Unidirectional heterologous receptor desensitization between both the fMLP and C5a receptor and the IL-8 receptor

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Abstract: During inflammation neutrophils receive multiple signals that are integrated, allowing a single modified response. One mechanism for this discrimination is receptor desensitization, a process whereby ligand-receptor binding is disassociated from cell activation. We examined the effect of heterologous receptor desensitization on neutrophil chemotaxis, calcium mobilization, and arachidonic acid production, using interleukin-8 (IL-8), C5a, and *N*-formylmethionyl-leucyl-phenylalanine (fMLP). We observed reciprocal inhibition with respect to chemotaxis. We demonstrated that homologous desensitization, with respect to the mobilization of intracellular calcium stores, lasted approximately 15 min. Heterologous desensitization between the fMLP receptor and the C5a receptor was reciprocal; either stimulant would diminish the cells' response to stimulation by the other for approximately 3–5 min. However, we observed a unidirectional heterologous desensitization of the IL-8 receptor by both the fMLP and the C5a receptor. This unidirectional heterologous desensitization was observed with respect to both calcium mobilization and arachidonic acid production (i.e., prestimulation of the IL-8 receptor had no effect on subsequent stimulation by either fMLP or C5a). *J. Leukoc. Biol.* 60: 88–93; 1996.

Key Words: *desensitization · neutrophils · chemotaxis · arachidonic acid · intracellular calcium*

INTRODUCTION

Inflammation results in the release of a variety of chemotactic and stimulatory factors from a number of different cell types. Polymorphonuclear leukocytes (PMNs), therefore, must differentiate these signals and be capable of modifying their responses when confronted with multiple stimulatory and inhibitory signals simultaneously. Prestimulation of PMNs with C5a or fMLP diminishes their chemotactic response not only to the initial stimulus (homologous desensitization) but also to the reciprocal stimulus (heterologous desensitization) [1, 2]. Not only is chemotaxis affected, but PMNs desensitized by prestimulation also fail to generate O_2^- and release granule marker enzymes [3, 4]. Homologous receptor desensitization is known to occur for many receptors (fMLP, C5a) through receptor internalization. However, Yagawa and co-workers [5, 6] found no inhibition of fMLP and wheat germ agglutinin binding following immune complex-mediated desensitization. Thus receptor recycling is not the mechanism of heterologous desensitization in this case.

Heterologous receptor desensitization appears to be specific for each receptor. Didsbury and co-workers [7] transfected human kidney cells with the C5a and fMLP receptors. Both receptors were able to initiate phospholipase C (PLC) activation as determined by the rise in intracellular Ca^{2+}. When the C5a and fMLP receptors were cotransfected they exhibited reciprocal heterologous receptor desensitization; however, neither affected, nor was affected by, stimulation of the native α_1-adrenergic receptor.

We examined the effect of heterologous receptor desensitization on PMN chemotaxis using three chemotactic peptides, IL-8, C5a, and fMLP. We observed that chemotaxis to a gradient stimulus was inhibited by a nongradient stimulus in a reciprocal fashion. In an attempt to delineate the location of the interruption in signal transduction, we examined two early events in PMN activation, intracellular calcium mobilization and arachidonic acid release. As expected, homologous receptor desensitization resulted in inhibition of calcium mobilization and arachidonic acid release. This inhibition lasted longer than 15 min. We also observed reciprocal heterologous desensitization between the fMLP receptor and the C5a receptor. However, we observed nonreciprocal heterologous desensitization between the IL-8 receptor and both the C5a and fMLP receptors, with respect to both calcium mobilization and arachidonic acid release.

Abbreviations: BSA, bovine serum albumin; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; IL-8, interleukin-8; PBS, phosphate-buffered saline; PLA_2, phospholipase A_2; PLC, phospholipase C; PMN, polymorphonuclear leukocyte; TCR, T cell receptor.

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METHODS

Isolation and preparation of human neutrophils

Neutrophils were obtained from fresh human blood using the method of Boyum [8]. ACD, anticoagulated blood was obtained from healthy donors and sedimented with 6% dextran (0.9% NaCl). Red blood cells were removed by hypotonic lysis, and neutrophils were isolated by density gradient centrifugation on a 10% Ficoll-Hypaque cushion. The resulting cells (98% neutrophils) were washed three times in phosphate-buffered saline (PBS) and transferred to the appropriate buffer for further studies.

Chemotaxis assay

The chemotaxis assays were performed in multiwell microchemotaxis chambers (Neuro Probe, Cabin John, MD) [9]. Human neutrophils were suspended in Hanks' balanced salt solution with 2% bovine serum albumin (BSA) at 4×10^6 cells/mL. For desensitization, the neutrophils were mixed with the desensitizing chemoattractant, 5×10^{-8} M fMLP or 5×10^{-9} M C5a or IL-8, and placed immediately in the upper chemotaxis chamber. The lower chamber contained an equal concentration of the desensitizing chemoattractant and the chemoattractant of interest, also at 5×10^{-8} M fMLP or 5×10^{-9} M C5a or IL-8. Random migration was determined using buffer alone as a control. The chambers were separated by a 200-μm-thick nitrocellulose filter, pore size 3 μm. The neutrophils were allowed to migrate for 13 min at 37°C. The filters were then fixed, stained with hematoxylin-eosin, and dehydrated and clarified with xylene. Migration of the leading front of neutrophils was assessed microscopically and expressed in micrometers. Net migration was the migration toward chemoattractant minus the random migration. Results were expressed as percent of control migration for each stimuli. Each experiment was performed in triplicate, and five fields per filter were examined.

Measurements of intracellular Ca^{2+}

Intracellular calcium determinations were performed using the fluorescent calcium indicator fluo-3 [10]. Purified neutrophils were suspended (1×10^7/mL) in buffer D (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 1 mM $CaCl_2$, and 1% BSA; pH 7.4) and incubated with 2 μM fluo-3 AM for 30 min at 37°C. The cells were washed three times with buffer D and maintained at 4°C until 3–5 min prior to use. Three to five minutes before each calcium determination, 1-mL aliquots of cells were brought to 37°C. Neutrophils (0.9 mL at 1×10^7/mL) were placed in an SLM 8000 spectrofluorometer (excitation wavelength 505 nm, emission wavelength 530 nm) and baseline was allowed to stabilize for 30 s. Varying concentrations of chemoattractants were added (0.1 mL) and fluorescence was monitored continuously for 150–180 s.

For assays examining the duration of either heterologous or homologous desensitization, five identical tubes were incubated and stimulated with desensitizing agent simultaneously. The initial sample was challenged with the second stimulating agent 150 s following the first agent and monitored continuously for 150 s. Subsequent tubes were stimulated and monitored every 150 s to determine the duration of desensitization.

Determination of arachidonic acid release

Purified PMNs (5×10^7 cells/mL) were incubated with 100 μCi (^3H) acid in buffer D containing 1% fatty acid-free BSA for 45 min at 37°C. Arachidonic acid was readily taken up by cells and incorporated into membrane phospholipids [11]. The cells were then washed three times and suspended in buffer D (10^7 cells/mL) containing 1% regular BSA and 0.01 mM unlabeled arachidonic acid, to remove unincorporated labels. Cells were stimulated for 5 min with the desensitizing stimulant and allowed to incubate for 7 min at 37°C. PMNs were then challenged with the second stimulus, 7 min, 37°C. Following centrifugation, the supernatants were collected and counted in a scintillation counter. Results were expressed as percent of total arachidonic acid released.

RESULTS

Neutrophils are known to migrate along a concentration gradient to a variety of chemotactic agents. Although most in vitro studies examining neutrophil chemotaxis are interested in specific responses to individual chemotactic agents, in vivo neutrophils encounter multiple signals simultaneously. Neutrophils must therefore process these multiple signals and elicit a single unified response. To better understand how neutrophils interpret multiple signals, we examined the effect of dual stimulation on PMN chemotaxis using three different chemotactic agents, fMLP, IL-8, and C5a. Human PMNs were simultaneously stimulated with two chemotactic agents, one as a gradient and the other at a static (nongradient) concentration. Static concentrations of both fMLP and C5a resulted in a statistically significant inhibition of the migration of human neutrophils to IL-8 (**Fig. 1A**). Likewise, fMLP and IL-8 inhibited chemotaxis toward C5a (Fig. 1C). IL-8 and C5a also resulted in a decrease in the migration of human neutrophils toward fMLP, even though the inhibition observed with C5a was not statistically significant (Fig. 1B). Thus a static concentration of a chemoattractant, such as IL-8, fMLP, or C5a, inhibits chemotaxis along a concentration gradient.

To determine whether the inhibition observed with chemotaxis correlated with receptor desensitization, we examined the effect of dual stimulation on intracellular calcium mobilization. Isolated PMNs were loaded with the calcium-sensitive fluorescent dye fluo-3 and stimulated with varying concentrations of fMLP, C5a, or IL-8. Intra-

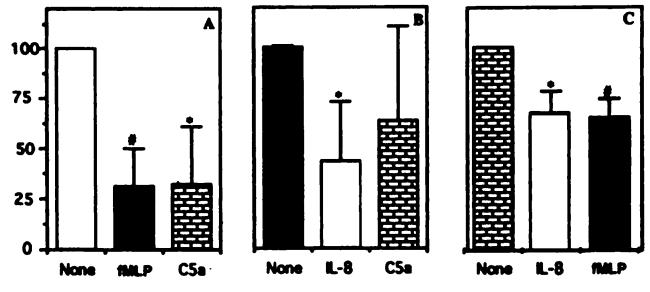

Fig. 1. Reciprocal inhibition of PMN chemotaxis by IL-8, C5a, and fMLP. Isolated human PMNs were mixed with the desensitizing stimulus (5×10^{-8} M fMLP or 5×10^{-9} M C5a or IL-8) immediately prior to being placed in the upper well of a chemotaxis chamber. The lower chamber contained an equal concentration of the desensitizing agent as well as the chemoattractant of interest. Net migration was determined (migration toward the chemoattractant minus random migration) microscopically, and results were expressed as a percentage of the control migration. Results are the mean and standard deviation of three separate experiments. (A) 5×10^{-9} M IL-8 is the gradient stimulus; (B) 5×10^{-8} M fMLP; (C) 5×10^{-9} M C5a. *$P \S 0.05$; #$P < 0.01$.

cellular calcium concentrations were monitored continuously on an SLM 8000 spectrofluorometer for 150 s. Cells stimulated with fMLP, C5a, or IL-8 all exhibited normal calcium transients. Prestimulation with either fMLP or C5a inhibited subsequent calcium transients to IL-8 given 150 s later (**Fig. 2A and C**). However, prestimulation with IL-8 had no effect on subsequent calcium transients elicited by either fMLP or C5a (Fig. 2B and D). The duration and the extent of IL-8 desensitization induced by fMLP and C5a were different. fMLP virtually abolished calcium transients in response to IL-8 for 12.5 to 15 min, while PMNs prestimulated with C5a showed a marked decrease in IL-8 responsiveness that was gradually restored with time, although normal levels were usually not reached at 12.5 to 15 min (**Fig. 3**).

Reciprocal heterologous desensitization was observed between the fMLP and C5a receptors with respect to calcium mobilization (**Fig. 4**). Prestimulation of either receptor resulted in decreased responsiveness to stimulation by the other receptor. The time course of desensitization lasted 5 to 7.5 min.

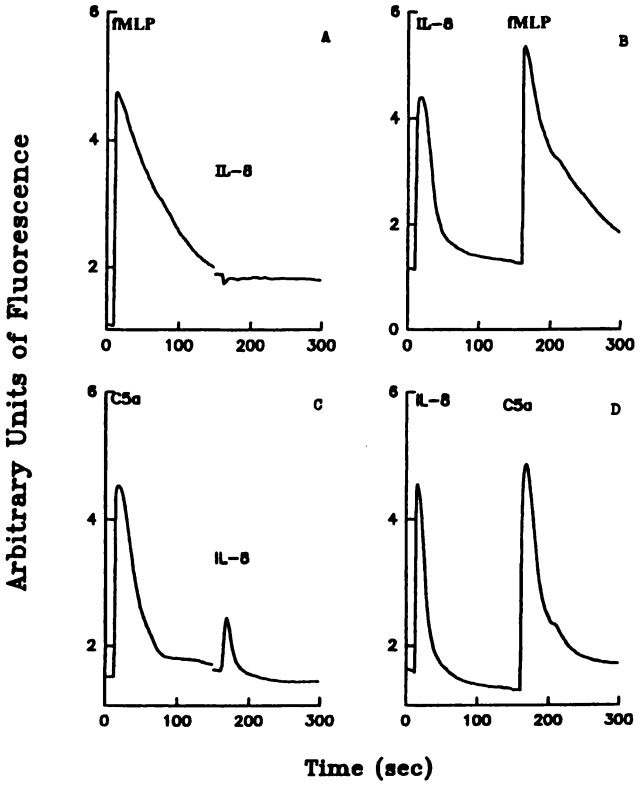

Fig. 2. Nonreciprocal Inhibition of IL-8-stimulated calcium mobilization by C5a and fMLP. Isolated PMNs (0.9×10^7/mL), loaded with the calcium-sensitive fluorescent dye fluo-3, were stimulated with 10^{-8} M fMLP (A), C5a (C), or IL-8 (B, D). Intracellular calcium concentrations were monitored continuously for 150 s on an SLM 8000 spectrofluorometer (excitation 505 nm, emission 530 nm). The cells were then challenged with 10^{-8} M IL-8 (A, C), fMLP (B), or C5a (D) and monitored an additional 150 s. Results are representative tracings from at least three separate donors.

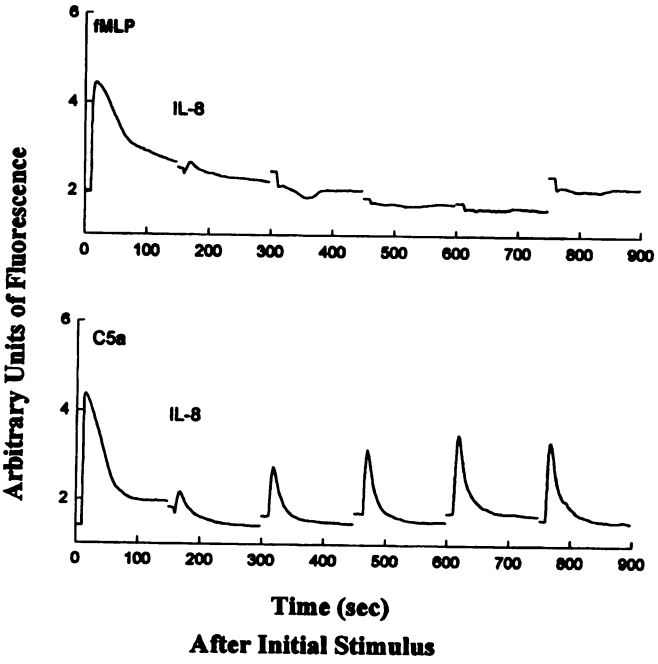

Fig. 3. Duration of fMLP- and C5a-induced desensitization of IL-8 receptor. Five aliquots of fluo 3-labeled PMNs were stimulated simultaneously with 10^{-8} M fMLP or C5a (top and bottom, respectively). At 150-s intervals an aliquot of cells was challenged with 10^{-8} M IL-8. Intracellular calcium concentrations were monitored continuously on an SLM 8000 spectrofluorometer. Results are representative tracings from at least three separate experiments from two different donors.

Desensitization was dependent on the concentrations of both the initial and secondary stimulus. Although decreasing concentrations of fMLP between 10^{-7} and 10^{-9} gave the same rise in intracellular calcium, they exhibited decreasing ability to desensitize and block subsequent calcium transients induced by IL-8 (**Fig. 5A–D**). Similar results were observed with C5a (data not shown).

Homologous receptor desensitization was observed with respect to stimulation of the fMLP, IL-8, and C5a (data not shown) receptors. Prestimulation with any of the chemoattractants examined inhibited calcium transients to subsequent rechallenge with the same stimuli. This desensitization was still present 12.5 to 15 min following the initial stimuli (data not shown).

To further localize where in the signal transduction pathway desensitization occurs, we examined the effect of receptor desensitization on arachidonic acid release from human neutrophils. PMNs whose membrane phospholipids were prelabeled with [^{3}H]arachidonic acid were stimulated with IL-8, C5a, or fMLP 7 min prior to rechallenge with IL-8, C5a, or fMLP, and the percent of total arachidonic acid released was determined. Arachidonic acid release due to stimulation by IL-8 was inhibited heterologously by prestimulation with fMLP and C5a and homologously by IL-8 prestimulation (**Fig. 6A**). Only homologous desensitization was observed for the C5a receptor (Fig. 6B). There was no significant effect of desensitization on arachidonic

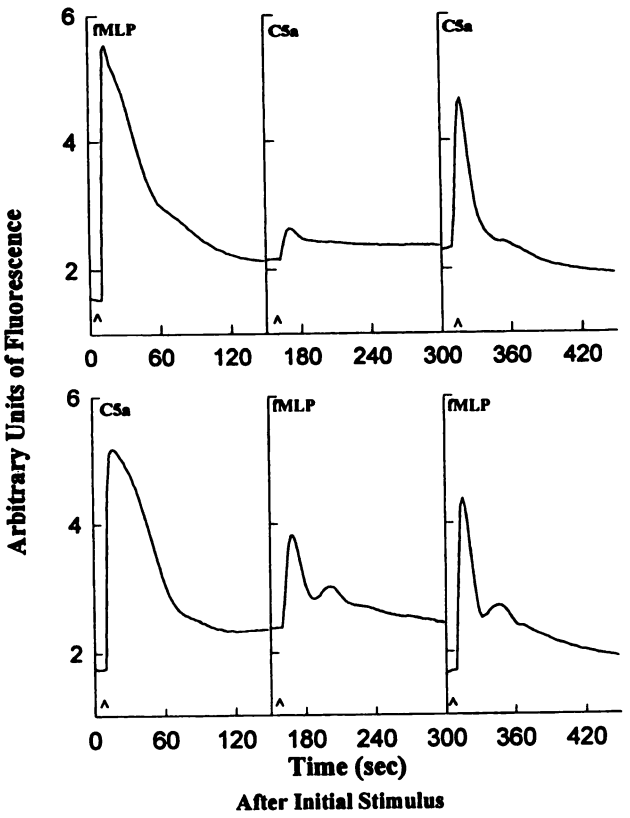

Fig. 4. Reciprocal inhibition of calcium mobilization induced by fMLP and C5a. Isolated PMNs were stimulated with 10^{-8} M fMLP (top) or C5a (bottom). The cells were rechallenged 150 or 300 s following the initial stimulus with 10^{-8} M C5a (top) or fMLP (bottom). Intracellular calcium concentrations were monitored continuously for 450 s on an SLM 8000 spectrofluorometer. Results are representative tracings from two separate donors.

acid production with respect to the fMLP receptor (Fig. 6C).

DISCUSSION

We examined the effect of heterologous receptor desensitization on PMN chemotaxis, calcium mobilization, and arachidonic acid production, using three chemotactic peptides: IL-8, C5a, and fMLP. We observed that a nongradient stimulus inhibited chemotaxis to a gradient stimulus in a reciprocal fashion. All three chemotactic peptides examined inhibited chemotaxis to the other stimuli.

In an attempt to localize where, during the signal transduction process, desensitization occurs, we examined intracellular calcium mobilization and arachidonic acid production in PMNs stimulated sequentially with different chemotactic peptides. We observed that the inhibition of chemotaxis was independent of calcium mobilization and arachidonic acid production. There was a unidirectional inhibition of both calcium mobilization and arachidonic acid production between both the fMLP and C5a receptors and the IL-8 receptor. Thus, IL-8 inhibited chemotaxis to both C5a and fMLP receptor stimulation despite normal generation of calcium transients and arachidonic acid production. Previous reports indicated that chemotaxis was not a calcium-dependent cellular function [12]. However, these studies also indicated that it was independent of the activation of phospholipase A_2 and the generation of arachidonic acid.

Norgauer and co-workers [13] have demonstrated that while both Il-8 and fMLP activate the mobilization of Ca^{2+} from intracellular stores, only fMLP triggers the secondary influx from the extracellular space. Interestingly, this correlated with differences in the metabolism of phosphorylated phosphoinositides as well as the formation of phosphatidic acid. Because phospholipid metabolism is an integral part of PMN activation, different levels or states of activation may represent one mechanism for the control of different neutrophil functions and may at least partially explain why although IL-8 is a potent chemoattractant it is a weak activator of the respiratory burst.

Unlike homologous desensitization, heterologous desensitization is not thought to be secondary to receptor recycling. Yagawa and co-workers [5, 6] described normal fMLP binding despite immune complex-mediated desensitization. Heterologous desensitization also appears to be specific for each receptor. Didsbury and co-workers [7] showed that although transfected fMLP and C5a receptors

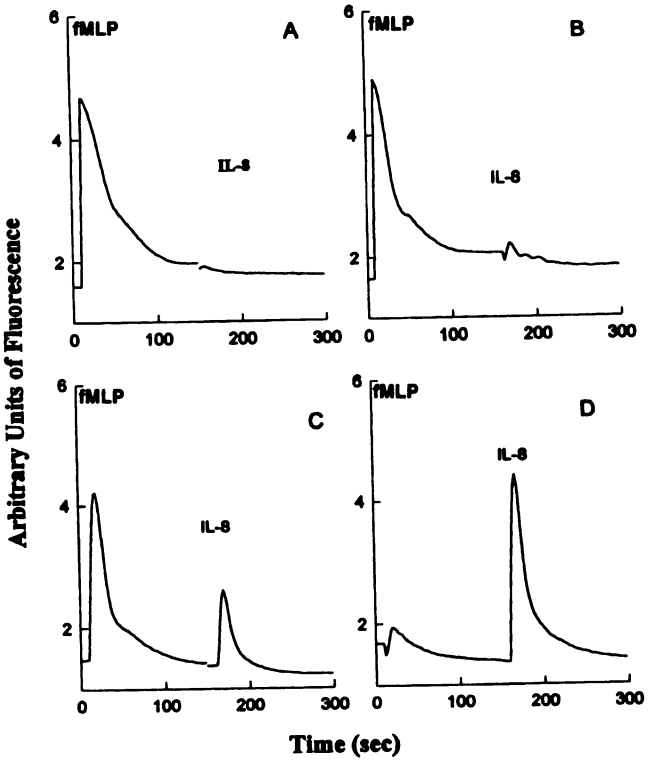

Fig. 5. Concentration-dependent heterologous desensitization between fMLP and IL-8 receptors. Fluo 3-labeled PMNs were stimulated with decreasing concentrations of fMLP (A, 10^{-7} M; B, 10^{-8} M; C, 10^{-9} M; D, 10^{-10} M) 150 s prior to stimulation with 10^{-8} M IL-8. Intracellular calcium concentrations were monitored continuously. Results are representative tracings from at least three separate donors.

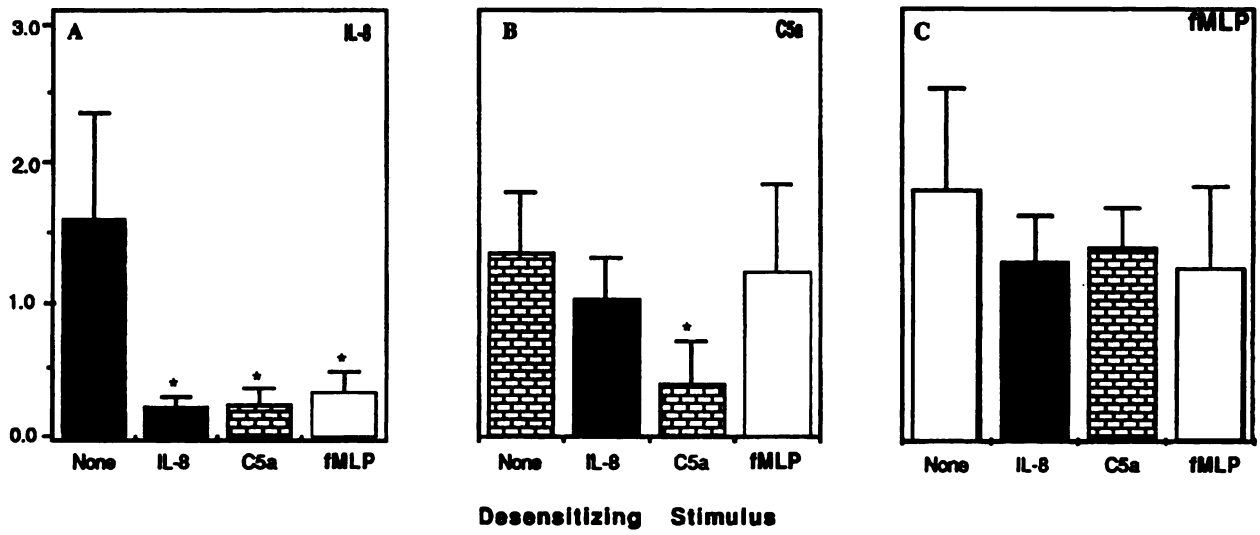

Fig. 6. Effect of desensitization on arachidonic acid production in human neutrophils. Human neutrophils were incubated with [^{3}H]arachidonic acid to label membrane phospholipids. The cells were then stimulated with 10^{-8} M IL-8, fMLP, or C5a (desensitizing stimulus) and incubated for 7 min prior to subsequent challenge with 10^{-8} M IL-8, C5a, or fMLP (secondary stimulus). Following a 7-min incubation, the cells were pelleted and the supernatants were assayed for the release of [^{3}H]arachidonic acid. Secondary stimulus: A, IL-8; B, fMLP; C, C5a. Results are the mean and standard deviation of at least five experiments done in duplicate. $^*P < 0.05$.

cross-desensitized each other, both were unaffected by stimulation of a native receptor from kidney cells. Because signal transduction, as measured by Ca^{2+} transients, was maintained across cell types, certain common components must exist. Thus receptor-receptor distinction must exist with respect to desensitization. Our results further demonstrate this phenomenon, indicating that a hierarchy may actually exist between receptors. Both arachidonic acid release and calcium mobilization in response to IL-8 stimulation were inhibited by previous stimulation with either fMLP or C5a. Desensitization, with respect to calcium mobilization, was more profound following fMLP stimulation as compared with C5a. Furthermore IL-8 showed no desensitization of either the fMLP or C5a receptor.

A similar nonreciprocal heterologous desensitization has been described with the CD3–T cell receptor (TCR) complex and the pertussis toxin receptor [14]. The authors propose that signal transduction through the pertussis toxin receptor is dependent on secondary stimulation of CD3-TCR complex distal to its antigen binding domain. Thus, regulation of the CD3-TCR complex would also regulate the pertussis toxin receptor. This is not the case for the fMLP and C5a receptors, because regulation would have to be through both receptors simultaneously and be distinct from the control pathway for chemotaxis. Heterologous desensitization for the fMLP, C5a, and IL-8 receptors is thought to involve receptor phosphorylation via second messanger-activated kinases, such as protein kinases A and C [15–20]. Interestingly, Metzner et al. [21] have demonstrated that IL-8 actually up-regulates fMLP-dependent generation of superoxide anion presumably through the increased expression of fMLP receptors.

We demonstrated that homologous desensitization, with respect to the mobilization of intracellular calcium stores, lasted more than 12.5–15 min. These finding are consistant with the findings that ligand-receptor binding results in the internalization of the receptor and a decrease in surface receptor numbers [22, 23]. As expected, we also observed homologous desensitization with respect to arachidonic acid production for IL-8 and C5a. Although we were unable to demonstrate homologous desensitization for the fMLP receptor, we suspect that this may be secondary to the constraints of our studies. As with other measures of PMN activation, the time course and total release of arachidonic acid in response to fMLP were greater than those observed for C5a and IL-8 (data not shown).

Confirming reciprocal heterologous desensitization between the fMLP receptor and the C5a receptor, with respect to intracellular Ca^{2+} transients (i.e., either stimulant would diminish the cells' response to stimulation by the other for approximately 3–5 min), we took advantage of the difference in the time course of desensitization between C5a, fMLP, and IL-8. We examinined the effect of dual stimulation on the activation of PLA_2 and the generation of arachidonic acid. PLA_2 metabolism has been implicated in both the respiratory burst and PMN degranulation [24–27]. As predicted, there was a correlation between desensitization of intracellular Ca^{2+} transients and desensitization of arachidonic acid release. We chose a time interval between stimuli of 7 min. At this interval both fMLP and C5a desensitize IL-8-mediated activation of PLC and PLA_2.

Reciprocal desensitization between the fMLP and C5a receptors lasted only 3 to 5 min with respect to the rise in intracellular Ca^{2+}. In conjunction with this, no desensitization was observed between these receptors with respect to arachidonic acid generation. IL-8, on the other hand, exhibited only homologous desensitization with respect to both PLC and PLA_2 activation.

Homologous receptor desensitization is known to be linked to receptor availability. However, the mechanism of heterologous desensitization is linked to receptor phosphorylation [15–17]. Our chemotaxis data indicated that the inhibition was reciprocal. Yet, two separate markers of early PMN activation, intracellular calcium concentration and arachidonic acid release, demonstrated nonreciprocal desensitization. Desensitization is also concentration dependent for both primary and secondary stimulants, indicating that while the receptor's availability is unaffected, its affinity or ability to transduce the signal may be altered. As both PLA_2 and PLC are activated through separate G proteins, one potential level of control for desensitization would be proximal to their activation. Tomhave and coworkers [28] have demonstrated that desensitization is also proximal to phospholipase D activation. Thus heterologous desensitization probably involves many levels of control and may very well be distinct for each receptor pair.

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