Neutral endopeptidase (EC 3.4.24.11, NEP) is an integral membrane protein of human neutrophils. NEP is identical with the common acute lymphoblastic leukemia antigen (CALLA) of leukemic cells. The expression of NEP on the surface of neutrophils is down-regulated by endocytosis which can be induced by phorbol 12-13-acetate (PMA) at 37 °C. The activity of the enzyme on the surface of intact cells decreases by 76% within 5 min. The activity can be recovered, however, if the cells are lysed within 5 min of the endocytosis. After 30 min, only 32% of the NEP activity is present in the neutrophil lysates. The loss of activity is presumably due to proteolytic inactivation. Diacylglycerol and monoclonal antibody to CALLA/NEP also induce internalization of NEP. PMA induces endocytosis even at 4 °C, but NEP is not inactivated at that temperature. The disappearance of NEP activity after adding PMA was inhibited by various agents.

Among the most active were the phospholipase inhibitor 4-bromophenacyl bromide and a combination of the serine protease and cathepsin inhibitors, disopropylfluorophosphate and N-ethylmaleimide. The employment of fluorescent monoclonal antibody confirmed the down-regulation and internalization of NEP antigen on the neutrophils. Since NEP inactivates chemotactic peptides and thereby affects chemotaxis of neutrophils, NEP is on the plasma membrane of intact cells in inflammation.

**Experimental Procedures**

**Materials**

The following reagents, inhibitors, and enzymes were obtained from Sigma: Histopaque 1077, PMA, retinal, adenosine-5' monophosphate, chymostatin, N-ethylmaleimide, 1-oleoyl-2-acetyl-rac-glycerol (analogue of diacylglycerol; DAG), superoxide dismutase, catalase, melittin, and diisopropylfluorophosphate (DFP). Lysolecithin was from Behring Diagnostics. The elastase inhibitor methoxy-Suc-Ala-Ala-Pro-Val-CH$_2$Cl (CK20) and the NEP substrate glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamide (MNA) were from Enzyme Systems Products. Phosphoramidon was from Peninsula Laboratories and Ficoll from Pharmacia LKB Biotechnology Inc. Monoclonal antibody to NEP and fluorescein isothiocyanate (FITC) labeled antibody to CALLA. The other chemicals used were of reagent-grade obtained commercially.

**Methods**

Preparation of Human Neutrophils—Neutrophils were isolated from 40-60 ml of fresh heparinized human blood by centrifugation over Histopaque-1077 cushions (22). Lymphocytes and plasma were removed after centrifugation at 400 X g for 30 min. The resulting
granulocyte/erythrocyte pellets were diluted to twice the original volume with phosphate-buffered saline, pH 7.2, and then treated with 1.5% dextran and left at 4 °C for approximately 1 h to sediment erythrocytes. The supernatant was then centrifuged at 200 × g for 10 min. The cell pellet was resuspended in cold H2O for 30 s to lyse the contaminating erythrocytes, and isotonicity was restored with an equal volume of 7.4% NaCl. The neutrophils were harvested by centrifugation at 200 × g for 10 min and washed twice in phosphate-buffered saline. This procedure yielded approximately 2 × 10^6 cells from 10 ml of whole blood.

**Fractionation of Neutrophils**—Purefied neutrophils, resuspended in 1 ml of resuscitation buffer (100 mM KCl, 3 mM NaCl, 1 mM ATP, 3.5 mM Na2HPO4, 10 mM PIPES, pH 7.3), were lysed by nitrogen cavitation. This was done in a nitrogen bomb at room temperature (22, 23) for 20 min at 350 p.s.i. with constant stirring. The cavitate was collected into EGTA (1.25 mM final), diluted with an equal volume of 0.78 M sucrose, and centrifuged at 800 × g for 15 min at 4 °C to remove unbroken cells and nuclear material. The supernatant (S1) was recentrifuged at 15,000 × g for 20 min at 4 °C to obtain a granular pellet (P2) and a supernatant containing membrane particles (S2). The membrane pellet (P2) was obtained by centrifugation of the S2 fraction at 100,000 × g for 1 h at 4 °C. The P3 fraction was resuspended in 0.34 M sucrose, 0.1 M MES buffer, pH 6.5, and stored at −70 °C until use.

**Immunofluorescent Staining of Cells**—Neutrophils (3 × 10^6/ml) were incubated at 37 °C in a shaking water bath in the presence or absence of PMA (300 ng/ml) in Tyrodes’ solution containing 0.1% bovine serum albumin. At specified times, aliquots of 10^6 cells were withdrawn and added to 10 volumes of ice-cold PBS, pH 7.2, containing 0.1% NaN3. After 10 min the cells were washed once by low speed centrifugation (4 °C, 5 min) and the cell pellet resuspended in 0.2 ml of FITC-labeled anti-CALLA diluted 1:20 in the same buffer. After 30 min on ice, the cells were washed three times as described and fixed with 1% paraformaldehyde for 10 min and examined immediately with an Olympus fluorescent microscope equipped with epillumination optics and a silicon intensification television camera (Dage, Inc., Michigan City, IN). Substitution of FITC-labeled mouse IgG for CALLA was used as a negative control. Image processing was done by a Hughes Aircraft instrument. Both the unprocessed and processed images were recorded with a half inch video cassette recorder. The final images were recorded directly from a high resolution monitor with a 35-mm camera. In order to make all images comparable, the silicon intensification television camera was operated in the manual mode at constant gain and voltage settings. The final image was the real time average of 12 consecutive video frames.

**Enzyme Assays**—The hydrolysis of the MNA by NEP was determined in a recording spectrophotometer (9, 12, 24). The reaction was done in the presence and absence of the specific inhibitor phosphoramidon (1 μM) and only the activity of the reaction inhibited by phosphoramidon was attributed to NEP. With intact cells, this was about 80-90% of the total activity.

**Neutrophils** (3 × 10^6/ml) were suspended in Hank’s balanced salts solution (29), pH 7.4, and divided into aliquots. Samples were incubated for various times at 37 or 4 °C, either on a rotating turntable in an incubator or in a shaking-water bath with PMA and/or the various agents used. At the end of the incubation period, samples were centrifuged in a microcentrifuge for 1 min (16,000 × g). The supernatants were removed and saved, and the pellets were washed in Hanks’ balanced salts solution and resuspended in 250 μl of 0.1 M MES, pH 6.5. Intact or sonicated cells were assayed for NEP activity with 0.04 mM MNA (9, 12).

The activity of the membrane marker 5'-nucleotidase was determined with adenosine-5'-monophosphate substrate by a modification of the reaction described by Gerlach and Hiby (95).

**RESULTS**

**Endocytosis**—Intact neutrophils in a large number of samples cleaved the NEP substrate MNA (0.04 mM) at a rate of 0.8–1.6 nmol/h/10^6 cells. The activity of NEP on the surface of neutrophils was stable for the duration of the experiments when cells were kept in suspension for 30–60 min. The activity on the intact cells, however, was reduced within 5 min to 23.8 ± 3% (±S.E.; n = 9; control = 100%) after adding 300 ng/ml PMA. In 30 min the activity dropped to 18.3 ± 1.7% (±S.E., n = 36; Fig. 1).

**PMA** can activate neutrophils with the subsequent generation or release of potentially noxious agents such as H2O2, superoxide, hydroxyl radicals, hypochlorous acid, and the proteolytic enzymes elastase and cathepsin G (22, 26-32). We therefore tested a variety of agents and enzyme inhibitors to establish whether the profound loss of NEP activity on neutrophils can be attributed to any of the substances generated or released. The decrease in NEP activity by added PMA was not affected when the following agents were incubated with neutrophils individually or in combination, when so indicated: 1) catalase (25 μg/ml); 2) superoxide dismutase (100 μg/ml)+ catalase (25 μg/ml); 3) methionine (5 mM); 4) taurine (5 mM); 5) formyl-Met-Leu-Phe (1 μM) ± cytochalasin B (5 μg/ml); 6) substance P (0.1 mM); 7) CK20 (10 μM); 8) chymostatin (0.1 mM); 9) ethyl alcohol (1%); 10) methyl alcohol (1%); 11) acetylsalicylic acid (0.1 mM); 12) indomethacin (20 μM); 13) H2O2 (0.03–0.3%); 14) lidocaine (0.01–1 mM); 15) bradykinin (0.1 mM); 16) chloroquine (1 mM); 17) leupeptin (50 μM); 18) captopril (0.1 mM); 19) retinal (100 μM); 20) cortisone (2–20 μM).

The reasons for using the above agents follow (19, 27–32): reagents 1 and 2 destroy generated H2O2 and superoxide; 3 and 4 trap hypochlorous acid; 5 and 6 activate the neutrophils; 7 inhibits elastase; 8 inhibits cathepsin G; 9 and 10 scavenge hydroxyl radicals; 11 and 12 inhibit cyclooxygenase; 13 possibly oxidizes NEP directly; 14 acts on the cell membrane; 15 activates phospholipases; 16 inhibits phospholipidase A2; 17 inhibits proteolytic enzymes; 18 inhibits angiotensin converting enzyme and/or activate some SH-dependent enzymes; 19 releases O2; and 20 is anti-inflammatory.

PMA had no effect on the NEP activity when the isolated membrane fraction (P2) was used. Thus, the diminished NEP activity caused by PMA in the intact cells was not due to a direct effect of PMA on NEP. In addition, the loss of NEP activity was not due to release of the enzyme from the cell surface, as NEP activity did not increase in the supernatants obtained from cells treated with PMA.

Since PMA is known to cause endocytosis of receptors (19–21), we tried to recover NEP activity by sonicating the cells. As shown in Fig. 1, although the apparent activity on the cell...
The activity was recovered in the sonicated cells, suggesting sonication. However, after 30 min of incubation, only 32% of the activity was recovered from phosphatidylinositol by phospholipase D. Decreased within 5 min to about 24%, NEP activity was fully recovered at this time point when the cells were lysed by sonication. After 30 min of incubation, only 32% of the activity was recovered in the sonicated cells, suggesting irreversible inactivation of the enzyme after endocytosis.

The effect of DAG (200 μM), an analogue of diacylglycerol which is released from phosphatidylinositol by phospholipase C (19, 32), was similar to that of PMA. In the presence of DAG, 29% of the neutrophil NEP activity remained after 30 min.

When neutrophils were incubated with PMA at 4 °C, NEP activity dropped to 7% of the initial value after 30 min. By lysing the cells with sonication, the activity was completely recovered (128%). Thus, although endocytosis occurred at 4 °C, NEP was not inactivated.

Exposure to PMA also decreased the activity of the plasma membrane marker 5′-nucleotidase in intact neutrophils by 75.8% after 30 min of incubation at 37 °C. This supports the contention that PMA can induce endocytosis of membrane bound enzymes.

Blocking Endocytosis—The effect of PMA on NEP activity was fully or partially blocked by the agents listed in Table I. The table shows the percent activity of NEP retained on the cell membrane of neutrophils after 30 min of incubation at 37 °C; the activity in absence of PMA was taken as 100%. In this series of experiments, PMA reduced NEP activity by 81%. Endocytosis was completely blocked by 4-bromophenacyl bromide, an inhibitor of phospholipase A2 (33), Lyssolecithin or melittin (34–36) partially solubilized NEP from the cell membrane but blocked the internalization of the remaining membrane-bound NEP activity by 90%. Phenybutazone (33) and sodium azide partially protected NEP at the concentrations employed. DFP and N-ethylmaleimide also blocked the disappearance of NEP almost completely, if given in combination. Individually, the two agents were less effective. In 30 min NEP activity on the intact cells decreased to 80 or 43%, when NEM or DFP were incubated with PMA-treated cells alone.

Monoclonal Antibody—Since antibodies to cell surface proteins induce endocytosis in neutrophils, we tested the effect of the J5 monoclonal antibody to NEP (or CALLA; 37) on NEP activity in neutrophils. The antibody did not inhibit NEP activity of the isolated neutrophil membrane fraction (P3) after 30 min at 37 °C or after 18 h of incubation at 4 °C. The J5 antibody (67 μg/ml), however, decreased NEP activity on intact neutrophils to 79.3 ± 4.6% (±S.E., n = 5) of the control value after 5 min and to 61.8 ± 3.2% (n = 5) after 30 min of incubation at 37 °C (Fig. 2). Since the monoclonal antibody did not inhibit NEP directly on the isolated membrane fragments, but decreased the activity on intact cells in a time-dependent manner, we conclude that this loss was also due to endocytosis.

When intact neutrophils were stained with FITC-labeled J5 monoclonal antibody, a uniform fluorescent surface staining pattern was observed (Fig. 3A). Within 5 min after treatment with PMA (300 ng/ml), the distribution of NEP (CALLA) on the surface of neutrophils was patchy and/or capped (Fig. 3B). This pattern presumably was not antibody-induced since the antibody staining procedure was performed later at 4 °C in the presence of sodium azide. After 30 min the cells stained weakly for surface NEP antigen, suggesting that it had been down-regulated (Fig. 3C). The redistribution and apparent internalization of NEP from the cell surface was not observed when cells were incubated with the chemotactic peptide N-formyl-Leu-Leu-Phe-Leu-Tyr-Lys (1 μM; data not shown).

**DISCUSSION**

The presence of NEP on the plasma membrane of neutrophils and cytoplasts has been shown by enzyme assay and by immunohistochemistry using immunogold in transmission (9, 38) and scanning electron microscopy (6). In pilot studies, NEP antigen was also detected in intracellular granules of
The biological importance of the phenomena described may be related to chemotaxis and inflammation. It was reported recently that a chemotactic peptide is rapidly internalized by neutrophils via non-coated pits (43). NEP on the cell membrane effectively cleaves the chemotactic peptide N-formyl-Met-Leu-Phe (12, 13). It was postulated that this peptide has to be cleaved by NEP to induce chemotaxis. Of the neuropeptides that may contribute to the inflammatory syndrome, bradykinin and substance P are also substrates of NEP (3, 4). Substance P is cleaved by intact neutrophils mainly by released cathepsin G but also by NEP, although to a lesser extent. Bradykinin is inactivated only by NEP on these cells (44), thus down-regulation of NEP in this setting may also be important.

These experiments showed that neutrophil activation or antigen-antibody reaction triggers internalization of NEP which can be followed by inactivation. In contrast to many receptors, which are recycled (20, 45), NEP is unlikely to be reactivated if and when it reappears on the cell membrane. Thus this process may negatively affect chemotaxis.

REFERENCES

* E. G. Erdos and R. A. Skidgel, unpublished results.