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The 47-kDa protein involved in the NADPH:O_2 oxidoreductase activity of human neutrophils is phosphorylated by cyclic AMP-dependent protein kinase without induction of a respiratory burst

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When human neutrophilic granulocytes are stimulated with chemoattractants or phorbol esters, these cells respond with a so-called respiratory burst: such stimuli induce the activation of a NADPH:O_2 oxidoreductase, which converts oxygen into superoxide. This activation coincides with the phosphorylation of a number of proteins, amongst which a 47-kDa phosphoprotein. Neutrophils from patients with the autosomal form of chronic granulomatous disease (CGD) fail to mount a respiratory burst and concomitantly lack phosphorylation of the 47-kDa protein. We have shown this protein to be a substrate for protein kinase C. In the present paper we describe the phosphorylation of the 47-kDa phosphoprotein by cyclic AMP-dependent protein kinase. For these studies, we used neutrophil cytoplasts, i.e., neutrophils devoid of nucleus and granules, but with an intact NADPH:O_2 oxidoreductase. Addition of dibutyryl cyclic AMP (Bt_2cAMP) to intact human neutrophil cytoplasts resulted in an increase in protein phosphorylation. Among the phosphorylated proteins is a 47-kDa phosphoprotein. Increased protein phosphorylation was also observed upon addition of Bt_2cAMP to neutrophil cytoplast lysates. In lysates of neutrophil cytoplasts from patients with the autosomal form of CGD, phosphorylation of the 47-kDa protein was absent. This finding (confirmed by analysis on two-dimensional gels) indicates that the 47-kDa phosphoprotein, relevant for the NADPH:O_2 oxidoreductase, is a substrate for the cAMP-dependent protein kinase. Unlike phorbol ester-induced phosphorylation, Bt_2cAMP-induced phosphorylation is not accompanied by initiation of a respiratory burst. This observation demonstrates that 47-kDa phosphoprotein phosphorylation can be uncoupled from respiratory burst activity and indicates that other modifications of the NADPH:O_2 oxidoreductase are required for induction of activity.

Abbreviations: BSA, bovine serum albumin; CGD, chronic granulomatous disease; cAMP, cyclic 3′,5′-adenosine monophosphate; Bt_2cAMP, dibutyryl cyclic 3′,5′-adenosine monophosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,-*N*′,*N*′-tetraacetic acid; fMLP, formylmethionylleucylphenylalanine; Hepes, 4-(-2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecylsulfate; PBS, phosphate-buffered saline SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

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Introduction

Neutrophilic granulocytes play a major role in the host defense against micro-organisms [1,2] and have been implicated in inflammatory events [2]. Upon activation by serum-opsonized particles, neutrophils release lysosomal enzymes [1] and show a 20- to 30-fold increase in oxygen consumption (the respiratory burst). The NADPH:O_2 oxidoreductase responsible for the respiratory burst activity is not sensitive to inhibitors of the mitochondrial respiration [3]. The oxygen-derived products of this enzyme (i.e., superoxide and hydrogen peroxide [4,5]) give rise to the formation of highly bactericidal agents. The importance of this defense mechanism is shown by the observation that a complete absence of respiratory burst activity results in an incapacity to kill certain bacteria and fungi [6,7]. This defect is the molecular basis for chronic granulomatous disease (CGD), in which the absence of NADPH:O_2 oxidoreductase activity is correlated with recurrent bacterial and fungal infections.

Not only serum-opsonized particles, but also soluble stimuli, such as the chemoattractant formylmethionylleucylphenylalanine (fMLP) [8] and the tumor promoter phorbol 12-myristate 13-acetate (PMA) [9] are able to induce a respiratory burst in neutrophils. PMA acts directly on the phospholipid-dependent protein kinase C [10]. For this reason, and because all stimuli seem to induce diacylglycerol [11–13] formation (the natural activator of protein kinase C), it is thought that protein kinase C activation is instrumental in the generation of the respiratory burst. Remarkably, the chemoattractant fMLP also induces increased levels of cyclic AMP (cAMP) [14], a second messenger that seems to have an inhibitory effect on the activation of the NADPH:O_2 oxidoreductase [14]. Increased levels of cAMP are probably the result of an inhibitory action of the fMLP-induced increase in intracellular free Ca^{2+} on the cAMP phosphodiesterase [15]. It has been suggested that the elevation of intracellular cAMP shortly after the addition of the fMLP stimulus affects neutrophil responses mainly by inhibiting the fMLP-induced phosphoinositide cycle and by reducing the amount of diacylglycerol produced [16].

One of the substrates of protein kinase C that has been shown to be important in the functioning of the NADPH:O_2 oxidoreductase is a 47-kDa phosphoprotein [17]. A defect in this 47-kDa protein, as monitored by the absence of its phosphorylation in activated neutrophils of autosomal-CGD patients, correlates with an absence of respiratory burst activity, both in intact cells [17,18] and in a cell-free reconstitution system [19]. Because a sustained rise in cAMP is an inhibitory signal for respiratory burst activity induced by fMLP, it is of interest to know whether the cAMP-dependent kinase, apart from its effect on phosphoinositide metabolism, also affects 47-kDa protein phosphorylation. To study this question, we used neutrophil cytoplasts (granulocytes devoid of granules and nucleus) that have intact NADPH:O_2 oxidoreductase but lack many proteins that are irrelevant for the activation or the functioning of the NADPH:O_2 oxidoreductase [20]. We show that the cAMP-dependent kinase indeed phosphorylates the 47-kDa protein in intact neutrophil cytoplasts and in a cell-free system. Cytoplasts from autosomal-CGD patients show a decreased level of phosphorylation. Our results indicate that the 47-kDa phosphoprotein functionally associated with the respiratory burst enzyme is a substrate of cAMP-dependent protein kinase. The resulting phosphorylation of the 47-kDa protein, however, is not accompanied by initiation of the respiratory burst.

Materials and Methods

Bovine serum albumin, dibutyryl 3′,5′-cyclic AMP, cAMP-dependent kinase, phenylmethylsulfonylfluoride and acetyl-L-leucyl-L-leucyl-L-arginine (leupeptin) were obtained from Sigma, St. Louis, MO, U.S.A. [^{32}P]Orthophosphate (7.4 GBq/mmol) and [γ-^{32}P]ATP (11 TBq/mmol) were purchased from the Amersham International, Amersham, U.K. ATP was from Boehringer, Mannheim, F.R.G. Triton X-100 was obtained from J.T. Baker, Deventer, The Netherlands. Ampholines were obtained from LKB, Bromma, Sweden. XAR or XRP films were obtained from Kodak, Rochester, U.S.A.

Preparation of neutrophils. Neutrophils were prepared from 200–500 ml of fresh, citrated blood. The blood was diluted three times with

phosphate-buffered saline (PBS; 140 mM NaCl/9.2 mM Na_2HPO_4/1.3 mM NaH_2PO_4, pH 7.4) and centrifuged over isotonic Percoll of 1.076 g/cm^3 (1000 × *g*, 20 min, 20 °C). The erythrocytes in the pellet fraction were lysed with NH_4Cl at 4 °C as described previously [21].

Preparation of neutrophil cytoplasts and cytoplast lysates. Cytoplasts were prepared from neutrophils exactly as described by Roos et al. [20]. To obtain lysates, the cytoplasts were suspended at $200 \cdot 10^6$ per ml in a sonication medium consisting of 20 mM Tris-HCl (pH 7.6), 5 mM β-mercaptoethanol, 1 mM PMSF, 100 μM leupeptin, 2 mM EGTA, 10 mM EDTA, 50 mM NaF and 0.25 M sucrose. After sonication of 3 × 10 s at 4 °C, the cytoplast lysate was separated into a 100000 × *g* pellet and a supernatant fraction by centrifugation. The supernatant fraction was used for lysate phosphorylation studies. These fractions could be stored at −70 °C for 1 month without significant loss of cAMP-dependent protein kinase activity.

Classification of the CGD patients. CGD patients were classified as autosomal recessive according to criteria set forth in the complementation studies described by Hamers et al. [22].

[^{32}P]Orthophosphate incorporation in intact cytoplasts. Incorporation of ^{32}P-labeled orthophosphate was measured essentially as described in Ref. 18. Cytoplasts were suspended at 10^8/ml in label-incorporation medium consisting of 120 mM NaCl, 12 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 20 mM Hepes and 0.1% (w/v) bovine serum albumin (pH 7.2). [^{32}P]Orthophosphate was added (0.5 mCi/ml) to this medium, and the cytoplasts were incubated at 37 °C for 45–60 min. At the end of the incubation period, the cytoplast suspension was diluted four times with label-incorporation medium, and 0.4-ml samples were transferred to prewarmed Eppendorf vials, containing Bt_2cAMP. The incubation was continued for another 5 min and was stopped by centrifugation of the cytoplasts (10000 × *g*, 5 s) and removal of the medium. The pellet was dissolved in Laemmli sample buffer [23] and kept at 70 °C for 15 min.

Protein phosphorylation in a cell-free system. To assay protein phosphorylation, we added 10 μl of lysate to 100 μl of the following mixture: 30 mM Tris-HCl (pH 7.4), 2 mM EGTA, 7.5 mM $MgCl_2$ and 10 μM [γ-^{32}P]ATP (200 Ci/mmol). The endogenous kinase was stimulated by addition of 1–100 μM of Bt_2cAMP. The reaction was allowed to proceed at 30 °C for 5 min and was stopped by the addition of Laemmli sample buffer, followed by an incubation at 70 °C for 15 min.

Solid-phase assay for phosphorylation of the 47-kDa protein. The solid-phase phosphorylation assay was performed essentially as described by Valtora et al. [24]. Cytoplast lysates were separated on a 5–15% (w/v) polyacrylamide gel and blotted onto nitrocellulose. The nitrocellulose sheets were then incubated for 60 min in a 'blocking solution' consisting of 0.4% (w/v) Ficoll 400 dissolved in 200 mM NaCl, 50 mM Tris-HCl (pH 7.4) supplemented with 0.1% (v/v) Triton X-100. The relevant 47-kDa region on the nitrocellulose sheet was cut out, to reduce the volume of the subsequent incubation with the kinase. The nitrocellulose pieces were then incubated in a solution consisting of 125 mM NaCl, 10 mM $MgCl_2$, 0.1 mM β-mercaptoethanol, 0.1% (v/v) Triton X-100, 10 μM [γ-^{32}P]ATP (200 Ci/mmol) and Bt_2cAMP (100 μM) plus cAMP-dependent kinase (5 μg). The phosphorylation reaction was carried out for 30 min at 30 °C in a final volume of 0.4 ml. The reaction was terminated by extensive washing of the nitrocellulose samples in blocking buffer in the absence of Ficoll 400. After three washes (30 min each), the blot was finally washed in 10% (w/v) trichloroacetic acid supplemented with 100 mM sodium phosphate.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was performed as described by Vasilov et al. [25], using a modification of the method described by O'Farrell [26]. For isoelectric focussing in tube gels, LKB ampholines were used at the following concentrations: pH 3.5–10, 1% (v/v); pH 5–7, 4% (v/v); pH 7–9, 1% (v/v). The isoelectric focussing gel was layered on a SDS-acrylamide gel (10% (w/v)) separation gel.

Autoradiography. For autoradiography, all samples were run on 5–15% polyacrylamide gels. Gels were stained for protein with Coomassie brilliant blue, dried and enclosed in a cassette with an intensifying screen and either an XRP or an XAR film. Exposure of the films took 2–5 days at −70 °C.

Results

Protein phosphorylation in intact cytoplasts

Cytoplasts were incubated for 45–60 min in [^{32}P]orthophosphate-containing medium. At the end of this incubation period cytoplasts were still responsive to various stimuli [18]. To provoke a sustained activation of cAMP-dependent protein kinase, a permeant analog of cAMP, Bt_2cAMP, was used. Addition of Bt_2cAMP (to a final concentration of 0.25 or 0.50 mM) resulted in an increased phosphorylation of protein (see Fig. 1). Amongst the phosphoproteins was a 47-kDa protein. The relative molecular mass of this protein is identical to the 47-kDa protein that is phosphorylated after phorbol 12,13-dibutyrate treatment of cytoplasts [18]. The addition of Bt_2cAMP did not induce respiratory burst activity in intact neutrophil cytoplasts (results not shown). Hence, phosphorylation of the 47-kDa protein does not result, per se, in initiation of respiratory burst activity.

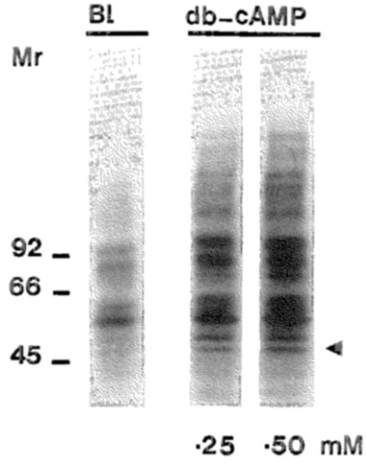

Fig. 1. Autoradiograph of neutrophil cytoplast phosphoproteins after addition of Bt_2cAMP (db-cAMP). Bt_2cAMP (0.25 or 0.50 mM) was added to ^{32}P-labeled cytoplasts and the incubation was continued at 37 °C for 5 min. The incubation was stopped by centrifugation of the cytoplasts (10000 × *g*) and, after removal of the medium, by addition of Laemmli sample buffer to the pellet. The solubilized pellet was subjected to SDS-PAGE and autoradiography. This phosphorylation pattern is representative of three experiments. The triangle indicates the position of the 47-kDa phosphoprotein.

Protein phosphorylation of a cell-free system

We also tested Bt_2cAMP-induced phosphorylation in a cell-free system. This system has the advantage of free accessibility of the protein kinase to Bt_2cAMP. The addition of Bt_2cAMP (1–100 μM final concentration) led to a dose-dependent increase in lysate protein phosphorylation, including a 47-kDa phosphoprotein (see Fig. 2). For comparison, a parallel incubation with phorbol dibutyrate as activator of protein kinase C was performed (see Fig. 2 second lane). Despite the limited protein resolution in a one-dimensional SDS-PAGE system, it is clear that Bt_2cAMP induced phosphorylation of some specific proteins. These are marked with arrows in Fig. 2. The phosphorylation of the 47-kDa protein in the presence of phorbol dibutyrate, however, appears to be much more pronounced. In intact cytoplasts this discrepancy in phosphorylation intensity is even greater (not shown).

To investigate whether the protein phosphorylations induced by Bt_2cAMP involved the 47-kDa phosphoprotein functionally coupled to the NADPH:O_2 oxidoreductase activity, we tested protein phosphorylation in cytoplast lysates obtained from the neutrophils of two autosomal CGD patients. After stimulation of the endogenous protein kinase with 100 μM Bt_2cAMP, we observed a phosphoprotein pattern comparable to that of the control lysate, except for the phosphorylation of the 47-kDa protein (see Fig. 2, last lane). The latter protein was hardly phosphorylated, as was previously observed with phorbol dibutyrate as an activator [18].

Protein phosphorylation in a solid phase assay

The observation that Bt_2cAMP induces 47-kDa protein phosphorylation in intact cytoplasts and in cytoplast lysates does not prove that cAMP-dependent protein kinase is able to phosphorylate this protein. This phosphorylation could be the consequence of another Bt_2cAMP-mediated event. Therefore, we tested 47-kDa protein phosphorylation using a solid-phase assay [24] with purified cAMP-dependent protein kinase after blotting of the 47-kDa protein onto nitrocellulose. In this assay system, interference of other kinases is excluded. Addition of the purified kinase also resulted in phosphorylation of the 47-kDa protein

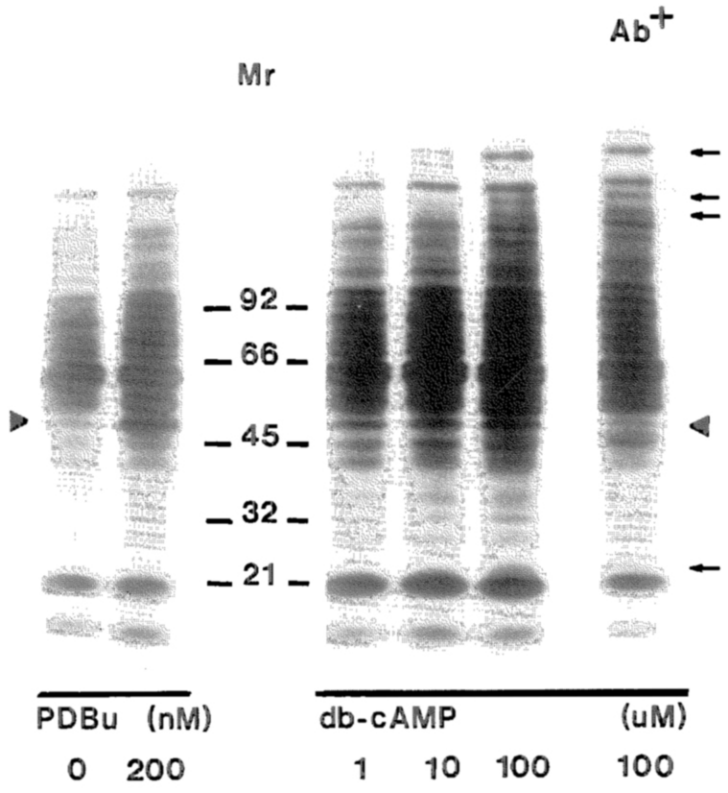

Fig. 2. Autoradiograph of phosphorylated neutrophil cytoplast lysate protein. Either Bt_2cAMP (db-cAMP) (at the concentrations indicated) or phorbol dibutyrate (PDBu) (200 nM) plus phosphatidylserine and phosphatidylcholine liposomes (1.2 and 0.4, μg respectively, dispersed in 25 μl of H_2O and sonicated for 2 min) was added to cytoplast lysates (see Materials and Methods). In the last lane, a cytoplast lysate of an autosomal cytochrome-b_{558}-positive CGD patient (Ab^+) was used. The phosphorylation reaction was started by the addition of [^{32}P]ATP and was continued for 5 min at 30 °C. The reaction was stopped by the addition of Laemmli sample buffer. The preparations were subjected to SDS-PAGE followed by autoradiography. These phosphoproteins patterns are representative of four experiments with two autosomal CGD patients. ▶ indicates the position of the 47-kDa phosphoprotein, the arrows mark the position of unique Bt_2cAMP-induced protein phosphorylations.

(see Fig. 3). Again, relevance of phosphorylation was shown by the observation that phosphorylation of the 47-kDa protein from autosomal CGD neutrophils was greatly reduced.

Two-dimensional gel-electrophoretic analysis of protein phosphorylation

To analyze the phosphoprotein pattern in more detail, we used two-dimensional gel electrophoresis in the separation of cytoplast lysate proteins. We compared phorbol dibutyrate-induced phosphorylation with Bt_2cAMP-induced phosphorylation and focussed on the 47-kDa level in both control and autosomal CGD cytoplast lysates. The autoradiographs of these gels show that with Bt_2cAMP, phosphorylation of the 47-kDa protein occurs in various spots with different p*I* values. The positions of these labeled spots resemble those observed after induction of phosphorylation by phorbol dibutyrate (see Fig. 4). With the autosomal CGD cytoplast lysate, no protein phosphorylation is observed in the 47-kDa protein region.

Effect of Bt_2cAMP on phorbol dibutyrate-induced respiratory burst activity in human neutrophils

Because Bt_2cAMP-dependent protein kinase acts directly on the 47-kDa protein, we tested whether Bt_2cAMP-mediated phosphorylation would affect the phorbol dibutyrate-induced respiratory burst activity in neutrophils. Both mediators bypass signal transduction pathways. Ad-

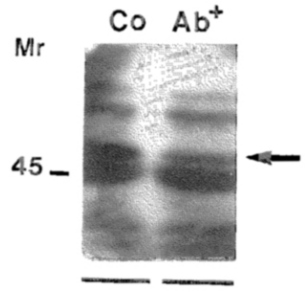

Fig. 3. Phosphorylation of the 47-kDa phosphoprotein by purified cAMP-dependent protein kinase in a solid-phase assay. Cytoplast lysates obtained from either control donors or autosomal CGD patients were subjected to SDS-PAGE followed by blotting of the protein onto a nitrocellulose sheet. The 47-kDa region was cut out and the cellulose matrix was saturated with Ficoll. Purified cAMP-dependent kinase was then added in the presence of Bt_2cAMP (100 μM) and [^{32}P]ATP. After an incubation at 30 °C for 30 min, the reaction was stopped by extensive washing of the nitrocellulose sheet in the presence of 0.1% Triton X-100. Subsequently, the remaining free label was removed by washing in 10% (w/v) trichloroacetic acid supplemented with 100 mM phosphate. This experiment represents one out of two with one autosomal CGD patient. Co represents the control lysate and Ab$^+$ represents the lysate from autosomal CGD patients. The arrow indicates the position of the 47-kDa phosphoprotein.

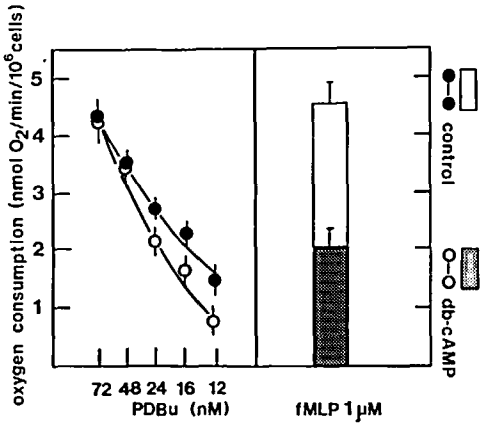

Fig. 5. Effect of Bt_2cAMP (db-cAMP) on respiratory burst activity in human neutrophils as induced by phorbol dibutyrate (PDBu) or fMLP. Neutrophilic granulocytes ($3 \cdot 10^6$ per ml) were incubated with 1mM Bt_2cAMP for 5 min at 37 °C in Hepes label-incorporation medium supplemented with 1.2 mM phosphate. After the preincubation interval, either fMLP or phorbol dibutyrate was added at the concentrations indicated in the figure and oxygen consumption was measured with a Clark-type oxygen electrode. The results shown represent the mean value (±S.D.) obtained from four experiments.

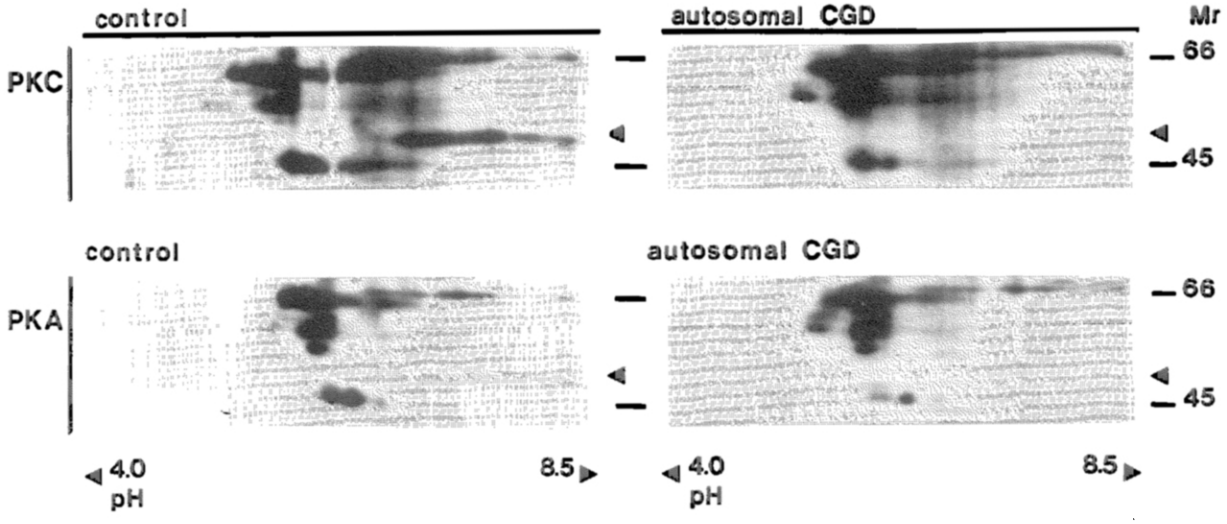

Fig. 4. Autoradiograph of neutrophil cytoplast phosphoproteins from control donors and autosomal CGD patients after separation by two-dimensional gel electrophoresis. Phosphorylation of protein and two-dimensional gel electrophoresis were carried out as described in Materials and Methods and in the legend to Fig. 2. The phosphoprotein patterns presented are representative of four similar experiments with two different patients. The arrows indicate the position of the 47-kDa phosphoprotein. The lower panel represents phosphorylations mediated by cAMP-dependent protein kinase (PKA) after the addition of Bt_2-cAMP, the upper panel represents phosphorylations mediated by protein kinase C (PKC) after the addition of phorbol dibutyrate.

dition of 1 mM Bt_2cAMP 4 min prior to the addition of phorbol dibutyrate had no significant effect on the respiratory burst activity induced by 100 nM phorbol dibutyrate. Only at very low doses of phorbol dibutyrate was some inhibition observed (see Fig. 5). In contrast, the fMLP-induced respiratory burst was found to be inhibited by Bt_2cAMP, even at optimal concentrations of fMLP (see Fig. 5).

Discussion

This study shows that the 47-kDa phosphoprotein functionally involved with NADPH:O_2 oxidoreductase activity is a substrate for the cAMP-dependent protein kinase. In intact neutrophil cytoplasts, in a cell-free system and in a solid-phase assay with purified cAMP-dependent kinase, addition of Bt_2cAMP resulted in phosphorylation of a 47-kDa protein. When neutrophil cytoplasts from autosomal-CGD patients were tested under similar conditions, phosphorylation of this protein did not occur. This latter finding indicates that the 47-kDa protein phosphorylated by Bt_2cAMP-dependent protein kinase is the 47-kDa phosphoprotein functionally involved in the respiratory burst enzyme. Although some phosphorylation of the autosomal CGD 47-kDa protein was detectable in the solid-phase assay, the two-dimensional gel electrophoretic analysis showed complete absence of phosphoprotein spots in the 47-kDa protein region. Possibly, some non-enzymic association of ATP with protein occurs in the solid-phase assay.

In intact cytoplasts and in the lysate-phosphorylation assay, Bt_2cAMP-induced phosphorylation of the 47-kDa protein was never as pronounced as phosphorylation induced by phorbol dibutyrate. Especially in intact cytoplasts, Bt_2cAMP-induced 47-kDa protein phosphorylation was weak, although in this case insufficient activation of the kinase due to limited permeation of the activator could also play a role. The phosphorylation induced by Bt_2cAMP, however, occurred in identical phosphoprotein spots as those induced by phorbol dibutyrate. Thus, both cAMP-dependent protein kinase and protein kinase C have access to the same 47-kDa phosphoprotein. Whether the two kinases phosphorylate similar serine or threonine residues is not known. A simple phosphopeptide map analysis (V_8 proteinase treatment followed by SDS-gel electrophoresis), resulting in a single 21-kDa phosphopeptide, did not reveal a difference in phosphopeptides (results not shown).

The importance of the observation that the 47-kDa phosphoprotein is a substrate for cAMP-dependent protein kinase is not yet clear. Addition of Bt_2cAMP to neutrophil cytoplasts at a concentration that induces protein phosphorylation did not induce a respiratory burst. This clearly implies that 47-kDa protein phosphorylation and initiation of respiratory-burst activity are not always coupled. This lack of correlation (unlike phosphorylation induced by protein kinase C) can be explained in two ways. First, cAMP-dependent protein kinase phosphorylates the 47-kDa protein at amino-acid residues distinct from those phosphorylated by protein kinase C. This has been shown to occur in some phosphoproteins [27,28]. This possibility is intriguing because it would point to the existence of a positive (protein kinase C-mediated) and a negative (cAMP-dependent protein kinase-mediated) phosphorylation signal. In resting neutrophil cytoplasts, the protein might be under negative control of cAMP-dependent protein kinase. This speculative model would explain why in intact neutrophil cytoplasts we find so little (extra) incorporation of [^{32}P]phosphate into the 47-kDa protein. However, preliminary analysis of the 47-kDa phosphoprotein after either cAMP-induced or phorbol dibutyrate-induced phosphorylation did not substantiate this hypothesis. In addition, Bt_2cAMP had only a moderate effect on the phorbol dibutyrate-induced respiratory burst.

A second, likely explanation for the lack of correlation between 47-kDa protein phosphorylation and respiratory burst activity is that apart from 47-kDa protein phosphorylation, another signal is obligatory for the initiation of the respiratory burst activity; this signal is not generated upon activation of cAMP-dependent protein kinase. The second signal has to be a protein kinase C-mediated modification other than 47-kDa protein phosphorylation.

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