Pharmacological control of neutrophil-mediated inflammation: Strategies targeting calcium handling by activated polymorphonuclear leukocytes

Gregory R Tintinger
Helen C Steel
Annette J Theron
Ronald Anderson

Medical Research Council Unit for Inflammation and Immunity, Department of Immunology, University of Pretoria and Tshwane Academic Division of the National Health Laboratory Service, Pretoria, South Africa

Abstract: Unlike most other effector cells of the innate, as well as the adaptive immune systems, the neutrophil is a relatively undiscerning aggressor with scant regard for damage limitation. Although this highly combative, professional phagocyte has become increasingly implicated in the immunopathogenesis of many acute and chronic inflammatory disorders, of both infective and noninfective origin, effective pharmacological strategies to counter neutrophil aggression have remained elusive. Activation of neutrophils results in rapid mobilization of both stored and extracellular Ca^{2+}, resulting in abrupt, usually transient increases in cytosolic Ca^{2+}, which precede, and are a prerequisite for activation of the Ca^{2+}-dependent pro-inflammatory activities of these cells. Mobilization of Ca^{2+} by, and restoration of Ca^{2+} homeostasis to activated neutrophils are multistep processes which present a number of potential targets, some well recognized and others novel and unconventional, for the pharmacological control of neutrophil-mediated inflammation. Uncovering these targets represents the primary focus of this review.

Keywords: calcium, cyclic AMP, NADPH oxidase, neutrophils, sodium-calcium exchanger

Introduction

Polymorphonuclear leukocytes, of which the neutrophil granulocyte is the most abundant, are key components of the innate immune system. These cells are activated and recruited to sites of infection by inflammatory stimuli generated by microbial pathogens, or by the interaction of these with pattern recognition molecules present on/in neighboring tissue cells such as epithelial cells, mast cells and tissue macrophages. Notwithstanding their abruptly mobilizable arsenal of antimicrobial agents, which include antimicrobial peptides/proteins, enzymes, bioactive lipids, and reactive oxidant species (ROS) (Anderson et al 1998; Theron et al 2002; Hatanaka et al 2004), these cells also have biosynthetic capability, albeit limited (Cassatella 1999; Witko-Sarsat et al 2000), which enables them to produce chemokines/cytokines, especially interleukin-8 (IL-8) and tumor necrosis factor-alpha (TNF-α). Acting in concert, TNF-α and IL-8 amplify neutrophil-mediated inflammatory responses by promoting extravasation and accumulation of neutrophils at sites of tissue injury and infection through induction and upregulation of expression of endothelial adhesion molecules and chemotaxis, respectively. Neutrophil migration and localization are further potentiated by leukotriene B_4 (LTB_4), an endogenously generated bioactive lipid with potent chemoattractant activity (Chen et al 2006).

Although extremely effective in eradicating microbial pathogens, the neutrophil, with its arsenal of indiscriminate oxidants and proteases, poses a potential threat to bystander host cells and tissues in the vicinity of the inflammatory reaction. Accordingly, neutrophil influx must be efficient, protective and promptly downregulated. Nevertheless, there is increasing awareness of the involvement of inappropriate

Correspondence: Gregory R Tintinger
Department of Immunology, PO Box 2034, Pretoria 0001, South Africa
Tel +27 12 354 6883
Fax +27 12 323 0732
Email grtintinger@gmail.com

activation of neutrophils in the etiology of many acute/hyper acute and chronic inflammatory disorders of both infective and noninfective origin, important examples of which are shown in Figure 1, with the airways and cardiovascular system being particularly vulnerable.

Responsiveness to corticosteroids

Few currently available therapeutic agents, including corticosteroids, effectively control the harmful pro-inflammatory activities of neutrophils. Indeed, insensitivity to corticosteroids appears to be a feature of those disorders in which the neutrophil is the predominant offender.

The apparent insensitivity of neutrophils is attributable to the coexistence of several different resistance mechanisms in these cells. Firstly, in contradistinction to other types of immune and inflammatory cells, glucocorticoids delay neutrophil apoptosis. This antiapoptotic effect of glucocorticoids in neutrophils is achieved by a mechanism which involves sustained expression of the antiapoptotic Bcl-2 family protein, Mcl-1L (Sivertson et al 2007). Secondly, neutrophils contain high levels of the functionally inactive beta isoform of the glucocorticoid receptor (GR), the synthesis of which is further upregulated on exposure of the cells to IL-8 (Strickland et al 2001), rendering them even less sensitive to corticosteroids. Although not yet described in neutrophils, the activity of the enzyme histone deacetylase, which is recruited by activated GRs as a mechanism of repression of expression of multiple inflammatory genes, is decreased in macrophages and circulating mononuclear leukocytes of patients with COPD and severe asthma respectively (reviewed in Barnes 2007). Thirdly, many of the proinflammatory activities of neutrophils, including generation of ROS, release of granule proteases, and generation of prostanoids, eicosanoids, and platelet-activating factor (PAF) occur within seconds of receptor-mediated activation of these cells and are independent of *de novo* protein synthesis.

Clearly the identification of novel targets for effective neutrophil-directed anti-inflammatory chemotherapy is a priority.

Calcium and neutrophils

Interaction of neutrophil membrane receptors with chemoattractants, opsonized particles, or endothelial adhesion molecules, results in abrupt, transient increases in cytosolic Ca^{2+} which precede, and are a prerequisite, for initiation of the proinflammatory activities of these cells. Ca^{2+}-activatible inflammatory functions include generation of superoxide by the membrane-associated electron transporting NADPH oxidase, adhesion to vascular endothelium, degranulation, activation of cytosolic phospholipase A_2 and 5-lipoxygenase, as well as synthesis of IL-8. Because of this critical dependence of activation of the proinflammatory activities of neutrophils on Ca^{2+}, the mechanisms utilized by these cells to mobilize and dispose of the cation represent attractive, and in several cases, novel potential targets for neutrophil-directed anti-inflammatory chemotherapy.

Calcium handling by activated neutrophils

A model of calcium mobilization and restoration of calcium homeostasis in activated human neutrophils is presented in Figure 2 and discussed below. Release of Ca^{2+} from intracellular stores following receptor-mediated activation of

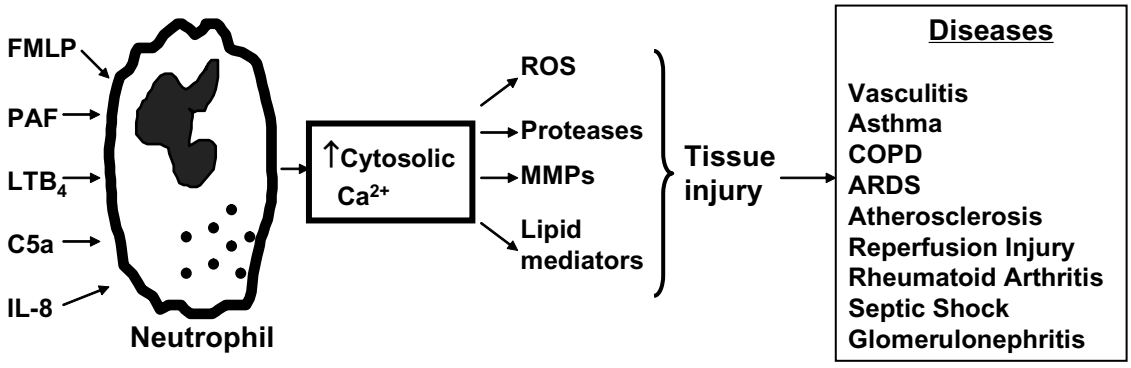

Figure 1 Activation of neutrophils by chemoattractants such as FMLP, PAF, C5a, and LTB_4 increases cytosolic Ca^{2+} concentrations with resultant generation of toxic reactive oxygen species (ROS) and release of proteases, matrix metalloproteinases (MMPs) and lipid mediators. The tissue injury that may be associated with release of these harmful molecules into the vicinity of innocent bystander host tissues contributes to the pathogenesis of numerous diseases, including chronic obstructive pulmonary disease (COPD) and the acute respiratory distress syndrome (ARDS).

Abbreviations: FMLP, N-formylated peptides/polypeptides; LTB_4, leukotriene B_4; PAF, platelet-activating factor.

neutrophils with various stimuli, including chemoattractants such as N-formylated peptides/polypeptides (FMLP), C5a, leukotriene B_4 (LTB_4), PAF, or IL-8, occurs rapidly, reaching peak values within several seconds of ligand-receptor binding which are 5–10-fold above the basal value of about 100 nM (Favre et al 1996). The receptors for the aforementioned chemoattractants belong to the 7-transmembrane, G-protein-coupled family of receptors. Occupation of these receptors, which are regulated by various Gα and Gβγ subunits, results in activation of the β isoforms of

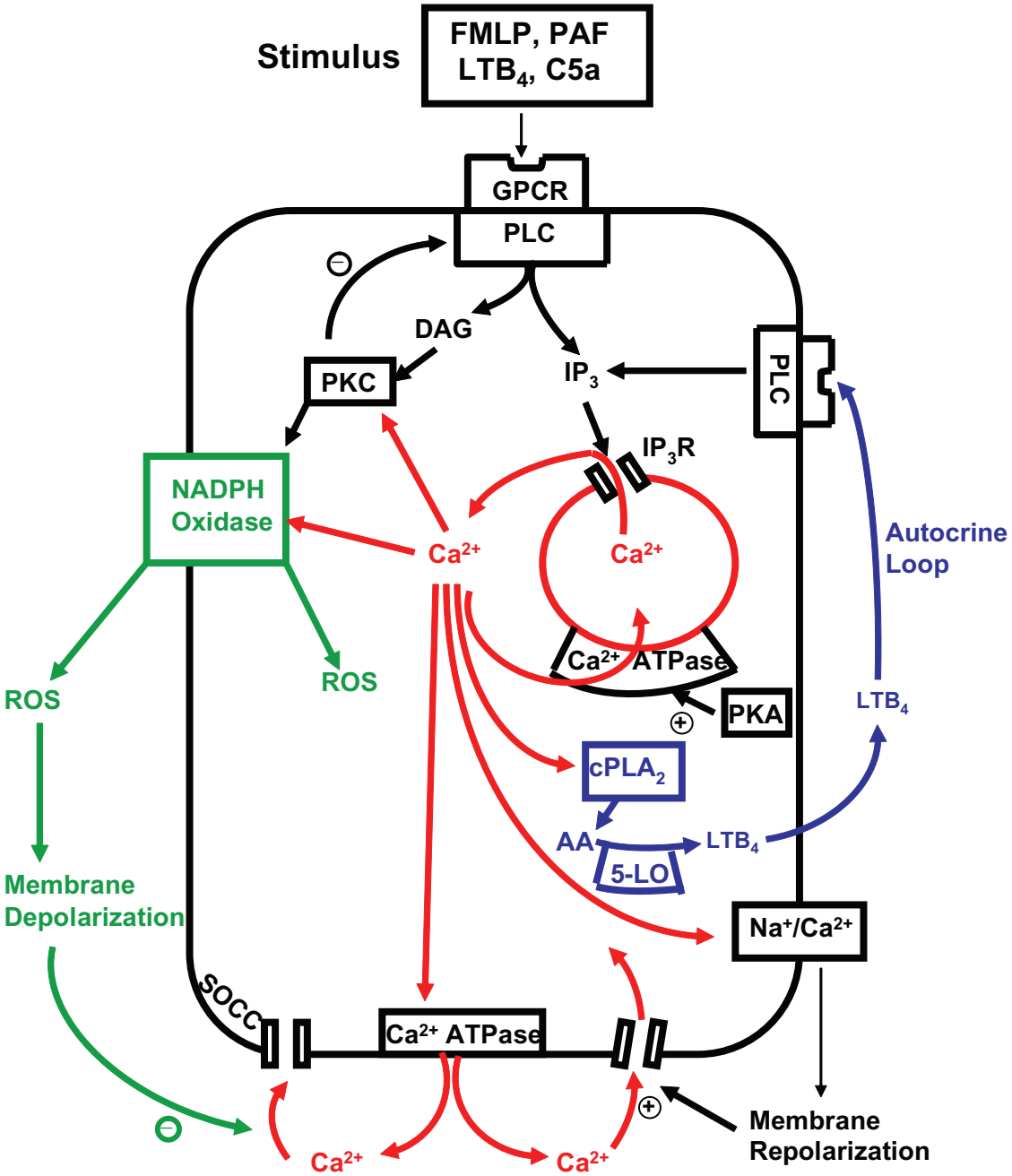

Figure 2 Calcium-mobilizing stimuli interact with membrane G-protein coupled receptors (GPCR) to activate phospholipase C (PLC) generating inositol triphosphate (IP_3) which interacts with IP_3 receptors (IP_3R) releasing Ca^{2+} from storage vesicles. Cytosolic Ca^{2+} activates cytosolic phospholipase A_2 ($cPLA_2$) which mobilizes arachidonic acid (AA) for the 5-lipoxygenase (5-LO) pathway. The AA metabolite leukotriene B_4 (LTB_4) is actively transported to the cell exterior where it binds to its receptor to activate PLC, completing a positive feedback autocrine loop. Ca^{2+} released into the cytosol is rapidly extruded from the cell by the plasma membrane Ca^{2+} ATPase and resequestered into storage vesicles by the protein kinase A (PKA)-sensitive endomembrane Ca^{2+} ATPase. Protein kinase C (PKC) activated by Ca^{2+} and diacylglycerol (DAG) facilitates assembly and activation of NADPH oxidase on the outer membrane which generates reactive oxygen species (ROS) with concomitant membrane depolarization. The depolarized membrane potential delays Ca^{2+} entry through store operated channels (SOCCs) until the Ca^{2+}-activatible Na^+/Ca^{2+} exchanger, operating in reverse mode, mediates recovery of the membrane potential promoting Ca^{2+} reuptake via SOCCs. PKC down-regulates PLC as part of a negative feedback loop to terminate IP_3 production.

phospholipase C (PLC), which in turn mediate production of inositol-1,4,5-triphosphate (IP_3) by hydrolysis of phosphatidylinositol-4,5,-biphosphate (Ali et al 1998; Yue et al 1998). IP_3 interacts with Ca^{2+}-mobilizing receptors on intracellular storage vesicles, resulting in discharge of stored Ca^{2+} into the cytosol. Only modest increases in IP_3 of around 15% of maximal are required to mobilize the total pool of stored Ca^{2+}. The duration of the peak increase in cytosolic Ca^{2+} varies according to the type and concentration of the chemoattractant, but is usually brief, being followed by a progressive decline in the concentration of cytosolic Ca^{2+} with a return to basal values within several minutes. The duration of the peak cytosolic Ca^{2+} response, as well as the rate of decline in the concentration of cytosolic Ca^{2+}, are determined by at least 4 mechanisms. These are: i) shuttling of Ca^{2+} between the stores and the cytosol (ie, repetitive bouts of release from, and resequestration of Ca^{2+} into stores (Anderson et al 2005); ii) activation of a secondary wave of Ca^{2+} influx due to endogenously-generated LTB_4 by chemoattractant-activated neutrophils (Steel et al 2007); iii) the efficiency of the systems which promote clearance of Ca^{2+} from the cytosol (Anderson et al 1998; Steel and Anderson 2002); and iv) the efficiency of the systems which regulate the time of onset, rate and magnitude of influx of extracellular cation (Tintinger et al 2001a).

Restoration of Ca^{2+} homeostasis

Rapid and efficient removal of Ca^{2+} from the cytosol of activated neutrophils ensures that activation of the cells is brief, thereby avoiding Ca^{2+} overload and hyperreactivity. This is achieved primarily by removal of Ca^{2+} from the cytosol by two adenosine triphosphate (ATP)-driven Ca^{2+} pumps operating in unison. These are the plasma membrane Ca^{2+}-ATPase and the endomembrane Ca^{2+}-ATPase, which mediate Ca^{2+} efflux and resequestration respectively. These two Ca^{2+} pumps appear to contribute equally to the removal of Ca^{2+} from the cytosol of activated neutrophils (Anderson and Goolam Mahomed 1997; Pettit and Hallett 2000).

The plasma membrane Ca^{2+}-ATPase of neutrophils is upregulated by calmodulin, acidic phospholipids, and polyunsaturated fatty acids, all of which shift the pump to a higher affinity for Ca^{2+}, resulting in enhanced maximal velocity (Carafoli et al 1992).

Apart from causing activation of PLC and release of stored Ca^{2+}, activation of neutrophils with chemoattractants such as FMLP is also accompanied by transient activation of adenylate cyclase (Iannone et al 1989; Theron et al 2002).This results from the interaction of adenosine, generated by dephosphorylation of adenylates, presumably by ecto-5'nucleotidase, with G-protein/adenylyl cyclase-coupled adenosine receptors (AR) of the $A2_A$ subtype on the neutrophil membrane (Iannone et al 1989; Theron et al 2002), resulting in activation of adenosine 3',5'-cyclic monophosphate (cAMP)-dependent protein kinase A (PKA). Phospholamban, a polypeptide regulator of the endomembrane Ca^{2+}-ATPase, undergoes PKA-mediated phosphorylation which results in up-regulation of the Ca^{2+} sequestering/resequestering activity of the pump (Chu et al 2000).

Importantly, efficient clearance of Ca^{2+} by the plasma membrane and endomembrane Ca^{2+}-ATPases is facilitated by the membrane depolarizing action of NADPH oxidase which restricts the influx of extracellular Ca^{2+}. NADPH oxidase undergoes Ca^{2+}-dependent activation on exposure of neutrophils to chemoattractants such as C5a, FMLP, and LTB_4, but not PAF or IL-8 (Steel and Anderson 2002; Guichard et al 2007), as well as to opsonized antigens. Activation of NADPH oxidase is accompanied by an abrupt and steep decrease in membrane potential which results primarily from the electrogenic properties of the oxidase (Tintinger et al 2001a). When the cells are depolarized, the driving force for entry of Ca^{2+} is abolished because the electrical component of the electrochemical gradient promoting Ca^{2+} entry is markedly reduced. Consequently, NADPH oxidase-mediated membrane depolarization enables the plasma membrane and endomembrane Ca^{2+}-ATPases to mediate clearance of Ca^{2+} from the cytosol of activated neutrophils, unhindered by influx of extracellular Ca^{2+}.

Influx of Ca^{2+}

Depletion of intracellular Ca^{2+} stores following receptor-mediated activation is followed by refilling of the stores, a process known as store-operated Ca^{2+} influx, or capacitative Ca^{2+} influx (Parekh and Penner 1997). In neutrophils, the time of onset and rate of store-operated Ca^{+} influx are determined primarily by the duration and intensity of activity of NADPH oxidase. In the case of cells activated with Ca^{2+}-mobilizing stimuli which are inefficient activators of the oxidase, such as PAF (Steel and Anderson 2002), influx of Ca^{2+} occurs rapidly, overwhelming the Ca^{2+}-ATPases, resulting in prolonged elevations in peak cytosolic Ca^{2+} concentrations. On the other hand, exposure of the cells to Ca^{2+}-mobilizing activators of the oxidase, such as FMLP, is accompanied by efficient clearance of store-derived cytosolic Ca^{2+}, in the setting of a gradual influx of Ca^{2+}, the rate of which is superimposable on that of membrane repolarization (Tintinger and Anderson 2004).

While the magnitude and duration of the maximal membrane depolarization responses of neutrophils activated with FMLP are determined by the intensity and duration of activation of NADPH oxidase, as well as by the counteracting effects of an efflux of protons from the cells (Schrenzel et al 1998; Bánfi et al 2000), until relatively recently less was known about the mechanisms which contribute to membrane repolarization. We have identified a role for the electrogenic Na^+/Ca^{2+} exchanger operating in reverse mode in mediating membrane repolarization in activated neutrophils (Tintinger and Anderson 2004).

As opposed to being a major transporter of extracellular Ca^{2+} for store refilling, the primary role of the exchanger when operating in reverse mode is to mediate recovery of the membrane potential which is necessary to drive the influx of Ca^{2+} through store-operated Ca^{2+} channels (Tintinger and Anderson 2004). However, the Na^+/Ca^{2+} exchanger is vulnerable to oxidative inactivation, being sensitive to the phagocyte-derived oxidants, hydrogen peroxide, and especially hypochlorous acid (Coetzee et al 1994; Tintinger et al 2007). The efficiency of the exchanger in activated neutrophils is therefore dependent on its level of exposure to phagocyte-derived oxidants.

Store-operated Ca^{2+} channels

Until very recently, the precise molecular identity of the store-operated Ca^{2+} channels operative in human neutrophils and other cell types had not been conclusively established. One particular family of nonvoltage-activated Ca^{2+} channels which had attracted considerable attention and interest was the family of transient receptor potential (TRP) channels (Elliot 2001; Li et al 2002). Although two members of this family, LTFP2 and TRP6, have been described in neutrophils (Li et al 2002; Heiner et al 2003), the characteristics of these channels are not entirely compatible with those of putative, prototype store-operated Ca^{2+} channels. However, in the past two years the identities of the major components of store-operated Ca^{2+} channels have been elucidated. These are the proteins Stim1 (and possibly Stim2), and Orai1 (and possibly Orai2 and 3), which function as the Ca^{2+} sensing and channel-forming proteins, respectively (Spassova et al 2006). Stim1 is located in the endoplasmic reticulum where it binds reversibly to Ca^{2+}. Following store depletion, dissociation of Ca^{2+} causes Stim1 to redistribute within the endoplasmic reticulum to areas which are in close proximity to Orai1 within the plasma membrane. Stim1 then activates the Ca^{2+}-selective Orai channels by a mechanism which remains to be elucidated (reviewed by Putney 2007).

Neutrophil-directed anti-inflammatory strategies

Notwithstanding the increasing awareness of the anti-inflammatory potential of macrolide antimicrobial agents (Simpson et al 2008), several other categories of anti-inflammatory agents have recently been described which have the potential to target neutrophils. These agents, described in detail elsewhere (Barnes 2007), include: i) antagonists of receptors for chemoattractants such as C5a, IL-8, and LTB_4; ii) antagonists of endothelial adhesion molecules; iii) inhibitors of pro-inflammatory enzymes and transcription factors such as phosphoinositide 3-kinase, 38 mitogen-activated protein kinase, and nuclear factor kappa B; and iv) activators of histone deacetylase 2 which is recruited by activated glucocorticoid receptors to switch off multiple proinflammatory genes.

In addition to these, recent insights into the mechanisms utilized by activated neutrophils to mobilize both intracellular and extracellular Ca^{2+}, as well as to restore Ca^{2+} homeostasis to the cells present a number of novel targets which are amenable to pharmacological control. In some cases target enhancement of activity improves the efficiency of clearance of Ca^{2+} from the cytosol, while in others this is achieved by target inhibition. Targets in the former category include endomembrane Ca^{2+} ATP-ases, NADPH oxidase, myeloperoxidase, and protein kinase C, while in the latter category, phospholipase C, the Na^{2+}/Ca^{2+} exchanger, 5'-lipoxygenase, and store-operated Ca^{2+} channels represent attractive targets.

Ca^{2+} mobilization and restoration of Ca^{2+} homeostasis as targets for neutrophil-directed anti-inflammatory chemotherapy

Inhibitors of phospholipase C

The importance of PLC as a potential target is underscored by the critical involvement of IP_3, not only in mediating the release of Ca^{2+} from intracellular stores, but also in sustaining elevations in cytosolic Ca^{2+} by promoting shuttling of the cation between the stores and the cytosol (Anderson et al 2005) and possibly by initiating influx of Ca^{2+} (Ma 2000; Bolotina 2004). Furthermore, diacylglycerol (DAG) generated by PLC activates protein kinase C (PKC), which in turn promotes the assembly and activation of NADPH oxidase (Tauber 1987).

Although pharmacological inhibitors of PLC and antagonists of IP_3 receptors such as U73122 and 2-aminoethoxydiphenyl borate respectively, are effective

in experimental systems, no such inhibitors are available for clinical use. However, PLC activity is inhibited in various cell types such as vascular endothelial cells (Avdonin and Ryan 2000) and platelets (Murphy et al 1991) by a negative feedback loop involving PKC. This feedback inhibition on PLC by PKC appears to be operative in human neutrophils as we have demonstrated recently that in the presence of PKC inhibitors, such as GF109203X, IP_3, and cytosolic Ca^{2+} concentrations in chemoattractant-activated neutrophils reach higher peaks and remain elevated longer than those measured in untreated cells (unpublished observations). This finding is important as it provides insight into the physiological mechanisms that down-regulate PLC in activated neutrophils and has implications for the design of pharmacological strategies targeting PKC, as inhibitors of this enzyme may paradoxically enhance the Ca^{2+}-dependent proinflammatory activities of phagocytes. In contradistinction to the paucity of clinically useful PLC or PKC inhibitors, the endomembrane Ca^{2+} ATPase is considerably more amenable to pharmacologic interventions.

Upregulation of endomembrane Ca^{2+} ATP-ases

The activity of the Ca^{2+} resequestering endomembrane Ca^{2+} ATP-ases can be upregulated by the cAMP-sensitive enzyme, protein kinase A (PKA) (Chu et al 2000). PKA-mediated enhancement of the activity of the endomembrane Ca^{2+} ATP-ase markedly accelerates the clearance of cytosolic Ca^{2+} following release of the cation from storage vesicles (Anderson et al 1998; Tintinger et al 2001b). The apparent sensitivity of PKA to cAMP has been exploited with the introduction of a wide range of pharmacologic agents, all of which share the ability to elevate intracellular cAMP concentrations. This can be achieved by several mechanisms, including activation of adenylate cyclase, or inhibition of phosphodiesterase enzymes (PDEs) responsible for the metabolism of cAMP (Moore and Willoughby 1995). Adenylate cyclase is activated via G-protein-coupled receptors linked to membrane associated β-adrenergic, adenosine (A_{2A}) and prostaglandin E_2 receptors. PDE isoenzymes of various classes have been identified in neutrophils and PDE 4B2 is probably the most abundant of these (Wang et al 1999). The relative tissue specificity of PDE 4 isoenzymes for inflammatory cells has enabled some degree of pharmacological targeting of neutrophils by new generation agents such as roflimulast (Sanz et al 2007) and cilomilast (Baumer et al 2007).

The validity of this pharmacologic approach has been confirmed *in vitro* as cAMP-elevating agents markedly suppress a range of Ca^{2+}-dependent proinflammatory activities of neutrophils, including adhesion to vascular endothelium (Bloemen et al 1997), oxidant production (Tintinger et al 2000), as well as release of proteases, eicosanoids, and cytokines (Moore and Willoughby 1995; Tintinger et al 2000). Furthermore, numerous clinical trials are ongoing or have been completed in which the efficacy of these agents has been verified *in vivo* (Pauwels et al 1997; Sullivan et al 2001; Vignola 2004). Less well documented, however, is the role of cGMP during the restoration of Ca^{2+} homeostasis in activated neutrophils. Intracellular cGMP concentrations can be increased by activation of guanylate cyclase, or by inhibition of PDEs that metabolize cGMP, predominantly PDEs 3 and 5 (Torphy 1998). The endomembrane Ca^{2+}-ATPase has been identified as a putative target of cGMP acting via protein kinase G (Ay et al 2006), although other mechanisms may exist whereby cGMP accelerates the clearance of cytosolic Ca^{2+}. In this regard, cGMP may elevate intracellular cAMP levels by effectively acting as a competitive antagonist of PDE 3-mediated hydrolysis of cAMP (PDE 3 hydrolyzes both cyclic nucleotides) (Boswell-Smith et al 2006). The selective PDE 3 inhibitor, cilostazol, significantly attenuated FMLP-mediated adhesion of neutrophils to human umbilical endothelial cells *in vitro* (Yang et al 2006), and suppressed the cough threshold in elderly asthmatics (Ishiura et al 2005).

Inhibitors of 5-Lipoxygenase

Leukotrienes, such as LTB_4, are produced by activated neutrophils consequent to upregulation of the 5-lipoxygenase (5-LO) pathway (Peters-Golden and Henderson 2007). Phospholipase A_2 hydrolyzes membrane phospholipids liberating arachidonic acid, the substrate for 5-LO, and in the presence of calcium and 5-lipoxygenase-activating protein (FLAP), activated 5-LO generates leukotriene A_4, which in neutrophils is converted to LTB_4. The diverse role of LTB_4 in promoting inflammation includes recruitment of inflammatory cells (De Caterina and Zampolli 2004), activation of cytosolic PLA_2 and degranulation (Boyce 2007), as well as the release of cytokines and matrix metalloproteinases, thus playing a role in immunoregulation (Rola-Pleszczynki et al 1986; Ford-Hutchinson 1990; Leppert et al 1995). In addition to the physiological responses above, LTB_4 also participates in a positive feedback autocrine loop which activates phospholipase C and triggers sustained elevations of cytosolic Ca^{2+} in PAF-activated neutrophils (McDonald et al 1994; Steel et al 2007). Intracellular LTB_4 derived from 5-LO is actively transported to the cell exterior where

it interacts with high affinity LTB_4 receptors (BLT1) linked to PLC on the neutrophil plasma membrane. This interaction induces a secondary pulse of IP_3 which mediates sustained release of Ca^{2+} into the cytosol and this in turn potentiates Ca^{2+}-dependent pro-inflammatory responses (Surette et al 1999; Steel et al 2007).The validity of pharmacological anti-inflammatory strategies targeting LTB_4 is underscored by recent evidence for the involvement of this leukotriene in the pathogenesis of bronchial asthma (De Caterina and Zampolli 2004), COPD (Marian et al 2006), and inflammatory arthritis (Chen et al 2006). Importantly, the imidazole antimycotic, itraconazole, antagonizes the effects of LTB_4 in PAF-activated neutrophils by inhibiting 5-LO (Steel et al 2007). The concentrations of itraconazole required to inhibit 5-LO are similar to those achieved in the plasma of patients receiving this antimycotic (Kageyama et al 1999). Interestingly, other agents in this class, fluconazole and flutrimazole, may also possess anti-inflammatory properties as the former decreased mortality in critically ill patients by a mechanism unrelated to its antifungal properties (Jacobs et al 2003), while the latter inhibited arachidonic acid-induced ear edema in a murine model (Merlos et al 1996). Other 5-LO inhibitors such as zileuton, which inhibits production of both LTB_4 and the cysteinyl leukotrienes, LTC_4 and LTD_4, by activated eosinophils, have shown efficacy in clinical trials of patients with bronchial asthma (Peters-Golden and Henderson 2007).

This is in keeping with the well established pathogenetic role of cysteinyl leukotrienes in inflammatory airway diseases (Arm 2004), and although neutrophils do not generate LTC_4, or LTD_4, receptors for cysteinyl leukotrienes, namely $CysLT_1$ and $CysLT_2$ are present on the plasma membrane of these cells. The physiological significance of these receptors is unknown, but we have found recently that cysteinyl leukotrienes act as receptor-mediated priming agents for human neutrophils *in vitro*. Pretreatment of neutrophils with these agents augments the subsequent responses to FMLP with marked increases in oxidant production and elastase release compared with cells activated with the chemoattractant alone (unpublished observations). This finding is likely to be of importance given the widespread use of $CysLT_1$ receptor antagonists such as montelukast, pranlukast and zafirlukast, all of which may modulate cysteinyl leukotriene-mediated priming of neutrophils in a clinical setting.

NADPH oxidase

The membrane-associated NADPH oxidase complex is assembled on the plasma membrane and becomes incorporated into the phagocytic vacuole following phagocytosis of microbial pathogens. When activated, the oxidase generates highly ROS by transferring electrons from NADPH to molecular oxygen. This electrogenic transfer of electrons results in significant depolarization of the resting membrane potential. The oxygen radicals formed during this process include superoxide anions, hydroxyl radicals, hydrogen peroxide and hypochlorous acid (Nagata 2005), all of which may participate in the killing of microbial pathogens. However, these toxic molecules have also been implicated in the pathogenesis of numerous diseases as their release to the extracellular environment may damage innocent bystander host tissues (Moraes et al 2006). Although inhibition of the oxidase may abolish the generation of toxic ROS, this also abrogates the membrane depolarization response and dissipates the electrical gradient required to restrain Ca^{2+} entry into leukocytes. Veritably, neutrophils lacking a functional oxidase such as those of patients with chronic granulomatous disease (CGD), are inherently predisposed to Ca^{2+} overload (Tintinger et al 2001a; Rada et al 2003) with consequent exaggeration of Ca^{2+}-dependent proinflammatory activity, including increased release of proteolytic enzymes (Tintinger et al 2001a) and cytokines (Bylund et al 2007). NADPH oxidase thus fulfills dual roles as destroyer of microbial pathogens and in protecting the cell from Ca^{2+} flooding of the cytosol mediated by its important membrane depolarizing action. Therefore, somewhat paradoxically, enhancing the activity of the oxidase may uncover unforeseen anti-inflammatory properties (Hallett 2003; Hultqvist et al 2006).

In addition to mediating membrane depolarization, recent evidence suggests that the oxidase also indirectly modulates the rate of neutrophil membrane repolarization (Tintinger et al 2007). In this regard, myeloperoxidase (MPO) and hypochlorous acid have emerged as important regulators of the neutrophil membrane potential following activation of NADPH oxidase.

Myeloperoxidase and hypochlorous acid

Myeloperoxidase is stored in tertiary granules in the neutrophil cytosol and following the formation of phagocytic vacuoles is released into the phagosome, together with ROS generated from the concomitant activation of NADPH oxidase. Hydrogen peroxide thus formed acts as a substrate for MPO which catalyzes its conversion to the highly reactive oxidant, hypochlorous acid (HOCl). In the presence of MPO inhibitors such as sodium azide or 4-aminobenzoyl hydrazide (ABAH), the magnitude of neutrophil membrane

depolarization in response to activating stimuli such as FMLP is not altered. However, the rate of recovery of the membrane potential is significantly accelerated (Tintinger et al 2007) and this is associated with an increased rate and magnitude of Ca^{2+} reuptake. Thus, inhibition of HOCl generation promotes rapid recovery of the membrane potential and accelerates the rate of capacitative Ca^{2+} entry, underscoring the regulatory roles of MPO and HOCl during these events. This may paradoxically represent a physiological anti-inflammatory activity of HOCl given the calcium requirements of numerous pro-inflammatory neutrophil functions. Lending support to this contention, exaggerated inflammatory responses have been observed in the skin and lungs of MPO-deficient mice exposed to injurious ultraviolet light and irradiation, respectively (Milla et al 2004; Komatsu et al 2006).

Inhibition of store-operated calcium channels

The IP_3-mediated release of Ca^{2+} from neutrophil storage vesicles is associated with activation of Ca^{2+} entry channels on the plasma membrane of these cells in order to facilitate store refilling. The mechanism of activation of these store-operated channels (SOCCs) remains controversial, although recent evidence suggests a role for proteins Stim 1 and Orai 1 (Spassova et al 2006). Given the elusive nature of the structure and mechanism/s of activation of these channels, it has been difficult to design pharmacologic inhibitors of SOCCs. However, the recent advances in our understanding of these channels, notably Stim 1 and Orai 1 (Putney 2007), may pave the way for the development of agents that antagonize this mechanism of Ca^{2+} reuptake. Regardless of the precise identity of, or coupling mechanisms that activate SOCCs, the rate of Ca^{2+} entry through these channels is regulated by the electrochemical gradient for Ca^{2+} across the plasma membrane (Geiszt et al 1997). Accordingly, at depolarized potentials, Ca^{2+} reuptake is opposed by the electrical gradient and is only initiated during the recovery phase of the membrane potential. Crucially, the rate of membrane repolarization is modulated by the activity of the plasma membrane Na^+/Ca^{2+} exchanger.

Inhibition of the Na^+/Ca^{2+} exchanger

The reverse mode of the Na^+/Ca^{2+} exchanger is activated by the rapid rise in cytosolic Ca^{2+} consequent to release of the cation from stores. When operating in reverse mode, the exchanger extrudes 3 Na^+ ions for each Ca^{2+} ion entering the cell (Blaustein and Lederer 1999), with a resultant net loss of positive charge. Although proton efflux from the cell is the primary charge compensating mechanism (Schrenzel et al 1998; Bánfi et al 1999), the electrogenic Na^+/Ca^{2+} exchanger contributes to recovery of the membrane potential (Tintinger and Anderson 2004) and represents a target amenable to pharmacologic intervention. Inhibitors of the reverse mode of the Na^+/Ca^{2+} exchanger such as KB-R7943 markedly retard the rate of membrane repolarization with consequent attenuation of the rate and magnitude of Ca^{2+} reuptake (Tintinger and Anderson 2004). It is likely that delayed Ca^{2+} entry with incomplete refilling of Ca^{2+} storage vesicles mediated by inhibitors of the exchanger may down-regulate the proinflammatory activity of neutrophils. Agents that inhibit the reverse mode of the Na^+/Ca^{2+} exchanger including KB-R7943 and SN-6 have shown promise in animal models of ischemia-reperfusion injury (Iwamato 2007), a condition in which activated neutrophils are considered to play a central pathogenetic role (Buras and Reenstra 2007). Interestingly, the expression of Na^+/Ca^{2+} exchanger genes is upregulated in leukocytes from patients with cardiac failure (Seiler et al 2004).

Conclusions

Advances in our understanding of the physiologic mechanisms pertaining to calcium handling by activated neutrophils have facilitated recognition of novel pharmacologic strategies designed to suppress the pro-inflammatory activities of these cells. The distinct potential for agents such as imidazole antimycotics, antagonists of cysteinyl leukotriene receptors, inhibitors of the Na^+/Ca^{2+} exchanger, long-acting beta-adrenergic receptor agonists and phosphodiesterase inhibitors to modulate neutrophil-mediated inflammation *in vivo*, creates exciting new opportunities for researchers in this important field.

Disclosure

The authors declare no conflicts of interest.

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