http://www.jbc.org/cgi/content/full/272/42/26247

Volume 272, Number 42, Issue of October 17, 1997 pp. 26247-26252

Monochloramine Inhibits Phorbol Ester-inducible Neutrophil Respiratory Burst Activation and T Cell Interleukin-2 Receptor Expression by Inhibiting Inducible Protein Kinase C Activity*

(Received for publication, May 1, 1997, and in revised form, August 3, 1997)

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ABSTRACT

Monochloramine derivatives are long lived physiological oxidants produced by neutrophils during the respiratory burst. The effects of chemically prepared monochloramine (NH2Cl) on protein kinase C (PKC) and PKC-mediated cellular responses were studied in elicited rat peritoneal neutrophils and human Jurkat T cells. Neutrophils pretreated with NH2Cl (30-50 μM) showed a marked decrease in the respiratory burst activity induced by phorbol 12-myristate 13-acetate (PMA), which is a potent PKC activator. These cells, however, were viable and showed a complete respiratory burst upon arachidonic acid stimulation, which induces the respiratory burst by a PKC-independent mechanism. The NH2Cl-treated neutrophils

showed a decrease in both PKC activity and PMA-induced phosphorylation of a 47-kDa protein, which corresponds to the cytosolic factor of NADPH oxidase, p47phox. Jurkat T cells pretreated with NH2Cl (20-70 μM) showed a decrease in the expression of the interleukin-2 receptor alpha chain following PMA stimulation. This was also accompanied by a decrease in both PKC activity and nuclear transcription factor-kappa B activation, also without loss of cell viability. These results show that NH2Cl inhibits PKC-mediated cellular responses through inhibition of the inducible PKC activity.

INTRODUCTION

Neutrophils play an important role in the defense against bacterial infections as well as other inflammatory responses. Many different stimuli activate neutrophils, which show a series of responses such as the respiratory burst, cell shape change, aggregation, degranulation, and phospholipid turnover (1). The respiratory burst is a rapid increase in non-mitochondrial oxygen consumption, in which large amounts of superoxide anion (Obardot 2) are produced by the electron transport complex NADPH oxidase (2, 3). Hydrogen peroxide is formed by the dismutation of Obardot 2, and HOCl is formed from H2O2 and Cl- by myeloperoxidase (4, 5). Chloramines are another type of oxidant produced in significant quantities by activated neutrophils (6). They are formed by the non-enzymatic reaction (I) of HOCl with many endogenous amines such as taurine, ammonia, lysine, and the amino termini of polypeptides (6, 7). Taurine is present in neutrophils at 10-20 mM (8).

R-NH2 + HOCL --→ R-NHC1 + H20

REACTION 1

Chloramines are less reactive than HOCl, and membrane-impermeable chloramines are relatively long lived oxidants (9). Chloramines react preferentially with sulfhydryls and thioethers (10), and some chloramines such as monochloramine (NH2Cl) are membrane-permeable (11). Neutrophils in concentrations found in blood (2-3 × 106/ml) may easily produce 100 μM chloramine in a short term culture (12). Moreover, chloramines show distinct biological effects such as inhibition of DNA repair (12), inhibition of the generation of macrophage inflammatory mediators (13), and

detachment of cultured myocytes (14). Recently we showed that membrane-permeable chloramines derived from the respiratory burst are the primary cause of accelerated turnover of glutathione in activated neutrophils (15). Considering these characteristics, chloramines may have signaling functions in inflammation (9).

One of the key enzymes of the NADPH oxidase activation is protein kinase C (PKC)1 (16). PKC was originally reported as a ubiquitous, Ca2+- and phospholipid-dependent serine/threonine kinase, which is activated transiently (17). To date, 12 isoforms of PKC have been reported, and they are classified into three groups, conventional, novel, and atypical, based on their primary structure and cofactor requirement (18, 19). Neutrophil activation by physiological stimuli such as formyl peptide or complement fragment C5a begins with interaction with their specific surface receptors (20). This interaction leads to the activation of phospholipase C, which generates two second messengers, inositol 1,4,5-trisphosphate and 1,2-diacylglycerol. These messengers together lead to the activation of PKC. Among the PKC isoforms, PKCbeta is suggested to be responsible for the neutrophil respiratory burst activation (21, 22). Activated PKC phosphorylates one of the cytosolic components of NADPH oxidase, p47phox (23, 24). The phosphorylated p47phox translocates and interacts with membrane component of NADPH oxidase (25, 26), which results in the assembly of the active enzyme.

The tumor promoter phorbol 12-myristate 13-acetate (PMA) induces a PKC-dependent respiratory burst (1, 26). PMA has a structural similarity with the endogenous PKC agonist diacylglycerol and causes a direct and more prolonged activation of PKC (17). PMA effectively activates neutrophil NADPH oxidase, and the cells produce maximal amounts of superoxide (26). However, this PKC-mediated activation pathway is not the only way of NADPH oxidase activation. Some stimuli such as arachidonic acid (27) or sodium dodecyl sulfate (SDS; 28) also induce a respiratory burst without requiring PKC (29). Arachidonic acid directly interacts with p47phox, changes its conformation, and enables it to assemble active NADPH oxidase (30). Moreover, arachidonic acid-induced respiratory burst in a cell-free reconstituted system is independent of PKC-cofactors (Ca2+ and ATP) and is not inhibited by PKC inhibitor (29). Therefore, arachidonic acid is a PKC-independent inducer of respiratory burst.

PKC also has a pivotal role in the activation of T cells. T cells are stimulated by the interaction of a specific antigen with T cell receptor-CD3 complex. This interaction activates the intracellular kinase cascade (31), one of which is mediated by PKC. Activated kinase cascade activates transcription factors (31), which initiate transcription and expression of a variety of molecules including interleukin-2 (IL-2) and the high affinity IL-2 receptor alpha chain (IL-2Ralpha). Because IL-2 is a major T cell growth factor, the coordinate production of IL-2 and IL-2 receptor is crucial for T cell proliferation and the immune response (32). T cell stimulation with PMA alone is enough for IL-2Ralpha expression (33), and PKCalpha is responsible for this effect (34). Expression of IL-2Ralpha is transcriptionally regulated (35) and involves nuclear transcription factor (NF)-kappa B activation (36). NF-kappa B activation comprises the phosphorylation of its inhibitory protein Ikappa B (37). The activated NF-kappa B translocates from the cytosol to the nucleus (38), where it binds to the kappa B consensus sequence of IL-2Ralpha gene promoter (36). This binding is essential for NF-kappa B-regulated gene expression (39, 40).

In this study we show that PMA-induced PKC activity is inhibited by NH2Cl in neutrophils and Jurkat T cells. This inhibition of PKC activity results in distinct biological effects, notably inhibition of the respiratory burst in neutrophils and inhibition of IL-2Ralpha expression in Jurkat T cells. These results suggest that chloramines may have a physiological function in regions where the respiratory burst is activated.

EXPERIMENTAL PROCEDURES

Materials

PMA, arachidonic acid, and PKCbeta were obtained from Sigma. Dulbecco's phosphate-buffered saline (Ca2+- and Mg2+-free, pH 7.4; PBS) and the PKC assay system were from Life Technologies, Inc. NF-kappa B consensus oligonucleotide was from Promega (Madison, WI). T4 polynucleotide kinase and protein molecular weight markers were from Boehringer Mannheim. Phycoerythrin-labeled anti-human CD25 (IL-2Ralpha) antibody was from Immunotech (Westbrook, ME). Calyculin A was from Calbiochem. [gamma -32P]ATP and [32P]orthophosphoric acid were from NEN

Life Science Products. Rabbit polyclonal antibody to human p47phox (raised against glutathione S-transferase-p47phox fusion protein) was a generous gift from Dr. Bernard M. Babior. All other reagents were of analytic grade or better.

Cell Preparation

Neutrophils

Rat elicited peritoneal neutrophils were prepared from Sprague-Dawley male rats (250-350 g, from Bantin and Kingman, Fremont, CA) as described previously (15, 41). The collected cells contained about 80% neutrophils based on microscopic examination, and the cell viability was more than 95%, which was assessed by the trypan blue exclusion test.

Jurkat T Cells

Jurkat T cell is a cell line of human acute T cell leukemia and was obtained from American Type Culture Collection (clone E6-1, Bethesda, MD). The cell culture medium was RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 1% (w/v) penicillin-streptomycin, 110 mg/liter sodium pyruvate, and 2 mM L-glutamine (from University of California, San Francisco, cell culture facility), and cells were grown in humidified air containing 5% CO2 at 37 °C.

Chloramine Preparation and Measurement

Taurine chloramine and monochloramine (NH2Cl) were prepared as described previously (15, 42). The chloramine concentration was determined by 2-nitro-5-thiobenzoate assay (42).

Pretreatment of the Cells with Chloramine

Neutrophils were suspended in PBS (5 × 106 cells/ml), and NH2Cl or taurine chloramine was added to get the final concentration of 10-70 μM. After the incubation for 10 min at 37 °C, neutrophils were separated from the PBS by centrifugation and used as described below. Jurkat T cells were suspended in the fresh cell culture medium (1 × 106 cells/ml), and NH2Cl was added to 20-70 μM. In contrast to PBS, this medium alone consumed added NH2Cl. After the incubation for 30 min at 37 °C, Jurkat T cells were used

without washing because the medium contained no more detectable chloramine.

Measurement of the Respiratory Burst

Neutrophils were resuspended in the assay mixture (1.25 × 106 cells/ml, 1.1 mM p-hydroxyphenylacetate, 50 μg/ml superoxide dismutase, 50 μg/ml horseradish peroxidase, in PBS), and they were stimulated with either PMA (300 nM, final concentration) or arachidonic acid (100 μM), which was previously dissolved in ethanol at 300 μM and 100 mM, respectively. Preliminary study showed that these concentrations were optimal. The respiratory burst was monitored continuously at 37 °C by H2O2 formation as described previously (15, 43).

Measurements of PKC Activity

PKC activity was measured just after the chloramine exposure using a PKC assay system from Life Technologies, Inc. Partial purification with an anion exchange column was omitted because a preliminary study using crude and partially purified samples showed similar results. Acetylated synthetic peptide from myelin basic protein 4-14 (Ac-Gln-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu) was used for the substrate, which is specific for conventional PKCs (44). Specificity for PKC was established by running the control sample using PKC-specific inhibitor peptide PKC(19-36) (45) for each sample. PKC activity was expressed as pmol of 32P incorporated/min/106 cells.

Phosphorylation of 47-kDa Protein

Phosphorylation of 47-kDa protein in neutrophils was studied as described previously (46), except for the following. Before NH2Cl treatment, neutrophils (1 × 108/ml) were incubated with [32P]orthophosphoric acid (500 μCi/ml) at 30 °C for 30 min in 138 mM NaCl, 2.7 mM KCl, and 7.5 mM D-glucose, adjusted to pH 7.5. After the incubation, neutrophils (5 × 106/ml) were added to 30-70 μM NH2Cl in PBS and incubated for 10 min at 37 °C. Then the cells were stimulated with 300 nM PMA for 90 s. The reaction was stopped by the addition of trichloroacetic acid (10% w/v), centrifuged, and the total precipitated protein was dissolved in SDS-PAGE sample buffer. The pH was adjusted to about 7 by NaOH.

Protein samples were analyzed by SDS-PAGE using 7.5% gel, stained with Coomassie Brilliant Blue R-250, and dried, and autoradiography was performed.

Phosphorylation of the 47-kDa protein was also studied in the cell-free system using exogenous PKCbeta . Cytosolic fractions were prepared from NH2Cl (50 μM)-treated and control neutrophils as described previously (47). Then p47phox was partially purified by passing through a DE52 ion exchange column as described previously (48). Immunoblot analysis showed that virtually all immunoreactive p47phox was recovered from the pass-through fraction. The reaction mixture contained 0.5 mg/ml partially purified protein, 5 units/ml PKCbeta , 0.3%(w/v) Triton X-100 mixed micelles containing 10 μM PMA, and 0.28 mg/ml phosphatidylserine (prepared as described in Ref. 49), 20 mM MgCl2, 1 mM CaCl2, 20 μM ATP (containing 100 μCi/ml [gamma -32P]ATP), and 20 mM Tris-HCl (pH 7.5), incubated at 30 °C for 7 min. The reaction was stopped by the addition of SDS-PAGE sample buffer containing 1 μM staurosporine and 1 mM ATP and analyzed by SDS-PAGE and autoradiography.

Immunoblot analysis of p47phox was performed following SDS-PAGE and Western blotting using anti-p47phox antibody. The optical density of the autoradiogram and immunoblot was measured by a Shimadzu CS-9301 PC densitometer and expressed as percent of positive control.

Measurements of IL-2Ralpha Expression

After 30 min of NH2Cl incubation, Jurkat T cells were stimulated with PMA (100 nM) dissolved in dimethyl sulfoxide. Control cells were treated with the same volume of dimethyl sulfoxide (0.1% v/v) without PMA. The cells were maintained in culture condition for 24 h after PMA treatment. Then the cells were washed with PBS and immunostained with phycoerythrin-labeled anti-human IL-2Ralpha antibody and analyzed at excitation 488 nm and emission 575 nm using EPICS-Elite flow cytometer (Coulter, Miami, FL). Data were collected from gated viable cell populations.

Electrophoretic Mobility Shift Assay for NF-kappa B

Nuclear protein extraction was performed 2 h after PMA stimulation as described previously (50, 51). In some experiments, calyculin A (20 nM) was also used as a stimulant, and nuclear proteins were extracted 30 min after the stimulation. Electrophoretic mobility shift assay was performed as described previously (52, 53).

Statistical Analysis

Results were tabulated for the indicated number of experimental samples. Mean and standard deviations are presented. Group means were compared using Student's t test for unpaired samples with a two-tailed distribution using Microsoft Excel.

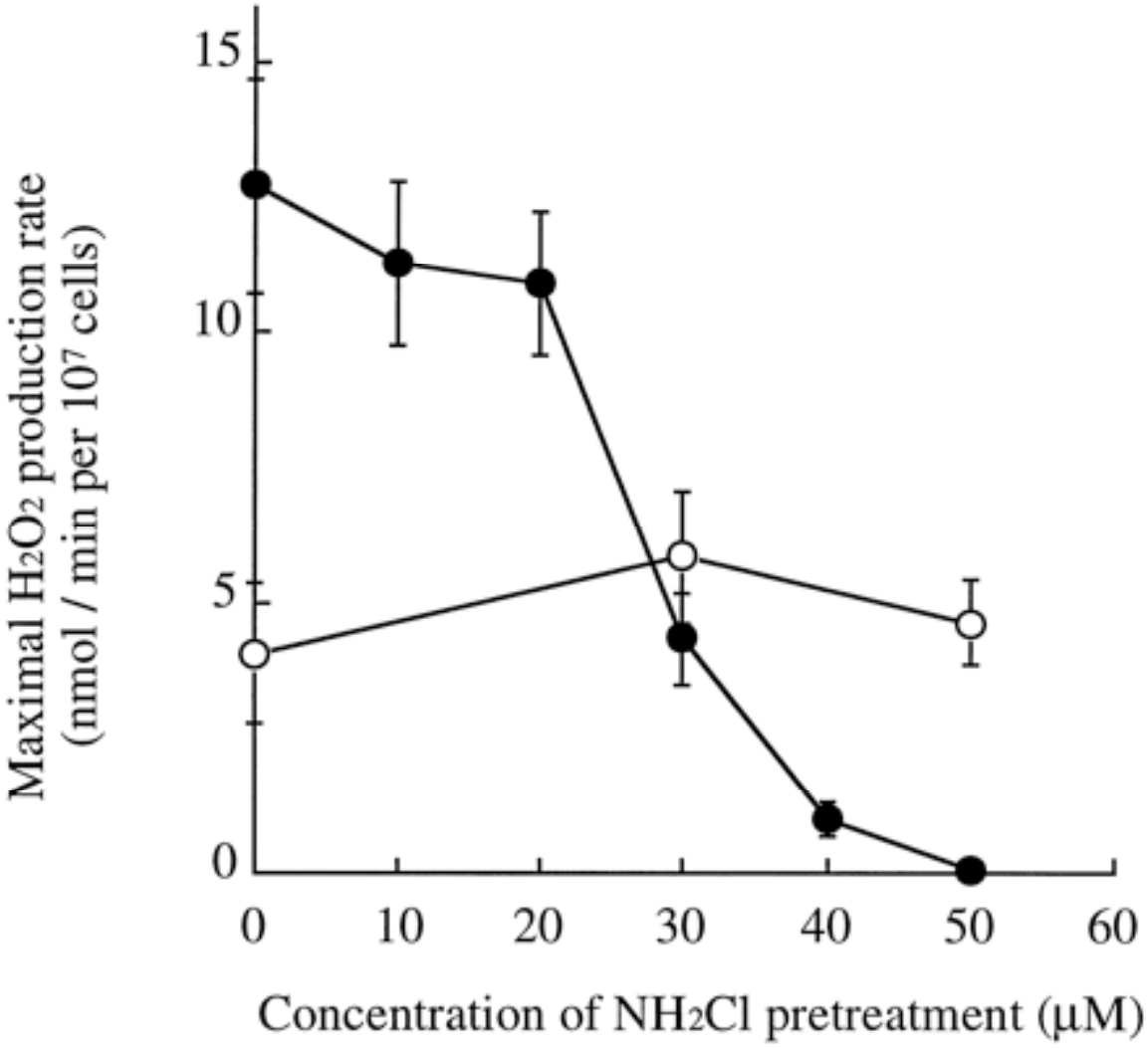
RESULTS

Effects of Chloramine Pretreatment on the Neutrophil Respiratory Burst

Under resting conditions, rat peritoneal neutrophils showed no detectable respiratory burst, as measured by H2O2 production. When cells were stimulated with PMA, which is a potent PKC activator, they showed a marked respiratory burst, whose rate was maximal for several minutes (15). The PMA-induced respiratory burst was inhibited significantly at 30 μM NH2Cl pretreatment, and it was abolished at 50 μM NH2Cl pretreatment (Fig. 1). In contrast, the neutrophils pretreated with 30-50 μM NH2Cl showed a complete respiratory burst when stimulated with arachidonic acid, which is a PKC-independent stimulant. In the control cells, the maximal rate of H2O2 production by arachidonic acid stimulation was lower than that which occurred by PMA stimulation (Fig. 1). This is not surprising because different stimuli cause a different degree of respiratory burst even at optimal conditions (26). Membrane-impermeable taurine chloramine at 50 μM had no inhibitory effects on the PMA-induced respiratory burst. At a higher NH2Cl concentration (70 μM) the arachidonic acid-induced respiratory burst was also inhibited (data not shown).

Fig. 1. Effects of NH2Cl pretreatment on the neutrophil respiratory burst activity induced by PMA or by arachidonic acid. Neutrophils (5 × 106 cells/ml) were pretreated with the indicated concentrations of NH2Cl for 10 min at 37 °C, and the cells were collected by centrifugation. Then the cells were resuspended in the assay mixture and stimulated with PMA (300 nM) or with arachidonic acid

(100 μM). The maximal rate of H2O2 production was measured. bullet , PMA-stimulated respiratory burst (significantly decreased from the 0 μM samples at 30 μM and higher; $p < 0.05$)). open circle , arachidonic acid-stimulated respiratory burst (no significant difference from the 0 μM samples; $p < 0.05$). Results are the mean ± S.D. for four determinations:



To assess whether the respiratory burst suppression by NH2Cl was caused by neutrophil death, cell viability was evaluated by trypan blue exclusion (Table I). Control cells showed 96% viability. Neutrophils treated with up to 50 μM NH2Cl showed more than 95% viability, not significantly different from the controls. Therefore, the suppression of PMA-induced respiratory burst by NH2Cl was not caused by neutrophil death.

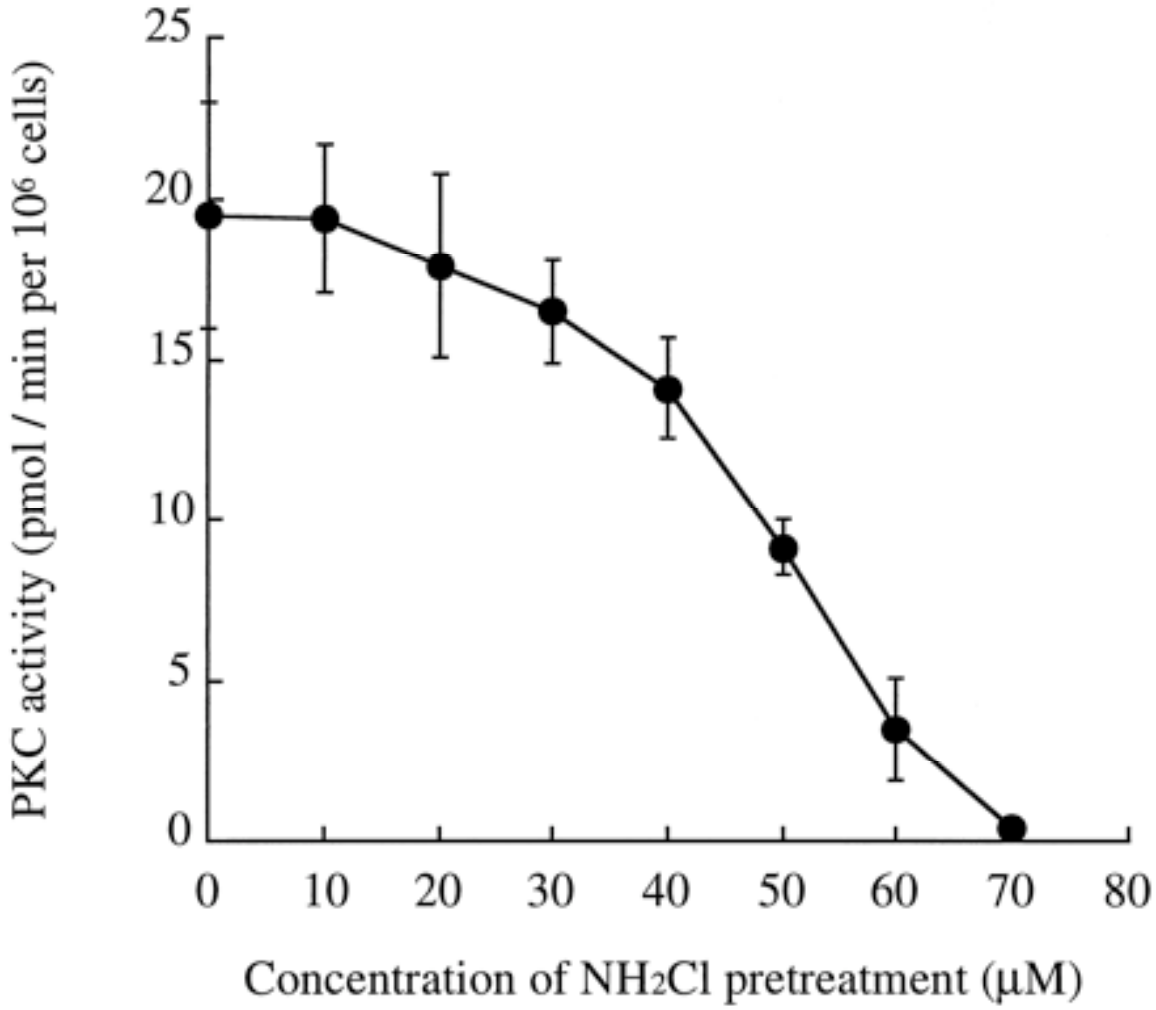
Table I. Cell viability after NH2Cl treatment
Neutrophils (5 × 106 cells/ml) were incubated with the indicated concentrations of NH2Cl for 10 min at 37 °C in PBS. Jurkat T cells (1 × 106 cells/ml) were incubated with the indicated concentrations of NH2Cl for 30 min at 37 °C in a fresh cell culture medium. Cell viability was studied by the trypan blue exclusion test. Mean ± S.D. for four determinations are shown. *Significantly different from the 0 μM samples ($p < 0.05$).

	Cell Viability	
NH2Cl concentration	Neutrophils	Jurkat T cells
μM	%	
0	96 ± 1	96 ± 2
20	95 ± 2	97 ± 1
30	96 ± 1	97 ± 1
50	95 ± 1	96 ± 2
70	92 ± 1*	97 ± 1

PKC Activity in NH2Cl-pretreated Neutrophils

Because PMA induces a respiratory burst by a PKC-dependent mechanism, we measured the inducible PKC activity in the NH2Cl-pretreated neutrophils. The inducible PKC activity decreased in the NH2Cl-pretreated neutrophils (Fig. 2). Neutrophils treated with 50 μM NH2Cl resulted in the PKC activity being decreased to 47% of the control cells under maximal activation. Taurine chloramine at 50 μM showed no inhibition of the respiratory burst and no inhibitory effects on PKC.

Fig. 2. Changes in the PKC activity in NH2Cl-treated neutrophils. Neutrophils (5 × 106 cells/ml) were treated with the indicated concentrations of NH2Cl for 10 min at 37 °C, and the cells were collected by centrifugation. PKC was extracted and measured as described under "Experimental Procedures" (significantly decreased from the 0 μM samples at 40 μM and higher; $p < 0.05$). Results are the mean ± S.D. for four determinations:

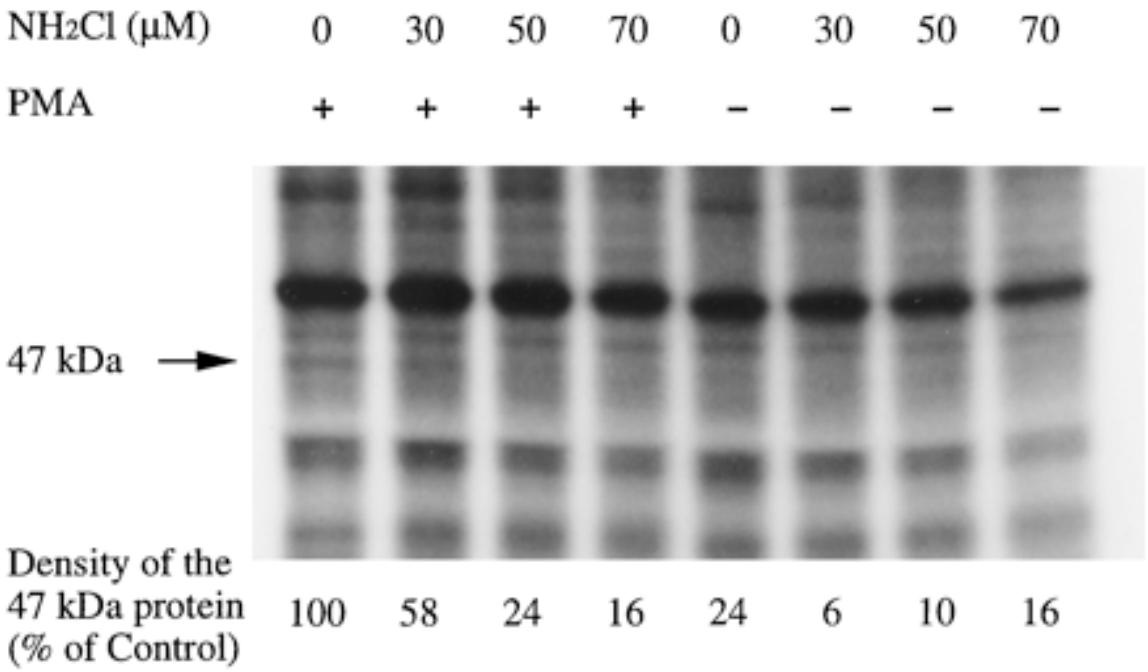


Phosphorylation of the 47-kDa Protein in NH2Cl-pretreated Neutrophils

PKC-catalyzed phosphorylation of p47phox is essential for the PMA-induced respiratory burst (54). The phosphorylation was assessed by detecting the 32P incorporation into 47-kDa protein in the whole cell system. Anti-human p47phox antibody reacted with a protein at this position. Although resting neutrophils did not show an apparent 32P incorporation into 47-kDa protein, PMA stimulation of the control cells resulted in a distinct 32P incorporation (Fig. 3), and this is consistent with similar studies with guinea pig neutrophils (46). Monochloramine-pretreated neutrophils showed a decrease in the phosphorylation of 47-kDa protein upon PMA stimulation. Pretreatment with 50 μM NH2Cl resulted in marked decrease in 32P incorporation (Fig. 3). Neutrophils pretreated with 30 μM NH2Cl showed a lower

phosphorylation of 47-kDa protein than control cells (Fig. 3), despite the statistically insignificant decrease in PKC activity (Fig. 2). These results were consistent with the significant decrease in respiratory burst activity in these cells (Fig. 1).

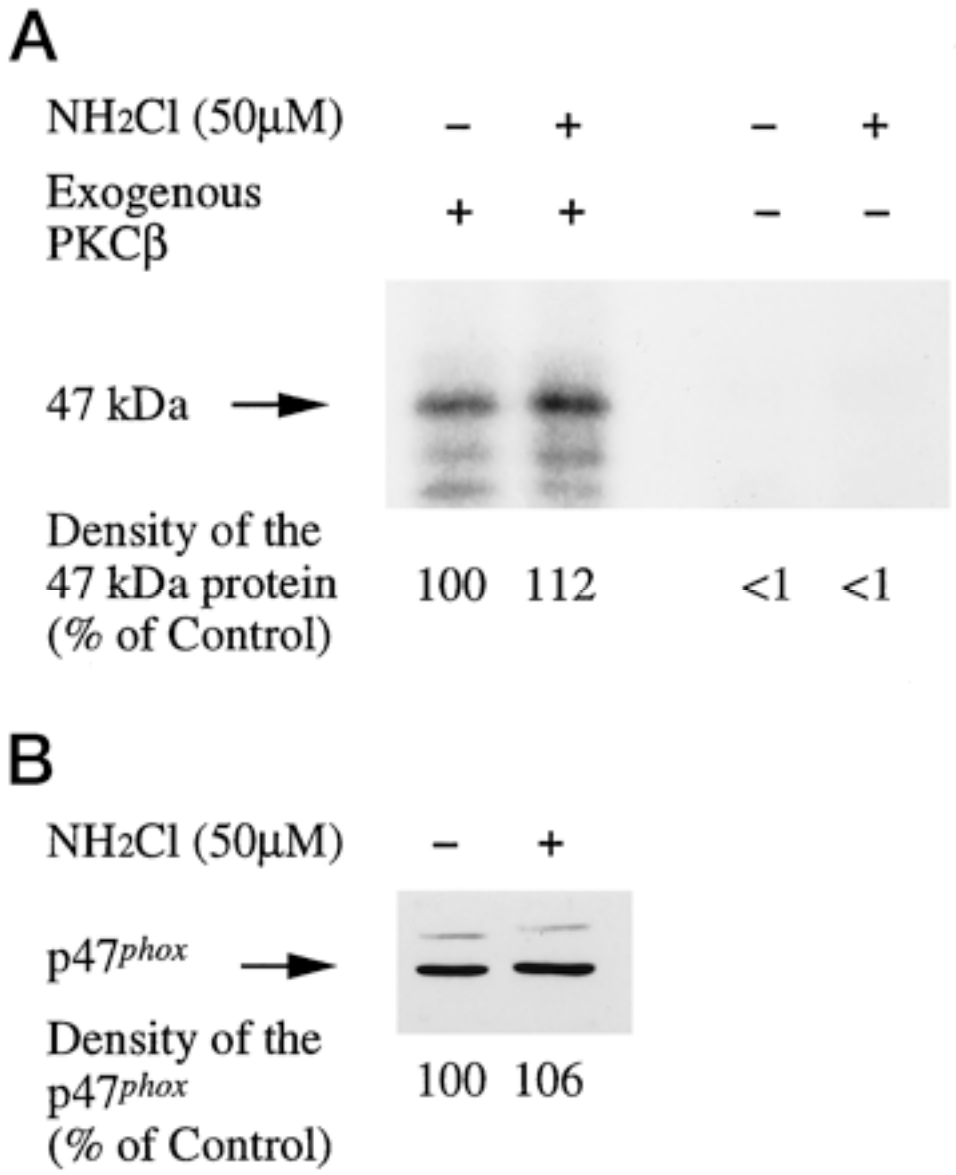
Fig. 3. Phosphorylation of the 47-kDa protein in NH2Cl-pretreated neutrophils. Neutrophils were prelabeled with [32P]orthophosphoric acid and then treated with NH2Cl. Cells were then stimulated with PMA (300 nM) for 90 s, and the reaction was stopped by trichloroacetic acid (10% w/v). Protein samples were analyzed by SDS-PAGE and autoradiography. Results are representative data from four independent experiments:



The phosphorylation of the 47-kDa protein was also studied in a cell-free system using exogenous PKCbeta , to study if NH2Cl damaged p47phox so that it could not be phosphorylated by PKC. The 47-kDa protein from NH2Cl (50 μM)-treated and the control cells were equally well phosphorylated by exogenous PKCbeta (Fig. 4A). Immunoblot analysis showed that these two samples contained comparable amounts of immunoreactive p47phox (Fig. 4B).

Fig. 4. Exogenous PKCbeta -catalyzed phosphorylation of the 47-kDa protein in a cell-free system. Panel A, P47phox was partially purified from the control and NH2Cl (50 μM)-treated neutrophils.

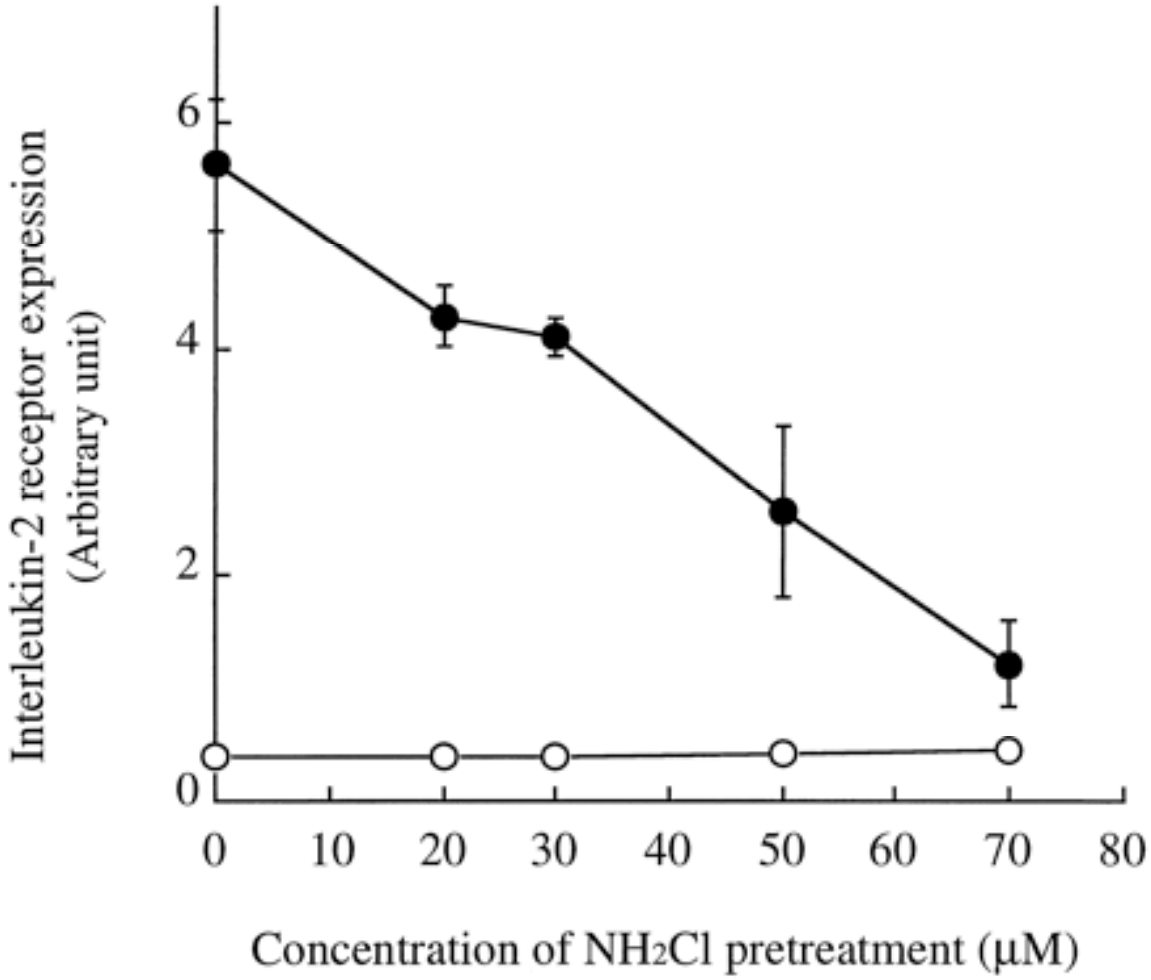
Then it was phosphorylated by exogenously added PKCbeta . The incorporation of 32P into 47-kDa protein was analyzed by SDS-PAGE and autoradiography. Panel B, immunoblot analysis. Partially purified p47phox samples were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunostained with anti-human p47phox antibody. Results are representative data from four independent experiments.



Suppression of IL-2Ralpha Expression in NH2Cl-pretreated Jurkat T Cells

To study whether the effect of NH2Cl on PKC is limited to neutrophils or whether it is a more general phenomenon involving other cell types, we studied the effect of NH2Cl on the PMA-induced IL-2Ralpha expression in Jurkat T cells. Unstimulated Jurkat T cells showed almost no IL-2Ralpha expression, but 24 h after PMA stimulation IL-2Ralpha expression was markedly enhanced. Monochloramine pretreatment (20-70 μM) decreased the PMA-induced IL-2Ralpha expression (Fig. 5). The NH2Cl pretreatment did not alter cell viability even 24 h after PMA stimulation (data not shown).

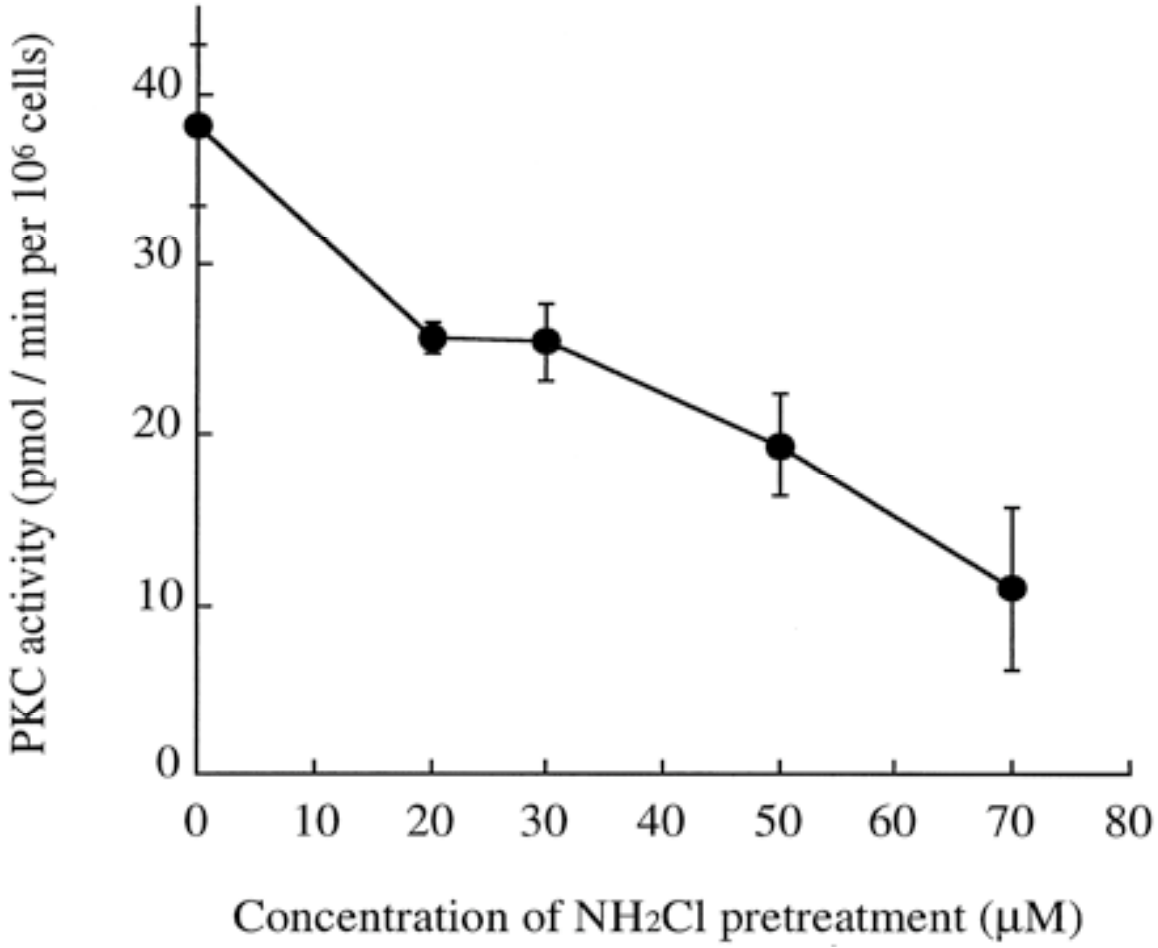
Fig. 5. Down-regulation of PMA-induced IL-2Ralpha expression by NH2Cl pretreatment in Jurkat T cells. Jurkat T cells were treated with NH2Cl for 30 min at 37 °C in a CO2 incubator. Then the cells were stimulated with PMA (100 nM) and allowed to express IL-2Ralpha for 24 h. The cells were collected and immunostained with phycoerythrin-labeled anti-IL-2Ralpha antibody and analyzed by flow cytometer. bullet , PMA-stimulated cells (significantly decreased from the 0 μM samples at 20 μM and higher; $p < 0.05$). open circle , cells without PMA stimulation. Results are the mean ± S.D. for three determinations:



PKC Activity in NH2Cl-treated Jurkat T Cells

To correlate the IL-2Ralpha expression with the inhibition of PKC activation, we measured the inducible PKC activity in NH2Cl-treated Jurkat T cells. Fig. 6 demonstrates that 20-70 μM NH2Cl inhibited inducible PKC activity in Jurkat T cells. Because the cell viability did not change (Table I), this inhibition of PKC activation was not the result of cell death.

Fig. 6. Changes in the PKC activity in NH2Cl-treated Jurkat T cells. Jurkat T cells (1 × 106 cells/ml) were treated with NH2Cl for 30 min at 37 °C, and the cells were collected by centrifugation. PKC was extracted and measured as described under "Experimental Procedures" (significantly decreased from the 0 μM samples at 20 μM and higher; $p < 0.05$). Results are the mean ± S.D. for four determinations:

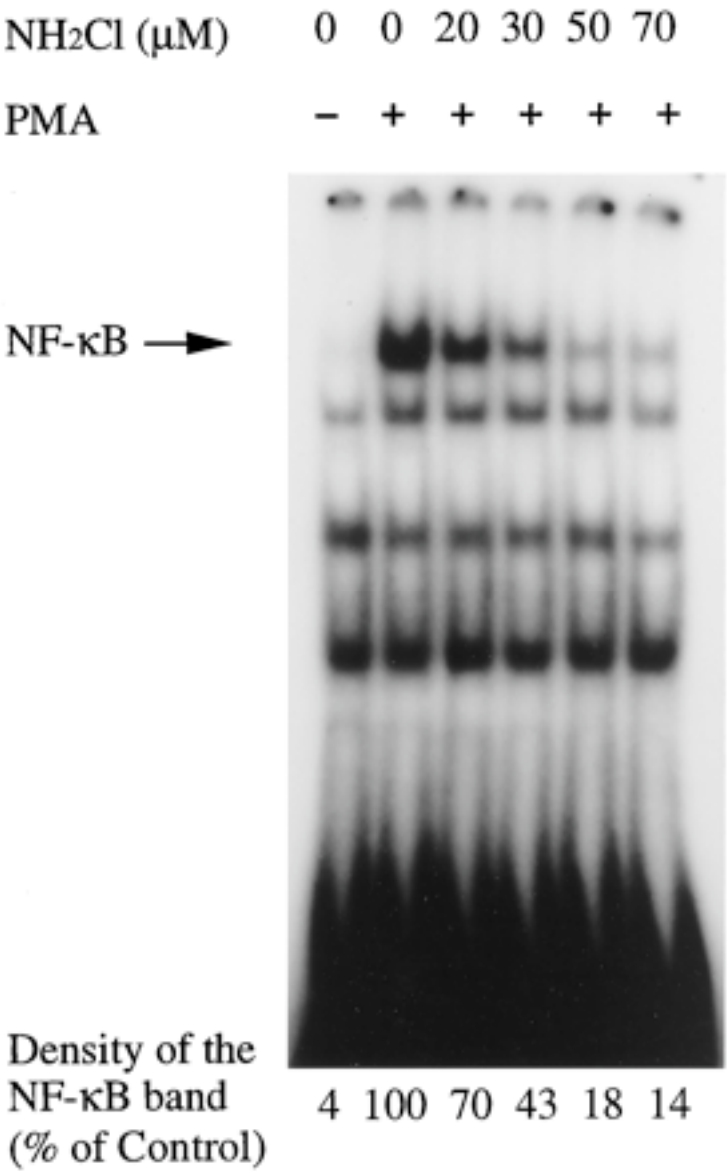


NF-kappa B Activation

The PMA-induced IL-2Ralpha expression is a multistep response involving kinase cascade activation, NF-kappa B activation, mRNA synthesis, and protein synthesis (35, 38). We studied NF-kappa B activation to see if the inhibited PKC activity fails to activate the step after PKC activation. NF-kappa B activation precedes IL-2Ralpha expression in Jurkat T cells (36). Consistent with previous reports (40, 55), PMA induced marked activation of NF-kappa B in Jurkat T cells. Pretreatment of Jurkat T cells with NH2Cl inhibited PMA-induced NF-kappa B activation (Fig. 7). The inhibition of IL-2Ralpha expression, NF-kappa B activation, and PKC activation by NH2Cl all occurred at similar (20-70 μM) NH2Cl concentrations (Figs. 5, 6, 7).

Fig. 7. Inhibition of PMA-induced NF-kappa B activation by NH2Cl pretreatment in Jurkat T cells. Jurkat T cells in fresh medium (1 ×

106 cells/ml) were treated with NH2Cl at 37 °C for 30 min. Then the cells were stimulated with PMA (100 nM) and incubated for 2 h before nuclear protein extraction. Results are representative data from four independent experiments:



To study if NH2Cl directly damaged NF-kappa B protein in a way that NF-kappa B cannot be activated, the NH2Cl (50 μM)-pretreated Jurkat T cells were stimulated with calyculin A, another inducer of NF-kappa B. Calyculin A treatment efficiently induced NF-kappa B activation in both the NH2Cl-pretreated and the control cells (data not shown).

DISCUSSION

When neutrophils were pretreated with 50 μM NH2Cl, the PKC-dependent stimulant PMA could not induce respiratory burst, yet the PKC-independent stimulant arachidonic acid induced a complete respiratory burst. This finding strongly suggests that the target of NH2Cl was PKC and not NADPH oxidase. Indeed, NH2Cl treatment resulted in a decrease in the inducible PKC activity in neutrophils. Membrane-impermeable taurine chloramine at 50 μM had no inhibitory effect on either the respiratory burst or the PKC activity. This suggests that the target for chloramine was not located on the outer cell membrane. PKC activity is one of the rate-limiting factors of the respiratory burst activity. It has been reported that the neutrophil respiratory burst correlates directly with the intracellular level of PKC (56). Moreover, PKC inhibitors such as staurosporine and H-7 inhibit both phosphorylation of 47-kDa protein and superoxide production in a dose-dependent manner (57). Therefore, the inhibition of inducible PKC activity appears to be the cause of inhibition of PMA-induced respiratory burst in NH2Cl-pretreated cells.

Neutrophils pretreated with 50 μM NH2Cl showed virtually no respiratory burst upon PMA stimulation but still possessed 47% of their PKC activity. Phosphorylation of p47phox is a requirement of PMA-stimulated respiratory burst activation (54). We measured the phosphorylation of protein of 47-kDa molecular mass. Antibodies against human p47phox reacted with a protein at this position. After a 50 μM NH2Cl pretreatment, neutrophils showed a marked decrease in the phosphorylation of the 47-kDa protein, despite the remaining PKC activity. This result suggests that the phosphorylation of p47phox was decreased in the NH2Cl-treated neutrophils, which results in the inhibition of the respiratory burst. We further studied if NH2Cl directly damaged p47phox so that it could not be phosphorylated by PKC. In a cell-free system, exogenously added PKCbeta phosphorylated equally well the 47-kDa protein from the control and NH2Cl-treated neutrophils. Both samples contained comparable amounts of immunoreactive p47phox. This result suggests that even after NH2Cl (50 μM) treatment, p47phox can be phosphorylated when catalyzed by intact PKCbeta . Therefore, the decrease in the phosphorylation of the 47-kDa protein appears to be caused by the decrease in the PKC activity in NH2Cl-treated neutrophils.

One explanation for the discrepancy between the 47-kDa protein phosphorylation and the remaining PKC activity is that neutrophils contain phosphoprotein phosphatases that reverse the action of PKC (58, 59). During the PMA-stimulated respiratory burst, p47phox undergoes a continual cycle of phosphorylation and dephosphorylation (60). Under optimally stimulated conditions the phosphorylation reaction by PKC predominates, which maintains the p47phox in a phosphorylated state (60) and keeps the NADPH oxidase in the active state. Therefore, if PKC activity decreases, the dephosphorylation reaction by phosphatase may predominate, and p47phox cannot be maintained in a sufficiently phosphorylated state. Another possible explanation is that NH2Cl may alter the substrate specificity of PKC. In this case, the PKC-catalyzed phosphorylation of p47phox may be impaired more severely than that of myelin basic protein peptide. This possibility should be studied further. In any case, it is likely that the remaining PKC activity was insufficient to keep the p47phox phosphorylated enough to allow assembly of an active NADPH oxidase. This would result in the observed substantial inhibition of the respiratory burst activity.

Monochloramine-induced suppression of inducible PKC activity was not restricted to neutrophils. At 20 μM, NH2Cl inhibited the PMA-induced IL-2Ralpha expression, PKC activation, and the NF-kappa B activation in Jurkat T cells. Although there are many steps that could result in the inhibition of IL-2Ralpha expression, we measured NF-kappa B activation because NF-kappa B is activated by the phosphorylation of its inhibitory protein Ikappa B (37), and NF-kappa B activation precedes IL-2Ralpha gene expression in Jurkat T cells (38). The inhibition of PKC activation and NF-kappa B activation occurred at similar concentrations of NH2Cl, which suggests that the inhibited PKC activity failed to activate NF-kappa B. We studied further if the decrease in NF-kappa B activation was the result of direct damage of NF-kappa B protein caused by NH2Cl. Calyculin A is a potent inhibitor of phosphoprotein phosphatase types 1 and 2A, and it activates NF-kappa B through Ikappa B phosphorylation and degradation in Jurkat T cells (61). Monochloramine treatment (50 μM) did not affect the calyculin A-induced NF-kappa B activation. This result showed that NF-kappa B in NH2Cl-treated Jurkat T cells was still inducible and suggests that NF-kappa B protein was not a direct target of NH2Cl. Because the

NF-kappa B activation is reported to regulate IL-2Ralpha gene expression in Jurkat T cells (38), inhibition of PKC activation by NH2Cl seems to be the primary cause of the inhibition of IL-2Ralpha expression.

Among the PKC isoforms, at least conventional types of PKCs (alpha , beta I, beta II, and gamma) are likely to be the target of NH2Cl. In the neutrophils, PKCbeta appears to be responsible for NADPH oxidase activation because PKCbeta migrates to the membrane fraction upon stimulation (21) and is able to phosphorylate p47phox (22). Although T cells contain both PKCalpha and PKCbeta as well as other PKCs (31), PKCalpha has been suggested to be responsible for IL-2Ralpha expression (34). We used a synthetic myelin basic protein peptide as a substrate for the measurement of PKC activity. This peptide is a good substrate for conventional PKCs but not for novel or atypical PKCs (44). However, it is yet to be resolved whether chloramines also affect novel and/or atypical PKCs.

During the respiratory burst, a variety of chloramine derivatives are detected in the extracellular medium (7). These chloramines are mostly hydrophilic, low molecular weight, mono-N-chloramine derivatives (7) and not likely to be membrane-permeable. However, membrane-permeable chloramines are also likely to be produced because normal human plasma contains 20-40 μM NH4+(62). When 2-3 × 106/ml of neutrophils, which is comparable to the concentration in blood, are activated with PMA, the chloramine concentration reaches as high as 100 μM in a short term culture (12). As the neutrophil concentration can be much higher in inflammatory sites, our chloramine concentration (20-70 μM) may well be physiologically attainable. Membrane-impermeable chloramines are long lived with an apparent half-life of about 18 h in vitro (9). These chloramines can migrate from their origin to the adjacent or even distant tissues. Moreover, these chloramines react with NH4+ to yield the membrane-permeable NH2Cl (7). The plasma NH4+ concentration increases substantially in some human conditions such as liver failure (63). Such conditions may be favorable for NH2Cl production.

We showed in this work that NH2Cl inhibited PKC-dependent activation both in neutrophils and Jurkat T cells. PKC, functionally impaired by chloramine, was not sufficiently activated upon PMA

stimulation, resulting in suppression of PKC-mediated cellular responses. Considering the central importance of PKC in cellular signaling pathways, respiratory burst-derived chloramines may function as signal modulating molecules in regions where neutrophils exhibit respiratory burst.

FOOTNOTES

* This work was supported by National Institutes of Health Grant GM27345.The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; IL-2, interleukin-2; IL-2Ralpha , interleukin-2 receptor alpha chain; NF-kappa B, nuclear factor kappa B; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

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