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REVIEW

Signal Transduction in Neutrophil Chemotaxis

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Abstract—This review discusses current knowledge on signal transduction pathways controlling chemotaxis of neutrophils and similar cells. Most neutrophil chemoattractants bind to seven-transmembrane-helix receptors. These receptors activate trimeric G proteins of the G_i class in neutrophils to initiate chemotaxis. Phospholipases Cβ, phosphoinositide 3-kinase γ, and PH domain-containing proteins play various roles in signaling further downstream. The actin cytoskeleton is crucial for cell motility, and is controlled by Rho family GTP-binding proteins. PIP 5-kinase, LIM kinase, myosin light chain kinase and phosphatase, or WASP-like proteins may be important links between Rho GTPases and actin during chemotaxis. Newly emerging ideas on the regulation of the "compass" of chemotaxing cells, which may involve Cdc42 and certain PH domain-containing proteins, are also presented.

Key words: neutrophil chemotaxis, signal transduction, G protein-coupled receptors, PI3 kinases, Rho family proteins, actin polymerization

I. PHYSIOLOGY AND MORPHOLOGY OF NEUTROPHIL MOTILITY

Neutrophils (polymorphonuclear leukocytes) constitute more than half of circulating white blood cells in humans and provide a major defense system against microorganisms. Their crucial role in innate immunity is highlighted by a severe susceptibility to bacterial infections in patients with neutrophil disorders such as various forms of neutropenia [1], leukocyte adhesion deficiency [2], or chronic granulomatous disease [3]. On the other hand, hyperactivated neutrophils cause pathologies. Reperfusion injury [4], vasculitis [5], adult respiratory distress syndrome [6], or glomerulonephritis [7] demonstrate the medical importance of neutrophil overactivation.

Neutrophils possess a large armament of antibacterial activities including phagocytosis [8], production of oxygen radicals [9], and secretion of various degrading enzymes [10]. Resting nonadherent neutrophils are spherical cells of about 7 μm in diameter [11]. Upon stimulation, they markedly change their shape, forming asymmetric protrusions called pseudopodia, which drive cell migration when in contact with a substratum. Physiologically, before migration through a tissue to the source of infection, a circulating neutrophil has to cross the blood vessel endothelium (Fig. 1). This preferentially happens in the postcapillary venules and involves several steps [12]: attachment to and rolling on the endothelium followed by firm adhesion and finally transendothelial migration or diapedesis. For the latter the neutrophil has to pull itself between endothelial cells through a hole that is several times narrower than the neutrophil diameter, a phenomenon demonstrating remarkable flexibility of the membranes and cytoskeleton of the neutrophil (Fig. 1).

The actin cytoskeleton is absolutely required for cell crawling, which is the main type of cell motility in multicellular organisms. Newly polymerized actin filaments are enriched in the leading edge of a migrating cell [13, 14], and pseudopod formation correlates temporarily with increases in filamentous actin [15]. Preventing actin polymerization abolishes chemotaxis [16-19]. These and other data have led to a widely accepted model that actin polymerization is the driving force of the cell leading edge protrusion [20, 21].

Neutrophil motility has been modeled *in vitro* on two-dimensional surfaces or in three-dimensional gels. These studies and investigation of neutrophils in suspension have revealed striking periodicities in neutrophil behavior upon stimulation (reviewed in [22]). When stimulated in suspension, neutrophils change their shape every eight seconds [23]. This is reflected by an eight second periodicity in neutrophil motility on a substratum

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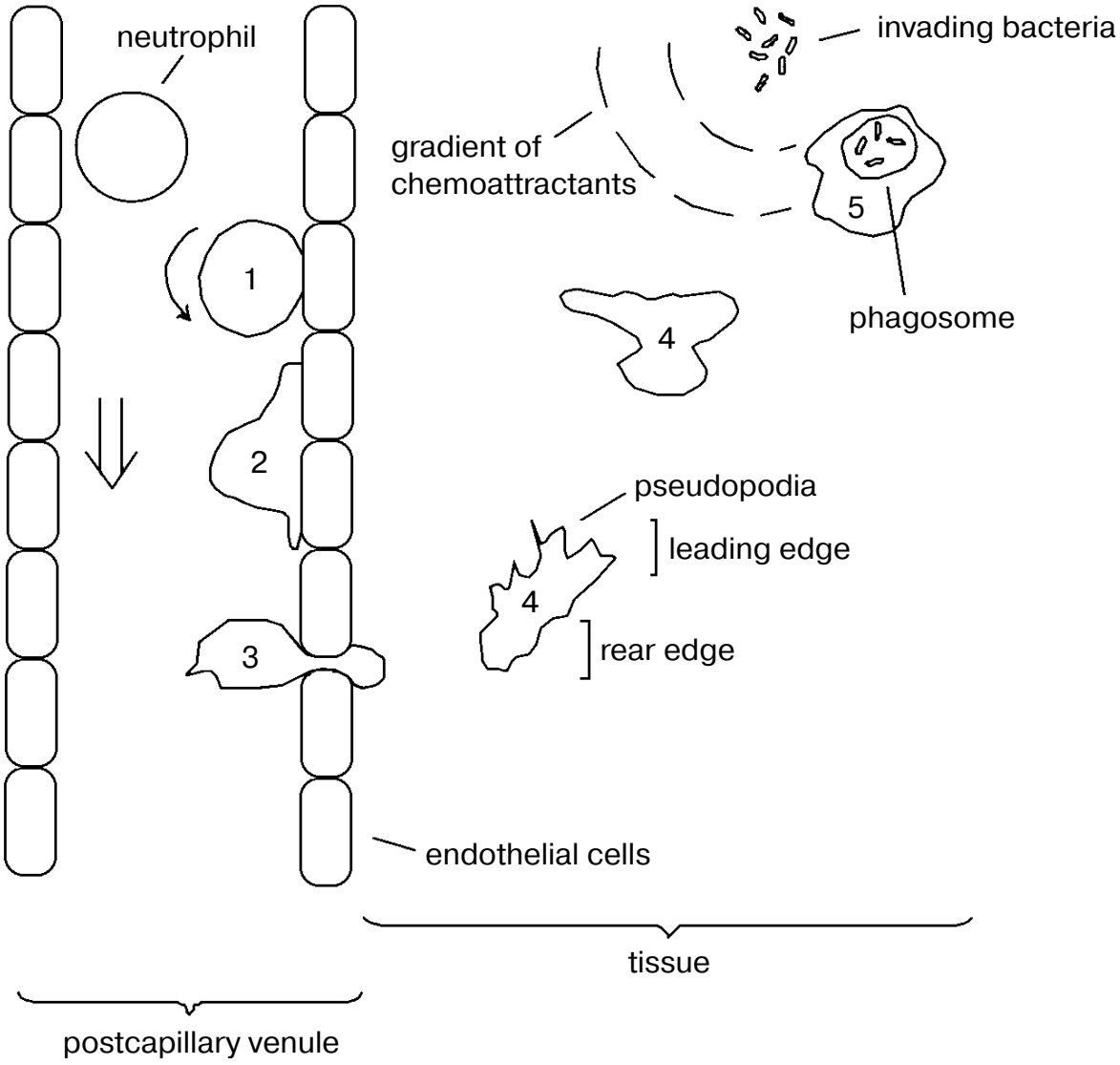

Fig. 1. Scheme of neutrophil emigration from a blood vessel to a tissue infected with bacteria. After receiving a chemoattractant signal, a circulating neutrophil passes through several stages (numbered on the scheme) of interaction with neighboring cells. These stages include: 1) rolling of the neutrophil on the surface of endothelial cells, a process mainly mediated by selectins; 2) firm integrin-mediated adhesion of the neutrophil to the endothelial cells; 3) neutrophil diapedesis or transmigration through the endothelial layer; 4) chemotaxis through the post-endothelial tissue to the source of bacterial infection; and 5) killing of bacteria by phagocytosis, production of oxygen radicals, and release of the antibacterial granule content.

[24, 25] and is achieved by oscillatory protrusion and retraction of pseudopodia. In addition to these small-scale oscillations, crawling neutrophils also pause and re-establish the direction of migration every 45-60 sec, this being mediated by cell repolarization and probably necessary for correct perception of a gradient [22]. Thus neutrophil migration seems to be timed by two superimposed molecular clocks enabling cell response to a multitude of stimuli.

II. CHEMOATTRACTANTS AND RECEPTORS IN NEUTROPHIL MOTILITY

1. "Non-classical" chemoattractants and their receptors. *In vitro* studies have identified a multitude of agents inducing neutrophil chemotaxis (directed migration) or chemokinesis (random migration). Although "classical" chemoattractants bind to G protein-coupled receptors, there are other reported chemotactic or

chemokinetic molecules which act on different receptors.

In addition to their well-known role in hematopoiesis [26, 27], granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been reported to activate chemokinesis but not chemotaxis in neutrophils [28-30]. These cytokines bind to monomeric (G-CSF) or heterodimeric (GM-CSF) non-catalytic receptors that in turn activate Ser/Thr/Tyr kinases called Janus kinases (Jaks) [31].

TNFα (tumor necrosis factor α) induces neutrophil chemotaxis [32-34] via TNF receptor clustering and signal transduction through multiple protein–protein interactions. Neutrophils possess two TNF receptors [35]: a p75 TNF receptor (CD120b) propagates the signal through TNF-receptor-associated proteins (TRAPs), while death-domain proteins are involved in signal transduction by a p55 receptor (CD120a) [36, 37]. It is not known which pathway induces neutrophil chemotaxis, although the p55 receptor was recently shown to modulate chemotactic responses in macrophages [38]. Conflicting reports exist as to whether lymphotoxin (TNFβ), which shares the same p75 TNF receptor with TNFα, is chemotactic for neutrophils [39, 40]. Recently, soluble Fas ligand has been shown to induce neutrophil chemotaxis [41, 42]. Fas (Apo1/CD95) is another representative of TNF-like receptors and when activated induces apoptosis in many cells upon signaling through death domain proteins (reviewed in [37]). The chemotactic activity of the Fas ligand has been shown to occur at concentrations incapable of inducing neutrophil apoptosis [42] and through a death domain-independent mechanism [41].

Platelet-derived growth factor [43] and insulin [44] were reported to induce neutrophil chemotaxis and chemokinesis, respectively. They activate members of the receptor tyrosine kinase superfamily. Upon ligand binding, such receptors homodimerize and autophosphorylate, creating docking sites for highly specific signal transducers [45].

The most potent chemoattractant for neutrophils identified so far, inducing chemotaxis at femtomolar concentrations, is the transforming growth factor β (TGFβ). TGFβ binds to a serine kinase receptor inducing phosphorylation of intracellular proteins called Smads (for a review see [46]). TGFβ is a "pure" chemoattractant since unlike classical chemoattractants it does not stimulate other neutrophil activities besides chemotaxis [47, 48]. Surprisingly, TGFβ-directed chemotaxis is sensitive to pertussis toxin [49], implying an involvement of trimeric G proteins, also crucial for the signaling induced by "classical" chemoattractants (see below).

2. G protein-coupled receptor agonists as neutrophil chemoattractants. Based on their molecular nature, one can distinguish five groups of "classical" leukocyte chemoattractants acting through G protein-coupled (serpentine, seven-transmembrane-helix) receptors [50] (Fig. 2). These would include: 1) *N*-formylated peptides, such as fMLP, derived from bacterial proteins [51]; 2) platelet-activating factor (PAF) produced by activated platelets, neutrophils, and other cells [52]; 3) leukotriene B4 (LTB4) derived from arachidonic acid metabolism and produced by various myeloid cells [53]; 4) C5a anaphylotoxin obtained after cleavage of the complement protein C5 [54], and 5) chemokines, a family of about 100-amino acid-long proteins with four conserved cysteines linked by disulfide bonds, which are produced locally in many tissues [55]. Based on whether the first two conserved cysteines are adjacent or separated by one amino acid, CC and CXC chemokine subfamilies are defined [56]. At excessive concentrations, these chemoattractants induce a wide range of responses in neutrophils including phagocytosis, respiratory burst, degranulation, intracellular Ca^{2+} increase, and protein synthesis, while chemotaxis requires the lowest (typically nanomolar) concentrations of the stimulators.

Schematic structures of the representatives of the five groups of neutrophil chemoattractants are shown in Fig. 2. The receptors for many of these molecules have been cloned. Besides the apparent differences of their ligands, all of them belong to the same subfamily of seven-transmembrane-helix receptors based on sequence homologies [57]. In addition to features typical of serpentine receptors such as an extracellular N- and intracellular C-terminus, seven transmembrane domains (TM), and extracellular (e1-e3) and intracellular (i1-i3) loops (see Fig. 3, [58, 59]), the chemoattractant receptors share other structural similarities. These unusually small serpentine receptors (ca. 350 amino acids) have an S-S bridge between e1 and e2, multiple Ser/Thr phosphorylation sites in the C-terminus, highly acidic N-terminus, and basic sequences in the short i3 [57, 60]. High affinity ligand binding is mediated by the extracellular face of the receptor, namely by the N-terminus (binding of C5a to C5aR [61], IL-8 to CXCR1 [62, 63], or MCP-1 to CCR2 [64]), by the e1 (fMLP binding by FPR [65]), e2 (binding of GROα and NAP2 by CXCR2 [63]), or e3 (binding of MIP-1α by CCR1 [64]). In addition to the high affinity ligand-binding site on the N-terminus, the existence of low affinity sites on the extracellular loops has been demonstrated for some chemoattractant receptors [63, 64]. Interestingly, these low affinity sites have been shown to be sufficient for signal transduction through the receptor [63, 64]. These data might indicate that no matter where on the receptor the high affinity ligand-binding site is, ligand interaction with the extracellular loops of the receptor will be crucial for the activation of the latter. This seems reasonable since G protein-coupled receptor activation is mediated by a change in relative orientation of the transmembrane domains, especially of TM3 and TM6 [66-68]. This reorientation depends on conserved Asp in TM2 and AspArgTyr tripeptide at the TM3–i2 interface [57, 69, 70] and unmasks G protein-binding sites of i2 [71, 72], i3 [73], or the C-terminus [71].

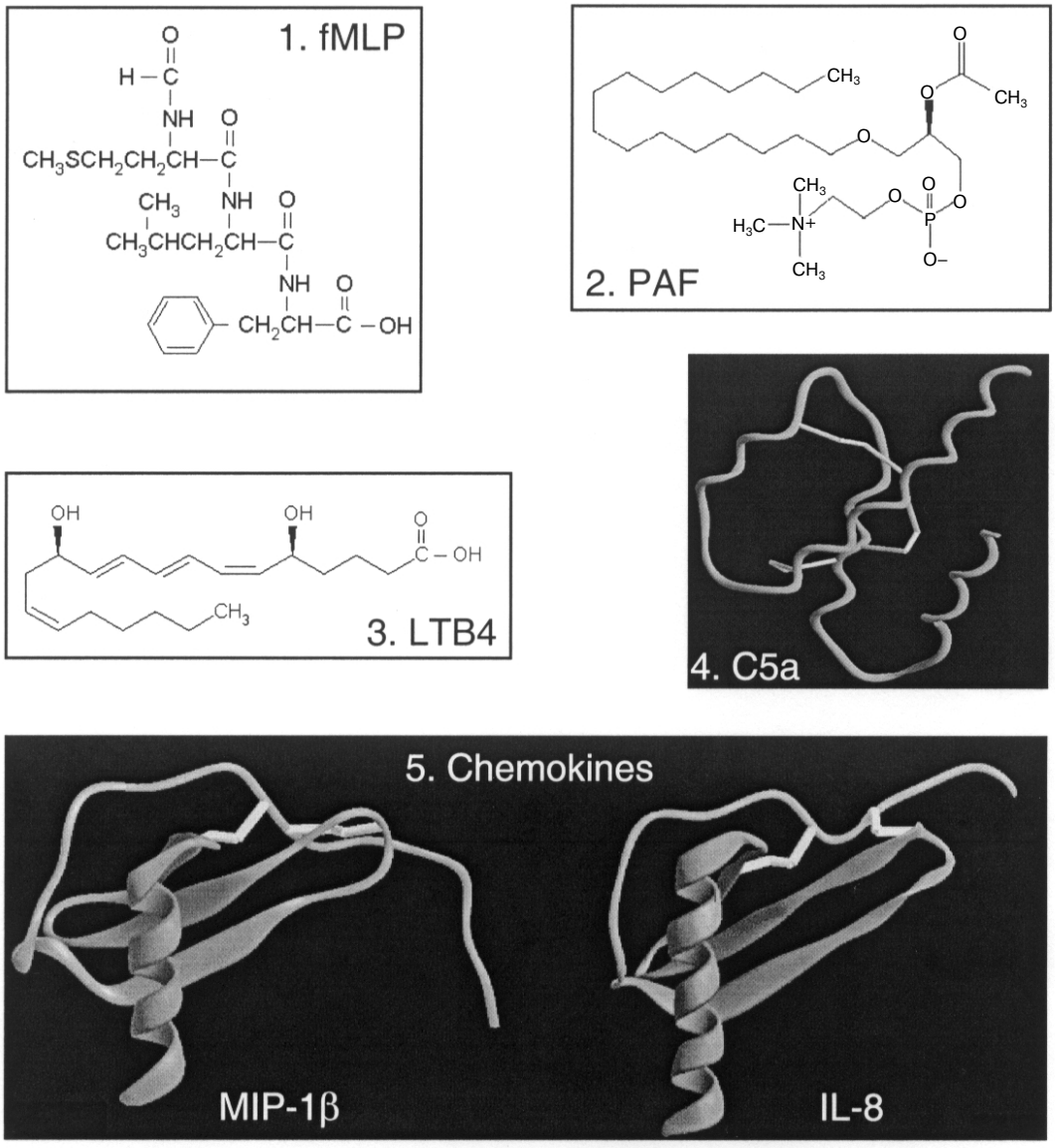

Fig. 2. Structures of representatives of five groups of chemoattractants for neutrophils acting through G protein-coupled receptors: 1) fMLP (*N*-formyl-methionyl-leucyl-phenylalanine) chemical structure. Note the *N*-formyl group capping the N-terminus of the peptide (to the top), which is a characteristic of bacterial but not eukaryotic proteins; 2) PAF (platelet-activating factor) chemical structure; 3) LTB4 (leukotriene B4) chemical structure; 4) C5a anaphylotoxin solution structure; 5) a CC chemokine (MIP-1β, macrophage inflammatory protein 1β) and a CXC chemokine (IL-8, interleukin-8) solution structures are presented. 3D-images of the chemokines are based on published NMR structures: [297] (C5a), [298] (MIP-1β), and [229] (IL-8).

III. SIGNALING DOWNSTREAM OF G PROTEIN-COUPLED CHEMOATTRACTANT RECEPTORS IN NEUTROPHILS

1. Activation of Trimeric G Proteins

Signaling through chemoattractant serpentine receptors in neutrophils is sensitive to pertussis toxin [74-76], indicating the involvement of G_i trimeric G proteins, such as $G_{\alpha i2}$ and $G_{\alpha i3}$, downstream from the receptors [77]. Trimeric G proteins are membrane-bound complexes consisting of a GDP-binding α-subunit and a βγ unit [78]. Upon serpentine receptor activation, the $G_{\alpha\beta\gamma}$ heterotrimer binds to the receptor, mainly through the α-subunit [66]. This induces GDP to GTP exchange on the α-subunit, resulting in dissociation of the βγ heterodimer from G_α-GTP. When GTP is hydrolyzed to GDP and phosphate due to intrinsic GTPase activity of the α-sub-

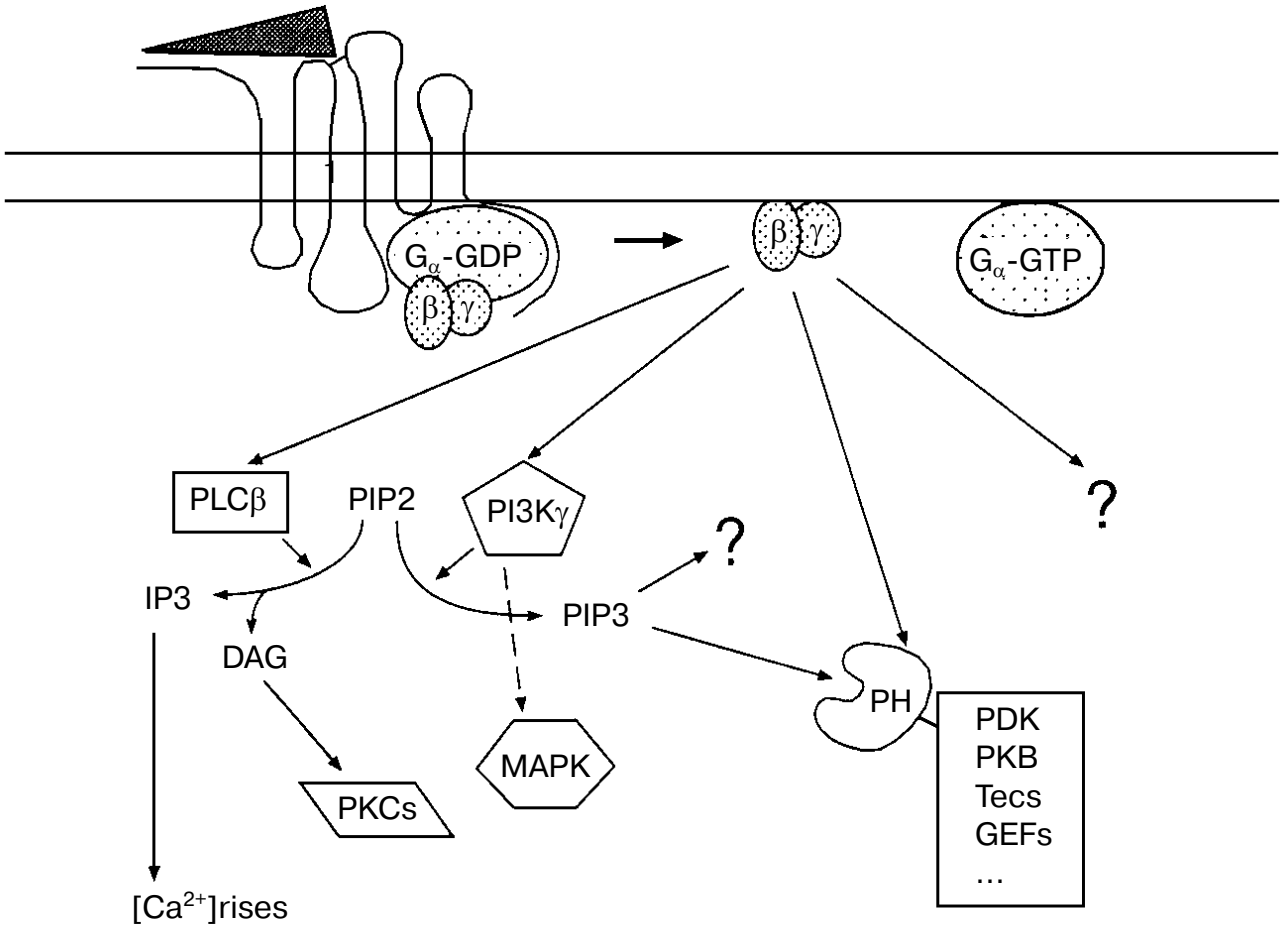

Fig. 3. Scheme of signaling events initiated by activation of a seven-transmembrane-helix receptor. Only the βγ-mediated signaling is shown. See text for description. G_α-GDP and G_α-GTP: GDP- or GTP-bound α subunit of trimeric G proteins. βγ: βγ subunits of the trimeric G proteins. PLCβ: phospholipase Cβ. PIP2: phosphatidylinositol 4,5-bisphosphate. IP3: inositol 3,4,5-trisphosphate. DAG: diacylglycerol. PKCs: protein kinases C. PI3Kγ: phosphoinositide 3-kinase γ. PIP3: phosphatidylinositol 3,4,5-trisphosphate. PH: pleckstrin homology domain. PDK: phosphoinositide-dependent kinase. PKB: protein kinase B. GEFs: guanine nucleotide exchange factors.

unit, the G protein complex reassociates and is ready to receive a new signal [78].

The βγ heterodimer of trimeric G proteins, rather than the GTP-loaded α-subunit, transduces the signal from seven-transmembrane-helix receptors to chemotaxis in motile cells [79]. Experiments confirming this were done in cultured lymphocyte- or fibroblast-like cells transfected with chemoattractant serpentine receptors. Agonist-induced chemotaxis of these cells was completely prevented by pertussis toxin or by βγ-sequestering proteins, such as G_α of transducin or βARK-ct [80, 81]. In contrast, agonist-induced intracellular calcium mobilization, inhibition of adenylate cyclase, or MAPK activation was only partially decreased by the βγ-sequestration [80, 81]. As soon as $G_{\beta\gamma}$ is released, $G_{\alpha i}$ is no longer required for activation of chemotaxis [82]. The pivotal role of βγ heterodimers has been demonstrated for signaling in chemotaxis of *D. discoideum* and directed growth in yeasts [83, 84]. Finally, certain mutations of the β3 subunit can enhance neutrophil chemotaxis [85].

2. Signaling Downstream from Trimeric G Proteins

2.1. Phospholipase Cβ–protein kinase C pathway. Phospholipases Cβ, namely PLCβ1-3, but not PLCβ4, can interact directly with and be activated by βγ heterodimers [86] through the PH domain or a region in the catalytic domain [87, 88]. $G_{\alpha i}$, unlike α-subunits of some pertussis toxin-insensitive G proteins, cannot activate PLCβ [88]. PLCβ catalyzes hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 3,4,5-trisphosphate (IP3) and diacylglycerol (DAG) [88]. The latter can activate classical (α, β, γ) and novel (δ, ε, η, θ) but not atypical (τ, ζ) isoforms of protein kinase C (PKC) [89]. PLCβ2 is expressed in hematopoietic cells [90] and is

responsible for 90% of the fMLP-induced IP3 production and consequently 70% of the [Ca^{2+}] rise in neutrophils [91]. The fMLP-induced respiratory burst is severely decreased in the PLCβ2 gene-less neutrophils [91] and absolutely absent in neutrophils from the PLCβ2/3 double knock-out mice [92], supporting the key role of DAG-stimulated PKCs in respiratory burst activation [93]. However, despite a prevention of the $\alpha_M\beta_2$ (Mac-1, CD11b/CD18) integrin upregulation [91], neutrophil chemotaxis was not impaired by PLCβ2 or PLCβ3 gene deletion [91, 92]. This confirms previous reports that PIP_2 breakdown is not necessary for neutrophil chemotaxis [94, 95]. Hence, any role of DAG-dependent PKCs in chemotaxis signaling in neutrophils downstream from PLCβ2 can be excluded. Reports of neutrophil chemotaxis sensitivity to PKC inhibitors [96-98] can thus be explained by proposing an involvement of PKC isoform(s) in some signaling events located further downstream. Since neutrophils can chemotax efficiently even when intracellular [Ca^{2+}] rises are prevented [95], Ca^{2+}-insensitive novel or atypical PKCs might be involved in chemotaxis. Of these, expression of PKC δ and ζ has been observed in neutrophils [99, 100]. Broad range inhibitors blocking PKC δ but not ζ were shown to prevent chemoattractant-induced polarization but not actin polymerization of neutrophils [96, 98, 101]. Neutrophils translocate PKC δ to the particulate fraction 45 sec after fMLP stimulation [99], coinciding with the time of cell polarity development (see [22]). In contrast, PKC ζ was shown to control neutrophil chemotaxis at the actin polymerization level and to be translocated to the plasma membrane within 10 sec after cell stimulation [97]. These data might indicate that different PKC isoforms regulate neutrophil chemotaxis at different levels: PKC ζ involved in switching on the immediate motile response, and a novel PKC like PKC δ regulating the choice of the migrating neutrophil where to go. However, these roles of PKCs must lie quite downstream in the signaling cascades, and they might be controlled by PDK-1 or small GTPases (see below).

2.2. PI-3 kinase γ pathway. Phosphoinositide 3-kinases (PI3Ks) are lipid kinases catalyzing phosphate addition to the third position of the inositol ring of phosphatidylinositol (PI), PI-4-P, and PI-4,5-P_2 [102]. The best-studied PI-3 kinase, PI3Kα, is implicated in a variety of signaling cascades downstream from receptor tyrosine kinases [102]. In contrast, PI3Kγ, which is strongly expressed in hematopoietic cells including neutrophils [103, 104], is suggested to be activated by G protein-coupled receptors [105, 106]. This is mediated by the βγ heterodimers; their binding to and activation of PI3Kγ can be achieved directly [106, 107] or through the PI3Kγ adapter protein p101 [108, 109].

The mechanism of PI3Kγ activation by $G_{\beta\gamma}$ may be a mere translocation of PI3Kγ to the plasma membrane, where it gets access to its lipid substrates. Indeed, targeting of PI3Kγ to the membrane was sufficient for constitutive PIP_3 production [110]. However, the ability of PI3Kγ to phosphorylate proteins has recently been shown necessary and sufficient in signaling leading to MAPK activation [110]. The detailed mechanism of activating the Ser-Thr protein kinase activity of PI3Kγ is not clear, but it requires a cytosolic rather than a membrane localization of the PI3Kγ [110, 111]. It has been recently proposed that PI3Kγ may serve as a functional homolog of the Ste5 yeast scaffold protein [112, 113]. Ste5 is indispensable for the G protein-coupled receptor pheromone signaling in yeast. Through multiple protein–protein interactions, Ste5 organizes a complex of *Saccharomyces cerevisiae* MEKK, MEK, and MAPK, which activates MAPK and leads to initiation of the mating process in yeasts [114].

The highly specific for PI3Ks covalent inhibitor wortmannin [115, 116] was used to assess PI3K involvement in neutrophil functions. Wortmannin could inhibit fMLP-induced actin polymerization [117] and cross-linking [118] by ca. 20 and 50%, respectively. Wortmannin was also shown to inhibit IL-8-induced neutrophil adhesion [119]. Depending on the experimental set-up, neutrophil motility can be wortmannin-insensitive (fMLP and IL-8 as stimuli [43]), completely prevented by wortmannin (IL-8 [120] or fMLP [118] as stimuli), or reduced by 50% by the inhibitor (MIP-2, CINC-1, and PAF as stimuli [98]). Among other neutrophil responses to chemoattractants, respiratory burst and exocytosis have been shown wortmannin-sensitive [117, 121].

Activation of PI3Ks other than PI3Kγ by trimeric G proteins has been proposed [122-125]. To clarify the role of PI3Kγ in chemoattractant-induced neutrophil responses, we and others have generated PI3Kγ gene-deficient mice [92, 104, 126]. PI3K$\gamma^{-/-}$ neutrophils completely lack chemoattractant-induced PIP_3 production and PKB activation, and fMLP-induced respiratory burst is also strongly affected in PI3Kγ-null cells. Upon stimulation with IL-8 and fMLP, these cells polymerize less actin and adhere worse to fibronectin than wild-type neutrophils [104, 127]. Most importantly, chemotaxis *in vitro* and *in vivo* is severely impaired in PI3Kγ-null neutrophils and macrophages, demonstrating a crucial role of PI3Kγ in cell motility [92, 104, 126].

2.3. PH domain-containing kinases. Several proteins possess a ca. 100-amino acid-long domain called the pleckstrin homology (PH) domain [128]. PH domains of different proteins have a weak sequence homology but a high degree of conservation of the 3D structure [129]. Some PH domains have been shown to interact with phosphorylated lipids (including the products of PI3 kinases) and βγ heterodimers of trimeric G proteins [128, 130]. They can thus occupy upstream positions in signaling cascades to transduce signals from G proteins or phospholipid kinases to the cytoplasm.

2.3.1. PH domain-containing serine/threonine protein kinases. Phosphoinositide-dependent kinase-1 (PDK-1) [131] has been shown to possess a PH domain that is indispensable for its activation by the lipid products of PI3K [132, 133]. PDK-1 has been proposed to phosphorylate PKCs [134-136], including PKC δ and ζ, whose possible role in controlling neutrophil chemotaxis was discussed above. PDK-1 contributes to the activation of $p70^{S6K}$ [137], protein kinase A [138], and protein kinase B (PKB) [132, 139]. The latter, which is activated in neutrophils upon their stimulation with chemoattractants [104], can also be directly activated via its PH domain by the lipid products of PI3K [140, 141]. Neutrophil and macrophage PKB activation by chemoattractants is lost in PI3Kγ gene knockout mice [104]. Whether this is the reason for a decrease in chemotactic properties of PI3Kγ-null cells remains to be elucidated.

2.3.2. PH domain-containing tyrosine kinases. The Tec family of cytoplasmic PH domain-containing tyrosine kinases includes Tec, Btk (Bruton's tyrosine kinase), Itk (Tsk), Bmx, and Txk kinases (reviewed in [142]). These proteins are predominantly expressed in hematopoietic cells where their primary role is believed to be the control of cell differentiation downstream from cytokine or similar receptors [143]. The βγ heterodimers of trimeric G proteins have been shown to activate Tsk and Btk through interaction with their PH domains [144, 145]. Phospholipid binding by the Btk PH domain was also shown [146, 147]. Tec is expressed in a wide range of hematopoietic cells including neutrophils [148], and Bmx is predominantly expressed in granulocytes [149, 150]. The Tec family protein kinases could potentially be involved in chemotaxis signaling downstream of $G_{\beta\gamma}$ or PI3Kγ, which would explain the reports of sensitivity of neutrophil migration to tyrosine kinase inhibitors [98, 151, 152]. Recent experiments in cell cultures [153] have shown that Btk may be involved in PI3K-mediated Rac activation and lamellopodial formation downstream from G-protein coupled receptors.

IV. ROLE OF SMALL GTP-BINDING PROTEINS OF THE RHO FAMILY IN NEUTROPHIL MOTILITY

1. Rho family G proteins, their regulators, and actin cytoskeleton control. Rho family GTPases together with Ras-, Rab-, Ran-, and Arf-like proteins constitute the superfamily of small GTP-binding proteins. The mammalian Rho family now includes Rho (RhoA-C), Rac (Rac1-3), and Rnd (Rnd1-3) isoforms, as well as Cdc42, TC10, RhoD, RhoG, and RhoH proteins (for a recent review, see [154]). Like other G proteins, Rho family members are active when bound to GTP. Hydrolyzing it to GDP, they become inactivated, while GDP to GTP exchange renders them active again. This on–off switch is controlled by several regulatory proteins (reviewed in [155]). GTPase activating proteins (GAPs) stimulate GTP hydrolysis on Rho proteins and convert them to the switched-off state. The opposite role is played by guanine nucleotide exchange factors (GEFs), which facilitate the GDP to GTP substitution on Rho proteins. Finally, guanine nucleotide dissociation inhibitors (GDIs) are able to "freeze" Rho proteins in GDP- and, with a lower affinity, GTP-bound forms, counteracting the effects of other regulators. Additionally, GDIs can shuttle the Rho proteins from or to the plasma membrane, an important site of their action [156].

Several functions of Rho proteins were found in different cells. Transcription regulation by Rac and Cdc42 via the control of Jun kinase is rather ubiquitous [157], while some other activities, like granulocyte respiratory burst activation by Rac isozymes [158], are more restricted. However, the overwhelming role of Rho proteins, established in all eukaryotic cells tested, is the control of the actin cytoskeleton [159, 160]. The classical pattern of the cytoskeleton regulation by Rho proteins has been obtained in studies with fibroblasts. In this system, cell stimulation with a seven-transmembrane-helix receptor agonist LPA induces formation of stress fibers, thick long bundles of actin-myosin filaments necessary for firm cell adhesion to the substratum. The action of LPA could be mimicked by microinjection of a constitutively active form of RhoA protein; moreover, inhibiting RhoA could prevent stress fiber formation by LPA [161]. Fibroblast stimulation with growth factors or a seven-transmembrane-helix receptor agonist bombesin induced lamellopodia and membrane ruffling, key structures in cell motility, and this action was shown to be transduced by Rac1 protein [162]. Finally, another serpentine receptor ligand bradykinin induces long finger-like protrusions called filopodia in fibroblasts, and Cdc42 was identified as a key regulator of this phenomenon [163, 164]. The differential action of Rho, Rac, and Cdc42 on the fibroblast actin cytoskeleton was reproduced in other cell systems, such as mast cells [165], epithelial cells [166], macrophages [167], and neurons [168].

Several neutrophil functions dependent on the actin network were shown to be under regulation of Rac, Rho, and Cdc42 proteins, which are activated upon neutrophil stimulation with seven-transmembrane-helix receptor agonists [169-171]. Thus, in addition to trimeric G proteins, a role of a small G protein in fMLP- or GTPγS-induced actin polymerization was suggested by studies in permeabilized neutrophils [172, 173]. In a cell-free system from neutrophil cytosol this small G protein was shown to belong to the Rho family, since it was inactivated by recombinant RhoGDI and *Clostridium difficile* toxin B, both specific inhibitors of Rho family proteins [174]. While this G protein was suggested to be [175] or to be not [174] Cdc42, Rho activation could account for about half of the GTPγS-induced actin polymerization [176]. Inhibition of Rho by C3 toxin eliminates neu-

trophil chemotaxis [177] and adhesion [178]. An effector of Rho, Rho-kinase (also called ROCK, or ROCK I, or ROKα) [179], is present in neutrophils and is necessary for neutrophil polarization and chemokinesis [180]. Rac2, constituting more than 96% of Rac proteins in neutrophils [181], plays a crucial role in transducing fMLP, LTB4, and IL-8 signaling to actin polymerization and chemotaxis [182]. These results were obtained by gene knockout studies in mice and are paralleled by a report of a human neutrophil chemotactic deficiency associated with a Rac2 mutation [183]. p21-activated kinases (PAKs), well-established targets of Rac and Cdc42 [184], are rapidly activated and translocated to the neutrophil lamellopodia upon chemoattractant stimulation [185, 186]. Altogether these data highlight a key role of Rho family proteins in transducing signaling to chemotaxis in neutrophils, supporting the results obtained with other leukocytes [187, 188].

2. Connecting serpentine receptor activation to Rho proteins. How is activation of G protein-coupled receptors linked to Rho family proteins? Several possibilities exist, although none of them have been proved to be involved in chemotaxis signaling in mammalian cells. An intriguing connection between the α subunit of G_{13} protein and Rho has been recently proposed [189, 190]. There, the direct binding of p115 RhoGEF to $G_{\alpha 13}$ has been shown to stimulate the p115 RhoGEF-mediated GDP–GTP exchange on Rho [190]. The interaction is achieved via the RGS (Regulators of G protein Signaling) domain of p115 RhoGEF, which can activate GTP hydrolysis on $G_{\alpha 12}$ and $G_{\alpha 13}$ [189]. p115 RhoGEF plays a crucial role in Rho activation downstream from $G_{\alpha 13}$ during development [191]. However, as explained above, G_i but not G_{12} or G_{13} trimeric G proteins direct chemotaxis in neutrophils; moreover, their βγ subunits are crucial for chemotaxis.

Most GEFs for Rho family proteins identified so far contain a PH domain [192], which is necessary for the cellular functioning of GEFs Dbl [193], Lbc [194], and Tiam-1 [195]. The Dbl PH domain was shown to bind $G_{\beta\gamma}$ *in vitro* [196] and *in vivo*, but this binding was not sufficient for Dbl activation [197]. In transfected COS-7 fibroblasts, chemoattractant receptor signaling to actin polymerization was proposed to be mediated by a $G_{\beta\gamma}$–PI3K$_\gamma$–Vav–Rac pathway, implicating the PH domain of the GEF Vav in its coupling to PI3K$_\gamma$ [198]. Although PH domains of the Rho GEFs Tiam-1 [147] and Vav [199] were shown to bind lipid products of PI3 kinases, this binding occurs with relatively low affinity and specificity [130, 200] and is not confirmed in physiologic assays [200, 201].

In yeasts, a connection of $G_{\beta\gamma}$ with Cdc24, the GEF for the small G protein Cdc42, regulates the directed growth [84]. This connection is achieved by a multiprotein assembly, where the pivotal role is played by the scaffold protein Far1 [202]. This essential protein binds Cdc24, recognizing a stretch of amino acids that is also present in mammalian Dbl and lies outside of the PH and Dbl homology domains [84, 202]. No functional homologs of Far1 have been identified so far in higher eukaryotes. However, recent data on the ability of certain proteins (Nef, EPS8-E3B1) to activate Rac and Cdc42 by PH domain-independent interactions with their exchange factors Vav [203] and Sos-1 [204] indicates that a link between trimeric and small G proteins mediated by protein–protein interactions might be involved in chemotaxis signaling in mammalian cells.

3. Connecting Rho proteins with the actin cytoskeleton. Several dozens of putative Rho protein targets have been identified [155, 205], and more appear every month. Some were proposed based on physical interactions in two hybrid systems, others established in functional assays. Despite the abundance of the announced Rho protein effectors, few links to actin rearrangements have been clearly described. We will concentrate on some Rho protein–actin cytoskeleton connections discovered recently, which could have implications in signal transduction to chemotaxis in motile cells (Fig. 4).

3.1. Rac and Rho can control actin-binding proteins by regulation of PIP_2 synthesis. Rho and Rac were shown to regulate PI(4,5)P_2 synthesis in fibroblasts and platelets, respectively [206, 207]. This is achieved by a physical interaction of a type I phosphatidylinositol-4-phosphate (PIP) 5-kinase with the small GTPases, which occurs *in vitro* in a GTP-independent manner [208, 209]. *In vivo*, Rac and PIP5-kinase form a multiprotein complex including also a diacylglycerol kinase and RhoGDI [210]. Rho-kinase, a downstream target of Rho, was also shown to activate PIP5-kinase [211]. These findings are important since a multitude of actin binding proteins can be regulated by phosphoinositides in general and PIP_2 specifically (reviewed in [212]). For example, capping proteins gelsolin and CapZ are dissociated from actin filaments under certain conditions *in vitro* by addition of PIP_2 micelles, allowing fast barbed-end actin polymerization [213, 214]. Moreover, phosphoinositide delivery to permeabilized platelets induced cellular F-actin decapping [207], while overexpression of PIP5-kinase in fibroblasts led to massive actin polymerization [215]. A link between Rac and gelsolin was reported based on an inability of fibroblasts from gelsolin knockout mice to exert Rac-transduced cytoskeletal changes [216]. These data have led to a model, where Rho GTPase constitutive interaction with the PIP5-kinase results upon cell activation in increased production of PIP_2, which in turn may decap actin filaments allowing their elongation at the plasma membrane [217, 218]. However, in contrast to platelets, most of the cells including neutrophils respond with a rapid decrease and not increase of PIP_2 when stimulated [219]. While local increases in PIP_2 upon neutrophil stimulation are not excluded, this model remains neither proved nor disproved for neutrophils.

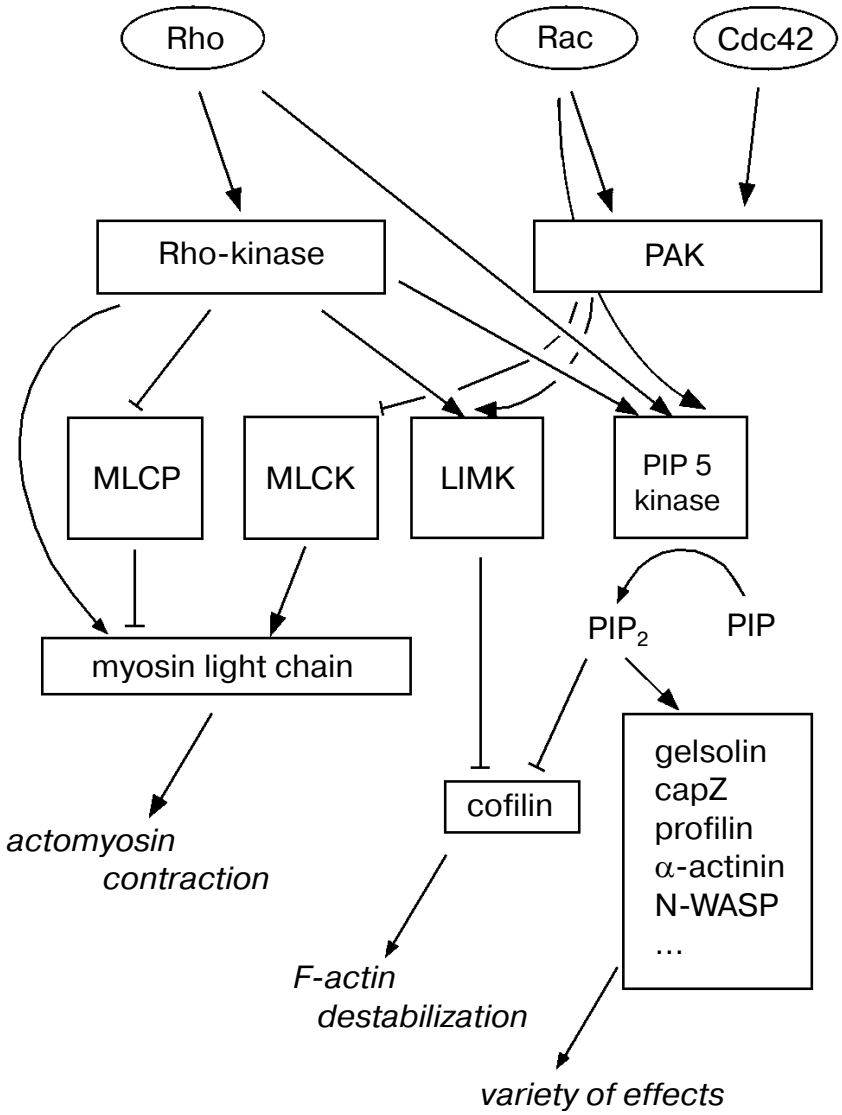

Fig. 4. Rho transduces many of its signals through Rho-kinase, while Rac and Cdc42 — through PAK (p21-activated kinase). Rac and Rho regulate activity of phosphatidylinositol-4-phosphate 5-kinase (PIP 5-kinase) inducing production of PIP_2. This in turn controls the activity of many actin-binding proteins, such as gelsolin or cofilin. Cofilin activity is also controlled by phosphorylation. Rac via PAK and Rho via Rho-kinase phosphorylate LIM kinase (LIMK), which in turn phosphorylates cofilin on Ser3. This leads to cofilin inactivation and prevention of depolymerization of actin filaments. Myosin phosphorylation status is also under Rho family control. Rho-kinase can induce phosphorylation of Ser19 of the myosin II light chain either directly or phosphorylating and thus inhibiting the myosin light chain phosphatase (MLCP). Increase in the myosin II light chain phosphorylation leads to an increase in myosin-dependent contractility. An opposite action is played by Rac, which through PAK (p21-activated kinase) can inhibit the myosin light chain kinase (MLCK).

3.2. Rac and Rho can stabilize actin filaments inducing phosphorylation of cofilin. Cofilin is an essential protein ubiquitously expressed in eukaryotes (see [220] for a review). Cofilin overexpression stimulated cell migration in *Dictyostelium* amoebae [221] and neurite outgrowths in neurons [222]. Cofilin and other members of its family are unique in their ability to increase the treadmilling of actin filaments [223]. Additionally, cofilin was shown to sever actin filaments, creating free barbed ends [224]. Cofilin activities are controlled by phosphorylation on Ser3 [225]. The kinase phosphorylating and inactivating cofilin was demonstrated to be LIM-kinase 1 [226, 227] or LIM-kinase 2 [228]. Overexpression of LIMK-1 in transfected cells led to accumulation of the actin cytoskeleton and reversed cofilin-induced actin depolymerization; moreover, LIMK-1 mediated actin rearrangements downstream from Rac and insulin [226, 227]. Rac-induced activation of LIMK-1 is indirect and

may be mediated by PAK1, a long-known target of Rac and Cdc42. PAK1 was shown to phosphorylate LIMK-1 and thus increase its ability to phosphorylate cofilin; Rac and Cdc42 were able to stimulate the PAK1 association with LIMK-1 [229]. Moreover, dominant negative LIMK-1 interfered with the Cdc42-, Rac-, and PAK1-induced cytoskeletal changes in BHT cells [229]. In addition to Rac and Cdc42, Rho was also shown to induce cofilin phosphorylation in cultured cells via LIMK-1; this effect is mediated by Rho-induced activation of Rho-kinase, which in turn phosphorylates LIMK-1 [230]. Thus, in cell cultures different Rho proteins seem to converge their signaling pathways to stimulate LIMK-1 and deactivate cofilin, which may lead to prevention of F-actin depolymerization and result in F-actin increase. Since different Rho proteins exert different effects on the actin cytoskeleton, this pathway must act together with other pathways that are used unequally by various Rho proteins. In highly motile cells like neutrophils, half of cofilin is phosphorylated under resting conditions and cell stimulation leads to its rapid dephosphorylation [231]. This is accompanied by cofilin redistribution from the cytoplasm to F-actin rich membrane ruffles [232, 233], where its activity might be required for rapid reorganization of the actin cytoskeleton.

3.3. Regulation of myosin phosphorylation by Rho proteins. Myosins are motor proteins indispensable for muscle contraction, cellular trafficking, and cell motility [234, 235]. Disruption of myosin II in *D. discoideum* leads to defects in cytokinesis and fruit body development [236, 237], while yeast myosins are crucial for cytokinesis and the actin cytoskeleton organization [238, 239]. In a chemotacting neutrophil, myosin is localized in the lamellopode [14], but is excluded from the filopodia [240]. Myosin inhibitors prevent neutrophil transmigration across colonic epithelial cells [241] and oscillatory shape changes in neutrophils stimulated with LTB4 and PAF [242]. Phosphorylation has been shown to control the activities of all myosins studied so far. In the case of the myosin II light chain (MLC), its phosphorylation on Ser19 results in an increase of the actin-activated ATPase activity of the myosin and thus in stimulated contraction [243]. In motile cells [244, 245] including neutrophils [13], MLC is phosphorylated upon stimulation, implying a role of myosin phosphorylation in cell migration. The phosphorylation state of MLC has been shown to be controlled by several enzymes, Rho-kinase, an effector of Rho, being one of them [246, 247]. Rho-kinase has also another way of stimulating MLC phosphorylation: it can phosphorylate MLC phosphatase, rendering it inactive [248]. The differential regulation of MLC phosphorylation may also explain the long-known fact that Rho on one hand and Rac or Cdc42 on the other have antagonistic effects on cytoskeleton in many systems. Indeed, a recent work has shown that PAK, an effector of Rac and Cdc42, inactivates MLC kinase [249], the major regulator of MLC phosphorylation identified in a multitude of cell types [243]. Thus the myosin II-based contractility can be counter-regulated by the Rac/Cdc42-PAK and Rho-Rho-kinase pathways, which might thus operate such a complex phenomenon as cell motility.

3.4. Kinase-independent regulation of the actin cytoskeleton. In the examples listed above, lipid or protein kinases were proposed to be downstream targets of Rho proteins. In addition to the above-mentioned, a tyrosine kinase [250], PKCζ [97, 251], and other kinases [205] were proposed to act downstream of Rho proteins in different systems. However, in permeabilized neutrophils GTPγS induces actin polymerization in a manner apparently independent of ATP [173]. Similarly, in a cell-free system from neutrophil cytosol, lipid and protein kinases are not involved in propagation of the signal from Rho proteins to actin polymerization [174]. Instead, a negative regulation of a non-kinase protein called CIP4 may be involved [176]. CIP4 is capable of binding to Rho and Cdc42 in a GTP-dependent manner [252]. Several other non-kinase targets of Rho proteins have been proposed (reviewed in [205]), like the Wiskott–Aldrich syndrome protein (WASP) [253] and its relatives N-WASP and Scar/WAVE.

3.5. Cdc42 can induce actin polymerization activating Arp2/3 via N-WASP (Fig. 5). In a cell-free system from *Xenopus* oocytes, exogenous Cdc42 was shown to induce *de novo* actin polymerization [254, 255]. Using a biochemical approach, Kirschner and coworkers have demonstrated that N-WASP and Arp2/3 complex are necessary and sufficient to transduce the signal from GTP-loaded Cdc42 to actin [256, 257], for the first time reconstituting a molecular link between a Rho protein and induction of actin polymerization. A role of the Arp2/3 complex in Rho-protein induced actin polymerization was also demonstrated in extracts of *Acanthamoeba* [258]. Arp2/3 is a complex of seven proteins of molecular weights ranging from 16 to 47 kD, two of which are actin-related proteins 2 and 3, hence the name. The complex is ubiquitously expressed in eukaryotic cells and has been purified from amoebae [259], yeasts [260], *Xenopus* [256], and human platelets [261] and neutrophils [262]. Arp2/3 can bind to the sides of actin filaments, bundling them [263, 264]. Moreover, Arp2/3 can establish branching points, so called Y-junctions, on the filaments [265] resulting in extremely branched filament organization in the leading edge of some cells [266]. But the key function of Arp2/3 is to nucleate actin filaments [265, 267], bypassing the rate-limiting step in *de novo* actin polymerization (see [268]). Bound with high affinity to the pointed end of the filament, Arp2/3 allows rapid elongation and branching at the barbed end [269]. The ability of Arp2/3 to nucleate barbed-end actin polymerization is dramatically increased by WASP [270] and its relatives Scar/WAVE [271] and N-WASP [257], which bind the p21 subunit of

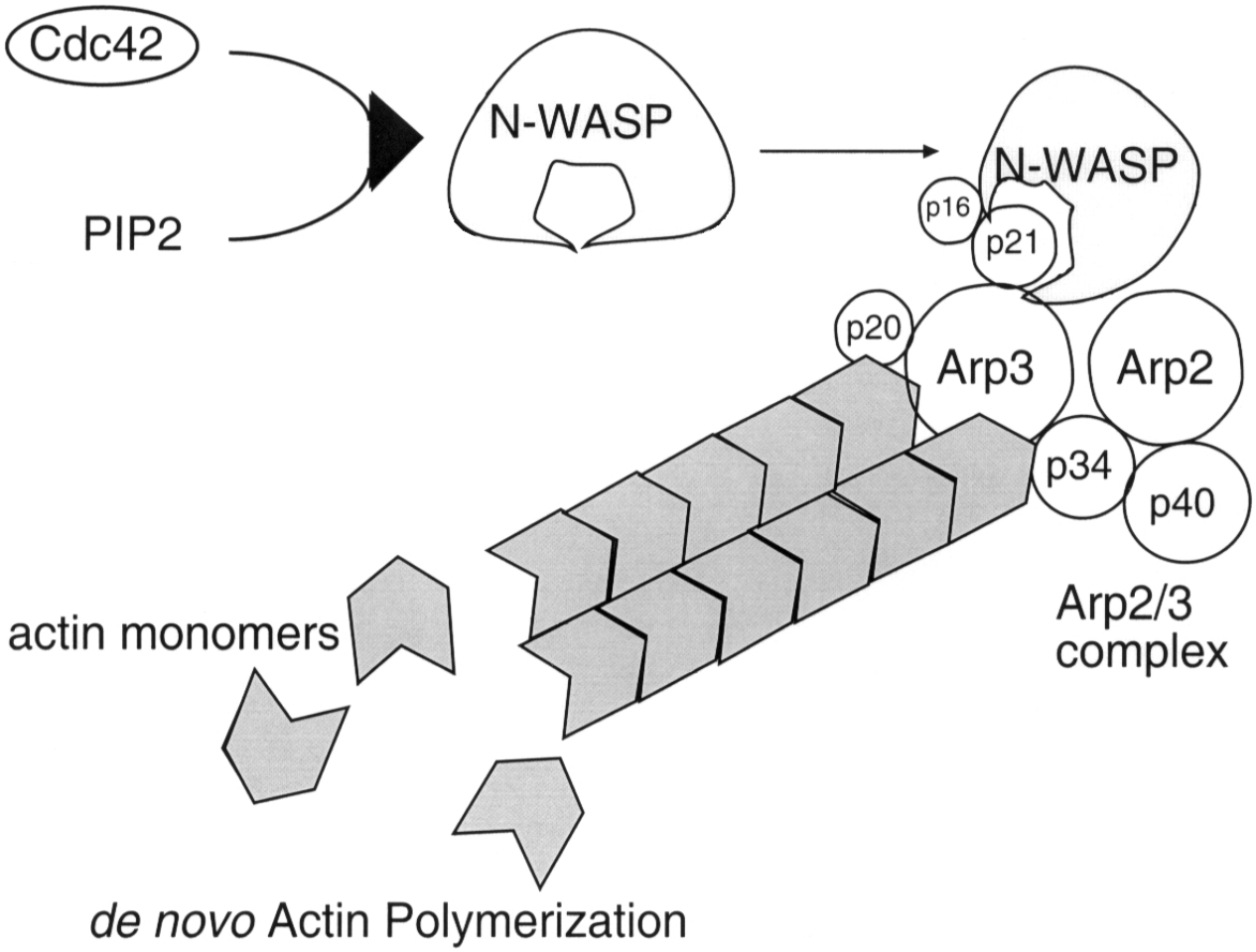

Fig. 5. Cdc42 can stimulate *de novo* actin polymerization via a kinase-independent mechanism. Cdc42 (together with PIP_2) induces a conformational change in N-WASP, unmasking the binding site for p21 of Arp2/3. Activated Arp2/3 then forms nuclei for new actin filaments, which are rapidly elongated at their barbed ends. The topology of the subunits of Arp2/3 is based on the published model [263].

Arp2/3 [272]. WASPs are targets of GTP-loaded Cdc42 and Rac and induce actin polymerization when overexpressed in cultured cells [273-278]. The fact that the C-terminal halves of Scar/WAVE and N-WASP are more potent in Arp2/3 activation is explained by the finding that the Arp2/3-binding domain of these proteins is normally hidden [279]. Binding of GTP-Cdc42 and phosphoinositides was shown to unmask the Arp2/3-activating capacity of the full-length N-WASP, which leads to rapid actin polymerization, constructing a new paradigm of Rho-protein-induced actin polymerization at the plasma membrane [257, 277]. The relevance of this paradigm in regulation of neutrophil chemotaxis is not clarified yet. N-WASP expression is limited to brain, heart, and lung [276], while Scar/WAVE expression is strictly limited to brain [280]. Although WASP is expressed in hematopoietic cells [253], neutrophils from Wiskott–Aldrich syndrome patients chemotax normally, in contrast to macrophages [281]. Recently, two new WASP family members, WAVE2-3, have been identified, of which WAVE2 is expressed ubiquitously including in peripheral blood leukocytes [282].

V. "MOTOR" VERSUS "COMPASS" ACTIVATION IN NEUTROPHIL MOTILITY

Previous sections described various proteins mediating motility signaling in neutrophils and similar systems. Several of these proteins have been proven crucial for cell migration not only *in vitro*, but also in multicellular organisms (see, e.g., [283-285]). However, speaking about a migration deficiency *in vivo*, one must distinguish between an inability of a cell to move in general and the loss of the sense for directional cell movement. The latter is hardly less important for an organism, since the ability

of cells to arrive at right places is indispensable in, e.g., tissue and organ development or inflammation.

To chemotax, a cell must be positioned in a gradient of a chemotactic substance, in which it moves towards the increasing concentrations of the chemoattractant. Understanding of how a cell decodes this gradient information only recently has started to emerge. The first clues came from studies with *Dictyostelium* amoebae, whose migratory features are very close to those of neutrophils. Application of gradients of cAMP induces cell polarization and chemotaxis in *Dictyostelium* amoebae (reviewed in [286]). This is mediated by a G protein-coupled receptor, and the βγ part of the trimeric G protein was shown crucial for the signal transduction [287]. Despite a capacity of migrating amoebae to detect very shallow differences in the chemoattractant concentrations across the cell diameter, both the cAMP receptor and the βγ subunits of the G protein are distributed evenly on the cell surface [288, 289]. Which part of the signal transduction machinery thus can reflect and amplify the unequal concentrations of the chemoattractant around the cell? The answer came when cellular localization of a protein called CRAC was studied in *Dictyostelium*. CRAC has a PH domain and is not necessary for chemotaxis *per se*, but is required for activation of the adenylyl cyclase in stimulated cells and propagation of the cAMP wave in the aggregating slime mold [286]. CRAC is cytoplasmic in a resting cell, and its rapid and transient translocation to the plasma membrane was detected upon cAMP addition [290]. The most striking result came when amoebae were placed in gradients of the chemoattractant. There CRAC was selectively translocated to the leading edge of the cell, where the maximal concentration of cAMP was provided [290]. Amazingly, disruption of the actin cytoskeleton did not prevent the selective CRAC translocation. Amoebae pretreated with latriculin A, a toxin sequestering monomeric actin and leading to F-actin depolymerization, could still "sense" the direction to the maximal chemoattractant concentration, despite their round morphology and inability to move [290]. This data could allow for the first time a distinction between activation of the "motor" (inevitably involving the actin cytoskeleton) and the "sensor" in chemotaxis.

The slime mold results were further developed in neutrophil-differentiated HL60 cells [291]. As mentioned above, chemoattractants induce rapid activation of a PH domain-containing kinase PKB in neutrophils [104], and PKB was shown necessary for *Dictyostelium* chemotaxis [292, 293]. It has been recently shown that, similarly to CRAC in *Dictyostelium*, PKB is vectorially translocated to the plasma membrane of neutrophils upon addition of chemoattractants, reflecting the chemoattractant gradient formed outside the cells [291]. Interestingly, the gradient of intracellular PKB distribution was more than sixfold steeper than that of the provided chemoattractant gradient [291], indicating amplification of the positional information by the cellular signaling mechanisms. The parallels with the *Dictyostelium* data are expanded by the finding that vectorial PKB translocation is also independent of the cytoskeleton and may occur even in immobile neutrophils [291].

What can induce selective membrane translocation of proteins like CRAC and PKB in activated cells? Both proteins have PH domains; that of PKB is known to bind specifically PIP_3 [140, 141], while it is not clear whether $G_{\beta\gamma}$ or phosphoinositides serve as a ligand for the PH domain of CRAC [128, 130]. However, the βγ heterodimers, although crucial for the signaling, appear not to be by themselves the binding site for CRAC on the plasma membranes [290]. Moreover, chemoattractant-induced membrane translocation of CRAC and PKB is insensitive or very weakly sensitive to the inhibitors of PI3 kinases [290, 291]. Instead, inhibition of a Rho family protein in neutrophils by *Clostridium difficile* toxin B prevented PKB translocation [291]. This protein is very likely to be Cdc42 due to its role in cell polarization in other systems. Thus, in macrophages, colony stimulating factor (CSF)-induced locomotion was aborted by inhibition of Rho and Rac, but not Cdc42 [294]. Instead, Cdc42 inhibition prevented the cells from directed migration in the gradients of CSF, turning macrophage chemotaxis into random migration [294]. Similarly, certain yeast mutants defective in Cdc42 or its guanine nucleotide exchange factor Cdc24 have normal cytoskeleton and mating pheromone-induced growth, but cannot orient that growth towards the pheromone gradient [84, 295]. Cdc42 is also crucial for polarization of T cells towards the antigen-presenting cell [296]. Intriguingly, inhibition of Rho family proteins in neutrophils prevented PKB activation initiated by chemoattractants like fMLP or C5a, but left intact insulin-induced PKB activation [291]. Given the fact that insulin can stimulate random migration but not chemotaxis in neutrophils [44], one could indeed speculate that a protein like Cdc42 is a crucial component of the neutrophil "compass" but dispensable for the locomotion. Interestingly, such "compass" activation downstream from Cdc42 must be different from described above pathways for the control of cytoskeleton by Cdc42, due to independence of PKB and CRAC membrane translocation from actin [290, 291].

VI. CONCLUDING REMARKS

Chemotaxis is a multicomponent process built up of a combination of many individual cell responses that are orchestrated in a virtually obscure way by a moving cell. Their activation is initiated by chemoattractant receptors, most of which in neutrophils belong to the superfamily of seven-transmembrane-helix receptors. To induce chemotaxis, these receptors activate trimeric G_i proteins, dissociating them to $G_{\alpha i}$ and $G_{\beta\gamma}$ subunits. The latter hands

downstream the chemotaxis-signaling courier. PI3Kγ is one of the proteins taking it up, but other proteins doing the same await identification. Through some more yet unrevealed partners, the signal achieves the members of Rho family G proteins. Through effectors like Rho-kinase, PAK, and WASP, Rho GTPases regulate a multitude of actin binding proteins. Myosin, gelsolin, cofilin, and Arp2/3 are among them, whose activities culminate at highly controlled in time and space actin rearrangements, staying behind cell motility.

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