FUNCTIONAL CHARACTERIZATION OF EQUINE NEUTROPHILS IN RESPONSE TO CALCIUM IONOPHORE A23187 AND PHORBOL MYRISTATE ACETATE

by

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Veterinary Medical Sciences

(ABSTRACT)

Equine neutrophils (PMN) play a critical role in inflammatory processes in horses. The objective of this study was to characterize equine PMN function ex vivo following stimulation with calcium ionophore A23187 (A23187) and phorbol myristate acetate (PMA). These stimulants trigger different branches of the PMN activation process that occurs in vivo. Equine PMN were isolated from the whole blood of six clinically normal geldings using a one-step discontinuous Percoll gradient technique. Neutrophil aggregation, degranulation, and superoxide anion production were evaluated in assay systems which had previously been established to quantitate PMN function. Dose-response curves for A23187 and PMA were derived for the three functions. Results indicate

that equine PMN aggregation and superoxide anion production are more responsive to activation by PMA as the maximum change in percent transmittance and maximum nanomoles of superoxide anion produced following PMA stimulation (60.8% and 10.4nmols/10^6cells, respectively) were greater than those values stimulated by A23187 (41.5% and 5.2nmols/10^6cells, respectively). However, degranulation was found to be more responsive to A23187 stimulation (maximum percent degranulation= 56.1%) than to PMA stimulation (maximum percent degranulation= 30.7%).

Dose-response curves following A23187 and PMA stimulation revealed that superoxide anion production had the lowest threshold concentration among the three functions. Degranulation had the highest threshold concentration among the three functions for both stimulants.

Results indicate that equine PMN functions differ in their dependence on second messengers in the activation pathway. These functions also occur in a dose-dependent manner and differ in the threshold concentrations required for their stimulation.

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INTRODUCTION

The objective of this study was to measure normal equine neutrophil functions in response to various stimuli and to define the dose-response relationships for these stimuli in neutrophil function assays which had previously been established. The selected stimuli trigger different branches of the activation process of neutrophils that occurs naturally in horses. Characterization of the equine neutrophil response and establishing these baseline dose-response curves will lend insight into investigations of mechanisms of action of inflammatory mediators produced in horses and thus, will increase knowledge about the pathophysiology of inflammation in horses.

Inflammation, characterized by heat, redness, swelling, and pain, results following tissue injury and is a vital prelude to restoration of the tissue. Leukocyte accumulation at the injury site is a primary factor in this repair process as leukocytes defend the body against invading microorganisms and other foreign material. Neutrophils (polymorphonuclear leukocytes, PMN) are the first leukocytes to arrive at injury sites, and their principal function is phagocytosis of foreign matter. Following their stimulation by chemoattractants, PMN aggregate near the injured sites, release hydrolytic enzymes

and other proteases from intracellular granules, and produce reactive oxygen species which are bactericidal agents important to intracellular degradation of microbes and foreign material. However, excessive stimulation of PMN and extracellular release of these bactericidal agents can lead to damage of host tissues.

PMN are easily accessible from blood or tissues of mammals and are commonly used ex vivo to study processes of cell biology such as receptor regulation and cellular activation. Their role in inflammation and other immunologic processes is also the subject of much research. Species differences have been demonstrated in such studies as PMN from many different species have been examined. These species differences are evident by the susceptibility and resistance of different species to different pathogens. The literature must often be carefully evaluated to determine which species has been studied. Human neutrophil responses have been studied extensively (Abramson,J.S. et al.,1993 for a review). However, equine PMN responses have not been characterized ex vivo to such an extent. Further studies of equine PMN are needed to understand their normal activities and inflammatory responses. This knowledge will be beneficial to studies of inflammatory mediators in normal equine neutrophil responses and in studies of possible neutrophil dysfunction in horses.

LITERATURE REVIEW

I. INFLAMMATION

A. Introduction

Inflammation, the body's fundamental protective response to tissue irritation and injury, is essential in eliminating the causes and the effects of the injury such as microorganisms, necrotic cells and damaged tissue (Kumar,et al.,1992). An organism could not survive injury without the protection supplied by inflammation. Heat, redness, swelling, and pain are classical signs of inflammation and were first described by Cornelius Celsus in the first century A.D. Later, a fifth sign, loss of function, was named by Virchow (Macleod,1973)(Gilman,1989). The acute inflammatory response consists of the following events which lead to development of the cardinal signs: 1) increased vascular permeability; 2) leukocyte adherence to vascular endothelium; 3) diapedesis of leukocytes through vessel walls; 4) accumulation of leukocytes in the injured area; and 5) phagocytosis and degradation of bacteria and other foreign material by leukocytes (Macleod,1973). Mediation of these responses is known to be partially humoral, arising from substances causing the injury, microbial products, or substances released due to injury to or stimulation of the host's cells (Evans, et al.,1992) (Kumar,et al.,1992) (Macleod,1973). The stimulus initiates a cascade of

events which activate cellular responses and leads to the elaboration of the leukocytes' role in inflammation.

The endothelium of the microvasculature plays a central role in acute inflammation. Following injury, there is an initial transient constriction of the microvasculature followed by dilatation. This vascular response may be initiated by endothelial damage, enzymes, toxic oxygen species or chemical mediators released by leukocytes and other cells which are involved in the inflammatory process. Increased vascular permeability results as activated endothelial cells contract and pull away from their attachments to each other. The intercellular spaces formed in the endothelium allow the flow of fluid out of the capillary lumen and into tissue spaces. Visible edema and swelling result from the subsequent accumulation of local protein-rich exudate.

These changes in the endothelium also result in the release of soluble factors from the endothelial cells which are chemotactic for specific leukocytes. Therefore, during these vascular changes, leukocytes marginate out of the central axis of cellular flow toward the endothelium in a process called margination. They move along an increasing concentration gradient toward the appropriate site. Leukocytes begin to adhere to the vascular endothelium through interaction between receptors on both cellular surfaces. Expression of these receptors follows stimulation of both cell

types. Following adherence, leukocytes insert pseudopodia between adjacent endothelial cells, squeeze between them, and emigrate into the surrounding tissue along the chemotactic chemical gradient. Margination, emigration, and chemotaxis therefore promote the accumulation of leukocytes. The accumulated leukocytes phagocytize foreign material at the site of insult where the concentration of chemoattractant is largest. Engulfed particulate matter is incorporated into a phagosome within the phagocyte cytoplasm, where it is enzymatically destroyed. Thus, phagocytosis by accumulated leukocytes promotes neutralization and elimination of infectious agents and disposal of damaged tissue.

B. Neutrophils

Neutrophils (polymorphonuclear leukocyte, PMN), the most abundant of the leukocytes, are the first leukocytes to arrive at a site of injury: thus, PMN accumulation is the principal histological indicator of inflammation (Evans,et al.,1992) (Kumar,et al.,1992)(Macleod,1973). Following stimulation, the activated PMN perform the following inflammatory responses: 1) adherence to endothelium; 2) emigration through capillary walls; 3) chemotaxis; 4) aggregation; 5) phagocytosis; 6) degranulation; and 7) generation of toxic oxygen species. Each of these responses is initiated as the PMN moves along an

increasing chemical gradient toward the site of insult.

PMN are short-lived and incapable of cell division so they must be continually supplied from the bloodstream during prolonged inflammation. They are not capable of extensive protein synthesis so most of the substances needed to aid their defense of the body are preformed and stored intracellularly. Granules in their cytoplasms contain enzymes such as myeloperoxidase, elastase, and lysozyme which aid degradation of phagocytized material and thus help in defending the body.

Phagocytosis begins with cellular attachment of a particle to be ingested, followed by invagination of the PMN plasma membrane around the particle. A phagosome is formed, detaches and moves to the center of the cell where it contacts and fuses with preformed granules in the cytoplasm. The granules contain hydrolytic enzymes, such as those named above, and other bactericidal proteins important in killing and digestion of phagocytized material. Following fusion of the phagosome and granules to form a phagolysosome, the enzymes and bactericidal agents are released into the phagolysosome where they act on the engulfed material without disrupting cellular integrity and function. Thus PMN have partially degranulated and the phagocytized material has been degraded.

Inflammatory stimuli also activate the respiratory burst

in which PMN oxygen consumption increases. Membrane-bound NADPH oxidase is activated to produce toxic oxygen metabolites, such as superoxide anion and hydrogen peroxide, which are active in microbial killing.

PMN accumulation at such injury sites and their release of bactericidal agents serve important functions in host defense. However, if inappropriately activated, PMN may actually contribute to tissue damage: so-called immunologically-induced injury (Korchak,H.,1989). Enzymes from granules and oxygen metabolites degrade and solubilize protein and carbohydrates and thus, are capable of host tissue injury and destruction (Leff,J.A.,et al.,1993). Thus, PMN may cause damage to host tissues when lytic enzymes and free radicals are released into the extracellular environment during phagocytosis. Escape of these substances from PMN contributes to PMN disintegration, host tissue damage, and perpetuation of the inflammatory reaction due to release of additional chemotactic products.

PMN inflammatory responses are triggered and activated by agents released from foreign organisms or host's cells following injury or perturbation. Studies of these responses require knowledge about the activation pathway which occurs in PMN. This activation pathway in PMN is known to involve enzyme activation and production of second messengers.

II. NEUTROPHIL ACTIVATION

A. Introduction

PMN are capable of responding to many stimuli including leukotrienes, complement factors, and bacterial polypeptides including formyl-methionyl-leucyl-phenylalanine (FMLP). Once activated, their responses include aggregation, degranulation, and superoxide anion production (Korchak,H.,1989). PMN activation occurs via receptor-ligand-induced changes in the cell membrane followed by activation of enzymes responsible for second messenger production (Fig. 1). The second messengers act synergistically to optimally stimulate PMN functions in response to stimulus concentrations which are present in vivo following injury or irritation. Two specific second messengers, inositol-1,4,5-triphosphate and diacylglycerol, are produced and act in separate but synergistic pathways to activate cellular functions. Inositol-1,4,5-triphosphate stimulates an increase in intracellular calcium which subsequently leads to activation of the PMN functions as diacylglycerol binds protein kinase C and results in its activation. Activation of protein kinase C results in the stimulation of PMN functions by phosphorylating essential enzymes, many of which remain to be defined.

Analysis of PMN function ex vivo utilizes either synthetic ligands or the activation pathways involving these

PMN ACTIVATION

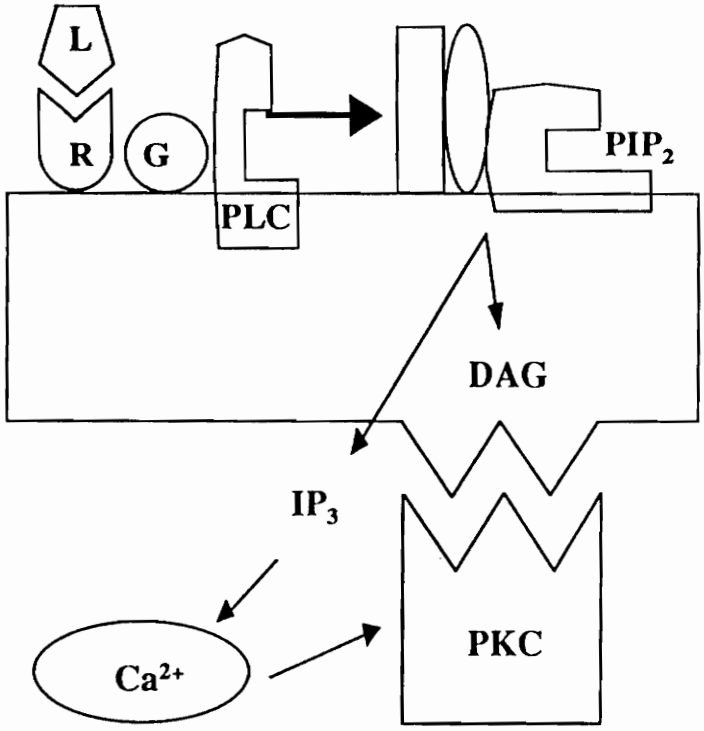

Fig. 1 Model of PMN activation following stimulation with ligands which bind surface receptors. The sequence of events is depicted: a) occupation of the specific receptor (R) by the ligand (L) results in conformational change in the receptor that is transmitted to phospholipase C (PLC) by a G-protein (G); b) activated phospholipase C catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). The former mobilizes calcium from intracellular storage sites and the latter perturbs the membrane; c) protein kinase C (PKC) associates with the perturbed membrane region in the presence of calcium and becomes activated.

second messengers by using substances which cause the same responses in the cell. Substances which bind surface receptors, activators of protein kinase C and calcium ionophores are all utilized to mimic PMN activation which occurs in vivo.

B. In vivo

Activation of PMN is a receptor-mediated event and is initiated by the binding of stimulant molecules to specific receptors on PMN surfaces. Receptors have been identified for the following ligands: 1) formyl-methionyl-leucyl-phenylalanine (FMLP); 2) split complement factors such as C5a and C3b; and 3) arachidonic acid metabolites such as leukotriene B_4. Binding of surface receptors by these ligands causes a conformational change in the receptor and initiates a cascade of events leading to cellular activation.

Following ligand-induced conformational change in the receptor, intramembrane interaction between the receptor and a guanine nucleotide-binding protein (G protein) is altered (Omann et al,1987)(Korchak,H.,1989). G proteins are responsible for transferring information from the receptor to the effector enzymes or to ion channels of the PMN (Korchak,H.,1989). When activated by altered receptor conformation, the G protein dissociates into two subunits, G_i-alpha and beta- gamma subunits. The G_i- alpha subunit (so-

called because its production consequently inhibits adenyl cyclase whose action leads to inhibition of PMN function) binds and activates an intracellular phospholipase C that releases inositol-containing phospholipids from the cell membrane. The beta- gamma subunit acts in a separate signal-transducing pathway. It binds a stimulatory G protein of the adenyl cyclase system thus, preventing activation of adenyl cyclase by the stimulatory protein. This prevents the inhibition of PMN responses by increased cyclic AMP produced by activated adenyl cyclase.

Activation of phospholipase C by G_i- alpha subunit triggers the hydrolysis of phosphatidyl-inositol-4,5-bisphosphate. Inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) are released following this reaction (Badwey,J.A.,et al.,1986).

IP_3 functions in stimulating the cellular response by inducing a change in calcium concentration in cells. Changes in IP_3 concentration correlate with changes in intracellular calcium concentration in stimulated PMN (Omann et al.,1987). Studies using permeabilized PMN preparations have shown, in the absence of a receptor-ligand complex, that IP_3 causes the release of intracellular calcium when IP_3 has access to intracellular stores of calcium. Thus, IP_3 is a confirmed second messenger of cellular activation as increased intracellular calcium has been shown to be necessary for PMN

functions.

Mobilization of intracellular calcium may be necessary for subsequent changes in membrane permeability to calcium (Omann et al., 1987) as release of calcium from intracellular stores is followed by prolonged calcium entry into the cell. Increased intracellular calcium may possibly open a pathway for calcium entry into the smooth endoplasmic reticulum (SER) from outside the cell. Subsequently, this calcium is released into the cytosol through IP_3-activated channels (Putney,J.W.,1988) (Korchak,H.,1989) in the SER membrane.

Intracellular calcium concentrations are important in cellular functions largely because of the activation of calcium-dependent enzymes. The function of PMN aggregation may occur in response to increased expression or affinity of receptors for ligands or other substances which promote PMN adherence. Increased expression of PMN receptors, including integrins such as CD11b/CD18 and leukocyte adhesion molecule-1 (LAD-1), may be in response to activation of calcium-dependent enzymes which promote expression of the receptors. The calcium-dependent enzymes may stimulate the translocation of these receptors from their intracytoplasmic stores to the plasma membrane. Increased affinity of receptors for their ligands may result as a consequence of the excitatory state of the cell.

Degranulation, another important PMN function, has been

found to be preceded by an increase in cytosolic free calcium and a translocation of cell membrane-associated calcium (Smith,R.J.et al.,1988). Intracellular calcium may directly alter cytoskeletal components and may also affect cytoskeletal phosphorylation by activating calcium-dependent protein kinases (Omann,et al.,1987). Contraction of microfilaments in the cytoskeleton, altering PMN shape and permitting secretion, involves phosphorylation of the myosin light chain by a calcium-dependent kinase. Granules move in response to the reaction and may fuse with phagosomes or cell membranes to release contents.

Calcium, by regulating calmodulin, has been shown to promote superoxide anion production by activating NAD kinase in equine PMN (Heyneman,R.A.,1987). Calmodulin, a calcium-dependent regulatory protein, becomes activated when it binds to calcium. Following calcium binding, calmodulin is able to bind and activate NAD kinase which phosphorylates NADH to NADPH, making more NADPH available for oxidation. Increased NADPH is available for the NADPH oxidase reaction, subsequently, triggering more NADPH oxidase activity and making more electrons available for oxygen reduction in activated PMN.

Diacylglycerol (DAG), the second product of phospholipase C action, also has an important second messenger role in cellular activation. DAG causes changes in membrane potential

and shape of PMN but not in cytosolic calcium concentration (Smith,R.J.,et al,1988). DAG is the physiological activator of the calcium-activated, phospholipid-dependent protein kinase, protein kinase C (PKC). Increasing levels of DAG causes translocation of PKC to the cell membrane with subsequent activation of the enzyme. This hypothesis that the enzyme is translocated is supported by the fact that PKC activity in resting PMN is greater in the cytosol while PKC activity in stimulated PMN is greater in the membrane-associated fraction (Christiansen, et al.,1988).

Activation of PKC plays a major role in the expression of the PMN inflammatory responses such as aggregation, degranulation and superoxide anion production by catalyzing the phosphorylation of key enzymes and proteins in the cell activation process. Enhanced phosphorylation of many cytoskeletal and membrane proteins has been demonstrated in activated PMN but initiation of aggregation or degranulation has not been associated with phosphorylation of any specific enzyme in this group (Korchak, H.,1989) (Omann,et al.,1987). It could be that PKC alone initiates activation of these two functions. PKC has been implicated in phosphorylation of a component in the NADPH oxidase system which results in superoxide anion production (Badwey, et al.,1986) as discussed above for equine PMN (Heyneman,R.A.,1987).

Increased intracellular calcium levels and increased PKC

activity in activated PMN are mutually synergistic in eliciting the inflammatory actions (Korchak,H.,1989). Neutrophils which have been activated by ligands have enhanced calcium mobilization and protein phosphorylation. Actin polymerization, which is important in cell secretion, requires actin-binding proteins which are calcium and phosphorylation-dependent (Omann,et al.,1987). Ligands binding receptors on the cells trigger both signals to optimally activate PMN responsiveness. Ligand-induced increases in diacylglycerol were shown to be decreased when calcium was depleted in PMN (Korchak et al.,1988). The subsequent production of superoxide anion in these PMN was diminished compared to cells in calcium medium in the absence of chelators to deplete calcium. This demonstrated a sensitivity of diacylglycerol to calcium and, thus, sensitivity of cellular stimulation to both second messengers.

Modulation of the pathways involving either or both of these second messengers with inflammatory mediators would reveal the activation mechanisms which affect PMN contribution to inflammatory diseases. It may be that controlling one branch of the activation pathway while leaving the other unaffected leads to desired PMN function with optimal defense potential without increasing tissue damage caused by PMN. The mechanisms by which substances activate the PMN inflammatory response can be described and ways to control them can be

investigated. Valuable information is provided to the understanding of the biochemical mechanisms that convert PMN from a resting state to an activated state in which toxic substances are produced and released.

C. Ex vivo

Analysis of PMN function ex vivo is based on selective activation utilizing several stimulants, including formyl-methionyl-leucyl-phenylalanine (FMLP), concanavalin A (Con A), calcium ionophore A23187 (A23187), and phorbol myristate acetate (PMA). These specific activators possess different modes of action in the PMN activation pathway and may also differ in the functions which are activated. Ligands which bind PMN surfaces induce conformational changes in the receptor and elicit cellular activation similar to that which occurs in vivo. Other stimulants which increase intracellular calcium levels, such as calcium ionophores, or activate PKC directly, such as phorbol esters, are commonly used ex vivo to trigger PMN activation. These stimulants mimic only portions of the activation cascade that occurs in vivo as they act at points later in the activation pathway which do not involve a cell surface receptor and only activate a particular branch of that pathway.

FMLP, a formyl peptide, directly activates PMN by binding

to its surface receptors and activating phospholipase C through a G protein (Omann et al,1987)(Korchak,H.,1989). Formylpeptides were originally isolated from bacterial cultures which were chemotactic for PMN and these peptides were found to be the smallest fragment of protein from these filtrates which remained chemotactic (Schiffman, E.,et al.,1975). Early studies showed that the formylpeptides were able not only to induce chemotaxis but also to cause granule enzyme secretion and superoxide anion generation in the respiratory burst (reviewed by Becker,E.L.,1987).

Both signals, increased intracellular calcium and PKC activation, play roles in optimal PMN activation in response to FMLP binding to the PMN surface. Following FMLP stimulation, both IP_3 and DAG are increased (Liang,S.et al.,1990). When PMN were pretreated with ionomycin, an ionophore which increases intracellular calcium in PMN, FMLP elicited degranulation and superoxide anion production with no additional increase in intracellular calcium (Omann et al., 1987), proving that FMLP causes an increase in intracellular calcium comparable to that initiated by an ionophore. When a calcium chelator was added to PMN which were stimulated with FMLP, superoxide anion production was reduced but not ablated as compared to that produced by FMLP-stimulated cells in the absence of the chelator, suggesting that another mechanism of cellular activation, in addition to increased intracellular

calcium, is participating in FMLP stimulation. Thus, both second messengers, IP_3 and DAG are important to optimal PMN stimulation with FMLP.

Concanavalin a (Con A), a plant protein of the lectin family, is commonly used ex vivo for its ability to stimulate PMN activity such as aggregation, degranulation, and superoxide anion production (Reeke,et al.,1974)(Gunther,et al.,1973). Con A is a large, multi-subunit protein which interacts with saccharide-containing cell surface receptors through hydrogen-bonding. Binding of Con A to its receptors forms a dense collection of the receptors and the lectin in the PMN surface called a cap. In low doses Con A forms caps with its receptors while at higher doses the cap formation is inhibited and PMN activity is not initiated. Both Con A and its receptor are internalized following cap formation. This internalization alters distribution and mobility of various receptor molecules on the cell surface. This alteration in receptor conformation in the membrane results in PMN activation, but has been shown to involve a mechanism which does not involve G proteins (Liang,S. et al.,1990).

Con A stimulates aggregation possibly by cross-linking receptors between PMN leading to their adherence. Selective discharge of specific granules occurs also with Con A stimulation, following an increase in the number of microtubules in the PMN (Hoffstein,S. et al.,1976). Con A has

also been used to stimulate superoxide anion production by PMN (Liang,S. et al.,1990)(Brown,G. et al.,1991)(Kaplan,H. et al.,1984). A rise in intracellular calcium, which may be involved in myosin light-chain phosphorylation during capping, occurs following Con A binding to its receptors. Rickard, et. al.(1985) showed that this rise in calcium is due to induction of a net influx across the plasma membrane and may be due to IP_3 formation.

Stimulants which activate PMN without ligand-surface receptor interaction, alternatively, cause calcium mobilization and PKC activation. Calcium ionophores and PKC activators can bypass the surface receptor which is G-protein-coupled and directly activate the end responses of cell-cell aggregation, degranulation, and superoxide anion generation (Korchak, 1989).

Calcium ionophore A23187 (A23187), a naturally occurring carboxylic ionophore, has strong stimulative effects on PMN aggregation, degranulation (Wright,et al.,1977)(Abrams,et al.,1983) and superoxide anion production (Kaplan,H.B.,et al.,1982)(McPhail,et al.,1983). A23187 permits the transport of calcium ions across biological membranes by forming a lipid-soluble complex with the cation (Pressman, 1976). The ionophore has a linear backbone with oxygen-containing heterocyclic rings, a carboxyl group at the head of the molecule and an ammonium group at the tail. The head-to-tail

hydrogen bonding that occurs in solution causes the formation of a cavity between the oxygen atoms of the rings. The protonated ionophore will diffuse to one interface within a membrane and release its proton as it only forms complexes with cations in its deprotonated anionic state. The charged ionophore is trapped due to its polarity and binds with calcium cations via ion-dipole interaction. After diffusing to the opposite interface as a neutral, lipid-soluble ionophore-cation complex, the compound releases the cation. The anion of the ionophore is formed again and combines with a proton. The neutral ionophore can diffuse back to the original interface for another proton-cation exchange.

The equilibrium attained through the calcium ionophore A23187 is driven by concentration gradients, thus calcium ionophore activity is affected by the presence or absence of calcium ions in the medium. In a calcium-containing medium, calcium ions are transported into the cell and an increase in intracellular calcium results. The influx of calcium ions and increase in cytosolic calcium ions causes direct activation of the PMN. In calcium-free medium, A23187 causes release of proteins from SER (Wileman, T., et. al.,1991) and no subsequent stimulation of PMN function. Stimulation of PMN may not be possible if intracellular calcium can not be increased using extracellular sources as Rickard, et. al. proved that the rise in intracellular calcium necessary for

PMN activation is largely dependent on calcium influx at the plasma membrane.

Phorbol myristate acetate (PMA), a phorbol ester which is a tumor promoter, has strong stimulatory effects on PMN aggregation, degranulation (Wright,D.G.,et al.,1977) and superoxide anion production (McPhail,L.C.,et al.,1983). The phorbol ester acts by directly binding to protein kinase C (PKC) at the same site that DAG binds and elicits the enzyme's translocation to the plasma membrane. Stimulation of PMN with PMA has been shown to cause an increase in membrane PKC activity and a decrease in cytosolic PKC activity (Christiansen,et. al.,1988). PMA was also compared to 1-oleoyl-2-acetyl-rac-glycerol (OAG), another PKC activator, in this study by Christiansen, et. al. Superoxide anion production following PMA stimulation (1µg/ml) was found to be greater than the production stimulated by OAG even though they equally activate PKC. It is suggested that PMA must induce other effects in addition to PKC activation. In the same study human PMN were pre-incubated with A23187 and subsequently activated with PMA. Superoxide anion production was equal to that produced when PMA alone was used to stimulate the cells. It seems that PMA was cancelling the effects of A23187 by an unknown mechanism, and that PMA activity is not solely associated with changes in intracellular calcium concentration. The mechanism of PMA

stimulation involves PKC activation and another mechanism which does not include increasing intracellular calcium. In further support of this hypothesis, Rickard, et. al.(1985) reported that another PKC activator, TPA (12-O-tetradecanoylphorbol-13-acetate), which is also a phorbol ester, abolishes the rise in intracellular calcium that follows Con A stimulation of porcine neutrophils and inhibits the rise of intracellular calcium induced by A23187 (10nM). TPA was found to stimulate a calcium efflux mechanism in porcine PMN. This extrusion was interpreted as being activation of a plasma-membrane calcium efflux mechanism. The known effect of TPA on PKC suggests a mechanism involving protein phosphorylation, possibly involving direct phosphorylation of the calcium-ATPase or of an unknown regulatory protein. TPA has structural similarities with diacylglycerol, the physiological activator of PKC, and PKC is the only cellular binding site for phorbol esters so far described. Raised intracellular calcium concentrations are also observed during cell activation and the two effects appear to act synergistically in stimulation of cell function. PKC-mediated activation of calcium efflux is an apparent paradox. However, delayed activation of efflux may be involved as a negative feed-back mechanism in modulation of stimulated influx and in recovery from raised intracellular calcium. This same mechanism can be assumed to be true for

other phorbol esters which are DAG analogues, such as PMA.

Christiansen, et al.(1988) showed that both PMA and A23187 increased membrane-associated PKC activity and decreased cytosolic activity of the enzyme in human PMN, proving that both stimulants induce translocation of PKC to the membrane fraction from the cytosol. This supports the hypothesis that A23187, causing an increase in calcium intracellularly, has a positive effect on the stimulation of PKC by its activators.

Levels of membrane-associated PKC due to A23187 and PMA stimulation were not compared in the Christiansen, et. al. study, but it is assumed that PMA would cause the greater translocation since activation of this enzyme is its primary action. Translocation induced by A23187 was found to be strictly dependent upon extracellular calcium concentration while PMA activation of translocation was not dependent.

Knowledge about mechanisms which lead to increased intracellular calcium and protein kinase C activation facilitates ex vivo investigation about the activation of PMN functions. This knowledge in combination with an understanding about the processes which lead to expression of PMN function directs studies which examine methods of controlling or promoting PMN immune functions during inflammatory reactions.

III. NEUTROPHIL INFLAMMATORY FUNCTIONS

A. Introduction

Neutrophils play a predominant role in host defense because they rapidly migrate to areas of infection and destroy foreign matter. Specific chemoattractants, released by bacteria or generated by host cells, spread out from the injury site and come into contact with PMN. When PMN encounter low concentrations of these stimulants in the bloodstream they are activated to move along a increasing concentration gradient to the endothelium which is closest to the injury site. PMN roll along the endothelium and adhere to areas of highest concentration of chemoattractants. PMN move between endothelial cells and enter the extracellular matrix as they continue to move along the chemical gradient to areas of higher concentration of stimulant. In this way PMN are able to migrate to the inflammatory site and induce microbial and foreign matter destruction in the area of highest stimulant concentration. This destruction may occur by oxidative or non-oxidative mechanisms or by a combination of both. Oxidative mechanisms are mediated by toxic oxygen metabolites and non-oxidative mechanisms involves secretion of lysosomal enzymes.

B. Adherence and Aggregation

Chemotactic factors liberated at injury sites stimulate

PMN to marginate out of the main axis of blood flow toward the endothelial surfaces. PMN are induced to adhere to the endothelium and aggregate together at these sites. This aggregation increases the number of PMN near sites of inflammation within the microcirculation, thereby providing a greater number of responding cells in the appropriate area.

When PMN are activated the expression of certain plasma membrane receptors, known as adhesion molecules, is increased (Hoffstein et al.,1982). The predominant adhesion molecules are called LeuCAMs (leukocyte adhesion molecules) and include lymphocyte function-associated antigen (LFA-1,CD11a/CD18), MAC-1 (CD11b/CD18) and glycoprotein (gp) 150,95 (CD11c/CD18) (Springer, T.A.,1990). LFA-1 binds specifically to intercellular adhesion molecules (ICAM-1 and ICAM-2) which are expressed on many different cell types, including endothelial cells. MAC-1 binds ICAM-1 and activated complement product, C3b.

Selectins, adhesion molecules on the surface of PMN and endothelial cells, also facilitate PMN localization. L-selectin is found on PMN and is required for PMN emigration at inflammatory sites. P-selectin is expressed on endothelial cells following upregulation by thrombin, histamine and peroxides (Rosales, C. et al.,1993). It binds to its ligand on circulating PMN to enable an early step in leukocyte adhesion to endothelium at sites of inflammation. E-selectin,

also synthesized by endothelial cells in response to interleukin-1 (IL-1) and tumor necrosis factor (TNF), recognizes a ligand on PMN and assists their adhesion to endothelium near inflammatory sites. The specific ligands for these selectins on PMN surfaces are as yet unknown.

Two mechanisms activated during inflammation seem to be involved in directing PMN into tissues at sites of inflammation. One is immediate, involves no protein synthesis, and results in transient increases of adhesiveness of both PMN and endothelium. The second is activated one to two hours after inflammation begins and requires protein synthesis and expression of new endothelial proteins that promote binding of both activated and resting PMN.

When PMN are exposed to chemoattractants and other active substances, they very rapidly become more adhesive for endothelium whether or not the endothelium is stimulated. This interaction is mediated by a qualitative change in affinity of the adhesion molecules during PMN activation and does not require the increase in adhesion molecule expression by PMN which also accompanies activation. This rapid binding of PMN to endothelium is mediated by P-selectin since monoclonal antibodies against P-selectin prevent this early adhesion (Geng,J.,et al.,1990). This binding is dependent on extracellular calcium ions and the qualitative change in affinity may be due to phosphorylation.

Thrombin and histamine activation of endothelium causes the release of platelet-activating factor (PAF) and secretion of P-selectin. PAF activates expression of the integrins on PMN surfaces and leads to enhanced adhesion between PMN and endothelium (Lorant,D.E.,et al.,1991). This adhesion requires active participation of both cell types. After exposure of endothelium to interferon, TNF or LPS for four to twenty-four hours, synthesis of ICAM-1 and E-selectin is induced and PMN adhesion is promoted further.

Leukocyte accumulation at inflammatory sites follows this model for in vivo binding: in the absence of an inflammatory signal, PMN are carried along in the circulation at a rate determined by blood flow. Upon recognition of endothelium at an inflamed site through selectin interaction, PMN are slowed and begin to adhere to the endothelial surfaces. This adhesion results in PMN rolling at a much slower rate than the rate of blood flow. Finally, PMN expression of adhesion molecules, activated by increased concentration of stimulant, causes interaction with endothelium ICAMs, resulting in stronger adhesion that eventually stops PMN rolling and allows transendothelial migration.

During the adhesion process, surface charges on PMN surfaces are also altered. The changes in surface charge follow secondary granule secretion and allow static interaction and thus, promote aggregation of many PMN

together. This aggregation occurs in the area of highest chemoattractant concentration present in the microvasculature where PMN need to emigrate across the endothelium nearest injured areas.

Once PMN are out of circulation, they encounter a complex array of molecules in the extracellular matrix (ECM) during their emigration to an inflammatory site. Various ECM proteins have significant effects on PMN function (Brown,E.J.,1986). A specific receptor named leukocyte-response integrin (LRI) has been identified and named as the receptor for a peptide sequence in the proteins of the ECM. Binding of proteins to this receptor on PMN increases phagocytosis. Monoclonal antibodies for this receptor inhibited increased phagocytosis stimulated by fibronectin, collagen type IV, von Willebrand's factor and fibrinogen (Gresham, H.D.,et al.,1989).

C. Non-oxidative killing or Degranulation

Three types of granules have been identified in PMN but all types do not exist in every species. Azurophilic (primary), specific (secondary), and C-particle granules (tertiary) are the three types of granule populations identified in human PMN which are important to PMN function in killing and digestion of microorganisms (Woessner, 1992). The

azurophilic granules contain elastase, myeloperoxidase, lysozyme, bactericidal/permeability-increasing protein (B/PI), chymotrypsin-like cationic protein (CLCP), and defensins. Specific granules contain lactoferrin, lysozyme, and collagenase which are important in PMN migration through tissue, and they are the first type of granule released when PMN are stimulated. C-particle granules have not been completely characterized but are known to contain gelatinase.

Phagocytosis at an injury site depends on the formation of a phagosome by PMN pseudopods which become polarized toward a particle and surround it. Digestion occurs following fusion between phagosomes and cytoplasmic granules which contain lytic enzymes necessary for degradation. Fusion of granules with phagosomes containing phagocytosed foreign material allows the release of the enzymes into phagosomes where they are needed. The release of enzymes causes the degradation of the cell membranes of foreign organisms but may also lead to damage of normal tissue if released outside the PMN (Abrams,W. et al.,1983).

Degranulation may occur before complete fusion of pseudopod arms around the foreign particle and the granule contents are released extracellularly by exocytic degranulation. Normal host tissue is exposed to and damaged by extracellular release of these substances. Damaged and degraded host tissue causes elaboration of inflammatory

mediators which trigger PMN inflammatory responses further, and, consequently, inflammation is intensified.

Azurophilic granules release myeloperoxidase, elastase, collagenase, B/PI and CLCP which are important in degrading foreign matter such as bacterial cell walls at a site of inflammation and which also contribute to host tissue injury. The release of these granules is most important to characterizing PMN function as these are released at the site of injury or inflammation and are probably more involved in elaborating the inflammatory process than specific granule contents.

D. Oxidative-dependent killing and Superoxide anion production

PMN bactericidal capacity partially depends on generation of toxic oxygen metabolites. When PMN are stimulated, oxygen consumption increases in a phenomenon that is called the respiratory burst (see Evans, et al.,1992 for a review). During their respiratory burst PMN consume oxygen which is converted to free radicals capable of killing ingested microorganisms. Superoxide anion, the initial conversion product in oxygen reduction, is an oxygen metabolite secreted by PMN (Henson,P., et al.,1987) and is the precursor for a

series of reactions that produce lethal oxygen metabolites. Superoxide anion production occurs following activation of NADPH oxidase complex in the phagolysosome membrane.

Superoxide anion has a single, unpaired electron in its outer shell which makes it a highly reactive and unstable molecule. The outer shell freely accepts another electron from molecules such as free acids, amines and other molecules. Reduction of superoxide results in oxidation of the electron donors and production of another reactive oxygen metabolite, hydrogen peroxide, in the dismutation reaction. Various oxidized biomolecules which are products of free radical reactions have been detected in inflammatory exudate (Winyard,et al.,1992).

Superoxide anions exert toxic actions on bacteria and host cells by altering energy-producing systems, lipid membrane continuity, cell surface receptor structure, and/or DNA integrity (Winyard,et al.,1992). Energy systems of mitochondria and the endoplasmic reticulum are disrupted when superoxide anion oxidizes enzymes or products, making them unavailable in the reactions. Lipid membranes are easily oxidized by superoxide anion due to readily released electrons from acidic side chains present on the phospholipid molecules. Surface receptors are affected when superoxide anion affects cell membrane integrity, altering normal arrangement of the receptors on the membrane and changing their conformation.

Superoxide anion causes single-stranded breaks in DNA structure which prevents DNA transcription and protein synthesis. These alterations in normal cell structure and function result in cell death.

Activation of NADPH oxidase is part of the controlled acute inflammatory response to bacterial invasion (Winyard,et al.,1992) but excess activity of this enzyme leads to tissue destruction. The mechanisms of action of oxygen radicals in inducing tissue injury parallel those for bacterial destruction, as discussed above. These include damage to proteins (intracellular and extracellular), membrane lipids, and nucleic acids. Lipid peroxidation of host cell membranes alters structural integrity and may perturb the microenvironment of membrane-bound proteins; thus altering their function (Leff, et. al.,1993). These proteins may be involved with ion transport or other important cellular functions. Oxygen radicals may also alter the structure and function of RNA and DNA. All mammalian tissues contain the antioxidant enzymes, superoxide dismutase and catalase, but these enzymes may be overwhelmed during intense local production of toxic oxygen species.

The NADPH oxidase system and granule constituents are used in a cooperative manner by PMN to destroy foreign material (Weiss, S.J.,1989). This combination of PMN responses can also lead to normal tissue damage by PMN and

constitutes their ultimate destructive potential. A popular proposal has been the concept of a 'frustrated phagocyte' in which the PMN can not phagocytose large substances, and accordingly, generates increasing amounts of enzymes and oxygen radicals continually and may release them into the extracellular space (Henson, P.M.,1972). Following activation, the NADPH oxidase system generates large quantities of superoxide anion, and almost simultaneously, PMN granules fuse with the external membrane at sites of activation and release the enzymes and superoxide anions into the extracellular environment. Hydrogen peroxide, which is rapidly produced in the dismutation of superoxide anion, combines with myeloperoxidase from PMN primary granules to oxidize halides, such as chloride anion, to their hypohalous acids. Thus, hypochlorous acid (HOCl), one of the most toxic products of PMN, is considered the major product of oxidative metabolism. This acid is a highly reactive oxidant that rapidly attacks many biomolecules.

IV. SPECIES VARIATION

A. Introduction

Evaluation of studies of the inflammatory process have demonstrated the multiple differences that exist among animal species, particularly in PMN responses (Bertram,1985) (Styrt,B.,1989). Appreciation of interspecies differences in PMN functions is important to the interpretation and application of ex vivo and in vivo studies of both pathogenesis and treatment of inflammatory disorders in animals. Examples of such differences include: 1) the ability of PMN from various species to react to certain stimulants and the corresponding responses elicited; 2) different enzymes and relative activities of enzymes; and 3) types and number of granules present. Existence of these differences may relate to the susceptibility or resistance of some species to specific pathogens.

The discussion which follows gives a brief overview of major comparative studies and hopefully points to the inadequacy of information about ex vivo studies involving equine neutrophils specifically.

B. Reaction to specific stimulants

Neutrophils from all species aggregate and adhere to surfaces, migrate in response to chemotactic stimuli, degranulate into phagocytic vacuoles or into the extracellular

environment, ingest foreign particles and generate oxygen metabolites. The heterogeneity among species occurs principally in the pathways by which these functions are accomplished and in the corresponding biological responsiveness to different stimuli.

Receptors for ligands which stimulate PMN activation have been characterized on PMN surfaces of a number of species. Human PMN can be activated by FMLP to migrate along a chemotactic gradient, degranulate, and produce oxygen metabolites. PMN from rabbits (Becker,1987), guinea pigs (Becker,1987), and mice (Styrt,et al.,1988) also respond to FMLP whereas PMN from cattle (Forsell,et al.,1985), pigs (Chenoweth,et al.,1980), dogs and cats (Stickle,et al.,1985) have no apparent response to FMLP. Equine PMN have been shown to have a secretory response but not a chemotactic response to FMLP (Snyderman,et al.,1980). Zinkl and Brown (1982) showed that horse PMN do have a weak chemotactic response to FMLP only at the very high concentration of 10^{-4}M. Different techniques were utilized by these two groups studying equine PMN to measure PMN chemotaxis which could explain this discrepancy.

The CD18 group of adhesion-related PMN membrane surface antigens has been identified in a number of species including mice, human beings, dogs, cattle and horses (Bochsler,et al.,1990). This family of molecules, including Mac1, LFA1,

and p150, appears to be necessary for a variety of ex vivo PMN functions including aggregation, adherence and chemotaxis. The use of monoclonal antibodies has allowed characterization of these molecules and so far shows some overlap and conservation of the structure of the molecules between species such as human and horse (Bochsler et al.,1990).

C. Functional Responses

Chemotaxis, which involves recognizing chemotactic signals, adhering to surfaces, and rearrangement of the cytoskeleton to produce directed movement, can be induced by many factors including FMLP and the complement fragment C5a. Human, bovine, equine, feline, canine and murine PMN all show bipolar shape changes or directed migration in response to complement, activated serum or activated plasma. Porcine PMN migrate toward products of filtrates of bacterial cultures but bovine PMN do not; however the bovine PMN do show shape changes. FMLP does not induce chemotaxis in bovine or swine PMN. Equine PMN may or may not have a chemotactic response to FMLP based on the assay procedure. More research involving equine PMN would help to clarify this question.

Oxidative metabolism generates reactive metabolites from atmospheric oxygen and contributes to PMN bactericidal function. The basic enzyme system generating these metabolites in the respiratory burst seems to be highly

conserved among mammalian species but heterogeneity is seen in requirements of its activation. Activity of the system is associated with assembly of NADPH oxidase complex from components present in the membrane, cytosol and granules of the resting PMN. Thus, plasma membranes and phagosome membranes are important to activity of the system as documented in PMN from humans, guinea pigs and cattle only. A low potential cytochrome B appears to be important in the NADPH oxidase system in human, bovine, equine and porcine PMN. Translocation of the cytochrome B has been documented in human and bovine PMN only. FMLP elicits the respiratory burst in human and guinea pig PMN, but not in ovine, bovine or porcine PMN. Calcium ionophore A23187 does not stimulate the respiratory burst in human and bovine PMN (Brown and Roth, 1991). The respiratory burst of equine PMN has not been previously evaluated in response to calcium ionophore A23187.

D. Granule enzymes and relative activities

Cytoplasmic granules which contain substances that are important to microbicidal activity of PMN are an essential characteristic of the cells. Species differences have been reported in the number of granule types, identifiable contents and circumstances under which these contents are released.

Human PMN contain three types of granules: 1) primary or azurophilic granules which contain myeloperoxidase and

lysosomal hydrolases; 2) secondary or "specific" granules which contain lactoferrin, cytochrome b and vitamin B12-binding protein; and 3) tertiary or C-particle granules which have been found to contain gelatinase. Azurophilic granules and specific granules can be identified in most species. A third type of PMN granule reported in cattle, rabbits, sheep, goats and dogs may or may not be comparable in content and function with the tertiary granules of human PMN.

Lysozyme, a characteristic granule enzyme of human PMN, is found in both azurophil and specific granules. Cattle, sheep, goats and cats are among the species which have negligible PMN lysozyme activity. Other species including horses, dogs, and rats show 10-20% of the lysozymal activity of human PMN.

The characteristic enzyme of the azurophil granule, myeloperoxidase, plays an important role in oxygen-dependent microbicidal activity of PMN. Activity of this enzyme is significantly lower in most species, including horses, than that present in human PMN. Exceptions include dogs and other primates (Rausch et al.,1975).

Major species differences again are suggested involving the granule enzyme elastase. Equine (vonFellenberg, et al.,1985) and porcine (Chibber, et al.,1983) PMN are reported as having one third the elastase activity as human PMN.

Several types of specific microbicidal proteins, in

addition to the enzymes discussed above, have been characterized in association with PMN granules and are probably important to optimal PMN bactericidal function. Many species differences have been reported involving these enzymes and are further reviewed in Styrt,B.(1989). Additional comparisons may help define the importance of granule components in defense against specific pathogens and its correlation with relative resistance or vulnerability of different species to different infectious processes.

E. Abnormalities of neutrophil function

Several defects of neutrophil function have been recognized and described. Chediak-Higashi syndrome (CHS) in human beings, characterized by the presence of large aberrant granules in multiple cell types including PMN, has analogues in cattle, mice, mink, whales and cats. Increased susceptibility to infection of humans with CHS is apparently reproduced in the bovine and murine CHS but not in feline CHS. Abnormal lysosomal calcium transport has been found in human and murine CHS but the cause of the abnormality in CHS is still being investigated. Such a disease of PMN has not been described in horses.

The human deficiency of the CD18 family of membrane adhesion molecules has recently been found to have an analogue in dogs and cattle. Human, canine and bovine patients are

susceptible to recurrent infection when afflicted with this deficiency in molecules which are vital for normal PMN migration.

Chronic granulomatous disease, the best characterized human PMN defect in which the respiratory burst and cytochrome b are defective or absent, does not yet seem to have an analogue in other species. The most common human PMN defect, myeloperoxidase deficiency, also has not been documented in other species.

F. Conclusion

The marked differences involving PMN function among the species (receptors present on surfaces, distribution and amounts of lysosomal enzymes, response to various chemoattractants, and even defects of function) suggests variations which make interspecies extrapolation of studies of neutrophil function difficult. These variations could account for differing vulnerabilities of various species to a given infection. Thorough studies and characterization of equine PMN should be completed to facilitate an understanding of the basic pathophysiology of inflammation in the horse.

MATERIALS AND METHODS

REAGENTS

Reagents were purchased from the following suppliers: Hank's Balanced Salt Solutions (HBSS) and calcium- free modified Hank's Balanced Salt Solutions (CFMH), Percoll, phorbol myristate acetate (PMA, MW=616.8), calcium ionophore A23187 (A23187, MW=523.6), superoxide dismutase (SOD), cytochrome c from horse heart and trypan blue from Sigma Chemical Company (St. Louis, MO); and pure acridine orange from Polysciences, Inc. (Warrington, PA).

HBSS and CFMH powders were dissolved in de-ionized water, sterile filtered and stored at 0^{0}C. These buffers were made fresh each week. A23187 and PMA were dissolved in 99.9% industrial grade DMSO and stored at -20^{0}C in 1 mg/ml aliquots. Acridine orange was dissolved in CFMH and stored at 0^{0}C in a dark container at 1 mg/ml concentration. SOD was dissolved in HBSS and stored at -20^{0}C in 15000 U/ml aliquots.

Animals:

Six mixed-breed geldings, aged 3 to 5 years, were used for this study. The horses were maintained on pasture at Virginia Polytechnic Institute and State University, and had

been in residence for greater than one year. They were fed a cracked corn and molasses mixture, and water was available ad libitum. All horses had been dewormed with ivermectin within six to eight weeks prior to the study and vaccinated against tetanus, eastern and western equine encephalomyelitis, influenza and equine rhinopneumonitis two months before the study. Prior to the study, all horses were determined to be healthy, based on normal physical examinations and total leukocyte counts. Blood collection was performed while the horses were at rest. Blood samples were collected and assays were conducted from April through August.

PMN Isolation:

PMN were isolated using a modification of the methods of isopyknic sedimentation on discontinuous Percoll gradients previously described by Pycock,J.F., et al., (1987) and Sedgwick, A.D., et al.(1987). First, the osmolarity of Percoll was adjusted to 280-300 milliosmoles per liter by mixing 440 ml Percoll and 40 ml 10X CFMH. This diluted solution of Percoll was isosmotic with CFMH. Two Percoll solutions, fifty-nine percent and seventy-five percent Percoll, were made daily using isosmotic Percoll solution and CFMH. The fifty-nine percent layer was made by mixing 11.8mls of isosmotic Percoll and 8.2mls CFMH and the seventy-five percent solution was made by mixing 15mls isosmotic Percoll

and 5mls CFMH.

Ten ml of 59 percent Percoll were placed in a clear, conical centrifuge tube and, using a 30 ml syringe and 25 gauge spinal needle, 7.5 ml of 75 percent Percoll were underlaid to produce discontinuous Percoll gradients. One gradient was prepared for each 25 ml blood collected.

Blood samples were collected by jugular venipuncture into sodium citrate (aggregation and degranulation assays) or sodium-EDTA (superoxide anion assay) evacuated blood collection tubes (Becton Dickinson Vacutainer Systems, Rutherford, New Jersey). A total of fifty ml of blood were required for each of the aggregation and degranulation assays. One hundred ml of blood were required for each superoxide anion assay. Blood was transported to the laboratory within thirty minutes and care was taken not to expose it to temperature extremes.

Total leukocyte counts were attained using an automatic cell counter (Coulter Counter Model ZF, Coulter Electronics, Hialeah, Florida). Differential leukocyte counts were determined using a Wright's- stained smear.

After centrifuging the blood tubes at 200 x g for 15 min, plasma was removed by aspiration from the tubes, and leukocyte-rich buffy coats were collected. Collection of the buffy coats was performed within one hour of blood collection. The buffy coat layer was diluted 1:1 with CFMH. The diluted

buffy coat mixture was layered onto the Percoll gradients (5 ml per gradient).

Gradients were centrifuged at 1000 x g for 40 min at 22°C. PMN sedimented to the interface of the 59% and 75% layers. The fifty-nine percent layer was removed by aspiration to within 0.5cm of the PMN layer and discarded. PMN were collected with a plastic Pasteur pipette, washed twice by dilution with CFMH followed by centrifugation for 15 min at 200 x g at 22°C and finally resuspended in 0.5 ml HBSS for the aggregation and superoxide anion assays or 0.5 ml CFMH for the degranulation assay.

A fifty µl aliquot of the PMN isolate was diluted 1:10 in HBSS and counted using a Coulter Counter Model ZF (Coulter Electronics, Hialeah, Florida) to determine PMN concentration. Cell viability was assessed using the trypan blue exclusion method (Freshney, R.I.,1987) prior to initiating the experiments. Briefly, 20 µl of a 1:10 dilution of the PMN isolate were mixed with 20 µl 0.4% trypan blue. Ten µl of the mixture were dispensed on a hemocytometer and 200 cells were counted. An additional 100 µl aliquot of isolate was diluted to 300cells/µl in a final volume of 0.5ml and cytocentrifuged (Shandon Cytospin 2, Shandon, Inc., Pittsburgh, Pennsylvania). The preparation was stained with Wright's stain and differential counts were conducted, as previously described for whole blood, to determine the PMN purity of the isolate.

All assays were completed within eight hours of blood collection.

PMN Aggregation:

Equine PMN aggregation was measured using a platelet aggregometer/strip recorder (Payton Aggregation Module/ Dual Channel, Payton Associates, Inc., Buffalo, New York) as previously described (Slauson, D.O., et al., 1987). Briefly, the minimum and maximum light transmission limits (0% transmittance and 100% transmittance) were calibrated with PMN suspensions containing $1.1x10^7$ cells/ml and $2.5x10^6$ cells/ml, respectively. The instrument was adjusted to produce full scale deflection between these two calibration solutions. All aggregation assays were performed at 37^0C in siliconized cuvettes with teflon-coated stir bars mixing at 600 rpm. For each concentration of each stimulant tested, 450µl PMN in HBSS ($1.1x10^7$ cells/ml) were placed in a cuvette, and allowed to warm and stir for a one minute equilibration period. Then fifty µl of stimulant were added and the recorder was activated to measure the response. HBSS was used as the negative control. Stimulants were diluted in HBSS so that fifty microliters produced the desired final concentration. Pilot studies tested multiple concentrations above and below those which yielded the maximum and minimum responses,

respectively, to bracket the range of concentrations producing the dose-dependent curve. Additional concentrations were selected to characterize the linear relationship between the minimum and maximum responses of change in percent transmittance. The final A23187 (MW=523.6) concentrations evaluated were 0.48, 0.95, 1.4, 1.9, 4.8 and 9.5μM. The final PMA (MW=616.8) concentrations evaluated were 0.002, 0.004, 0.008, 0.02, 0.04, 0.08 and 0.2μM.

Aggregation was allowed to proceed until the recording clearly leveled off or began to descend. Change in percent transmittance was recorded for each run. An increase in percent transmittance indicated induction of aggregation by stimulants (Fig. 2).

Paired samples were evaluated at different times to eliminate time as a possible variable in PMN response. In one channel responses to stimulants were evaluated starting at the lowest concentration and increasing the concentration for each consecutive sample. In the other channel responses to stimulants were evaluated starting with the highest concentration of stimulants and decreasing the concentration in each consecutive sample. The mean of the two responses for each concentration of stimulant was calculated and recorded as the change in percent transmittance.

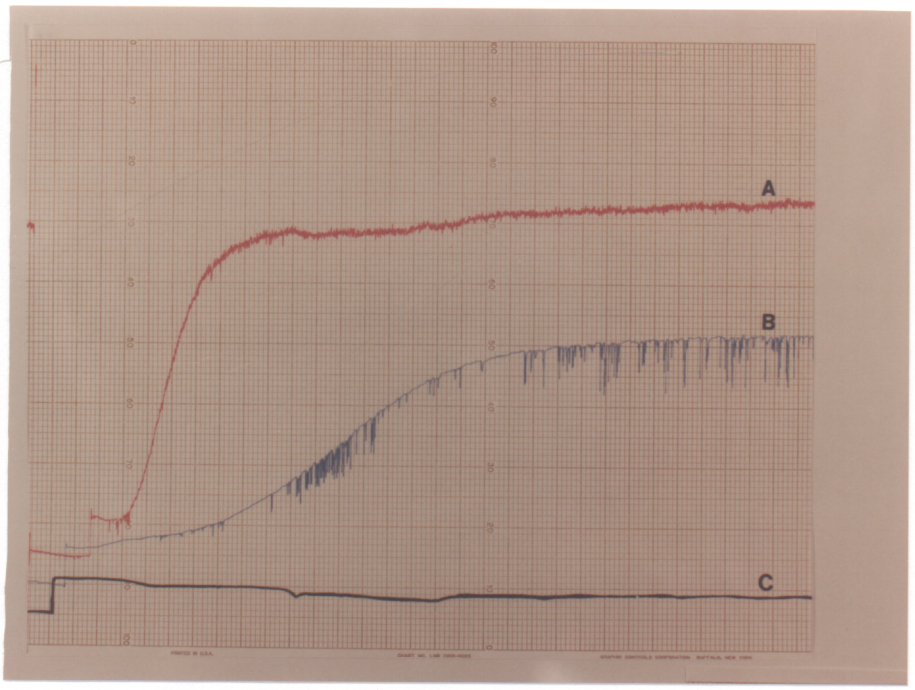

Fig. 2 Dose-dependence of percent transmittance in PMA-treated PMN (A: 0.081μM PMA, B: 0.016μM PMA, C: HBSS). The control result from this experiment was traced onto the results of the two stimulated samples for demonstration purposes.

PMN Degranulation:

A flow cytometric method was used to assess degranulation by stimulated PMN as previously described (Abrams, W.R.,et al.,1983). Acridine orange was used as a fluorescent label of azurophilic granules in PMN. Briefly, PMN ($2x10^7$cells/ml) were mixed gently with 10 µg/ml acridine orange at 37^0C for 15 minutes. Cells were washed to remove excess acridine orange. Stained cells were resuspended in HBSS to maintain a concentration of $2x10^7$cells/ml. Then stained cells (0.125ml) were added to 0.375ml of HBSS which contained appropriate concentrations of stimulants and incubated 30 minutes at 37^0C. Final cell concentration in the incubating microcentrifuge tubes was $5x10^6$cells/ml with a final volume of 0.5mls. Pilot studies were conducted which tested multiple concentrations equal to and above that which produced the maximum response as well as concentrations equal to and less than that which produced the minimum response. Concentrations were selected to characterize the dose-dependent relationship between the minimal and maximal responses. Final A23187 concentrations were 0.48, 0.95, 1.4, 1.9, 4.8, 7.2, 9.5, 19 and 48µM. Final PMA concentrations were 0.004, 0.008, 0.02, 0.04, 0.057, 0.08, 0.2 and 0.41µM. HBSS was used as a negative control.

Intracellular fluorescence due to acridine orange was measured by a Coulter EPICS 752 Flow Cytometer and Cell Sorter (Coulter Electronics, Hialeah, Florida) equipped with an

INNOVA 90 Argon Laser (Coherent, Inc., Palo Alto, California). The laser provided a 488 nm excitation wavelength at 300 mW. Fluorescence emission greater than 600 nm was recorded and the data were analyzed by a MDADS Computer System (Coulter Electronics, Hialeah, Florida). The intensity of each sample was represented as a histogram with fluorescence expressed as the mean channel value measured between 0 and 255 channels. The mean channel value was calculated from fluorescence measurements of at least 10,000 cells per sample. Each cell was placed in a channel based on its relative fluorescence. The number of cells in each channel was multiplied by the respective channel value for all channels and these products were added to calculate total fluorescence intensity (FI) of the sample.

Equation 1.

FI for channel = number cells in channel x channel number

Total FI = sum of FI for all channels (0-255)

Mean channel value (MCV) for the individual sample was calculated according to equation 2.

Equation 2.

MCV = Total FI / total number cells counted

The mean channel value decreased as the fluorescence of cells in the sample decreased. The decreased fluorescence indicated degranulation of PMN induced by stimulants(Fig.3).

The difference between the control's MCV and each stimulated sample's MCV was divided by the control's MCV and multiplied by 100 to calculate percent degranulation (% degranulation).

Equation 3.

$$\% \text{ degranulation} = \frac{(\text{control MCV} - \text{sample MCV})}{\text{control MCV}} \times 100$$

Percent degranulation was calculated for each concentration of each stimulant.

Superoxide anion production by PMN:

A microassay was used for the measurement of superoxide anion produced by stimulated PMN, as previously described (Pick,E., et al.,1981). Briefly, PMN ($5x10^5$ cells/well) were combined with a detection solution containing cytochrome c (160μM) and A23187 or PMA in 96-well microtiter plates. Superoxide dismutase (SOD)(300 Units/ml) was included in the

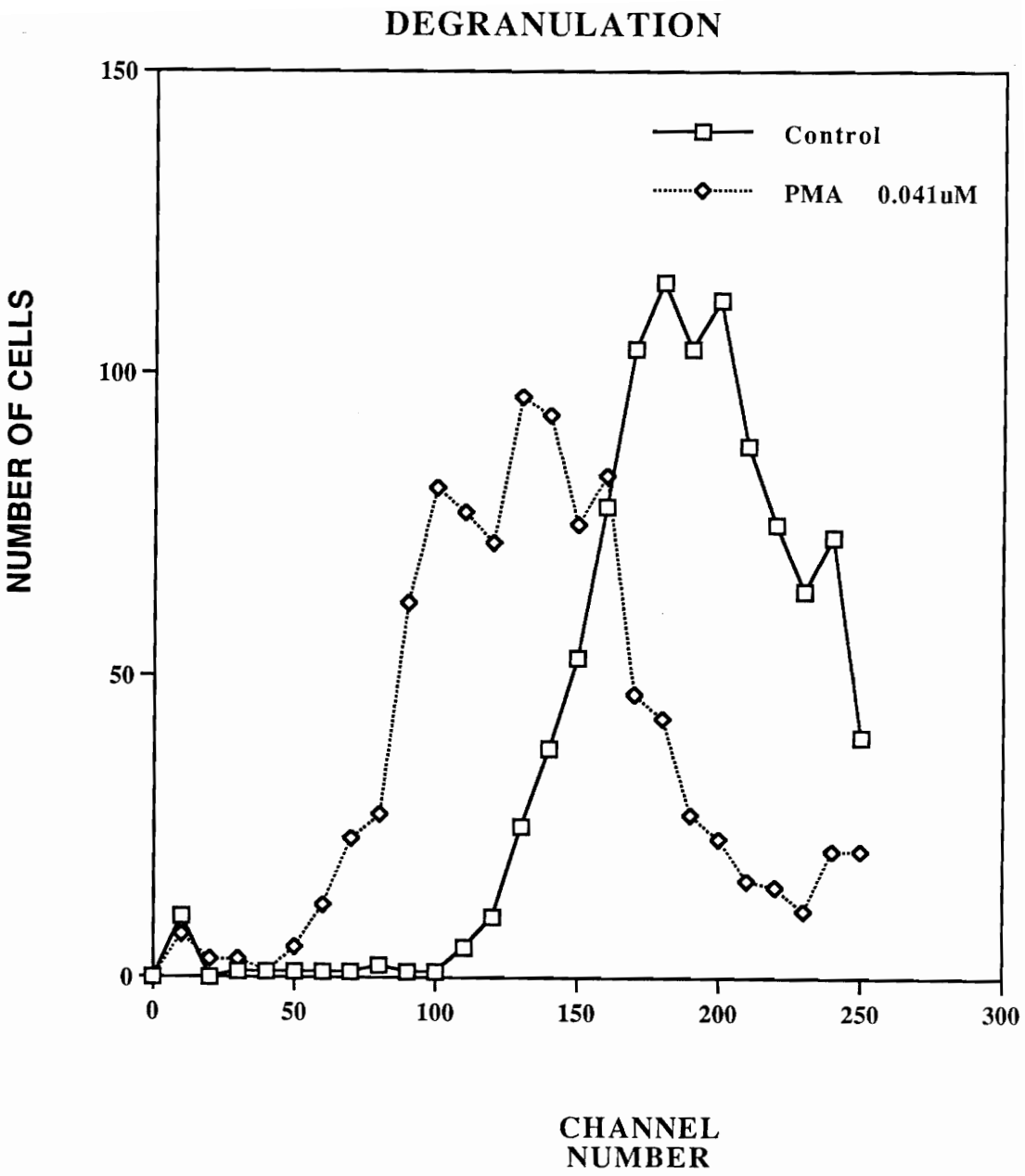

Fig. 3 Degranulation histograms of control PMN and PMN stimulated with 0.041μM PMA. As PMN degranulated following stimulation the mean channel value for the sample decreased.

detection solution of half of the wells. Measurement is based on the difference in absorbances between stimulated cells in the presence and absence of SOD. This difference represents the increase in absorbance of cytochrome c at 550nm due to superoxide anion reduction. Reduction of cytochrome c by superoxide anion is prevented in the presence of SOD as SOD converts superoxide anion to hydrogen peroxide. Increase in reduced cytochrome c concentration was used to determine the amount of superoxide anion generated.

Pilot studies evaluated the response at multiple concentrations equal to and greater than that which produced the maximum response and also at multiple concentrations equal to and less than that which produced the minimum amount of superoxide anion. A range of concentrations was bracketed. This demonstrated the dose-response relationship which exists with these stimulants for this function. Additional concentrations were selected to further characterize the direct relationship between the minimum and maximum responses. Final A23187 concentrations in the wells were 0.18, 0.23, 0.31, 0.38, 0.47, 0.70, 0.93, 1.9 and 9.3μM. Final PMA concentrations were 0.0002, 0.0005, 0.002, 0.005, 0.02, 0.05, 0.2 and 0.5μM in the wells.

Plates were incubated at 37°C for 30 minutes and then read with a microplate reader (UVmax Kinetic Microplate Reader, Molecular Devices Corporation, Menlo Park,

California). The extinction coefficient for the absorption of reduced cytochrome c which equals $2.1x10^{4}M^{-1}cm^{-1}$ was used in the Lambert-Beer law **(A = a x b x c)** to determine the amount of superoxide anion actually produced (Robyt,et al.,1987).

Equation 4.

$$c = A / (a \times b)$$

A = difference in absorbances of SOD+ and SOD- wells containing the same concentration of stimulant

a = extinction coefficient ($2.1x10^{4}M^{-1}cm^{-1}$)

b = pathlength (cm) (0.25cm for this study)

c = concentration(M) of reduced cytochrome c

There is a direct 1:1 relationship between the amount of cytochrome c that is reduced and the amount of superoxide anion produced as there is an exchange of only one electron. The concentration of reduced cytochrome c equals the concentration of superoxide anion. The molar concentration was converted to nanomoles (nmoles) of superoxide anion per million cells (10^{6} cells) using the final volume in the wells and the number of cells in each well ($5x10^{5}$ cells/well).

The difference in absorbance between SOD+ and SOD- wells and, thus, superoxide anion production by stimulated PMN increased as concentration of stimulant was increased (Table

1).

The pathlength for the microplate reader in this assay was determined according to an applications note from Molecular Devices Corporation. Briefly, concentrations of cytochrome c (60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160 and 170μM) were prepared, assuring that the concentration used in these assays (160μM) was included in the range. Their absorbances were measured using a spectrophotometer and the microplate reader. The spectrophotometer absorbances were plotted versus microplate reader absorbances and the slope determined. The slope multiplied by 1 cm is the pathlength for the microplate reader assay. The slope and pathlength for our experiment was determined to be 0.25cm.

DATA ANALYSIS

The mean response induced by each concentration of each stimulant in each assay was calculated for six horses and plotted versus log stimulant concentration. Maximum responses induced by A23187 and PMA for each assay were compared using the Student's t-test.

Percent of maximum response (% max. response$_c$) values at each concentration of each stimulant were calculated using the means (n=6) for each stimulant concentration and the mean for

TABLE 1. MEAN ABSORBANCE VALUES FOR ONE A23187 SUPEROXIDE ANION ASSAY

A23187 conc. (μM)	Absorbance (SOD+)	Absorbance (SOD-)	O_2-/well (nmols)
0.18	0.440	0.445	0.09
0.23	0.438	0.484	0.88
0.31	0.400	0.497	1.85
0.38	0.442	0.552	2.09
0.47	0.425	0.572	2.81
0.70	0.411	0.560	2.84
0.93	0.378	0.563	3.52
1.9	0.410	0.577	3.18
9.3	0.401	0.544	2.71

the concentration that produced the maximum response (n=6) according to equation 5.

Equation 5.

$$\%\ \text{max. response}_c = \frac{\text{mean response}_c}{\text{mean maximum response}} \times 100$$

In all assays the mean percent of maximum response at each concentration for each stimulant for the six horses was calculated and plotted versus log concentration of A23187 and PMA.

Analysis of Response versus Log Concentration Curves:

Each mean percent of maximum response was transformed to a probit value.

Equation 6.

$$\text{PROBIT} = 5 + \frac{[\%\text{max. response}_c - (\%\text{max. response}_{mean})]}{\text{std(\%max. responses)}}$$

$\%\text{max. response}_{mean}$= the mean of all responses for all stimulant concentrations in the assay

std(%max. responses)= the standard deviation of all responses

for all stimulant concentrations in the assay

Linear regression analysis of probit values of percents of maximum response versus log concentration values was conducted for the mean percent of maximum response at each concentration for each stimulant in each assay. Only values between the minimum and maximum responses for each assay were included in the analysis. Diminished responses which occurred at higher concentrations were assumed to represent a loss of functionality in the PMN.

Probit values for 0%, 50% and 99% of maximum response were calculated as below.

Equation 7.

$$\text{Probit}_x = 5 + \frac{[\{X\%\} - (\%\text{max. response}_{\text{mean}})]}{\text{std(\%max. responses)}}$$

Effective concentrations for each stimulant at 0%(threshold), 50% and 99% of maximum response (EC_0, EC_{50}, and EC_{99}, respectively) for the assays were calculated using the resulting slope (m) and intercept (b) of each assay-stimulant combination.

Equation 8.

$$\log (EC_x) = (PROBIT_x - b)/m$$

The assumptions of the model are that a linear relationship exists between the two variables and that there is a normal distribution of percents of maximum responses for each log concentration. Residuals were calculated and plotted against log concentration to assure that the assumptions were appropriate and a reasonable model had been fit to the data.

Confidence intervals (95%) were calculated for the probits of 0%(threshold), 50% and 99% of maximum response for each assay-stimulant combination by using the following formula (Kleinbaum,D.G.,et al.,1988):

Equation 9.

$$\mathbf{PROBIT_x \pm t_{n-1,1-(\alpha/2)} \times (S/n^{1/2})}$$

t= value from t-distribution table for n-1 degrees of freedom

α= 0.05 when determining 95% confidence intervals

$S/n^{1/2}$= standard error of mean Probit estimate

These ninety-five percent confidence intervals of probits for these percents of maximum response were used to determine the ninety-five percent confidence interval for EC_0, EC_{50} and EC_{99} by reflection onto the x-axis.

RESULTS

PMN Isolation:

Isolated PMN preparations were greater than or equal to 98±1% pure as determined by differential count. Viability was 99% as determined by trypan blue exclusion. Erythrocyte contamination was minimal.

PMN morphology in the isolates was comparable to normal PMN morphology in blood smears (Fig.4). Resting PMN were round and had segmented nuclei. Stimulated PMN were markedly different in morphology as evidenced by cellular polarization, ruffled membranes and enlarged, swollen nuclei, which had a high degree of heterogeneity (Fig.5).

PMN Aggregation:

Dose-response relationships for equine PMN aggregation following both A23187 and PMA stimulation are reported in Fig. 6 and Tables 2 and 3. Results are reported as the mean ± standard error of the mean (Mean ±SEM).

No change in percent transmittance occurred at the lowest concentration of A23187 tested (0.48μM). The mean maximum change in percent transmittance (41%±4.4) occurred at a concentration of 4.8μM. The lowest PMA concentration tested (0.002μM) did not stimulate a change in percent transmittance.

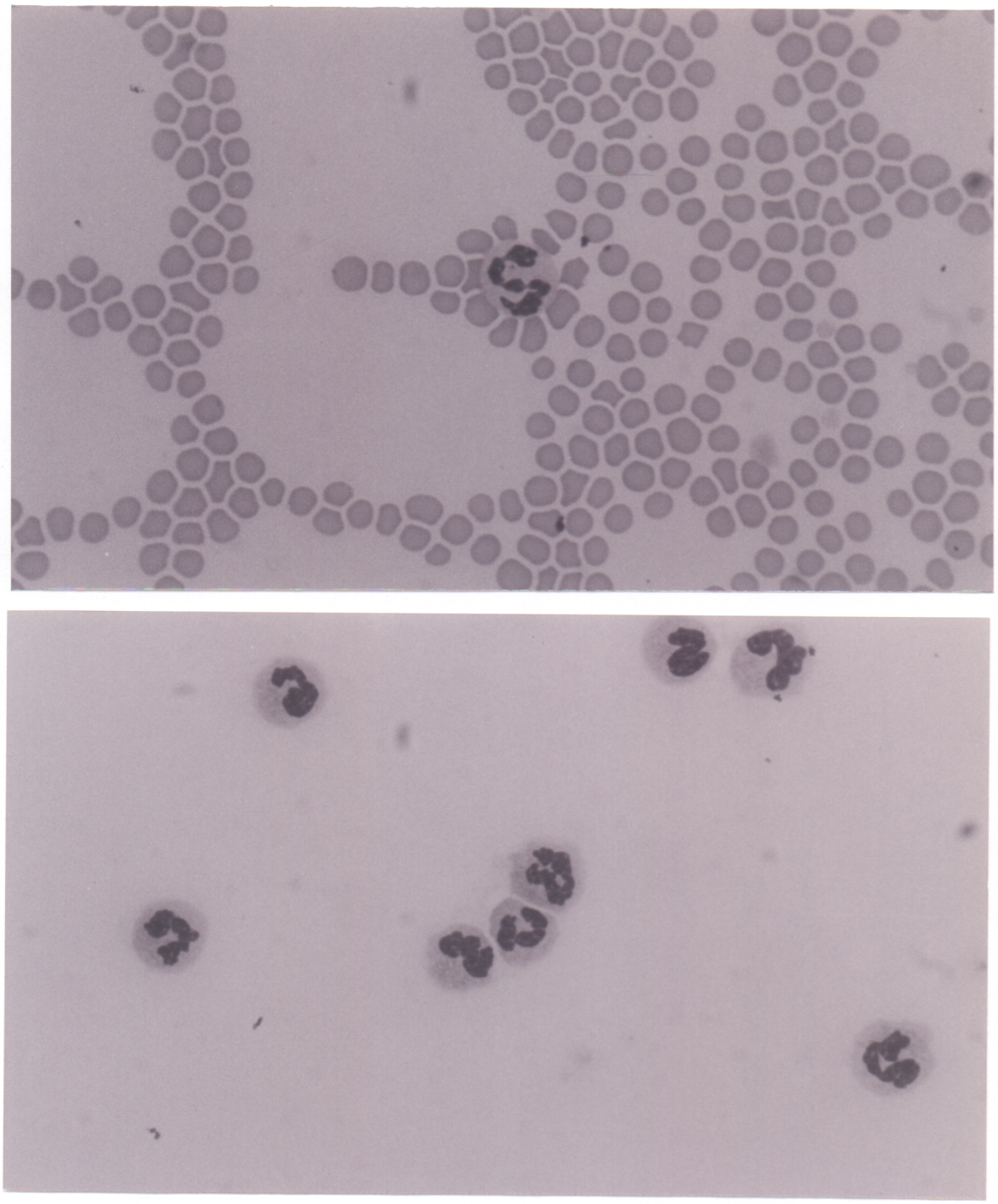

Fig. 4 A. (top) PMN in a whole blood smear. (x400) B. (bottom) PMN isolate. (x400)

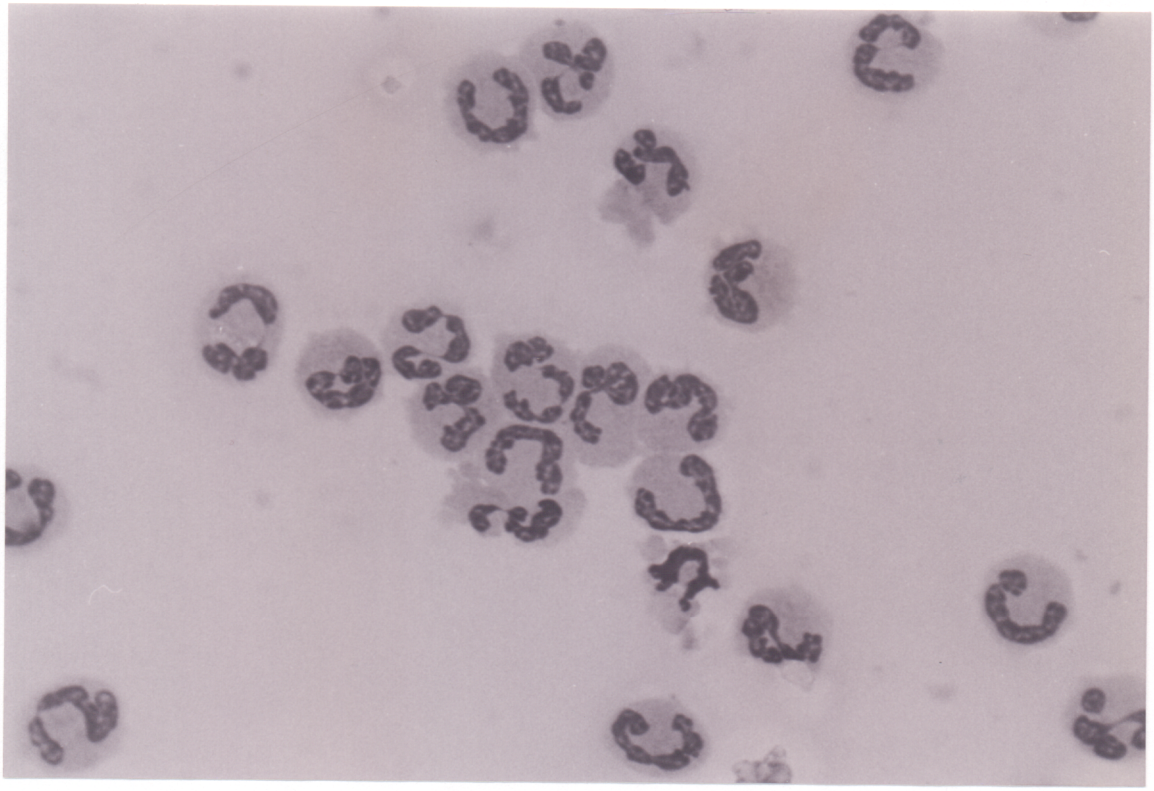

Fig. 5 PMN isolate exposed to 1.91μM A23187. (x400)

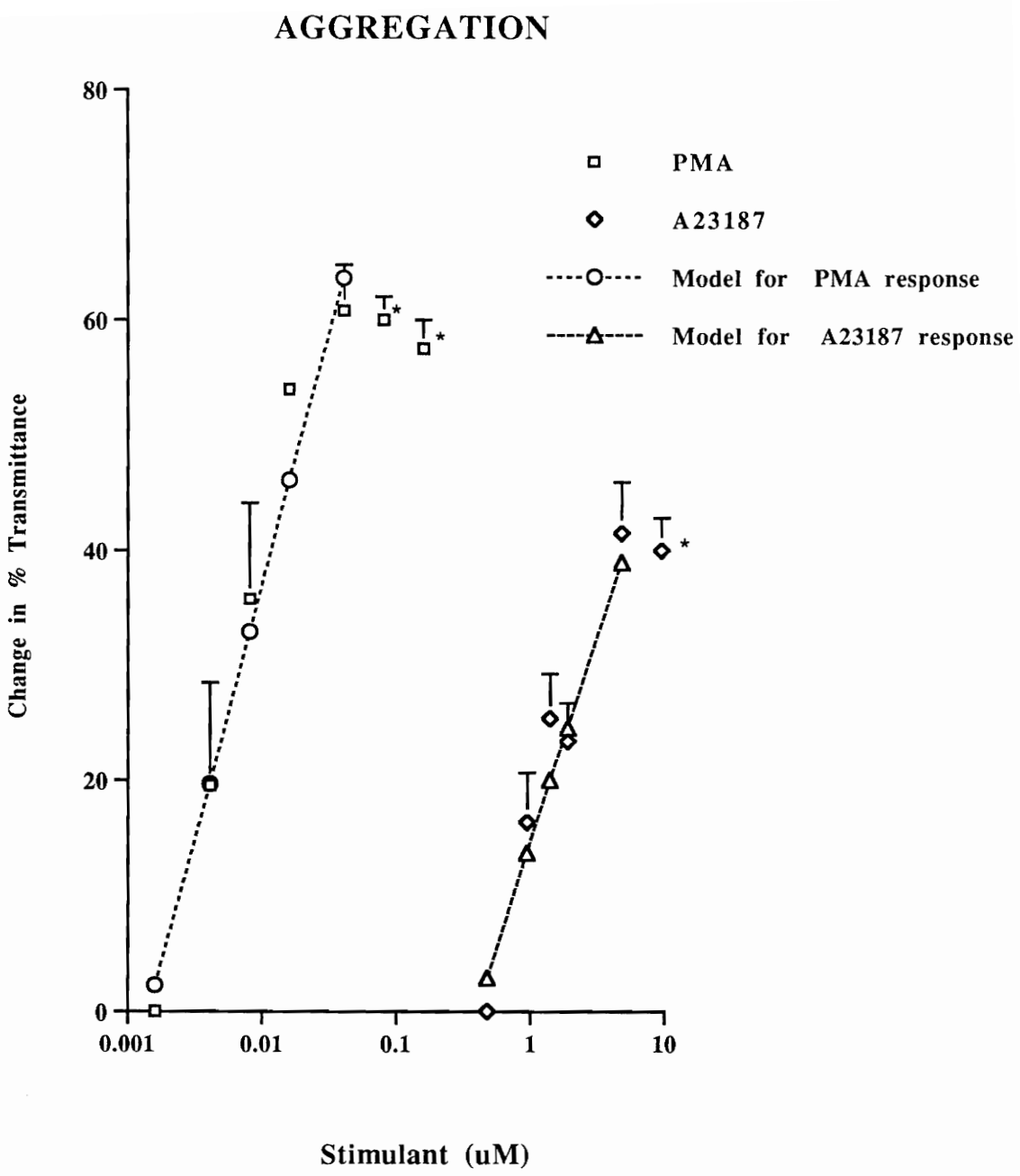

Fig. 6 Change in percent transmittance versus log concentration of A23187 and PMA. These dose-response curves are the average response of six horses and the vertical bars represent the standard errors of the mean.
* These points were not analyzed by linear regression analysis.

The mean maximum change in percent transmittance (61%±4.0) occurred at a concentration of 0.041μM. The mean maximum change in percent transmittance caused by PMA was significantly greater than the mean maximum change in percent transmittance caused by A23187 stimulation ($P<.01$) (Fig. 6 and Tables 2 and 3).

Only probit values for the percents of maximum response at 0.48μM to 4.8μM A23187 were used in the linear regression analysis to determine the best model for the data (Fig. 7). Only values for percents of maximum response at 0.002 to 0.041μM PMA were analyzed in the linear regression analysis (Fig. 8). Linear regression analysis of probit values of the percents of maximum response versus log concentration resulted in $R^2 = 0.95$ for A23187 (Table 4) and $R^2 = 0.97$ for PMA (Table 5) indicating linearity between the two variables. Plots of residuals versus log A23187 concentration and log PMA concentration indicated that the model of a linear relationship between the x and y variables is reasonable and that it is reasonable to assume that a normal distribution for percent of maximum response and, thus, change in percent transmittance, exists for each concentration.

Effective concentrations of A23187 for 0%(threshold), 50% and 99% of maximum response were 0.40, 1.49 and 5.46μM, respectively (Table 4). PMA effective concentrations for 0%(threshold), 50% and 99% of maximum response were 0.0014,

0.007 and 0.034μM, respectively (Table 5).

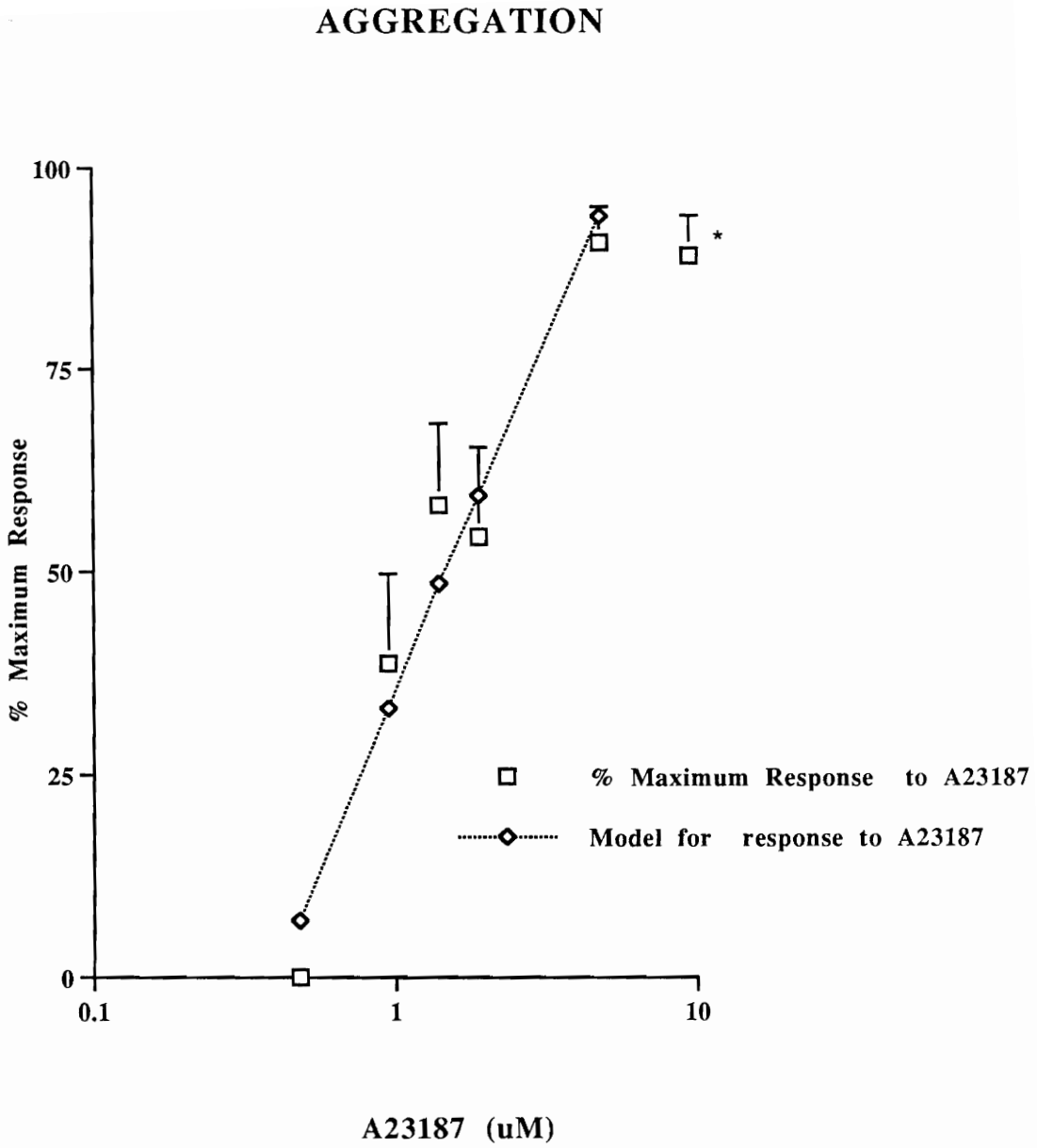

Fig. 7. Percent of maximum response versus log A23187 concentration for the aggregation assay. The graph is the average response of six horses and the vertical bars represent the standard errors of the mean.
* This point was not analyzed by linear regression analysis.

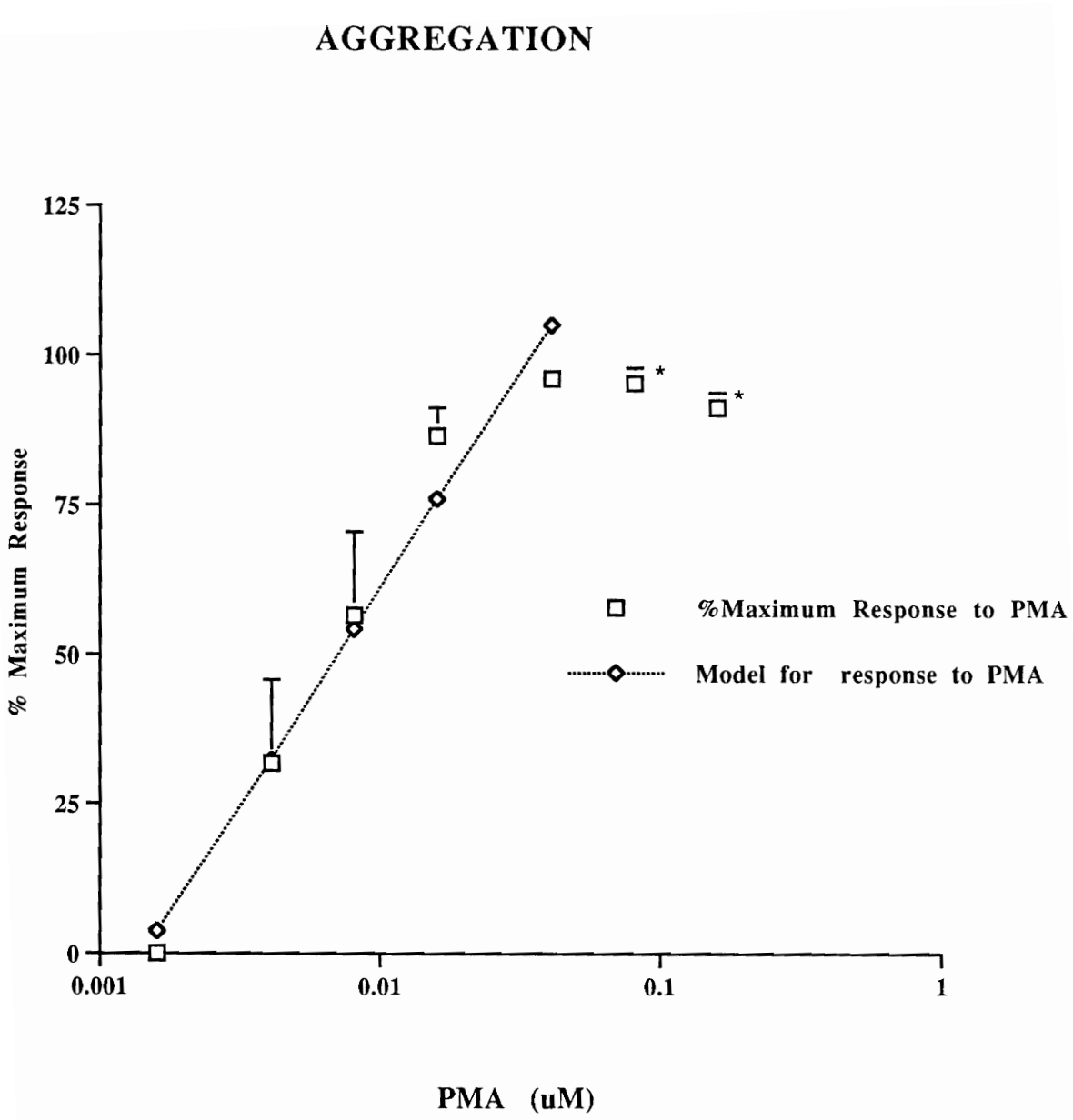

Fig. 8 Percent of maximum response versus log PMA concentration for the aggregation assay. The graph is the average response of six horses and the vertical bars represent the standard error of the mean.
* These points were not analyzed by linear regression analysis.

PMN Degranulation:

Dose-response curves were derived for both A23187 and PMA in the degranulation assay (Fig. 9 and Tables 2 and 3). Results are reported as the mean ± the standard error of the mean (Mean ±SEM). The lowest concentration of A23187 tested (0.48μM) activated only an average 3%(±1.8) degranulation. The mean maximum percent degranulation (56%±2.6) occurred at a concentration of 48μM which was the highest A23187 concentration tested. The lowest PMA concentration tested (0.004μM) caused an average 1.4%(±2.6) degranulation while the mean maximum percent degranulation (31%±4.6) occurred at 0.16μM PMA. Mean maximum percent degranulation for A23187 was significantly greater than the mean maximum percent degranulation caused by PMA ($P<.01$) (Fig. 9 and Tables 2 and 3).

Only probit values for percents of maximum response at 0.48μM to 7.2μM A23187 were included in the linear regression analysis (Fig. 10). Only probit values for percents of maximum response at 0.004μM to 0.16μM PMA were utilized for linear regression analysis of probit values versus log concentration of PMA (Fig. 11). Linear regression analysis of the values of the percents of maximum response versus log concentration of stimulant resulted in $R^2 = 0.94$ for A23187 (Table 4) and $R^2 = 0.93$ for PMA (Table 5). Assumptions for these models of percents of maximum response versus log

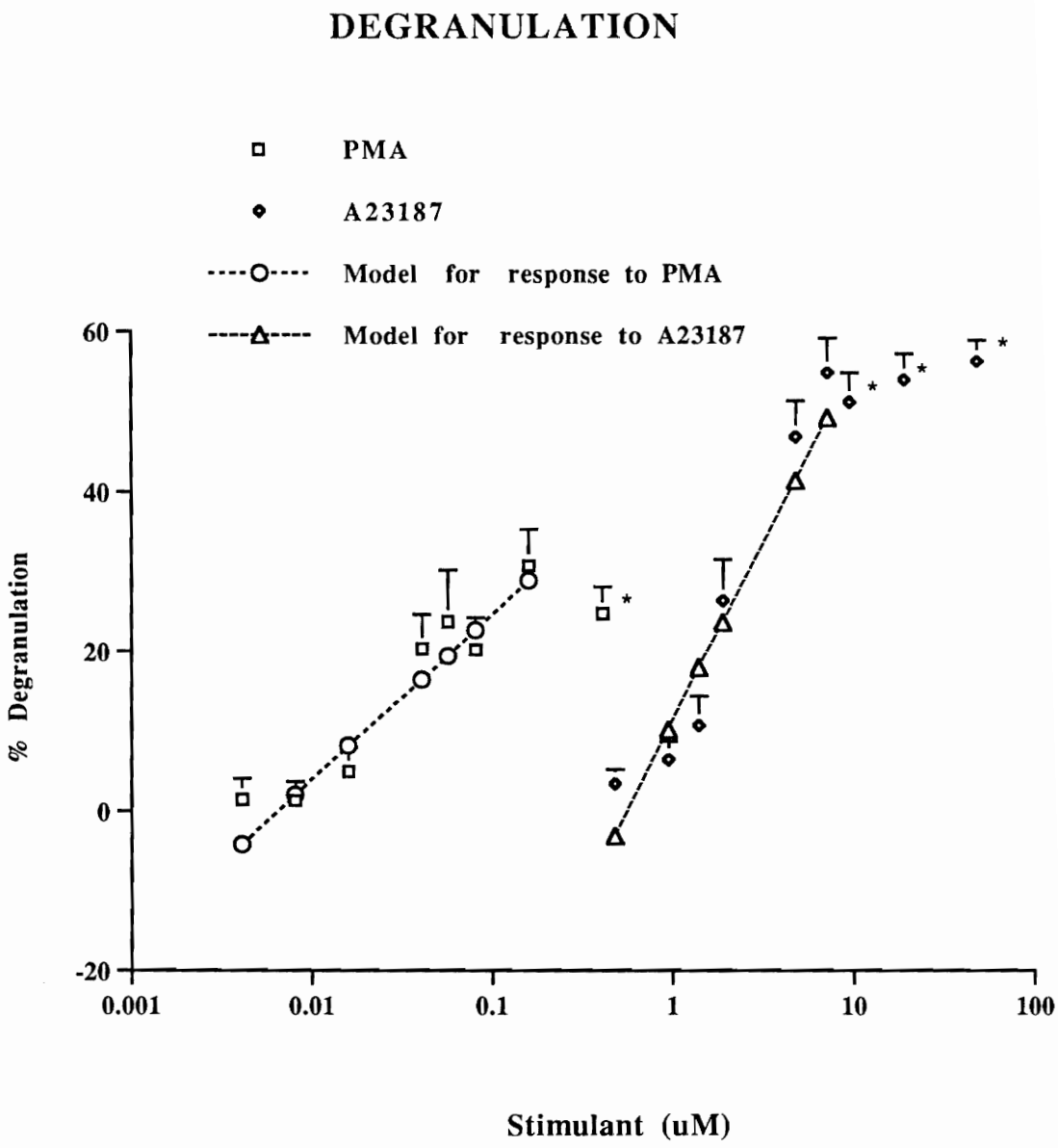

Fig. 9 Percent degranulation versus log concentration of A23187 and PMA. The graphs are the average response of six horses and the vertical bars represent the standard errors of the mean.

* These points were not analyzed by linear regression analysis.

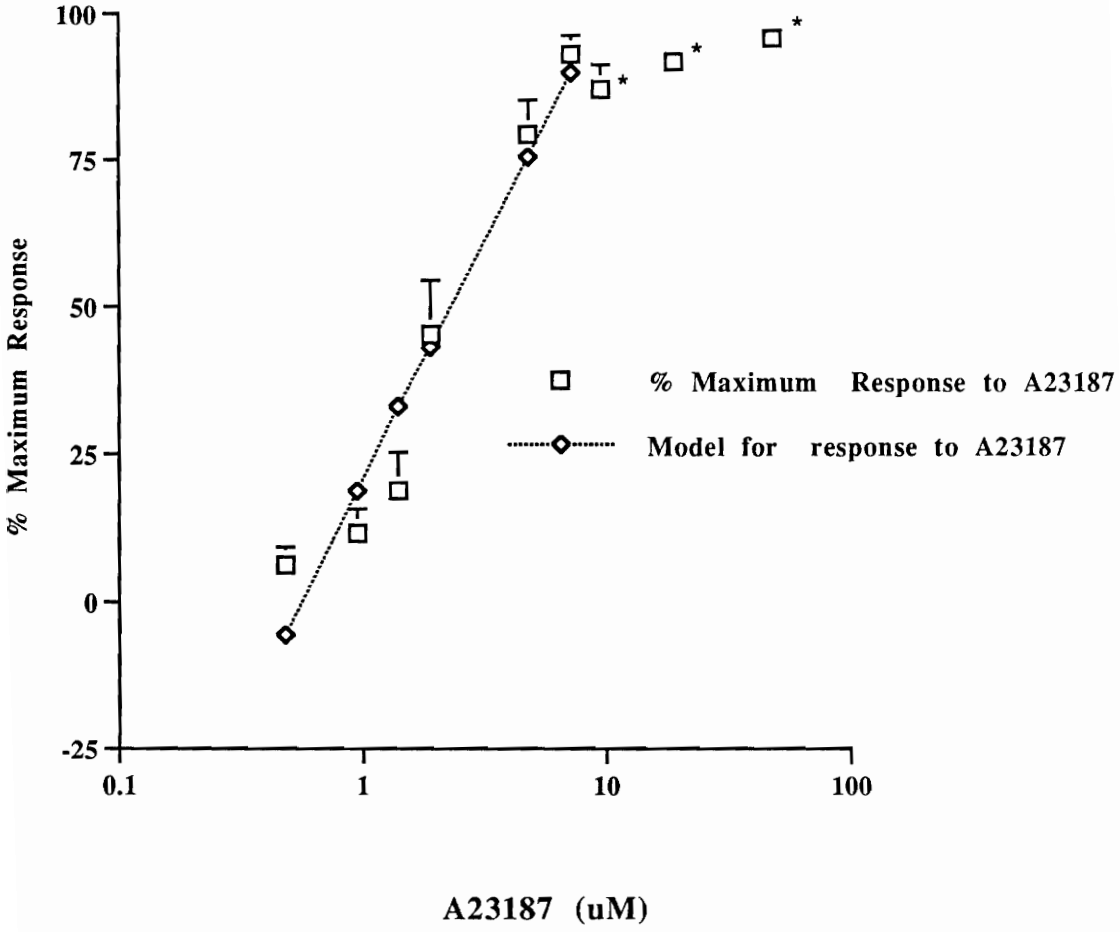

Fig. 10 Percent of maximum response versus log A23187 concentration. The graph represents the average response of six horses and the vertical bars represent the standard error of the mean.

* These points were not analyzed by linear regression analysis.

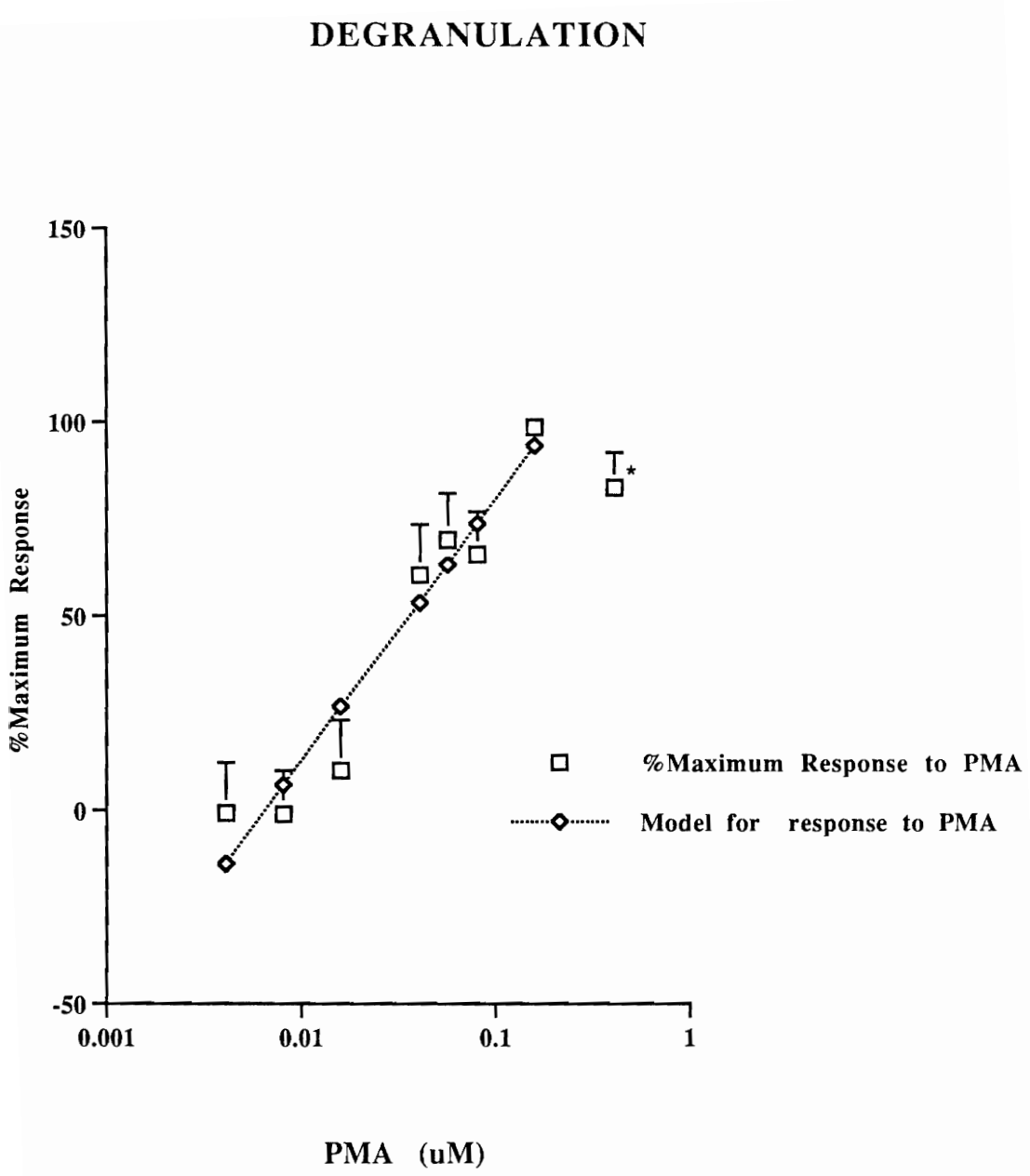

Fig. 11 Percent of maximum response versus log PMA concentration. The graph depicts the average response of six horses and the vertical bars represent the standard error of the mean.

* This point was not analyzed by linear regression analysis.

concentration of A23187 and PMA are reasonable as the plots of residuals versus log concentration indicated.

Percents of maximum response of 0%(threshold), 50% and 99% were induced by 0.56, 2.32 and 9.31μM, respectively, of A23187 (Table 4). PMA effective concentrations for 0%(threshold), 50% and 99% of maximum response were 0.006, 0.036 and 0.19μM, respectively (Table 5).

Superoxide anion production by PMN:

Superoxide anion production by equine PMN following both A23187 and PMA stimulation was dose-dependent (Fig. 12 and Tables 2 and 3). Results are reported as the mean ± the standard error of the mean (Mean ±SEM). The lowest A23187 concentration tested (0.18μM) induced production of a mean 0.9 (±0.6) nanomoles of superoxide anion per million PMN. Mean maximum production which was 5.2 (±0.75) nanomoles of superoxide anion per million PMN occurred at 0.93μM A23187. PMN superoxide anion production at the lowest PMA concentration tested (0.16nM) was 0.64 (±0.2) nanomoles per million PMN. The mean maximum production was 10 (±1.1) nanomoles per million PMN which was induced by 0.49μM PMA. The mean maximum superoxide anion production by PMA was significantly greater than that which was produced by A23187 stimulation ($P<.01$) (Fig. 12 and Tables 1 and 2).

Only probit values of percents of maximum response at

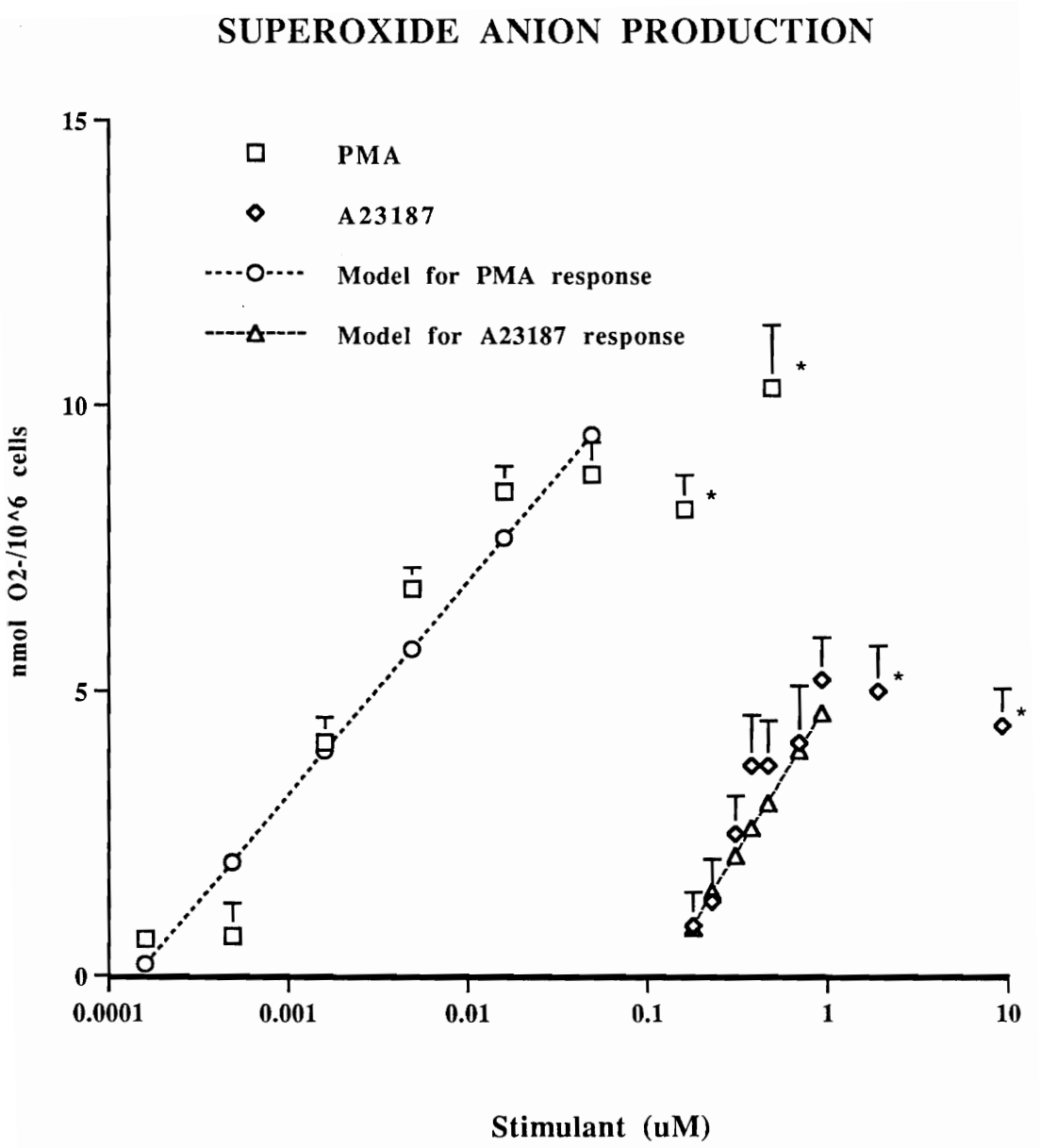

Fig. 12 Superoxide anion production (nmols O_2-/10^6cells) versus log concentration of A23187 and PMA. The graphs represent the mean response of six horses and the vertical bars depict the standard errors of the means.
* These points were not analyzed by linear regression analysis.

0.18μM to 0.93μM A23187 were used in the linear regression analysis (Fig. 13). Only probit values of percents of maximum response at 0.00016μM to 0.049μM PMA were analyzed by linear regression analysis (Fig. 14). Linear regression analysis of probits of percents of maximum response versus log concentration A23187 resulted in R^2 = 0.94 (Table 4). The same analysis of probits of percents of maximum response versus log concentration PMA resulted in R^2 = 0.94 (Table 5). Plots of residuals versus log concentration A23187 and log concentration PMA indicated that linearity and normal distribution were reasonable assumptions.

In this assay percents of maximum response of 0%(threshold), 50% and 99% were induced by 0.12, 0.39 and 1.2μM A23187, respectively (Table 4). Effective concentrations of PMA for 0%(threshold), 50% and 99% of maximum response were 0.0001, 0.003 and 0.076μM, respectively (Table 5).

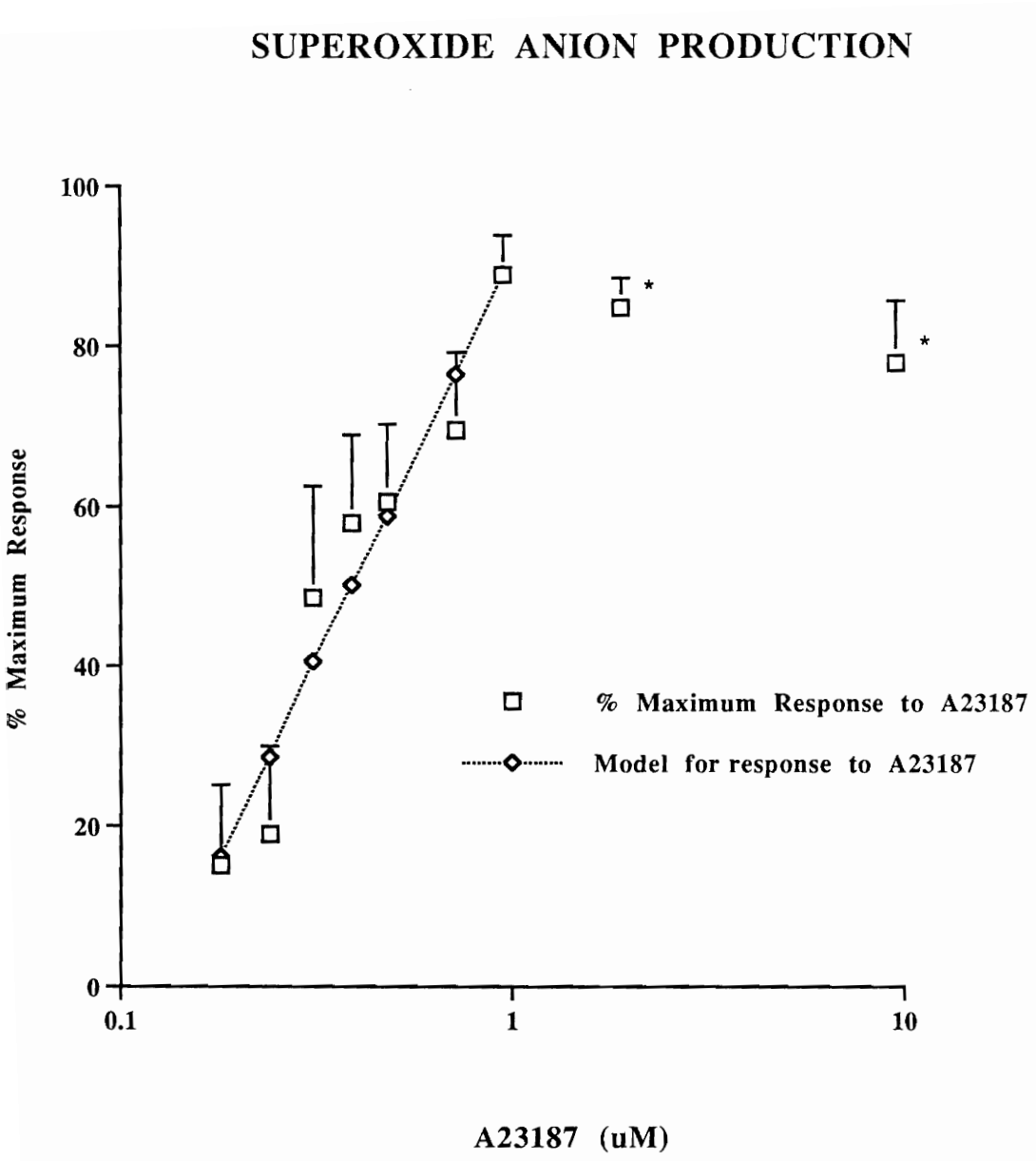

Fig. 13 Percent of maximum response versus log A23187 concentration for superoxide anion production. The graph demonstrates the average response of six horses and the vertical bars represent the standard errors of the means. * These points were not analyzed by linear regression analysis.

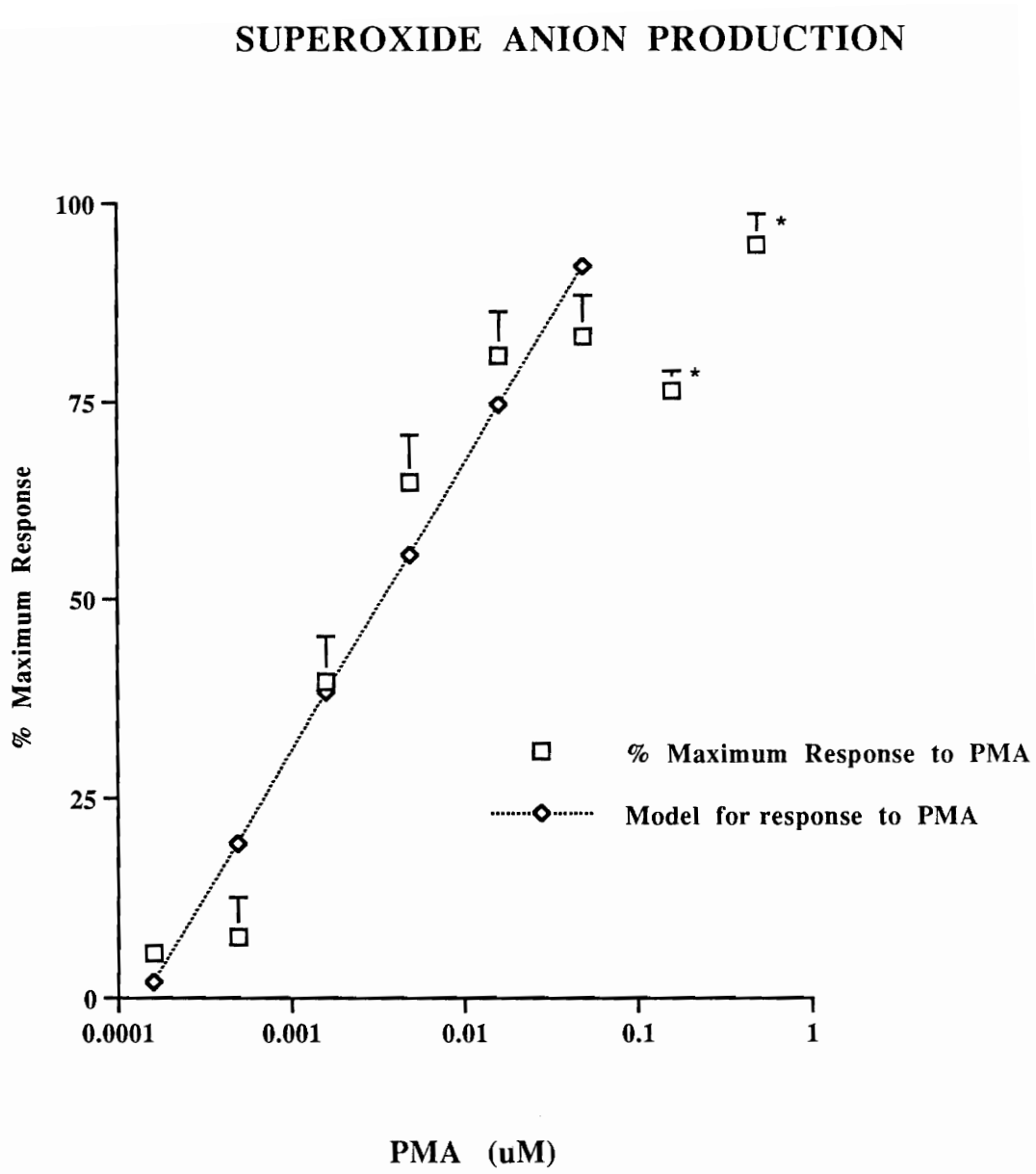

Fig. 14 Percent of maximum response versus log PMA concentration for superoxide anion production. The graph depicts the average response of six horses and the vertical bars represent the standard errors of the means.
* These points were not analyzed by linear regression analysis.

TABLE 2.

CALCIUM IONOPHORE STIMULATION IN THREE ASSAYS

AGGREGATION

Change in %Transmittance

CAl conc (uM)	Horse #0729	Horse #0731	Horse #0804	Horse #0805	Horse #0806	Horse #0807	Mean	SEM
0	0	0	0	0	0	0	0.00	0.00
0.48	0	0	0	0	0	0	0.00	0.00
0.95	9.25	0	24	16	20	29	16.38	4.28
1.43	18	10.3	36	29	26	33	25.38	3.94
1.91	12.5	20	26	23	21	38	23.42	3.45
4.8	54	48	51	35	31	30	41.50	4.37
9.5	50	41	34	46	35	34	40.00	2.79

DEGRANULATION

% Degranulation

Cal conc. (uM)	Horse #0716	Horse #0717	Horse #0721	Horse #0722	Horse #0723	Horse #0724	Mean	SEM
0	0	0	0	0	0	0	0	0
0.48	-4.03	3.39	5.40	9.38	3.10	3.10	3.39	1.78
0.95	-2.05	3.62	9.82	9.84	13.35	3.79	6.39	2.29
1.43	-0.40	3.38	9.91	23.01	8.38	19.99	10.71	3.74
1.91	21.35	16.61	18.00	41.66	16.81	43.49	26.32	5.19
4.8	63.02	32.62	38.84	54.59	43.94	46.98	46.66	4.46
7.2	71.43	50.25	63.45	50.41	43.60	49.15	54.72	4.28
9.5	65.78	46.75	45.96	54.44	39.96	53.12	51.00	3.65
19	69.18	49.74	54.20	51.35	45.71	52.61	53.80	3.30
48	65.76	46.66	56.79	55.25	52.31	59.57	56.06	2.65

SUPEROXIDE ANION PRODUCTION

nmol O2-/10^6 cells

Cal conc. (uM)	Horse #0602	Horse #0609	Horse #0610	Horse #0611	Horse #0614	Horse #0616	Mean	SEM
0.18	0.18	-0.31	3.48	0.06	0.24	1.65	0.88	0.59
0.23	1.76	1.22	2.18	-1.29	-0.06	4.08	1.32	0.76
0.31	3.70	0.78	2.99	0.60	2.37	4.90	2.56	0.68
0.38	4.19	3.08	4.70	2.85	0.36	6.87	3.67	0.89
0.47	5.62	3.10	5.23	2.23	0.81	5.03	3.67	0.79
0.7	5.69	3.93	3.93	1.72	1.55	8.02	4.14	1.00
0.93	7.04	5.41	5.58	4.74	1.80	6.46	5.17	0.75
1.87	6.36	3.90	5.20	5.34	1.77	7.36	4.99	0.80
9.35	5.43	5.01	4.55	6.21	1.64	3.71	4.42	0.65

TABLE 3.

PHORBOL MYRISTATE ACETATE STIMULATION IN THREE ASSAYS

AGGREGATION

Change in % Transmittance

PMA con (uM)	Horse #0624	Horse #0729	Horse #2804	Horse #2805	Horse #2806	Horse #2807	Mean	SEM
0	0	0	0	0	0	0	0.00	0.00
0.0016	0	0	0	0	0	0	0.00	0.00
0.0041	0	44	31.5	0	0	42	19.58	8.93
0.0081	23	50	46	0	46	50	35.83	8.28
0.016	52	57	55	51	55	54	54.00	0.89
0.041	79	54	58	52	58	64	60.83	4.00
0.081	67	62	57	53	58	63	60.00	2.03
0.16	66	56.5	52.5	51	64	55	57.50	2.51

DEGRANULATION

% Degranulation

PMA con (uM)	Horse #0623	Horse #0625	Horse #0630	Horse #0701	Horse #0707	Horse #0702	Mean	SEM
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.0041	-1.87	-7.43	-0.03	3.44	2.61	11.69	1.40	2.60
0.0081	-3.93	-6.97	3.16	2.35	5.04	8.50	1.36	2.35
0.016	-0.82	-6.77	6.80	12.28	5.80	12.25	4.92	3.07
0.041	20.58	0.52	18.90	31.56	24.87	25.09	20.25	4.34
0.057	17.81	4.69	11.08	34.09	48.09	26.48	23.71	6.50
0.081	23.40	8.89	7.57	32.71	23.08	25.64	20.21	4.05
0.16	31.29	13.81	29.30	35.23	48.18	26.25	30.68	4.59
0.41	31.30	11.82	20.81	34.35	21.10	28.67	24.67	3.40

SUPEROXIDE ANION PRODUCTION

nmol O2-/10^6 cells

PMA con (uM)	Horse #0409	Horse #0412	Horse #0413	Horse #0415	Horse #0421	Horse #0414	Mean	SEM
0.00016	1.25	0.46	0.80	0.98	0.04	0.30	0.64	0.18
0.00049	0.92	1.78	-2.00	1.49	1.43	0.58	0.70	0.57
0.0016	3.15	5.53	2.57	4.25	4.35	4.82	4.11	0.44
0.0049	7.65	7.79	5.55	6.48	6.07	7.21	6.79	0.37
0.016	6.96	8.97	9.12	8.89	7.54	9.80	8.55	0.44
0.049	9.51	8.60	9.65	8.42	6.51	10.39	8.85	0.55
0.16	8.26	8.40	8.78	9.66	5.43	8.85	8.23	0.59
0.49	10.12	11.21	12.90	12.54	5.73	9.58	10.35	1.06

TABLE 4.
Linear Regression Analysis of Probits versus Log Concentration of Calcium Ionophore A23187*
(n=6)

	R^2	(μM) EC_0	EC_{50}	EC_{99}
Superoxide anion production	0.94	0.12 (0.08-0.19)	0.39 (0.25-0.60)	1.20 (0.78-1.85)
Aggregation	0.95	0.40 (0.22-0.70)	1.49 (0.84-2.65)	5.46 (3.08-9.69)
Degranulation	0.94	0.56 (0.26-1.19)	2.32 (1.09-4.92)	9.31 (4.39-19.76)

* Confidence intervals (95%) for effective concentrations for threshold, 50% of maximum response and 99% of maximum response (EC_0, EC_{50}, EC_{99}) are reported in parentheses.

TABLE 5.
Linear Regression Analysis of Probits versus Log Concentration of Phorbol Myristate Acetate*
(n=6)

	R^2	EC_0	(μM) EC_{50}	EC_{99}
Superoxide anion production	0.94	0.0001 (0.00003-0.0007)	0.003 (0.0007-0.016)	0.076 (0.02-0.35)
Aggregation	0.97	0.0014 (0.0007-0.0028)	0.007 (0.004-0.014)	0.034 (0.017-0.067)
Degranulation	0.93	0.006 (0.002-0.018)	0.036 (0.013-0.100)	0.19 (0.07-0.54)

* Confidence intervals (95%) for effective concentrations for threshold, 50% of maximum response and 99% of maximum response (EC_0, EC_{50}, EC_{99}) are reported in parentheses.

DISCUSSION

Responses of equine neutrophils were evaluated following stimulation with calcium ionophore A23187 and phorbol myristate acetate. The purpose of this study was to characterize and compare the responses initiated by these two stimulants in assays of aggregation, degranulation and superoxide anion production. Acting on different components of signaling, A23187 and PMA bypass the surface receptor-ligand binding step in initiating PMN activation. Calcium ionophore A23187 causes increases in intracellular calcium and PMA activates protein kinase C. Other investigations have shown that these two mechanisms of cellular activation act synergistically when PMN are stimulated by ligands which bind specific receptors (Badwey, et al.,1986)(Korchak,1989)(Omann, et al.,1987). If suboptimal concentrations of an activator of PKC and of calcium ionophore A23187 are added to cells simultaneously, a synergistic stimulation of superoxide anion release occurs (Badwey, et al.,1986). Increases in intracellular calcium that follow stimulation of PMN with ligands may play a role in initiating superoxide anion production and other cellular functions by increasing the sensitivity of cellular PKC to diacylglycerol (Badwey, et. al.,1986). Results of our study suggested that the relative importance of calcium mobilization and protein kinase C

activation varies according to the end response.

Results of the aggregation assay indicated that PKC activation causes a greater aggregatory response than increases in intracellular calcium does. We consider two explanations for the difference in maximum aggregatory response. First, a wide range of PKC isoenzymes are affected and activated by PMA (Pontremoli, S., et al.,1990) whereas A23187 is thought to facilitate activity of calcium-dependent PKC isoenzymes (reviewed in McPhail, et al.,1993). Second, A23187 forms a channel in the PMN membrane to allow calcium ion flow into the cell. It may be that, at the higher concentrations of A23187, the channels occupy space that would otherwise be available for expression of adhesion molecules, and less aggregation is possible.

In the PMN degranulation assay calcium ionophore A23187 stimulates a greater response because it initiates two events in cellular activation and PMA may only initiate one event. Calcium ionophore A23187 stimulated PMN degranulation by increasing the amount of intracellular calcium and enhancing PKC activation. The fact that PMA elicits degranulation supports the hypothesis that phosphorylation by PKC of key enzymes or molecules, such as actin-myosin complexes, is necessary for degranulation. Alternatively, the channels formed by A23187 in PMN plasma membranes constitute activated sites in the membrane. Granules may fuse with the activated

sites and release their contents. As more channels are formed, more granules fuse with the plasma membrane and release their contents. Finally, high concentrations of PMA may secondarily reduce intracellular calcium concentrations. Phorbol esters activate calcium ion transporters, which are ATP-dependent and located in the plasma membrane and in a non-mitochondrial intracellular membrane pool (Rickard, et al.,1985). During the changes in ionic balance which occur with PMN activation, PMA may act to decrease intracellular calcium to pre-stimulation levels to help restore homeostatic concentrations of the cation. This results in less calcium being available for the calcium-dependent kinases in their phosphorylation of microfilament components; therefore, less degranulation would be possible with PMA activation.

In the superoxide anion production assay PKC activation caused a greater response than increasing intracellular calcium did. Two possible explanations follow that may clarify why PMA can stimulate the production of a greater quantity of superoxide anion than A23187 can. As stated previously, PMA may activate multiple isoenzymes of PKC (Pontremoli, S., et al.,1990) which simultaneously may trigger different components of the assembly and activation of NADPH oxidase, thus enhancing the stimulation of the enzyme complex. Second, A23187 may cause too much membrane discontinuity so that not as many NADPH oxidase systems can be assembled in the

membrane and, therefore not as much superoxide anion is produced.

We also compared our calculated values between the assays but within a stimulant. These analyses indicate that superoxide anion production is initiated at a lower concentration of stimulant than aggregation and degranulation (Table 4 and 5 and Fig. 15 and 16). The theory of PMN activation in inflammatory reactions is that as PMN are exposed to a low concentration of stimulus in the circulation at the endothelial cells closest to the injury, expression of adhesion molecules on the PMN surface is increased first to facilitate PMN localization in the area where they are needed. However, there are several possible explanations for why the threshold concentration for superoxide anion production in this study is lower than threshold concentration for aggregation: 1) PMN leak superoxide anion in their resting state and only small increases in stimulant concentration will augment the production of the anion; 2) PMN may begin assembly and activation of the NADPH oxidase complex to produce superoxide anion at the earliest point in the inflammatory process; 3) the responses to natural ligands are mediated by interactions at the G-protein level. This second level of regulation of concentration versus response is bypassed when second messengers are activated directly; and 4) the true threshold for aggregation may be below the sensitivity of our

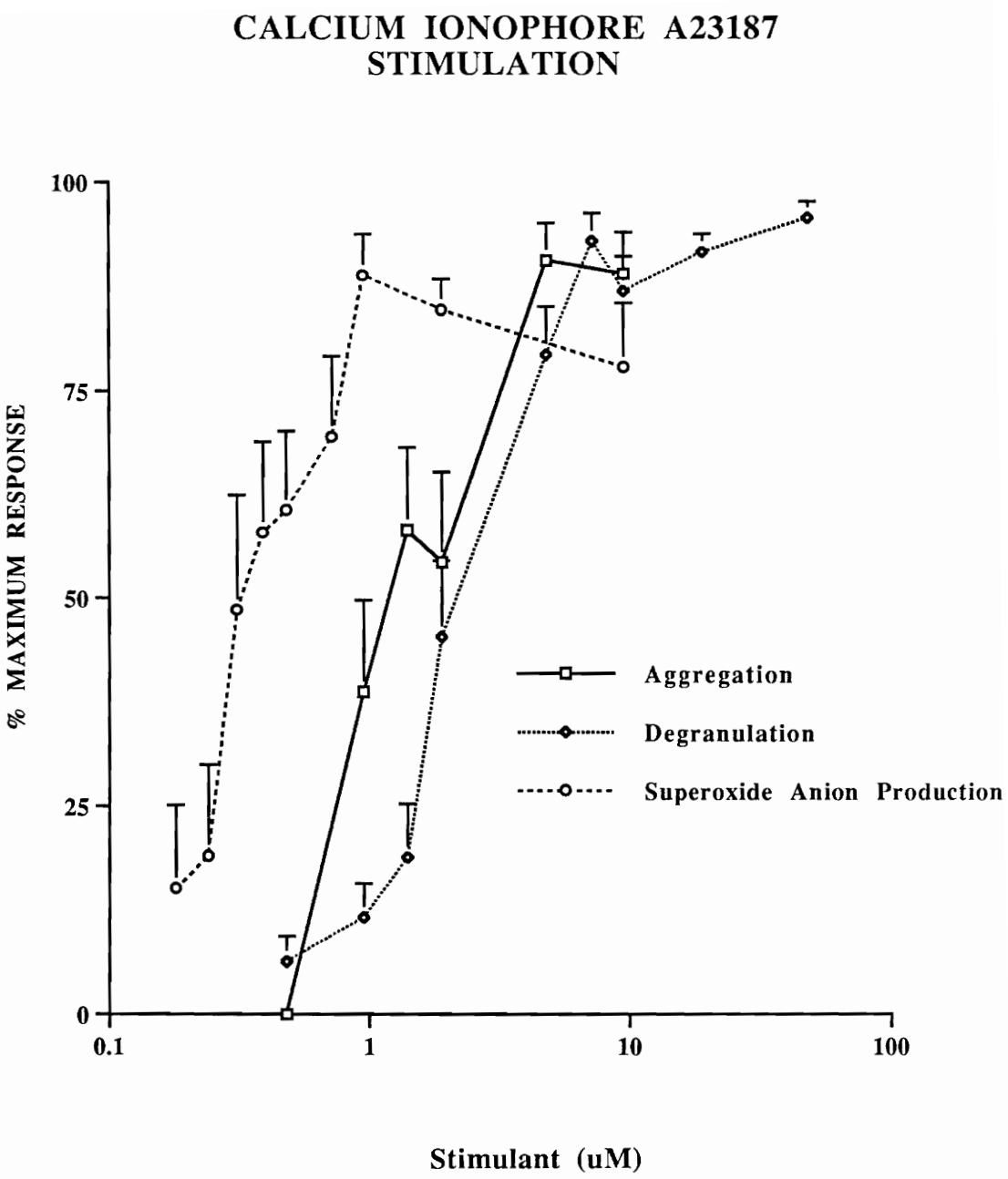

Fig. 15 Percent of maximum response versus log calcium ionophore A23187 concentration.
Vertical bars represent the standard errors of the means.

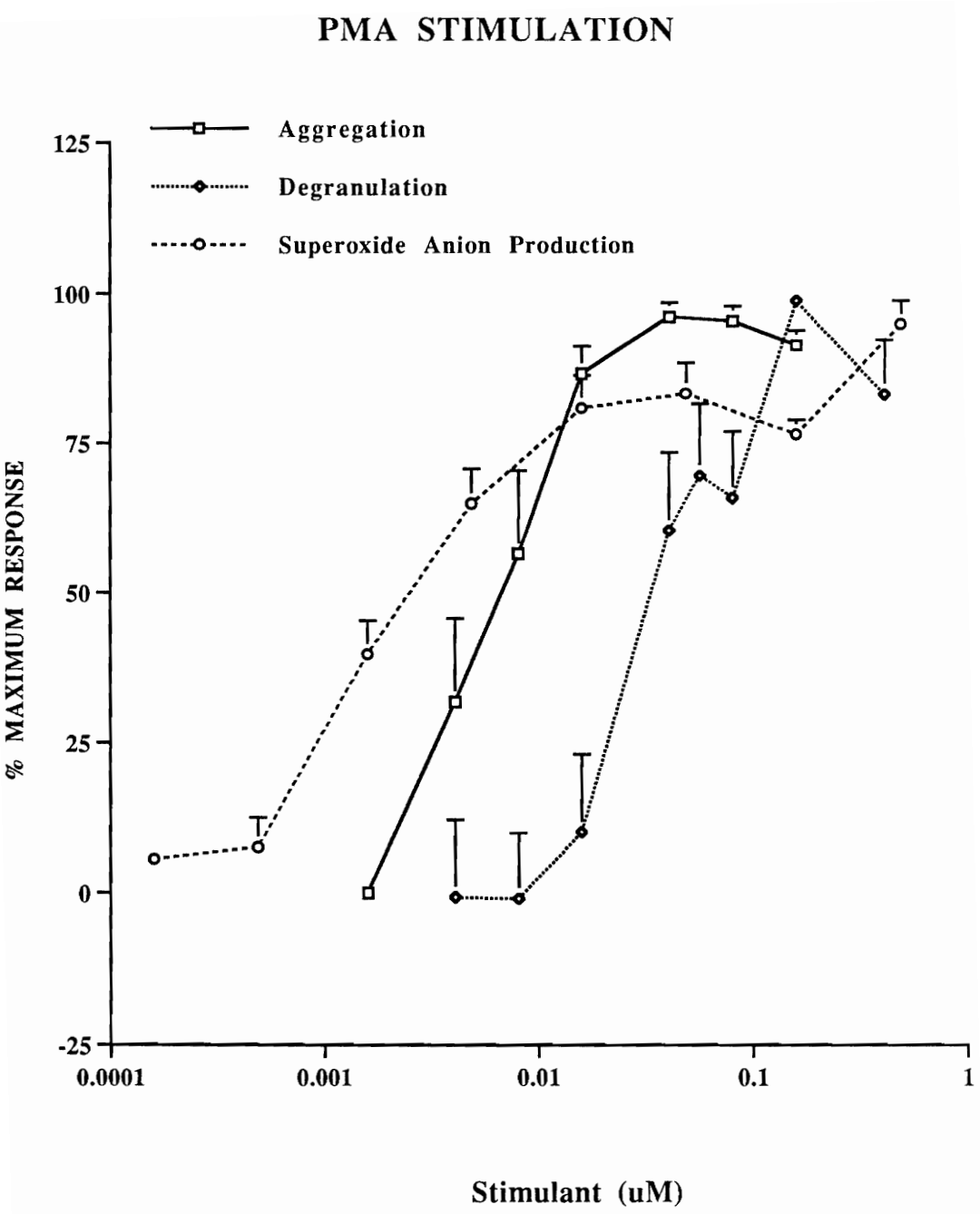

Fig. 16 Percent of maximum response versus log phorbol myristate acetate concentration.
Vertical bars represent the standard errors of the means.

assay system.

Following PMA stimulation, the effective concentration for maximum response in the aggregation assay was less than the same value for superoxide anion production. Figure 16 demonstrates that the slope of the dose-response curve for aggregation is steeper than the slope for superoxide anion production. This change in slope implies that the mechanistic relationship between PKC activation and aggregation is different from that for superoxide anion production.

Threshold concentrations for degranulation were significantly higher than threshold concentrations for superoxide anion production. This may be a reflection of the specificity of our assay system for azurophilic granules. Degranulation of azurophilic granules occurs in inflamed tissues in the horse where PMN are phagocytizing bacteria and other foreign materials which have stimulated production of high concentrations of inflammatory mediators. Assembly and activation of the NADPH oxidase systems and superoxide anion production require degranulation of specific granules which occurs at much lower concentrations than release of azurophilic granules (Wright, D.G., et al.,1977). The threshold for specific granule exocytosis may equal the threshold for superoxide anion production. In the present study, the assay system measured degranulation of azurophilic granules, not specific granules, and the threshold was

significantly greater than the threshold concentration for superoxide anion production.

The objective of this thesis was to investigate functional responses of equine neutrophils to varying concentrations of known second messenger activators, PMA and A23187. Specific knowledge of the dose-response relationships for these substances will facilitate future investigations of other stimulants and inhibitory substances. These investigations can now be more readily based on subthreshold, threshold, half- or maximal activation of second messenger systems. This information should also prove valuable in detecting and characterizing equine neutrophil dysfunctions. Although undetected in the equine population to date, conditions similar to bovine leukocyte adhesion molecule deficiency, human chronic granulomatous disease (NADPH oxidase complex deficiency/defect), and various granule deficiencies likely exist. They may have gone unreported to date for lack of specific information about normal equine neutrophil function.

CONCLUSION

Dose-response curves for calcium ionophore A23187 and phorbol myristate acetate were derived for three equine neutrophil functions ex vivo. Equine neutrophil aggregation and superoxide anion production are more responsive to protein kinase C activation by PMA and degranulation is more responsive to increases in intracellular calcium initiated by A23187. The results may be used in further research to evaluate the effects of other inflammatory mediators on PMN function and responses; studying antiinflammatory drugs and mechanisms; and characterizing possible defects in equine neutrophil function.

APPENDIX

Con A and FMLP were tested in the degranulation assay. Con A (0.1-30µg/ml) and FMLP (0.006-5µM) did not cause degranulation in these conditions. Con A stimulates degranulation of specific granules (Hoffstein,S.,et al.,1976) but not degranulation of azurophilic granules which were important in this study. FMLP (0.001-1µM) caused degranulation of specific granules from equine PMN (Snyderman,R.,et al.,1980) by binding receptors on the cell membrane. Higher concentrations of these stimulants may be necessary for azurophilic degranulation and should be tested in this system.

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