutes at 37^0C. Total intracellular calcein fluorescence of each well (λ = 485nm excitation, λ = 530nm emission) was measured using a fMax fluorescence plate reader (Molecular Devices, Sunnyvale, CA) before and after washing with 150μL PBS. Percent adhesion was calculated by dividing the fluorescence after washing by the fluorescence before washing. In preliminary experiments, fluorescence was shown to be linearly related to cell number (data not shown).

Respiratory burst assay – PMN respiratory burst activity was measured using a previously described assay (Zhou and Brown 1993) adapted for equine PMN (Jones *et al* 2001). The PMN were suspended in Kreb's – Ringer's phosphate glucose buffer (KRPG) at a concentration of $2.5X10^6$ cells/mL and treated with vehicle control or various cAMP modulators at the indicated concentrations for 20 minutes at 37^0C. Following this incubation, 20μL of the PMN solution was added to wells of a substrate-coated microtiter plate containing 80μL of a reaction mixture consisting of 0.2mM scopoletin, 2.5mM NaN3, and 2.5U/mL HRP and the corresponding concentration of the appropriate cAMP modulator in KRPG. In the case of PMA-stimulated PMN, the reaction mixture also contained 175ng/mL PMA. The resulting solution in each well was 100μL containing $5X10^5$ cells/mL in KRPG plus the desired concentration of cAMP modulator, 80μM scopoletin, 1mM NaN_3, 1U/mL HRP and, when required, 70ng/mL PMA. The fluorescence was measured using a fMax fluorescence plate reader (λ = 360nm excitation, λ = 460nm emission) immediately and after a 90 minute incubation at 37^0C protected from light.

HRP catalyzes a reaction between the peroxide generated by the PMN and scopoletin in the reaction mixture, resulting in a loss of scopoletin fluorescence. The fluorescence decay is proportional to the peroxide produced and therefore can be used to measure respiratory burst activity. The nanomoles of peroxide released was calculated from the fluorescence decay as previously described (Zhou and Brown 1993).

Results

Cyclic AMP inhibits equine PMN adhesion to immune complexes - Elevation of intracellular cAMP generally inhibits PMN activation by a variety of agonists, including IC (Bloemen *et al* 1997, Derian *et al* 1995, Condino-Neto *et al* 1991, Nielson *et al* 1990). Activation of PMN by IC is most efficient when the IC are immobilized on a surface. Activation of PMN by immobilized IC requires adhesion and this adhesion is mediated by β2 integrins (Jones *et al* 2001, Jones *et al* 1998). There is accumulating evidence that cAMP inhibits the activation of β2 integrin-mediated adhesion in leukocytes. Thus, in order to begin to define the mechanism of cAMP-induced suppression of equine PMN activation by IC, we sought to determine whether cAMP inhibits β2 integrin-dependent adhesion to IC.

Equine PMN adhered avidly to immobilized IC and PMA stimulated adhesion to the β2 integrin substrate FCS (Figure 1). Elevation of intracellular cAMP concentrations with the cell permeant stable cAMP analog 8-Br-cAMP inhibited equine PMN adhesion to immobilized IC. In contrast to adhesion to IC, 8-Br-cAMP did not inhibit PMA-induced adhesion of equine PMN to FCS, demonstrating that cAMP elevation does not globally inhibit PMN adhesion and is not generally toxic to equine PMN. Moreover, the

inhibitory effect of 8-Br-cAMP on equine PMN adhesion to IC was reversed by the addition of the apparently specific PKA inhibitor KT5720, which inhibits the catalytic site of PKA, demonstrating that PKA activity is necessary for cAMP to inhibit adhesion to IC.

We demonstrated previously that adhesion of equine PMN to immobilized IC is dependent on β2 integrins (Jones *et al* 1998). Therefore, the ability of pharmacologic cAMP to inhibit adhesion to immobilized IC raised the possibility that the cAMP-PKA signaling axis is an important negative regulator of β2 integrin activation. To examine this possibility, we determined whether inhibition of PKA activity with KT5720 was sufficient to activate adhesion. KT5720 treatment activated adhesion of PMN to the β2 integrin substrate FCS in the absence of any other stimulus (Figure 1). KT5720 stimulated PMN adhesion in a dose dependent manner, with maximal adhesion observed at a concentration of 25μM (data not shown). Pharmacologic cAMP had no significant effect on KT5720-induced adhesion to FCS. KT5720-induced adhesion was completely inhibited by the anti-β2 integrin Mab IB4, demonstrating that inhibition of PKA specifically activates adhesion mediated by β2 integrins (data not shown). KT5720 treatment significantly increased adhesion of equine PMN to immobilized IC and had little effect on PMA-induced adhesion.

Inhibition of adhesion by pharmacologic modulation of intracellular cAMP concentration - The intracellular concentration of cAMP in PMN can be pharmacologically modulated via several different mechanisms. Ligation of β2 adrenergic receptors triggers cAMP production by stimulating adenyl cyclase activity.

The subsequent hydrolysis of cAMP can be prevented by pharmacologic inhibition of the enzyme phosphodiesterase. Because pharmacologic elevation of cAMP by phosphodiesterase inhibition or β2 adrenergic receptor stimulation is a potentially useful clinical therapeutic strategy, we sought to determine whether modulation of intracellular cAMP concentration by either mechanism affected PMN adhesion to IC.

Treatment of PMN with the relatively non-specific phosphodiesterase inhibitor pentoxifylline resulted in a dose-dependent inhibition of IC-induced PMN adhesion with an IC_{50} of 1mM (Figure 2). Rolipram, which is specific for the predominant equine neutrophil phosphodiesterase (PDE4), similarly inhibited equine PMN adhesion to IC with an IC_{50} of 1.25mM. The β2 adrenergic agonists clenbuterol and isoproterenol also inhibited equine PMN to IC in a dose-dependant manner (IC_{50} = 0.5mM, and IC_{50} = 15mM, respectively). Like pharmacologic cAMP, inhibition of phosphodiesterase activity or β2 adrenergic stimulation had little effect on PMA-induced adhesion, further demonstrating that PMA-stimulated PMN adhesion is cAMP-insensitive.

The finding that either PDE inhibition or β2 adrenergic stimulation inhibited adhesion to IC raised the possibility that co-treatment with a PDE inhibitor and a β2 adrenergic agonist would enhance the ability of either agent alone to inhibit PMN adhesion. To address this question, the effect of a sub-optimal concentration of PDE inhibitor on the dose response curve of clenbuterol or isoproterenol was determined (Figure 3). Co-treatment of PMN with either pentoxifylline or rolipram enhanced the ability of both clenbuterol and isoproterenol to inhibit equine PMN adhesion to IC.

Co-treatment with any combination of PDE and β2 adrenergic agonist resulted in significantly ($p<0.05$) greater inhibition of adhesion than was predicted for that

combination of cAMP modulators if the effects were additive (Table 1). These results demonstrate that combined treatment of equine PMN with PDE inhibitors and β2 adrenergic agonists synergistically inhibit adhesion to IC.

Inhibition of respiratory burst activity by pharmacologic modulation of intracellular cAMP concentration –PMN require β2 integrin-mediated adhesion to become optimally activated. Without this adhesion, PMN will not activate the phagocyte oxidase complex, activate phagocytosis, or degranulate in response to a variety of stimuli, including IC (Brown 1997). Thus, we examined the effect of modulation of intracellular cAMP concentrations on respiratory burst activity, a potent PMN inflammatory response.

As with IC-stimulated adhesion, clenbuterol, pentoxifylline or rolipram treatment inhibited IC-induced respiratory burst activity in equine PMN in a dose dependent manner (clenbuterol IC_{50} = 0.5mM, pentoxifylline IC_{50} = 0.5mM, rolipram IC_{50} = 0.5mM) (figure 4). Isoproterenol was excluded from the respiratory burst activity experiments because background fluorescence from the drug interfered with the assay. Pentoxifylline and clenbuterol had no significant effect on respiratory burst activity of PMN activated by PMA at the doses examined. Interestingly, rolipram did inhibit respiratory burst activity in PMA-stimulated PMN in a dose dependent manner. This inhibition of respiratory burst activity by rolipram on PMA-stimulated PMN is also evident in human PMN (data not shown).

Given the synergistic effect on inhibition of IC-stimulated PMN adhesion of PDE inhibitors and β2 adrenergic agonists, we determined if a similar effect could be observed in IC-stimulated PMN respiratory burst activity. Co-treatment of PMN with a sub-

optimal dose of either PDE inhibitor augmented the clenbuterol-induced inhibition of respiratory burst activity (Figure 5). As with adhesion to IC, the measured inhibition of respiratory burst activity co-treated with a PDE inhibitor and clenbuterol was greater ($p<0.05$) than the predicted effect if the inhibition was additive (Table 2).

The affect of IC density on the sensitivity of PMN adhesion to cAMP inhibition – cAMP may regulate adhesion to IC by inhibiting signals initiated by IC that activate β2 integrins to enable adhesion or by independently inhibiting β2 integrin signaling necessary for normal adhesion. To characterize the role of cAMP in the mechanism regulating adhesion of equine PMN to immobilized IC, we determined whether the signal strength (IC density) altered the IC_{50} for pentoxifylline. As the density of IC decreased, the IC_{50} for pentoxifylline also decreased (Figure 6). The effect of decreased IC density on pentoxifylline IC_{50} is unlikely to be due to a decrease in adhesive strength due solely to a loss of any adhesive capacity of the IC substrate bound to FcγR, since equine PMN have no detectable adhesion to any density of IC in the absence of β2 integrin ligation (Jones *et al* 2001). Thus, the loss of adhesive strength must be β2 integrin-dependent. These data suggest that cAMP inhibits the signals initiated by IC ligation of FcγR that activate β2 integrin avidity and adhesion rather than β2 integrin signaling. This is supported by the fact that PMA-induced adhesion is unaffected by pharmacologic cAMP, PDE inhibition, or β2 adrenergic stimulation.

Discussion

In the absence of any stimuli, PMN exist in a quiescent state in the circulation and tissues. Several different molecules can transform PMN to an activated state, including chemokines, cytokines, bacterial products, complement fragments and IC. Activated PMN will respond in different manners depending on the stimulus. Specific stimuli may induce chemotaxis, degranulation, adhesion, phagocytosis, production of reactive oxygen intermediates or cytokines. Integrin-dependent adhesion is required for PMN activation by IC (Jones *et al* 2001, Jones *et al* 1998, Tang *et al* 1997, Gresham *et al* 1991). When the Fc portion of an IgG molecule bound to antigen binds to FcγR, the receptor initiates a signaling cascade termed an "inside-out" signal. This signal results in activation of αMβ2 integrins to a high-avidity state, ligand binding, and adhesion, which are required for optimal activation of PMN effector functions (Brown 1997).

We have now demonstrated that cAMP and PKA have a role in regulating this inside-out signal during adhesion of equine PMN to IC. Elevation of cytosolic cAMP inhibits β2 integrin-dependent adhesion to IC and activation of respiratory burst activity. Indeed, inhibition of phosphodiesterase activity coupled with stimulation of β2 adrenergic receptors synergistically inhibited both adhesion and adhesion-dependent activation of the phagocyte oxidase, supporting the conclusion that these agents both act via elevating cytosolic cAMP. Inhibition of PKA rescues adhesion in PMN treated with cAMP, demonstrating that the cAMP effect is dependent on activation of PKA. Moreover, the IC_{50} for inhibition of adhesion by pentoxifylline is dependent on signal strength, as would

be expected if cAMP regulates Fc receptor mediated inside-out signaling during integrin activation. Elevation of cAMP has no effect on PMA-induced adhesion, ruling out an effect of cAMP on integrin-generated outside-in signals as an explanation of our results.

The role of cAMP and PKA in integrin-dependent PMN activation has not been completely determined. It is speculated that basal PKA activity in the quiescent PMN prevents the cell from developing an activated phenotype in the absence of an appropriate stimulus. It has also been shown that PKA negatively regulates the activity of RhoA, a member of the Rho family of small GTPases (Lang *et al* 1996). RhoA appears to be a critical element of the inside-out signals that activate integrin avidity in PMN. In this model, inhibition of PKA results in activation of RhoA and thus activation of β2 integrin avidity and adhesion.

Our work has demonstrated an IC_{50} ranging from 0.5mM to 15mM for each of the cAMP modulators examined. Previously published data describe the IC_{50} for isoproterenol and rolipram in fMLP-induced human PMN adhesion as 51.5μM and 0.023μM, respectively (Derian *et al* 1995). Denis and Riendeau (1999) reported IC_{50} for inhibition of fMLP-induced LTB4 production by human PMN of isoproterenol, rolipram and pentoxifylline as 0.35μM, 0.026μM and 20μM, respectively. These differences in IC_{50} suggest that the sensitivity of fMLP-receptor signaling and FcR signaling to cAMP are quite different. The reported rolipram IC_{50} for inhibition of PDE activity from equine PMN preparations is 1.2μM (Rickards *et al* 2000). The difference between the IC_{50} for FcR-induced adhesion and respiratory burst activity may reflect an inability for rolipram to efficiently penetrate the PMN cell membrane and achieve high concentrations in the cytosol.

β2 adrenergic agonists and PDE inhibitors increase intracellular cAMP concentrations by different means. Stimulation of the β2 adrenergic receptors on the surface of the PMN results in an increase in cAMP synthesis, where as inhibition of PDE decreases hydrolysis of cAMP and result in a build up of cAMP in the cytosol. Since PDE are found in virtually all cell types of the body, it may be beneficial from a therapeutic standpoint to use PDE inhibitors targeted to isoforms specific to PMN. Several different PDE have been identified and PDE4 has been shown to be the predominant type in human PMN (Rickards *et al* 2000). The equine PMN also primarily uses PDE4 for cAMP hydrolysis (Rickards *et al* 2000). We were able to inhibit IC-induced adhesion and the subsequent respiratory burst activity in equine PMN with either β2 adrenergic receptors or PDE inhibitors. Our study supports the view that PDE4 is the most functionally relevant PDE in equine PMN, because the PDE4 inhibitor rolipram inhibited adhesion to IC and activation of the respiratory burst as well or better than the non-specific PDE inhibitor pentoxifylline. The synergism observed between PDE inhibitors and β2 adrenergic agonists in this study most likely results from a synergistic effect on intracellular cAMP concentrations.

The inhibitory effect of rolipram upon PMA-induced respiratory burst activity was unexpected. Rolipram has been shown to be relatively specific for human PDE4 (Rickards *et al* 2000). Interestingly, rolipram inhibited respiratory burst activity without affecting adhesion in both human and equine PMA-stimulated PMN. These data suggest that PDE4 has a specific role in PMA-induced respiratory burst activity distinct from activation of integrins. It is puzzling that pentoxifylline, a non-specific PDE inhibitor, did not have a significant effect on PMA-induced respiratory burst activity. Perhaps the

PDE4 specific activity of pentoxifylline is not sufficient to inhibit the activation of the phagocyte oxidase by PMA at the concentrations used in these experiments. It remains possible that rolipram has activity other than its PDE4 effects that result in the inhibition of PMA-induced respiratory burst activity.

While the inflammatory response of PMN is crucial to combat infection, it can at times be too vigorous and cause significant damage to host tissues (Dallegri and Ottonello 1997, Brown and Lindberg 1996, Berton *et al* 1992). This study supports the concept that β2 adrenergic agonists and PDE inhibitors might have clinically relevant, anti-inflammatory effects in horses with IC-associated disease. We have shown that the density of IC present has an effect on the strength on PMN adhesion, most likely due to the strength of signaling within the PMN. We do not know the density of IC deposited in the tissues of horses with IC disease, but it is conceivable that in inflammatory conditions associated with IC deposition, the IC density in the tissue could be such that a therapeutic level of the β2 adrenergic agonists and/or PDE inhibitors could be achieved. Moreover, our data suggest that the combination of a PDE inhibitor and a β2 adrenergic agonist may allow us to achieve more potent inhibition of PMN adhesion activation and effector functions while minimizing the toxicity associated with these drugs.

References

Berton G, Laudanna C, Sorio C, Rossi F. 1992. Generation of signals activating neutrophil functions by leukocyte integrins: LFA-1 and gp150/95, but not CR3, are able to stimulate the respiratory burst of human neutrophils. *J. Cell. Biol.* 116:1007-1017

Berton G, Yan SR, Fumagalli L, Lowell CA. 1996. Neutrophil activation by adhesion: mechanisms and pathophysiological implications. *Int. J. Clin. Lab. Res*. 26:160-177

Bloemen PG, van den Tweel MC, Henricks PA, Engels F, Kester MH, van de Loo PG, Blomjous FJ, Nijkamp FP. 1997. Increased cAMP levels in stimulated neutrophils inhibit their adhesion to human bronchial epithelial cells. *Am. J. Physiol.* 272:L580-L587

Brown E. 1997. Neutrophil adhesion and the therapy of inflammation. *Semin. Hematol.* 34:319-326

Brown EJ, Lindberg FP. 1996. Leucocyte adhesion molecules in host defence against infection. *Ann. Med.* 28:201-208

Condino-Neto A, Vilela MM, Cambiucci EC, Ribeiro JD, Guglielmi AA, Magna LA, De Nucci G. 1991. Theophylline therapy inhibits neutrophil and mononuclear cell chemotaxis from chronic asthmatic children. *Br. J. Clin. Pharmacol.* 32:557-561

Dallegri F, Ottonello L. 1997. Tissue injury in neutrophilic inflammation. *Inflamm. Res.* 46:382-391

Denis D, Riendeau D. 1999. Phosphodiesterase 4-dependent regulation of cyclic AMP levels and leukotriene B4 biosynthesis in human polymorphonuclear leukocytes. *Eur. J. Pharmacol.* 367:343-350

Derian CK, Santulli RJ, Rao PE, Solomon HF, Barrett JA. 1995. Inhibition of chemotactic peptide-induced neutrophil adhesion to vascular endothelium by cAMP modulators. *J. Immunol*. 154:308-317

Graham IL, Lefkowith JB, Anderson DC, Brown EJ. 1993. Immune complex-stimulated neutrophil LTB4 production is dependent on β2 integrins. *J. Cell Biol.* 120:1509-1517

Gresham HD, Graham IL, Anderson DC, Brown EJ. 1991. Leukocyte adhesion deficient (LAD) neutrophils fail to amplify phagocytic function in response to stimulation: evidence for CD11b/CD18-dependent and -independent mechanisms of phagocytosis. *J. Clin. Invest.* 88:588-597

Jones SL, Knaus UG, Bokoch GM, Brown EJ. 1998. Two signaling mechanisms for the activation of αMβ2 avidity in polymorphonuclear neutrophils. *J. Biol. Chem.* 273:10556-10566

Jones SL., Sharief Y, Chilcoat CD. 2001. Signaling mechanism for equine neutrophil activation by immune complexes. *Vet. Immunol. Immunopathol.* 82:87-100

Kato K, Sautes-Fridman C, Yamada W, Kobayashi K, Uchiyama S, Kim H, Enokizono J, Galinha A, Kobayashi Y, Fridman WH, Arata Y, Shimada I. 2000. Structural basis of the interaction between IgG and Fcγ receptors. *J. Mol. Biol.* 295:213-224

Lang P, Gesbert F, Delespine-Carmagnat M, Stancou R, Pouchelet M, Bertoglio J. 1996. Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. *EMBO J* 15:510-519

Nagahata H, Kehrli ME Jr., Murata H, Okada H, Noda H, Kociba GJ. 1994. Neutrophil function and pathologic findings in Holstein calves with leukocyte adhesion deficiency. *Am. J. Vet. Res*. 55:40-48

Nielson CP, Vestal RE, Sturm RJ, Heaslip R. 1990. Effects of selective phosphodiesterase inhibitors on the polymorphonuclear leukocyte respiratory burst. *J. Allergy Clin. Immunol.* 86:801-808

Ninomiya N, Hazeki K, Fukui Y, Seya T, Okada T, Hazeki O, Ui M. 1994. Involvement of phosphatidylinositol 3-kinase in Fc gamma receptor signaling. *J. Biol. Chem.* 269:22732-22737

Rickards KJ, Page CP, Lees P, Cunningham FM. 2000. Phosphodiesterase activity in neutrophils from horses with chronic obstructive pulmonary disease. *Vet. Immunol. Immunopathol.* 76:319-330

Tang T, Rosenkranz A, Assmann KJM, Goodman MJ, Gutierrez-Ramos JC, Carroll MC, Cotran RS, Mayadas TN. 1997. A role for Mac-1 (CDIIb/CD18) in immune complex-stimulated neutrophil function *in vivo*: Mac-1 deficiency abrogates sustained Fcγ

receptor-dependent neutrophil adhesion and complement-dependent proteinuria in acute glomerulonephritis. *J. Exp. Med.* 186:1853-1863

Zhou MJ, Brown EJ. 1993. Leukocyte response integrin and integrin associated protein act as a signal transduction unit in generation of a phagocyte respiratory burst. *J. Exp. Med.* 178:1165-1174

Table 1. *Synergistic effect of PDE inhibitors combined with a β2 adrenergic agonist on inhibition of adhesion in IC-stimulated PMN* – PMN were treated with the indicated combinations of PDE inhibitors and β2 adrenergic agonists and adhesion was measured as described in "Materials and Methods". The measured inhibition of adhesion for each treatment combination is reported as the percent reduction of adhesion as compared to control (untreated) PMN adhesion, where 100% is complete inhibition of adhesion. The expected inhibition for each combination of PDE inhibitor and β2 adrenergic agonist was calculated by adding the percent reduction of control PMN adhesion resulting from treatment with the PDE inhibitor and the β2 adrenergic agonist separately. The measured inhibition of adhesion was significantly less than the expected inhibition of adhesion for all combinations of PDE inhibitor and β2 adrenergic agonist examined ($p<0.05$ determined by ANOVA).

TABLE 1

β2 Adrenergic Agonist	Phosphodiesterase Inhibitor	Measured	Expected
clenbuterol 0.5mM	pentoxifylline 0.5mM	84.7 ± 3.3%	20.4 ± 8.4%
clenbuterol 0.5mM	rolipram 0.5mM	73.7 ± 4.5%	21.5 ± 11.8%
isoproterenol 10mM	pentoxifylline 0.5mM	31.2 ±1.4%	12.6 ± 7.1%
isoproterenol 10mM	rolipram 0.5mM	65.2 ± 6.8%	13.7 ±12.5%

Table 2. *Synergistic effect of PDE inhibitors and Clenbuterol on inhibition of respiratory burst activity in IC-stimulated PMN* – PMN were treated with the indicated combinations of PDE inhibitors and clenbuterol and respiratory burst activity was measured as described in "Materials and Methods". The measured inhibition of respiratory burst activity for each treatment combination is reported as the percent reduction of respiratory burst activity as compared to control (untreated) PMN respiratory burst activity, where 100% is complete inhibition of respiratory burst activity. The expected inhibition for each combination of PDE inhibitor and clenbuterol was calculated by adding the percent reduction of control PMN respiratory burst activity resulting from treatment with the PDE inhibitor and clenbuterol separately. The measured inhibition of respiratory burst activity was significantly less than the expected inhibition of respiratory burst activity for all combinations of PDE inhibitor and clenbuterol examined ($p<0.05$ determined by ANOVA).

TABLE 2

β2 Adrenergic Agonist	Phosphodiesterase Inhibitor	Measured	Expected
clenbuterol 0.5mM	pentoxifylline 0.5mM	61.7 ± 5.2%	10.4 ± 12.9%
clenbuterol 0.5mM	rolipram 0.5mM	67.4 ± 7.2%	18.4 ± 10.8%

Figure 1. *Cyclic AMP and PKA regulate PMN adhesion to IC* – PMN (2 X 10^6 cells/mL) loaded with calcein were treated with KT5720 (25uM), 8Br-cAMP (5mM), KT5720 (25uM) + 8Br-cAMP (5mM) or vehicle control (DMSO) for 20 minutes at 37^0C and then allowed to adhere to 96-well plates coated with IC or FCS for 30 minutes at 37^0C. Some PMN were treated with 70ng/mL PMA at the time of addition to the FCS-coated wells. The data are the mean $\pm$ standard error of triplicate wells, reported as % adhesion, the percentage of cells that remained adherent after washing as described in "Materials and Methods." Data are representative of three separate trials. "*" signifies values that are statistically different ($p<0.05$) from the untreated group, as determined by Student's T-test.

FIGURE 1

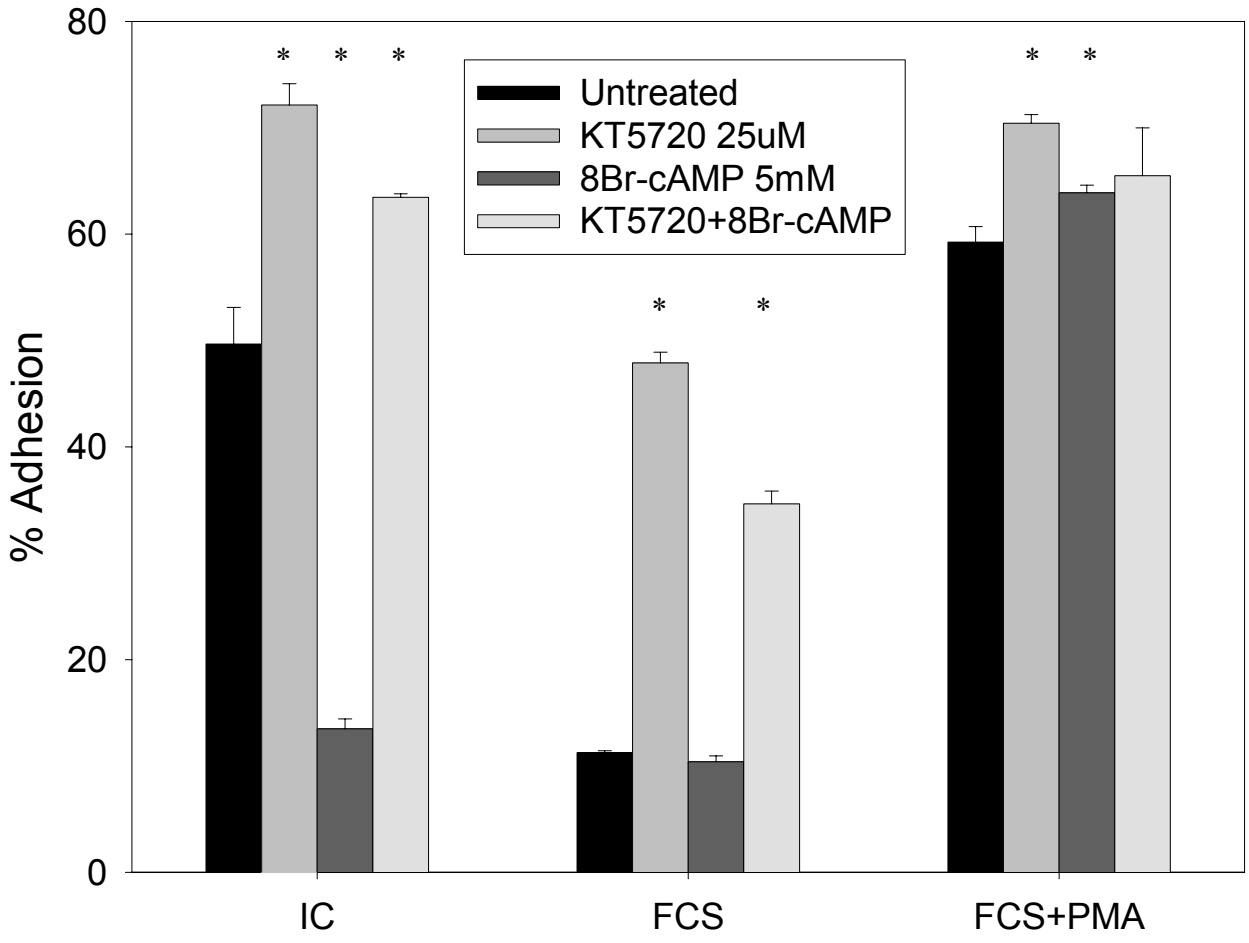

80
60
40
20
0
% Adhesion
Untreated
KT5720 25uM
8Br-cAMP 5mM
KT5720+8Br-cAMP
IC
FCS
FCS+PMA

Figure 2. *Dose dependent effects of cAMP modulators on PMN adhesion* – PMN were treated with various doses of isoproterenol (A), clenbuterol (B), rolipram (C), or pentoxifylline (D) for 20 minutes at 37^0C and then allowed to adhere to 96-well plates coated with IC or FCS for 30 minutes at 37^0C. Some PMN were treated with 70ng/mL PMA at the time of addition to the FCS-coated wells. Adhesion was determined as in figure 1. The data are the mean $\pm$ standard error of triplicate wells. Data are representative of three separate trials.

FIGURE 2

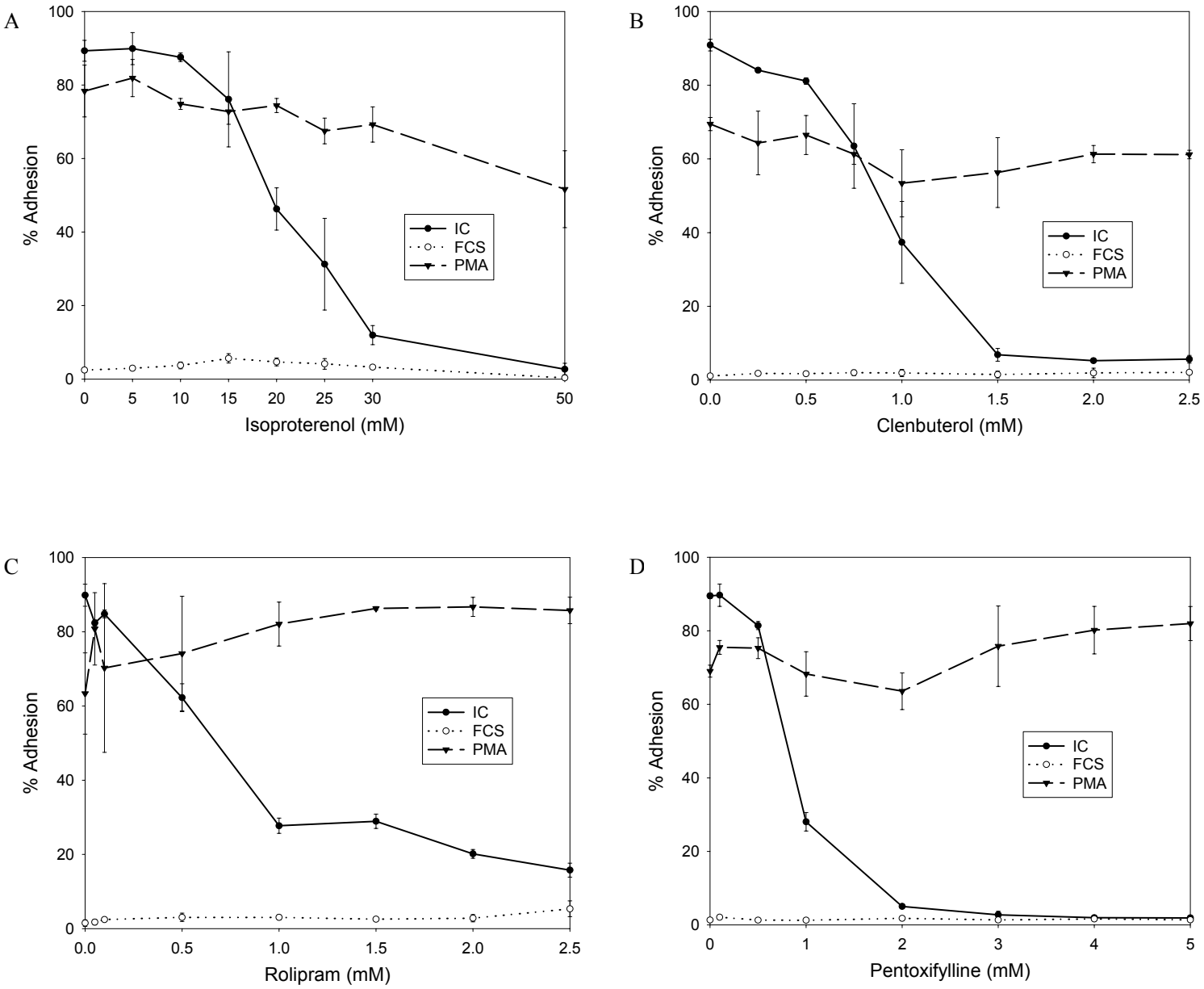

A
% Adhesion
Isoproterenol (mM)
IC
FCS
PMA
B
% Adhesion
Clenbuterol (mM)
IC
FCS
PMA
C
% Adhesion
Rolipram (mM)
IC
FCS
PMA
D
% Adhesion
Pentoxifylline (mM)
IC
FCS
PMA

Figure 3. *Effects of PDE inhibition on theβ2 adrenergic agonist dose response in PMN adherent to IC* – PMN were treated with various doses of isoproterenol (A and C) or clenbuterol (B and D) alone (labeled "control") or in combination with rolipram 0.5mM (A and B), or pentoxifylline 0.5mM (C and D) for 20 minutes at 37^0C and then allowed to adhere to 96-well plates coated with IC. Adhesion was determined as in figure 1. The data are the mean $\pm$ standard error of triplicate wells. Data are representative of three separate trials.

FIGURE 3

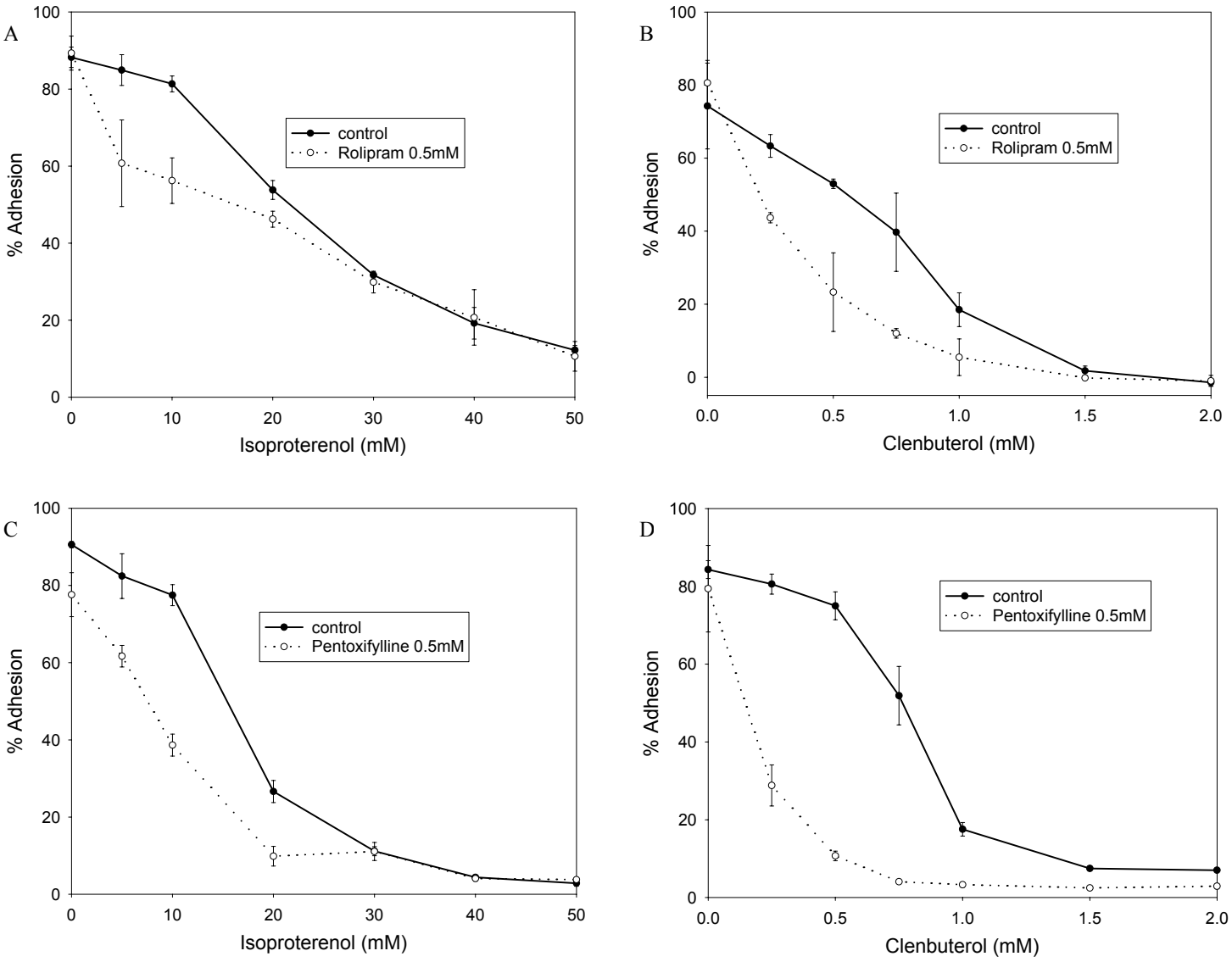

A
% Adhesion
Isoproterenol (mM)
control
Rolipram 0.5mM
B
% Adhesion
Clenbuterol (mM)
control
Rolipram 0.5mM
C
% Adhesion
Isoproterenol (mM)
control
Pentoxifylline 0.5mM
D
% Adhesion
Clenbuterol (mM)
control
Pentoxifylline 0.5mM

Figure 4. *Dose dependent effects of cAMP modulators on PMN respiratory burst activity* – PMN (2.5 X 10^6 cells/mL) were treated with various doses of pentoxifylline (A), clenbuterol (B) or rolipram (C) for 20 minutes at 37^0C. 20μL of cells was added to wells of 96-well plates coated with IC or FCS and containing 80μL of reaction mixture described in "Materials and Methods." Some PMN were treated with 70ng/mL PMA at the time of addition to the FCS-coated wells. The data are the mean $\pm$ standard error of triplicate wells, reported as nmole of peroxide produced. Data are representative of three separate trials.

FIGURE 4

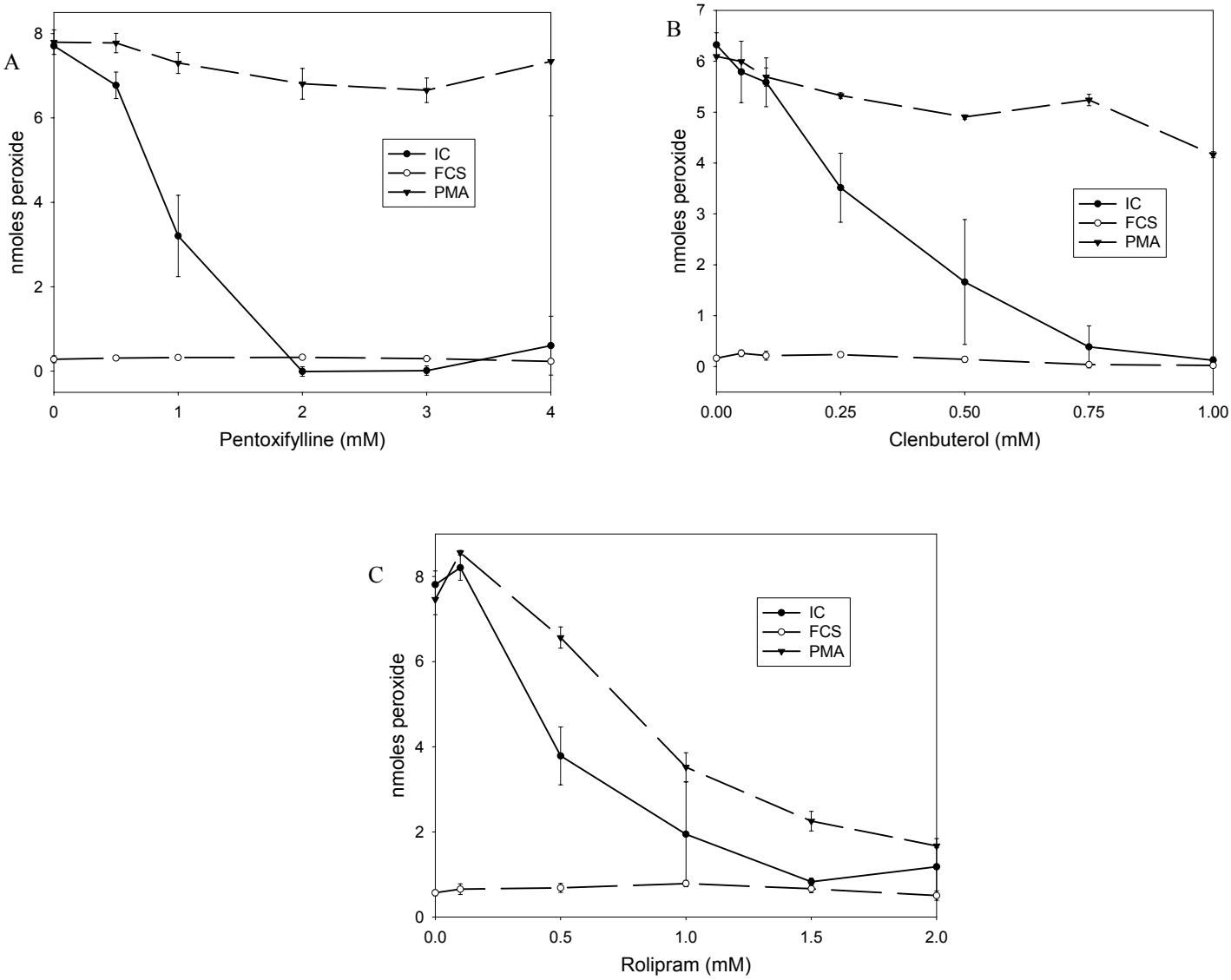

A
nmoles peroxide
Pentoxifylline (mM)
IC
FCS
PMA
B
nmoles peroxide
Clenbuterol (mM)
IC
FCS
PMA
C
nmoles peroxide
Rolipram (mM)
IC
FCS
PMA

Figure 5. *Addition of a PDE inhibitor on the dose dependent effects of clenbuterol on IC-induced PMN respiratory burst activity* - PMN were treated with various doses of clenbuterol alone (labeled "control") or in combination with rolipram 0.05mM (A) or pentoxifylline 0.1mM (B) for 20 minutes at 37^0C. 20μL of cells was added to wells of 96-well plates coated with IC and containing 80μL of reaction mixture. Respiratory burst activity was measured as in figure 4. The data are the mean $\pm$ standard error of triplicate wells. Data are representative of three separate trials.

FIGURE 5

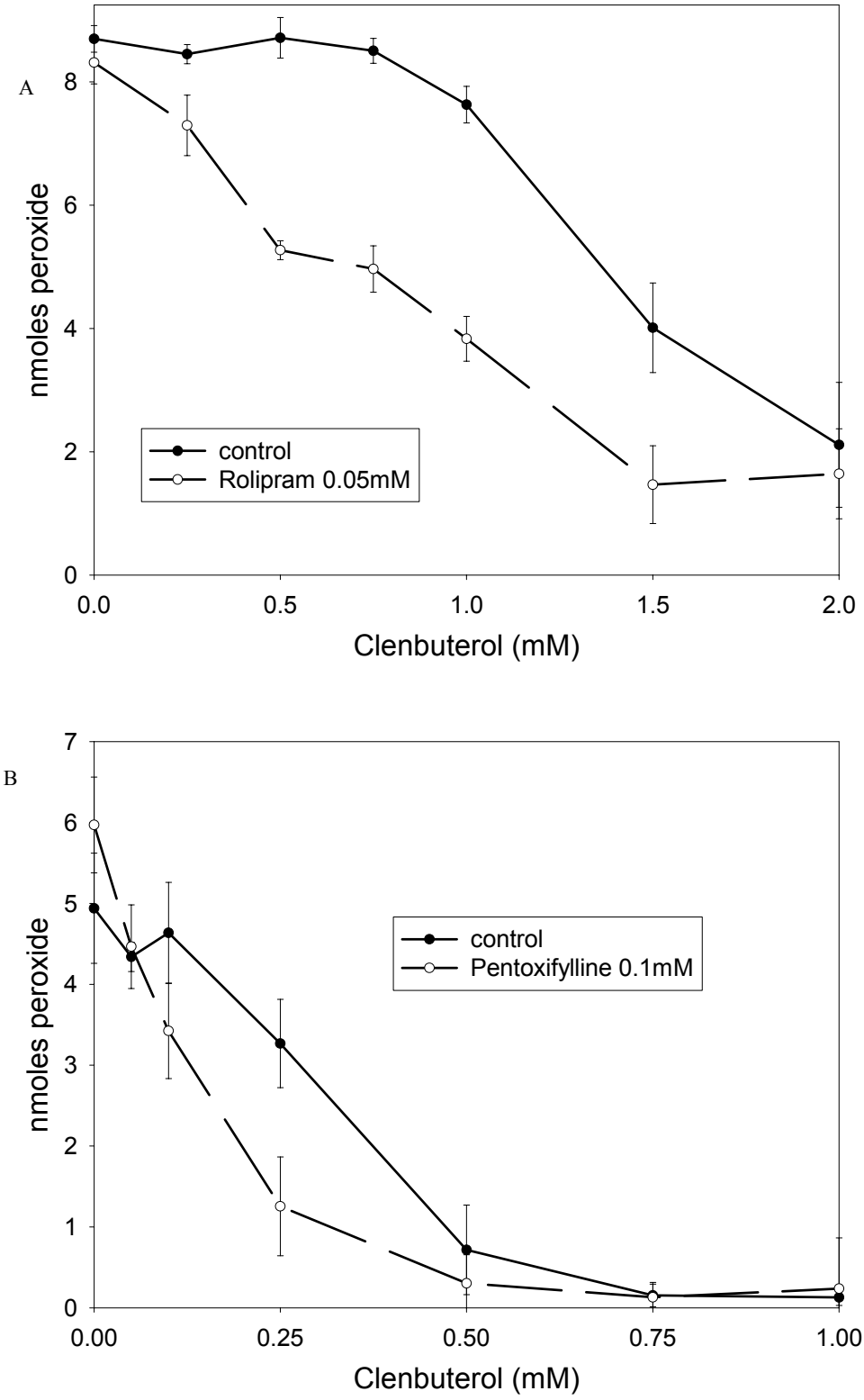
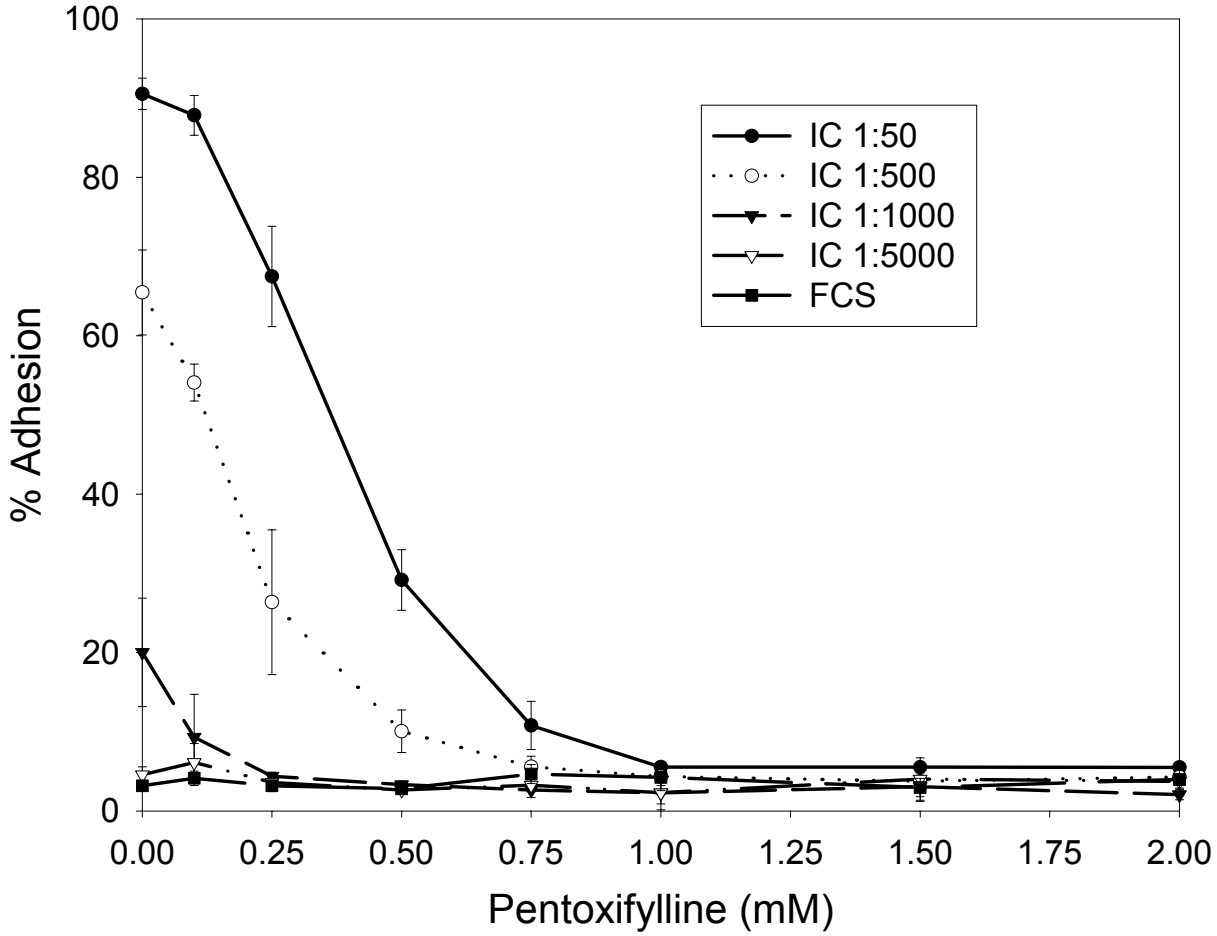

A
nmoles peroxide
0
2
4
6
8
0.0
0.5
1.0
1.5
2.0
Clenbuterol (mM)
control
Rolipram 0.05mM
B
nmoles peroxide
0
1
2
3
4
5
6
7
0.00
0.25
0.50
0.75
1.00
Clenbuterol (mM)
control
Pentoxifylline 0.1mM

Figure 6. *Dose dependent effects of IC on induction of PMN adhesion*- PMN were treated with various doses of pentoxifylline for 20 minutes at 37^0C and then allowed to adhere to 96-well plates coated with FCS or the indicated densities of IC for 30 minutes at 37^0C. Adhesion was determined as in figure 1. The data are the mean $\pm$ standard error of triplicate wells. Data are representative of three separate trials.

FIGURE 6


100
80
60
40
20
0
% Adhesion
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00
Pentoxifylline (mM)
IC 1:50
IC 1:500
IC 1:1000
IC 1:5000
FCS

CHAPTER 3

Protein Kinase A Regulates β2 Integrin Avidity and Subsequent Activation of Neutrophils via Modulation of Myosin Light Chain Phosphorylation

Abstract

β2 integrins are adhesion molecules on the surface of neutrophils. Affinity activation of β2 integrins consists of transportation of pre-formed integrins to the cell surface and a conformational change in the integrin to a high-binding state. Upon binding ligand, β2 integrins initiate a signaling cascade that results in activation of the neutrophil to a pro-inflammatory state. We have previously shown that enhanced protein kinase A (PKA) activity via increased intracellular cAMP concentrations will attenuate β2 integrin-dependent adhesion and subsequent integrin-dependent respiratory burst activity in stimulated neutrophils. We have also demonstrated that inhibition of PKA in quiescent neutrophils activates β2 integrin-dependent adhesion. PKA has been shown to inactivate myosin light chain kinase (MLCK). Myosin light chain (MLC) phosphorylation is crucial for actin-myosin complex formation, which is required for stability and contraction of the actin cytoskeleton in neutrophils. We hypothesize that the inhibitory effect of PKA upon β2 integrin affinity activation is via its inhibition of MLCK. In this study, we demonstrate via flow cytometric detection of antibodies directed against β2 integrins that pharmacologic inhibition of PKA activity results in overall increased β2 integrin expression on the neutrophil surface, as well as increased expression of the activated form of the integrin. This upregulation and activation of β2 integrins due to inhibition of PKA is abolished by pharmacologic MLCK inhibition. Inhibition of MLCK also blocked β2 integrin-dependent neutrophil adhesion achieved by inhibition of PKA, as well as neutrophil migration along towards a PKA inhibitor. These findings demonstrate that PKA regulation of β2 integrin affinity activation and subsequent neutrophil activation is via an MLCK-dependent pathway.

Introduction

Neutrophils, or "polymorphonuclear cells" (PMN) are a critical component of innate immunity due to their protection against local infection and systemic dissemination of pathogens. The transition of PMN from a quiescent, monitoring phenotype to an aggressive pro-inflammatory state is referred to as phagocyte activation. Migration and activation of PMN is mediated through a set of molecules called integrins (Jones *et al* 2001, Jones *et al* 1998, Tang *et al* 1997, Graham *et al* 1993). Integrins are heterodimeric glycoprotein cell surface adhesion molecules, consisting of an α and a β sub-unit, found on many different cell types and are involved in binding the cell to other cells or to extracellular matrix. The β2 class of integrins, specifically integrins αLβ2 and αMβ2, is by far the most prevalent class of integrin found on the PMN cell surface (Jones *et al* 1998, Tang *et al* 1997, Nagahata *et al* 1994 Graham *et al* 1993, Gresham *et al* 1991). These integrins can be found in two distinct states; low and high affinity for ligand. In the resting PMN, β2 integrins are in a low affinity state and will bind ligand poorly. When the PMN receives certain extracellular pro-inflammatory signals, the β2 integrins cluster and become activated to a high affinity state and subsequently bind strongly to their specific ligands (Jones *et al* 2001, Ninomiya *et al* 1994). This up-regulation of integrins is termed "avidity activation". The integrin-ligand interaction is critical for the migration of PMN to sites of inflammation and also plays a key role in phagocytosis (Brown and Lindberg 1996, Berton *et al* 1996). The biochemical pathways initiated by

pro-inflammatory mediators and resulting in avidity activation are often referred to as an "inside-out signal".

β2 integrins also play a key role in phagocyte activation (Brown 1997). When β2 integrins bind ligand, they initiate a signaling cascade via the cytoplasmic tail of their β sub-unit (Carman and Springer 2003, Ley 2002, Travis *et al* 2003). This "outside-in signal" results in the transformation of resting PMN to a pro-inflammatory state. It has been previously demonstrated by our laboratory and others that by blocking β2 integrin binding of ligand in the presence of strong pro-inflammatory extracellular signals, phagocyte activation of PMN can be prevented (Brown 1997). This effect can also be witnessed clinically in patients afflicted with leukocyte adhesion molecule deficiency (LAD), where due to a lack of β2 integrins and the subsequent inability to undergo phagocyte activation, these individuals cannot mount an adequate innate immune response (Anderson *et al* 1986, Anderson *et al* 1984, Nathan *et al* 1989, Nathan and Sanchez 1990, Graham *et al* 1993, Gresham *et al* 1991, Shappell *et al* 1990). From the clinical and experimental data, it is clear that β2 integrins are potent regulators of phagocyte activation and therefore of the innate immune system.

Cyclic AMP (cAMP) is a "second messenger" molecule involved in many intracellular signaling pathways and has been previously shown to mediate anti-inflammatory signals in PMN. The anti-inflammatory effects of cAMP have been shown to repress PMN inflammatory functions, such as adhesion, phagocytosis and respiratory burst activity initiated by many different activators of PMN (Bloemen *et al* 1997, Derian *et al* 1995, Condino-Neto *et al* 1991, Nielson *et al* 1990). We have demonstrated in our laboratory, in both human and equine PMN, that treatment of PMN with either a cell

permeant analog of cAMP or substances that increase intracellular cAMP concentrations will inhibit PMN phagocyte activation in response to several pro-inflammatory mediators (Jones 2002, Chilcoat *et al* 2002). Interestingly, elevation of intracellular cAMP concentrations had no significant effect upon PMN phagocyte activation mediated by phorbol myristate acetate (PMA), a potent pro-inflammatory mediator. This inconsistency in the effect of cAMP supports the previously determined concept that different pro-inflammatory mediators trigger their effects through different signaling pathways.

Protein kinase A (cAMP-dependent protein kinase, PKA) is a common effector of cAMP signaling. PKA is a heterotetrameric protein comprised of two cAMP-binding regulatory subunits (PKAr) and a catalytic subunit (PKAc). Binding of cAMP by the PKAr results in release of the PKAc, which is subsequently able to phosphorylate specific substrates. A kinase anchoring proteins (AKAPs) bind PKA and are responsible for PKA localization in different compartments of the cytosol (Edwards and Scott 2000). We have previously demonstrated that inhibition of PKA with the specific inhibitor KT5720 will block the cAMP-derived inhibition of phagocyte activation in PMN (Jones *et al* 2001, Jones 2002, Chilcoat *et al* 2002). In fact, we have shown that KT5720-induced inhibition of PKA in PMN is sufficient to induce avidity activation of β2 integrins but not subsequent phagocyte activation in the absence of a pro-inflammatory stimulus. It can therefore be stated that inhibition of PKA does not directly activate PMN, but specifically activates β2 integrins and subsequent integrin-mediated adhesion, which can in turn support phagocyte activation. These findings suggest that the

inhibition of PMN due to cAMP is mediated through PKA and that the constitutive PKA activity in the quiescent PMN plays a role in maintaining PMN integrins in the inactivated state. It is unknown at this time if PMN phagocyte activation via pro-inflammatory mediators is achieved by directly down-regulating PKA activity or if the inhibitory activity of PKA is exceeded by the signal of the pro-inflammatory mediators. Recent work in our laboratory has demonstrated that global inhibition of PKA by KT5720 results in a loss of fMLF-induced chemotaxis in PMN, however exposure of PMN to a gradient of KT5720 results in polarization and chemotaxis of the PMN along the KT5720 gradient (Jones unpublished data). These findings suggest that PKA activity varies within the PMN cytosol based on its distribution to the leading or trailing edge of the polarized cell.

The actin cytoskeleton plays a critical role in both avidity activation of integrins as well as subsequent phagocyte activation of PMN (Gresham *et al* 1991, Shappell *et al* 1990). The actin cytoskeleton serves as a scaffold and clusters integrins with other cell surface receptors, activatable enzymes, and signal transduction molecules and stabilizes focal adhesions (Graham *et al* 1993, Weber *et al* 1996, Detmers *et al* 1990, Anderson *et al* 1986). Coordinated cell movement requires directed contraction of the actin cytoskeleton. The actin cytoskeleton is also utilized in the process of degranulation, which delivers preformed integrins to the cell surface in the face of avidity activation. We have recently shown that global inhibition of PKA prevents F-actin reorganization and subsequent PMN polarization, while exposure of PMN to a gradient of KT5720 induces PMN polarization (Jones unpublished data). These findings demonstrate that PKA plays a role in maintaining PMN quiescence by regulating cytoskeletal activity.

Myosin light chain (MLC) is a subunit of the myosin complex and phosphorylation of MLC at a serine residue at position 19 is required for formation of the actin-myosin complex, which results in stabilization and contraction of the actin cytoskeleton (Bresnick 1999). This phosphorylation of MLC is catalyzed by myosin light chain kinase (MLCK). MLCK contains a calmodulin (CaM) binding region, and the activity of MLCK requires Ca^{2+}/CaM binding. The CaM binding site of MLCK includes a PKA phosphorylation site, and studies have determined that phosphorylation of MLCK at this site by PKA results in inhibition of PKA activity resulting in decreased MLC phosphorylation (Howe 2004, Smith *et al* 2003).

This paper addresses our hypothesis that PKA inhibits β2 integrin affinity and avidity, and subsequent PMN activation, via inhibition of MLC phosphorylation by MLCK.

Materials and methods

Reagents - Powdered phosphate buffered saline (PBS) and Hank's balanced salt solution (HBSS) were purchased from Gibco BRL, Grand Island, NY. Ficoll-Paque PLUS, dextran, and ECL chemiluminescence detection reagents were obtained from Amersham Pharmacia Biotech, Piscataway, NJ. Calcein was purchased from Molecular Probes, Eugene, OR. KT5720 and the MLCK inhibitory peptide were purchased from Alexis Corporation, San Diego, CA. A scrambled version of the MLCK inhibitory peptide (MLCK scrambled) was generated by Synpep Corp., Dublin, CA. Bovine fetal calf serum (FCS) was obtained from HyClone, Logan UT. Diisopropylfluorophosphate and ML-7 were purchased from Calibiochem Corp., La Jolla, CA. The bicinchinonic acid (BCA) assay kit was purchased from Pierce, Rockford, IL. Heparin was purchased from

Elkins-Sinn, Inc., Cherry Hill, NJ. Polyacrylamide was purchased from National Diagnostics, Atlanta, GA. The sodium dodecyl sulfate, 2-mercaptoethanol, Triton-X-100 and sodium azide were purchased from Fisher Biotech, Fair Lawn, NJ. The polyvinylidene fluoride membrane was purchased from Millipore, Billerica, MA. Aprotinin, leupeptin, phenylmethylsulfonyl fluoride, pepstatin, iodoacetamide, bovine serum albumin (BSA), monoclonal mouse anti-myosin light chain (MLC) IgM, horseradish peroxidase (HRP)-conjugated anti-mouse IgM, and FITC-conjugated F(ab')2 sheep anti-mouse IgG were purchased from Sigma, St Louis, MO. Mouse anti-phospho-MLC IgG and HRP-conjugated anti-mouse IgG were purchased from Cell Signaling Technology, Beverly, MA. The anti-β2 integrin monoclonal antibody IB4 (Wright *et al* 1983) was purified, and F(ab')2 was prepared as described (Zhou and Brown 1994). The monoclonal antibody CBRM1/5 binds a neoepitope on activated αMβ2 integrins and was kindly provided by Timothy Springer (Harvard Medical School).

PMN isolation – Heparinized whole blood was collected from healthy adult donors via cephalic venipuncture. The blood was mixed with dextran (final concentration 1.4% dextran) and then allowed to settle for 60 minutes, after which time the leukocyte rich plasma was collected and layered onto 5mL of Ficoll-Paque PLUS in 15mL conical tubes. The plasma was centrifuged @ 1800RPM for 20 minutes at room temperature. The residual erythrocytes were lysed utilizing a hypotonic saline lysis solution and the remaining PMN were re-suspended in Hank's balanced salt solution containing 20 mM HEPES and 8.9 mM sodium bicarbonate without calcium or magnesium (HBSS),

yielding PMN that were greater than 98% pure and greater than 99% viable (data not shown).

Adhesion assay – 96-well microtiter plates (Immulon 2HB, Dynex Technologies, Inc.) were coated with 50μL 5% FCS for 2 hours. PMN adhesion was quantified as previously described (Jones *et al* 2001). PMN were suspended in HBSS at a concentration of $1X10^7$ cells/mL. PMN were then incubated with 1μg/mL calcein at room temperature for 30 minutes. Following incubation with calcein, the cells were washed once with HBSS. The PMN were then re-suspended in HBSS containing 1mM Ca^{2+} and 1mM Mg^{2+} (HBSS++) at a concentration of $2X10^6$ cells/mL and treated with vehicle control or various PKA and MLCK inhibitors at the indicated concentration for 30 minutes at 37^0C. 50μL of the PMN solution was then added to the appropriate wells of a substrate-coated microtiter plate and incubated at room temperature for 10 minutes to allow the PMN to settle to the bottom of the wells. Total intracellular calcein fluorescence of each well (λ = 485nm excitation, λ = 530nm emission) was measured using an fMax fluorescence plate reader (Molecular Devices, Sunnyvale, CA) before and after washing with 150μL PBS. Percent adhesion was calculated by dividing the fluorescence after washing by the fluorescence before washing. In preliminary experiments, fluorescence was shown to be linearly related to cell number (data not shown).

Migration assay – PMN were suspended in HBSS at a concentration of $1X10^7$ cells/mL and incubated with 1μg/mL calcein at room temperature for 30 minutes. Following incubation with calcein, the cells were washed once with HBSS. The PMN were then re-

suspended in HBSS++ with 2% FCS at a concentration of $1X10^6$ cells/mL and treated with vehicle control, KT5720 and MLCK inhibitors at the indicated concentrations for 30 minutes at 37^0C. 20μL of the PMN suspension was added to the upper wells of a 96-well ChemoTx chemotaxis plate (Neuro Probe, Inc., Gaithersburg, MD) with a pore size of 2μm and 30μL of vehicle control, KT5720 and MLCK inhibitors was placed in the bottom wells at the indicated concentrations to stimulated PMN migration across the membrane. The plate was incubated for 30 minutes at 37^0C and then the total intracellular calcein fluorescence of each well (λ = 485nm excitation, λ = 530nm emission) was measured using an fMax fluorescence plate reader (Molecular Devices, Sunnyvale, CA). 20μL of the PMN suspension was added to the bottom wells of the plate to serve as positive control for fluorescence detection. The percent of PMN that underwent migration was calculated as the fluorescence of the experimental well divided by the fluorescence of the positive control wells X 100%.

Flow cytometry – PMN were suspended in HBSS containing 1mM Ca^{2+} (HBSS+) at a concentration of $4X10^6$/mL and treated with vehicle control, KT5720 and ML-7 at the indicated concentrations for 30 minutes at 37^0C. The PMN were then washed and resuspended with chilled PBS containing 1% FCS and 0.1% sodium azide (wash buffer). The PMN were then incubated with the indicated primary antibody for 40 minutes on ice, washed twice and resuspended with chilled wash buffer. The PMN were subsequently incubated with FITC-conjugated anti-mouse F(ab')2 for 20 minutes on ice. The PMN were washed twice with chilled wash buffer to remove any unbound antibodies and the PMN were then suspended in PBS. The relative fluorescence of 10,000 gated PMN was

then measured using a FACSCalibur flow cytometer (Becton-Dickinson, San Diego, CA).

MLC phosphorylation analysis – PMN were suspended in HBSS++ at a concentration of $4X10^6$ cells/mL and treated with vehicle control, KT5720 and MLCK inhibitors at the indicated concentrations for 30 minutes at 37^0C. The PMN were centrifuged at 6000RPM for 10 minutes and resuspended in chilled lysis buffer containing 0.5% Triton-X-100, 10ug/mL aprotinin/leupeptin, 1mM phenylmethylsulfonyl fluoride, 0.1mg/mL pepstatin, 1mM iodoacetamide, and 5mM diisopropylfluorophosphate. The PMN were agitated on ice for 30 minutes and then centrifuged at 14000RPM and 4^0C for 10 minutes. The supernatant was collected, an aliquot of each sample was saved for total protein quantification via bicinchinonic acid (BCA) assay, and the remainder of each sample was diluted with an equal volume of Lammeli buffer with 5% 2-mercaptoethanol and boiled for 5 minutes. The equal total protein content of each lysate was loaded on a 12% SDS-polyacrylamide gel and subsequently resolved via SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membranes were blocked with BSA and then probed with either mouse anti-phospho-MLC IgG or mouse anti-MLC IgM. Unbound antibody was removed via washing of the membrane and the membrane was then probed with an appropriate horseradish peroxidase (HRP) conjugated secondary antibody. The secondary antibody was detected via chemiluminescence using the ECL detection kit and exposure of Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY). The films were digitized using a ScanJet 5100C (Hewlett-Packard, Palo Alto, CA) and the optical

density of each band was determined using ONE-Dscan 2.05 for Windows (Scanalytics, Inc., Fairfax, VA).

Statistical analysis – Significance of difference between data points was determined using student's T-test. Findings were considered significantly different with $P < 0.05$.

Results

PKA negatively regulates MLC phosphorylation via a MLCK – dependent pathway.

Previous studies have determined that PKA can phosphorylate, and thereby inactivate MLCK. To determine if MLC phosphorylation is regulated by PKA in PMN, PMN were isolated from peripheral blood and treated with the PKA inhibitor KT5720 at varying concentrations for 30 minutes at 37ºC, and MLC phosphorylation was determined via Western Blot. Inhibition of PKA by KT5720 resulted in a dose dependant increase in MLC phosphorylation (figure 1). Since this increase in phosphorylated MLC could be due to an increase in phosphorylation or a decrease in phosphatase activity, we assessed the effect of the MLCK inhibition on KT5720-induced MLC phosphorylation. PMN were co-treated with KT5720 (10μM) and either the pharmacologic MLCK inhibitor ML-7 (10μM), or a MLCK inhibitory peptide (10μM) and subsequently lysed, and the lysates were resolved by SDS-PAGE (figure 2). The KT5720-induced upregulation of MLC phosphorylation was partially prevented by pretreatment with either MLCK inhibitor. A scrambled version of the MLCK inhibitory peptide (10μM) had no effect upon KT5720-induced MLC phosphorylation. These findings demonstrate that the regulatory effect of PKA upon MLC phosphorylation requires the activity of MLCK.

PKA regulates PMN migration via a MLCK – dependent pathway.

MLC phosphorylation is required for completion of the actin-myosin complex and subsequent cytoskeletal stability and contraction, and this stability and contraction are required for the firm adhesion and extravasation of leukocytes. Since PKA regulates MLC phosphorylation in PMN, we examined the role of PKA in PMN chemotaxis. Based on previous work that demonstrates that MLC phosphorylation regulates PMN migration and our findings that demonstrate that KT5720 stimulates MLC phosphorylation, we next addressed whether the ability of a gradient of KT5720 to stimulate chemotaxis is dependent on MLCK activity. Exposure of PMN to a gradient of the PKA inhibitor KT5720 resulted in a dose-dependent stimulation of PMN migration (figure 3). To determine if MLCK activity is required for the inhibitory of effect of PKA upon chemotaxis, PMN were pretreated with an MLCK inhibitor prior to the induction of chemotaxis with KT5720. Pre-treatment of PMN with either ML-7 (10μM) or the MLCK inhibitory peptide (10μM) resulted in inhibition of PMN chemotaxis. PMN migration was not stimulated by a gradient of ML-7 or the inhibitory peptide, and direct treatment of PMN with KT5720 did not stimulate migration (data not shown). The affect of MLCK inhibition on KT5720-induced chemotaxis was found to be dose dependent for both ML-7 (figure 4) and MLCK inhibitory peptide (figure 5) and chemotaxis was stimulated by KT5720 (25μM). The scrambled control peptide hade no effect upon KT5720–induced chemotaxis, demonstrating that the activity of the inhibitory peptide is specific to its sequence.

PKA regulates β2 integrin - mediated PMN adhesion via a MLCK – dependent pathway.

β2 integrin - mediated adhesion is critically required for PMN extravasation and migration. We have previously demonstrated that treatment of PMN with the PKA inhibitor KT5720 will initiate PMN adhesion to the β2 integrin substrate, FCS. Given this, we elected to determine if the ability of MLCK inhibitors to abolish KT5720-induced PMN migration is due to an inhibition of β2 integrin-dependent adhesion. Minimal adhesion was detected in untreated PMN, while adhesion was induced in a dose dependent manner by the PKA inhibitor KT5720 (figure 6). PMN were treated MLCK inhibitors prior to KT5720 treatment to examine the role of MLC phosphorylation in KT5720-induced PMN adhesion. Pretreatment of the PMN with ML-7 (10μM) inhibited the ability of KT5720 to stimulate adhesion. To determine if the effect of ML7 on KT5720-induced adhesion is dose dependent, PMN were pretreated with a range of doses of ML-7 and adhesion to FCS was stimulated by KT5720 (25μM) (figure 7). ML-7 had no effect upon adhesion in the cells lacking KT5720 treatment, while ML-7 inhibited β2 integrin–mediated adhesion in the KT5720-treated PMN in a dose dependant manner. MLCK inhibitory peptide inhibited β2 integrin–mediated adhesion in PMN treated with KT5720 (25μM) in a dose dependant manner similar to the effect demonstrated with ML-7 (figure 8). The control peptide had no effect upon KT5720–induced PMN adhesion. Both the active and control peptides had no effect upon unstimulated PMN (data not shown).

PKA regulates β2 integrin activation upon the surface of PMN via a MLCK – dependent pathway.

β2 integrin activation is required for PMN adhesion and migration. Treatment of PMN with KT5720 results in a dose-dependent increase in the number of β2 integrin molecules on the surface of individual PMN, as well as an increase in αMβ2 affinity. To determine whether the ability of KT5720 to activate β2 integrins is dependent on MLCK activity, we examined whether KT5720-induced β2 integrin expression and affinity activation was affected by inhibitors of MLCK. Treatment of PMN with KT5720 (25μM) resulted in upregulation of β2 integrin expression. ML-7 (10μM) had no effect upon β2 integrin expression upon unstimulated PMN, whereas co-treatment with ML-7 and the PKA inhibitor KT5720 resulted in inhibition of the upregulation of KT5720-induced upregulation of β2 integrin expression (figure 9). Moreover, inhibition of MLCK by ML-7 (10μM) resulted in inhibition of both the quantity of PMN expressing high affinity β2 integrins (figure 10) as well as the number of high affinity β2 integrin molecules per cell (figure 11) stimulated by the PKA inhibitor KT5720.

Discussion

Since phosphorylation of MLCK by PKA results in the loss MLCK's kinase activity, we hypothesized that the regulation of β2 integrins and the subsequent inhibition of PMN activation by PKA is dependent upon PKA inhibition of MLC phosphorylation. While MLCK does phosphorylate MLC, the phosphorylation state of MLC is also directly

affected by other kinases and by myosin light chain phosphatase (Howe 2004, Smith *et al* 2003, Chew *et al* 1998, Lamb *et al* 1988, Blue *et al* 2002). Western blot analysis of lysates of PMN treated with the PKA inhibitor KT5720 detected increased concentrations of phosphorylated MLC (pMLC). To assess the role of MLCK in this finding, we treated PMN with the pharmacologic MLCK inhibitor ML-7 in combination with KT5720 and witnessed a decrease in the pMLC concentration in the co-treated cells, demonstrating that MLCK activity is required for the increase of pMLC caused by PKA inhibition. Stated otherwise, MLCK activity is required for the inhibitory activity of PKA upon MLC activity. The MLCK inhibitor ML-7 is relatively specific at the concentrations examined, however to confirm our findings we repeated the experiment with a commercially available MLCK inhibitory peptide consisting of amino acids 11-19 of MLC which contains the catalytic site of MLCK and acts as a competitive inhibitor. The results of these experiments were similar to those performed with the ML-7, confirming our initial data. A control peptide was generated containing a randomized sequence of the inhibitory peptide, and this peptide had no affect upon MLC phosphorylation.

It has been previously determined that MLC phosphorylation is required for leukocyte chemotaxis (Adachi *et al* 2003, Klemke *et al* 1997). We have demonstrated that exposure of PMN to a gradient of KT5720 results in polarization of PMN and migration towards the PKA inhibitor. Our data presented here show that MLCK activity is required for this PKA regulation of PMN chemotaxis. MLC must be phosphorylated to allow actin polymerization, and the subsequent cytoskeletal rearrangement is required for both PMN polymerization and contraction. Our laboratory has determined that while exposure of PMN to a gradient of KT5720 results in cell polarization, global exposure to the PKA

inhibitor does not, suggesting that PKA activity must be inhibited at the leading edge but intact at the trailing edge of the cell to provide polarity and directional movement (Jones unpublished data).

To address the PKA-MLCK affects upon β2 integrins, we next looked at PMN adhesion to FCS which is a β2 integrin-dependent event in PMN. As previously observed, inhibition of PKA resulted in PMN adhesion to the β2 integrin substrate FCS (Chilcoat *et al* 2002, Jones *et al* 2002). Pre-inhibition of MLCK abolished the KT5720-induced adhesion, demonstrating the requirement for MLCK activity in the PKA regulation of β2 integrin-dependent adhesion. β2 integrins are anchored to the actin cytoskeleton and the clustering of integrins is mediated by cytoskeletal rearrangement. This clustering aids in adhesion by both increasing the number of integrin-ligand "anchors" at the site of adhesion as well as grouping, and therefore intensifying, the β2 integrin-mediated signaling that occurs subsequent to integrin-ligand binding (Stewart *et al* 1998). To further examine the effect of PKA and MLCK upon β2 integrins, we analyzed the surface expression of β2 integrins after treatment with KT5720. PKA inhibition resulted in an increase in both the total number and activation state of β2 integrins upon the surface of PMN. Co-inhibition of PKA and MLCK resulted in no change in β2 integrin expression, demonstrating the need for MLCK activity in the PKA regulation of β2 integrins. Upregulation of β2 integrin expression on the surface of PMN requires mobilization of preformed integrins to the cell surface, a function requiring MLC activity to move granules through the cytosol. Activation of β2 integrins requires integrin clustering which is also dependent upon reorganization of the cytoskeleton, and therefore, MLC.

The data presented here further demonstrates that β2 integrin activation and subsequent conversion of PMN to a pro-inflammatory state is regulated by PKA, and we have now seen that this regulation is mediated by regulation of MLCK activity. As we gain a greater knowledge of the physiologic mechanisms that regulate inflammation, we will become better equipped to therapeutically modulate the inflammatory response in the face of disease.

References

Adachi T, Stafford S, Kayaba H, Chihara J, Alam R. 2003. Myosin light chain kinase mediates eosinophil chemotaxis in a mitogen activated protein kinase–dependent manner. *J. Allergy Clin.Immunol.* 111: 113-116

Anderson D, Schmalstieg F, Arnaout M, Kohl S, Dickey W, Abramson J, Springer T, Boxer L, Hollers J, Smith C. 1984. Abnormalities of polymorphonuclear leukocyte function associated with a heritable deficiency of high molecular weight surface glycoproteins (GP138): common relationship to diminished cell adherence. *J. Clin. Invest.* 74: 536-551

Anderson D, Miller L, Schmalstieg F, Rothelein R, Springer T. 1986. Contributions of the Mac-1 glycoprotein family to adherence-dependent granulocyte functions: structure-function assessments employing subunit- specific monoclonal antibodies. *J. Immunol.* 137: 15-27

Berton G, Yan S, Fumagalli L, Lowell C. 1996. Neutrophil activation by adhesion: mechanisms and pathophysiological implications. *Int J Clin Lab Res* 26:160

Bloemen P, van den Tweel M, Henricks P, Engels F, Kester M, van de Loo P, Blomjous F, Nijkamp F. 1997. Increased cAMP levels in stimulated neutrophils inhibit their adhesion to human bronchial epithelial cells. *Am. J. Physiol.* 272: L580-L587

Blue E, Goeckeler Z, Jin Y, Hou L, Dixon S, Herring B, *et al*. 2002. 220-and 130-kDa MLCKs have distinct tissue distributions and intracellular localization patterns. *Am J Physiol Cell Physiol* 282:C451-60

Bresnick A. 1999. Molecular mechanisms of nonmuscle myosin-II regulation. *Curr.Opin. Cell Biol. 11*, 26-33

Brown E, 1997. Neutrophil adhesion and the therapy of inflammation. *Semin. Hematol.* 34: 319-326

Brown E, Lindberg F. 1996. Leucocyte adhesion molecules in host defence against infection. *Ann Med.* 28:201

Carman C, Springer T. 2003. Integrin avidity regulation: are changes in affinity and conformation underemphasized? *Curr.Op. Cell Biol.* 15:547-556

Chew T, Masaracchia R, Goeckeler Z, Wysolmerski R. 1998. Phosphorylation of non muscle myosin II regulatory light chain by p21-activated kinase (γ-PAK). *J. Muscle Res Cell Motil.*19:839-854

Chilcoat C, Rowlingson K, Jones S. 2002. The effects of cAMP modulation upon the adhesion and respiratory burst activity of immune complex-stimulated equine neutrophils. *Vet Immunol Immunopath.* 88: 65-77

Condino-Neto A, Vilela M, Cambiucci E, Ribeiro J, Guglielmi A, Magna L, De Nucci G. 1991. Theophylline therapy inhibits neutrophil and mononuclear cell chemotaxis from chronic asthmatic children. *Br. J. Clin. Pharmacol*. 32: 557-561

Derian C, Santulli R, Rao P, Solomon H, Barrett J. 1995. Inhibition of chemotactic peptide-induced neutrophil adhesion to vascular endothelium by cAMP modulators. *J. Immunol*. 154: 308-317

Detmers P, Lo S, Olson-Egbert E, Walz A, Baggiolini M, Cohn Z. 1990. *J. Exp. Med.* 171: 1155-1162

Edwards A, Scott J. 2000. A-kinase anchoring proteins: protein kinase A and beyond. *Curr Opin Cell Biol* 12:217

Graham I, Lefkowith J, Anderson D, Brown E. 1993. Immune complex-stimulated neutrophil LTB4 production is dependent on β2 integrins. *J. Cell Biol.* 120: 1509-1517

Gresham H, Graham I, Anderson D, Brown E, 1991. Leukocyte adhesion deficient (LAD) neutrophils fail to amplify phagocytic function in response to stimulation: evidence for CD11b/CD18-dependent and -independent mechanisms of phagocytosis. *J. Clin. Invest.* 88: 588-597

Howe A. 2004. Regulation of actin-based cell migration by cAMP/PKA. *Biochimica et Biophysica Acta Mol Cell Res.* 1692:159-174

Jones S, Knaus U, Bokoch G, Brown E. 1998. Two signaling mechanisms for the activation of αMβ2 avidity in polymorphonuclear neutrophils. *J. Biol. Chem*. 273:10556-10566

Jones S, Sharief Y, Chilcoat C. 2001. Signaling mechanism for equine neutrophil activation by immune complexes. *Vet. Immunol. Immunopathol*. 82: 87-100

Jones S. 2002. Protein kinase A regulates ß2 integrin avidity in neutrophils. *J Leuk Biol.* 71:1042-1048

Klemke R, Cai S, Giannini A, Gallagher P, Lanerolle P, Cheresh D. 1997. Regulation of Cell Motility by Mitogen-activated Protein Kinase. J. Cell Biol. 137: 481-492

Lamb N, Fernandez A, Conti M, Adelstein R, Glass D, Welch W, Feramisco J. 1988. Regulation of actin microfilament integrity in living nonmuscle cells by the cAMP dependent protein kinase and the myosin light chain kinase. *J Cell Biol.* 106: 1955-1971

Ley K. 2002. Integration of inflammatory signals by rolling neutrophils. *Imm. Rev.* 186:8

Nagahata H, Kehrli M, Murata H, Okada H, Noda H, Kociba G. 1994. Neutrophil function and pathologic findings in Holstein calves with leukocyte adhesion deficiency. *Am. J. Vet. Res.* 55: 40-48

Nathan C, Srimal S, Farber C, Sanchez E, Kabbash L, Asch A, Gailit J, Wright S. D. 1989. Cytokine-induced respiratory burst of human neutrophils: dependence on extracellular matrix proteins and CD11/CD18 integrins. *J. Cell Biol.* 109: 1341-1349

Nathan C, Sanchez E. 1990. Tumor necrosis factor and CD11/CD18 (beta 2) integrins act synergistically to lower cAMP in human neutrophils. *J. Cell Biol.* 111: 2171-2181

Nielson C, Vestal R, Sturm R, Heaslip R. 1990. Effects of selective phosphodiesterase inhibitors on the polymorphonuclear leukocyte respiratory burst. *J. Allergy Clin. Immunol.* 86: 801-808

Ninomiya N, Hazeki K, Fukui Y, Seya T, Okada T, Hazeki O, Ui M. 1994. Involvement of phosphatidylinositol 3-kinase in Fc gamma receptor signaling. *J. Biol. Chem.* 269: 22732-22737

Shappell S, Toman C, Anderson D, TaylorA, Entman M, Smith C. 1990. Mac-1 (CD11b/CD18) mediates adherence-dependent hydrogen peroxide production by human and canine neutrophils. *J. Immunol.* 144: 2702-2711

Smith A, Bracke M, Leitinger B, Porter J, Hogg N. 2003. LFA-1-induced T cell migration on ICAM-1 involves regulation of MLCK-mediated attachment and ROCK-dependent detachment. *J Cell Sci.* 116:3123-3133

Stewart M, McDowall A, Hogg N. 1998. LFA-1-mediated adhesion is regulated by cytoskeletal restraint and by a $Ca2^{+}$-dependent protease, calpain. *J Cell Biol* 140: 699-707

Tang T, Rosenkranz A, Assmann K, Goodman M, Gutierrez-Ramos J, Carroll M, Cotran R, Mayadas T. 1997. A role for Mac-1 (CDIIb/CD18) in immune complex-stimulated neutrophil function *in vivo*: Mac-1 deficiency abrogates sustained Fcγ receptor-dependent neutrophil adhesion and complement-dependent proteinuria in acute glomerulonephritis. *J. Exp. Med.* 186: 1853-1863

Travis M, Humphries J, Humphries M. 2003. An unraveling tale of how integrins are activated from within. *Trends Pharmacol Sci* 24:192–197

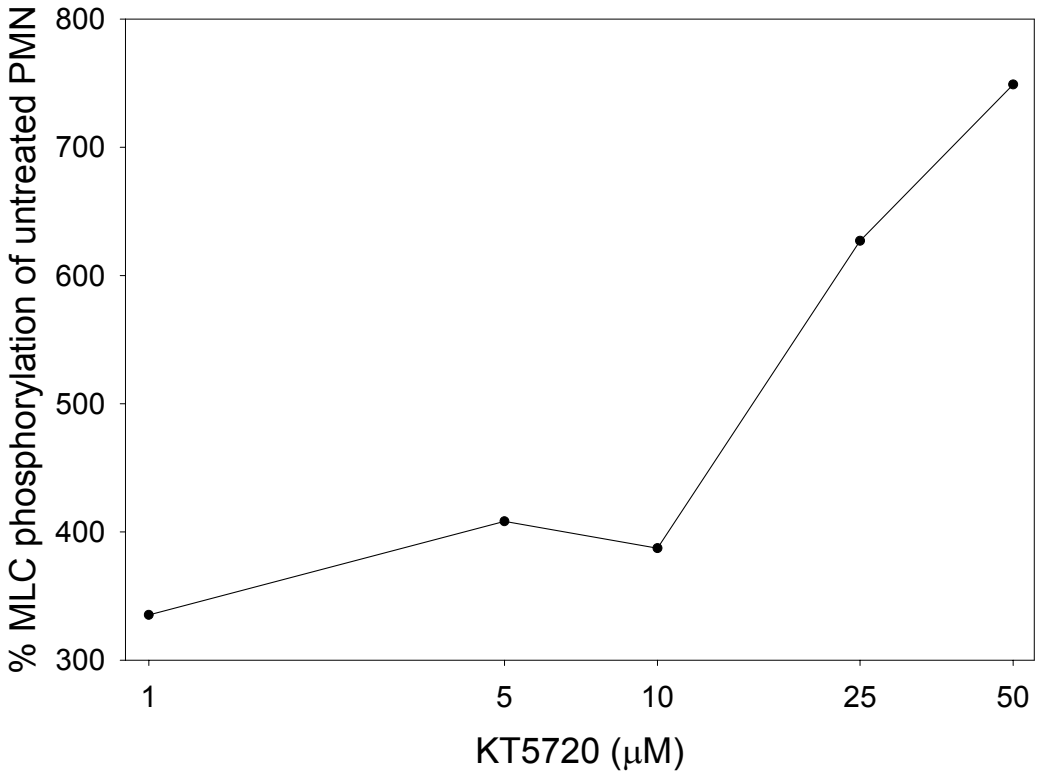
Weber C, Kitayama J, Springer T. 1996. *Proc. Natl. Acad. Sci. U. S. A.* 93: 10939-10944

Wright S, Rao P, Wesley C, van Voorhis W, Craigmyle L, Iida K, Talle M, Westberg E, Goldstein G, Silverstein S. 1983. Identification of the C3bi receptor of human monocytes and macrophages by using monoclonal antibodies *Proc. Natl. Acad. Sci. USA* 80:5699-5703

Zhou M, Brown E. 1994. CR3 (Mac-1, CD11b/CD18) and FcγRIII cooperate in generation of a neutrophil respiratory burst: requirement for FcRγRII and tyrosine phosphorylation *J. Cell Biol.* 125:1407-1416

Figure 1. *Treatment of PMN with the PKA inhibitor KT5720 results in a dose-dependent Phosphorylation of MLC* - PMN were treated with the indicated concentration of KT5720 or vehicle alone for 30 minutes at 37^0C and subsequently lysed. The cleared lysate was resolved via SDS-PAGE and blotted with anti-phospho-MLC antibodies and detected with HRP-conjugated secondary antibodies. The blot was stripped and reprobed with antibodies against any phosphorylation state of MLC and detected with HRP-conjugated secondary antibodies. The relative densities (as compared to the vehicle treated group) of each phospho-MLC band were determined and are shown in the graph. Data are representative of three separate trials.

Figure 1


% MLC phosphorylation of untreated PMN
800
700
600
500
400
300
1
5
10
25
50
KT5720 (μM)

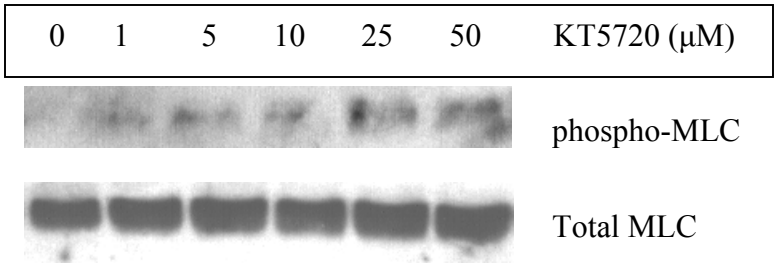
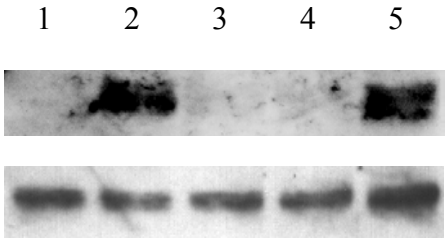

0 1 5 10 25 50 KT5720 (μM)
phospho-MLC
Total MLC

Figure 2. *KT5720-induced MLC phosphorylation is prevented by inhibition of MLCK*-PMN were treated with vehicle or the 10μM KT5720 alone or with 10μM ML-7, 10μM MLCK inhibitory peptide or 10μM scrambled control peptide for 30 minutes at 37^0C and subsequently lysed. The cleared lysate was resolved via SDS-PAGE and blotted with anti-phospho-MLC antibodies and detected with HRP-conjugated secondary antibodies. The blot was stripped and reprobed with antibodies against any phosphorylation state of MLC and detected with HRP-conjugated secondary antibodies. The relative densities (as compared to the vehicle treated group) of each phospho-MLC band were determined and are shown in the graph. Data are representative of three separate trials.

Figure 2



Lane 1	untreated
Lane 2	10μM KT5720
Lane 3	10μM KT5720 + 10μM ML-7
Lane 4	10μM KT5720 + 10μM MLCK peptide
Lane 5	10μM KT5720 + 10μM control peptide

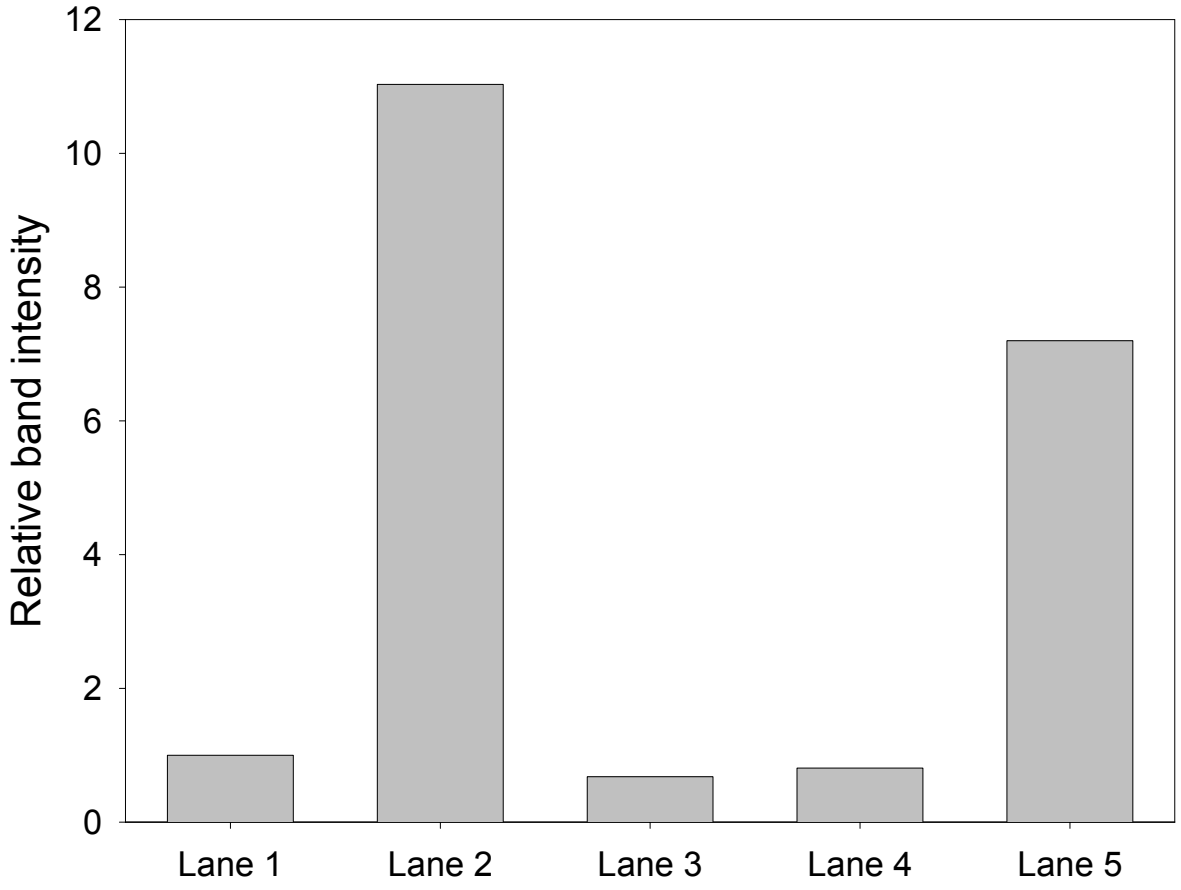
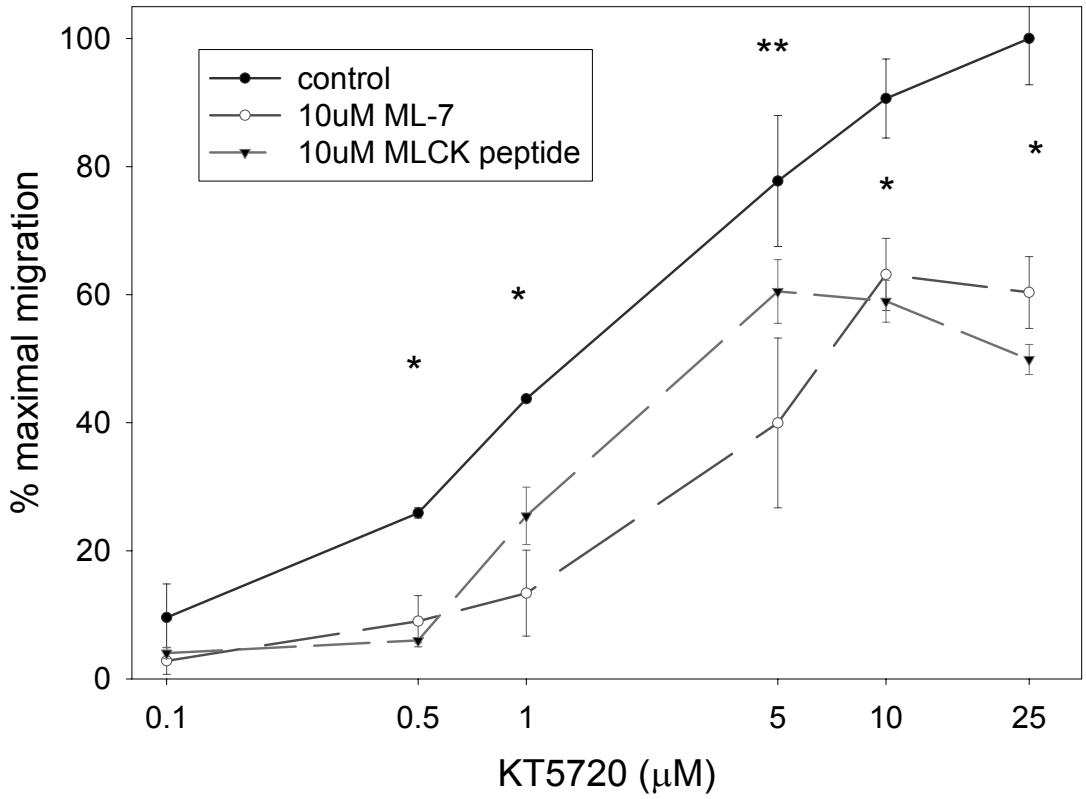


Figure 3. *KT5720-induced PMN migration is decreased by inhibition of MLCK*- PMN loaded with calcein were treated with the vehicle, 10μM ML-7 or 10μM MLCK inhibitory peptide for 30 minutes at 37^0C. PMN were added to the upper wells of a 96-well chemotaxis plate and the indicated concentrations of KT5720 were placed in the bottom wells to stimulated PMN migration across the membrane. The plate was incubated for 30 minutes at 37^0C and then the calcein fluorescence of each well was measured. PMN suspension was added to a set of bottom wells of the plate to serve as positive control for fluorescence detection. The fraction of PMN that underwent migration was calculated as the fluorescence of the experimental well divided by the fluorescence of the positive control, and these values were normalized to the maximal migration induced by KT5720. The data are the mean $\pm$ standard deviation of triplicate wells. Data are representative of three separate trials. "*" signifies values that are statistically different ($p<0.05$) between the no inhibitor and both ML-7 and MLCK peptide treated groups, as determined by Student's T-test. "**" signifies values that are statistically different ($p<0.05$) between the no inhibitor and ML-7 treated group.

Figure 3


control
10uM ML-7
10uM MLCK peptide
% maximal migration
KT5720 (μM)
0
20
40
60
80
100
0.1
0.5
1
5
10
25
*
*
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*
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Figure 4. *Inhibition of KT5720-induced PMN migration by ML-7 is dose-dependent*- PMN loaded with calcein were treated with the indicated concentrations of ML-7 for 30 minutes at 37^0C. PMN were added to the upper wells of a 96-well chemotaxis plate and 25μM KT5720 was placed in the bottom wells to stimulated PMN migration across the membrane. The plate was incubated for 30 minutes at 37^0C and then the calcein fluorescence of each well was measured. PMN suspension was added to a set of bottom wells of the plate to serve as positive control for fluorescence detection. The fraction of PMN that underwent migration was calculated as the fluorescence of the experimental well divided by the fluorescence of the positive control, and these values were normalized to the maximal migration induced by KT5720. The data are the mean $\pm$ standard deviation of triplicate wells. Data are representative of three separate trials.

Figure 4

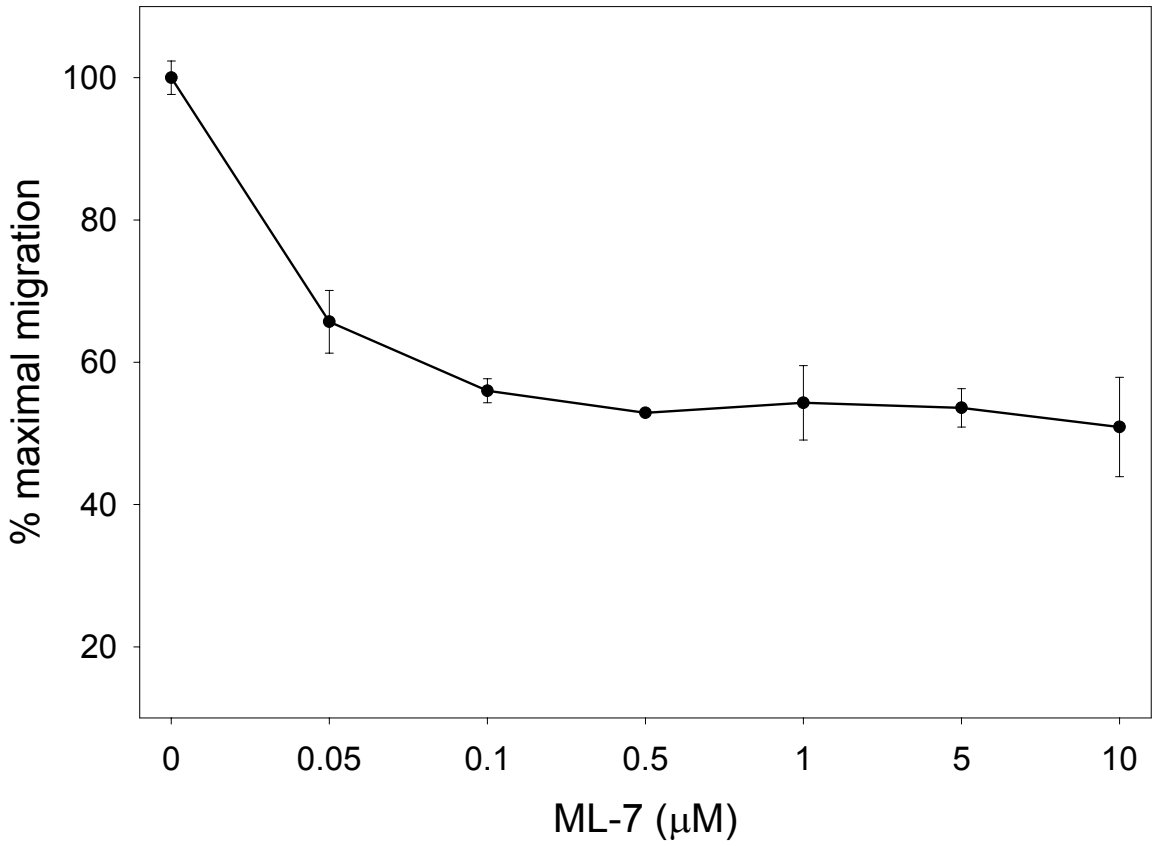


Figure 5. *Inhibition of KT5720-induced PMN migration by MLCK inhibitory peptide is dose-dependent*- PMN loaded with calcein were treated with the indicated concentrations of MLCK inhibitory peptide or scrambled control peptide for 30 minutes at 37^0C. PMN were added to the upper wells of a 96-well chemotaxis plate and 25μM KT5720 was placed in the bottom wells to stimulated PMN migration across the membrane. The plate was incubated for 30 minutes at 37^0C and then the calcein fluorescence of each well was measured. PMN suspension was added to a set of bottom wells of the plate to serve as positive control for fluorescence detection. The fraction of PMN that underwent migration was calculated as the fluorescence of the experimental well divided by the fluorescence of the positive control, and these values were normalized to the maximal migration induced by KT5720. The data are the mean $\pm$ standard deviation of triplicate wells. Data are representative of three separate trials. "*" signifies values that are statistically different ($p<0.05$) between the MLCK peptide and control peptide treated groups, as determined by Student's T-test.

Figure 5

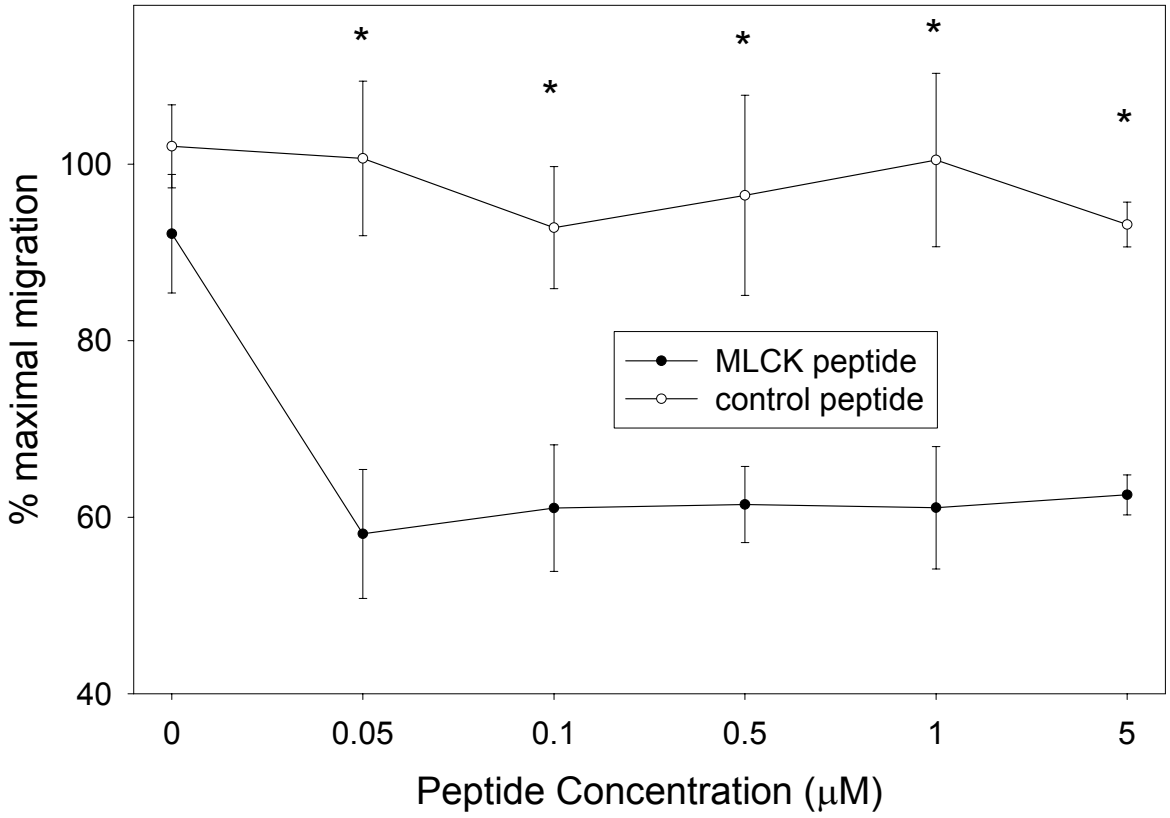

*
*
*
*
*
100
80
60
40
% maximal migration
MLCK peptide
control peptide
0
0.05
0.1
0.5
1
5
Peptide Concentration (μM)

Figure 6. *KT5720-induced PMN adhesion is inhibited by ML-7* – Calcein loaded PMN were treated with vehicle or the indicated concentrations of KT5720 in the presence or absence of 10μM ML-7 at for 30 minutes at 37ºC and were subsequently added to FCS-coated wells of a microtiter plate and incubated at room temperature for 10 minutes. The data are the mean $\pm$ standard deviation of triplicate wells, reported as % adhesion, the percentage of cells that remained adherent after washing as described in "Materials and Methods." The data are the mean $\pm$ standard deviation of triplicate wells. Data are representative of three separate trials. "*" signifies values that are statistically different ($p<0.05$) between the ML-7 treated and untreated groups, as determined by Student's T-test.

Figure 6

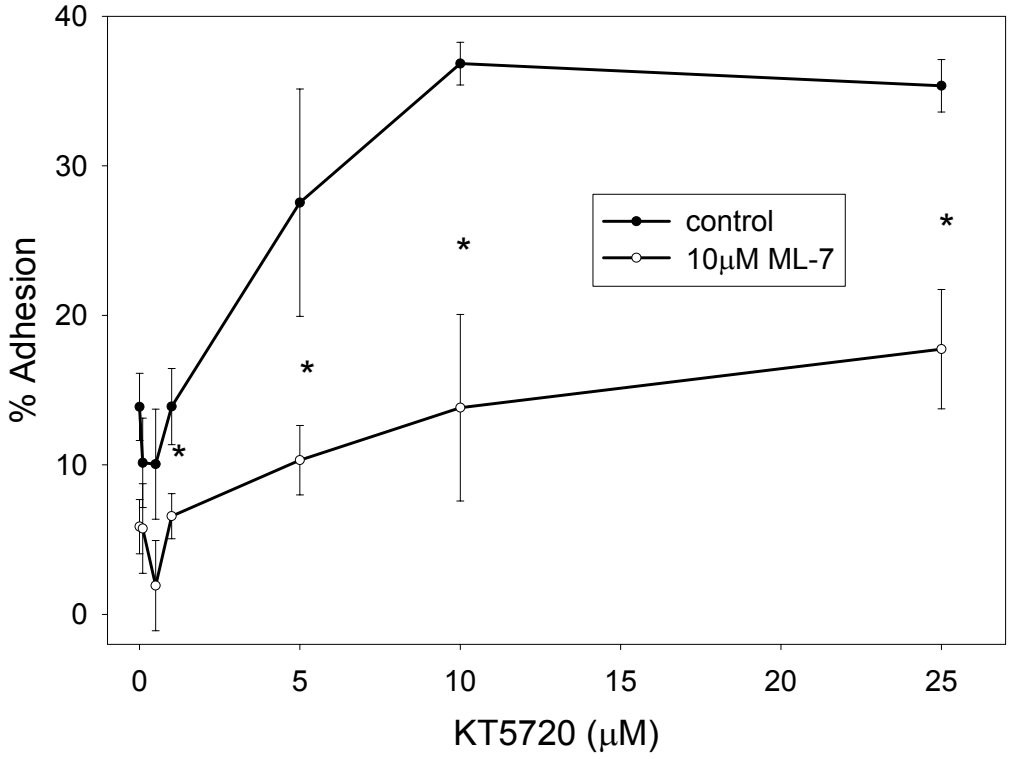


Figure 7. *ML-7 inhibition of KT5720-induced PMN adhesion is dose-dependent*– Calcein loaded PMN were treated with vehicle or the indicated concentrations of ML-7 in the presence or absence of 25μM KT5720 at for 30 minutes at 37ºC and were subsequently added to FCS-coated wells of a microtiter plate and incubated at room temperature for 10 minutes. The data are the mean ± standard deviation of triplicate wells, reported as % adhesion, the percentage of cells that remained adherent after washing as described in "Materials and Methods." The data are the mean ± standard deviation of triplicate wells. Data are representative of three separate trials.

Figure 7

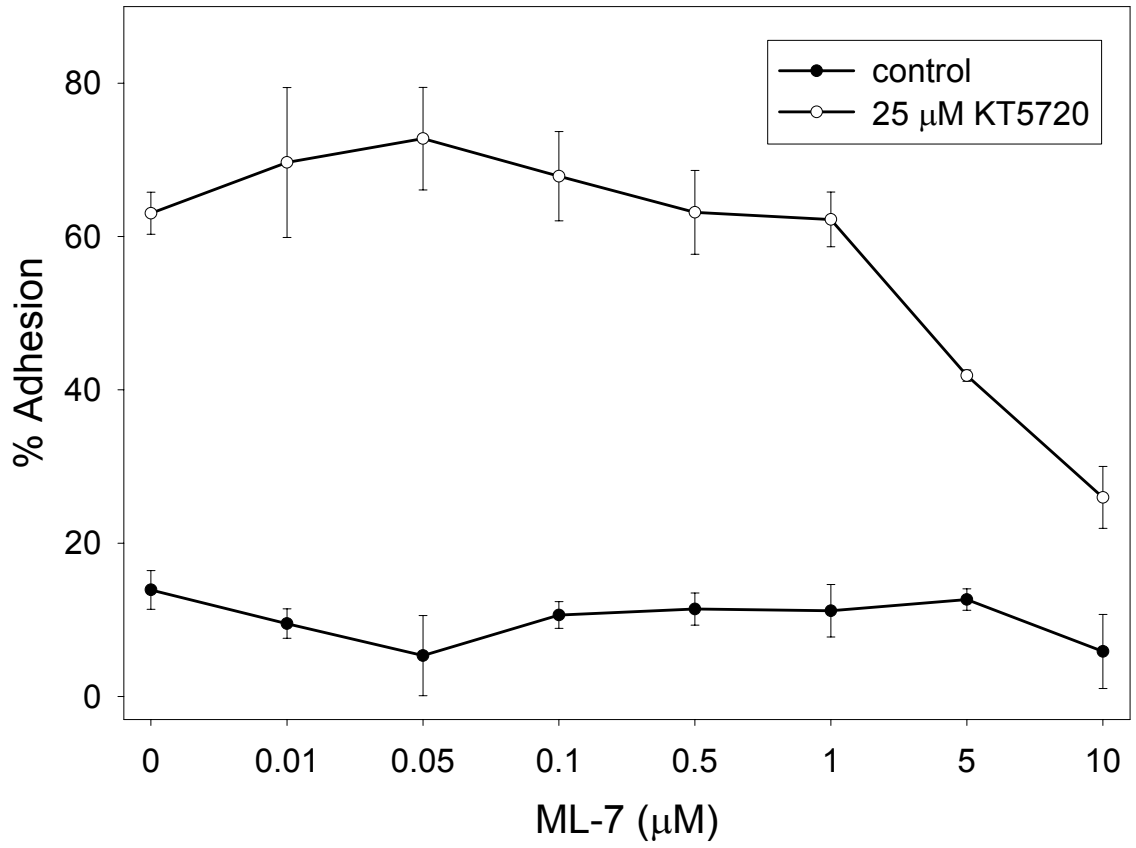

control
25 μM KT5720
% Adhesion
80
60
40
20
0
0
0.01
0.05
0.1
0.5
1
5
10
ML-7 (μM)

Figure 8. *MLCK inhibitory peptide inhibition of KT5720-induced PMN adhesion is dose-dependent*– Calcein loaded PMN were treated with vehicle or the indicated concentrations of either MLCK inhibitory peptide or scrambled control peptide in the presence or absence of 25μM KT5720 at for 30 minutes at 37ºC and were subsequently added to FCS-coated wells of a microtiter plate and incubated at room temperature for 10 minutes. The data are the mean ± standard deviation of triplicate wells, reported as % adhesion, the percentage of cells that remained adherent after washing as described in "Materials and Methods." The data are the mean ± standard deviation of triplicate wells. Data are representative of three separate trials. "*" signifies values that are statistically different ($p<0.05$) between the MLCK peptide and scrambled peptide treated groups, as determined by Student's T-test.

Figure 8

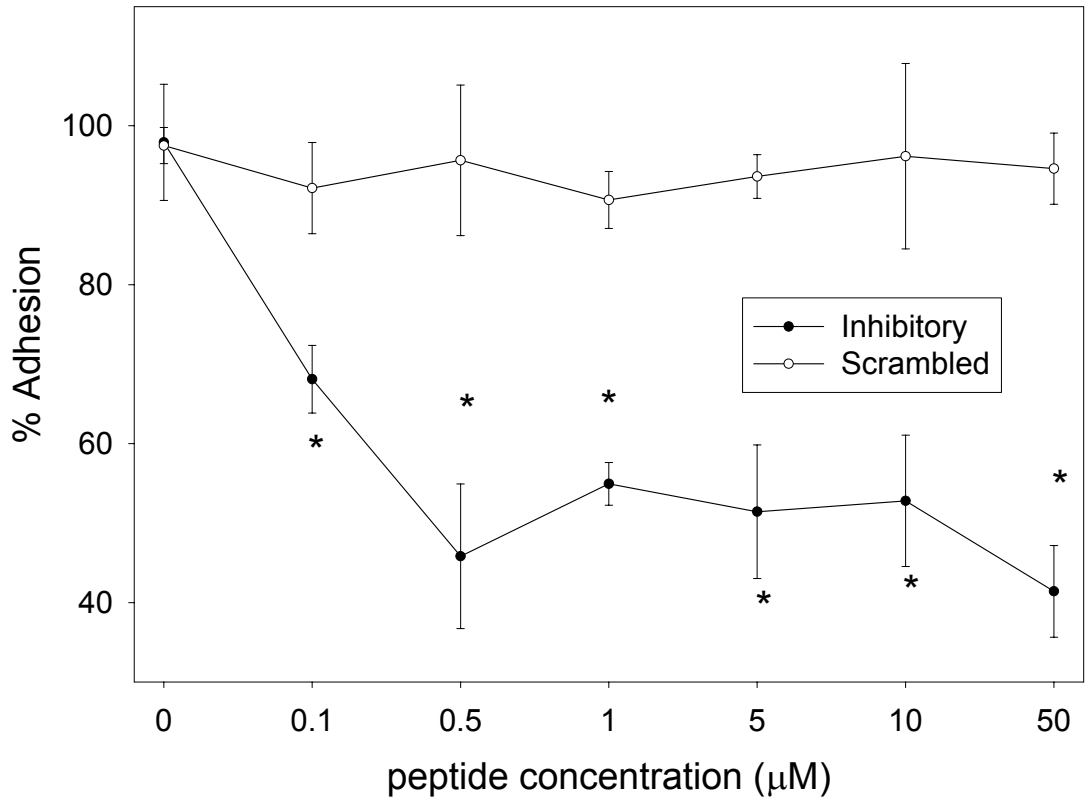

100
80
60
40
% Adhesion
Inhibitory
Scrambled
*
*
*
*
*
*
0
0.1
0.5
1
5
10
50
peptide concentration (μM)

Figure 9. *Treatment of PMN with the PKA inhibitor KT5720 results in up-regulation of ß2 integrin expression via an MLCK-dependent pathway*– PMN were treated with vehicle control, 25µM KT5720, 10µM ML-7 or a combination of 25µM KT5720 and 10µM ML-7 for 30 minutes at 37ºC. The PMN were then incubated with the anti-ß2 integrin antibody IB4 for 40 minutes on ice, washed and subsequently incubated with FITC-conjugated anti-mouse F(ab')2 for 20 minutes on ice. The PMN were washed and the fluorescence of 10,000 gated PMN was then measured. The reported relative fluorescence represents the mean fluorescence intensity of each treatment group normalized to the mean fluorescence intensity of the untreated control group. The data are the mean $\pm$ standard deviation of three separate trials. "*" signifies values that are statistically different ($p<0.05$) from the untreated group, as determined by Student's T-test.

Figure 9

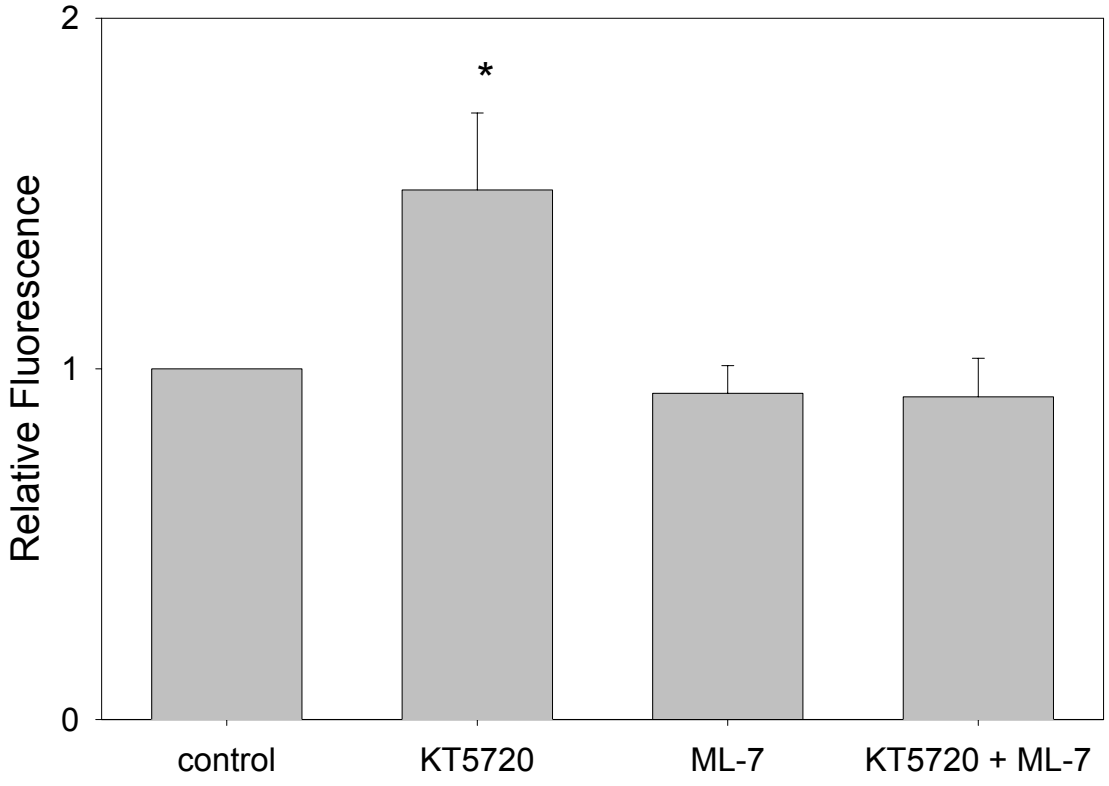


Figure 10. *Treatment of PMN with the PKA inhibitor KT5720 results in a dose-dependent activation of ß2 integrins via an MLCK-dependent pathway*– PMN were treated with the indicated concentration of KT5720 in the presence or absence of 10μM ML-7 for 30 minutes at 37ºC. The PMN were then incubated with the anti-high affinity ß2 integrin antibody CBRM1/5 for 40 minutes on ice, washed and subsequently incubated with FITC-conjugated anti-mouse F(ab')2 for 20 minutes on ice. The PMN were washed and the relative fluorescence of 10,000 gated PMN was then measured. The unstimulated PMN demonstrated a uniform low fluorescence intensity when compared to that seen in the KT5720 stimulated populations. The PMN were gated to low and high fluorescence intensity based upon the fluorescence of the unstimulated PMN, and the data represent the percent of the total PMN expressing high fluorescence consistent with an increase in the presence of high affinity β2 integrins. The data are the mean $\pm$ standard deviation of three separate trials. "*" signifies values that are statistically different ($p<0.05$) between the ML-7 treated and untreated groups, as determined by Student's T-test.

Figure 10

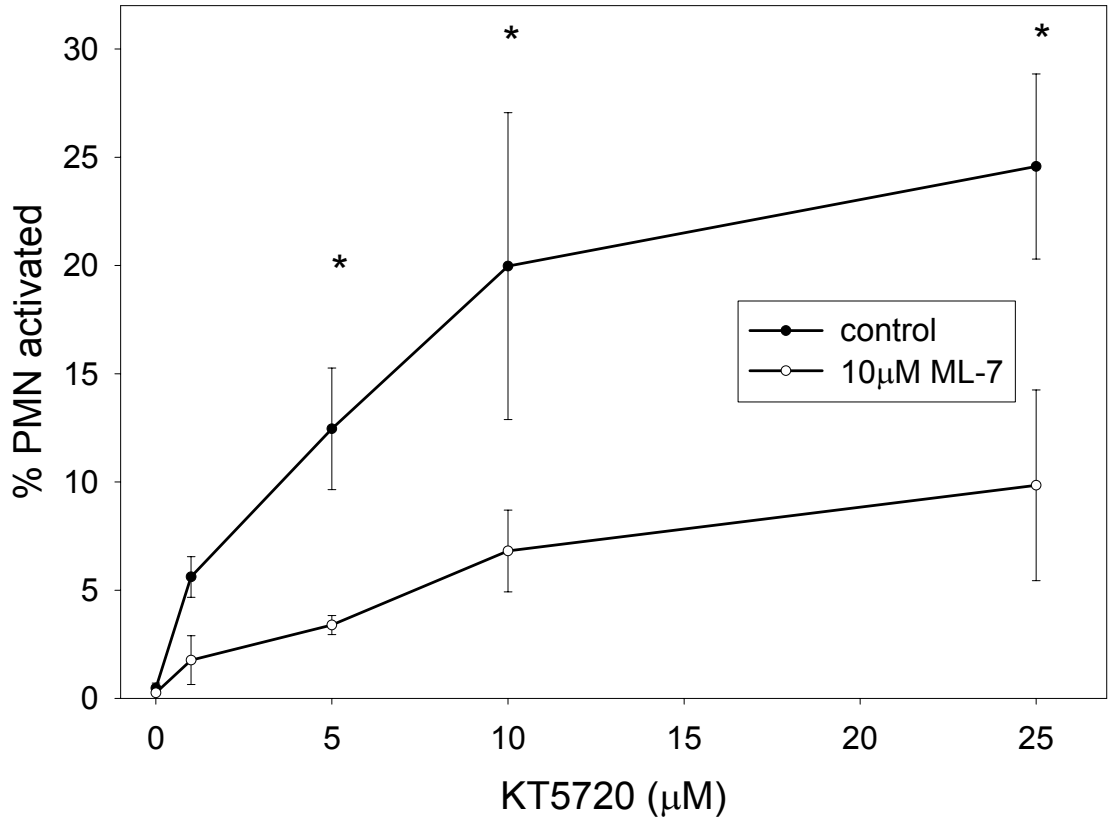
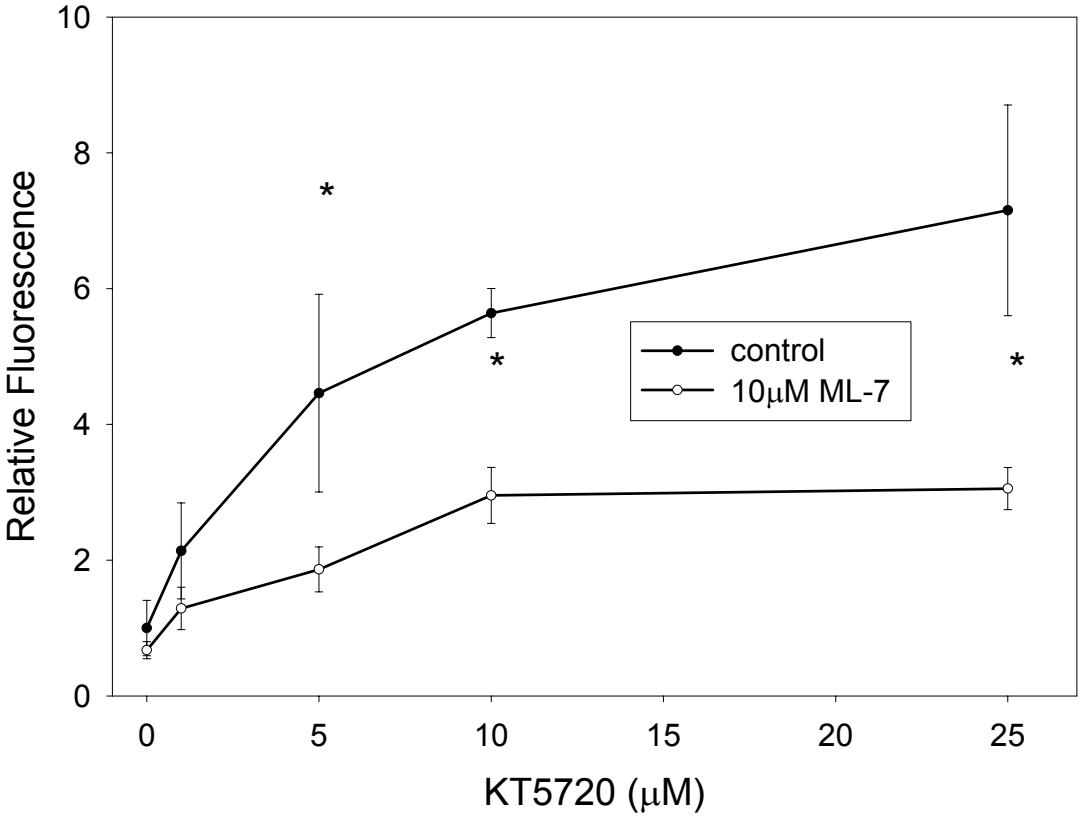


Figure 11. *Treatment of PMN with the PKA inhibitor KT5720 results in a dose-dependent activation of ß2 integrins via an MLCK-dependent pathway*– PMN were treated with the indicated concentration of KT5720 in the presence or absence of 10µM ML-7 for 30 minutes at 37ºC. The PMN were then incubated with the anti-high affinity ß2 integrin antibody CBRM1/5 for 40 minutes on ice, washed and subsequently incubated with FITC-conjugated anti-mouse F(ab')2 for 20 minutes on ice. The PMN were washed and the relative fluorescence of 10,000 gated PMN was then measured. The reported relative fluorescence represents the mean fluorescence intensity of each treatment group normalized to the mean fluorescence intensity of the untreated control group. The data are the mean $\pm$ standard deviation of three separate trials. "*" signifies values that are statistically different ($p<0.05$) between the ML-7 treated and untreated groups, as determined by Student's T-test.

Figure 11


Relative Fluorescence
KT5720 (μM)
control
10μM ML-7
*
*
*
0
2
4
6
8
10
0
5
10
15
20
25

SUMMARY

We have demonstrated in the preceding experiments:

1. Inhibition of PKA will induce β2 integrin-dependent adhesion in PMN.
2. Augmentation of cAMP will inhibit β2 integrin-dependent adhesion in IC stimulated PMN, and this effect requires PKA activity.
3. Upregulation of intracellular cAMP by either β adrenergic agonists or PDE inhibitors results in inhibition of β2 integrin-dependent adhesion and subsequent respiratory burst activity in IC stimulated PMN.
4. Co-treatment of IC stimulated PMN with β adrenergic agonists and PDE inhibitors has a synergistic effect upon inhibition of β2 integrin-dependent adhesion and subsequent respiratory burst activity.
5. Inhibition of PKA results in MLCK-dependent phosphorylation of MLC in PMN.
6. Inhibition of PKA results in MLCK-dependent, β2 integrin-dependent migration in PMN.
7. Inhibition of PKA results in MLCK-dependent, β2 integrin-dependent adhesion in PMN.
8. Inhibition of PKA results in MLCK-dependent upregulation of β2 integrin expression on PMN.
9. Inhibition of PKA results in MLCK-dependent affinity activation of β2 integrin on PMN.

CONCLUSION

To test our hypothesis that PKA inhibits β2 integrin affinity and avidity, and subsequent PMN activation, via inhibition of MLC phosphorylation by MLCK, we first set out to demonstrate the effect of PKA upon β2 integrin-dependent adhesion. We observed that inhibition of PKA induced β2 integrin-dependent adhesion of PMN to FCS, suggesting that constitutive PKA activity in the quiescent PMN prevents this adhesion. To confirm this, we augmented PKA activity via treatment of PMN with a cell permeant analog of cAMP and observed the effect upon β2 integrin-dependent adhesion upon stimulation of the PMN with IC. As previously demonstrated, stimulation of PMN with IC induced β2 integrin-dependent adhesion and we observed that augmentation of PKA activity prevented this adhesion, further strengthen the role of PKA in inhibiting β2 integrin adhesion. The effect of the cAMP analog upon IC stimulated PMN was blocked by inhibition of PKA activity, demonstrating that this inhibitory effect of cAMP is mediated through PKA.

The inhibitory effect of cAMP upon adhesion witnessed with the cAMP analog was also seen with pharmacologic augmentation of intracellular cAMP by both adrenergic agonists, which increase cAMP generation, and PDE inhibitors, which prevent cAMP degradation. Since β2 integrin-dependent adhesion is required for further phagocyte activation in the face of stimulation of PMN through the FcγR, we examined the effect of PKA upon respiratory burst activity subsequent to IC induced β2 integrin-dependent adhesion. As expected, pharmacologic augmentation of cAMP resulted in an inhibition of respiratory burst activity, demonstrating that PKA activity can prevent phagocyte activation to pro-inflammatory stimuli which require β2

integrin-dependent adhesion for PMN activation. Interestingly, the adrenergic agonists and PDE inhibitors had a synergistic effect upon the inhibition of both β2 integrin-dependent adhesion and the subsequent respiratory burst activity in PMN. A possible mechanism for this synergistic effect is that the PDE inhibitors prevent the degradation of the increased cAMP concentrations generated by the adrenergic stimulation, resulting in higher cAMP concentrations obtained from either pharmacologic agent alone.

Since β2 integrins both generate and are affected by intracellular signals, it can be difficult to determine if an inhibitory event is upon β2 integrin aviditiy activation or upon the subsequent integrin-generated outside-in signal. To assess the role of PKA upon integrin activation, we examined the effect of PKA upon β2 integrin expression upon the surface of PMN. We found that inhibition of PKA activity resulted in an upregulation of β2 integrin expression on PMN, demonstrating that the constitutive PKA activity of quiescent PMN prevents upregulation of β2 integrins. Further examination of these PKA-inhibited PMN showed that not only were the number of β2 integrins increased on the cell surface, but the integrins expressed high affinity for ligand, demonstrating that PKA prevents integrin affinity activation in the resting PMN.

We had hypothesized that PKA exerted its inhibitory effects upon the β2 integrins via the activity of MLCK upon MLC and the subsequent alteration of the actin cytoskeleton. To address this, we first needed to confirm that PKA could affect MLCK activity in PMN. We inhibited PKA activity in PMN and witnessed an increase in MLC phosphorylation. To ensure that this effect was via MLCK and not

due to alterations in MLC phosphatase activity or phosphorylation of MLC by kinase other than MLCK, we demonstrated that inhibition of MLCK activity blocked the PKA-mediated upregulation of MLC phosphorylation. These findings confirm that PKA can inhibit MLCK and prevent the subsequent MLC activation required for cytoskeletal rearrangement in PMN.

To determine if the PKA inhibition of β2 integrin-dependent adhesion is via inhibition of MLCK activity, we demonstrated that the PMN adhesion generated by PKA inhibition could be blocked by inhibition of MLCK activity. Similar to β2 integrin-dependent adhesion, we found that the β2 integrin-dependent migration of PMN induced by inhibition of PKA activity is also blocked by inhibition of MLCK. These findings show that PKA inhibits both β2 integrin-dependent adhesion and migration in quiescent PMN via inhibition of MLCK. Finally, we examined the role of MLCK activity in PKA regulation of β2 itegrin avidity activation. We found that both the upregulation of β2 integrin expression and affinity upon PMN caused by inhibition of PKA activity could be blocked by inhibition of MLCK, demonstrating that the inhibitory effect of PKA upon β2 integrin avidity activation in PMN is via MLCK.

We have demonstrated here that the constitutive activity of PKA prevents β2 integrin avidity activation, β2 integrin-dependent adhesion and the subsequent phagocyte activation of PMN. Further, we have shown that these effects of PKA are mediated through the inhibition of MLCK activity, supporting our hypothesis.