Characterization of Calcineurin in Human Neutrophils

INHIBITORY EFFECT OF HYDROGEN PEROXIDE ON ITS ENZYME ACTIVITY AND ON NF-κB DNA BINDING*

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We describe here a novel calcineurin activity in neutrophil lysates, which is dependent on Ca2+, inhibited by trifluoperazine, and insensitive to okadaic acid. Immunoblotting experiments using a specific anti-serum recognized both the A and B chains of calcineurin. Neutrophils treated with cyclosporin A or FK 506 showed a dose-dependent inhibition of calcineurin activity. The effect of oxidant compounds on calcineurin activity was also investigated. Neutrophils treated with hydrogen peroxide (H2O2), where catalase was inhibited with aminotriazole, exhibited a specific inhibition of calcineurin activity. However, the addition of reducing agents to neutrophil extracts partially reversed the inhibition caused by H2O2. A similar inhibitory effect of H2O2 on calcineurin activity was observed to occur in isolated lymphocytes. This is the first demonstration that redox agents modulate calcineurin activity in a cellular system. In addition, electrophoretic mobility shift assays revealed that lipopolysaccharide-induced activation of NF-κB in human neutrophils is inhibited by cell pretreatment with H2O2 in a dose-dependent manner. These data indicate that calcineurin activity regulates the functional activity of lipopolysaccharide-induced NF-κB/Rel proteins in human neutrophils. These data indicate a role of peroxides in the modulation of calcineurin activity and that the H2O2-dependent NF-κB inactivation in neutrophils occurs in concert with inhibition of calcineurin.

Calcineurin (CN)† has recently been established as a key enzyme in the signal transduction cascade leading to T cell activation (1–4), and an important regulator of transcription factors such as NF-AT, NF-κB, and AP-1, which are involved in the expression of a number of important T cell early genes, i.e. interleukin-2, tumor necrosis factor-α, and interleukin-2R (5–8). CN, also known as phosphatase 2B, is a calcium/calmodulin-dependent serine/threonine phosphatase (9–11) and is composed of the following two subunits: a 59-kDa catalytic subunit (CNA), which contains a calmodulin-binding domain and an autoinhibitory region, and a 19-kDa intrinsic calcium-binding regulatory subunit (CNB) (12–14). Human CN possesses a Fe-Zn active center. The assignment of stoichiometric amounts of Zn2+ and Fe3+ in the CNA center is based on atomic absorption experiments (15). The same assignments were made for the di-metal site in the structure of some CNAs (16). The central role of CN in T cell signaling was appreciated by its identification as the target of the immunosuppressive drugs cyclosporin A (CsA) and FK 506 (1–4). The phosphatase activity of CN is inhibited by either drug when complexed to intracellular binding proteins (immunophilins), i.e. CsA to cyclophilin and FK 506 to the FK 506-binding protein 12 (FKBP12), respectively. Neither drug nor immunophilin alone bind to or affect the activity of CN (1). This phosphatase is expressed ubiquitously in eukaryotic cells. In mammals, CN is most abundant in the brain (17) but has also been detected in T cells (1–4). On the other hand, it is known that NF-AT-mediated transactivation depends on the CN activity (18, 19). Other findings suggest that NF-κB activity is also under CN control (20–22).

In neutrophils, only indirect evidence has been presented on the occurrence of the phosphatase CN. The treatment of these cells with inhibitors of CN (e.g. CsA and FK 506) inhibited the neutrophils chemokinesis on vitronectin matrix (23, 24). Furthermore, intracellular calcium and CN regulate neutrophil motility on vitronectin through a receptor identified by antibodies against the integrins αv and β3 (25, 26). The first purpose of the present work was to assess the presence of CN in neutrophils using as a substrate a specific peptide corresponding to the phosphorylation site of the RII subunit of cyclic AMP-dependent protein kinase.

Additionally, reactive oxygen intermediates (ROI) have been implicated in mediating signal transduction by a variety of stimuli in lymphoid cells, and transcription factors seem to be responsible for the inducible expression of a number of genes in response to oxidative stress (27, 28). In this context, the addition of H2O2 to the culture medium has been shown to activate NF-κB (29). Hydroxyl radicals produced from H2O2 cannot function as diffusible intracellular messengers, since they can react with the nearest molecule in a nonspecific fashion. A drofluorescein diacetate; EMSA, electrophoretic mobility shift assay; LPS, lipopolysaccharide.

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† The abbreviations used are: CN, calcineurin; NF-AT, nuclear factor of activated T cells; NF-κB, nuclear factor κB; CsA, cyclosporin A; CNA, calcineurin A subunit; CNB, calcineurin B subunit; ROI, reactive oxygen intermediates; H2O2, hydrogen peroxide; pVifh, sodium oxodiperoxid 1,10-phenanthroline/veranavate(V); HBSS, Hanks’ balanced salt solution; BSA, bovine serum albumin; DTT, dithiothreitol; PMSE, phenylmethylsulfonyl fluoride; PDTC, pyrrolidine dithiocarbamate; AMT, 3-amino-1,2,4-triazole; PAGE, polyacrylamide gel electrophoresis; DFP, diisopropyl fluorophosphate; H2DCFDA, 2',7'-dichlorodihydro-
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more suitable ROI messenger would be the less reactive H₂O₂. However, arguments against ROI involvement in NF-κB activation have been published (30–32), and despite the fact that phorbol 12-myristate 13-acetate-dependent NF-κB stimulation is cancelled by antioxidants, it has been recently shown that phorbol 12-myristate 13-acetate does not increase intracellular ROI (33). Peroxide-mediated stimulation of NF-κB appears to be cell line-specific, since N-acetylcysteine, an antioxidant, elicited up-regulation of NF-κB binding activity in monocoyte-derived macrophages (34). Moreover, NF-κB is not the only nuclear factor whose activity is altered by H₂O₂. In a fashion similar to that described for NF-κB, antioxidant treatment does not prevent the activation of NF-κB by pV(phyn). Therefore, we have also used pV(phyn) as another tool to study the effect of oxidative stress on calcineurin activity.

In summary, previous data suggest that, first, CN modulates the DNA binding activity of essential transcription factors (e.g., NF-AT and NF-κB), and second, ROI regulates positively or negatively those transcription factors. However, a link between both signals, that is CN and ROI, is lacking. In this paper we address this question and the implications of the ROI as universal messenger to activate NF-κB.

EXPERIMENTAL PROCEDURES

Cell and Reagents—Neutrophils were isolated from fresh heparinized blood of healthy human donors by dextran sedimentation, followed by Ficol-Paque gradient centrifugation and hypotonic lysis of residual erythrocytes as indicated (38). Neutrophils were washed twice in Hanks’ balanced salt solution (HBSS), suspended at a density of 1 × 10⁷ cells/ml in HBSS supplemented with 0.1 mg/ml BSA, and maintained at 37 °C for 1–2 h. Peripheral blood lymphocytes were obtained from heparinized venous blood of normal volunteers by Ficol-Paque centrifugation.

Dextran T-500 was obtained from Pharmacia Biotech (Barcelona, Spain). Ficol-Paque, HBSS, and RPMI 1640 were obtained from Bio-Whittaker (Verviers, Belgium). Ca²⁺ and FK 506 were kindly provided by Dr. S. F. Borel (Sandoz Ltd., Basel, Switzerland) and Fujisawa GmbH (München, Germany), respectively. Chemicals were of analytical grade from Merck (Darmstadt, Germany). Dithiothreitol (DTT) and phenylmethanesulfonyl fluoride (PMSF) were obtained from Boehringer Mannheim (Barcelona, Spain). Bovine serum albumin (BSA), okadaic acid, trifluoroacetic acid, sodium pyruvate, and pyridyl diithiocarbamate (PDTC), hydrogen peroxide (30% v/v), 3-amino-1,2,4-triazole (amino triazole, AMT), soybean trypsin inhibitor, leupeptin, aprotinin, Nonidet P-40, and goat anti-rabbit IgG conjugated to horseradish peroxidase were purchased from Sigma (Madrid, Spain). Rabbit anti-bovine calcineurin IgG was kindly provided by C. B. Kee. The synthetic peptide used as a substrate for calcineurin was purchased from Peninsula Laboratories (Belmont, CA). pV(phyn) was synthesized as described previously (37). [γ³²P]ATP was obtained from New England Life Sciences. 2-Mercaptoethanol, SDS, acrylamide, N,N’-methylene-bisacrylamide, Coomassie Brilliant Blue R-250, and blotting nitrocellulose membranes were purchased from Bio-Rad. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and 4-iodophenol were freshly dissolved in 100 mM phosphate buffer, pH 7.0, containing 5% trichloroacetic acid. The reaction mixture was passaged through a 500-μl column of activated Dowex cation-exchange resin, and free inorganic phosphate was quantitated in the eluate by scintillation counting. It was verified that at 15 min of incubation time the assay was linear. Assays were performed in triplicate, and the counts/min measured in blank assay lacking cell lysate were subtracted. Data are expressed as the number of picomoles of [³²P]PO₄ released in 15 min per mg of protein.

2-P-Labeled Phosphopeptides—The synthetic peptide (Asp-Leu-Asp-Val-Arg-Gly-Arg-Val-Val-Val-Ala-Ala-Val) corresponding to a segment of the RII subunit of CAMP-dependent kinase (41), was phosphorylated on the unique serine residue by the catalytic subunit of cAMP-dependent protein kinase using [γ³²P]ATP, essentially as described (40), and used as phosphatase substrate. The specific activity of fresh preparations of [³²P]labeled phosphopeptide was about 500 μCi/μmol peptide.

Calcineurin Phosphatase Assay—Cells (7 × 10⁶ cells/ml) were lysed for 30 min on ice in 100 μl of buffer B (50 mM Tris, pH 7.5, 0.1 mM EGTA, 1 mM EDTA, 0.5 mM dithiothreitol, 50 μg/ml PMSF, 50 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and disrupted by sonication. Cell debris was removed by centrifugation at 4 °C for 10 min at 12,000 × g, and supernatant was used as the source of CN.

The specific activity of freshly prepared CN phosphatase activity was measured using an assay adapted from Hubbard and Klee (42), basically as described (22). Neutrophils (7 × 10⁶ cells/ml) were incubated for 2 h at 37 °C in the presence or absence of drugs as indicated in the text. Reaction mixtures containing 2 μM [³²P]labeled phosphopeptide, 500 mM sodium acetate (added to inhibit PP-IA and PP-2A type phosphatase activities), and 20 μl of cell lysate (about 80 μg of protein) were incubated in a total volume of 60 μl of assay buffer C (20 mM Tris, pH 8.6, 100 mM NaCl, 6 mM MgCl₂, 0.5 mM dithiothreitol, and 0.1 mM CaCl₂, or 5 mM EDTA, as indicated in the figures) for 15 min at 30 °C. After this time, reactions were terminated by the addition of 0.5 ml of 100 mM potassium phosphate buffer, pH 7.0, containing 5% trichloroacetic acid.

The reaction mixture was passed through a 500-μl column of activated Dowex cation-exchange resin, and free inorganic phosphate was quantitated in the eluate by scintillation counting. It was verified that at 15 min of incubation time the assay was linear. Assays were performed in triplicate, and the counts/min measured in blank assay lacking cell lysate were subtracted. Data are expressed as the number of picomoles of [³²P]PO₄ released in 15 min per mg of protein.

Western Blot Analysis—Cells (7 × 10⁶ cells/ml) were lysed for 30 min on ice in 100 μl of buffer A (see above). Lysates were clarified by centrifugation at 4 °C for 2 min at 12,000 × g. Protein concentrations in the lysates were determined by the Bradford method (42), using BSA as a standard. For the Western blot analysis of CN subunits A and B, neutrophil lysates were subjected to 12.5% SDS-PAGE followed by electrophoretic blotting onto nitrocellulose using the Bio-Rad Mini-blotting apparatus. Filters were blocked for 1 h in TBS (150 mM NaCl, 50 mM Tris-HCl, pH 7.5) containing 3% BSA. Furthermore, they were rinsed twice with TBS containing 0.1% Tween 20 (TBST), and they were incubated overnight with rabbit anti-bovine calcineurin IgG diluted 1:1000 in TBST. After three washes in TBST, the filters were incubated for 90 min with horseradish peroxidase-conjugated goat anti-rabbit IgG (1,5000 dilution). Filters were then washed twice with TBS and then twice with TBST for 10 min each time. The bound secondary antibody was detected by enhanced chemiluminescence (43). Briefly, the membranes were incubated for 1 min in 1 ml of fresh luminescent reagent solution, composed of 10 ml Tris-HCl, pH 8.5, 2.25 ml luminol, 0.015% (v/v) H₂O₂, and 0.45 ml 4-iodophenol, the latter acting as an enhancer of the chemiluminescence reaction (44). The concentrations of luminol, H₂O₂, and 4-iodophenol were determined to be optimal for maximum light production. Luminol and 4-iodophenol were freshly prepared in 10 ml of 10 mM Tris-HCl, pH 8.5. Luminol was previously dissolved in 50 μl of 1 M NaOH. The use of Me₂SO as solvent should be...
Calcineurin Is Present in Neutrophil Lysates—CN is a well characterized phosphatase that plays an important role in T cell activation pathways (1–4). In this work we have characterized the presence of CN activity and immunoreactive CN protein in human neutrophils. In crude neutrophil lysates, specific CN activity was observed, and a linear appearance of product during the assay was obtained in the range of 20–75 μg of lysate proteins (Fig. 1A). The time course of CN activity is shown in Fig. 1B. Clearly, 32P release increased linearly along assay time until 15 min, and then a slow activity was found. Fig. 1 (inset) illustrates an immunoblotting analysis of CN expression in lymphocytes (lane 1) and different amounts of human neutrophil lysates (lanes 2–4). We used an antiserum that recognizes both the A (59 kDa) and B (19 kDa) chains of CN, confirming that both subunits are expressed in human neutrophils. As shown, neutrophil CNB migrates as a 16-kDa band in SDS-polyacrylamide gels, and it is detected along with a predominant CNA band migrating at 59 kDa. A third band, detected at 57 kDa in lymphocytes extracts, is probably a proteolytic fragment of CNA generated during preparation of the cell lysates. In some preparations of neutrophil lysates, a similar band of 55–57 kDa was also found (data not shown). Next experiments were addressed to analyze the regulation of CN activity in crude neutrophil lysates. Fig. 2 illustrates that the dephosphorylation of the CN-specific substrate peptide by crude neutrophil lysates was Ca2+-dependent, as well as insensitive to okadaic acid, a potent and specific inhibitor of phosphatases 1A and 2A (reviewed in Ref. 47). When 500 nM okadaic acid was included in the assays, nearly all of the remaining phosphatase activity was Ca2+-dependent and could be eliminated by substituting 5 mM EGTA for Ca2+ (Fig. 2). In contrast, the okadaic acid-sensitive component was resistant to EGTA, which is consistent with the reported Ca2+-independence of phosphatases 1A and 2A (17). CN activity was abrogated in nominally calcium-free medium and in the presence of a known inhibitor of calmodulin, trifluoperazine (48). However, trifluoperazine did not inhibit calcium-independent, okadaic acid-sensitive phosphatases from neutrophil lysates (Fig. 2).
as picomoles of phosphate released per min per mg of protein. Three experiments were performed with similar results.

Together, these data indicate that a specific Ca$^{2+}$/calmodulin-dependent phosphatase activity is present in the neutrophil lysates.

Inhibition of Calcineurin Activity in Isolated Neutrophils Treated with Immunosuppressive Drugs—As previously indicated (1–4), CsA and FK 506 can now be used as tools to elucidate the participation of CN on signal transduction processes. To assess whether treatment with these drugs inhibits CN activity, neutrophils were incubated with different concentrations of FK 506 and CsA for 2 h, and phosphatase activity was measured in cell lysates. Both agents effectively inhibited Ca$^{2+}$-dependent phosphatase activity, as shown in Fig. 3. These results indirectly suggested that the drug-sensitive phosphatase present in neutrophils is CN. Furthermore, in drug titration experiments both FK 506 and CsA inhibited CN activity in a concentration-dependent fashion. IC$_{50}$ values determined for CN inhibition were approximately 0.5 ng/ml for FK 506 and 5 ng/ml for CsA. This greater sensitivity to FK 506 than to CsA exhibited by neutrophil CN is similar to that previously described for lymphocyte CN (1–4).

Effect of Oxidants on Calcineurin Activity—As previously indicated, there is a clear relationship between the oxidative stress and activation or suppression of transcription factor activity (27–29). Since it has been shown that H$_2$O$_2$ suppresses the transcriptional activation of NF-AT (35) and that NF-AT is able to directly interact with CN (18, 19), we have explored whether H$_2$O$_2$ could alter CN activity. Fig. 4 illustrates that preincubation of neutrophils with H$_2$O$_2$ alone had no effect on this phosphatase. However, the preincubation of cells with AMT, an inhibitor of catalase, for 30 min and the further addition of H$_2$O$_2$ produced a clear decrease in CN activity. Together, these data suggest that the inhibition of CN by H$_2$O$_2$ in a neutrophil cell-free system may be reversed by the further addition of Fe$^{2+}$, DTT, or ascorbate, all of which act as reductants. A
previous report on the protective effect of these agents was documented in a partially purified CN assay activity from brain (50). Thus, our results are more consistent with a reversible modification of the catalytic center of CN elicited by H$_2$O$_2$. H$_2$O$_2$ rapidly diffuses away from and into cells. In order to analyze the ability of neutrophils to internalize exogenous H$_2$O$_2$, intracellular oxidant levels were monitored by measuring the fluorescence of H$_2$DCFDA, a highly fluorescent probe sensitive to peroxides (51). Cells labeled with H$_2$DCFDA displayed a significant increase in DCF fluorescence upon incubation with H$_2$O$_2$ ($154.5 \pm 3.2$ fluorescence units in 100 $\mu$m H$_2$O$_2$-treated cells versus $12.7 \pm 1.1$ units in control cells). In other experiments, the treatment of neutrophils with a variety of physiological and pharmacological stimuli revealed that agents that promoted an increase of intracellular H$_2$O$_2$ also induced a dose-dependent inhibition of CN activity in human neutrophils. Notably, both tumor necrosis factor-\(\alpha\) (50 ng/ml) and interferon-\(\gamma\) (50 units/ml) elicited an inhibition of CN activity of about 25% after 2 h of treatment. Increased times of tumor necrosis factor-\(\alpha\) treatment (e.g. 8 h) resulted in an enhanced inhibitory effect (of about 35%) on CN activity. However, LPS (100 ng/ml), platelet-activating factor, or glucocorticoids were without effect on CN activity (data not shown).

To investigate whether the H$_2$O$_2$ effect was specific, we also analyzed the CN activity with a new peroxovanadium compound, pV(phen), which causes intracellular oxidative stress and induces strong protein tyrosine phosphorylation (37). The treatment of neutrophils with pV(phen) caused a dose- and time-dependent inhibition of CN activity (Fig. 7). After 60 min of incubation of the cells with 50 $\mu$m pV(phen), an inhibition of about 60% was observed, with an apparent IC$_{50}$ of about 30 $\mu$m. Therefore, we provide evidence that the observed inhibition of CN by H$_2$O$_2$ could be the result of oxidative stress. Under these conditions (e.g. in the presence of H$_2$O$_2$ and pV(phen)), the cell viability was determined to be about 90%, and hence significant cytotoxic effects can be ruled out. Also, a strong
increase in the intracellular phosphotyrosine levels was detected in neutrophil lysates after incubation of the cells with PV(phen),\textsuperscript{2} in agreement with previous reports on B lymphocytes (37).

EMSA analyses were carried out in order to test whether the changes in CN activity promoted by H\textsubscript{2}O\textsubscript{2} were accompanied by an alteration of the DNA binding activity of two transcription factors, namely NF-AT and NF-\textk{B}. No binding activity was detected on neutrophil nuclear extracts when the NF-\textk{B} probe was used. However, activated NF-\textk{B} was detectable as a uniquely positioned band in assays of nuclear extracts from human neutrophils stimulated with LPS (1 \textmu g/ml) (Fig. 8). On the basis of the results from McDonald et al. (46) and of supershift assays using anti-p50 and anti-p65 antibodies (data not shown), we interpreted the upper band as corresponding to the activated form of NF-\textk{B} (i.e. the p50/p65 tetramer). Gel retardation analysis of extracts from neutrophils stimulated with LPS and different doses of H\textsubscript{2}O\textsubscript{2} demonstrated that H\textsubscript{2}O\textsubscript{2} selectively inhibits the activation of NF-\textk{B}, resulting in a gradual decrease of the intensity of the p50/p65 band, as H\textsubscript{2}O\textsubscript{2} was increased. As a negative control, we also analyzed the effect of CsA (1 \textmu g/ml) (lane 1). In agreement with previous data from other cell lines (1–4), the p50/p65 band was only barely detectable in extracts from CsA/LPS-treated neutrophils. These results are consistent with an NF-\textk{B} activity regulated by CN in neutrophils, in agreement with previous reports (20–22). Present data concerning H\textsubscript{2}O\textsubscript{2} inhibition of CN in neutrophils and lymphocytes suggest that previous evidence on H\textsubscript{2}O\textsubscript{2}-stimulated NF-\textk{B} activity (27, 29) can be interpreted as the result that peroxides, above a threshold level, are able to bypass the CN modulatory step and regulate transcription factors activity independently of CN.

**DISCUSSION**

Only a few reports concerning CN in neutrophils and presenting indirect evidence of its presence in these cells have been published (23–26). All of them deal with neutrophil motility on vitronectin and its inhibition by CsA and FK 506 (23–26), a couple of well known inhibitors of CN activity. Here we have analyzed accurately, using a synthetic peptide corresponding to the phosphorylation site of the RII subunit of cyclic AMP-dependent protein kinase, the presence of CN in human neutrophils. Lysates from neutrophils dephosphorylate this substrate in a dose- and time-dependent manner. CN activity in neutrophils is insensitive to okadaic acid, whereas other phosphatases (e.g. 1A and 2A) are sensitive to this inhibitor (47). In the presence of okadaic acid the CN activity from neutrophil lysates is both Ca\textsuperscript{2+}-dependent and Ca\textsuperscript{2+}-sensitive to inhibition by trifluoperazine, a calmodulin inhibitor. In addition, we present evidence that two well known immunosuppressants and inhibitors of CN activity in lymphocytes, CsA and FK 506 (1–4), potently depressed CN activity in neutrophils. It is well accepted that CN plays an important role as a prominent component of the calcium signaling pathway in T cells, by acting as an obligatory step between immunosuppressive drugs (e.g. CsA and FK 506) and some transcription factors (e.g. NF-AT and NF-\textk{B}) (1–4, 20, 21). The mechanism by which CN activates NF-\textk{B} seems rather complex. It has been shown that the coexpression in transfected cells of the activated CN and activated p21ras could mimic T cell receptor signaling during NF-AT induction, both acting as cooperative partners during T cell activation (52). Also, recent data show that CN forms a complex with cytosolic NF-AT4 (an isoform of NF-AT), which is transported to the nucleus where CN continues to dephosphorylate NF-AT4 (53). On the other hand the important role of ROI in the regulation of some transcription factors, mainly...
NF-κB, is stressed by its activation in response to the addition of H$_2$O$_2$ (29), although doubts that it represents a universal phenomenon has been raised (30–32, 34). Moreover, an inverse relationship between ROI and NF-AT has also been pointed out, since low levels of H$_2$O$_2$ can actively suppress the transcription activity of NF-AT and the expression of interleukin-2 mRNA (35). These studies indicate that CN is a key component of the T cell signal transduction cascade and that oxidative signals can positively or negatively regulate transcription factor activity (54, 55). However, a deep knowledge of the molecular mechanism connecting both components (i.e. CN and oxidative signals) is lacking. We present here evidence for the first time that human neutrophils treated with H$_2$O$_2$ or pV(phen) exhibit a suppression of CN activity and that H$_2$O$_2$ effect required the previous inhibition of catalase activity. Only when catalase was inhibited by AMT a clear decrease of CN activity in the presence of exogenous H$_2$O$_2$ was observed. As expected, we have observed that the preincubation of neutrophils with the antioxidant PDTC cancelled the inhibition of CN activity by H$_2$O$_2$. As a preliminary effort toward the elucidation of the functional consequences of H$_2$O$_2$-dependent CN inhibition, we focused our attention on the transcription factor NF-AT as a potential target. However, we were unable to find any NF-AT DNA binding activity in nuclear extracts from neutrophils (data not shown). This fact closely agrees with previous results from immunoblotting analysis pointing out the absence of NF-AT proteins in neutrophils (56). Subsequent experiments were thus addressed to analyze whether H$_2$O$_2$-dependent CN inactivation could affect NF-κB activation. Conflicting results have been published on the presence or absence of NF-κB in human neutrophils. Browning et al. (57) did not observe any NF-κB activation in these cells. However, Cassatella and co-workers (46) described the presence of NF-κB subunits as well as the existence of NF-κB DNA binding activity in human neutrophils. We have followed the methodology described by the latter authors, with minor modifications, and have detected NF-κB DNA binding activity in fresh human neutrophils, together with its inhibition by H$_2$O$_2$. These data indicate that NF-κB activation is modulated by CN activity in human neutrophils.

Indirect evidence that CN is an enzyme sensitive to its redox environment has been reported, based on the fact that superoxide dismutase protects CN from spontaneous inactivation in brain crude extracts (50). This inactivation was interpreted as resulting from oxidative damage of the Fe-Zn active center of CN (50). Since there is a good evidence that CN is an Fe-Zn-containing enzyme (16), the hypothetical mechanism that can be proposed for this oxidative damage is that H$_2$O$_2$ and pV-(phen) could modify the redox state of the Fe-Zn center in the catalytic site and thereby inactivate the enzyme. This mechanism is in agreement with the observation that the reactivation of H$_2$O$_2$-inhibited neutrophil CN requires the addition of reducing agents, such as Fe$_{2+}$, ascorbate, and DTT (Fig. 6), as it was also previously demonstrated for a preparation of CN from brain (50). The observed inhibition of CN in intact neutrophils by oxidants, such as H$_2$O$_2$ and pV(phen), represents a novel mechanism of action for these agents. The pV(phen) molecule presents a dual activity, acting both as an intracellular oxidant and as an inhibitor of phosphotyrosine phosphatase (37). Evidence also has been presented that the activities of both protein tyrosine phosphatase and protein phosphatase 2A were reduced after H$_2$O$_2$ treatment of intact Jurkat T cells (58). Previously it has been described that CN has a regulatory phosphorylation site that is phosphorylated by the Ca$_{2+}$-independent form of calmodulin-kinase II. This phosphorylated CN exhibits a 50% decrease in its $V_{\text{max}}$ and 2-fold increase in the $K_v$ values (59). Thus, a hypothetical model in which oxidants modulate CN activity through phosphorylation of its regulatory site can therefore be postulated in the light of present data.

An apparent discrepancy between CN inhibition by H$_2$O$_2$ and other oxidant species, described here, and the protection exerted on CN by superoxide dismutase (50), which converts anion superoxide on H$_2$O$_2$, may be raised. In this context, however, the role of catalase and peroxidases, as detoxicant enzymes that degrade H$_2$O$_2$, should be introduced. These enzymes are also mutually protective, and therefore synergistic, when both O$_2^-$ and H$_2$O$_2$ are being made. In fact, experimental evidence (Fig. 4) illustrates the absence of effect by exogenous H$_2$O$_2$ added alone and the requirement of AMT to inhibit intracellular catalase and to detect H$_2$O$_2$-dependent CN inactivation. These data indirectly provide the notion of catalase as an additional protecting enzyme for CN against ROI inactivation. The implication of this suggestion is that, under normal physiological conditions, the cellular CN may be relatively less susceptible to molecular oxidative damage by ROI. Conversely, in pathological process, such as inflammation and reperfusion injury, or situations characterized by an inhibition of ROI-detoxicant enzymes, the CN inactivation by oxidant species may take place, and it could be a relevant process in the context of the oxidative stress state.

In summary, available evidence indicates that CN activity is a redox-sensitive step in cellular signaling cascades and suggests that the inhibition of NF-AT from lymphocytes (35) and NF-κB from neutrophils (present work) by H$_2$O$_2$ is elicited through inactivation of CN.

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