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Review Article

Ca^{2+}, calpain and 3-phosphorylated phosphatidyl inositides; decision-making signals in neutrophils as potential targets for therapeutics

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Abstract

The chemical signals within neutrophils that control their behaviour are complex and these signals control the complex activity of neutrophils with precision. Failure of neutrophils to reform their anti-bacterial activity would lead to infection, while over-activity of neutrophils may lead to tissue damage and inflammatory disease. The identity of some of the intracellular signals is becoming clear and insights into the potential for interplay between them are being sought. Although it is well established that cytosolic free Ca^{2+} plays a role, it is only recently that the importance of intracellular protease, calpain, and the 3-position phosphorylated phosphatidyl inositides is becoming recognised. In this review these three key signals are discussed as potential therapeutic targets for the modulation of neutrophil activity.

Introduction

Neutrophils are the most numerous phagocytic white cells of the blood, out-numbering all other whites put together. They represent the front line of defence against infection, operating before the need for antibody production. Their main role is to recognise and kill infecting microbes, by phagocytosis. To achieve this, these cells have two characteristics: firstly, they are autonomous cells, which migrate from the blood through the extravascular space when signalled to do so by the presence of infecting microbes; secondly, they are armed with the machinery necessary to kill the microbe. These two characteristics pose a potential threat to the host, as the possibility exists that they could inappropriately unleash their killing power against surrounding host tissue. The mechanism that controls the behaviour of these cells must therefore be reliable, so that damage to host tissues is limited, and also be sufficiently flexible to enable these cells to be effective. This autonomy of action has presented problems both in understanding signalling within these cells and also in devising ways in which they may be controlled pharmacologically. Clearly, a hypothetical drug that could inhibit all neutrophil activity would suppress a pathological inflammatory process but would also produce a greater threat by leaving the patient open to infection. For example, the genetic condition, chronic granulomatous disease, where the neutrophil oxidase is ineffective and neutrophil function does not result in effective microbial killing, is fatal. An anti-inflammatory therapeutic clearly must have more subtlety than simply a suppression of neutrophil activity.

Another theoretical possibility for therapy lies in increasing the efficacy of neutrophils. If this were achieved, enhanced neutrophil effect could either complement antibiotics or, preferably, replace them. The latter possibility would eliminate the danger of antibiotic resistance in microbes.

For whatever ends neutrophil behaviour is to be manipulated pharmacologically, a clear prerequisite for designing or searching for such a drug is that the intracellular signals that control neutrophil behaviour are identified. In this review, we aim to point to cytosolic Ca^{2+} signalling, calpain activation and 3-position phosphorylated phosphatidyl inositides as key signalling components in neutrophils.

The role of cytosolic Ca^{2+} signalling

As in many other cell types, cytosolic free Ca^{2+} plays an important role in signalling activity in neutrophils. Surprisingly, in each neutrophil activity, adhesion, morphological

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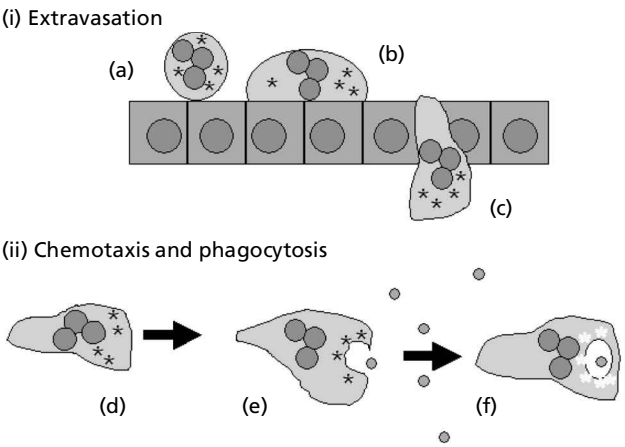

Figure 1 The neutrophil programme. The sequence shows the inactive oxidase molecules (*) within the cytosol of the rolling neutrophil (i) in the blood stream (a) as it is transformed through adherence to the endothelium (b) and transmigration (c); and (ii) in the extravascular space during chemotaxis (d), phagosome formation around a microbe (e) and, finally, oxidase activation as the phagosome closes (f).

change and polarization, extravasation, chemotaxis, phagocytosis, oxidase activation and apoptosis (Figure 1), there is evidence to implicate cytosolic Ca^{2+} as the trigger. Clearly, cytosolic free Ca^{2+} alone cannot signal all these events as there would be continual cross-talk between stimuli and their response outcomes. However, it is now becoming clear that the role of Ca^{2+} is more limited physiologically and that complexity of signalling is introduced by localisation of some signals and the affinity of some Ca^{2+} binding proteins.

Classical receptors

The receptors for all the neutrophil chemoattractants are similar, having seven transmembrane (7TM) domain structure. These are classical pharmacological receptors. Their activation results in the elevation of cytosolic free Ca^{2+} through the text-book route, namely the trimeric GTP-protein route, which couples receptor occupancy to phospholipase PLC-β (phospholipase C) activity. The enzyme generates IP_3 (inositol trisphosphate) and diacylglycerol from PIP_2 (phosphatidyl inositol bisphosphate). IP_3 (or InsP(3)) diffuses away from the plasma membrane through the cytosol to a single juxta-nuclear site within the neutrophil, from where Ca^{2+} is released (Pettit et al 1997; Pettit & Hallett 1998). Blockade of IP_3 receptors results in an inhibition of Ca^{2+} release by these stimuli (Davies-Cox et al 2001). However, it is unlikely that this mechanism plays a part in neutrophil chemotaxis because: the geometry of a single centrally located Ca^{2+} release site would not permit the polarity in signalling that would be required for chemotaxis; the concentration of chemoattractant required to induce the Ca^{2+} signal is several orders of magnitude higher than that required for chemotaxis or actin polymerisation (Al-Mohanna & Hallett 1990); during chemotaxis no Ca^{2+} signals are generated (Laffafian & Hallett 1995); neutrophils from PLC-β knock-out mice fail to signal Ca^{2+} in response to chemoattractants yet their ability for chemotaxis is unimpaired (Li et al 2000). Another reason that this route is unlikely to be involved is that Ca^{2+} influx is triggered following Ca^{2+} store release. Although the mechanism that connects the release of Ca^{2+} from stores to the opening of Ca^{2+} channels at the plasma membrane is not clear, it probably involves a diffusible factor, as yet unidentified (but named Ca^{2+}-influx factor), which is generated during the release of Ca^{2+} from the central Ca^{2+} store (Davies & Hallett 1995). The diffusion of this factor would be uniform throughout the neutrophil and thus also destroy the polarisation of intracellular chemoattractant signalling.

Non-classical receptors

Neutrophils also express other receptors, which may be described as non-classical. These retain information about the locality of stimulation and do so via cytosolic free Ca^{2+} signalling. Whereas the 7TM receptors can be thought of as classical pharmacological receptors, where occupancy of a crucial site on the receptor provides the stimulus, the non-classical receptors do not require such a binding (Figure 2). Instead, the cell is activated when these receptors are immobilised relative to each other. In fact, experimentally these receptors can be immobilised in a number of ways (e.g., using anti-antibody-antibodies or covalently cross-linking them). This explains why these receptors can be promiscuous, having more than one structurally unrelated ligand. The β2 integrin, for example, binds to RGD sequences on extracellular proteins, to iC3b (the complement derived opsonin) and also to ICAM-1 (intercellular adhesion molecule-1). The intracellular signal, however, is generated only when the ligand (and hence the bound receptor) is immobilised. In this way, neutrophils distinguish between free iC3b and that bound to the microbe. The Fc receptors are similarly of the non-classical, immobilisation-activated type. Again, this permits neutrophils to be stimulated by antibody bound to a microbe while existing in an environment full of free antibody.

The signal generated by cross-linking these non-classical receptors is an elevation of cytosolic free Ca^{2+} but the mechanism for this has yet to be established. However, it is possible that IP_3 generation, perhaps from PLC-γ, is involved. However, the amount of IP_3 generated by cross-linking stimulation is very small (Rosales & Brown 1992; Fallman et al 1993) and the microinjection of heparin (which blocks IP_3 receptors) fails to inhibit Ca^{2+} signalling by β2 integrin (Dewitt et al 2002). The possibility thus exists that intracellular signals other than IP_3 mediate Ca^{2+} by this route. cADP ribose or NAADP (Partida-Sanchez et al 2001; Dewitt et al 2002), or PI-3 kinases (which generate PIP_3), may be involved. Whatever the mechanism for its generation, the Ca^{2+} signal itself is tightly coupled to the events associated with β2 integrin-mediated phagocytosis.

When iC3b-opsonised particles are presented to neutrophils with a micro-manipulator, a small localised Ca^{2+} change occurs at the site of particle attachment and cup formation as a result of store release, but rapid engulfment

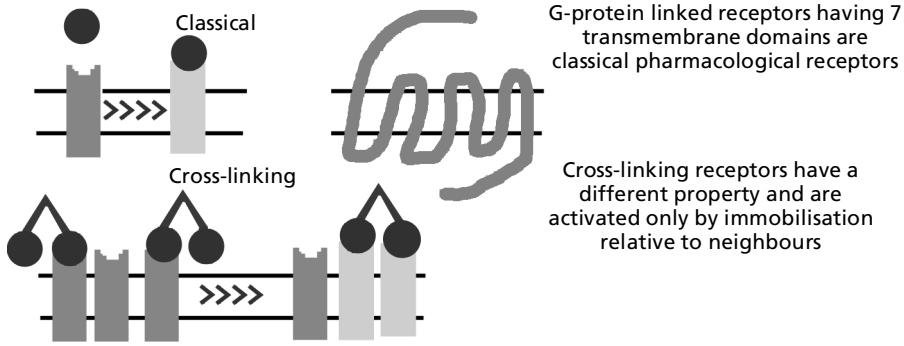

Figure 2 Receptor types on neutrophils.

of the particle requires a large change in cytosolic free Ca^{2+} throughout the cytosol (Dewitt & Hallett 2002). This global Ca^{2+} signal results from Ca^{2+} influx into the cell through open Ca^{2+} channels and leads to the liberation of a fraction of β2 integrin receptors that were initially immobile on the neutrophil surface. These freed β2 integrin molecules are then available for binding to the particle and so phagocytosis is accelerated (Dewitt & Hallett 2002). The Ca^{2+} influx signal that is triggered by β2-integrin-mediated phagocytosis has two temporally separated components, the first part of which occurs during β2 integrin engagement as the phagocytic cup forms, and does not trigger oxidation of the particle (Dewitt et al 2003). However, the second part of the global Ca^{2+} signal, which is triggered about the time of phagosomal closure, causes an abrupt activation of the oxidase (Dewitt et al 2003). Surprisingly, although this second Ca^{2+} signal is not restricted to the region of the phagosome, oxidase activation is only triggered locally in the phagosome, with no evidence of activation at other sites in the neutrophil. This points to a dual control of oxidase activation, with Ca^{2+} controlling the timing of oxidase activation but slower and more localised molecular events, which involve oxidase assembly and PI(3)P generation (see later), determining the site of oxidase activation.

The role of calpain

The calpains are intracellular Ca^{2+}-activated cysteine proteases. They are composed of two subunits, the larger containing the catalytic site and both having Ca^{2+} binding EF hands. In neutrophils, the dominant form is μ-calpain (Lokuta et al 2003), which is activated by about 30–50 μM Ca^{2+}, whereas another ubiquitous calpain, m-calpain, which is activated by 1 mM Ca^{2+}, is not found (Lokuta et al 2003). Calpain has a number of potentially important proteolytic targets in neutrophils (Table 1). The most important substrates are involved in controlling β2-integrin-mediated events such as extravasation and phagocytosis and may include talin. The mobility of β2 integrins on the neutrophil surface is extremely limited (Dewitt & Hallett 2002) and is probably immobilised by being bound to the underlying cytoskeleton through talin. Cleaved talin probably binds to the cytosolic tail of β2 integrin as it does to β3 integrin (Yan et al 2001) and affects the affinity of the integrin for its substrate (Tadokoro et al 2003). Once talin is cleaved by calpain, β2 integrin molecules are able to cluster and participate in signalling. It has been shown that inhibition of calpain activation prevents neutrophil trans-endothelial migration (Noble et al 1998), adhesion to endothelial cells (Anderson et al 2000) and phagocytosis of iC3b-opsonised particles during phagocytosis (Dewitt & Hallett 2002). All these effects can be attributed to an inhibition of Ca^{2+}-induced liberation of β2 integrin. Since bulk cytosolic free Ca^{2+} within neutrophils is never sufficiently high enough to activate calpain (bulk level $\approx$1 μM, $K_{0.5}$ (calpain activation) $\approx$50 μM), the possibility exists that during Ca^{2+} influx there is a high level of sub-plasma-membrane Ca^{2+}. Indeed, it has been shown that during Ca^{2+} influx, the sub-plasma-membrane Ca^{2+} reaches more than 50 μM (Davies & Hallett 1998). This would explain why Ca^{2+} influx is required for phagocytic

Table 1 Some cytosolic calpain substrates related to cell shape change and phagocytosis.

Substrate	Function	References
Talin	Links cytosolic tail of β-integrins to actin	Goll et al (2003), Tadokoro et al (2003)
Myosin X	Binds PI(3,4,5) and actin	Berg et al (2000), Cox et al (2002)
WASP (Wiskoft-Aldrich syndrome protein)	Actin polymerisation	Scherbina et al (2001)
RhoA	Actin assembly	Kulkarni et al (2002)

acceleration and would also restrict calpain activation to the strategic location just beneath the plasma membrane. There may also be cross-talk between β2 integrin mobility and the other opsonin, namely antibody. Jongstra-Bilen et al (2003) recently showed, in a myeloid cell line, that engagement of Fc receptors also leads to an enhanced mobility of β2 integrin. They concluded, however, that the effect was not dependent on Ca^{2+} as it could not be inhibited by increasing intracellular Ca^{2+} chelation using BAPTA. In view of the high and localised Ca^{2+} signal required for calpain activation, this is not conclusive evidence against a role for Ca^{2+} or calpain in this system and it is probably similar to that described for neutrophils. It has also been suggested that calpain is active within resting neutrophils (Lokuta et al 2003) and that this proteolytic activity exerts an inhibitory effect on chemotaxis. It has been reported that inhibition of this proteolytic activity accelerates chemotaxis in neutrophils (Lokuta et al 2003). This is surprising, especially as calpain inhibition is inhibitory for chemotaxis by other cell types (Glading et al 2002), as it may be expected that integrin engagement would be a prerequisite for chemotaxis.

3-Position phosphorylated phosphatidyl inositides

It is well established that phosphorylation of phosphatidyl inositol (PI) at position 4 and 5 on the inositol ring to produce PI(4,5)P2 is a key step in cell signalling, because it is the substrate for IP_3 generating phospholipases. However, there is now clear evidence that PI-3 kinases, which phosphorylate at the 3 position, are also very important in neutrophils. Neutrophil chemotaxis is impaired in mice deficient in the gene for PI-3 kinase-γ (Hirsch et al 2000; Li et al 2000; Sasaki et al 2000; Stephens et al 2002). This points to a role for $PI(3,4,5)P_3$ in chemotaxis and myeloid cell polarisation (Rickert et al 2000; Servant et al 2000; Stephens et al 2002; Weiner et al 2002). The evidence for such a role in neutrophil polarisation comes from the use of PH-Akt-GFP constructs, which bind $PI(3,4,5)P_3$ and possibly other lipids and translocate to the leading edge of myeloid (neutrophil-like) cells during chemotaxis polarisation (Rickert et al 2000; Servant et al 2000). However, there is also a linkage to integrin and Ca^{2+} signalling. The activity of PI-3 kinase increases during β2 integrin engagement (Traynor-Kaplan et al 1989) and inhibitors of the enzyme prevent Ca^{2+} signalling (Vossebeld et al 1997). It has also recently been shown that PI(3,4,5)P3 introduced into the inner leaflet of the plasma membrane of neutrophils results in complex Ca^{2+} signalling (Tian et al 2003) and that the neutrophils then polarise with exogenous PIP_3 accumulating at the rear (Tian et al 2003). This is in contrast to the location of endogenously produced PIP_3, which is generated and remains at the front of polarising neutrophil-like HL60 cells (Rickert et al 2000; Servant et al 2000; Stephens et al 2002; Weiner et al 2002). The precise role of PI(3,4,5)P3 thus remains unclear, but PI-3 kinase activity clearly underlies the ability of neutrophils to polarise.

Another 3-position phosphorylated phosphoinisotide that has been implicated in neutrophil activity is PI(3)P — a monophosphate on the 3 position is also important in neutrophil behaviour. Unlike PIP_3, which is recognised by PH domains, this lipid is bound by a related domain, PX. It has been shown that p47phox, a component of the neutrophil oxidase, has a PX domain (Ellson et al 2001; Kanai et al 2001) and that after phagosome formation PI(3)P accumulates around the phagosome (Ellson et al 2001). As PI(3)P accumulates in the phagosome, it is thought that this event underlies the assembly of the oxidase at the correct location (i.e., only within the phagosomal membrane) and thus its activation by Ca^{2+} results only in phagosomal activity.

Potential therapeutic targets

The previous brief discussion of some of the key signalling events in neutrophil behaviour has highlighted three steps that may be beneficially targeted for therapy. These are Ca^{2+}-channel opening, which underlies Ca^{2+} influx, calpain activation, which is a consequence of Ca^{2+} influx, and 3-position phosphorlyated phosphatidyl inositides.

Ca^{2+}-channel opening and Ca^{2+} influx

The small amount of intracellularly stored Ca^{2+} in neutrophils that is released by IP_3 or other intracellular messengers acts as a trigger for the larger Ca^{2+} signal, which is derived from Ca^{2+} influx (Hallett & Lloyd 1997) and it is this that is responsible for a number of the activities of neutrophils (Pettit & Hallett 1996; Hallett & Lloyd 1997; Bei et al 1998; Dewitt & Hallett 2002). The mechanism for this coupling between the release of Ca^{2+} from intracellular Ca^{2+} storage sites and influx is unclear, but it is often referred to as capacitative Ca^{2+} influx. This coupling depends on the release of Ca^{2+} regardless of the mechanism for this release. For example, when Ca^{2+} is experimentally allowed to leak from the stores by the inhibition of the SERCa pumps, using for example thapsigargin or cyclopiazonic acid, the plasma membrane channels are also open. Until recently, there has been surprisingly little established about the nature of the Ca^{2+} channels in neutrophils. von Tscharner et al (1986) showed that the addition of f-met-leu-phe to membrane patches did not cause Ca^{2+}-channel opening, revealing that the opening was not directly coupled to receptor occupancy, but required cytosolic components (which were missing from the patch). The Ca^{2+} channels are not voltage-sensitive channels and are insensitive to inhibitors of such channels (Demaurex et al 1994), although membrane potential may play a role (DiVirgilio et al 1987), especially when generated by the activity of the NADPH oxidase (Hallett 2003; Rada et al 2003).

The channels have two types of channel current, with conductance of 18–25 pS and 4–6 pS, and are poorly selective for Ca^{2+}, being equally permeable to K^+, Na^+ and Ca^{2+}, but not Cl^- (von Tscharner et al 1986). It has recently been established that the myeloid channels belong to the TRP family (Heiner et al 2003a, b) and that LTRPC2 (long-TRP channel 2), which has the required electrophysiological properties, is found in both neutrophil-like HL60 cells and neutrophils (Heiner et al 2003a, b).

Inhibitors of this channel would be of obvious importance in controlling neutrophil-driven inflammation. Experimentally, these Ca^{2+} channels are blocked by Ni^{+} ions, but the pharmaceutical industry has produced only one organic inhibitor of the channels, namely SKF 96365 (Merritt et al 1990), which is structurally related to econazole and itraconazole. This blocks Ca^{2+} influx into neutrophils but may act at a similar site to econazole, which is claimed to be an inhibitor of CIF activity (Randriamampita & Tsein 1993). The capacitative influx route is also inhibited by some inhibitors of the P450 system (Montero et al 1992). Recently, another compound, LOE 908, has been shown to inhibit Ca^{2+} influx into myeloid cells (Krautwurst et al 1993) and a number of cell types with a pharmacology that suggests that it may also inhibit this non-specific Ca^{2+} channel (Miwa et al 2000).

It is unlikely that any of the present agents will be useful therapeutically as their specificity for neutrophils is limited. However, the discovery that the LTRPC2 channel is the Ca^{2+} influx route for neutrophils may open up a route to discovering more selective neutrophil Ca^{2+}-channel blockers.

μ-*Calpain*

The structure of calpain is well established (Lin et al 1997) although the molecular mechanism by which calpain is activated and the interplay between the Ca^{2+}-binding EF hands remains a subject of study (Goll et al 2003). There are inhibitors of both the protease catalytic site (e.g. calpeptin, ALLN and Z-Leu-Leu-CHO and the Ca^{2+}-binding site, PD150606 (Goll et al 2003)). This is therefore a well-defined target for therapeutics. There are several reports of the beneficial anti-inflammatory effects of inhibiting calpain activity. For example, in animals, calpain inhibition attenuates neutrophil-mediated myocardial ischaemia–reperfusion injury (Ikeda et al 2002). Another example is that neutrophil aberration in essential hypertension (Fardon et al 2001) reduces renal ischaemia–reperfusion injury (Chaterjee et al 2001) and reduces neutrophil-mediated colon injury caused by dinitrobenzene sulfonic acid (Cuzzocrea et al 2001). There are also reportedly selective inhibitors of μ-calpain (Lokuta et al 2003), the form almost exclusively in neutrophils, which may have a selective therapeutic benefit.

PI-3 kinase

Inhibition of PI-3 kinase activity has profound effects on neutrophils. Firstly, Ca^{2+} signals associated with β-integrin or FcR cross-linking, as would occur during phagocytosis or transendothelial migration, are inhibited. Secondly, the ability to form pseudopodia or polarise is inhibited. PI-3 kinase activity can be inhibited experimentally by wortmannin or LY-294002. However, both these agents also have other inhibitory effects and there is no specificity of isotypes. However, recently an inhibitor specific for PI(3) kinase delta has been reported (Sadhu et al 2003). This inhibitor was shown to inhibit the ability of neutrophils to migrate or orientate correctly (Sadhu et al 2003). Interestingly, there seemed to be an effect on the uropod with neutrophils forming exaggeratedly long tails (Sadhu et al 2003), an effect that may be related to the need to accumulate PI-3 kinase products at the uropod (Tian et al 2003).

Conclusion

In this short review, three crucial intracellular signalling steps have been highlighted as potential therapeutic targets for immunotherapy. While there is a long way to go, it is clear that as our understanding of the signalling within neutrophils increases, the number of potential therapeutic targets also increases. It is hoped that some of the proteins involved in these steps may be unique, or at least preferentially expressed in myeloid cells, so that they become suitable targets. Also as the number of targets increases so does the chance of hitting the bull's eye (i.e. finding a compound with the appropriate inhibitory action by mass screening of compounds). It is on these twin glimmers of light at the end of the long tunnel of research that the hope of finding selective neutrophil-based anti-inflammatory therapeutics is fixed.

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