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THE INTERACTION OF N-FORMYL PEPTIDE CHEMOATTRACTANT RECEPTORS WITH THE MEMBRANE SKELETON IS ENERGY-DEPENDENT

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Abstract—Desensitization of N-formyl peptide chemoattractant receptors (FPR) in human neutrophils is thought to be achieved by lateral segregation of receptors and G proteins within the plane of the plasma membrane resulting in an interruption of the signalling cascade. Direct coupling of FPR to membrane skeletal actin appears to be the basis of this process; however, the molecular mechanism is unknown. In this study we investigated the effect of energy depletion on formation of FPR-membrane skeleton complexes. In addition the effect of the protein kinase C inhibitor stauroporine and the phosphatase inhibitor okadaic acid on coupling of FPR to the membrane skeleton was studied. Human neutrophils were desensitized using the photoreactive agonist N-formyl-met-leu-phe-lys-N'-[^{125}I]2(p-azidosalicylamido)ethyl-1,3'-dithiopropionate (fMLFK-[^{125}I]ASD) after ATP depletion with NaF or after incubation with the respective inhibitors. The interaction of FPR with the membrane skeleton was studied by sedimentation of the membrane skeleton-associated receptors in sucrose density gradients. Energy depletion of the cells markedly inhibited the formation of FPR-membrane skeleton complexes. This does not appear to be related to inhibition of protein phosphorylation due to ATP depletion because inhibition of protein kinases and phosphatases had no significant effect on coupling of FPR to the membrane skeleton. We conclude, therefore, that coupling of FPR to the membrane skeleton is an energy-dependent process which does not appear to require modification of the receptor protein by phosphorylation.

Key words: Chemotactic receptors, G proteins, N-formyl peptides, signal transduction, desensitization, membrane skeleton, receptor-G protein coupling.

INTRODUCTION

Receptors for N-formyl peptide chemoattractants (FPR) are an important component of the body's first line of defence against invading microbes. These receptors on human neutrophils transduce signals via pertussis toxin-sensitive guanyl nucleotide-binding proteins (G-proteins) which activate phospholipase C [1, 2]. Typically, receptors of this family exhibit two affinity states for agonists with a high-affinity state representing receptor-G protein complexes. In the presence of GTP this complex is dissociated and the receptors are converted to a low-affinity state [3]. In addition, in desensitized cells a second high affinity state of FPR was identified that appears to be insensitive to GTP [4–6]. This desensitized receptor state has been called LRX [6] indicating coupling of the ligand–receptor complex to protein(s) different from G protein such proteins might be cytoskeletal elements.

Analogous to other G protein-coupled receptors, FPR exhibit the phenomenon of desensitization upon prolonged exposure to agonists. Several lines of evidence suggest that a new mechanism for desensitization of FPR may be operative in neutrophils, which is based on a lateral segrega-

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Abbreviations: FPR—N-formyl peptide chemoattractant receptors; *f*MLFK-[^{125}I]ASD—N-formyl-met-leu-phe-lys-N$^{\varepsilon}$-[^{125}I]2(p-azidosalicylamido)ethyl-1,3'-dithiopropionate; LRX—desensitized ligand–receptor complex.

tion of FPR and G proteins into different membrane domains (for review see [7, 8]). This lateral segregation could serve as a mechanism to immobilize receptors in a G protein-free membrane domain resulting in an interruption of the signalling cascade. Recently, it was shown that FPR in neutrophils associate with the membrane skeleton upon desensitization [9]. Direct binding of FPR to membrane skeletal actin [10] may be the basis for this linkage which could provide for a mechanism for FPR immobilization in the plane of the plasma membrane [8].

It is not clear whether coupling of FPR to the membrane skeleton is the event that characterizes the previously defined state LRX. The most striking feature of LRX is its energy-dependent formation [6]. The interaction of other membrane proteins with the membrane skeleton, e.g. Na^+, K^+-ATPase, has also been shown to be energy-dependent [11]. Therefore, we studied the effect of energy depletion on immobilization of FPR to the membrane skeleton. In addition, the effects of the nonspecific protein kinase C inhibitor staurosporine [12] and the serine/threonine phosphatase inhibitor okadaic acid [13] on FPR-membrane skeleton-coupling were tested because ATP depletion might also affect desensitization pathways that involve phosphorylation.

MATERIALS AND METHODS

Materials

Staurosporine and okadaic acid were purchased from Sigma, St Louis, MO. All other materials were from sources previously described [9, 14, 15] or highest purity available.

Cells and membranes

Human neutrophils were prepared as described by Parkos *et al.* [14]. Desensitized cells were prepared by incubation with the photoreactive agonist *f*MLFK-[^{125}I]ASD for 20 min at 15°C as described recently [9]. At the end of the incubation period the cell suspension was UV-irradiated which leads to photoincorporation of the ligand into the receptor protein. Plasma membranes of responsive and desensitized neutrophils were prepared by N_2 cavitation [9].

Inhibitor treatment

For energy depletion of neutrophils the protocol of Sklar *et al.* [6] was used. In brief, before neutrophils were desensitized the cells (10^8 cells/ml) were incubated for 15 or 60 min in Hanks buffer pH 7.4 in the presence or absence of 40 mM NaF. After this pretreatment cells were desensitized and membranes were prepared thereafter. The FPR linkage to the membrane skeleton was analysed in sucrose density gradients as described [9].

Neutrophils were incubated with staurosporine or okadaic acid for 10 min at 37°C at a concentration of 2 μM. After this incubation period cells were desensitized with *f*MLFK-[^{125}I]ASD at 15°C or immediately used for the preparation of plasma membranes (responsive cells).

Solubilization of membranes and velocity sedimentation of FPR

All methods for analysis of receptor coupling were performed exactly as described recently [9]. In brief, plasma membranes were solubilized in solubilization buffer (20 mM HEPES/3 mM $MgCl_2$, pH 7.4 containing 0.5% Triton X-100). The receptor coupling to the membrane skeleton was monitored by velocity sedimentation of membrane extracts on detergent-containing 5–20% sucrose density gradients. The gradients were fractionated and aliquots of the individual fractions were used for SDS-PAGE. The gels were dried for autoradiography and the autoradiograms were analysed with a Molecular Dynamics Computing Densitometer and the Image Quant software.

RESULTS AND DISCUSSION

Neutrophils were incubated with fluoride for 15 or 60 min at 37°C prior to desensitization with the photoaffinity ligand *f*MLFK-[^{125}I]ASD. This treatment has been shown to decrease the ATP level in neutrophils practically to zero [6]. In desensitized cells the majority of FPR was found in the pellets of sucrose density gradients that represent the membrane skeletal pellets [9]. As shown in Fig. 1, 75% of the FPR from desensitized neutrophils were found in the membrane skeletal pellet while after energy depletion with fluoride this portion is reduced to about 30%.

This result suggests that indeed formation of the membrane skeleton-coupled state of FPR is energy dependent. Therefore, the desensitized receptor state that has been defined as LRX [6]

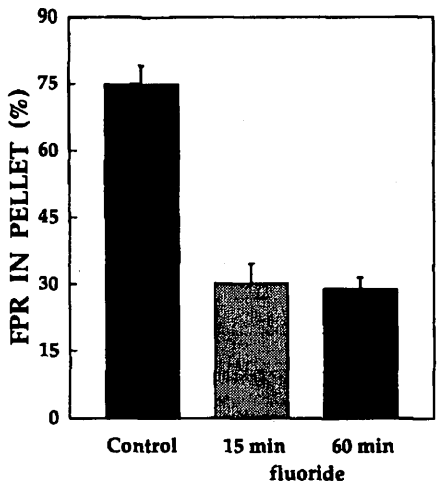

Fig. 1. Effect of energy depletion on FPR-MSK coupling. After energy depletion with 40 mM NaF, neutrophils were desensitized by incubation with *f*MLFK-[125I]ASD for 20 min at 15°C (for experimental details see [9]). In desensitized cells (control) 74.9 ± 4,3% of the FPR were found coupled to the membrane skeleton. After fluoride treatment for 15 and 60 min 30.2 ± 4.6% and 28.9 ± 2.7%, respectively, sedimented to the membrane skeletal pellet in sucrose density gradients. Values are means ± S.E.M. of four experiments.

might be identical to this FPR population that is immobilized to the membrane skeleton [9, 10]. The energy-dependent formation of the slowly dissociating form of desensitized FPR that is insensitive to guanine nucleotides is a peculiarity of this receptor and possibly of other chemoattractant receptors as well. According to our model of homologous FPR desensitization this receptor form is confined to a G protein-depleted membrane domain [7, 8]. The association of FPR to membrane skeletal actin, which is thought to serve as the basis for immobilization of receptors in the G protein-depleted membrane domain, might be the energy-requiring process.

It is conceivable, however, that after ATP depletion the membrane skeleton-coupled state cannot be formed because a phosphorylation step is involved in immobilization of FPR. The first step in rapid desensitization of β-adrenergic receptors, for instance, is phosphorylation of the receptor protein [16, 17] which then allows for binding of β-arrestin resulting in uncoupling of receptor and G protein [18]. Recently, agonist-dependent phosphorylation of FPR was demonstrated [19, 20]. Whether such a modification initiates binding of FPR to actin, which is thought to be critical for FPR desensitization [8–10], is not known. If such an analogy to the β-adrenergic receptor and rhodopsin system exists one might expect kinase and phosphatase inhibitors to affect FPR coupling to the membrane skeleton. In the case of the β-adrenergic receptor a receptor-specific kinase (β-adrenergic receptor kinase, βARK) has been shown to be responsible for homologous desensitization while protein kinase C- and protein kinase A-mediated phosphorylation appear to be responsible for heterologous desensitization [21]. Staurosporine is an inhibitor with IC_{50}-values for protein kinase A and protein kinase C below 10 nM [12]. However, it does not inhibit β-adrenergic receptor kinase at a concentration of 10 μM [22]. The recent finding that the FPR is phosphorylated mainly by a staurosporine-insensitive kinase [19, 20] suggests that a receptor-specific kinase might be involved in homologous desensitization. Accordingly, staurosporine had

Table 1. Effect of okadaic acid and staurosporine on FPR-MSK coupling

	Control	Okadaic acid	Staurosporine
Responsive cells	27.5± 8.4	31.7 ± 5.7	37.2 ±10.3
Desensitized cells	66.9 ± 11.3	54.7 ± 4.4	77.2 ± 9.9

Neutrophils were treated with 2 μM of the respective inhibitor as described in Materials and Methods. Values are given as percentage FPR found in the membrane skeletal pellet and are means of three to four experiments ± S.E.M.

no significant effect of FPR coupling to the membrane skeleton (Table 1). Therefore, a role for phosphorylation in FPR-membrane skeleton coupling cannot be excluded.

As another tool used to study phosphorylation as a possible mechanism for coupling of FPR to the membrane skeleton, we used the potent serine/threonine phosphatase inhibitor okadaic acid [13]. Okadaic acid has profound effects on protein phosphorylation in neutrophils [23]. If phosphorylation were a mechanism that triggers binding of FPR to the membrane skeleton, inhibition of phosphatases might potentiate desensitization. However, okadaic acid has been shown to cause a sustained formyl peptide-induced respiratory burst in human neutrophils [23]. In our study no significant effect of okadaic acid on FPR coupling to the membrane skeleton was detected (Table 1). These results suggest that phosphorylation is not a mechanism to induce coupling of FPR to membrane skeletal actin. Recently we have demonstrated that coupling of FPR to G proteins is not changed in desensitized neutrophils [24]. Taken together with the results of this study this confirms that lateral segregation of FPR and G protein is the crucial event for desensitization in human neutrophils that is apparently not triggered by receptor phosphorylation. Currently it is not clear what molecular mechanism causes the receptor to bind membrane skeletal actin instead of G protein after prolonged exposure to agonist. In addition to this novel mechanism, other mechanisms of desensitization that require receptor phosphorylation and subsequent binding of an arrestin-like protein in analogy to the β-adrenergic receptor or rhodopsin also may be operative for FPR.

In summary, our results are in agreement with the hypothesis that physical segregation of FPR from G protein within the plane of the plasma membrane may be the basis for desensitization [8]. We believe that coupling to the membrane skeleton is mediated by direct binding of FPR to actin [9, 10] and that this membrane skeleton-coupled state is identical to the previously defined desensitized receptor state LRX [6]. This study suggests that the energy-dependent formation of this state does not require phosphorylation of FPR as a molecular determinant for coupling to actin.

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