DEPENDENCE OF SUPEROXIDE ANION PRODUCTION ON EXTRACELLULAR AND INTRACELLULAR CALCIUM AND PROTEIN KINASE C IN BOVINE NEUTROPHILS

by

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ABSTRACT

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Dependence of Superoxide Anion Production on Extracellular and Intracellular Calcium and Protein Kinase C in Bovine Neutrophils

Calcium (Ca^{2+}) and protein kinase C (PKC) are believed to act as intracellular signals triggering the activation of NADPH oxidase in neutrophils leading to superoxide (O_2^-) generation. This was tested on bovine neutrophils by chelating extracellular and/or intracellular free Ca^{2+} and by measuring PKC activity when the cells were stimulated by phorbol myristate acetate (PMA) or opsonized zymosan (OZ). Chelation of extracellular Ca^{2+} with EGTA did not alter O_2^- production from PMA stimulated cells. However, it did cause a 64% decrease in O_2^- production in the neutrophils when stimulated with OZ. When intracellular Ca^{2+} was chelated with BAPTA/AM, there was a significant decrease in O_2^- generation following PMA activation. Yet, OZ activated cells, pre-treated with BAPTA/AM, showed an increase in the respiratory burst

proportional to the chelator's concentration. Moreover, although OZ was previously shown to increase O_2^- generation by neutrophils, no significant changes in PKC activity were observed. PMA stimulation led to an increase in PKC activity at the membrane level. Furthermore, treating the cells with calphostin C, a PKC activity inhibitor, caused a 69% decrease in O_2^- production demonstrating the involvement of PKC in PMA-stimulated cells. However, no differences were observed between the OZ activated cells incubated with the inhibitor and the control cells. These data provide evidence that activation of NADPH oxidase can be achieved by either a PKC-dependent or a PKC-independent pathway depending on the stimulatory agent.

RÉSUMÉ

Maîtrise en Science Sciences Animales

Brenda Allard

La Dépendance du Calcium et de la Protéine Kinase C sur la Génération de Superoxyde chez les Neutrophiles Bovins

Il est de toute croyance que le calcium (Ca^{2+}) et la protéine kinase C (PKC) sont des signaux intracellulaires conduisant au déclenchement de l'activation de la NADPH oxydase chez les neutrophiles, permettant la génération de superoxyde (O_2^-). Ceci a été vérifié sur les neutrophiles bovins en chélatant le Ca^{2+} extracellulaire et/ou intracellulaire et en déterminant l'activité de la PKC suite à l'activation des cellules à l'aide de l'acétate et myristate de phorbol (PMA) ou de zymosans opsonisés (ZO). La chélation du Ca^{2+} extracellulaire n'a causé aucune modification sur la production de O_2^- des cellules stimulées à la PMA. Cependant, elle a causé une diminution de 64% sur la génération de O_2^- chez les neutrophiles activés par les ZO. Lorsque le Ca^{2+} intracellulaire était chélaté à l'aide de BAPTA/AM, une diminution significative de la génération de O_2^- pouvait être

observée suite à leur stimulation par la PMA. Cependant, les cellules traitées à l'aide de BAPTA/AM et activées par les ZO ont augmenté la production de O_2^- de façon proportionnelle à la concentration du chélateur. Bien que les ZO aient augmenté la production de O_2^- des neutrophiles, aucun changement significatif de l'activité de la PKC n'a été observé. Les cellules stimulées à l'aide de la PMA ont démontré un accroissement de l'activité de la PKC au niveau de la membrane. De plus, traiter les cellules au calphostin C, un inhibiteur de la PKC, a causé une diminution de la production de O_2^- demontrant l'importance de la PKC. Cependant, aucune différence n'a été observée au niveau de la production de O_2^- entre les neutrophiles témoins et les neutrophiles traités au calphostin C lorsqu'ils étaient stimulés par les ZO. De plus, nos données fournissent l'évidence que l'activation de la NADPH oxydase peut être induite par un signal intracellulaire, soit dépendant ou indépendant de la PKC, selon l'agent stimulateur.

LIST OF ABBREVIATIONS

AA.....arachidonic acid
BAPTA/AM.....1,2-bis(o-aminophenoxy) ethane - N, N, N', N' - tetraacetic acid acetoxymethyl ester
Con-A.....concovalin A
Ca^{2+}.....free calcium
$[Ca^{2+}]_i$.....intracellular free calcium
$[Ca^{2+}]_o$.....extracellular free calcium
DBHQ.....2,5-di-tert-butylhydroquinone
DG.....diacylglycerol
DMSO..... dimethyl sulfoxide
DPBS..... Dulbecco's phosphate-buffered saline
DTT..... dithiothreitol
EDTA..... (ethylenedinitrilo) tetraacetic acid
EGTA.....ethylene glycol bis (ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ER.....endoplasmic reticulum
FAD.....flavin adenine dinucleotide
fMLP.....formyl-methionyl-leucyl-phenylalanine
HBSS.....Hank's balanced salt solution
H_2O_2.....oxygen peroxide
IP_3..... inositol 1,4,5-triphosphate
LTA..... lipoteichoic acid
O_2.....oxygen
O_2^-.....superoxide anion
OZ.....opsonized zymosan
PA.....phosphatidic acid
PAF.....platelet-activating factor
PIP_2.....phosphatidylinositol-4-5-bisphosphate
PKC.....protein kinase C
PLA_2.....phospholipase A_2
PMA.....phorbol myristate acetate
PMN.....polymorphonuclear neutrophils
SEM.....standard error of the mean
SOD.....superoxide dismutase
TG.....thapsigargin
ZO.....zymosan opsonisé

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TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

CHAPTER I. GENERAL INTRODUCTION

The cells and molecules constituting the immune system are essential for the body's resistance or elimination of abnormal cells and potentially harmful foreign substances. Among the immune cells, known as leukocytes, the polymorphonuclear neutrophils (PMNs) play an important role since they constitute the first line of defense within the body for mammalian organisms.

One of the critical functions involved in the killing ability of neutrophils is their generation of superoxide anions (O_2^-) and other related toxic oxygen metabolites. This phenomenon, known as the respiratory burst, results from assembly and activation of the enzyme NADPH oxidase which catalyses the conversion of oxygen (O_2) to O_2^-. The oxidase can be activated by both particulate and soluble stimulating agents. Yet, the mode of activation of the enzyme, the intracellular messengers, and the mechanisms leading to cellular responses remain unclear. Furthermore, the

respiratory burst has been extensively studied in humans whereas very few studies have been conducted with bovine neutrophils. However, certain experiments have clearly demonstrated that there are quantitative and qualitative differences in the NADPH oxidase activation between bovine and human neutrophils. Thus, studies of the respiratory burst in other species may have limited relevance to bovine. Furthermore, understanding the respiratory burst of bovine neutrophils may lead to novel therapeutic strategies, such as the prevention of mastitis.

Some experiments, using human neutrophils, have demonstrated the importance of either extracellular or intracellular calcium on production of O_2^-. In fact, the specific dependence of calcium (Ca^{2+}) (i.e. extracellular or intracellular) was shown to be related to the stimuli used. Similarly, the active form of protein kinase C (PKC) has also been shown to be required for the respiratory burst of human PMNs stimulated with certain activating agents.

The ultimate goal of this study is to determine the importance of intracellular and extracellular Ca^{2+} as well as PKC in the generation of O_2^- from bovine neutrophils activated by either a soluble stimulus (PMA, i.e. phobol myristate acetate) or a particulate stimulus (opsonized zymosan).

CHAPTER II. REVIEW OF LITERATURE

2.1. INTRODUCTION

"Immunity" is a term that refers to the body's capability to resist or eliminate potentially harmful foreign substances, including microbes, macromolecules such as proteins and polysaccharides as well as abnormal cells (Sherwood, 1988). The cells and molecules responsible for immunity are said to constitute the "immune system" (Abbas et al., 1991). These cells, known as leukocytes or white blood cells, defend the host in two different ways: (1) by engulfing and digesting foreign materials or abnormal cells through phagocytosis (natural immunity); and (2) by immune responses such as production of antibodies which mark the foreigner for destruction (acquired immunity) (Sherwood, 1988; Tortora, 1988). The collective and co-ordinated work of the leukocytes towards destruction of foreign substances comprises the "immune response" (Abbas et al., 1991).

Neutrophils constitute the first line of defense for mammalian organisms against foreign particles and abnormal cells (Thelen et al., 1993). Just like other white blood cells, neutrophils are alarmed by chemotactic agents that arise within infected tissues. Then they migrate through the wall of the local capillary vessels and accumulate at the lesion site (Meshulam et al., 1986; Styrt, 1989; Pike et al., 1991; Roth and Zwahlen, 1991; Thelen et al., 1993). Once the cells are at the site of acute inflammation, they ingest foreign particles and exocytose enzymes from the cytoplasmic granules and other storage organelles (Wilson et al., 1986, Young and Beswick, 1986; Styrt, 1989; Kessels et al., 1991; Pike et al., 1991; Baggiolini et al., 1993).

Another critical function involved in the killing ability of neutrophils is their production of O_2^- and related toxic oxygen metabolites which are found in the phagocytic vesicle and extracellular space (Dyer et al., 1985; Wilson et al., 1986; Kessels et al., 1991; Baggiolini et al., 1993). This phenomenon is referred to as the "respiratory burst"

which is the most characteristic property of phagocytes (Dyer et al., 1985; Baggiolini et al., 1993). The respiratory burst results from assembly and activation of a multicomponent enzyme called NADPH:O_2 oxidoreductase (also called NADPH oxidase) which catalyses the one-electron reduction of molecular oxygen to O_2^- (Baggiolini et al., 1993; Thelen et al., 1993). Thus, a striking feature of the NADPH oxidase activation is the marked increase in oxygen consumption (Dyer et al., 1985; Klebanoff, 1992). NADPH oxidase can be activated by chemotactic agonists or other inflammatory stimuli (Baggiolini et al., 1993). More details regarding the production of O_2^- will soon follow.

It is of interest to note that these neutrophil activities have been extensively studied in humans as compared to only a few studies in the domesticated species. However, enough work has been carried out to show that there are quantitative and qualitative differences in the neutrophil activation in bovine, ovine and porcine, compared to human neutrophils (Young and Beswick, 1986).

2.2. RESPIRATORY BURST

2.2.1. Stimulation

As mentioned previously, the enzyme system responsible for the production of O_2^- is an NADPH:O_2 oxidoreductase which is known to be dormant until the cells have been stimulated by particulate and/or soluble stimulating agents (Dyer et al., 1985; Wilson et al., 1986; Tarsi-Tsuk and Levy, 1990; Kessels et al., 1991; Thelen et al., 1993; Le Cabec and Maridonneau-Parini, 1995). Phagocytosis of bacteria, opsonized latex, or opsonized zymosan (yeast cell walls) can activate the respiratory burst (Young and Beswick, 1986). Contrarily, chemotactic agents such as C5a (constituent of activated serum or plasma), C3bi-coated erythrocytes, and formyl-methionyl-leucyl-phenylalanine (fMLP) have been shown to stimulate the respiratory burst via cell surface receptor mechanisms in human neutrophils (Wilson et al., 1986; Styrt, 1989). Thus, although phagocytosis is commonly associated with the respiratory burst, it is not a necessary step for O_2^- to be produced. The lipid diacylglycerol (DG) and the tumor promoter

phorbol myristate acetate (PMA), both known as activators of PKC, can also activate the respiratory burst (Cohen et al., 1982; Wilson et al., 1986) along with arachidonate and other unsaturated fatty acids (Dyer et al., 1985).

When activation of neutrophils is initiated by the binding of a chemotactic agonist to its receptor, it can be prevented by antagonists and interrupted by displacement of the agonist which indicates that the agonist-receptor complex must exist (Baggiolini et al., 1993). The mode of activation of the enzyme, the intracellular messengers, as well as the mechanisms leading to cellular responses, remain unclear. It is known, however, that some of the activated chemotactic receptors couple to G-proteins (GTP-binding proteins). This was proven by the inhibition with pertussis toxin (G-protein inhibitor) along with the observation that direct activation of G-proteins, using fluoride or the nonhydrolyzable GTP analogs, can mimic receptor-dependent stimulation (Baggiolini and Kernen, 1992; Baggiolini et al., 1993). Accumulating evidence suggests that all activators ultimately stimulate the same NADPH oxidase (McPhail and Snyderman, 1983). However,

different mediation mechanisms have been proposed and it appears that not all stimuli share a common pathway, as will be discussed further. Meanwhile, it is possible that the initial pathways converge in a final sequence of regulatory events.

One major process that enhances the responsiveness of neutrophils and other phagocytes is "priming" (Thelen et al., 1993). A number of cytokines as well as ATP, fMLP, PAF (platelet-activating factor), leukotriene B_4 and Ca^{2+} ionophores have been shown to prepare phagocytes for a rapid and more intense respiratory burst (Cohen et al., 1980; Cohen et al., 1982; Pozzan et al., 1983; Nishihira et al., 1986; Liang et al., 1990; Bian et al., 1991; Walker et al., 1991; Walz et al., 1991; Thelen et al., 1993). Priming is achieved at concentrations insufficient to cause O_2^- generation, however, it prepares the cells for exaggerated O_2^- responses after subsequent addition of an agonist. For example, Finkel et al. (1987) primed human neutrophils by briefly exposing these cells to low concentrations of ionomycin such as 10 nM and observed that these cells released almost six times more

O_2^- in response to fMLP compared to the neutrophils preincubated without ionomycin. Similar results were obtained in this study when PMA was used as the stimulating agent instead of fMLP. Furthermore, Walz et al. (1991) mentioned that Con-A was also shown to act in synergism with fMLP in inducing the respiratory burst.

Once again, there are clear differences among species with regard to priming and stimulation (Young and Beswick, 1986). An example of this is the incapability of bovine neutrophils to surmount chemotactic responses to formyl peptides (Carroll et al., 1982) although those are potent chemoattractants for other species (Styrt, 1989). Bovine neutrophils also have been reported to be incapable of binding C3bi-coated erythrocytes, which suggests that this species may not have a membrane glycoprotein analogous to the C3bi receptor of the human neutrophil (Styrt, 1989). These variations could be of great importance for understanding phylogenic differences in host defense. This also suggests

that studies of the inflammatory process in other species may have limited relevance to the bovine.

2.2.2. Resulting Metabolites

The NADPH:O_2 oxidoreductase is a short membrane-bound electron transport chain found exclusively in phagocytes and B-lymphocytes, that reduces extracellular oxygen to O_2^- using NADPH as the electron donor (Thelen et al., 1993) (Eq.1). This is the critical step in the respiratory burst since several other microbicidal oxidants will be generated following this step. This is due to the fact that O_2^- is unstable and dismutates rapidly to hydrogen peroxide (H_2O_2) and oxygen by being either a reductant or an oxidant (Klebanoff, 1992; Thelen et al., 1993). This reaction can occur spontaneously or can be catalyzed by the enzyme called superoxide dismutase (SOD) (Klebanoff, 1992). Three different SODs are known (copper-zinc SOD; manganese SOD; iron SOD) each varying in their metal component as well as their cellular distribution (Klebanoff, 1992). Once hydrogen peroxide has been generated, it can either dismutate (spontaneously or catalytically) into

water and oxygen, or serve as an oxidant for other reactions such as peroxidase-dependent ones (Salgar et al., 1991; Thelen et al., 1993) (Eq.2).

(1) $2\ NADPH + 4\ O_2 \rightarrow (2\ NADP^+) + 4\ O_2^- + 2H^+$

(2) $4\ O_2^- + 4\ H^+ \rightarrow 2\ O_2 + 2\ H_2O_2 \rightarrow 3\ O_2 + 2\ H_2O$

O_2^- and H_2O_2 are known to produce a series of secondary oxidative decarboxylation, oxidative deamination as well as peroxidative reactions which destroy both prokaryotic and eukaryotic cells (Freeman and Crapo, 1982; Klebanoff, 1992).

2.2.3. Activation of NADPH oxidase

Activation of the NADPH oxidase is brought about by rapid mobilization of soluble oxidase components from the cytosol to the plasma membrane (Kessels et al., 1991). Thus, the activated protein is membrane bound and evidently hydrophobic. According to a study conducted by Doussiere and Vignais (1985), the optimum pH of the NADPH oxidase in bovine neutrophils was 7.5 and its isoelectric point was at pH 5.0. So far, six components of the oxidase have been fully

characterized (Baggiolini et al., 1993). Three of them, the 91 kD glycoprotein, the 22 kD phosphoprotein (designated $gp91^{phox}$ and $p22^{phox}$ for phagocyte oxidase, respectively) which are subunits of cytochrome b_{558}, and the rapla, a member of the ras superfamily, are membrane-bound (Baggiolini et al., 1993; Le Cabec and Maridonneau-Parini, 1995), whereas the other three, $p47^{phox}$, $p67^{phox}$, and $p21^{rac}$ are cytosolic proteins (Rotrosen, 1992; Baggiolini et al., 1993; Le Cabec and Maridonneau-Parini, 1995). Volpp et al. (1989) estimated that 0.5% and 0.3% of neutrophil cytosolic protein was constituted of $p47^{phox}$ and $p67^{phox}$, respectively. Cytochrome b_{558}, for its part, is presumed to be the terminal electron-carrying component of the oxidase, as well as the donor of electrons to molecular oxygen due to its very low midpoint potential (-245 mV) (Rotrosen, 1992). It therefore allows the oxidase to reduce molecular oxygen to O_2^- (Rotrosen and Leto 1990; Rotrosen, 1992). Recently, an additional cytosolic protein, identified as $p40^{phox}$, has been suggested to modulate the NADPH oxidase activity (Le Cabec and Maridonneau-Parini, 1995). A flavin adenine dinucleotide (FAD) has further been suggested to be

involved with the NADPH oxidase. In fact, it is believed to serve as the redox centre linking the NADPH to cytochrome b_{558} (Rotrosen, 1992). Undoubtedly, all oxidase components associate together at the plasma membrane upon cell activation (Rotrosen, 1992; Thelen et al., 1993; Le Cabec and Maridonneau-Parini, 1995), except for $p21^{rac}$ whose recruitment to the membrane is still under investigation (Le Cabec and Maridonneau-Parini, 1995). A highly schematized model of the NADPH oxidase is presented in Figure 1.

Although all of the oxidase subunits are essential for its activation, some other factors are also very important. One of them is GTP. Baggiolini et al. (1993) studied the neutrophil cytosol's ability to reconstitute NADPH oxidase activity in the presence or absence of ATP and GTP. Their results showed that upon depletion in ATP and GTP, no NADPH oxidase activity could occur. However, it was fully restored by the addition of GTP or its nonhyrolyzable analogs, which indicates the oxidase activation's absolute dependence of GTP (Baggiolini, 1993).

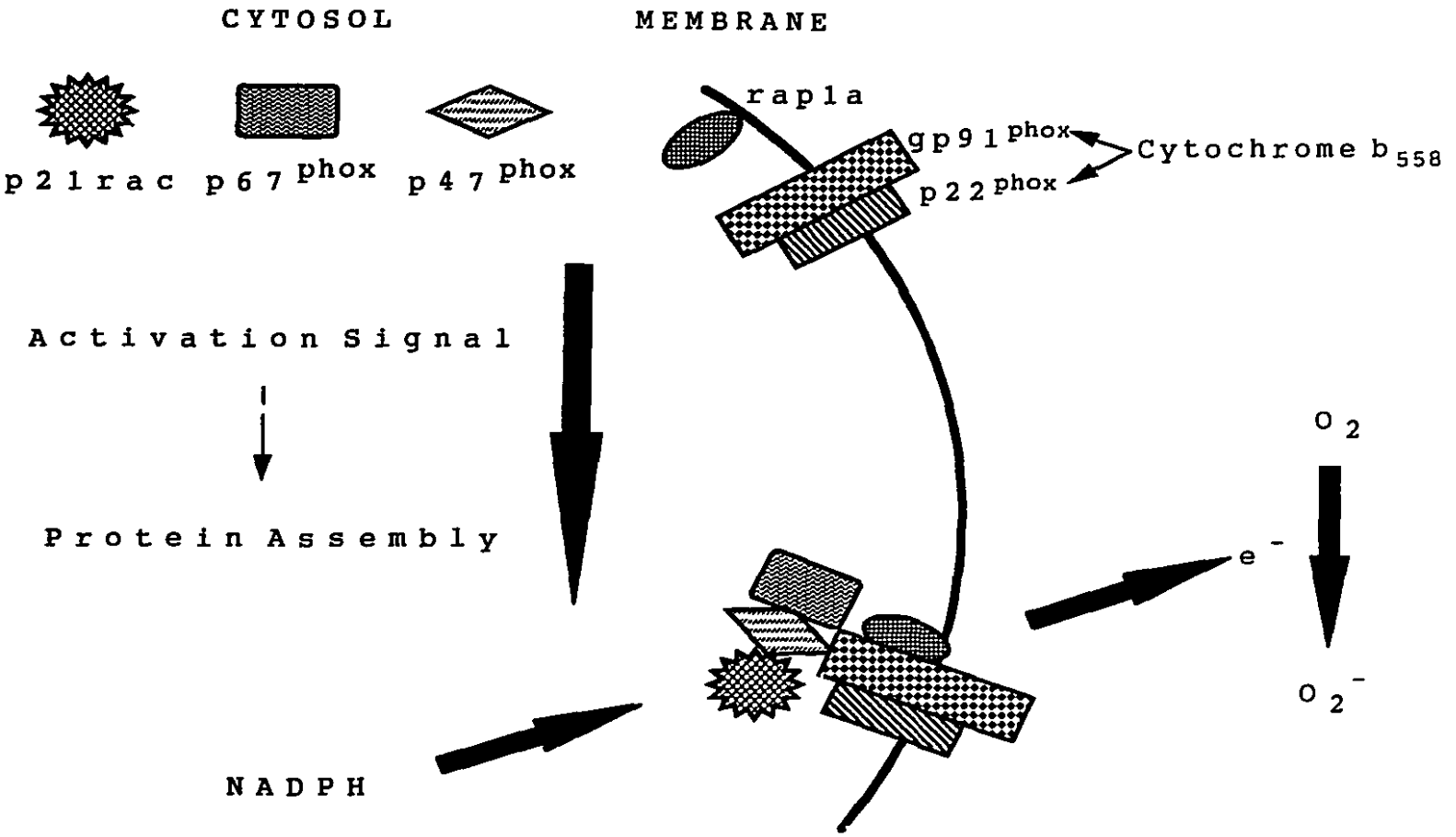

Figure 1. The NADPH oxidase. Stimulation of the cell will result in the assembly of cytosolic and membrane proteins at the membrane level. The assembled oxidase will further reduce O_2 to O_2^- using NADPH as the electron donor. (Figure adapted from Rotrosen, 1993)

The sequence of events is also essential for a proper activation of oxidase. Upon neutrophil stimulation, $p47^{phox}$ translocates from the cytosol to the plasma membrane where it binds to the cytochrome b_{558} and becomes part of the cytoskeleton-associated oxidase complex (Thelen et al., 1993). Furthermore, the cytochrome appears to regulate the membrane association of $p47^{phox}$ since this one failed to attach to the membrane in cytochrome b_{558}-deficient human neutrophils (Heyworth et al., 1989). The second cytosolic factor, $p67^{phox}$, then translocates from the cytosol to the membrane, where it binds to the oxidase complex (Thelen et al., 1993). Movement of $p67^{phox}$ to the membrane cannot occur in the absence of $p47^{phox}$ (Rotrosen, 1992) which, once again, suggests that NADPH oxidase activation also depends on the sequence of events that the enzyme subunits undergo.

In addition, multisite phosphorylation of the $p47^{phox}$ appears to be essential for the oxidase activation (Rotrosen and Leto, 1990). Phosphorylation of $p47^{phox}$ was studied by Rotrosen and Leto (1990) who incubated human neutrophils with

^{32}P for certain lengths of time followed by immunoprecipitation from the cells' cytosol and membrane fractions. After two-dimensional gel electrophoresis and autoradiography, they found that $p47^{phox}$ had not been phosphorylated in the resting cells. On the other hand, eight distinct p47 phosphoproteins were present in the cytosol of the PMA-activated neutrophils. In neutrophils from patients with cytochrome b_{558}-deficient chronic granulomatous disease, an inherited disorder characterized by the inability of phagocytes to express a respiratory burst, $p47^{phox}$ phosphorylation was incomplete. Similar results were obtained by another group of researchers (Okamura et al., 1988). Thus, the $p47^{phox}$ phosphorylation appears to be important in regulating the activity of the NADPH oxidase. Okamura et al. (1988) further revealed that $p47^{phox}$ was phosphorylated exclusively on serine residues. Since PKC is the best enzyme known to phosphorylate on serine and threonine residues (Thelen et al., 1993), and since $p47^{phox}$ was shown to be phosphorylated on serine, a PKC inhibitor is expected to inhibit the phosphorylation of $p47^{phox}$. Indeed, by using α-

tocopherol to inhibit PKC activity and phorbol myristate acetate (PMA) to stimulate guinea pig peritoneal neutrophils, Kanno et al. (1995) noted an inhibition of O_2^- production in cells treated with α-tocopherol versus non-treated ones. The authors further suggested that inhibition of phosphorylation of $p47^{phox}$ may be correlated with the inhibition of O_2^- generation in neutrophils by α-tocopherol. Recently, some data suggested that phosphorylation of proteins on tyrosine residues may also play an important role in activation of the NADPH oxidase (Le Cabec and Maridonneau-Parini, 1995). Thus, other protein kinases may also be involved in phosphorylating certain proteins for the proper enzyme activation. Of the few studies conducted on bovine neutrophils, phosphorylation of $p47^{phox}$ was also observed in response to a variety of activating agents such as PMA, 1-oleyl-2-aceylglycerol, ionomycin and latex beads (Gennaro et al., 1985; Gennaro et al., 1986).

2.3. CALCIUM

2.3.1. Cellular Role of Calcium

Most blood cells have the ability to accumulate Ca^{2+} owing to various organelles such as endoplasmic reticulum (ER), mitochondria, nucleus, calciosomes in neutrophils, and various vesicular and granular structures which may act as Ca^{2+} stores (Scharff and Foder, 1993). The exceptions are human and other mammalian erythrocytes which in fact lack cellular organelles (Scharff and Foder, 1993). It is widely recognized that Ca^{2+} plays a very important role in the regulation of many cellular functions. In fact, a certain level of cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) is required in cellular processes including membrane transport, secretion, proliferation, muscle contraction, and motility (Kuijpers et al., 1992; Marks and Maxfield, 1990). It has been observed that one of the first signs of cellular "distress" is an immediate increase in the plasma membrane permeability for Ca^{2+} which is followed by a high accumulation of Ca^{2+} by the cell (Borle, 1975).

2.3.1.1. Cell shape and movements

Certain cells are known to change shape during cell stimulation, and neutrophils also move toward a chemotactic source, all suggesting cytoskeletal rearrangements which in turn, requires Ca^{2+} (Scharff and Foder, 1993). In fact, during migration and adherence of human neutrophils, some changes in $[Ca^{2+}]_i$ can be observed, and migration can be reduced or even inhibited at suppressed $[Ca^{2+}]_i$ values (Marks and Maxfield, 1990). On the other hand, Kuijper and associates (1992) tested this hypothesis and found that a rapid rise of $[Ca^{2+}]_i$ did not constitute a prerequisite for neutrophil migration across resting or cytokine-activated human endothelial cells. Similarly, Zigmond et al. (1988) reported that migration of rabbit neutrophils was essentially independent of extracellular Ca^{2+} ($[Ca^{2+}]_o$). In addition, they observed that cells that had been Ca^{2+} permeabilized with an ionophore in the presence of the Ca^{2+} chelator EGTA (ethylene glycol bis (ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid) could still migrate.

2.3.1.2. Proliferation and differentiation

The involvement of Ca^{2+} in cell growth is very well documented and different growth factors have been shown to initiate cellular Ca^{2+} mobilization (Scharff and Foder, 1993). For example, Ca^{2+} mobilization is one of the earliest detectable events triggered in lymphocytes upon binding of a ligand, such as an antigen or a receptor antibody, to an appropriate receptor exposed on the outer cell surface (Gelfand et al., 1986; Gelfand, 1990). A study conducted by Wozniak et al. (1993) showed that stimulation of neutrophils with rhIL-8 increased $[Ca^{2+}]_i$ by increasing Ca^{2+} influx as well as mobilizing Ca^{2+} from internal stores. A study by Short et al. (1993) has revealed existance of a precise correlation between the ability of the cells to initiate DNA synthesis and the Ca^{2+} content of $[Ca^{2+}]_i$ pools. The authors used ionophores as well as two Ca^{2+} pump inhibitors thapsigargin (TG) and 2,5-di-tert-butylhydroquinone (DBHQ) in order to deprive the cells of Ca^{2+}. Intracellular Ca^{2+} pool content was then proven to be linked to the control of cell growth (Short et al., 1993).

2.3.1.3. Secretion and phagocytosis

The ingestion step of phagocytosis in neutrophils is apparently independent of Ca^{2+} (Scharff and Foder, 1993). On the other hand, fusion of specific granules with the phagosomal membrane requires elevated $[Ca^{2+}]_i$ (Jaconi et al., 1990). Production of O_2^- is also believed to require elevated levels of $[Ca^{2+}]_i$. This last point will be examined later.

2.3.2. Signal Transduction Sequence

Only a minor part (most often 10-20%) of the cellular Ca^{2+} is contained in the cytosol, and it is largely bound to either soluble cytosolic proteins or membrane surfaces (Pozzan et al., 1994). Thus, a Ca^{2+} signalling cascade has to be driven in order to elicit a rapid transient rise in free cytosolic Ca^{2+} concentration which is required in several cellular processes as previoulsy mentioned.

Upon receptor-ligand interaction and coupling to a guanosine 5'-triphosphate-binding protein (G protein), a phosphatidylinositol-specific phospholipase C is activated leading to subsequent hydrolysis of phosphatidylinositol-4-5-

bisphosphate (PIP_2) and generation of two second messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DG) (Pike et al., 1991; Baggiolini et al., 1993; Thelen et al., 1993). Following this step, IP_3 diffuses into the cytosol, and induces the release of Ca^{2+} from $[Ca^{2+}]_i$ pools leading to an increase in cytosolic Ca^{2+} concentration (Korchak et al., 1988a; Pike et al., 1991; Baggiolini et al., 1993; Thelen et al., 1993; Zweifach and Lewis, 1993). Due to its hydrophobic property, DG remains associated with the membrane where it participates in the activation of PKC (Pike et al., 1991; Baggiolini et al., 1993; Thelen et al., 1993). An additional source of DG is phosphatidic acid (PA) which is the product of phosphatidylcholine hydrolysis by phospholipase D (Thelen et al., 1993). In fact, this source of DG is said to be more important than the one resulting from PIP_2 hydrolysis (Thelen et al., 1993). A group of researchers (von Tscharner et al., 1986) prevented the influx by chelating $[Ca^{2+}]_o$ and depleting the intracellular stores in order to assess the role of Ca^{2+} mobilization. According to their results, the agonist-dependent rise in $[Ca^{2+}]_i$ reflects both the release from intracellular stores and the influx through the plasma

membrane which supports the signal transduction sequence described above.

Many cell types contain stores equipped with Ca^{2+} channels that open in the presence of cytosolic IP_3 (Scharff and Foder, 1993). One of the most popular Ca^{2+} stores is the endoplasmic reticulum which is believed to be the major intracellular source of the Ca^{2+} release into the cytosol, since it appears to be composed of multiple functional domains involved in rapid Ca^{2+} release and accumulation (Moore et al., 1987; Kass et al., 1989; Pozzan et al., 1994). The 260 kD Ca^{2+} channel molecule contains the IP_3 binding site near the NH_2-terminus and the transmembrane domains (at the carboxyl-terminus) are believed to be the seat of the Ca^{2+} channel (Scharff and Foder, 1993). Arachidonic acid also has the capability of inducing Ca^{2+} releases from $[Ca^{2+}]_i$ stores in a manner similar to that of IP_3 (Scharff and Foder, 1993). This was studied using different cells such as permeabilized human neutrophils (Beaumier et al., 1987) as well as lymphocytes and platelet membrane vesicles (Scharff and Foder, 1993).

Although it is generally accepted that IP_3 leads to the Ca^{2+} release from intracellular stores through IP_3-activated channels, the mechanisms that regulate sustained Ca^{2+} entry across the cytosolic membrane during the stimulation periods are still under debate (Zweifach and Lewis, 1993). The controversy centers on whether Ca^{2+} channels in the plasma membrane are opened by a signal generated by the IP_3-triggered depletion of Ca^{2+} stores (depletion hypothesis) or by IP_3 (IP_3 hypothesis) (Jaconi et al., 1993; Zweifach and Lewis, 1993).

2.3.3. Dependence of Superoxide Production on Extracellular and Intracellular Calcium Concentration

As mentioned previously, the pathways involved in the signal transduction of the respiratory burst in neutrophils are not completely understood. Nevertheless, it is important to understand the process of neutrophil activation since it may become the basis of novel therapeutic strategies, such as prevention, or cure of different diseases and infections.

To test for $[Ca^{2+}]_o$ dependence, the cells are usually incubated with EGTA (ethylene glycol bis (ß-aminoethyl ether)-

N,N,N',N'-tetraacetic acid) for chelation of Ca^{2+}. On the other hand, in order to test the importance of $[Ca^{2+}]_i$ in neutrophil activation, most studies were conducted using EGTA along with an $[Ca^{2+}]_i$ chelator. BAPTA/AM (1,2-bis(o-aminophenoxy) ethane - N, N, N', N' - tetraacetic acid acetoxymethyl ester) is a widely used $[Ca^{2+}]_i$ chelator (Meshulam et al., 1986; Korchak et al., 1988b; Sullivan et al., 1989; Liang et al., 1990; Marks and Maxfield, 1990; Tarsi-Tsuk and Levy, 1990; Kessels et al., 1991; Walker et al., 1991). BAPTA/AM belongs to the same family as EGTA however the methylene links between oxygen and nitrogen are replaced by benzene rings (Tsien, 1980). Another alternative for the $[Ca^{2+}]_i$ chelation is the use of the Ca^{2+} sensitive dyes Indo-1-AM (Pike et al., 1991), Fura-2-AM (Finkel et al., 1987; Korchak et al., 1988b; Kass et al., 1989; Dyer et al., 1994) or Quin-2-AM (Grzeskowiak et al., 1986; von Tscharner et al., 1986; Tarsi-Tsuk and Levy, 1990). Two other methods have also been used to verify the importance of $[Ca^{2+}]_i$ in neutrophil activation. One of them is by adding Ca^{2+} or an ionophore to alter the Ca^{2+} concentration in the cells (Walker et al.,

1991). Another is by monitoring the $[Ca^{2+}]_i$ during neutrophil activation (Liang, et al., 1990; Walz et al., 1991).

According to the different experiments conducted with human neutrophils, it can be concluded that at least three signal transduction sequences are involved in the induction of the respiratory burst. One of them is dependent on the $[Ca^{2+}]_i$ (Finkel et al., 1987; Korchak et al., 1988b), a second one is dependent on $[Ca^{2+}]_o$ (Maridonneau-Parini et al., 1986; Tarsi-Tsuk and Levy, 1990) while the third one is independent of Ca^{2+} (Grzeskowiak et al., 1986; Walker et al., 1991). The differences among the signal transductions are directly related to the stimuli used to activate neutrophils.

2.3.3.1. Calcium-dependent signal transduction

In 1983, Pozzan and associates used ionomycin, a Ca^{2+} ionophore, in human neutrophils to test whether an increase in $[Ca^{2+}]_i$ could lead to the production of O_2^-. Their results showed that this mineral had no direct ability to activate the NADPH oxidase. Later, this was further confirmed by other studies (Finkel et al., 1987; Liang et al., 1990). FMLP,

however, is known to activate the NADPH oxidase which is preceded by an increase in cytosolic Ca^{2+} (Pozzan et al., 1983; Grzeskowiak et al., 1986; Korchak et al., 1988a; Liang et al., 1990). Finkel et al. (1987) tested the dependence of the O_2^- production on Ca^{2+} in human neutrophils stimulated with fMLP. Their results showed that activation of the NADPH oxidase was dependent on an elevation in $[Ca^{2+}]_i$ when the cells were stimulated with fMLP, supporting the concept that Ca^{2+} can serve as a second messenger in this event. The following year, Korchak et al. (1988b) also demonstrated that O_2^- generation was inhibited when neutrophils were depleted of Ca^{2+} prior to fMLP stimulation. Additionally, they measured the DG concentration in the Ca^{2+}-depleted cells and noticed a decrease in this lipid. Therefore, they came to the conclusion that elevated DG and Ca^{2+} may be necessary for O_2^- generation.

Another stimulus, lipoteichoic acid (LTA) from Streptococcus faecalis, was proven to depend on an increase in $[Ca^{2+}]_i$ for the respiratory burst (Tarsi-Tsuk and Levy, 1990). However, this experiment was performed using human monocytes. Results showed that removal of $[Ca^{2+}]_o$ ions, in the presence of

EGTA, abolished the LTA-stimulated O_2^- production showing the dependence on $[Ca^{2+}]_o$. These results are in accordance with a similar study conducted by Maridonneau-Parini and associates (1986) who used opsonized zymosan (OZ) to stimulate human neutrophils.

2.3.3.2. Calcium-independent signal transduction

Although O_2^- production from fMLP-stimulated neutrophils has been shown to be Ca^{2+}-dependent, it has been demonstrated to be Ca^{2+}-independent when the cells were primed with PAF prior to the fMLP stimulation (Walker et al., 1991). These cells had been treated with BAPTA/AM, an $[Ca^{2+}]_i$ chelator, and did not show $[Ca^{2+}]_i$ increases. This suggests that there may be priming pathways that are independent of increases in $[Ca^{2+}]_i$. Following the same line, an experiment done with DG-primed human neutrophils and further activated with fMLP have shown that an increase in $[Ca^{2+}]_i$ is not strictly required by these cells to produce O_2^- (Liang et al., 1990). However, the same authors showed that EGTA inhibited both the $[Ca^{2+}]_i$ and the O_2^- responses of neutrophils primed with cytochalasin B and activated with either fMLP or Con A.

The tumor promoter, PMA, was shown to induce O_2^- generation in human neutrophils without any increase in cytosolic Ca^{2+} (Tarsi-Tsuk and Levy, 1990; Korchak et al.. 1988b). In fact, PMA did not trigger the breakdown of PIP_2, PIP, nor PI in an experiment by Korchak et al. (1988b). In addition, no increase in inositol phosphates after PMA stimulation is in accordance with its inability to elicit increases in cytosolic Ca^{2+} (Korchak et al., 1988b). Similar to PMA, synthetic diacylglycerol and its analogues, directly activate the PKC without any phosphoinositide hydrolysis and Ca^{2+} mobilization (Grzeskowiak et al., 1986; Meshulam et al., 1986).

2.4. PROTEIN KINASE C

2.4.1. Description and Activation of Protein Kinase C

Protein Kinase C is an enzyme which catalyses the phosphorylation of serine and threonine residues in proteins (Babior, 1988). PKC is the best-known enzyme acting on these two amino acid residues (Thelen et al., 1993). It has been described originally as a Ca^{2+}- and phospholipid-dependent kinase. However, recent research has showed that some isotypes

such as δ-, ϵ-, ζ- and η-isotypes are Ca^{2+}-independent, whereas the α-, β-, and γ-isotypes are Ca^{2+}-dependent (Thelen et al., 1993; Dyer et al., 1994). PKC has been studied extensively in neutrophils. These cells express both Ca^{2+}-dependent and Ca^{2+}-independent isoenzymes (Thelen et al., 1993). However, the function of these last ones in signal transduction is not clear and there is no evidence for a role of such isoenzymes in activation of the NADPH oxidase (Sharma et al., 1991).

PKC can be activated by diacylglycerol or phorbol esters such as PMA (May et al., 1985; Wolf et al., 1985a; Wolf et al., 1985b; Grzeskowiak et al., 1986; Wilson et al., 1986; Korchak et al., 1988a; Pike et al., 1991; Dyer et al., 1994; Kanno et al., 1995). Stimulation of human neutrophils leads to translocation of PKC from the cytosol to the plasma membrane (Nishihira et al., 1986; Thelen et al., 1993). This translocation process has been proven to be Ca^{2+} dependent as well as reversible (Wolf et al., 1985a; Wolf et al., 1985b; Thelen et al., 1993).

2.4.2. Dependence of Superoxide Production on Protein Kinase C

As mentioned previously, phosphorylations by PKC appear to be required for assembly and activation of the NADPH oxidase. In inactivated neutrophils, O_2^- generation can be elicited by chemotactic agonists (by selective interaction with surface receptors) or by phorbol esters and DG which act directly on PKC (Nishihira et al., 1986; Korchak et al., 1988a; Korchak et al., 1988b; Baggiolini et al., 1993; Kanno et al., 1995). In an experiment conducted by Korchak and associates (1988b), PMA bypassed the normal signalling pathway and triggered the respiratory burst without an increase in $[Ca^{2+}]_i$, DG, nor inositol phosphates. However, the respiratory response induced by phorbol esters or diacylglycerols was much slower in onset than that elicited by receptor agonists (Babior, 1988; Kessels et al., 1991). According to the authors, this indicates that PKC activation is only part of the signal transduction process. By treating human neutrophils with 1 mg/ml of the G-protein inhibitor, pertussis toxin, Liang et al. (1990) could not see any changes in the fMLP-induced increment in $[Ca^{2+}]_i$, but they noticed an inhibition of IP_3 generation (and DG by association) and a marked reduction

of the O_2^- production, suggesting that DG generation and PKC activation are important for the O_2^- generation. More recently, another group of researchers came to similar conclusions when they used α-tocopherol to inhibit PKC activity (Kanno et al., 1995). However, they used several stimuli such as the PKC activators (PMA and DG) and various other stimuli (OZ, fMLP, and sodium dodecyl sulfate) in order to activate the NADPH oxidase. Their results indicated that α-tocopherol could inhibit O_2^- generation of the PMA- and DG-activated cells through the inhibition of PKC but could not affect the other stimuli-dependent O_2^- production. This indicates that the sensitivity of O_2^- generation to α-tocopherol following stimulation differs according to the stimulus. Using H-7 to inhibit the PKC activity, Doré et al. (1991) also found that PMA activation of human neutrophils was dependent on PKC activity.

From these studies, it can be concluded that PKC activation is an important activation mechanism for the respiratory burst although it does not constitute the sole

activation mechanism. The importance of PKC in the NADPH oxidase activation may be due to the fact that one of the cytosolic components of this enzyme, $p47^{phox}$, contains several recognition sites for PKC and becomes rapidly phosphorylated after challenge with either phorbol esters or receptor agonists (Thelen et al., 1993; Kanno et al., 1995). Nevertheless, this cannot explain why production of O_2^- was still evident in neutrophils activated by particulate stimulating agents, such as opsonized particles, following inhibition of PKC activity using α-tocopherol (Kanno et al., 1995).

In summary, the respiratory burst from neutrophils is a very important defense mechanism against invading foreign substances. The processes for activation of the NADPH oxidase and the enzyme responsible for the reaction, are still unclear especially in bovine neutrophils. Some studies have shown that at least three signal transduction sequences can be involved in the induction of the respiratory burst. One transduction signal is independent of Ca^{2+}. A second possible signal

transduction is dependent on the $[Ca^{2+}]_i$ whereas a third one is dependent on the $[Ca^{2+}]_o$. The differences among the signal transductions all appear to be directly related to the stimuli used to activate the neutrophils. Furthermore, PKC appears to be required for O_2^- response in cells stimulated with PKC activators such as PMA and DG. On the other hand, particulate stimulating agents, including OZ and opsonized latex, were shown to be independent of PKC activation.

2.5. SIGNIFICANCE OF THIS RESEARCH PROJECT

Since bovine neutrophils have not been studied intensively, not much is known about the transduction of signals involved for their activation. Certain viruses or bacteria have been shown to cause tremendous economic losses in the dairy industry (Heyneman et al., 1989; Kehrli et al., 1990; Dyer et al., 1994). For example, bovine granulocytopathy syndrome has been reported as a cause of death in young Holstein cattle which is partly due to phagocytic and oxidative malfunctions of the neutrophils (Kehrli et al., 1990). Certain microbial virulence mechanisms also deactivate some part of the signal transduction in bovine alveolar

phagocytes, which could cause disfunction of the pulmonary phagocytes and thus enhance intrapulmonary microbial virulence (Dyer et al., 1994). Bovine mastitis due to bacterial infections is one of the most costly diseases in the dairy industry. Cows with phagocytic potential, along with elevated blood neutrophil numbers and a high O_2^- generation competence showed limited bacterial replication in the mammary gland following Escherichia coli induction (Heyneman et al., 1990). The authors further stated that the considerable variation in O_2^--producing capacity of blood neutrophils might be due to the underlying mechanisms, which are responsible for the variation in susceptibility to E. coli mastitis among individuals. Therefore, it is important to know more about the processes involved in the activation of neutrophils since it can become the basis of new therapeutic strategies.

CHAPTER III. MATERIALS AND METHODS

3.1. REAGENTS

Reagents and materials were purchased from the following sources: Dulbecco's phosphate-buffered saline (DPBS), Hank's balanced salt solution (HBSS), [Ser^{25}]PKC from GIBCO Laboratories (Grand Island, NY, USA); ^{125}I and ^{32}P- ATP from Amersham (Oakville, Ontario, Canada); HEPES, phorbol 12-myristate 13-acetate (PMA), superoxide dismutase (SOD), cytochrome C (horse heart), EGTA, Iodogen, NaCl, $CaCl_2$, aprotinin, benzamedine, Nonidet-P40, and diolein from Sigma Chemical Co. (St.Louis, MO, USA); $MgCl_2$ from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA); BAPTA/AM from Molecular Probes Inc. (Eugene, OR, USA); Calphostin C from Calbiochem (La Jolla, CA, USA); Zymosan from ICN Pharmaceuticals Canada Ltd (Montréal, Québec, Canada); Percoll from Pharmacia Biotech (Baie d'Urfé, Québec, Canada); (ethylenedinitrilo) tetraacetic acid (EDTA) and phosphoric acid from J.T. Baker Canada (Toronto, Ontario, Canada); dithiothreitol (DTT), pepstatin, and leupeptin from Boringer Mannheim Canada (Laval, Québec, Canada); P81 paper and DE52 from Fisher Scientific (Montréal,

Québec, Canada); phosphatidylserine from Avanti Polar-Lipids, Inc. (Alabaster, AL, USA); and Triton-X from BDH Inc. (Toronto, Ontario, Canada).

3.2. OPSONIZATION OF ZYMOSAN

One hundred mg zymosan was suspended in 10 ml of boiling water for 45 min. It was then centrifuged for 10 min at 250 x g and 4°C. The pellet was then resuspended in equal volume of DPBS (10 ml) and fresh cow serum (10 ml) followed by a 30 min incubation at 30°C using a shaking water bath. For a second time, centrifugation and resuspension of the pellet was performed as described above. The zymosan particles were then washed twice in DPBS, resuspended at a concentration of 30 mg/ml, aliquoted and stored at -20°C.

3.3. PREPARATION OF PERCOLL

The working solution of Percoll (1.077 g/ml; 100 ml) was prepared by adding 10 ml of 1.5 M sodium chloride and 35.24 ml of double distilled water to 54.76 ml of the Percoll stock solution and stored at 4°C.

3.4. ISOLATION OF NEUTROPHILS

Bovine blood was obtained from healthy cows by tail venipunction using heparinized tubes. The anti-coagulated blood diluted 1:2 with HBSS was layered over a column of Percoll's working solution in a screw cap test tube (29 x 114 mm) at a ratio of 3:2 (blood:Percoll). The tubes were then centrifuged at 2000 x g for 40 min at 4°C followed by the removal of the plasma and the buffy layer. The pellet fraction, containing erythrocytes and neutrophils, was treated for 8 min with Tris-NH_4Cl solution (170 mM Tris-Base, 160 mM NH_4Cl, 1 vol pellet:3 vol Tris-NH_4Cl) to lyse the erythrocytes. The remaining neutrophils were harvested after centrifugation of the mixture at 500 x g for 10 min at 4°C. The pellets were washed twice with HBSS and the cells were resuspended in HBSS. The viability and purity of PMNs isolated by this technique were >99% and >95%, respectively.

3.5. MEASUREMENT OF SUPEROXIDE PRODUCTION

Generation of O_2^- by neutrophils was assessed by the stimulus-induced reduction of ferricytochrome C. Superoxide anion has the ability to reduce cytochrome C resulting in a

stoichiometric change (Dyer et al., 1985). This reaction is the underlying principle for a quantitative spectrophotometric assay of O_2^- production by phagocytes (Dyer et al., 1985). After counting, the neutrophils were resuspended at 2.5 x 10^6 cells/ml or 4.5 x 10^6 cells/ml in HBSS containing 160 mM ferricytochrome C and activated by different doses of PMA or OZ, respectively. After incubation for 30 min at 37°C, the cells were removed by centrifugation at 10 000 x g for 45 sec. The absorbance at 550 nm of the supernatants was measured by a spectrophotometer (Spectronic 600, Milton Roy, NY). The content of the reference cuvette was identical to that in the sample cuvette except for the additional presence of SOD (300 U/ml). The amounts of O_2^- generated were calculated using an extinction coefficient of 21.1 mM^{-1} cm^{-1} at 550 nm (Massey, 1959; Pick, 1986) and were expressed as nmoles of reduced cytochrome C per 10^6 cells.

3.6. MODULATION OF EXTRACELLULAR CALCIUM

Extracellular Ca^{2+} depletion was performed using a modification of previously described techniques (Liang et al., 1990). The neutrophils were suspended for 15 min at room

temperature in HBSS containing 160 mM ferricytochrome C and 10 mM EGTA (pH 7.4). This concentration of the chelator was shown to reduce external Ca^{2+} to less than 5 nM when the cells were incubated for only 2 min (Liang et al., 1990). Following the 15 min incubation, the cells were stimulated with either 0.25 μM PMA or 1.5 mg/ml OZ and incubated at 37°C for 30 min. The experiment was followed by the measurement of O_2^-.

3.7. MODULATION OF INTRACELLULAR CALCIUM

3.7.1. Neutrophils stimulated with PMA

Intracellular Ca^{2+} depletion was performed using a modification of previously described procedures (Marks and Maxfield, 1990). The cells (2.5×10^6 cells/ml) were suspended in HBSS containing 160 mM ferricytochrome C and different doses of BAPTA/AM (50 and 165 μM). The concentrations of BAPTA/AM were chosen since 75 μM was shown to significantly diminish internal Ca^{2+} concentrations when human neutrophils were incubated for 30 min at room temperature (Marks and Maxfield, 1990). All tubes contained a total of 165 μM of dimethyl sulfoxide (DMSO). Ten mM EGTA was also present for certain samples, depending on the experiment. Under this

condition, $[Ca^{2+}]_o$ is not available to replace $[Ca^{2+}]_i$ that is chelated by BAPTA/AM (Meshulam et al., 1986). After a 30 min incubation at 37°C, allowing complete $[Ca^{2+}]_i$ chelation, the cells were stimulated with 0.25 μM PMA and incubated a second time under similar conditions. Measurements of O_2^- generation followed.

3.7.2. Neutrophils stimulated with opsonized zymosan

The neutrophils (4.5 x 10^6 cells/ml) were incubated for 30 min at 37°C in HBSS containing different doses of BAPTA/AM (10, 25, and 50 μM) and all tubes contained a total of 50 μM of DMSO. Certain batches of cells were supplemented with 10 mM EGTA (pH 7.4) depending on the experiment. The cells were washed twice with Ca^{2+}-free DPBS containing 1 mM EGTA in order to remove DMSO from the cells. In fact, no O_2^- production could be observed from the OZ-stimulated neutrophils when there was presence of DMSO in the tubes (data not shown). The neutrophils were then resuspended in HBSS with or without 10 mM EGTA (according to the experiment) and supplemented with 160 μM ferricytochrome C. Stimulation with 1.5 mg/ml OZ and

incubation for 30 min at 37°C were the subsequent steps followed by O_2^- production measurements.

3.8. PROTEIN KINASE C ACTIVITY MEASUREMENTS

PKC activity was assayed in neutrophil cytosol and membrane fractions using a modification of previously described techniques (Hannun et al., 1985; Liles et al., 1986). Briefly, neutrophils were isolated, according to the method mentioned earlier, and diluted to 2.5 x 10^7 cells/ml or 4.5 x 10^7 cells/ml which was followed by PMA or OZ stimulation for 15 min at 37°C. The cells were then collected by centrifugation (1,000 x g, 8 min, 4°C), snap frozen in liquid N_2, and stored at -70°C until the PKC assay. The cells were homogenized in 5 ml of ice cold buffer A (pH 7.4) consisting of 20 mM HEPES, 20 mM $MgCl_2$, 10 mM EGTA, 2 mM EDTA, 2 mM DTT, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 1 μg/ml aprotinin and 400 μg/ml benzamidine. High speed centrifugation (100,000 x g, 60 min, 4°C) was followed to separate the cellular cytosol fraction (supernatant) from the membrane fraction (pellet). The supernatants were applied to DE52 columns preequilibrated with buffer B (pH 7.4) consisting of 20 mM HEPES, 2 mM EGTA, 2

mM EDTA and 2 mM DTT. The membrane pellets were solubilized with ice cold buffer A with 1% (wt/vol) Nonidet-P40, kept on ice for 30 min, centrifuged for 30 min at 4°C (15,000 x g) and the supernatants were added to DE52 columns. The unbound proteins were then removed by washing the columns with buffer B and the fractions containing PKC were eluted with the same buffer containing 0.1 M NaCl. The PKC activity in the eluates was measured by the mixed micelle assay according to Hannun et al. (1985). The mixed micelle reaction determined the incorporation of ^{32}P-gamma ATP into [Ser^{25}PKC] substrate peptide. Briefly, reaction mixtures (1 mg/ml DTT, 34 mM NaCl, 11 mM $MgCl_2$, 30 mM HEPES, 700 μM EGTA, 700 μM EDTA, 3 mM $CaCl_2$ and 320 μM [Ser^{25}PKC]) with or without 60 μg/ml of phosphatidylserine and 500 μg/ml of diolein in 0.3% Triton-x were added to samples of cytosol and membrane fractions. The samples were then incubated for 10 min at 30°C and spotted onto p81 paper. The paper was washed using 1% phosphoric acid followed by determination of activity through scintillation counting. The protein concentrations of both fractions, i.e. cytosolic and membrane were quantified by the Bradford protein assay (Bradford, 1976).

3.9. PROTEIN KINASE C INHIBITION

Inhibition of PKC activity was performed according to the technique used by Kobayashi et al. (1989) and Bruns et al. (1991). Briefly, 1 μM Calphostin C was added to the cell suspension (HBSS + 160 μM ferricytochrome C) followed by an incubation at room temperature for 60 min under ordinary fluorescent light in order to activate Calphostin C. One μM of Calphostin C was used since this concentration was sufficient to inhibit close to 95% of the PKC activity (Kobayashi et al., 1989; Bruns et al., 1991). The neutrophils were then stimulated with either 0.25 μM PMA or 1.5 mg/ml OZ and incubated for another 30 min at 37°C after which measurements of O_2^- generation from these cells were performed.

10. ^{125}I-ZYMOSAN UPTAKE

^{125}I-zymosan uptake was assessed according to the procedure described by Phillips et al. (1992). Twenty mg of zymosan was incubated for 2 min at room temperature with 200 μCi of ^{125}I-iodine in the presence of 300 mg Iodogen in order to label the zymosan. The zymosan particles were then washed four times (1000 x g, 8 min) with DPBS to remove unbound ^{125}I-

iodine and further diluted with unlabelled zymosan to obtain a specific activity of 2 x 10^6 cpm/mg.

The uptake and binding of zymosan by control, EGTA-DMSO- or BAPTA/AM-treated cells was assessed by exposing the cells to 0.1 mg/ml of labelled zymosan at 37°C for 30 min. The neutrophils were then washed four times with DPBS to remove the free zymosan. Cell-associated radioactivity was determined by dissolving the neutrophils in 0.2 N NaOH and counting with a gamma-counter (LKB Wallac, 1275 Minigamma).

11. DATA PRESENTATION AND STATISTICS

Data are presented as mean and standard error of the mean (SEM) of 2 determinations from at least three independent experiments. Comparisons between groups were made using the multiple comparison test.

The two-way classification model was used i.e.:

$$y = \mu + trt_i + cow_j + e_{ij}$$

where μ is the mean, trt is the treatment given to the cells, cow is the cow effect and e is the error.

CHAPTER IV. RESULTS

4.1. SUPEROXIDE GENERATION IN PMA STIMULATED CELLS

The O_2^- production was measured in cells stimulated with various concentrations of PMA in order to determine an appropriate concentration of this stimulant to be used in the subsequent experiments. As shown in Figure 2, concentrations as low as 0.05 μM PMA led to a respiratory burst. However, the response was further increased when 0.25 μM and 1.0 μM PMA were used. Since the cells activated with 1.0 μM PMA did not show a significant difference in O_2^- production compared to those stimulated with 0.25 μM PMA, this last concentration was chosen to be used in the following experiments.

4.2. SUPEROXIDE GENERATION IN OPSONIZED ZYMOSAN STIMULATED CELLS

A similar experiment to the previous one was performed using different concentrations of OZ (Figure 3). As shown in Figure 3, there was a dose-dependent effect of OZ on O_2^- generation. It also appears that an OZ concentration of 3.0 mg/ml was similar to 1.5 mg/ml in its ability to cause the

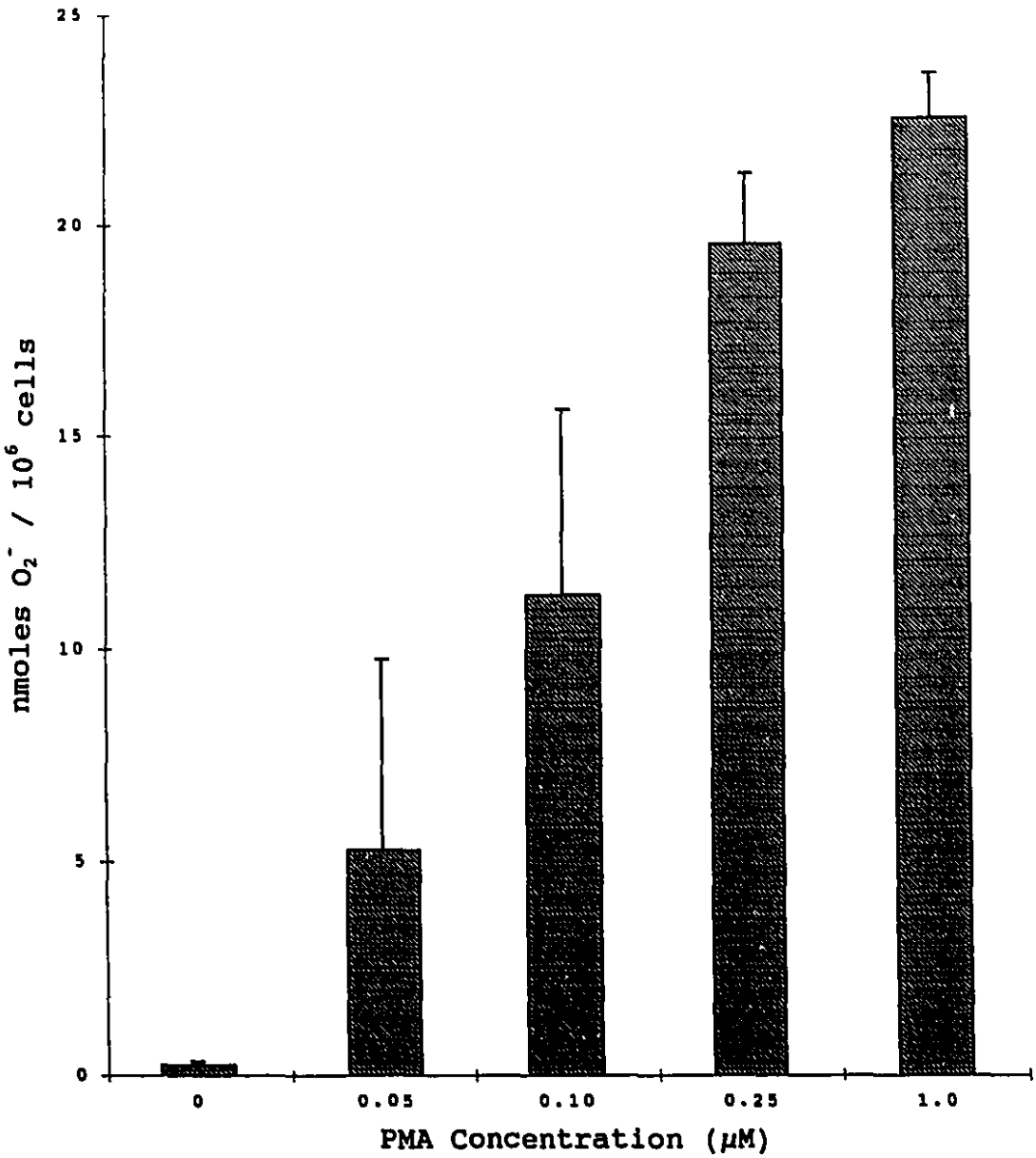

Figure 2. Superoxide anion generation triggered by PMA in bovine neutrophils. Neutrophils (2.5 x 10^6/ml) were activated with different concentrations of PMA (0, 0.05, 0.1, 0.25, 1.0 μM) for 30 min at 37°C. O_2^- production was assessed by the SOD-inhibitable reduction of cytochrome C. Results shown are means ± SEM for four independent experiments. Each experiment was performed in duplicate.

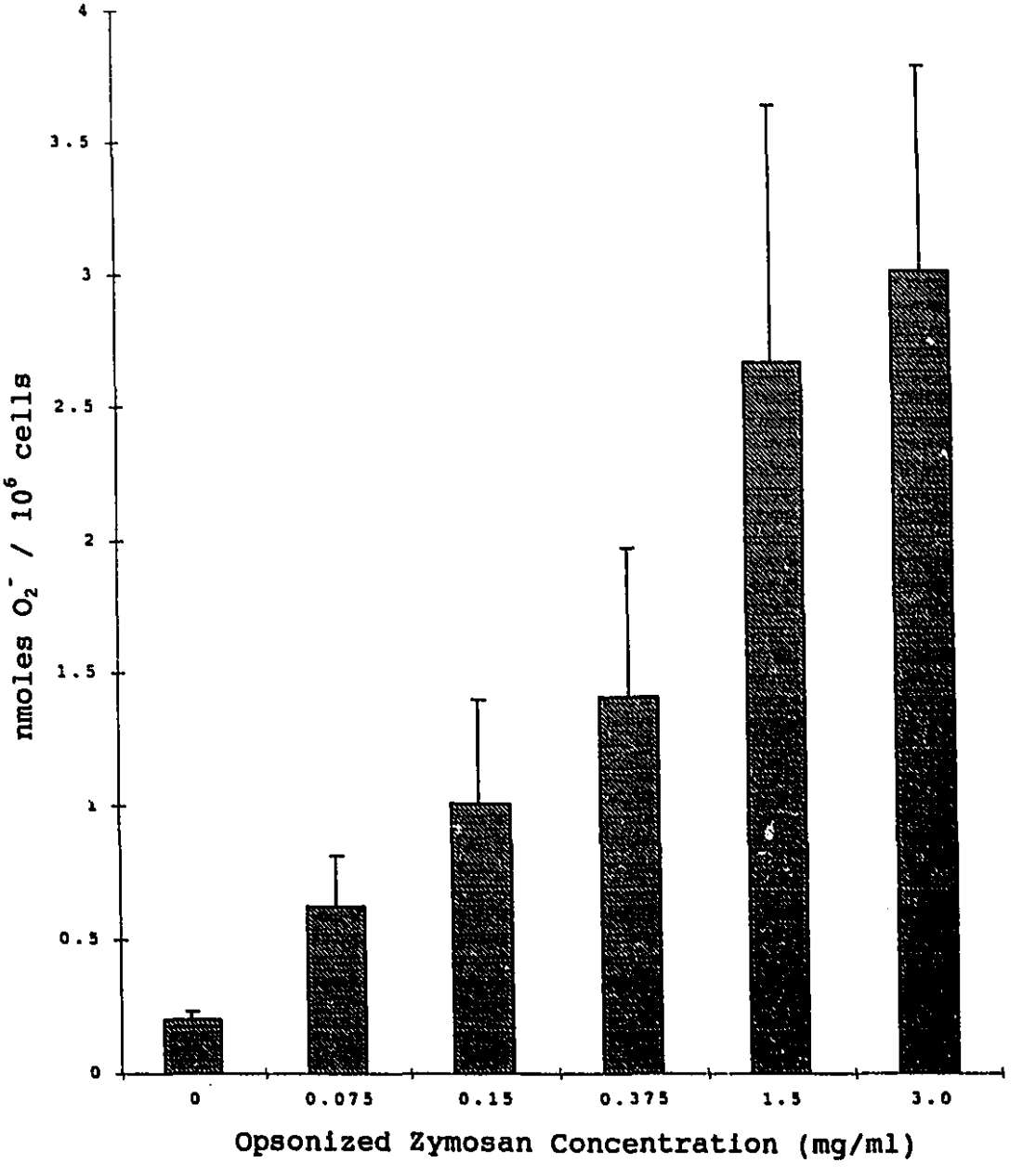

Figure 3. Superoxide anion generation triggered by opsonized zymosan in bovine neutrophils. Neutrophils (4.5 x 10^6/ml) were stimulated with various OZ concentrations (0, 0.075, 0.15, 0.375, 1.5, 3.0 mg/ml) for 30 min at 37°C. O_2^- production was assessed by the SOD-inhibitable reduction of cytochrome C. Results shown are means ± SEM for three independent experiments which were performed in duplicate.

generation of O_2^-. Consequently, 1.5 mg/ml OZ was chosen to be used for the following experiments.

4.3. REQUIREMENTS FOR EXTRACELLULAR CALCIUM IN SUPEROXIDE ANION RESPONSES IN PMA-STIMULATED CELLS

The importance of $[Ca_2^+]_o$ in the production of O_2^- when neutrophils were triggered by PMA was assessed using the Ca_2^+ chelator EGTA. As shown in Figure 4, stimulation of cells with PMA resulted in a high production of O_2^- (27.60 nmol/10^6 cells). Furthermore, the EGTA-treated cells generated O_2^- in quantities that were not statistically different from non-EGTA-treated cells. This demonstrated that chelation of $[Ca_2^+]_o$ did not have any effects on O_2^- production by PMA activated cells.

4.4. REQUIREMENT FOR EXTRACELLULAR CALCIUM IN SUPEROXIDE ANION RESPONSES IN OPSONIZED ZYMOSAN STIMULATED CELLS

Neutrophils stimulated with OZ clearly indicated NADPH activity through O_2^- production (5.86 nmol/10^6 cells; Figure 5). However, these OZ-activated cells showed a significant ($p,0.001$) decrease in O_2^- generation (2.13 nmol/10^6 cells) when $[Ca_2^+]_o$ was chelated prior to stimulation.

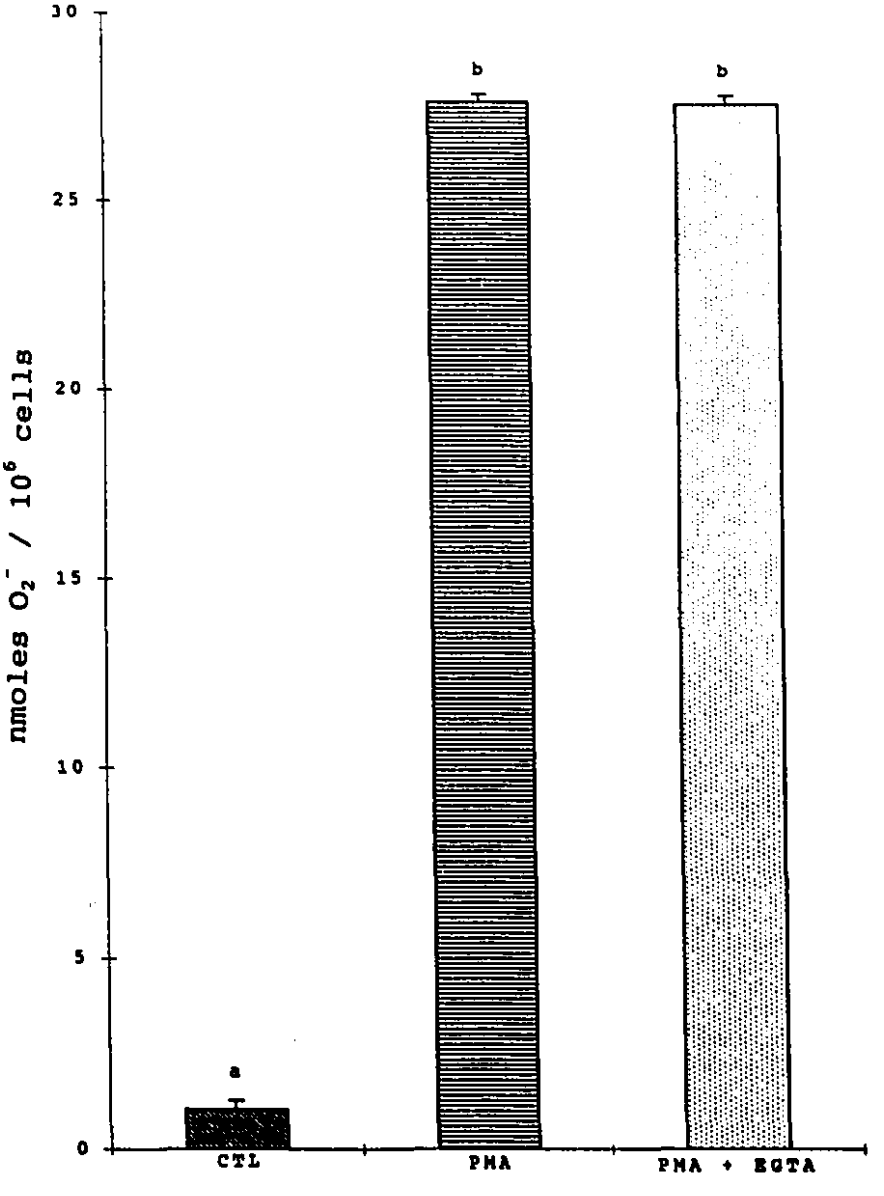

Figure 4. Effects of extracellular calcium chelation on PMA-induced response. Neutrophils (2.5 x 10^6/ml) were stimulated with or without PMA (0.25 μM) in the presence or absence of EGTA (10 mM) for 30 min at 37°C. O_2^- production was recorded as the SOD-inhibitable reduction of cytochrome C. The results shown are means ± SEM for duplicate determinations from an experiment which was representative of three experiments. Means with different letters are significantly different at $p < 0.001$.

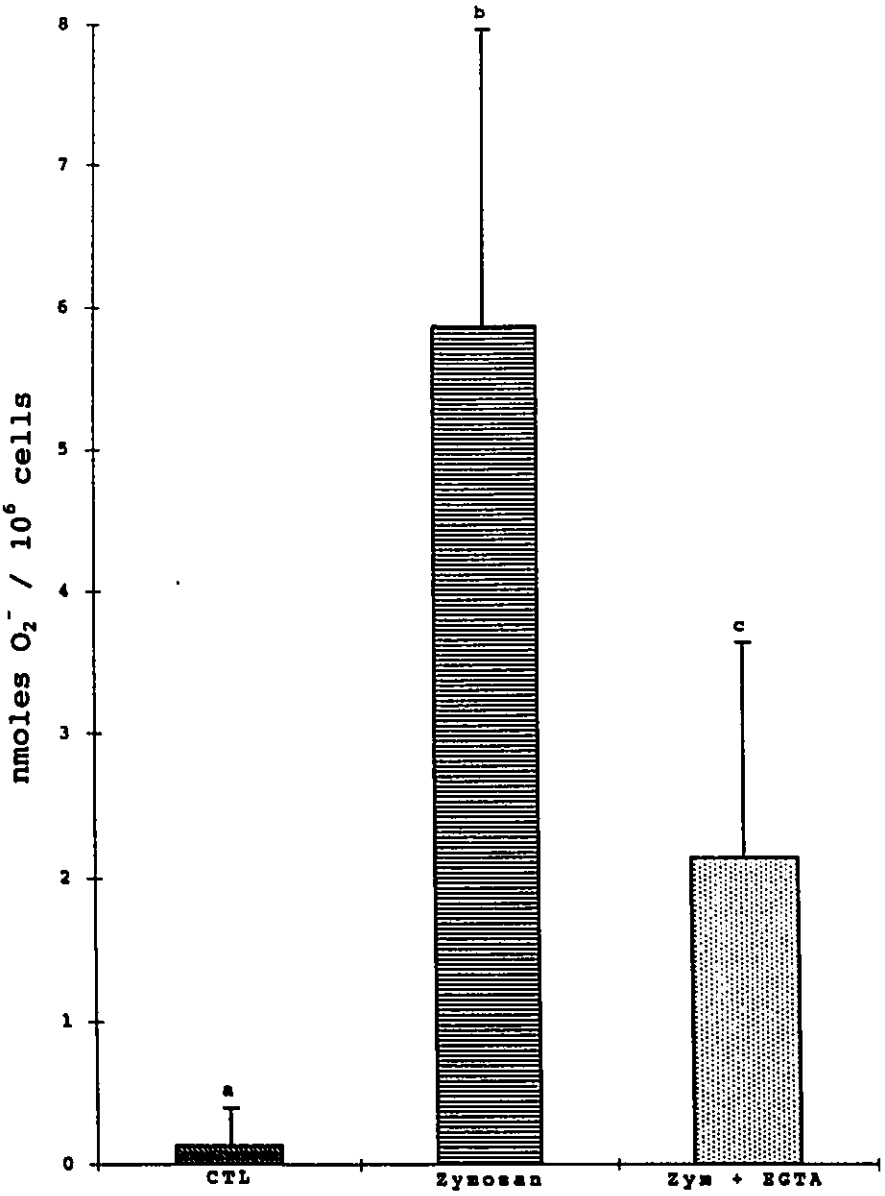

Figure 5. Effects of extracellular calcium chelation on zymosan-induced response. Neutrophils (4.5 x 10^6/ml) were activated with or without OZ (1.5 mg/ml) in the presence or absence of EGTA (10 mM) for 30 min at 37°C. O_2^- generation was measured as the SOD-inhibitable reduction of cytochrome C. The results in this figure are means ± SEM from five independent experiments. Means with different letters are significantly different at $p < 0.001$.

To verify whether or not the effects of EGTA were caused by a failure in the association of zymosan with the cells, measurements of the uptake of ^{125}I-labelled zymosan were performed (Figure 6). As shown, there was no difference in phagocytosis of zymosan between the untreated control and EGTA-treated neutrophils, suggesting that EGTA did not affect phagocytosis of OZ.

4.5. REQUIREMENTS FOR INTRACELLULAR CALCIUM IN SUPEROXIDE ANION RESPONSES IN PMA-STIMULATED CELLS

The requirements for $[Ca^{2+}]_i$ in PMA-activated cells were assessed by pre-loading the cells with different BAPTA/AM concentrations along with 10 mM EGTA. Extracellular Ca^{2+} chelation was performed to avoid any influx due to the low $[Ca^{2+}]_i$, thereby increasing free cytosolic Ca^{2+}. Comparison tests performed for O_2^- generation between BAPTA/AM-treated cells (165 μM) plus 10 mM EGTA and BAPTA/AM-treated cells (165 μM) without EGTA showed no significant differences, thus allowing us to use EGTA without influencing the true effects of BAPTA/AM (Figure 7).

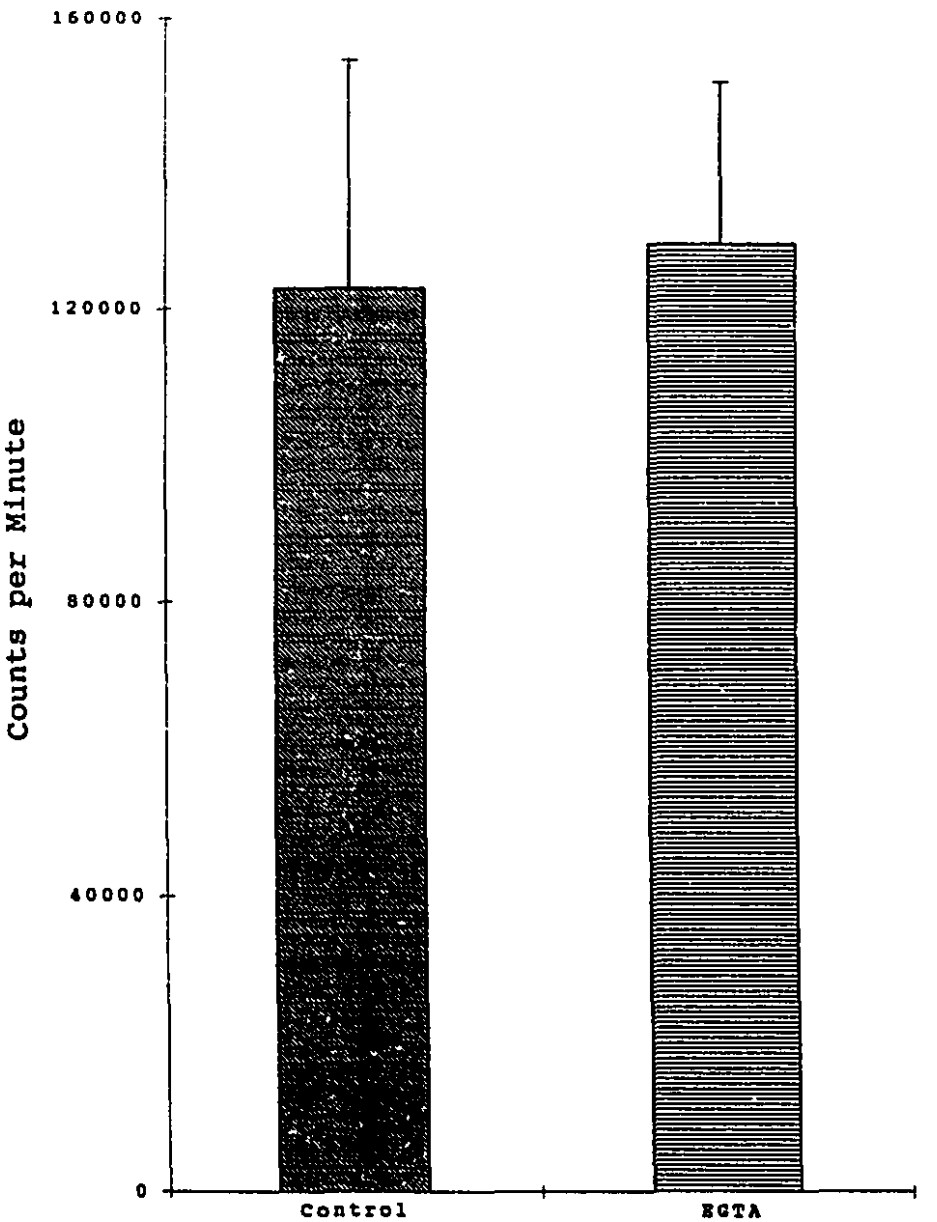

Figure 6. Uptake of ^{125}I-labelled opsonized zymosan by control and EGTA-treated cells. Neutrophils (4.5 x 10^5/ml) were incubated for 30 min at 37°C with or without EGTA. The uptake of zymosan was then measured by determining cell-associated radioactivity following exposure to 0.1 mg/ml (200,000 cpm/ml) ^{125}I-zymosan for 30 min at 37°C. The results in this figure are the mean ± SEM for duplicate determinations from three independent experiments.

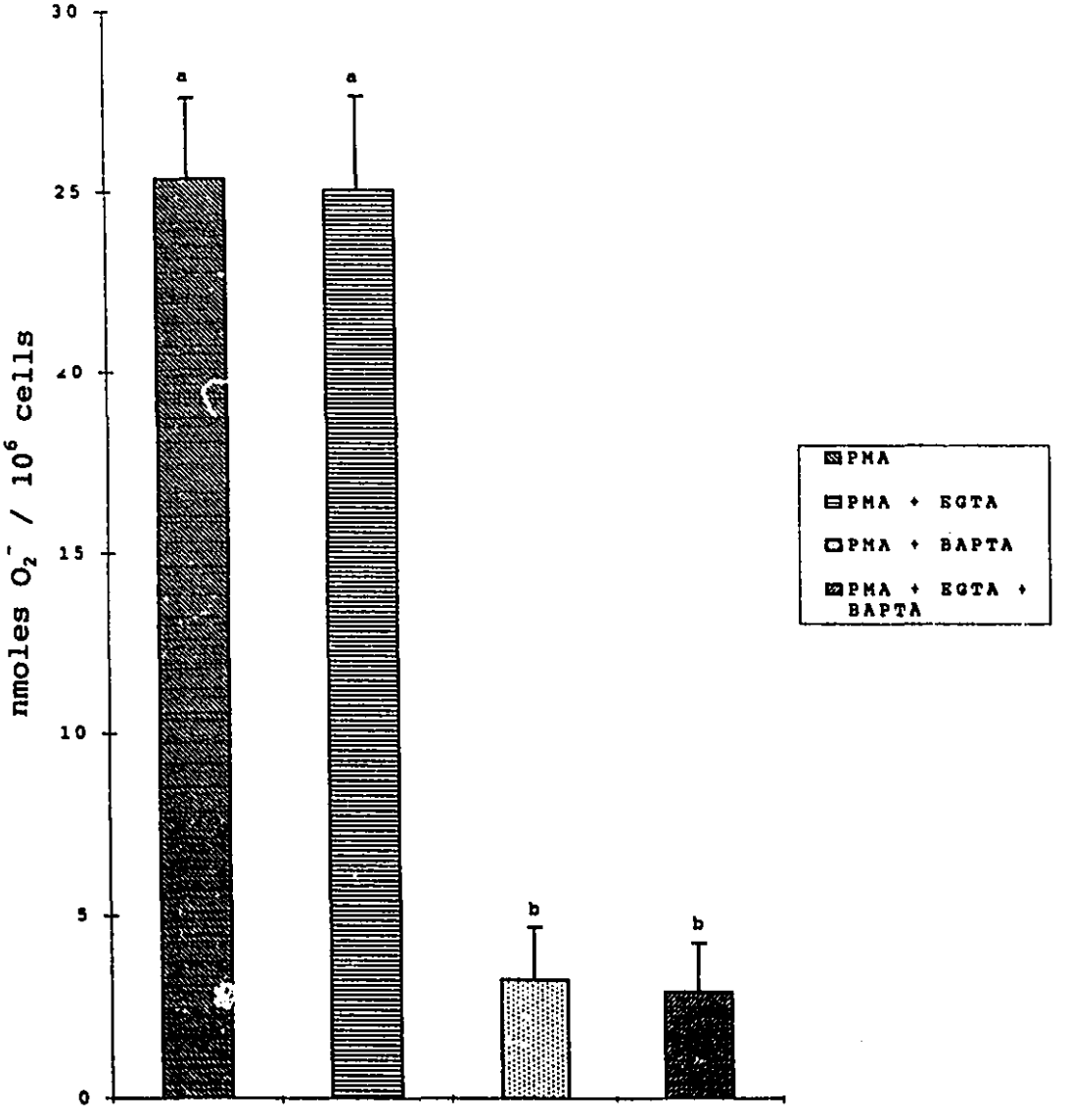

Figure 7. Effect of extracellular calcium chelation on PMA-induced response of neutrophils treated with or without BAPTA/AM. Neutrophils (2.5 x 10^6/ml) were incubated for 30 min at 37°C with or without BAPTA/AM (165 μM) in the presence or absence of EGTA (10 mM) prior to a PMA stimulation (0.25 μM) for 30 min at 37°C. Controls for BAPTA/AM-treated cells were incubated with DMSO (165 μM). O_2^- production was assessed by the SOD-inhibitable reduction of cytochrome C. The results shown are means ± SEM for three independent experiments which were accomplished in duplicate. Means with different letters are significantly different at $p < 0.001$.

Figure 8 clearly demonstrates that a lower $[Ca^{2+}]_i$, caused by elevated BAPTA/AM concentrations, led to a decrease respiratory burst induced by PMA. In fact, BAPTA/AM concentrations of 50 and 165 μM led to a reduction of O_2^- generation by 23.0 and 88.4%, respectively.

4.6. REQUIREMENTS FOR INTRACELLULAR CALCIUM IN SUPEROXIDE ANION RESPONSES IN OPSONIZED ZYMOSAN-STIMULATED CELLS

The O_2^- production was measured in BAPTA/AM-treated cells followed by OZ stimulation in order to assess the importance of $[Ca^{2+}]_i$. As with PMA activation, O_2^- generation was compared between 50 μM BAPTA/AM-treated cells plus 10 mM EGTA and 50 μM BAPTA/AM-treated cells without EGTA. As expected, chelating the $[Ca^{2+}]_o$ masked the effects of $[Ca^{2+}]_i$ chelation (Figure 9). These results demonstrate the importance of $[Ca^{2+}]_o$ for O_2^- production in OZ-stimulated cells as was observed in Figure 5. Therefore, EGTA was not used for the subsequent experiment which evaluated the dose-dependence effect of BAPTA/AM on O_2^- generation. Surprisingly, as can be observed in Figure 10, increased BAPTA/AM concentrations, leading to lower $[Ca^{2+}]_i$, caused the OZ-stimulated cells to generate greater amounts of O_2^-. Indeed, treatments with 10, 25, and 50 μM caused the

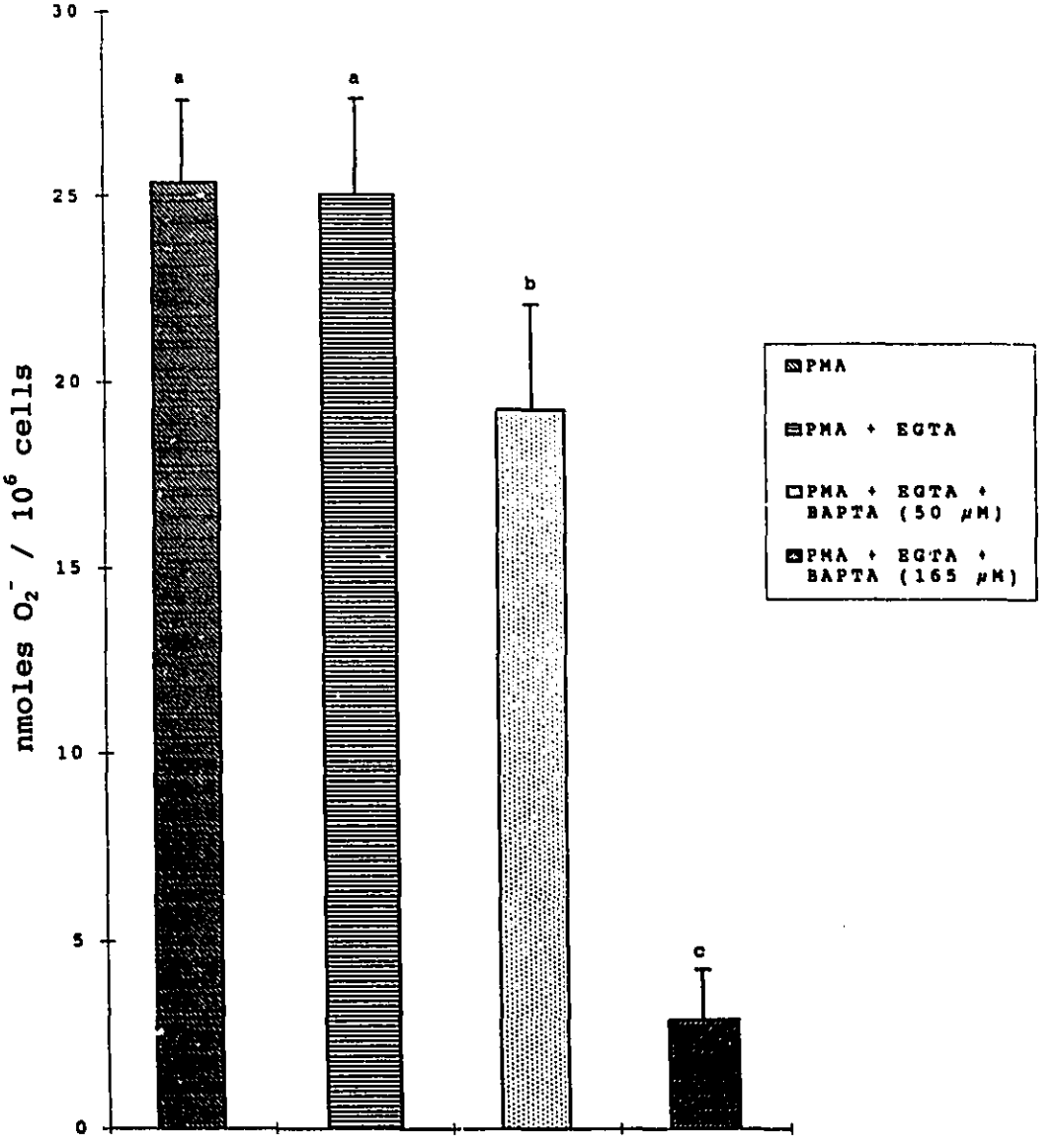

Figure 8. Superoxide anion responses of BAPTA/AM-loaded neutrophils exposed to PMA. Neutrophils (2.5 x 10^6/ml) were incubated for 30 min at 37°C with BAPTA/AM (50 μM, 165 μM) in the presence of EGTA (10 mM) after which they were stimulated with PMA (0.25 μM) for 30 min at 37°. All samples contained a total of 165 μM DMSO. O_2^- generation was recorded as the SOD-inhibitable reduction of cytochrome C. The results in this figure are means ± SEM from 3 independent experiments. Means with different letters are significantly different at $p < 0.001$.

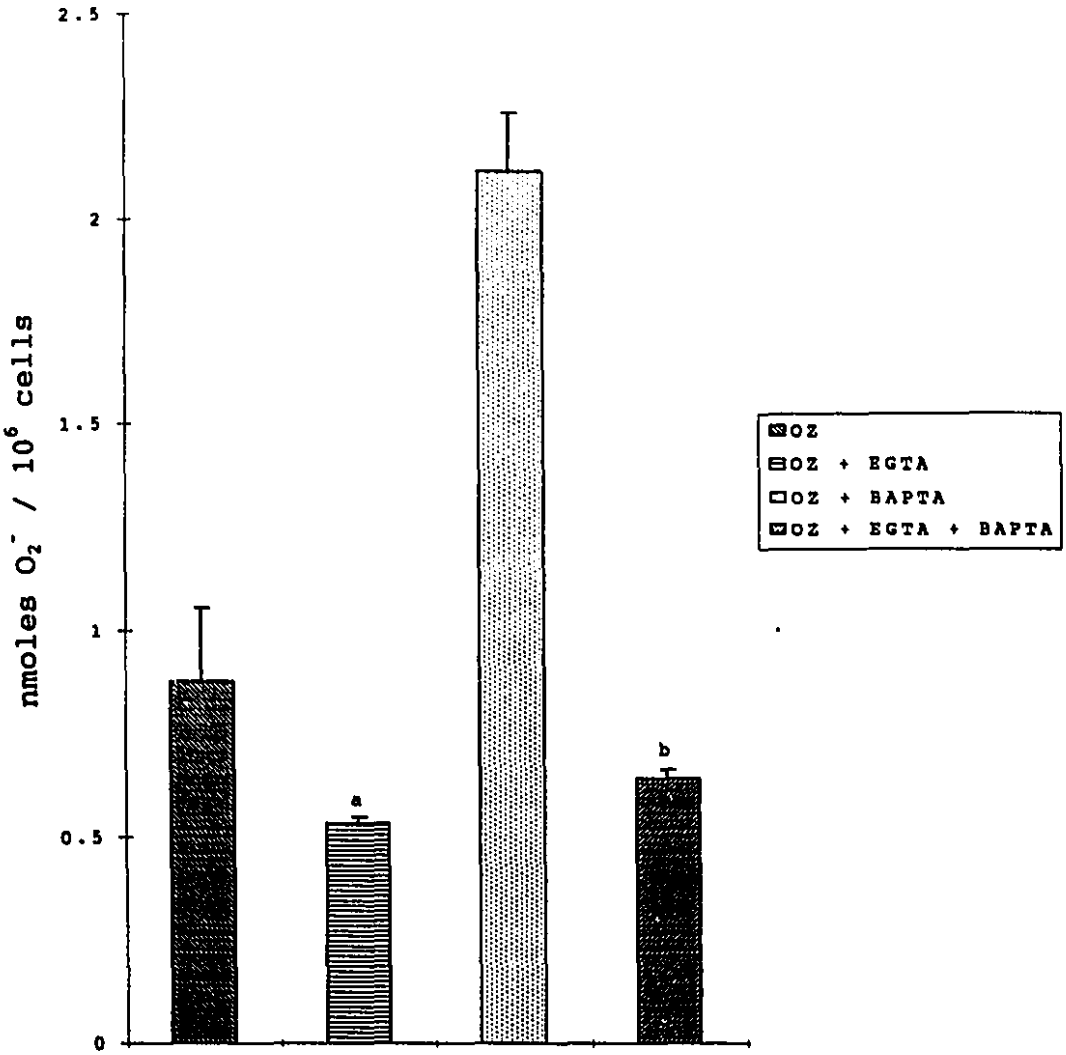

Figure 9. Effects of extracellular calcium chelation on opsonized zymosan-induced response of neutrophils treated with or without BAPTA/AM. Neutrophils (4.5×10^6/ml) were incubated for 30 min at 37°C with or without BAPTA/AM (50 μM) in the presence or absence of EGTA (10 mM). The cells were then washed twice and incubated for a further 30 min at 37°C with OZ (1.5 mg/ml). Controls for BAPTA/AM-treated cells were incubated with DMSO (50 μM). O_2^- production was measured by the SOD-inhibitable reduction of cytochrome C. Values are means ± SEM for duplicate determinations from an experiment which was representative of three done. [a]$p < 0.001$ for comparison to OZ-stimulated cells alone. [b]$p < 0.001$ for comparison to BAPTA/AM-treated cells activated by OZ in the absence of EGTA.

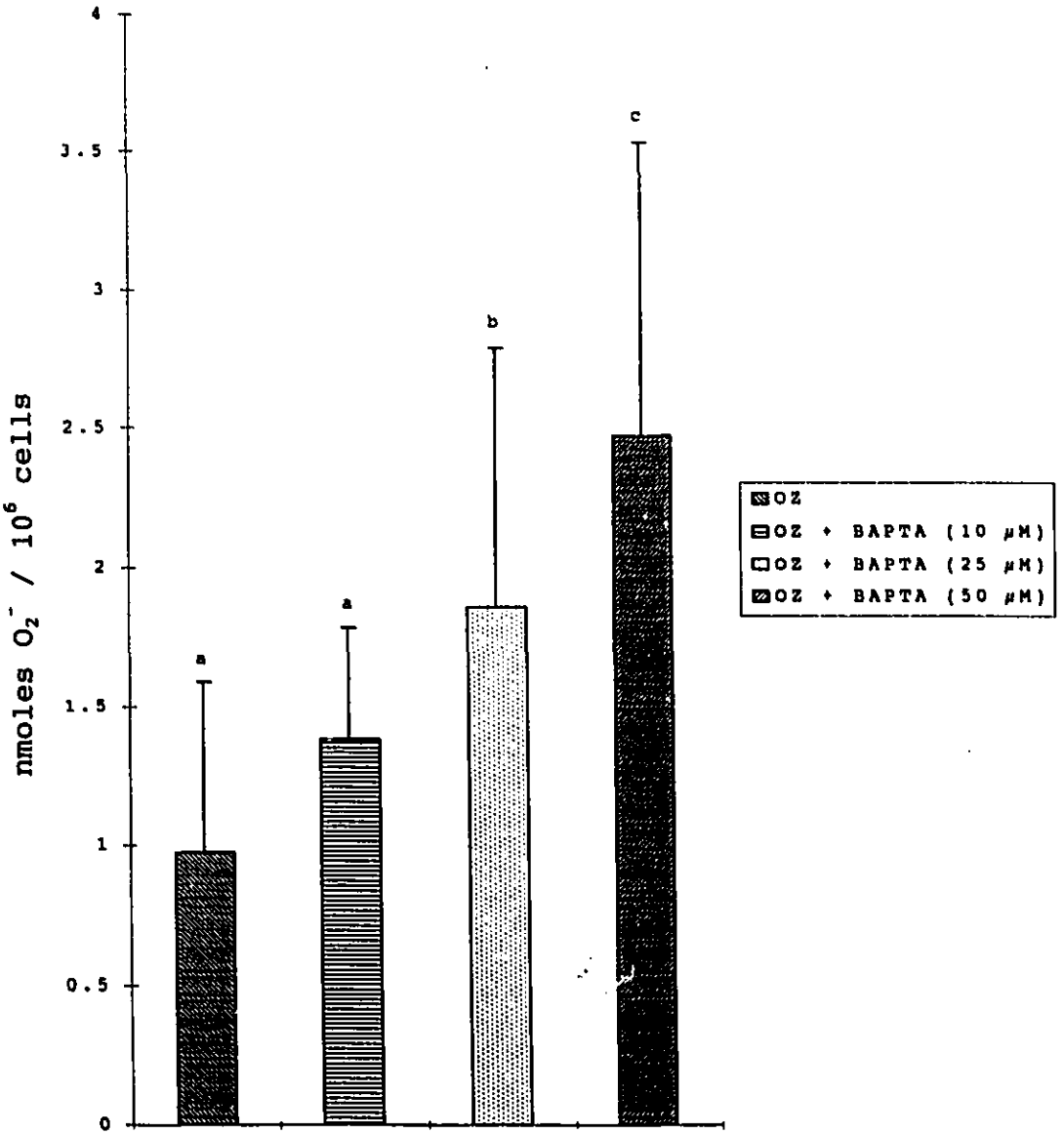

Figure 10. Superoxide anion generations of BAPTA/AM-loaded neutrophils exposed to opsonized zymosan. Neutrophils (4.5 x 10^6/ml) were incubated with BAPTA/AM (10 μM, 25 μM, 50 μM) for 30 min at 37°C. All samples contained a total of 50 μM DMSO. The cells were then washed twice followed by OZ-stimulation for 30 min (37°C). O_2^- production was reported as the SOD-inhibitable reduction of cytochrome C. Results shown are means ± SEM from 3 independent experiments. Means with different letters are significantly different from non-BAPTA/AM-treated cells activated by OZ. Means with different letters are significantly different at $p < 0.001$.

neutrophils to increase O_2^- production by 1.4, 1.9, and 2.5 fold, respectively. Furthermore, Figure 11 shows that there was no difference in phagocytosis of zymosan between control (DMSO adjusted) and BAPTA/AM-treated cells.

4.7. IMPORTANCE OF PKC ACTIVATION ON SUPEROXIDE ANION RESPONSES IN PMA- OR OPSONIZED ZYMOSAN-STIMULATED CELLS

Cellular cytosol fractions as well as membrane fractions were isolated following PMA- or OZ-stimulation in order to measure PKC activity. As expected, PMA significantly ($p < 0.01$) increased the protein activity at the membrane level (Figure 12). A slight increase (statistically not significant) was also seen in the cytosolic portion of the cells. Furthermore, OZ failed to activate the measured enzyme, PKC, as can be seen in Figure 12.

To further investigate the possible involvement of PKC in the response of neutrophils to PMA, the influence of 1 μM of the PKC inhibitor, Calphostin C, on the respiratory burst was determined. As shown in Figure 13, a 68.7% decrease in O_2^-

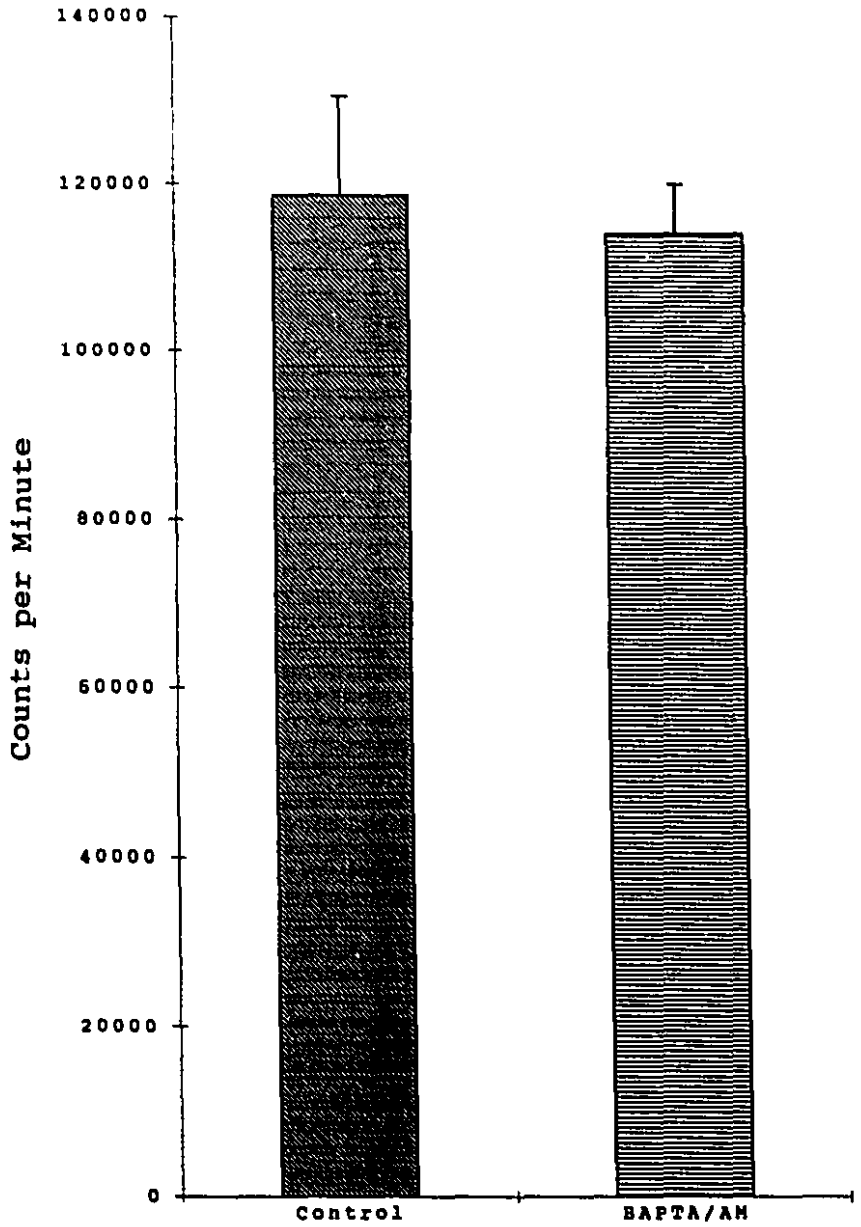

Figure 11. Uptake of ^{125}I-labelled opsonized zymosan by control and BAPTA/AM-treated cells. Neutrophils (4.5 x 10^5/ml) were incubated for 30 min at 37°C with either DMSO (50 μM) or BAPTA/AM (50 μM). The cells were then washed and the uptake of zymosan was measured by determining cell-associated radioactivity following exposure to 0.1 mg/ml (200,000 cpm/ml) ^{125}I-zymosan for 30 min at 37°C. The results in this figure are the mean ± SEM for duplicate determinations from three independent experiments.

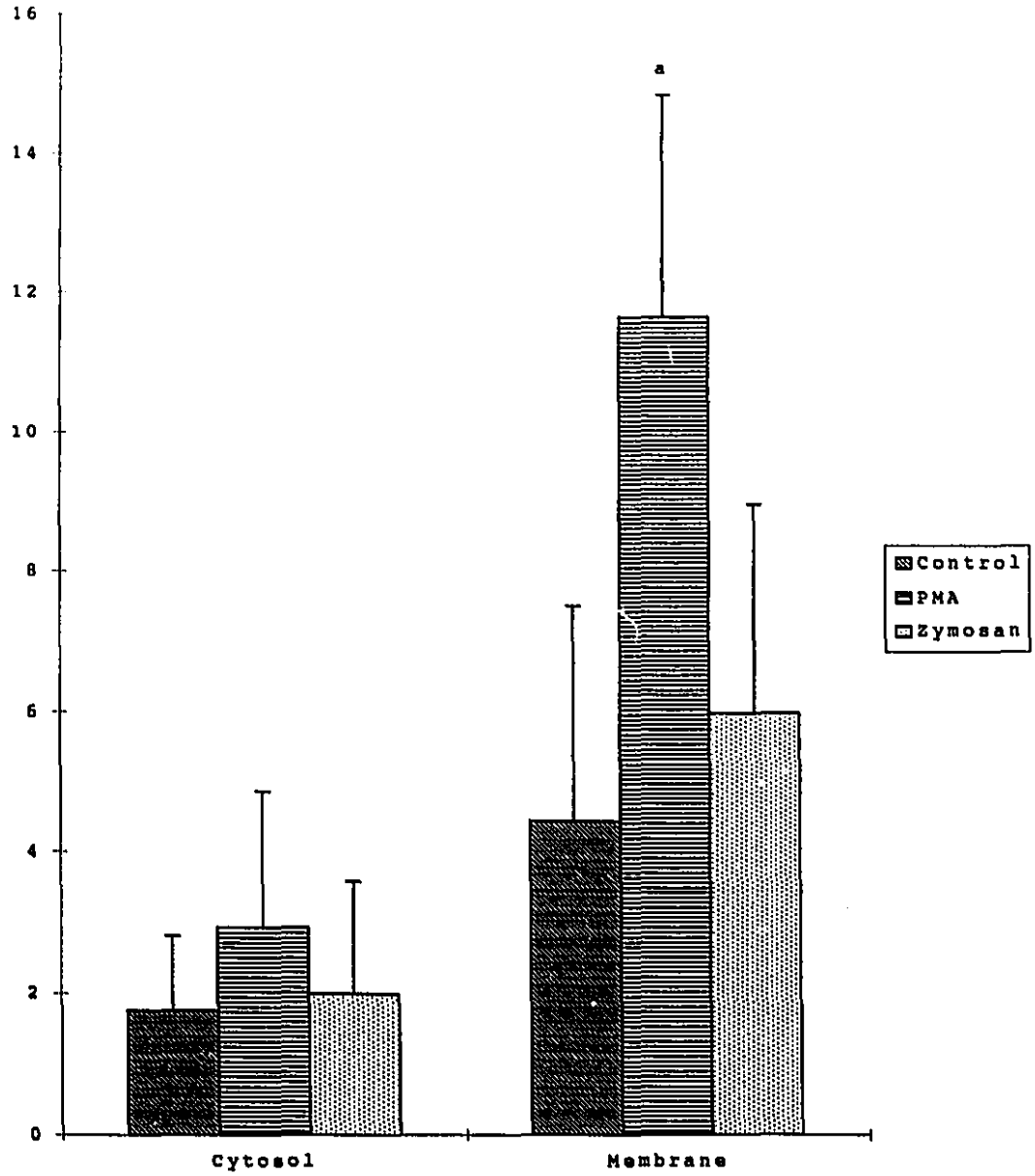

Figure 12. PKC activity of PMA- or opsonized zymosan-stimulated neutrophils. 2.5 x 10^7/ml and 4.5 x 10^7/ml were incubated at 37°C for 15 min with 2.5 μM PMA and 1.5 mg/ml OZ, respectively. An additional sample containing the control cells (2.5 x 10^7/ml) was also incubated under the same conditions with the exception of the non-addition of stimuli. PKC activity was measured as described under ''Materials and Methods''. The results are means ± SEM from 3 independent experiments. $^a p < 0.01$ for comparison to membrane fraction of unstimulated cells (control).

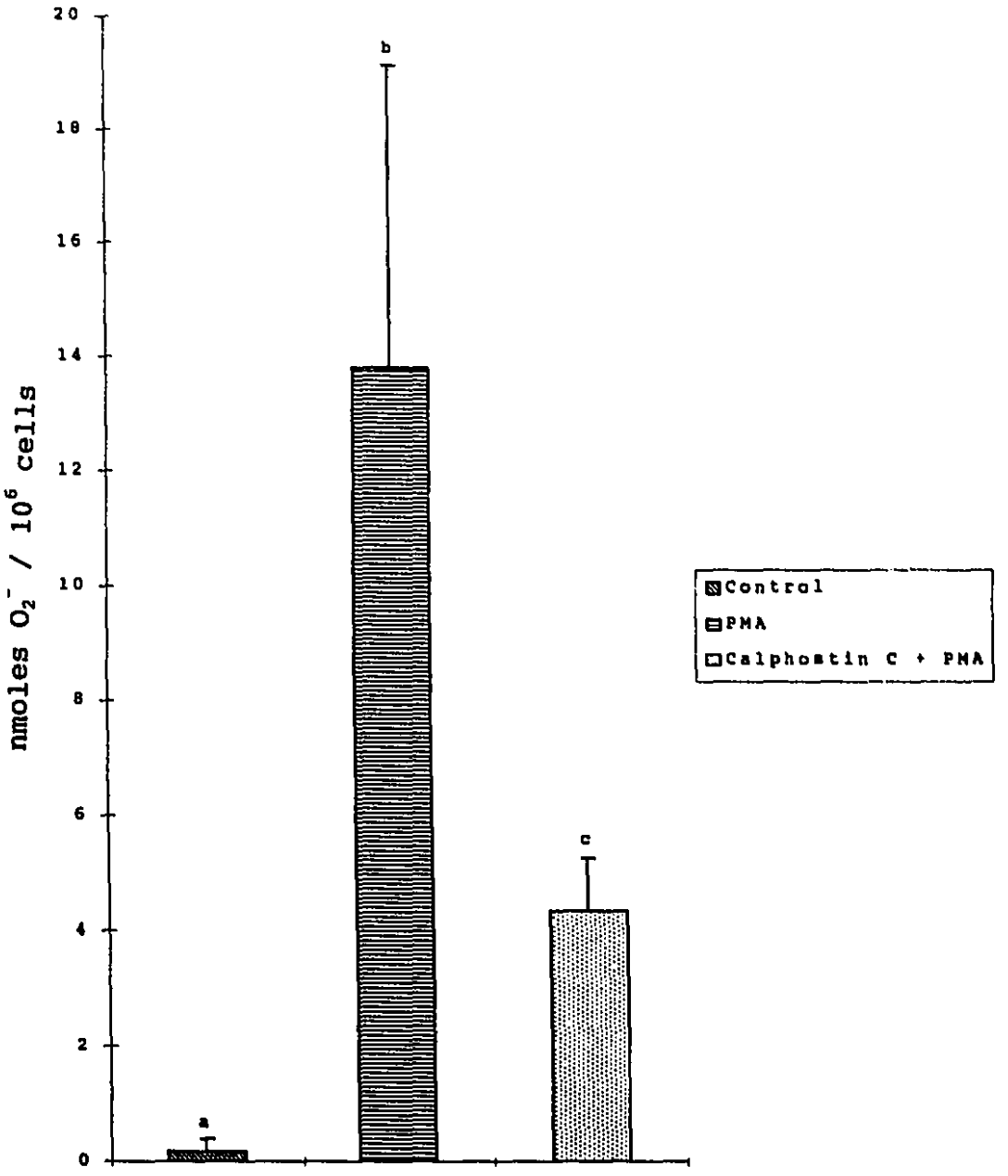

Figure 13. Superoxide anion responses of Calphostin C-treated neutrophils exposed to PMA. Neutrophils (2.5 x 10^6/ml) were treated with 1 μM Calphostin C for 60 min at room temperature under ordinary fluorescent light. The cells were then incubated with 0.25 μM PMA for 30 min at 37°C. O_2^- generation was measured according to the cytochrome C reduction. Results represent the means ± SEM of three independent assays. Means with different letters are significantly different at $p < 0.05$.

production occurred when the cells were pre-treated with 1 μM of the inhibitor.

Moreover, even though OZ did not cause any significant increase in PKC activity, a similar experiment was carried out in order to verify that O_2^- production was truly independent of PKC activity when OZ was the stimulating factor. The results obtained are shown in Figure 14 and confirm our assumption that OZ stimulation of NADPH oxidase activity is independent of PKC activation.

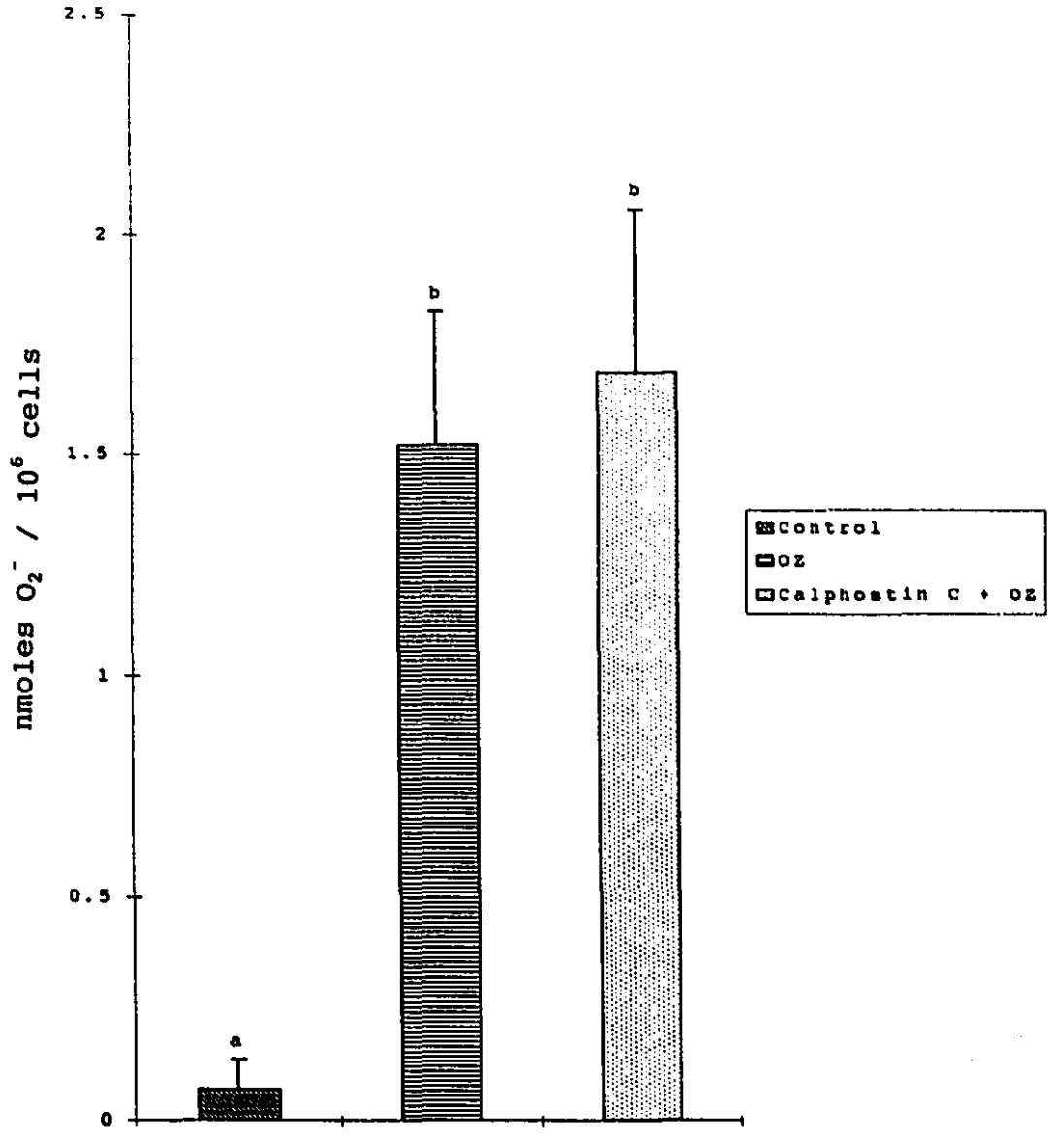

Figure 14. Superoxide anion responses of Calphostin C-treated neutrophils exposed to opsonized zymosan. Neutrophils (4.5 x 10^6/ml) were treated with 1 μM Calphostin C for 60 min at room temperature under ordinary fluorescent light. Following this, the cells were incubated at 37°C with OZ (1.5 mg/ml) for 30 min. O_2^- responses were assessed by the SOD-inhibitable reduction of cytochrome C. The presented results symbolize the means ± SEM of three individual experiments. Means with different letters are significantly different at $p < 0.001$.

CHAPTER V. DISCUSSION

The respiratory burst is a critical function involved in the killing ability of activated neutrophils (Baggiolini et al., 1993; Thelen et al., 1993). This study modified $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$ and monitored PKC activity and O_2^- production following stimulation by either a particulate or soluble agent to investigate parts of the signal transduction sequence in stimulated bovine neutrophils. The various findings from this investigation have unveiled several significant and previously unknown aspects of O_2^- production by bovine neutrophils.

Both PMA- and OZ-stimulated neutrophils have generated O_2^- at concentrations similar to those reported by others. In fact, Doré et al. (1991) stimulated bovine neutrophils for 10 min at 37°C with either 100 ng/ml (0.16 μM) or 500 ng/ml (0.81 μM) PMA and obtained approximate averages of 25 ± 6 and 24.5 ± 5.5 nmol $O_2^-/10^6$ cells, respectively. These results are comparable to those obtained in this study i.e. 19.5 ± 1.7 nmol $O_2^-/10^6$ cells and 22.4 ± 1.1 nmol $O_2^-/10^6$

cells using 0.25 μM and 1.0 μM PMA, respectively (Figure 2). Moreover, stimulation with 1.5 mg/ml OZ led to the generation of 2.67 ± 0.97 nmol $O_2^-/10^6$ cells which agrees with results by Young and Beswick (1986) who obtained 2.41 ± 0.51 nmol $O_2^-/10^6$ cells under similar conditions.

It is also interesting to note that PMA-stimulation led to a much higher O_2^- response compared to OZ-stimulation. No explanation for this phenomenon can be given at this time.

5.1. PMA AS A STIMULUS

This study showed that O_2^- generation following PMA stimulation was independent of the $[Ca^{2+}]_o$ since no decrease in the anion could be observed after chelating $[Ca^{2+}]_o$. These results are in accordance with those reported by Tarsi-Tsuk and Levy (1990) in human monocytes. They used the isotope $^{45}Ca^{2+}$ to measure the Ca^{2+} influx following PMA stimulation and did not observe any uptake in Ca^{2+} even though these cells showed a marked increase in cytochrome C reduction when compared with the control group. These results indicated that $[Ca^{2+}]_o$ was not needed for the monocytes to

produce O_2^- subsequent to PMA activation. Similar findings were obtained by Korchak and associates (1988b) who also measured the Ca^{2+} uptake of PMA activated human neutrophils using the same isotope i.e. $^{45}Ca^{2+}$. As expected, no Ca^{2+} uptake was observed although the cells generated significant amounts of O_2^-. Once again, it appears that neutrophils of both bovine and human origin do not require $[Ca^{2+}]_o$ to discharge O_2^- when stimulated with PMA.

In addition to the determination of Ca^{2+} influx, some authors also measured the changes in cytosolic Ca^{2+} subsequent to PMA stimulation. Using rabbit neutrophils, Sha'afi et al. (1983) reported that PMA induced an increase in oxygen consumption without a subsequent rise in the level of $[Ca^{2+}]_i$, as measured using the fluorescent probe Quin-2/AM. Comparable results were obtained with human neutrophils in a study performed by Pike et al. (1991) as well as the one conducted by Korchak and colleagues (1988b) who measured the cytosolic Ca^{2+} of the cells in the presence or absence of $[Ca^{2+}]_o$ using Fura-2/AM and EGTA. Their results revealed that O_2^- generation was independent of any $[Ca^{2+}]_i$

increase, either from a Ca^{2+} influx or from their release from Ca^{2+} stores, following a PMA activation of rabbit or human neutrophils. Further, Korchak et al., (1988b) also noted that the addition of PMA to the cells did not trigger an increase in inositol 1,4,5-triphosphate (IP_3), inositol biphosphate (PIP_2), or inositol phosphate (PI) which is concordant with the absence of an $[Ca^{2+}]_i$ increase.

According to the reported results described above, it would be reasonable to postulate that NADPH oxidase activation in human and rabbit neutrophils is independent of $[Ca^{2+}]_o$ as well as $[Ca^{2+}]_i$. However, the findings with bovine neutrophils have shown a different picture. A 23 and an 88% decrease in O_2^- generation in cells treated with EGTA and 50 or 165μM BAPTA/AM, respectively, were detected. The exact reason why bovine neutrophils are different from human or rabbit neutrophils in terms of $[Ca^{2+}]_i$ dependency is unknown. Young and Beswick (1986) demonstrated a qualitative and a quantitative difference in the respiratory burst of bovine, porcine and ovine neutrophils compared to human neutrophils. They have shown that the neutrophils from the various farm

animals could not be stimulated by fMLP to increase O_2^- production. This is in complete contrast to the responses of human neutrophils which generated significant amounts of O_2. In addition to this qualitative difference, quantitative differences in O_2^- generation between human and animal cells were also reported by Young and Beswick (1986). In fact, human neutrophils showed a much stronger respiratory burst subsequent to OZ stimulation in contrast to bovine neutrophils. Our results with $[Ca^{2+}]_i$ requirement for O_2^- production exemplified yet another aspect of the difference between human neutrophils and bovine neutrophils.

Our results indicated that $[Ca^{2+}]_i$ in the bovine cells could be a limiting factor for O_2^- production upon stimulation. On the other hand, human neutrophils were reported to be independent of intracellular and extracellular Ca^{2+}, as mentioned previously, preventing this mineral from being a limiting factor when stimulated with PMA. Furthermore, it was noted that modulation of $[Ca^{2+}]_i$ can alter the rate of O_2^- production in these cells even though they do not require the mineral when maximally stimulated

with PMA (Di Virgilio, et al., 1984). This could indicate that Ca^{2+} plays a role in the signal transduction of human PMNs but is not strictly necessary for the eventual respiratory burst.

The following three tentative suggestions could partially explain why bovine neutrophils would require an $[Ca^{2+}]_i$ increase whereas human neutrophils would not when activated with optimal amounts of PMA: 1) the requirement of $[Ca^{2+}]_i$ for the interaction between PMA and the receptor in bovine PMNs as opposed to no Ca^{2+} dependence for the same interaction in human neutrophils; 2) the pathway implicated in the signal transduction of the respiratory burst following PMA stimulation in human PMNs is different from the one involved in bovine neutrophils which is $[Ca^{2+}]_i$-dependent. Since human neutrophils were shown to express Ca^{2+}-dependent as well as Ca^{2+}-independent PKCs (Thelen et al., 1993), activation of these cells with PMA could turn on the Ca^{2+}-independent PKC, leading to the respiratory burst. However, to the best of our knowledge, no studies in bovine neutrophils have been conducted in order to verify whether

only Ca^{2+}-dependent PKC isoenzymes were expressed which might explain the absolute requirement for $[Ca^{2+}]_i$; 3) the variance of the NADPH oxidase multicomponents. Perhaps one or more of the human oxidase components is/are different from the bovine ones which might require a Ca^{2+} increase for ultimate NADPH oxidase activation. These speculations attempting to explain the difference in $[Ca^{2+}]_i$ requirements between bovine and human neutrophils warrant further investigation.

This study also demonstrated that PKC activity at the membrane level was significantly higher in PMA-stimulated cells than in the control cells. These results are in agreement with many reports on human neutrophils activated with either PMA or other phorbol esters (May et al., 1985; Wolf et al., 1985a; Wolf et al., 1985b). It is of interest to note that Wolf and associates (1985a) mentioned that PKC activators, like PMA, regulate membrane binding as well as the enzyme activity. On the other hand, Ca^{2+} was said to regulate PKC enzyme translocation (Korchak et al., 1988a) and its binding to the cytosolic membrane (Wolf et al.,

1985a). In fact, it was proven that the rate of membrane association of PKC in the presence of PMA and low Ca^{2+} levels is much slower than the rate obtained at much higher Ca^{2+} concentrations (Wolf et al., 1985a; Wolf et al., 1985b; Horn and Karnovsky, 1986; Phillips et al., 1987). Therefore, when no sufficient amounts of Ca^{2+} are found in the cells, PKC binding to the membrane and its eventual activation can be inhibited. However, these reports contradict the results obtained by several researchers following PMA-stimulation of human neutrophils in the absence of $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$.

In order to verify if PKC activity is essential in O_2^- generation when bovine neutrophils are activated by PMA, the PKC inhibitor, Calphostin C, was used. In fact, a significant decrease in respiratory burst was observed following stimulation of PMA in the Calphostin C-treated cells, when compared with the control cells. This confirms that activation of the PKC enzyme is essential for PMA-induced O_2^- production in bovine neutrophils. As mentioned previously, phosphorylation of some NADPH oxidase proteins is extremely important for the enzyme to be active and PKC

appears to be highly responsible for this reaction in both human neutrophils (Doré et al., 1991; Kanno et al., 1995) and bovine neutrophils (Gennaro et al., 1986). In fact, Kramer et al. (1988) and Okamura et al. (1988) have observed a rapid phosphorylation of $p47^{phox}$ upon incubation of human neutrophils' cytosol with purified PKC. Moreover, phosphorylation of $p47^{phox}$ was proven to be necessary for the assembly of the oxidase (Rotrosen and Leto, 1990; Nauseef et al., 1991), and the deactivation of the cells was accompanied by a return to the dephosphorylated state (Babior, 1988). The extent of the enzyme's assembly was also shown to closely match the intensity of the O_2^- generation (Heyworth and Segal, 1986; Okamura et al., 1988).

According to the similarities in the respiratory burst of our results and the numerous publications cited above, it would be reasonable to speculate that bovine neutrophils require PKC phosphorilation of some of the NADPH oxidase proteins, when stimulated with PMA, to produce O_2^-. Furthermore, the fact that PKC requires Ca^{2+} for its activation could explain, to some extent, the absence of a

respiratory burst obtained in the $[Ca^{2+}]_i$-depleted bovine cells.

5.2. OPSONIZED ZYMOSAN AS A STIMULUS

When OZ was used to stimulate the cells, different results were observed compared to PMA activation, which clearly indicated a different signal transduction pathway.

Firstly, bovine neutrophils activated with OZ showed a drastic 64% reduction in O_2^- when 10 mM EGTA was added to the cell suspension. Since no differences in phagocytosis could be found between the control cells and the EGTA-treated cells, it clearly indicated that signal transduction regarding the NADPH-oxidase activation of OZ-activated PMNs was $[Ca^{2+}]_o$-dependent. This was expected since the ingestion step of phagocytosis in human neutrophils was independent of Ca^{2+} (Scharff and Foder, 1993) and the following steps required elevations of $[Ca^{2+}]_i$: fusion of specific granules with the phagosomal membrane and production of O_2^- (Jaconi et al., 1990).

To the best of our knowledge, no studies have previously been conducted with bovine PMNs using similar conditions i.e. OZ-stimulation in the presence of EGTA. However, attempts can be made to compare our results with the ones by Maridonneau-Parini et al. (1986) who used OZ particles to stimulate the respiratory burst in human peripheral neutrophils. Their results showed that stimulation by OZ was $[Ca^{2+}]_o$-dependent which is concordant with our study. Furthermore, when they increased $[Ca^{2+}]_o$ using $CaCl_2$, to their EGTA-containing cell suspension, the O_2^- generation of these cells also increased proportionally. These results clearly show the $[Ca^{2+}]_o$-dependence of OZ-stimulated respiratory burst in human neutrophils which appears to be similar to bovine neutrophils.

Another study using a different receptor agonist was also performed on human peripheral blood monocytes and led to similar results (Tarsi-Tsuk and Levy, 1990). The authors used LTA from Steptococcus faecalis to stimulate the respiratory burst of the cells involved and noted a

significant dependence on $[Ca^{2+}]_o$ for the O_2^- generation which was also proportional to the $[Ca^{2+}]_o$.

Regarding $[Ca^{2+}]_i$, our study showed that as the BAPTA concentration increased, rendering $[Ca^{2+}]_i$ less accessible, a stronger respiratory burst occured. A possible explanation for these surprising results is a dependence of $[Ca^{2+}]_o$ influx of the OZ-activated neutrophils. In other words, a stronger Ca^{2+} influx, which could be caused by a low $[Ca^{2+}]_i$, would lead to a stronger respiratory burst. The reverse is also true. This speculation could explain the significant decrease in O_2^- production when a chelation of $[Ca^{2+}]_o$ was performed. There is a lack of direct evidence to support this speculation. However, as was previously mentioned, LTA might activate the NADPH oxidase in a way similar to OZ. Tarsi-Tsuk and Levy (1990) noted a significant increase in $^{45}Ca^{2+}$ uptake in the LTA-treated cells compared to control monocytes showing an importance for Ca^{2+} influx. The same authors also demonstrated that the $[Ca^{2+}]_i$ increase following LTA stimulation was mainly due to the Ca^{2+} influx since the addition of EGTA together with LTA did not increase $[Ca^{2+}]_i$.

From this, it can be said that O_2^- production by LTA-stimulated monocytes is Ca^{2+} influx-dependent since no Ca^{2+} release from Ca^{2+} stores could be observed and the lower the $[Ca^{2+}]_o$ was, the lower the respiratory burst would be. Similar mechanisms may work in bovine neutrophils in terms of the Ca^{2+} influx dependence of OZ-stimulated respiratory burst.

As expected, O_2^- production by OZ-activated cells was independent of PKC activation. This is in agreement with Kanno et al. (1995) who treated human neutrophils with α-tocopherol (vitamin E) in order to inhibit PKC prior to their stimulation with opsonized particles. Similar results were obtained in studies using H-7 to inhibit PKC activity (Maridonneau-Parini et al. 1986).

Since PKC is not involved in the OZ activation of neutrophils and phosphorylation is known to be important for the respiratory burst, it must be accomplished differently. One of the possibilities is the activation of protein-

tyrosine kinases (Le Cabec and Maridonneau-Parini, 1995) or protein-serine kinases. Another possibility is the inhibition of protein tyrosine phosphatases (Garcia-Morales et al., 1990; Stover et al., 1991).

According to Maridonneau-Parini and associates' results (1986), OZ caused a specific release of arachidonic acid (AA) which indicated the involvement of phospholipase A_2 (PLA_2) in the signal transduction leading to respiratory burst. Moreover, they noted a significant decrease in cytochrome C reduction in the cells treated with inhibitors of AA metabolisms (BW755C and indomethacin) or the ones incubated with mepacrine, a phospholipase inhibitor. From these, released AA, following OZ activation, was believed to play a mediating role in the NADPH-oxidase expression (Tarsi-Tsuk and Levy, 1990). These comments were consistent with those from other experiments (Cox et al., 1987; Muid et al., 1988). All of these studies can lead to certain speculations on the transduction signals involved in the stimulation of O_2^- production by OZ in bovine neutrophils. In fact, it seems reasonable to speculate that stimulation of

the neutrophils in our study by OZ could trigger the production of AA through PLA_2, consequently leading to the respiratory burst. Figure 15 summarizes the speculated pathway which could lead OZ to O_2^- production in bovine neutrophils.

A further justification for the proposal that OZ activates the NADPH-oxidase of bovine neutrophils by a PLA_2-mediated pathway is: oxidase activation by AA was shown to be PKC-independent (Cox et al., 1987) which is consistent with the results obtained by OZ stimulation.

In summary, this study showed that PMA-stimulated respiratory burst in bovine neutrophils was independent of $[Ca^{2+}]_o$, but dependent on $[Ca^{2+}]_i$. PMA was also shown to stimulate PKC which was necessary for an appropriate respiratory burst. Opsonized zymosan-stimulation was however proven to be dependent on $[Ca^{2+}]_o$. Extracellular Ca^{2+} did not affect phagocytosis of OZ. Moreover, as $[Ca^{2+}]_i$ decreased, O_2^- production increased. These suggest that OZ-induced O_2^- production depends on influx of $[Ca^{2+}]_o$. PKC activation, for

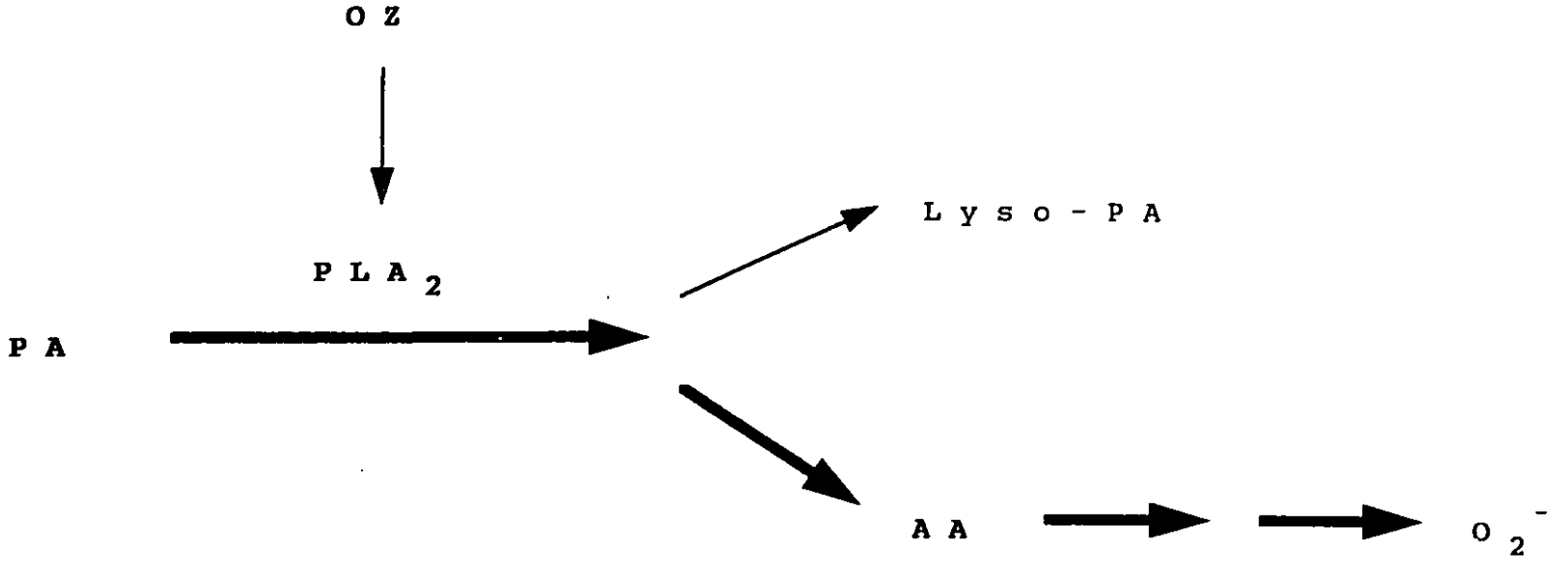

Figure 15. Generation of Superoxide in Bovine Neutrophils Stimulated with Opsonized Zymosan Particles. It is speculated that opsonized zymosan (OZ) would lead to the activation of phospholipase A_2 (PLA_2) which in turn would breakdown phosphatidic acid (PA) into lyso-phosphatidic acid (Lyso-PA) and arachidonic acid (AA). The released AA is then believed to lead to O_2^- generation.

its part, was absent and not required for an optimal OZ-induced respiratory burst.

CHAPTER VI. GENERAL CONCLUSIONS AND SPECULATIONS

Based on the results obtained in the present study, the following conclusions can be drawn:

6.1. PMA STIMULATION:

a) Superoxide production in bovine neutrophils is independent of $[Ca^{2+}]_o$.

b) Superoxide production in bovine neutrophils is dependent on the $[Ca^{2+}]_i$.

c) Active PKC is essential for O_2^- generation by bovine neutrophils.

These results imply that the signal transduction involved in activation of the NADPH oxidase following PMA stimulation requires sufficient amounts of $[Ca^{2+}]_i$ and the active form of PKC.

6.2. OPSONIZED ZYMOSAN STIMULATION:

a) Superoxide production in bovine neutrophils is dependent on the $[Ca^{2+}]_o$.

b) Superoxide production in bovine neutrophils is dependent on the Ca^{2+} influx. This dependence was indicated by an increased O_2^- generation of intracellularly Ca^{2+} chelated cells which, consequently, provoked an increase in Ca^{2+} influx.

c) Active PKC is not required for O_2^- generation by bovine neutrophils.

These results demonstrate that the signal transduction implied in the NADPH oxidase activation subsequent to OZ stimulation necessitate $[Ca^{2+}]_o$ and its influx.

REFERENCES

Abbas, A.K., Lichtman, A.H., and Pober, J.S. (1991) Cellular and molecular immunology. W.B. Saunders Company. Philadelphia, PA, USA.

Babior, B.M. (1988) Protein phosphorylation and the respiratory burst. Arch. Biochem. Biophys. 264:361-367.

Baggiolini, M., Boulay, F., Badwey, J.A., and Curnutte, J.T. (1993) Activation of neutrophil leukocytes: chemoattractant receptors and respiratory burst. FASEB J. 7:1004-1010.

Baggiolini, M. and Kernen, P. (1992) Neutrophil activation. Control of shape change, exocytosis and the respiratory burst. News Physiol. Sci. 7:215-219.

Beaumier, L., Faucher, N., and Naccache, P.H. (1987) Arachinonic acid-induced release of calcium in permeabilized human neutrophils. FEBS Lett. 221:289-292.

Bian, J., Ghosh, T.K., Wang, J.C., and Gill, D.L. (1991) Identification of intracellular calcium pools. Selective modification by thapsigargin. J. Biol. Chem. 266:8801-8806.

Borle, A.B. (1975) Methods for assessing hormone effects on calcium fluxes in vitro. Meth. Enzymol. 39:513-574.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Bruns, R.F., Miller, F.D., Merriman, R.L., Howbert, J.J., Heath, W.F., Kobayashi, E., Takahashi, I., Tamaoki, T., and Nakano, H. (1991) Inhibition of protein kinase C by

calphostin C is light-dependent. Biochem. Biophys. Res. Commun. 176:288-293.

Carroll, E.J., Mueller, R., and Panico, L. (1982) Chemotactic factors for bovine leukocytes. Am. J. Vet. Res. 43:1661-1664.

Cohen, M.S., Chovaniec, M.E., Wilson, M.K., and Newburger, P.E. (1982) Con-A-stimulated superoxide production by granulocytes: reversible activation of NADPH oxidase. Blood 60:1188-1194.

Cohen, M.S., Metcalf, J.A., and Root, R.K. (1980) Regulation of oxygen metabolism in human granulocytes: relationship between stimululs binding and oxidative response using plant lectins as probes. Blood 55:1003-1010.

Cox, J.A., Jeng, A.Y., Blumberg, P.M., and Tauber, A.I. (1987) Comparison of subcellular activation of the human neutrophil NADPH-oxidase by arachidonic acid, sodium dodecyl sulfate (SDS), and phorbol myristate acetate (PMA). J. Immunol. 138:1884-1888.

Di Virgilio, F., Lew, D.P., and Pozzan, T. (1984) Protein kinase C activation of physiological processes in human neutrophils at vanishingly small cytosolic Ca^{2+} levels. Nature 310:691-693.

Doré, M., Slauson, D.O., and Neilsen, N.R. (1991) Decreased respiratory burst activity in neonatal bovine neutrophils stimulated by protein kinase C agonists. Am. J. Vet. Res. 52:375-380.

Doussiere, J. and Vignais, P.V. (1985) Purification and properties of an O_2^--generating oxidase from bovine polymorphonuclear neutrophils. Biochem. 24:7231-7239.

Dyer, R.M., Benson, C.E., and Boy, M.G. (1985) Production of superoxide anion by bovine pulmonary macrophages

challenged with soluble and particulate stimuli. Am. J. Vet. Res. 46:336-341.

Dyer, R.M., Majumdar, S., Douglas, S.D., and Korchak, H.M. (1994) Bovine parainfluenza-3 virus selectively depletes a calcium-independent, phospholipid-dependent protein kinase C and inhibits superoxide anion generation in bovine alveolar macrophages. J. Immunol. 153:1171-1179.

Finkel, T.H., Pabst, M.J., Suzuki, H., Guthrie, L.A., Forehand, J.R., Phillips, W.A., and Johnston, Jr. R.B. (1987) Priming of neutrophils and macrophages for enhanced release of superoxide anion by the calcium ionophore ionomycin. Implications for regulation of the respiratory burst. J. Biol. Chem. 262:12589-12596.

Freemen, B.A. and Crapo, J.D. (1982) Biology of disease; Free radicals and tissue injury. Lab Invest. 47:412-425.

Garcia-Morales, P., Minami, Y, Luong E., Klausner, R.D., and Samelson, L.E. (1990) Tyrosine phosphorylation in T cells is regulated by phosphatase activity: studies with phenylarsine oxidase. Proc. Natl. Acad. Sci. 87:9255-9259.

Gelfand, E.W. (1990) Cytosolic calcium changes during T- and B-lymphocyte activation: biological consequences and significance. In: Current Topics in Membranes and Transport. Volume 35, Edited by A. Kleinzeller. Academic Press, Inc. San Diago, CA, USA. pp. 153-177.

Gelfand, E.W., Cheung, R.K., and Grinstein, S. (1986) Mitogen-induced changes in Ca^{2+} permeability are not mediated by voltage-gated K^{+} channels. J. Biol. Chem. 261:11520-11523.

Gennaro, R., Florio, C., and Romeo, D. (1985) Activation of protein dinase C in neutrophil cytoplasts. Localization

of protein substrates and possible relationship with stimulus-response coupling. FEBS Lett. 180:185-190.

Gennaro, R., Florio, C., and Romeo, D. (1986) Co-activation of protein kinase C and NADPH oxidase in the plasma membrane of neutrophil cytoplasts. Biochem. Biophys. Res. Commun. 134:305-312.

Grzeskowiak, M., Della Bianca, V., Cassatella, M.A., and Rossi, F. (1986) Complete dissociation between the activation of phosphoinositide turnover and of NADPH oxidase by formyl-methionyl-leucyl-phenylalanine in human neutrophils depleted of Ca^{2+} and primed by subthreshold doses of phorbol 12,myristate 13,acetate. Biochem. Biophys. Res. Commun. 135:785-794.

Hannun, Y.A., Loomis, C.R., and Bell, R.M. (1985) Activation of protein kinase C by Triton X-100 mixed micelles containing diacylglycerol and phosphatidylserine. J. Biol. Chem. 260:10039-10043.

Heyneman, R., Burvenich, C., and Vercauteren, R. (1989) Interaction between the respiratory burst activity of neutrophils leukocytes and experimentally induced Escherichia coli mastitis in cows. J. Dairy Sci. 73:985-994.

Heyworth, P.G. and Segal, A.W. (1986) Further evidence for the involvement of a phosphoprotein in the respiratory burst oxidase of human neutrophils. Biochem. J. 239:723-731.

Heyworth, P.G., Shrimpton, C.F., and Segal, A.W. (1989) Localization of the 47 kDa phosphoprotein involved in the respiratory-burst NADPH oxidase of phagocytic cells. Biochem. J. 260:243-248.

Horn, W. and Karnovsky, M.L. (1986) Features of the translocation of protein kinase C in neutrophils stimulated with the chemotactic peptide f-MET-LEU-PHE. Biochem. Biophys. Res. Commun. 139:1169-1175.

Jaconi, M.E., Lew, D.P., Carpentier, J.L., Magnusson, K.E., Sjogren, M., and Stendahl, O. (1990) Cytosolic free calcium elevation mediates the phagosome-lysosome fusion during phagocytosis in human neutrophils. J. Cell. Biol. 110:1555-1564.

Jaconi, M.E.E., Lew, D.P., Monod, A., and Krause, K.H. (1993) The regulation of store-dependent Ca^{2+} influx in HL-60 granulocytes involves GTP-sensitive elements. J. Biol. Chem. 268:26075-26078.

Kanno, T., Utsumi, T., Kobuchi, H., Takehara, Y., Akiyama, J., Yoshioka, T., Horton, A.A., and Utsumi, K. (1995) Inhibition of stimulus-specific neutrophil superoxide generation by alpha-tocopherol. Free Rad. Res. 22:431-440.

Kass, G.E.N., Duddy, S.K., Moore, G.A., and Orrenius, S. (1989) 2,5-Di-(tert-butyl)-1,4-benzohydroquinone rapidly elevates cytosolic Ca^{2+} concentration by mobilizing the inositol 1,4,5-Trisphosphate-sensitive Ca^{2+} pool. J. Biol. Chem. 264:15192-15198.

Kehrli, M.E., Schmalstieg, F.C., Anderson, D.C., Van Der Maaten, M.J., Hughes, B.J., Ackermann, M.R., Wilhelmsen, C.L., Brown, G.B., Stevens, M.G., and Whetstone, C.A. (1990) Molecular definition of the bovine granulocytopathy syndrome: identification of deficiency of the Mac-1 (CD11b/CD18) glycoprotein. Am. J. Vet. Res. 51:1826-1836.

Kessels, G.C.R., Roos, D., and Verhoeven, A.J. (1991) fMet-Leu-Phe-induced activation of phospholipase D in human neutrophils. Dependence on changes in cytosolic free Ca^{2+} concentration and relation with respiratory burst activation. J. Biol. Chem. 266:23152-23156.

Klebanoff, S.J. (1992) Oxygen metabolites from phagocytes. In: Inflammation: Basic Principles and Clinical Correlates. Second Edition. Edited by Gallin, J.L.,

Goldstein, I.M., and Snyderman, R. Raven Press. Ltd., New York, NY, USA. pp. 541-588.

Kobayashi, E., Nakano, H., Morimoto, M., and Tamaoki, T. (1989) Calphostin C (UCN-1028C), A novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. Biochem Biophys. Res. Commun. 159:548-543.

Korchak, H.M., Vosshall, L.B., Haines, K.A., Wilkenfeld, C., Lundquist, K.F., and Weissmann, G. (1988a) Activation of the neutrophil by calcium-mobilizing ligands. II. Correlation of calcium, diacyl glycerol, and phosphatidic acid generation with superoxide anion generation. J. Biol. Chem. 263:11098-11105.

Korchak, H.M., Vosshall, L.B., Zagon, G., Ljubich, P., Rich, A.M., and Weissmann, G. (1988b) Activation of the neutrophil by calcium-mobilizing ligands. I. A chemotactic peptide and the lectin concanavalin A stimulate superoxide anion generation but elicit different calcium movements and phosphoinositide remodeling. J. Biol. Chem. 263:11090-11097.

Kramer, I.M., Verhoeven, A.J., van der Bend, R.L., Weening, R.S., and Roos, D. (1988) Purified protein kinase C phosphorylates a 47-kDa protein in control neutrophil cytoplasts but not in neutrophil cytoplasts from patients with the autosomal form of chronic granulomatous disease. J. Biol. Chem. 263:2352-2357.

Kuijpers, T.W., Hoogerwerf, M., and Roos, D. (1992) Neutrophil migration across monolayers of resting or cytokine-activated endothelial cells. Role of intracellular calcium changes and fusion of specific granules with the plasma membrane. J. Immunol. 148:72-77.

Le Cabec, V. and Maridonneau-Parini, I. (1995) Complete and reversible inhibition of NADPH oxidase in human neutrophils by phenylarsine oxide at a step distal to

membrane translocation of the enzyme subunits. J. Bio. Chem. 270:2067-2073.

Liang, S., Woodlock,T.J., Whitin,J.C., Lichtman, M.A., and Segel,G.B. (1990) Signal transduction in N-Formyl-Methionyl-Leucyl-Phenylalanine and concanavalin A stimulated human neutrophils: superoxide production without a rise in intracellular free calcium. J. Cell. Physiol. 145:295-302.

Liles, W.C., Hunter, D.D., Meier, K.E., and Nathanson, N.M. (1986) Activation of protein kinase C induces rapid internalization and subsequent degradation of muscarinic acetylcholine receptors in neuroblastoma cells. J. Biol. Chem. 261:5307-5313.

Maridonneau-Parini, I., Tringale, S.M., and Tauber, A.I. (1986) Identification of distinct activation pathways of the human neutrophil NADPH-oxidase. J. Immunol. 137:2925-2929.

Marks, P.W. and Maxfield, F.R. (1990) Transient increases in cytosolic free calcium appear to be required for the migration of adherent human neutrophils. J. Cell. Biol. 110:43-52.

Massey, V. (1959) The microestimation of succinate and the extinction coefficient of cytochrome C. Biochem. Biophys. Acta 34:255-256.

May, W.S.Jr, Sahyoun, N., Wolf, M., and Cuatrecasas, P. (1985) Role of intracellular calcium mobilization in the regulation of protein kinase C-mediated membrane processes. Nature 317:549-551.

McPhail, L.C. and Snyderman, R. (1983) Activation of the respiratory burst enzyme in human polymorphonuclear leukocytes by chemoattractants and other soluble stimuli. Evidence that the same oxidase is activated by different transductional mechanisms. J. Clin. Invest. 72:192-200.

Meshulam, T., Proto, P., Diamond, R.D., and Melnick, D.A. (1986) Calcium modulation and chemotactic response: divergent stimulation of neutrophil chemotaxis and cytosolic calcium response by the chemotactic peptide receptor. J. Immunol. 137:1954-1960.

Moore,G.A., McConkey, D.J., Kass, G.E.N., O'Brien, P.J., and Orrenius, S. (1987) 2,5-Di(tert-butyl)-1,4-benzohydroquinone - a novel inhibitor of liver microsomal Ca^{2+} sequestration. FEBS Lett. 224:331-336.

Muid, R.E., Twomey, B., and Dale, M.M. (1988) The effect of inhibition of both diacylglycerol metabolism and phospholipase A_2 activity on superoxide generation by human neutrophils. FEBS Lett. 234:235-240.

Nauseef, W.M., Volpp, B.D., McCormick, S., Leidal, K.G., and Clark, R.A. (1991) Assembly of the neutrophil respiratory burst oxidase: protein kinase C promotes cytoskeletal and membrane association of cytosolic oxidase components. J. Biol. Chem. 266:5911-5917.

Nishihira, J., McPhail, L.C., and O'Flaherty, J.T. (1986) Stimulus-dependent mobilization of protein kinase C. Biochem. Biophys. Res. Commun. 134:587-594.

Okamura, N., Curnutte, J.T., Roberts, R.L., and Babior, B.M. (1988) Relationship of protein phosphorylation to the activation of the respiratory burst in human neutrophils. Defects in the phosphorylation of a group of closely related 48-kDa proteins in two forms of chronic granulomatous disease. J. Biol. Chem. 263:6777-6782.

Phillips, W.A., Croatto, M., Veis, N., and Hamilton, J.A. (1992) Protein kinase C has both stimulatory and suppressive effects on macrophage superoxide production. J. Cell. Physiol. 152:64-70.

Phillips, W.A., Korchak, H.M., and Johnston, R.B.Jr. (1987) Calcium induces an association of protein kinase C with neutrophil membranes. Fed. Proc. 46:2068.

Pick, E. (1986) Microassays for superoxide and hydrogen peroxide production and nitroblue tetrazolium reduction using an enzyme immunoassay microplate reader. Meth. Enzymol. 132:407-421.

Pike, M.C., Costello, K., and Southwick, F.S. (1991) Stimulation of human polymorphonuclear leukocyte phosphatidylinositol-4-phosphate kinase by concanavalin A and formyl-methionyl-leucyl-phenylalanine is calcium-independent. Correlation with maintenance of actin assembly. J. Immunol. 147:2270-2275.

Pozzan, T., Lew, D.P., Wollheim, C.B., and Tsien, R.Y. (1983) Is cytosolic ionized calcium regulating neutrophil activation? Science 221:1413-1415.

Pozzan, T., Ruzzuto, R., Volpe, P., and Meldolesi, J. (1994) Molecular and cellular physiology of intracellular calcium stores. Physiol. Rev. 74:595-636.

Roth, D.R. and Zwahlen, R.D. (1991) Intradermal neutrophil mobilization induced by complement fragments and endotoxin is more effective in neonatal than in adult cattle. Vet. Pathol. 28:446-448.

Rotrosen, D. (1992) The respiratory burst oxidase. In: Inflammation Basic Principles and Clinical Correlates, Second Edition, edited by J.I. Gallin, I.M. Goldstein, and R. Snyderman. Raven Press Ltd. New York, NY, USA. pp. 589-601.

Rotrosen, D. and Leto, T.L. (1990) Phosphorylation of neutrophil 47-kDa cytosolic oxidase factor. Translocation to membrane is associated with distinct phosphorylation events. J. Biol. Chem. 165:19910-19915.

Salgar, S.K., Paape, M.J., Altson-Mills, B., and Miller, R.H. (1991) Flow cytometric study of oxidative burst activity in bovine neutrophils. Am. J. Vet. Res. 52:1201-1207.

Scharff, O. and Foder, B. (1993) Regulation of cytosolic calcium in blood cells. Am. Phys. Soc. 73:547-582.

Sha'afi, R.I., White, J.R., Molski, T.F.P., Shefeyk, J., Volpi, M., Naccache, P.H., and Feinstein, M.B. (1983) Phorbol 12-myristate 13-acetate activates Rabbit neutrophils without an apparent rise in the level of intracellular free calcium. Biochem. Biophys. Res. Commun. 114:638-645.

Sharma, P., Evans, A.T., Parker, P.J., and Evans, F.J. (1991) NADPH oxidase activation by protein kinase C-isotypes. Biochem. Biophys. Res. Commun. 177:1033-1040.

Sherwood, L. (1989) Human Physiology; from cells to systems. West Publishing Company. St. Paul, MN, USA.

Short, A.D., Bian, J., Ghosh, T.K., Waldron, R.T., Rybak, S.L., and Gill, D.L. (1993) Intracellular Ca^{2+} pool content is linked to control of cell growth. Proc. Natl. Acad. Sci. 90:4986-4990.

Stover, D.R., Charbonneau, H., Tonks, N.K., and Walsh, K.A. (1991) Protein-tyrosine-phosphatase CD45 is phosphorylated transiently on tyrosine upon activation of Jurkat T cells. Proc. Natl. Acad. Sci. 88:7704-7707.

Styrt, B. (1989) Species variation in neutrophil biochemistry and function. J. Leuk. Biol. 46:63-74.

Sullivan, F., Fredette, J.P., Griffin, J.D., Leavitt, J.L., Simons, E.R., and Melnick, D.A. (1989) An elevation in the concentration of free cytosolic calcium is sufficient to activate the oxidative burst of granulocytes primed with recombinant human granulocyte-

macrophage colony-stimulating factor. J. Biol. Chem. 264:6302-6309.

Tarsi-Tsuk, D. and Levy, R. (1990) Stimulation of the respiratory burst in peripheral blood monocytes by lipoteichoic acid. The involvement of calcium ions and phospholipase A_2. J. Immunol. 144:2665-2670.

Thelen, M., Dewald, B., and Baggiolini, M. (1993) Neutrophil signal transduction and activation of the respiratory burst. Physiol. Rev. 73:797-821.

Tortora, G.J. (1988) Introduction to the Human Body. Harper and Row, Publishers, New York, NY, USA.

Tsien, R.Y. (1980) New calcium indicators and buffers with high selectivity against magnesium and protons: desigh, synthesis, and properties of prototype structures. Biochem. 19:2396-2404.

Volpp, B.D., Nauseef, W.M., Donelson, J.E., Moser, D.R., and Clark, R.A. (1989) Cloning of the cDNA and functional expression of the 47-kilodalton cytosolic component of human neutrophil respiratory burst oxidase. Proc. Natl. Acad. Sci. USA 86:7195-7199.

von Tscharner, V., Prod'hom, B., Baggiolini, M., and Reuter, H. (1986) Ion channels in guman neutrophils activated by a rise in free cytosolic calcium concentration. Nature 324:369-372.

Walker, B.A.M., Hagenlocker, B.E., and Ward, P.A. (1991) Superoxide responses to formyl-methionyl-leucyl-phenylalanine in primed neutrophils. Role of intracellular and extracellular calcium. J. Immunol. 146:3124-3131.

Walz, A., Meloni, F., Clark-Lewis, I., von Tscharner, V., and Baggiolini, M. (1991) $[Ca^{2+}]_i$ changes and respiratory burst in human neutrophils and monocytes

induced by NAP-1/Interleukin-8, NAP-2, and gro/MGSA. J. Leuk. Biol. 50:279-286.

Wilson, E., Olcott, M.C., Bell, R.M., Merrill, Jr.A.H., and Lambeth, J.D. (1986) Inhibition of the oxidative burst in human neutrophils by sphingoid long-chain bases. Role of protein kinase C in activation of the burst. J. Biol. Chem. 261:12616-12623.

Wolf, M., Cuatrecasas, P., and Sahyoun, N. (1985a) Interaction of protein kinase C with membranes is regulated by Ca^{2+}, phorbol esters, and ATP. J. Biol. Chem. 260:15718-15722.

Wolf, M., LeVinne III, H., May, W.S.Jr., Cuatrecasas, P., and Sahyoun, N. (1985b) A model for intracellular translocation of protein kinase C involving synergism between Ca^{2+} and phorbol esters. Nature 317:546-549.

Wozniak, A., Betts, W.H., Murphy, G.A., and Rokicinski, M. (1993) Interleukin-8 primes human neutrophils for enhanced superoxide anion production. Immunol. 79:608-615.

Young, S. and Beswick, P. (1986) A comparison of the oxidative reactions of neutrophils from a variety of species when stimulated by opsonized zymosan and f-Met-Leu-Phe. J. Comp. Path. 96:189-196.

Zigmond, S.H., Slonczewski, J.L., Wilde, M.W., and Carson, M. (1988) Polymorphonuclear leukocyte locomotion is insensitive to lowered cytoplasmic calcium levels. Cell Motil. Cytoskel. 9:184-189.

Zweifach, A., and Lewis, R.S. (1993) Mitogen-regulated Ca^{2+} current of T-lymphocytes is activated by depletion of intracellular Ca^{2+} stores. Proc. Natl. Acad. Sci. 90:6295-6299.