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Modulation of Human Neutrophil Responses to CD32 Cross-Linking by Serine/Threonine Phosphatase Inhibitors: Cross-Talk Between Serine/Threonine and Tyrosine Phosphorylation

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The interplay between serine/threonine and tyrosine phosphorylation was studied in human neutrophils. The direct effects of calyculin and okadaic acid, potent inhibitors of PP1 and PP2A serine/threonine phosphatases, on the patterns of neutrophil phosphorylation, and their effects on the responses of neutrophils to CD32 cross-linking were monitored. After a 2-min incubation with $10^{-6}$ M calyculin, a transient tyrosine phosphorylation of a subset of proteins, among which Cbl and Syk, was observed. After a longer incubation (>5 min) with calyculin, concomitant with an accumulation of serine and threonine phosphorylation, neutrophil responses to CD32 cross-linking were selectively altered. Tyrosine phosphorylation of Cbl in response to CD32 cross-linking was inhibited by calyculin, and this inhibition was linked with a slower electrophoretic mobility of Cbl as a consequence of its phosphorylation on serine/threonine residues. However, tyrosine phosphorylation of Syk and of the receptor itself were not affected. Furthermore, the mobilization of intracellular calcium stimulated by CD32 cross-linking was totally abrogated by calyculin. Finally, the stimulation of superoxide production observed in response to CD32 cross-linking was enhanced in calyculin-treated cells. These results suggest that serine/threonine phosphorylation events regulate the signaling pathways activated by CD32 cross-linking in neutrophils and identify a novel mechanism of modulation of the functional responsiveness of human neutrophils to CD32 cross-linking.

The phosphorylation-dephosphorylation of cellular proteins is a major regulatory mechanism for many eukaryotic signaling pathways. In human polymorphonuclear neutrophils (neutrophils) which form the first line of immune defense and are the first cells to arrive at sites of inflammation and infection, tyrosine phosphorylation has been intensively studied and has been shown to be rapidly stimulated in response to phagocytic particles (1, 2), cytokines (3–5) or pathogen-derived stimuli (6–8). Neutrophils express at least five different types of tyrosine kinases whose involvement in neutrophil activation has been documented: Src family kinases [p59/61hck (1), p58fgr (9–11), and p53/56fyn (12)], the Syk kinase (homologous to Zap 70 in other cells) (13), Pyk2 (14), Jak2 (15), and p93fes (16). The importance of these enzymes is underlined by the inhibitory effect of tyrosine kinase inhibitors on several functions including chemotaxis (17, 18), adherence (19), phagocytosis (20), and production of radical oxygen (21–23). Although early studies focused mainly on tyrosine kinases, several studies clearly indicate that phosphotyrosine phosphatases (PTP) also play a critical regulatory role in neutrophils. PTP are likely to be responsible for the transient nature of the tyrosine phosphorylation responses to agonists since pervanadate, a nonspecific PTP inhibitor, stimulates the intracellular accumulation of tyrosine-phosphorylated proteins (24). Overall, neutrophil PTP activity has been shown to decrease following stimulation with FMLP or phorbol esters (25, 26). Two membrane receptor-like PTPs, CD45 and CD148, have been linked to the regulation of neutrophil responses (27, 28). However, the role of soluble PTP in neutrophil signal transduction is poorly understood although two Src homology (SH) 2-containing phosphatases, namely, SHP-1 (29) and SHP-2 (30) are present in neutrophils. Serine and threonine phosphorylations are also involved in activated neutrophils. For example, p47phox, which is one of the five core components of the NADPH oxidase complex, is extensively phosphorylated on serine residues following activation of the complex (31). These serine residues have been mapped, and their phosphorylation is essential for membrane translocation and activation of the NADPH oxidase. Furthermore, serine/threonine phosphatases are involved in the deactivation of NADPH oxidase, as illustrated by the enhancement of $O_2^-$ production in FMLP-activated neutrophils treated with inhibitors of serine/threonine phosphatases (32, 33).

Okadaic acid and calyculin are two potent inhibitors of type 1 and type 2A (PP1 and PP2A) serine/threonine phosphatases that have been exploited to provide evidence for an interplay between serine/threonine and tyrosine phosphorylation signaling pathways in several cell types. In platelets, okadaic acid drastically inhibits...
thrombin-induced tyrosine phosphorylation and platelet aggregation (34). In several lymphoid cell lines, calyculin A induces dephosphorylation of tyrosine phosphorylated p130<sub>cas</sub> (35). In neutrophils, Brumell and Grinstein (36) showed that tyrosine phosphorylation is a necessary upstream event in the activation of two serine/threonine kinases PKδ5 and PK72. Furthermore, okadaic acid has been reported to enhance the increase in tyrosine phosphorylation observed in response to CD32 cross-linking (37) and to decrease the responses to PMA in LPS-primed neutrophils (38).

The aim of our study was to investigate the cross-talk between the serine/threonine and tyrosine phosphorylation signaling pathways in human neutrophils. We examined the direct effects of serine/threonine phosphatase inhibitors on the patterns of neutrophil serine/threonine and tyrosine phosphorylation as well as their effects on the responses elicited by CD32 cross-linking. Distinct time-dependent effects of serine/threonine phosphatase inhibitors were observed. Short-term treatment with calyculin induced transient increases in the tyrosine phosphorylation of a limited number of substrates including Cbl and Syk. On the other hand, longer incubation times with the inhibitors resulted in an inhibition of the tyrosine phosphorylation of Cbl and of the mobilization of calcium and in an increase in the superoxide production stimulated by the cross-linking of CD32. These effects were accompanied by a phosphorylation of Cbl on serine/threonine residues leading to a marked retardation of its electrophoretic mobility.

### Materials and Methods

#### Reagents

The enhanced chemiluminescence (Reagenoise) reagents used for Western blotting and [125I]orthophosphate (NEXO53H) were purchased from DuPont Pharmaceuticals (Mississauga, Ontario, Canada). Calyculin was purchased from Biomol (Plymouth Meeting, PA) and okadaic acid from Kamiya Biomedical (Seattle, WA). Dioxpropylfluorophosphosphate and cytochrome c were obtained from Sigma-Aldrich (Oakville, Ontario, Canada). Dextran, Sephadex G-10, and protein A-Sepharose were obtained from Pharmacia (Baie d’Urée, Quebec, Canada). Ficoll and the Mg<sup>2+</sup>-free HBSS were obtained from Wisent Canadian Laboratories (St. Bruno, Quebec, Canada). Alkaline phosphatase was purchased from Promega (Madison, WI).

#### Antibodies

F(ab')<sub>2</sub> fragments of Abs IV-3 (hybridoma obtained from American Type Culture Collection, Manassas, VA) were prepared essentially as described in the Pierce catalog (Rockford, IL). Briefly, the Abs were digested with pepsin (as pepsin beads) and intact Abs were eliminated by adding protein A and protein G beads. The integrity of the F(ab')<sub>2</sub> fragments was verified by their ability to label intact human neutrophils as determined by flow cytometry. Affinity-purified F(ab')<sub>2</sub> fragment goat anti-mouse IgG, F(ab')<sub>2</sub> fragment-specific (15-006-072), and peroxidase-labeled anti-mouse or anti-rabbit IgG Abs were obtained from Jackson ImmunoResearch (West Grove, PA). The anti-phosphotyrosine Ab (UBI 05-321, clone 4G10) was obtained from Upstate Biotechnology (Lake Placid, NY); the polyclonal anti-phosphothreonine Ab (P4A1603H) and the Mg<sup>2+</sup>-free HBSS were obtained from Wisent Canadian Laboratories (St. Bruno, Quebec, Canada). Alkaline phosphatase was purchased from Promega (Madison, WI).

#### Neutrophil purification

Neutrophils were obtained from healthy adult volunteers as described previously (8). Neutrophils were resuspended at a concentration of 40 × 10<sup>6</sup> cells/ml in HBSS containing calcium (1.6 mM). Before stimulation, the neutrophil suspensions were always incubated with 1 mM di-isopropylfluorophosphate for 10 min at room temperature.

#### Cell stimulation and lysis

Neutrophils were incubated with 10<sup>-6</sup> M calyculin or the diluent DMSO (final concentration, 0.1%) at 37°C for the time indicated. Cross-linking of CD32 was accomplished by the addition of F(ab')<sub>2</sub> goat anti-mouse F(ab')<sub>2</sub> fragments (cross-linker Ab; final concentration, 25 μg/ml) for 1 min, or for the times indicated, to neutrophil suspensions previously preincubated for 5 min at 37°C with 2.5 μg/ml of the F(ab')<sub>2</sub> fragment of Ab IV-3.

For the determination of the phosphorylation patterns in whole cells, 80 μl of the cell suspensions was directly transferred to tubes containing an equal volume of boiling 2× Laemmli’s sample buffer (1× is 62.5 mM Tris-HCl (pH 6.8), 4% SDS, 5% 2-ME, 8.5% glycerol, 2.5 mM orthovanadate, 10 mM paranitro-phenylphosphate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.025% bromophenol blue) and boiled for 7 min.

#### Immunoprecipitation

Lysates prepared as described above were filtered through Sephadex G-10 columns to remove the denaturing and the reducing agents, then Nonidet P-40, BSA, orthovanadate, aprotinin, and leupeptin (final concentrations, 1%, 0.005%, 2 mM, 10 μg/ml, and 10 μg/ml, respectively) were added. Lysates were incubated for 90 min with protein A-Sepharose prepared as follows: 1 μg of anti-Cbl Ab or 1 μg of anti-Syk Ab or 5 μg of anti-CD32 Ab was incubated with 50 μl of a 30% slurry of protein A-Sepharose for 1 h at 4°C with constant end-over-end mixing. This complex was washed once in washing buffer (62.5 mM Tris (pH 6.8), 1% Nonidet P-40, 1% glycerol, 2 mM orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 137 mM NaCl, and 0.001% BSA) and added to the lysates for immunoprecipitation. The agarose beads were thoroughly collected, and an aliquot for electrophoretic analysis. The beads were washed five times with the same washing buffer. A total of 40 μl of 2× sample buffer was added and the immunoprecipitates were boiled for 7 min.

#### Electrophoresis and immunoblotting

Samples were fractionated on 7.5%–20% SDS-polyacrylamide gel gradients. Proteins were then transferred onto Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Immunoblotting was performed as follows: nonspecific sites were blocked using either 1% fatty acid-free BSA (for anti-phosphothreonine blotting) or 2% gelatin (for all other Abs) in TBS-Tween (25 mM Tris-HCl (pH 7.8), 5 mM KCl, 10 mM glucose, and 20 mM HEPES (pH 7.4)) and resuspended at 40 × 10<sup>6</sup> cells/ml in the same buffer containing 0.4% McIlVain labeled phosphoric acid. After 1 h at 37°C, DMSO or calyculin was added for 30 min. The reactions were stopped by washing the radioactive cell suspensions twice with HBSS and mixing them with the same volume of boiling denaturing lysis buffer for immunoprecipitation as described above.

#### Calcium mobilization measurement

The cells (10<sup>7</sup> cells/ml) were incubated at 37°C with 1 μM fura-2-acetoxyethyl ester for 30 min. Neutrophils were washed twice in HBSS to remove extracellular calcium and resuspended at 5 × 10<sup>6</sup> cells/ml in HBSS transferred to the thermostated (37°C) cuvette compartment of a spectrofluorometer (SLM 8000; Aminco, Urbana, IL). The fluorescence of the cells was monitored at an excitation wavelength of 340 nm and an emission wavelength of 510 nm.
wavelength of 510 nm. The internal calcium concentrations were calculated as described by Grynkievicz et al. (41).

**Superoxide production measurement**

Superoxide production was monitored as already described (18) as the reduction of cytochrome c by a suspension of 10^7 neutrophils using a slight modification of the method described in Metcalf et al. (42). The absorption of cytochrome c was monitored at 550 nm and 540 nm, and the amount of superoxide anions produced was calculated from the difference between the OD at the two wavelengths using an extinction coefficient of 21.1.

**Results**

**Direct effects of calyculin on neutrophils**

Because calyculin is a potent serine-threonine phosphatase inhibitor, we first examined, by immunoblotting, its effects on the levels of serine and threonine phosphorylation in human neutrophils. We also monitored the tyrosine phosphorylation patterns, since calyculin has been shown to induce tyrosine phosphorylation in other systems (36, 43). Neutrophils were incubated with 1 µM calyculin or 0.1% DMSO as control; at each time indicated, a sample of cell suspension was transferred in the same volume of boiling sample buffer. The samples were electrophoresed and transferred to Immobilon PVDF membranes before being probed with either the anti-phosphothreonine, anti-phosphoserine, or anti-phosphotyrosine Abs (Fig. 1). These data make several points. First, as expected, serine and threonine phosphorylation levels gradually increase with the time of exposure of neutrophils to calyculin. Newly serine- or threonine-phosphorylated bands are detectable within 5 min of incubation with calyculin, and the phosphorylation levels increase gradually until 60 min, the longest time tested. A major threonine-phosphorylated band appeared in the 140-kDa region and other bands appeared in the 116-kDa, 60-kDa, and 40-kDa molecular mass ranges. The serine-phosphorylated bands are less well defined; many proteins appear newly serine-phosphorylated in the molecular range between 40 and 150 kDa. On the other hand, the antiphosphotyrosine blot indicates that calyculin rapidly and transiently increases the levels of tyrosine phosphorylation of a small number of substrates, prominent among which are bands in the 120-kDa, 85-kDa, 70-kDa, and 55-kDa regions. The tyrosine phosphorylation of these bands is detectable within 30 s of incubation with calyculin, reaches a maximal level at 2 min, then gradually declines, and is undetectable at times ≥10 min. We also observed the tyrosine phosphorylation of a 40-kDa substrate after 30 min or more of incubation with calyculin. This protein might be a member of the mitogen-activated protein (MAP) kinase family as previously suggested in neutrophils (36) and in adipocytes (44). Therefore, in human neutrophils, calyculin rapidly increases the tyrosine phosphorylation of a select subset of proteins. This is followed by a disappearance of the tyrosine phosphorylation which occurs concomitantly with an increase in overall serine and threonine phosphorylation. Time courses of control cells (0.1% DMSO) probed with the three Abs indicated that the phosphorylation levels (serine, threonine, and tyrosine) were not modified during the 60-min interval (data not shown). We also confirmed by trypan blue exclusion that neutrophil viability was not modified even after long exposure to DMSO or calyculin under our conditions. Furthermore, neutrophils can still respond to CD32 cross-linking following a 60-min exposure with calyculin or DMSO (see Fig. 4), further indicating that the cells remained functionally responsive.

We next wanted to characterize at least some of the tyrosine-phosphorylated substrates that rapidly responded to the addition of calyculin. Since Cbl and Syk, whose molecular masses are 120 kDa and 72 kDa, respectively, are prominent tyrosine-phosphorylated substrates in activated neutrophils (45–47), we asked whether these two proteins might correspond to the tyrosine-phosphorylated substrates observed in Fig. 1 in these molecular mass ranges. The denaturated lysates of cells incubated 2 min with calyculin were immunoprecipitated using anti-Cbl or anti-Syk Abs, and the immunoprecipitates were subjected to electrophoresis and immunoblotting using the anti-phosphotyrosine Ab. The results shown in Fig. 2A indicate that Cbl and Syk are tyrosine phosphorylated in response to calyculin. Reprobing the membranes with anti-Cbl or anti-Syk Ab (Fig. 2B) demonstrates that equal amounts of the respective proteins were immunoprecipitated and deposited in each lane. In our hands, Cbl immunoblots routinely identify a doublet at about 120 kDa which may reflect the presence of presently uncharacterized isoforms in human neutrophils.

**Effects of calyculin on neutrophil responses to CD32 cross-linking**

**Inhibition of CD32 cross-linking-induced tyrosine phosphorylation.**

Calyculin and okadaic acid have been shown to modulate agonist-induced tyrosine phosphorylation in several systems (37, 38, 48). This prompted us to study the effect of calyculin on CD32 cross-linking-induced tyrosine phosphorylation because this response is well characterized in human neutrophils. Neutrophils were preincubated with 10^{-6} M calyculin (or 0.1% DMSO as control) for...
various times before cross-linking CD32 for 1 min as described in Materials and Methods. The reactions were stopped by direct transfer of cell suspension aliquots to the same volume of boiling 2× Laemmli’s sample buffer. The tyrosine-phosphorylated proteins were analyzed using the anti-phosphotyrosine Ab, and the results of these experiments are illustrated in Fig. 3A. These data first illustrate that the characteristic increases in tyrosine phosphorylation induced by cross-linking CD32 was observed to be stable over the 60-min time period. In the absence of calyculin, as already described (46), CD32 cross-linking rapidly induced the tyrosine phosphorylation of several proteins among which were Cbl (120 kDa), Syk (72 kDa), and CD32 (40 kDa). After 5 min of incubation with calyculin, a slight and selective decrease in the tyrosine phosphorylation pattern in response to CD32 cross-linking was observed. More specifically, treatment with calyculin decreased the intensity of phosphorylation of substrates in the 120-kDa and 60- to 70-kDa regions, whereas a 140-kDa band appears. The other tyrosine-phosphorylated substrates in response to CD32 cross-linking, and most prominently those bands in the 70-kDa and 40-kDa regions are not affected. The inhibitory effect of calyculin increased with the incubation time. The effects of calyculin on the kinetics of the increases in tyrosine phosphorylation induced by cross-linking CD32 were examined next. Neutrophils were preincubated with calyculin for 20 min, and the time course of the increases in tyrosine phosphorylation in response to CD32 cross-linking was monitored. The results of these experiments are presented in Fig. 4A. The level of tyrosine phosphorylation of most of the substrates increased as rapidly as 20 s after CD32 cross-linking, reached a maximum between 40 s and 2 min, and then gradually decreased. After a 20-min preincubation with calyculin, the overall tyrosine phosphorylation response in response to ligation of CD32 was decreased. More specifically, and consistently with the results illustrated in Fig. 3A, the tyrosine phosphorylation of the 120-kDa band (Cbl) decreased whereas that of the 72-kDa and 40-kDa bands was not affected. Tyrosine phosphorylation of the two bands in the 60- to 70-kDa range was totally abrogated.

The protooncogene Cbl has previously been shown to comprise most, if not all, of the 120-kDa tyrosine-phosphorylated band observed in response to CD32 cross-linking (46). The loss of phosphorylation in the 120-kDa band was thus likely related to Cbl. More specifically, two possibilities may account for the observed effects of calyculin. First, calyculin may stimulate the degradation of Cbl, or, second, it may prevent its phosphorylation upon cross-linking of CD32. The former possibility was tested by reprobing the above membranes with anti-Cbl Abs. The results presented in Figs. 3B and 4, B and C, indicate that the integrity of Cbl was not affected by the calyculin treatment. However, within 5 min of the addition of calyculin, Cbl exhibited a slower electrophoretic mobility, and this shift increased with the duration of calyculin treatment. Thus, in the presence of calyculin, the increase in tyrosine phosphorylation of the 120-kDa band observed in response to CD32 cross-linking is inhibited, and this inhibition is concomitant with a marked electrophoretic shift of Cbl. The second possibility was tested by immunoprecipitating Cbl from lysates of neutrophils preincubated or not with calyculin for 20 min followed by CD32 cross-linking for 1 min, a time point that corresponded to the peak of tyrosine phosphorylation in neutrophils in response to CD32 cross-linking (Fig. 4A). The results shown in Fig. 4C indicate that Cbl tyrosine phosphorylation was decreased following calyculin

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Stimulation of the tyrosine phosphorylation of Cbl and Syk in human neutrophils by calyculin. The cells were incubated with calyculin (10⁻⁶ M) or DMSO (0.1% as control) for 2 min at 37°C. They were lysed and processed for immunoprecipitation (IP) with anti-Cbl (1 μg) or anti-Syk (1 μg) Ab under denaturing conditions as described in Materials and Methods. After electrophoresis and transfer to PVDF membranes, immunoblotting (Blot) using the anti-phosphotyrosine Ab was conducted (A). The membrane was then reprobed using anti-Cbl or anti-Syk Abs (B). The data shown are representative of three independent experiments.

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Tyrosine phosphorylation response induced by CD32 cross-linking in human neutrophils following incubation with calyculin. The cells were incubated with calyculin (10⁻⁶ M) for the times indicated. F(ab')₂ fragments of Ab IV.3 (2.5 μg/ml) were added for the last 5 min of incubation. Ligation of CD32 was then initiated by the addition of the cross-linker Ab (anti-F(ab')₂, 25 μg/ml) for 1 min. The cells were lysed in Laemmli’s sample buffer and processed for immunoblotting with anti-phosphotyrosine (A) or anti-Cbl (B) Abs. The data shown are representative of five independent experiments.
preincubation. Repробbing the membrane with anti-Cbl Ab (Fig. 4C) demonstrated that equal amounts of Cbl were immunoprecipitated and deposited in each lane and confirmed the electrophoretic shift of Cbl following calyculin treatment. Syk and the CD32 were immunoprecipitated under the same conditions as Cbl. The results in Fig. 4C clearly indicated that the stimulated tyrosine phosphorylation of Syk and CD32 in response to CD32 cross-linking was not affected by calyculin. Therefore, preincubation with calyculin specifically inhibited the tyrosine phosphorylation of Cbl. **Characterization of the electrophoretic shift of Cbl induced by calyculin.** Changes in electrophoretic migration are often caused by hyperphosphorylation which increases the apparent m.w. of the protein. This might explain the shift of Cbl since calyculin increased the level of serine and threonine phosphorylation of several proteins under our conditions with kinetics similar to those of the alteration of migration of the protooncogene (compare Figs. 1 and 3B). Two approaches were used to test this hypothesis. First, neutrophils were preincubated with [32P]orthophosphorus in the absence or presence of calyculin, Cbl was then immunoprecipitated, and the autoradiogram of the electrophoretic analysis of this immunoprecipitation is shown in Fig. 5A. Incorporation of 32P into Cbl was observed only in Cbl immunoprecipitates from calyculin-treated cells. This membrane was then reprobed for the presence of Cbl to show that equivalent amounts of Cbl were loaded in both lanes and that calyculin induced the previously described shift of electrophoretic mobility of Cbl. In an additional series of experiments, Cbl was immunoprecipitated from cells incubated for 30 min with DMSO or calyculin, and the immunoprecipitates were treated with alkaline phosphatase before electrophoretic analysis (Fig. 5B). A comparative examination of *lanes 1–4* indicates that the decreased electrophoretic mobility of Cbl induced by calyculin was significantly reduced following treatment with alkaline phosphatase. Furthermore, Cbl was immunoprecipitated from cells incubated for 30 min with DMSO or calyculin. Immunoblotting of the immunoprecipitates with anti-phosphoserine and anti-phosphothreonine Abs indicates that Cbl is serine phosphorylated and, to a lesser extent, threonine phosphorylated in response to treatment with calyculin (data not shown). Therefore, the calyculin-induced shift of Cbl was due, at least in part, to serine and/or threonine phosphorylation.

**Inhibition of calcium mobilization induced by CD32 cross-linking.** We next investigated the effects of calyculin on another response to CD32 cross-linking, namely, the mobilization of intracellular calcium. As illustrated in Fig. 6, cross-linking of CD32 induced after a lag time of about 20 s a transient increase in the concentration of cytoplasmic-free calcium. Calyculin, by itself, had little if any significant effect. On the other hand, preincubation with calyculin essentially abrogated the calcium response to the cross-linking of CD32.

**Stimulation of superoxide production induced by CD32 cross-linking.** Since superoxide production has been described following CD32 cross-linking (37), the effects of calyculin on this functional response of neutrophils was investigated. As illustrated in Fig. 7, we observed a very modest production of superoxide following CD32 cross-linking. However, calyculin, which by itself induces only a very slight production of superoxide, greatly enhanced that observed in response to CD32 cross-linking.

**Discussion**

The experiments described in the present manuscript were undertaken to investigate the interplay between serine/threonine phosphorylation and tyrosine phosphorylation in the regulation of neutrophil activation. Two types of effects of calyculin were observed. Short-term events (<5 min) were characterized by a rapid and transient induction of tyrosine phosphorylation of a few proteins among which were Cbl and Syk. Longer treatments with calyculin (>5 min)
min) led to a partial inhibition of tyrosine phosphorylation, an inhibition of intracellular calcium mobilization, and a potentiation of the production of superoxide anions in response to CD32 cross-linking. These events occurred concomitantly with a shift in the electrophoretic mobility of Cbl resulting from an increase in its level of serine/threonine phosphorylation.

All of the effects observed following calyculin treatment were also observed with okadaic acid, although to a lesser extent and with slightly slower kinetics (data not shown). Okadaic acid and calyculin inhibit PP2A with similar efficiency (IC50 of 0.5–1.0 nM) whereas calyculin is a better inhibitor of PP1 than okadaic acid (IC50 of 2 and 60–500 nM for calyculin and okadaic acid, respectively). The effects of calyculin described in the present study were detectable at 100 nM and optimal at 0.5–1 μM (data not shown). Although these concentrations are high, the lack of knowledge concerning the intracellular concentrations of the inhibitors precludes any firm conclusions to be drawn about the respective contributions of the two phosphatases to the effects observed. The permeability of neutrophil membranes for calyculin has never been tested directly. The rates of transport of okadaic acid and calyculin through erythrocyte membranes have been estimated (49), and the results indicate that the permeation of calyculin is much faster than that of okadaic acid. This is in agreement with our results since calyculin acted more rapidly and was more efficient than okadaic acid (data not shown).

Since calyculin is described as a serine/threonine phosphatase (PP1 and PP2A) inhibitor, the rapid (<5 min) increases in tyrosine phosphorylation were surprising. However, a similar effect has been observed in spermatozoa after a 5-min incubation with calyculin (43). Two proteins of molecular masses of 105 kDa and 81 kDa, localized in the flagellum of spermatozoa, were tyrosine phosphorylated in response to calyculin, and there was a concomitant increase of the level of sperm capacitation. In neutrophils, our study is the first to analyze the short-term effects of calyculin. However, earlier reports showed that calyculin by itself stimulated serine/threonine protein kinase activities as well as tyrosine phosphorylation after a 30-min incubation (36). These calyculin-induced kinase activities may account for the accumulation of phosphothreonine and phosphoserine shown in Fig. 1. The tyrosine phosphorylation pattern observed in the previous study (36) is consistent with our results since these authors observed a spontaneous

![FIGURE 5. Phosphorylation of Cbl in human neutrophils following incubation with calyculin. A. The cells were 32P labeled as indicated in Materials and Methods. DMSO (0.1%) or calyculin (10−6 M) was added for the last 30 min of the labeling. Cbl was immunoprecipitated, and the immunoprecipitates were analyzed by SDS-PAGE, transferred to Immobilon PVDF membranes, and the radioactivity levels incorporated into Cbl (autoradiogram) as well as the amount of Cbl (blot anti-Cbl) in the immunoprecipitates was detected by autoradiography and immunoblotting, respectively. B. The cells were incubated with DMSO (0.1%) or calyculin (10−6 M) for 30 min and Cbl was immunoprecipitated under denaturing conditions as described in Materials and Methods. Half of the immunoprecipitates was treated with alkaline phosphatase (10 U/ml) in a buffer containing 10 mM Tris (pH 8.0) for 30 min at 37°C before electrophoresis. After transfer, the PVDF membrane was immunoblotted with anti-Cbl Ab. The data shown are representative of three independent experiments.](http://www.jimmunol.org/)

![FIGURE 6. Effect of calyculin on the mobilization of calcium induced by CD32 cross-linking in human neutrophils. The cells (10^7/ml) were loaded with fura-2 as described in Materials and Methods. Calyculin (10−6 M) was added for the last 20 min, and the F(ab')_2 fragment of Ab IV.3 (2.5 μg/ml) for the last 5 min of fura-2 loading. The cross-linker Ab (anti-F(ab')_2, 25 μg/ml) or HBSS as control was then added (arrow). The data shown are representative of three independent experiments.](http://www.jimmunol.org/)
tyrosine phosphorylation and activation of MAP kinase which correspond to the 40-kDa tyrosine-phosphorylated substrate evident in Fig. 1C after a 30-min incubation with calyculin. Moreover, Haystead et al. (44) showed that okadaic acid activates MAP kinase in adipocytes as well. One explanation proposed by these authors is that PP2A acts as a tyrosine kinase as well as a serine/threonine phosphatase. This interpretation would be consistent with the findings of Cayla et al. (50) that PP2A possesses significant phosphotyrosine phosphatase activity, in vitro, under certain conditions. However, in contrast to the above data, our results indicate that the calyculin-induced tyrosine phosphorylation was rapid and transient. Therefore, the stimulation by calyculin of tyrosine phosphorylation in human neutrophils does not seem to reflect a simple inhibition of tyrosine phosphatases since this would be anticipated to generate time-dependent phosphotyrosine accumulation as we observed for phosphothreonine (Fig. 1A) and phosphoserine (Fig. 1B). Furthermore, the limited number of tyrosine-phosphorylated proteins in response to calyculin also argues against a generalized antiphosphotyrosine phosphatase mechanism of action of this compound.

The inhibition of PP2A and PP1 by calyculin may activate tyrosine kinases, either by increased phosphorylation of serine/threonine residues of the kinases themselves or of regulatory factors associated with their activity. In calyculin-treated neutrophils, detectable serine/threonine phosphorylation was dissociated in time from the tyrosine phosphorylation burst. Therefore, the available data do not provide evidence that the tyrosine phosphorylation peak induced by calyculin is a direct effect of serine/threonine phosphatase inhibition.

Immediately following calyculin addition in suspensions of neutrophils, we observed, within 2 min, the transient tyrosine phosphorylation of Cbl, Syk, and two other unidentified substrates of 85 kDa and 55 kDa. Although the rapidity of this event is characteristic of a response involving a membrane receptor, calyculin has never been shown to interact with such a cellular component. Furthermore, its overall hydrophobicity makes it cell permeant (49). However, we cannot rule out the possibility that calyculin interacts with a membrane component, namely, a tyrosine kinase or phosphatase, thus modifying its activity. The receptor-like tyrosine phosphatase CD45 is a membrane enzyme and it is the most abundant tyrosine phosphatase in neutrophils; it modulates the activity of the src family kinase (51, 52) and appears to have significant constitutive tyrosine phosphatase activity that may serve to repress neutrophil activation (53). The inhibition of this receptor-type tyrosine phosphatase might explain the tyrosine phosphorylation peak observed in response to calyculin. These considerations indicate that further investigations into the potential effects of calyculin and okadaic acid on the activity of CD45 are warranted.

The observation that Cbl and Syk are rapidly tyrosine phosphorylated in response to calyculin is of potential physiological relevance because these two proteins are thought to be intimately involved in neutrophil activation (39, 46, 47). In neutrophils, Cbl is tyrosine phosphorylated in response to a variety of agonists including CD32 cross-linking, opsonized bacteria and zymosan, GM-CSF, monosodium urate, and calcium pyrophosphate microcrystals (46). All of these agonists are known to activate various functions of neutrophils such as calcium mobilization, oxygen consumption, or phagocytosis. The signaling role of Cbl in different cell types has been related to its combination of potential tyrosine phosphorylation sites, proline-rich motifs, and leucine zipper domains (54) which have led to the present characterization of Cbl as an adapter molecule. Cbl has been shown to associate with several signaling proteins (55) including Syk. Cbl has been reported to be a substrate of Syk and to regulate its activity (56–58) but little is known about its role in neutrophils.

Exposure to calyculin for 10 min or longer induced an increase in serine and threonine phosphorylation. This is in accord with the property of this inhibitor. Previous reports, using pulse-chase experiments in electropermeabilized neutrophils, showed that this effect was due, at least in part, to an inhibition of dephosphorylation (32) and also, as already mentioned, to the activation of protein kinases (36). In the latter study, calyculin was found to greatly inhibit the FMLP-induced activities of pK65 and pK63 which belong to the p21-activated kinase (PAK) family of serine/threonine kinases (59, 60) as well as the FMLP-induced tyrosine phosphorylation of unidentified proteins of similar sizes. In response to CD32 cross-linking, we observed an inhibition of the tyrosine phosphorylation pattern when the cells are preincubated with calyculin. This calyculin-dependent inhibition of tyrosine phosphorylation in response to CD32 cross-linking was irreversible since the same effect was observed after a 2-min incubation with calyculin followed by washing of the cells and incubation for 20 min with HBSS before CD32 cross-linking (data not shown). However, the inhibition of the stimulation of tyrosine phosphorylation induced by calyculin was partial and substrate specific. Tyrosine phosphorylation of Cbl was decreased as was that of two unidentified substrates in the 60- to 70-kDa molecular mass range. On the other hand, the stimulated tyrosine phosphorylation of Syk (72 kDa) and of the CD32 itself was not affected by calyculin treatment. The present results thus differ from those of Liang and Huang (37) who observed an enhancement of the tyrosine phosphorylation of proteins in the 120-kDa and 75-kDa molecular mass range in response to CD32 cross-linking following okadaic acid (1 μM) treatment. The reasons for these discrepancies are not known.

CD32 is constitutively expressed on neutrophils. It has a membrane-spanning domain and an immunoreceptor tyrosine-based activation motif-containing cytoplasmic tail which has been shown to play an important signaling role (39). Although the signal transduction pathway in response to the ligation of this receptor is

**FIGURE 7.** Effect of calyculin on the superoxide production induced by CD32 cross-linking in human neutrophils. The cells (10^7/ml) were incubated with calyculin (10^-6M) for 20 min. The F(ab')2 fragment of Ab IV.3 (2.5 μg/ml) was added for the last 5 min and cytochrome c (130 μM) for the last minute of incubation. Superoxide production measurement was then initiated by addition of the cross-linker Ab (anti-F(ab')2, 25 μg/ml) to 1 ml of the cell suspension. The reactions were stopped on ice after 5 min. Means ± SEM of five separate determinations.
poorly understood in neutrophils, the data accumulated in other systems favor a model in which Cbl is a direct downstream target of Syk/Zap70, whereas members of the Src family of tyrosine kinases contribute indirectly to this process by the activation of Syk/Zap70. However, conflicting data argue against this model, and, in this context, the calycin-dependent inhibition of specific tyrosine-phosphorylated substrates, and of Cbl in particular, could help to elucidate the precise role of each of these proteins in this signaling pathway.

Several hypothesis may be proposed to explain the inhibitory effects of calycin on the responses to CD32 ligation (tyrosine phosphorylation and calcium mobilization). First, a serine/threonine phosphatase (PP1 and/or PP2A) may be required early in the CD32-signaling pathway leading to intracellular calcium mobilization and Cbl tyrosine phosphorylation. A similar hypothesis was proposed in macorphages where the addition of calycin results in sustained tyrosine phosphorylation of the MAP kinases and inhibition of LPS-induced expression of several genes (48). This hypothesis implies the involvement, in the CD32-signaling pathway, of at least one serine/threonine-phosphorylated protein, as negative regulator, upstream of Cbl tyrosine phosphorylation and calcium mobilization.

Second, a tyrosine phosphatase may be activated following serine/threonine phosphorylation in response to preincubation with calycin. Two receptor-type tyrosine phosphatases CD45 and CD148 are known to modulate the functional responses to CD32 cross-linking in neutrophils (27, 28). Additionally, the soluble phosphatases SHP-1 and SHP-2 are both present in neutrophils. Phosphorylation of PTPs on serine residues has been described (61, 62), but in only a few cases could these phosphorylation events be correlated with changes in the catalytic activity of the respective phosphatase. Stover and Walsh (63) analyzed the influence of phosphorylation of CD45 activity in vitro and found that sequential phosphorylation on tyrosine and serine residues was necessary for activation. On the other hand, CD148 is constitutively associated with a serine/threonine kinase which may control its activity (64).

Finally, since the modulation of CD32 responses by calycin is correlated with a detectable accumulation of serine/threonine-phosphorylated substrates, and more specifically of Cbl, these two events are likely to be linked. Cbl may be serine/threonine phosphorylated by one of the renaturable kinases activated by calycin (36). Alternatively, Cbl may be a substrate of protein kinase C as suggested by Liu et al. (65). Although in T cells, PMA induces serine/threonine phosphorylation of Cbl and its association with 14-3-3 proteins (66), the addition of PMA (100 nM, 0–30 min) did not alter the electrophoretic mobility of Cbl in neutrophils, and the protein kinase C inhibitor RO 31820 (0.33 mM) did not inhibit the changes in electrophoretic mobility of Cbl induced by calycin (data not shown). This posttranslational modification, illustrated by the electrophoretic shift, might limit the stimulated tyrosine phosphorylation of Cbl which, in turn, is likely to affect the association of Cbl with other proteins. The inhibition of the tyrosine phosphorylation of Cbl would then down-regulate the signaling pathway leading to functional responses to CD32 cross-linking. The inhibition of calcium mobilization illustrated in Fig. 6 argues in favor of this hypothesis.

The calycin-dependent increase of superoxide formation in response to CD32 cross-linking confirms the nontoxic effect of calycin. This increase may be explained by an inhibition of the dephosphorylation of p47phox which plays a critical role in activation and maintenance of superoxide anion generation. There are contradictory results on the role of phosphatases on superoxide formation (67, 68). These reports provide evidence for an heterogeneity in the signaling pathways of different neutrophil receptors. The results of the present investigation uncovered complex and unexpected interrelationships between the serine/threonine and the tyrosine phosphorylation-signaling pathways. They suggest that additional investigations directed at the elucidation, at the molecular levels, of the targets of calycin/okadaic acid are warranted to derive a comprehensive picture of signal transduction in neutrophils and by extension in other cell types.

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References


