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Review Article

Signalling in Neutrophils: A Retro Look

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This review presents a summary of signalling events related to the activation of human polymorphonuclear neutrophils by a variety of soluble and particulate agonists. It is not intended as a comprehensive review of this vast field or as a presentation of the multiple new aspects of neutrophil functions that are being documented at an ever faster rate. Its aim is rather to focus on multiple aspects of major signalling pathways that, in the view of this reviewer, are currently shadowed by present trends and to provide the core evidence for their implication and the limitations of our present knowledge. More specifically, this review starts with cell surface receptors and some of their functional and biological properties and then moves on to downstream transducers (G proteins) and effectors (the phosphoinositide, tyrosine kinases, and cyclic nucleotide pathways). Classical second messengers (calcium, protein kinase C, polyphosphoinositides, and cyclic nucleotides) are emphasized. It is hoped that this presentation will not only remind present-day investigators of the central role these pathways play in the regulation of the functional responsiveness of neutrophils, but that it will also highlight some of the areas deserving additional investigation.

1. Introduction

The role(s) of the polymorphonuclear neutrophil granulocyte (neutrophil) in the immune system is (are) rapidly evolving and expanding [1]. Long after having been recognized by Metchnikoff (cited in [2]) as a major player in the first line of defence of the organism, the multiple contributions of the neutrophil to the shaping of the immune response, both innate and acquired, are being recognized. Evidence for the phenotypic plasticity of the neutrophil as well as its functional diversity is rapidly accumulating [3]. This includes the realization that neutrophils can acquire the capacity to present antigens through the expression of MHC class II molecules [4, 5], to express functional elements of the T cell receptor [6], and to produce and secrete various cytokines and chemokines [7] that direct the differentiation and function of dendritic cells and T and B lymphocytes, that is, the effector arm of the immune system [1, 8].

In the following, I will review some of the early events related to signalling in neutrophils. In view of the width of the topic, the choices made will necessarily be arbitrary, and significant elements will be left out. The emphasis in this review will be on several aspects of signalling which have, to some extent, been relatively in the shadow recently with the recent emphasis on novel neutrophil functions [1]. It is intended to complement the information in the multiple excellent reviews on neutrophil signalling and functions (signalling: [9–11] and function: [8, 12–20]) that have recently been published.

2. Historical Considerations Related to Signalling

Detailed studies of the signalling mechanisms associated with the regulation of neutrophil functions began in earnest in the 1970s. These studies were greatly aided by the ready availability of the cells (the most numerous blood borne leukocytes) and the identification by Schiffmann and his colleagues of bacterial-related molecular signals (the formylated peptides) that directed the movement of these cells [21–23]. The latter breakthrough provided investigators with characterized agonists with which to stimulate the cells and the ability to identify specific biochemical events related to their functions. The functional identification of plasma membrane receptors and of specific signalling pathways

rapidly followed. Although much remains to be clarified, three decades of evidence have since accumulated concerning the biochemical pathways associated with various neutrophil responses. This has, *inter alia*, illustrated the complexity of the regulatory mechanisms and the limits of our present understanding and, in so doing, indicates the directions of future studies.

3. Signalling in Neutrophils

3.1. Receptors. Classical pharmacological evidence for the presence of receptors on the neutrophil plasma membrane rapidly followed the identification of chemotactic factors (formylated peptides, C5a, leukotriene B_4, and interleukin 8...). The subsequent development of molecular biology tools led to the molecular characterization of these entities. Receptors for chemotactic factors, opsonins, cytokines, Toll-like receptors, neuropeptides, and growth factors, among others, have since been identified on the plasma membrane of neutrophils.

These receptors belong to multiple categories. They include G protein- (most chemotactic factors), tyrosine kinase- (directly or indirectly) as well as adenylyl and guanylyl cyclase (the latter will not be dealt with in this review) coupled receptors.

While most, if not all, of these receptors are transmembrane proteins, signal transduction mediated by direct perturbation of the lipid bilayers (as opposed to interaction with surface protein receptors), possibly at the level of lipid rafts, cannot be excluded in some cases, for example, in response to monosodium urate crystals (the etiological agent of gout) [24].

3.1.1. Multiple Ligands for Same Receptors. While receptors recognize specific ligands, they may also recognize multiple ligands as exemplified by the chemokine receptors for CXCL8 (IL-8). For example, the CXCL8 receptor, CXCR1, binds and responds to CXCL8 as well as to CXCL7 (NAP-2) and CXCL1 (Gro-α). The formyl peptide receptor (FPR) binds not only formylated peptides but recognizes and responds also to the antimicrobial granule proteins cathepsin G [25] and to cryptic peptides of HIV-1 envelope proteins [26–28], while serum amyloid A [29] (see, however, [30, 31] for an opposing view) and the antimicrobial peptide LL-37 [32] interact with formyl peptide receptor-like 1 (FPRL1). Similarly, FcγRs may recognize acute phase proteins such as C-reactive proteins [33, 34] in addition to the Fc tail of immunoglobulins, and the danger signal HMGB1 may interact with the LPS receptor TLR4 [35].

3.1.2. Multiple G Proteins for Same Receptor. Additional levels of complexity are provided by the observations that receptors for the same ligands (e.g., CXCR1 and CXCR2) may couple to distinct G proteins [36] and that specific G protein subunits may mediate different functions [37].

3.1.3. Multiple Functions for Same Receptor. Signalling through a single receptor does not necessarily lead to the activation of the same intracellular pathways depending on the ligand recognized. For example, IL-8 and Gro-α may not be perceived equivalently by neutrophils. In human neutrophils, only Gro-α, but not IL-8, induces an influx of calcium. These results were confirmed in HEK 293 cells expressing CXCR1 or CXCR2 indicating that the consequences of the binding of IL-8 and Gro-α to CXCR2 are distinct [38].

3.1.4. Receptor Multimerization. The receptors expressed on neutrophils may function, at least in part, in a high-order complex involving homo- or heterodimerization that leads to distinct signalling outcomes [39]. Chemokine receptor trafficking to the cell membrane is likely to occur as oligomers [40]. The dimerization status of CXCR1 and CXCR2 is modulated both by receptor expression and by their ligands. For example, CXCL8 decreases heterodimerization of CXCR1 and CXCR2 while stabilizing homodimers and stimulating internalisation of both receptors [41]. Oligomerization may involve more than one receptor family, and interactions between the complement fragment receptor C5aR and CCR5 leading to cointernalization of the receptors have been described [42]. The mechanisms by which the GPI-linked FcγR CD16b transmits its signals to the interior of the cells are still unresolved, but they may involve the interactions of CD16b with the β_2 integrin CD11b [43–46].

3.1.5. Receptor Internalization. Internalization of many neutrophil receptors has been observed. This includes receptors for chemoattractants such as fMLF, C5a and IL-8 (e.g., [47]) as well as opsonins (CD32a, CD16b) [48, 49]. Different regions of the carboxy-terminus of the receptors have been identified that play a role in these events mediated in part by phosphorylation. For example, IL-8-dependent internalization of CXCR1 is dependent on a region of the carboxy terminal of the receptor that includes multiple phosphorylation sites. IL-8-induced CXCR2 internalization on the other hand relies on a different, membrane-proximal region of the carboxy-terminus [50]. These results are consistent with the observations that the internalization of CXCR1/2 that follows stimulation by IL-8 is decreased by truncation of the last (C-terminal) amino acids of the receptors [51–53]. Kinases such as the GRK family (and GRK2 in particular) and PKC have been reported to be involved in the regulation of the internalization of the receptors. The phosphorylation sites are likely to be located in the C-terminal region of the receptors. Arrestins and dynamin also play a role in the internalization of CXCR1/2 which appears to be independent of the interaction with heterotrimeric G proteins as FPR internalization is not affected by pertussis toxin [54]. Engagement of the opsonin receptor CD32a similarly leads to its internalization and subsequent proteosomal degradation [49].

Internalization of the chemoattractant receptors is transient and followed by a recycling to the plasma membrane while that of CD32a results in its proteasome-dependent degradation [48, 49]. The mechanisms regulating receptor recycling differ from those involved in their internalization; for example, recycling, as opposed to internalization, of CXCL8 receptors is phosphorylation independent [50, 53].

GPCR phosphorylation is likely to be mediated by G protein-coupled receptor kinase 2 [55]. The mechanisms regulating receptor recycling vary from receptor to receptor; C5aR recycling is inhibited potently by monensin, while that of FPR is not [56]. This indicates that cells may differentially regulate receptor recycling.

3.2. Coupling of Neutrophil Receptors. The major receptors on neutrophils (chemoattractant and opsonin receptors) are coupled to two principal intermediaries, heterotrimeric G proteins and tyrosine kinases. These systems serve to relay the information conveyed by the occupancy of the receptors to the cells' interior, provide an initial amplification stage, and are responsible for the appropriate activation of the required downstream effector systems.

3.2.1. Coupling to G Proteins. The receptors for most chemoattractants including those for formylated peptides, chemokines such as CXCL1,2 or 8 (CXCR1,2), the complement fragment C5a, and the lipid mediators platelet-activating factor (PAF) and leukotriene B_4 (LTB_4) are all coupled to heterotrimeric G proteins. Neutrophils express a variety of heterotrimeric G proteins and in particular those of the Gs, Gi, and Gq families. The best characterized functions of the chemotactic factor receptors are linked to the activation of G proteins of the Gi family and in particular Giα2 and Giα3. Experimentally, this is manifested by the sensitivity to inhibition by pertussis toxin, the toxin from *Bordetella pertussis* [57–59]. The inhibition by pertussis toxin of chemotaxis, degranulation, and the stimulation of the oxidative burst as well as of several signalling pathways (calcium mobilization, actin polymerization, and stimulation of tyrosine phosphorylation) provided the first indices of the involvement of Gi or Gi-like proteins in signal transduction in neutrophils [57, 60, 61]. The results of Damaj et al. [62] describing the inhibitory effects of peptides derived from Giα2 (but not Giα1 or Giα3) on the coimmunoprecipitation of CXCR1 and CXCR2 and Gia2 as well as on the mobilization of calcium induced by CXCL8 are consistent with this interpretation and additionally indicate that CXCR1 and CXCR2 specifically associate with Giα2. Pertussis toxin-insensitive G proteins of the Gq family (Gia16) are also expressed in neutrophils and may mediate some neutrophil responses [63]. Occupation of the chemoattractant receptors and activation of heterotrimeric G proteins follow the well described and by now canonical activation sequence of exchange of GDP for GTP on the α subunit of the G proteins, dissociation of the α and β-γ subunits, and interaction with downstream effectors.

The α and $\beta\gamma$ subunits of the heterotrimeric G proteins involved in the responses of human neutrophils are likely to be linked to specific signalling pathways (e.g., $\beta\gamma$ and PI 3-kinases [64, 65]). This remains to be elucidated in detail in the context of individual agonists.

3.2.2. Coupling to Tyrosine Kinases. The neutrophil opsonin receptors CD32a and CD16b do not interact, at least not directly, with heterotrimeric G proteins. Instead, their dimerization induced upon their engagement leads to the recruitment and activation of tyrosine kinases of three major families, Src, Syk, and Tec [66]. Enhanced levels of tyrosine phosphorylation upon engagement of CD32a or CD16b have been observed. Pharmacological as well as genetic information indicates that Src kinases are mobilized upon the engagement of CD32a and of CD16b upstream of Syk. The latter plays a crucial role in the regulation of phagocytosis [67–69]. The Src kinases implicated (Fgr, Hck, and Lyn), in particular in response to different modes of stimulation of CD32a and CD16b, remain to be unambiguously identified. Though a significant level of redundancy between Src family kinases is illustrated by the requirement for double or triple knockout of these kinases for the clear demonstration of functional effects [70, 71], it is also apparent that they control different and specific aspects of the internalization/recycling cycle in neutrophils [71, 72]. The activation of Tec family kinases in response to the engagement of CD32a and CD16 [73] as well as to stimulation with monosodium urate crystals has also been reported. The latter stimuli appear to rely specifically on Tec to mediate their phlogistic activity [74, 75].

Tyrosine kinases of the Src family are also activated upon occupation of chemoattractant receptors (e.g., [76]; see reviews [77, 78]). Direct evidence for the activation of Src family kinases (increased kinase activity, enhanced tyrosine phosphorylation) by chemotactic factors as well as inhibition of signalling and functional responses to these same agonists by potent Src family kinase inhibitors has been repeatedly described. As is the case for opsonin receptors, the Src family of tyrosine kinases play redundant roles in the responses to chemoattractants also [70, 71]. Silencing of single members of the Src family members has little if any effect on the functional responsiveness of neutrophils to chemotactic factors. On the other hand, while silencing of both Hck and Fgr failed to inhibit the mobilization of calcium, the phosphorylation of Akt and of MAPK ERK1 and ERK2 and neutrophil migration in response to fMLF, deficient signalling to the respiratory burst, the polymerization of actin, and the phosphorylation of vav1 and of the p21-activated kinases were observed [70].

The detailed molecular mechanisms by which G protein-coupled receptors activate Srk kinase remain to be satisfactorily characterized. In view of the inhibitory effects of pertussis toxin on the activation of Src kinases by chemotactic factors, it is highly likely that these kinases are responding either directly or indirectly to activated (GTP-bound, dissociated) heterotrimeric G proteins. G proteins and Src family kinases, as well as some chemoattractant receptors (e.g., BLT1 but not FPR [79]), have been observed in lipid rafts which may provide the proximity required for the activation of the latter.

Tyrosine kinases other than Src and Syk family kinases are also involved in the responses of human neutrophils to GPRs. Prominent among these are the Tec family kinases [80, 81]. On the other hand, Syk which plays a crucial role in the control of phagocytosis (see above) is not stimulated upon the activation of chemoattractant receptors. A complex interrelationship exists between the activation of Tec kinases and PI 3-kinases. The stimulation of the former is inhibited

by the pan-PI3kinase inhibitor wortmannin [80], while the accumulation of PtdIns(345)P3 in response to fMLF is itself decreased by the Tec kinase inhibitor LFM-A13 [81]. It should be pointed out however that direct effects of LFM-A13 on the binding of fMLF to FPR1 have not been rigorously ruled out and may explain the inhibition of the mobilization of calcium by the former in response to fMLF [78] which is not observed with a variety of potent tyrosine kinase inhibitors (e.g., PP1 and PP2 (Naccache et al., unpublished observations)). The activation of Syk by monosodium urate crystals, the etiological agent of gout, has also been reported [74, 82, 83]. It should be pointed that the activation of Tec family kinases and of Syk occurs downstream of that of Src family kinases.

Evidence for the involvement of the tyrosine kinase FAK, at least in murine systems, has been reported [84]. However, no direct evidence for the expression by FAK in human neutrophils is available, and FAK is abundantly expressed in platelets which are likely to contaminate to a significant extent most, if not all, neutrophil preparations routinely used.

Recent results indicate that the cytosolic tyrosine kinase c-Abl is critically involved downstream of Src kinases in response to the occupation and activation of β_2-integrins. The ubiquitous kinase c-Abl is implicated in regulating membrane ruffling, cell spreading, cytoskeletal dynamics, and extension of filopodia in various cell types and fibroblasts in particular. Pharmacological inhibition or genetic knockdown of c-Abl inhibited neutrophil recruitment in a thioglycollate-induced peritonitis as well as the *in vitro* chemotactic movement of neutrophils [85]. The activation of c-Abl appears specific to the engagement of β_2-integrins and is not observed in response to stimulation by fMLF in cells in suspension. It occurs downstream of Src kinases, and accordingly, inhibition of Src kinases decreases c-Abl activation [85], and tripartite complexes of Fgr or Hck and c-Abl have been detected in β_2-integrin immunoprecipitates [86]. The link between c-Abl and the cytoskeleton appears to involve the adaptor protein Vav1 whose tyrosine phosphorylation upon β_2-integrin activation is profoundly decreased upon the inhibition of c-Abl [85]. It remains to be understood why β_2-integrins but not fMLF (which both activate Src kinases) stimulate the c-Abl signalling pathway.

As described below, the activation of tyrosine kinases, in response to opsonin or chemoattractant receptors, plays major signalling roles in the regulation of the functional responsiveness of human neutrophils.

3.3. Downstream Effector Pathways. A number of well-characterized signalling pathways lay downstream from heterotrimeric G proteins and/or tyrosine kinases. These include the phospholipase C and the phosphatidylinositol 3-kinase pathways; the activation of either further leads to the stimulation of a variety of signalling intermediates.

3.3.1. Calcium. Transient increases in the cytosolic concentrations of free calcium are an early response of human neutrophils to most of their pathophysiological agonists [87]. Increases in free cytoplasmic calcium (up to close to 1 μM) are detected within seconds of stimulation by a variety of neutrophil agonists including chemotactic factors [88–90], opsonin receptor engagement, and phlogistic microcrystals. They last a few minutes before the cytosolic levels of calcium are restored to their prestimulation levels (about 0.1 μM). Longer lasting calcium oscillations driven by increased calcium influx have been detected upon stimulation of human neutrophils by immune complexes [91].

The stimulated increases in cytosolic free calcium play a central role in the initiation and modulation of the functional responsiveness of neutrophils having been linked to the regulation of most, if not all, neutrophil functions including chemotaxis, phagocytosis, degranulation, and the stimulation of the oxidative burst.

The mobilization of calcium in nonexcitable cells derives from a combination of release from intracellular stores and enhanced influx from the extracellular milieu. This, by now classical, paradigm was derived [88] before the role of Ins(3,4,5)P3 in the mobilization of intracellular calcium was discovered by Berridge and collaborators (reviewed in [36]).

The role of phospholipase C-derived inositol(1,4,5)-trisphosphate (Ins(1,4,5)P_3) in the release of calcium from intracellular stores is well established [36], though it should be noted that the nature of the endoplasmic reticulum-associated intracellular stores of calcium (originally termed calciosomes [92]) remains to be directly characterized in neutrophils.

The nature of the link, if any, between the emptying of the calcium stores into the cytoplasm and the regulation and nature of the plasma membrane calcium channels involved in the influx of calcium is another element that requires further investigation (see below).

(1) Phospholipase C. The increases in cytosolic free calcium are mediated by the activation of one or more phospholipase C (PLC), and the attendant hydrolysis of phosphatidylinositol(4,5)bisphosphate (PtdIns(4,5)P_2) leading to the generation of inositol(1,4,5)trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG). Ins(1,4,5)P_3 plays a major role in the release of calcium from intracellular stores through its binding to specific receptors located on the intracellular storage organelles. Neutrophils express both PLCβ and PLCγ isoforms of PLCs, and these are differentially activated depending on the type of receptors engaged (GPCR- versus tyrosine-kinase coupled, resp.).

(a) PLCβ. The evidence for the involvement of the G protein-coupled PLCβ in the mediation of the responses to the occupation of chemoattractant receptors derives from the evidence of the stimulated hydrolysis of PtdIns(4,5)P_2 [93] and the inhibitory effects of pertussis toxin on the responses of neutrophils to chemotactic factors [59, 60]. Little direct evidence for the stimulation of the activity of PLCβ is available, however. Nevertheless, the activation of a non-identified PLC by fMLF in rat neutrophils in the presence of GTPγS has been reported [94]. Most if not all chemotactic factors for neutrophils depend on PLCβ2 to mediate the responses to receptors for chemotactic factors [95]. It is possible that some of these receptors may be coupled, in addition to Gi, to G proteins of the Gq family. The lack of sensitivity of the responses to PAF to pertussis toxin [96]

and the ability of Gα16 (a member of the pertussis toxin-insensitive Gq family) to interact with the C5a receptor [97] support, *inter alia*, this possibility.

Silencing of PLCβ2 enhanced, unexpectedly, rather than inhibited the chemotaxis of neutrophils from knockout mice to fMLF and CXCL8 [98]. The mechanistic explanation for this (puzzling) observation is still lacking.

(b) PLCγ2. The occupation of tyrosine kinase-coupled receptors such as opsonin receptors (CD32a, CD16b, and CD11b) calls into action PLCs of the PLCγ family and PLCγ2 in particular as evidenced by its enhanced tyrosine phosphorylation upon receptor engagement [99–101].

The nature of the tyrosine kinase involved in the activation of PLCγ_2 upon the engagement of opsonin receptors in neutrophils remains to be unambiguously identified. The known activation of Src kinases by the engagement of these receptors, coupled to the inhibitory effects of highly specific Src kinase inhibitors on the responses, including the mobilization of calcium, makes it highly likely that one or more of the Src kinases may be involved. Lyn is likely to play a role in the phosphorylation of the ITAM of CD32a [76], possibly subsequently to the translocation of the latter to detergent-resistant domains where the kinase resides [102, 103]. Furthermore, direct evidence of the activation of Lyn upon the cross-linking of CD32a [104] and by particulate agonists (monosodium urate crystals) has also been reported [76]. The presently available data cannot define or exclude, however, contributions of the other members of the Src family of kinases to the responses to the engagement of opsonin receptors in human neutrophils.

(2) SOCE and Its Limitations. Calcium mobilization in human neutrophils in response to stimulation by most agonists, and in particular fMLF, conforms to the often described model in which Ins(3,4,5)P_3-dependent release of calcium from intracellular stores (and their concomitant emptying) leads to the opening of plasma membrane calcium channels and an influx of calcium from the extracellular milieu. This is usually referred to as the "store-operated calcium entry" (or SOCE) model [105]. The link between the emptying of the intracellular stores (and indeed their specific identity) and the opening of the plasma membrane calcium channels remains to be characterized in details although the contribution of endoplasmic reticulum-associated Stim-1 and plasma membrane-associated Orai proteins (forming the calcium channels termed I_{CRAC}) appears highly likely [106–109].

Evidence for means of mobilization of calcium (and in particular calcium influx) in human neutrophils other than through SOCE has also been obtained. For example, whereas engagement of CD32a leads to a rapid and robust mobilization of calcium in human neutrophils, this is not accompanied by a stimulation of calcium influx [110]. On a similar note, while both CXCL-8 and CXCL-1 bind to the same receptors on human neutrophils (CSCR8) and induce a quantitatively similar mobilization of calcium (though that induced by CXCL-1 is longer lasting), only the latter induces a detectable influx of calcium from the extracellular medium [38]. Furthermore, pharmacological evidence for ICRAC-independent means of mobilizing calcium in human neutrophils has also been reported [111–114]. This includes the observations that the piperazine compound ML-9 inhibits thapsigargin-induced but not the receptor-operated calcium influx and that the SOCE inhibitor Gd^{3+} only partially inhibits the influx of calcium induced by fMLF. Additionally, fMLF stimulates the influx of Sr^{2+} (an index of receptor-operated calcium entry or ROCE). These results indicate that the link between the emptying of the calcium stores and the opening of the calcium channels can be uncoupled and is dependent on specific, as yet unidentified, intracellular signals that deserve further examination. The potential contribution of arachidonate-regulated calcium channels (termed ARC) [115] as well as of TRP channels (known to be expressed in human neutrophils [116, 117]) to the SOCE-independent calcium influx deserves to be investigated in additional details and in particular in the context of stimulation by various chemoattractants which may depend differentially on distinct calcium channels [118].

(3) Protein Kinase C. In addition to generating the intermediate for the release of intracellular calcium, Ins(3,4,5)P_3, the activation of PLC leads to the formation of the bioactive lipid mediator diacylglycerol or DAG. One of the major signalling pathways dependent on the generation of DAG is that reliant on the activity of a family of serine/threonine kinases known as protein kinase C or PKC.

The PKC family comprises at least ten members subdivided among three subgroups depending on their cofactor requirements [119, 120]. The conventional PKCs (cPKC), of which at least two members are expressed in neutrophils (PKC-α and PKC-β), require binding of phosphatidylserine, DAG, and calcium for activation. Phorbol are esters can substitute for DAG [121]. Novel PKCs (nPKC), of which PKCδ is present in neutrophils, are calcium insensitive, but, as cPKC, DAG and phorbol ester sensitive. Finally, atypical PKCs (aPKCs) do not respond to calcium or to DAG. Whether they are expressed in human neutrophils remains to be rigorously established, although their presence in rat neutrophils has been reported [122].

The ability of phorbol esters to activate neutrophils has been reported as early as the mid-1970s [123–125]. In human neutrophils, phorbol esters, *inter alia*, potently activate the oxidative burst [126] and induce a limited but extensive degranulation of specific granules [125]. The stimulation of the NADPH oxidase by PKCs is closely related to their ability to phosphorylate $p47^{phox}$, one of the molecular components of this complex [127]. The selective secretion of specific granules induced by phorbol esters is itself likely the result of the lack of increases in the levels of cytoplasmic free calcium by this agonist [128] as the simultaneous addition of calcium ionophores results in complete neutrophil degranulation.

The use of phorbol esters has revealed intimate and complex relationships between PKCs and neutrophil activation. Preincubation with phorbol esters has complex and opposite effects on the subsequent functional responsiveness of neutrophils to chemotactic factors. Short incubation times and low concentrations (nM) of phorbol esters enhance the mobilization of calcium induced by fMLF [129] possibly,

though this has not been tested, by recruiting PLCβ in addition to the G protein-coupled PLCγ. On the other hand, high concentrations (μM) of phorbol esters inhibit potently the same responses [130] at the same time as they inhibit the stimulation of the GTPase activity of the α-subunit of Gi induced by fMLF [131]. It is tempting to extrapolate from these results and speculate, *in vivo*, the early phases of neutrophil recruitment which involve modest activation of chemotactic factor receptors and integrins (and a concomitant relatively weak stimulation of PKC) and that this may synergize to stimulate the functional responsiveness of neutrophils. On the other hand, upon prolonged stimulation of these two receptor types such as would be expected at the site of infection (and an accentuated activation of downstream effector pathways, including PKC), inhibition of chemotactic factor receptor-mediated signal transduction would contribute to neutrophil arrest and the performance of their phagocytosis-related functions.

Complex and incompletely understood relationships between the activation of PKCs and that of tyrosine kinases exist in neutrophils. Some of the earliest evidence for these interrelationships came from the observation that phorbol esters altered the pattern of tyrosine phosphorylation induced by fMLF [129]. The ability of phorbol esters to inhibit the activation of Lyn and the tyrosine phosphorylation of the p85 subunit of PI 3-kinase and its subsequent activation [132] provides evidence for a role of PKC upstream of the lipid kinases. These observations are consistent with the more recent findings of Popa-Nita et al. [83] that the tyrosine kinase Syk is a substrate of PKC and that the PKC-mediated serine phosphorylation of Syk is necessary for its interaction with the p85 subunit of PI 3-kinase and its activation. Interactions between PKCs and Src family tyrosine kinases have been reported in various other cellular systems [133–135].

The data described above indicates that PKC modulates the activity of tyrosine kinases. In cellular systems other than neutrophils, data have also been obtained indicating that PKCs can themselves be tyrosine phosphorylated and that these events modulate their activity [136, 137].

Some of the interrelationships just described may result from the direct association of PKCs and tyrosine kinases such as observed in platelets [138].

(4) Phosphatidylinositol 3-Kinases. A substantial body of evidence has implicated phosphatidylinositol 3-kinases (PI3Ks) in the regulation of the functional responsiveness of human neutrophils. This includes both direct measurements of the functional activation of these lipid kinases, measurements (direct and indirect) of the levels of the products generated upon their activation, the results of genetic manipulation in murine models, and the use of pharmacological agents.

Of the multiple PI3Ks, it is the class I PI3Ks (both class IA and IB) which are of direct relevance to the early events of the activation of human neutrophils [139, 140]. This subset of PI3Ks comprises tyrosine phosphorylation (class IA) and G protein (class IB) dependent members, namely, PI3Kα, PI3Kβ, and PI3Kδ for the former and PI3Kγ for the latter.

Direct evidence for the activation of PI3Ks is available. The initial indication of the implication of PI3Ks in the responses of human neutrophils came from the measurement of the stimulated accumulation of the product of PI3Ks, namely, PtdIns(3,4,5)P_3 [141, 142]. Accumulation of PtdIns(3,4,5)P_3 in response to the engagement of CD32a has also been reported [143]. Rapid activation (within seconds) of PI3Kγ by GPCRs (fMLF) has been documented [144].

The stimulation of the activity of PI3Ks may come directly upon the activation of G proteins through the interaction of the dissociated $\beta\gamma$ subunit with the regulatory p101 subunit as in the case of PI3Kγ [145] (with an additional contribution for optimal activation of GTP-Ras [146]) or secondarily to the activation of tyrosine kinases and Src family kinases in particular, upon the interaction of the SH2 domains of the p85 regulatory subunit of class IA PI3Ks with phosphotyrosines on adaptor proteins. GTP-Ras plays a role, here too, in the optimal activation of the p110 catalytic subunit. In the case of β2 integrins and FcγRs, these adaptor proteins include DAP12 and the Fcγ chain [147].

Pharmacological as well as genetic approaches have provided evidence for class I PI3Ks in the regulation of neutrophil adhesion, chemotaxis and recruitment, and phagocytosis and bactericidal activity (mostly through the regulation of the assembly and activation of the NADPH complex) [148–150]. Introduction of dominant negative mutants of p85 and of p110γ in the myeloid cell line PLB-985 differentiated to express a neutrophil-like phenotype indicates that only class IA PI3Ks are involved in the regulation of the chemotactic and oxidative responses to fMLF, though PI3Kγ is mostly responsible for the initial peak of accumulation of PtdIns(3,4,5)P_3 [151].

(5) Cyclic Nucleotides. Most neutrophil functions are inhibited by pharmacological manipulations that increase the levels of intracellular increase cAMP (e.g., [152–159]). Stimulation of neutrophils by various agonists leads to rapid and transient increases in cAMP [160–162]. The increases in cAMP may be due to an inhibition of phosphodiesterases [37] rather than to a stimulation of adenylyl cyclase. Evidence that the cAMP responses to the chemoattractant fMLF are secondary to the accumulation of adenosine has been obtained [163, 164]. The implication of the heterotrimeric G protein Gs, which is known to be present and active in neutrophils [165, 166], in the mediation of the increases of cAMP in neutrophils remains to be investigated. The role of cAMP in dampening neutrophil responses, while generally admitted, is however far from being completely understood as is the cAMP-mediated regulation of gene transcription in neutrophils [167, 168].

Very little is known about the potential involvement of cyclic GMP in the responses of human neutrophils though it is generally assumed to play a role opposite to that of cAMP in a manner consistent with the Yin-Yang hypothesis [169]. Accordingly, cyclic GMP enhances while cAMP inhibits neutrophil chemotaxis [165]. The intriguing results of Coffey et al. [170] linking the priming effects of GM-CSF to a stimulation of guanylyl cyclase coupled to an inhibition of adenylyl cyclase remain to be revisited.

(6) Other Signalling Pathways. The implication of various other signalling pathways in the regulation of the functional responsiveness of human neutrophils has been documented. These include, *inter alia*, the MAPK pathway, protein tyrosine phosphatases, serine/threonine phosphatases, small molecular weight G proteins of the Rho, and Rac and Arf families (and their relationship to the activation of the phospholipase D pathway and to the organization of the motor of the neutrophil, the actin cytoskeleton). Information about these events can be found in the reviews listed at the beginning of this paper.

4. Closing Remarks

As briefly summarized above as well as in the cited literature, a vast amount of detailed information about "signalling in neutrophils" is now available. The major pathways stimulated upon the engagement of a variety of surface receptors, with which the neutrophil is richly endowed, have been identified. Several central challenges remain however. As mentioned in the introduction, our understanding of the role(s) and function(s) of the neutrophil in innate as well as acquired immunity has vastly expanded in recent years. The manner in which this cell recognizes the clues that direct and control its functional and phenotypic plasticity, that is, the specific pathways which are called upon and their order and intensity, remain to be elucidated on an individual basis.

It is also clear that neutrophils will rarely, if ever, be exposed to a single class of agonists *in vitro* and that the extracellular environment and the extracellular matrix play major roles in directing and redirecting neutrophil responses (including the control of transcriptional programmes [171]). This is intimately related to the development of neutrophil subtypes and activated phenotypes that are likely to be encountered at inflammatory and tumor sites [172, 173]. This is another area that will require a sustained attention in coming years. A corollary of this is that the influence of other cell types, including endothelial cells, monocyte/macrophages, and platelets (e.g., [174–176]), as well as that of the variety of extracellular vesicles that have been identified by now (microvesicles, exosomes...) [177] on the functional responsiveness of neutrophil can no longer be ignored in the perspective of deriving a more complete picture of this cell's pathophysiological role(s).

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