A 23-kDa Protein as a Substrate for Protein Kinase C in Bovine Neutrophils. Purification and Partial Characterization†

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ABSTRACT: In $^{32}P_i$-loaded bovine neutrophils stimulated with phorbol myristate acetate (PMA), radioactivity was preferentially incorporated into a protein of low molecular mass, suggesting a PKC-dependent phosphorylation. This protein, termed 23-kDa protein, was predominantly localized in the cytosol. It was purified from bovine neutrophil cytosol by a series of chromatographic steps, including ion exchange on DE-52 cellulose and Mono Q, and filtration on Bio-Gel P60 in the presence of mercaptoethanol and urea. The apparent molecular mass of the purified protein, assessed by SDS–PAGE and mercaptoethanol by reference to protein markers, ranged between 20 and 23 kDa, depending on the percentage of polyacrylamide and conditions of migration. In the absence of mercaptoethanol, a dimer accumulated. Homogeneity of the 23-kDa protein was verified by 2D-PAGE analysis. Some properties of the 23-kDa protein, including its amino acid composition, were determined. Gel isoelectric focusing (IEF) of the purified 23-kDa protein followed by Coomassie blue staining allowed the visualization of four discrete protein bands with isoelectric points ranging between pH 6.3 and 6.7. Phosphorylation of the 23-kDa protein by [γ-^{32}P]ATP in the presence of bovine neutrophil PKC supplemented with Ca^{2+}, phosphatidylserine, and diacylglycerol or with PMA occurred on serine and required the presence of mercaptoethanol. The apparent K_M of ATP was 9 μM. The 23-kDa protein was also phosphorylated by PKM, the catalytic fragment of PKC obtained after removal of the regulatory domain, but not by cAMP-dependent protein kinase. IEF of the ^{32}P-labeled 23-kDa protein followed by autoradiography revealed four discrete bands with distinct isoelectric points similar to those of the bands stained by Coomassie blue after IEF on nonlabeled 23-kDa protein; the more acidic bands were the more labeled. The bands of the 23-kDa protein resolved by IEF and transferred to nitrocellulose showed ability to bind [^{35}S]GTP-γ-S. The immunoreactivity of antibodies raised in rabbits against the bovine neutrophil 23-kDa protein was demonstrated on immunoblots after SDS–PAGE. Binding of antibodies prevented the PKC-dependent phosphorylation of the protein. The antibodies reacted with a 23-kDa protein present in a bovine liver extract, possibly due to the macrographic Kupffer cells present in the liver tissue. No reaction occurred in extracts from bovine heart, skeletal muscle, platelets, and brain. Compared to human and rabbit neutrophils, the 23-kDa protein from bovine neutrophils appears to be overexpressed. A similarly sized protein from neutrophils, which is the regulatory light chain of myosin isolated from bovine neutrophil cytosol, was phosphorylated in the presence of [γ-^{32}P]ATP by neutrophil PKC, like the 23-kDa protein. Yet the two proteins differed in their amino acid composition, the nature of their phosphorylated amino acids, and the absence of immunological cross-reactivity. The 23-kDa protein differed also from several other proteins of similar molecular mass that have been identified in neutrophils, namely, calmodulin, the small subunit of the low-potential cytochrome *b*, and a low molecular weight protein which is ADP-ribosylated by the botulinum toxin.

Neutrophils are particularly rich in protein kinase C; in comparison to PKC,[1] cAMP- and cGMP-dependent protein kinases are present in lower levels (Helfman et al., 1983). During the course of bovine neutrophil PKC purification (Dianoux et al., 1989), it was observed that a 23-kDa protein comigrated with PKC and was readily labeled by [γ-^{32}P]ATP in the presence of PKC upon addition of Ca^{2+}, diacylglycerol, and phosphatidylserine. A protein of similar molecular weight present in human blood neutrophils, ascribed to a light chain of myosin, has recently been reported to be phosphorylated by PKC (Pontremoli et al., 1987a,b). We therefore decided to study in more detail the 23-kDa protein of bovine neutrophils to clarify its identity. In this paper, we describe the purification of this protein and its partial characterization. We show that the 23-kDa protein and the regulatory light chain of myosin are two distinct proteins. We further show that the 23-kDa not only behaves as a natural substrate for PKC but also has the ability to bind GTP in a specific manner.

Materials and Methods

Materials. The following materials were supplied by the companies indicated: [γ-^{32}P]ATP and [^{32}P]H_3PO_4, Amersham; [^{35}S]GTP-γ-S, NEN; histone type IIIS, phosphatidyl-

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[1] Abbreviations: PKC, protein kinase C; MLC20, myosin light chain of molecular mass of 20 kDa; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PMA, 4β-phorbol 12-myristate 13-acetate; ATP, adenosine 5′-triphosphate; PBS, phosphate-buffered saline; IEF, isoelectric focusing; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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serine, Mops, EDTA, EGTA, PMSF, leupeptin, soybean trypsin inhibitor, diolein, 4β-phorbol 12-myristate 13-acetate (PMA), guanidine hydrochloride, and bovine serum albumin from Sigma Chemical Co.; ATP, Boehringer, TPCK, TLCK, β-mercaptoethanol, SDS, and Coomassie brilliant blue R250 and G250, Serva; acrylamide and bis(acrylamide), BDH Biochemicals; standard molecular weight markers, Mono Q column, Sepharose 4B, and phenyl-Sepharose Cl-4B, Pharmacia; DEAE-cellulose (DE-52), Whatman; Bio-Gel P60, Bio-Rad; ultrafilters, Amicon; urea, pyridine, and hydrochloric acid, Merck; nitrocellulose BA83 and cellulose F1440 thin layers from Schleicher and Schuell; ampholines, LKB; goat antirabbit peroxidase linked IgG, Miles; sodium chloride, ammonium bicarbonate, and acetic acid, Prolabo.

Biological Preparations. Bovine neutrophils were routinely isolated from 10 L of fresh blood (Morel et al., 1985). Contamination by platelets was less than 1%. Platelets from bovine blood were prepared as described by White (1987). Cytosol from bovine neutrophils was obtained as follows. Neutrophils were suspended in 0.25 M sucrose, 10 mM Mops, pH 7.4, 2 mM EDTA, 10 mM EGTA, 50 mM β-mercaptoethanol, 1 mM PMSF, 1 μg/mL TPCK, 1 μg/mL TLCK, 1 μg/mL soybean trypsin inhibitor, and 10 μg/mL leupeptin (buffer A). The neutrophil suspension at a concentration of 2–3 × 10^8 cells/mL was subjected to ultrasonic irradiation for 4 × 15 s at 2–4 °C with a Branson sonifier at 60-W output. The homogenate was centrifuged for 1 h at 140000*g* in a Ti45 rotor (Beckman). The supernatant was referred to as neutrophil cytosol. Cytosols from bovine liver, heart, skeletal muscle, and brain were obtained after homogenization with a Potter apparatus, followed by sonic irradiation. The homogenate was centrifuged first at 10000*g* for 20 min. After a second centrifugation for 1 h at 140000*g*, the supernatant was recovered and termed cytosol. Cytoskeleton was prepared as described by Yassin et al. (1985) from neutrophils treated with 1% Triton X-100. Bovine neutrophil PKC and bovine brain PKC were purified as previously described by Dianoux et al. (1989).

Purification of 23-kDa Protein. Forty milliliters of bovine neutrophil cytosol obtained from 10 L of blood were loaded on a DE-52 cellulose column (10 × 2.7 cm). Elution was performed with a 400-mL linear (0–0.3 M NaCl) gradient in a buffer containing 10 mM Mops, pH 7.4, 2 mM EDTA, 2 mM EGTA, 10 mM β-mercaptoethanol, 10% ethylene glycol, 1 mM PMSF, and 10 μg/mL leupeptin or 1 μg/mL TLCK (buffer B). Two pools of eluted fractions were recovered. Pool 1 corresponded to fractions recovered between 0 and 0.08 M NaCl; pool 2, consisting of fractions recovered between 0.08 and 0.17 M NaCl, contained the bulk of the 23-kDa protein with numerous other proteins including PKC. This pool was concentrated and desalted by ultrafiltration on an Amicon YM-30 filter to about 2 mL, diluted 10 times with buffer B, and then loaded on a Mono Q column (HR 5/5) previously equilibrated with the same buffer. Elution was performed with 30 mL of a 0–0.3 M NaCl linear gradient. As for the DE-52 chromatography, the 23-kDa protein was collected in two pools corresponding to fractions recovered between 0 and 0.04 M NaCl (pool 1′) and between 0.04 and 0.1 M NaCl (pool 2′), respectively. The two pools contained some contaminants, including an abundant protein of molecular mass ≃ 7 kDa. Urea was used to resolve the 23-kDa protein from the 7-kDa protein by chromatography on Bio-Gel P60 as described under Results. The purified 23-kDa protein was then dialyzed extensively against 50 mM ammonium bicarbonate, pH 7.4, and stored at −20 °C.

The presence of the 23-kDa protein at the different steps of the purification procedure was detected after SDS–PAGE by Coomassie blue staining, as its absorbance of UV light was very low. The amount of 23-kDa protein was evaluated by the method of Bradford (1976) and after acid hydrolysis with 6 N HCl, 110 °C, for 18 h by its amino acid composition.

For amino acid sequence determination, the 23-kDa protein was electroeluted from the polyacrylamide gel to minimize amino-terminal blockage. This allowed us to avoid the last step of the purification procedure, which involved the use of urea, a compound that may produce traces of highly reactive cyanate (Hunkapiller et al., 1983). In brief, partially purified fractions obtained from the Mono Q chromatography were used. After concentration on a PM-10 filter, these fractions were subjected to SDS–PAGE (Laemmli & Favre, 1973). The 23-kDa protein was localized on the gel by weak staining with Coomassie blue R250 in water. After destaining by soaking in water, the band was cut out, sliced, and electroeluted under 80 V for 5 h at room temperature in 50 mM ammonium bicarbonate brought to pH 7.5 by addition of acetic acid and supplemented with 0.01% SDS.

Preparation of Bovine Neutrophil Myosin. The method described by Trotter and Adelstein (1979) for the preparation of rabbit alveolar macrophage myosin was utilized with some modifications. All operations were performed at 2–4 °C unless otherwise indicated. Bovine neutrophils in 40 mM Mops, pH 7.2, 20 mM KCl, 10 mM Mg-ATP, 5 mM β-mercaptoethanol, and 2 mM EGTA were homogenized in a Potter apparatus with a tightly fitted pestle. The homogenate was first centrifuged for 1 h at 12000*g*, and the Mg^{2+} concentration was adjusted to 50 mM by addition of powdered magnesium acetate. After mild stirring for 1 h, the precipitated actomyosin was spun down by centrifugation at 12000*g* for 8 h at room temperature. The pellet was suspended in 20 mM Mops, pH 7.4, and 5 mM β-mercaptoethanol. Ammonium sulfate was then added to a final concentration of 25%. The protein precipitate recovered by centrifugation for 1 h at 12000*g* was taken up in the minimal volume of 15 mM Tris-HCl, pH 7.4, 0.5 M NaCl, and 5 mM β-mercaptoethanol and dialyzed overnight at room temperature. The dialysate was applied onto a Sepharose 4B column (45 × 2 cm) equilibrated with the same buffer. The column was eluted at the rate of 17 mL/h. The fractions containing actomyosin were identified by migration of aliquots on SDS–PAGE and pooled. They could be stored at −20 °C or immediately processed for isolation of the regulatory light chain of myosin (MLC20).

Purification of the Regulatory Light Chain of Neutrophil Myosin (MLC20). The actomyosin was dissociated in 20 mM Mops, pH 7.4, and 10 mM β-mercaptoethanol by 0.05% SDS, 8 M urea, and 1 M guanidine for 2 h at room temperature. Tris-base powder was added to bring the pH to 8.0. The myosin heavy chain was precipitated with ethanol and discarded by centrifugation at 12000*g* for 15 min. The supernatant contained the two myosin light chains, which were separated as described by Hathaway and Haeberle (1983) in the case of chicken gizzard myosin light chains. In brief, the supernatant was dialyzed overnight at 4 °C against 20 mM Mops, pH 7.5, 1 mM EDTA, and 5 mM β-mercaptoethanol and then supplemented with NaCl to reach a final concentration of 1.5 M before being loaded on a phenyl-Sepharose column (1 × 0.5 cm) equilibrated with the same buffer. Elution with 5 mM β-mercaptoethanol resulted in the resolution of the regulatory light chain (MLC20) and the alkali light chain with molecular weights of 20 000 and 17 000, respectively. The isolated MLC20 was dialyzed against 50 mM

ammonium bicarbonate, pH 7.8, and stored at −20 °C.

Antibodies and Immunoblotting. Before immunization, serum was collected from rabbits and used as control. Polyclonal antibodies were raised by three subcutaneous injections at 2-week intervals of 200 μg of the 23-kDa protein dissolved in 500 μL of distilled water and 500 μL of Freund's complete adjuvant. Two weeks afterward, bleeding was performed.

Immunoblotting was carried out by the method of Towbin et al. (1979). After electrophoretic transfer onto 0.2-μm nitrocellulose sheets from slab gels and incubation with the anti 23-kDa protein serum diluted 500 times, the immunoreactive complex was detected with goat anti-rabbit IgG conjugated with peroxidase. The peroxidase activity was revealed with a solution of 50 mM diaminobenzidine and 50 mM sodium phosphate, pH 7.2, in the presence of H_2O_2.

Assay of PKC Activity. PKC activity was measured by enzymatic transfer of the terminal radiolabeled phosphate from [γ-^{32}P]ATP to histone type IIIS as described (Stasia et al., 1987). In brief, the medium contained 20 mM Mops, pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 25 μg of histone type IIIS, 20 μg/mL phosphatidylserine, 2 μg/mL diacylglycerol, and 10 μL of active PKC in a final volume of 100 μL. The reaction was started by addition of [γ-^{32}P]ATP (5×10^5 dpm) to give a final concentration of 70 μM. Control assays to test the activity of Ca^{2+} and phospholipid-independent kinase were systematically included, in which $CaCl_2$, diacylglycerol, and phosphatidylserine were replaced by 2 mM EGTA. Incubation was carried out at 30 °C for 6 min and was terminated by addition of 0.5 mL of ice-cold 10% trichloroacetic acid, followed by 0.5 mL of a 2 mg/mL bovine serum albumin solution. Protein was collected on Millipore HAWP 0.45-μm membrane filters and washed three times with 1 mL of ice-cold 10% trichloroacetic acid. Radioactivity of the filters was measured by liquid scintillation cocktail Ready Value from Beckman. A blank assay was performed with trichloroacetic acid and bovine serum albumin being added to the incubation medium prior to PKC. A unit of PKC activity corresponds to 1 nmol of $^{32}P_i$ incorporated into histone per minute.

In Vitro Phosphorylation of Proteins by PKC. Phosphorylation of proteins was performed under the same conditions as those used for the PKC assay, except that histone was replaced by the different proteins to be tested. The incubation with [γ-^{32}P]ATP, 10^6 dpm, final concentration 20 μM, lasted for 30 min at 30 °C. The ATP concentration was kept not too high to maintain the specific radioactivity at a value sufficiently high, yet this concentration was somewhat above the K_M values. The reaction was stopped by precipitation with ice-cold 25% trichloroacetic acid. The precipitate was washed with cold acetone and further processed for SDS–PAGE. The labeled proteins were detected by autoradiography. Routinely, exposure to the film was for 12–15 h.

In Vivo PKC-Dependent Phosphorylation of Proteins Induced by PMA. Bovine neutrophils (2×10^9 cells) were suspended in 15 mL of 10 mM Tris, pH 7.4, containing 0.15 M NaCl and 10 mM glucose and incubated with 2.5 mCi of neutralized $H_3{}^{32}PO_4$ at 37 °C for 30 min. The cells were then activated by 100 μg of phorbol myristate acetate (PMA) at 37 °C for different periods of time. The pellets recovered after a 10-min centrifugation at 400*g* were suspended in 0.25 M sucrose, 2 mM EDTA, 2 mM EGTA, 10 mM β-mercaptoethanol, 1 mM PMSF, and 10 μg/mL leupeptin and subjected to four successive sonic irradiations of 15 s separated by 30-s intervals in an ice bath by use of a Branson sonifier at 60-W output. A control experiment was conducted in the absence of PMA. The labeled proteins including the 23-kDa protein were analyzed by SDS–PAGE followed by autoradiography. Occasionally, the radiolabeled 23-kDa protein was purified.

Analysis of Phosphorylated Amino Acids. The 23-kDa protein and the regulatory light chain of myosin were phosphorylated by PKC in vitro and then precipitated by ice-cold 25% trichloroacetic acid. Precipitates were washed with acetone and solubilized in 100 mM *N*-methylmorpholine, pH 7.8. This was followed by a tryptic digestion for 24 h at 37 °C, using a trypsin to protein ratio of 1/5. The released peptides were hydrolyzed in 6 N HCl for 1 h at 110 °C as described by Hunter and Sefton (1980). Samples were spotted on thin-layer plates (F1440 cellulose) together with standard phosphorylated amino acids and then subjected to electrophoresis at 800 V for 1 h at 10 °C using a buffer containing pyridine, acetic acid, and water (1:10:89 v/v), pH 3.6. The plates were stained with ninhydrin to reveal amino acids and subsequently exposed to Fuji X-ray films.

SDS–Polyacrylamide Slab Gel Electrophoresis (SDS–PAGE). Prior to electrophoresis, protein samples were precipitated with 25% ice-cold trichloroacetic acid. The precipitate was washed with cold acetone and dissolved in 0.06 M Tris-HCl, pH 6.8, 15% (v/v) glycerol, 5% β-mercaptoethanol, 2.3% SDS, and 0.001% bromophenol blue. After incubation at 60 °C for 30 min, the solubilized samples were subjected to SDS–PAGE (Laemmli & Favre, 1973), using a 4% stacking gel and a 13% resolving gel. The molecular weight standards were phosphorylase *b* (M_r = 94 000), bovine serum albumin (M_r = 67 000), ovalbumin (M_r = 43 000), carbonic anhydrase (M_r = 30 000), soybean trypsin inhibitor (M_r = 20 000), and lactalbumin (M_r = 14 400). Gels were stained with Coomassie blue R250. For autoradiography, the gels were dried under vacuum and exposed to Fuji X-ray films.

Two-Dimensional Gel Electrophoresis (2D-PAGE). The method of O'Farrell (1975) was used with slight modification. The 23-kDa protein purified as described above was desalted and dissolved in 6.7 M urea, 1.5% Triton X-100, and 3% β-mercaptoethanol. IEF was carried out with an LKB Multiphor apparatus with slab gels consisting of 5% acrylamide supplemented with 8.4 M urea and 2% ampholines (pH range 3.5–9.5). After 1 h of prefocusing, about 20 μg of protein was loaded on the gel, and focusing was allowed to proceed for 2 h at 1500 V and 7 W. Bands of 1-cm width were then cut out and equilibrated in 2% SDS, 0.06 M Tris-HCl, pH 6.8, 5% mercaptoethanol, 15% glycerol, and traces of bromophenol blue for migration in the second dimension. SDS–PAGE in the second dimension was performed as described above with a separating gel of 13% acrylamide and a stacking gel of 4% acrylamide.

In some cases, only IEF was used, omitting the second dimension. This was the case when the isoelectric point of the 23-kDa protein was determined. A strip of the gel was cut into 5-mm pieces. The gel pieces were soaked in 1 mL of deionized and degassed water to solubilize the ampholines, and the pH of the solution was measured. IEF was also used to explore the binding efficiency of [^{35}S]GTP-γ-S to different forms of the 23-kDa protein and to assess the PKC-dependent phosphorylation of these different forms by [γ-^{32}P]ATP.

[^{35}S]GTP-γ-S Binding Assay. The purified 23-kDa protein separated by SDS–PAGE was electrotransferred onto a 0.2-μm nitrocellulose sheet for 2 h at 24 V in 0.012 M Tris, 0.096 M glycine, 0.05% SDS, and 20% methanol (Towbin et al., 1979). After transfer, the sheet was soaked in 100 mL of phosphate-buffered saline (PBS) consisting of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4, and supplemented with 0.05% Tween 20 (v/v) and 3 g of BSA

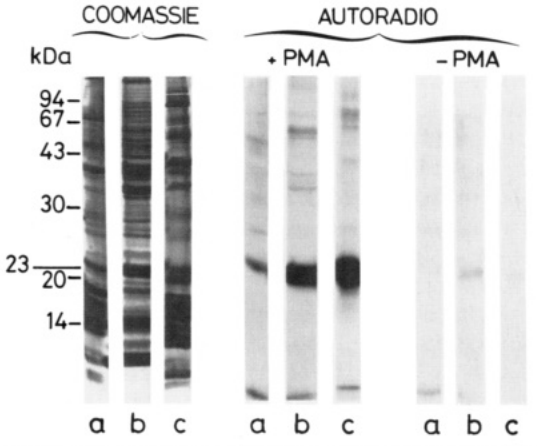

FIGURE 1: In vivo phosphorylation of the 23-kDa protein in membranes, cytosol, and cytoskeleton of bovine neutrophils. $^{32}P_i$-loaded bovine neutrophils were activated by incubation with PMA for 10 min. About 120 μg of protein of membranes (a), cytosol (b), and cytoskeleton (c) were submitted to SDS–PAGE, and the gels were stained with Coomassie blue R250. The radiolabeled products were revealed by autoradiography. A control experiment was conducted without PMA.

for 1 h at room temperature. It was washed thrice with PBS and incubated in the presence of 5 mM $MgCl_2$, 2 mM ATP, and 1 μM [^{35}S]GTP-γ-S (50 μCi) for 1 h at room temperature. This was followed by rinsing thrice in PBS for 10 min and in distilled water for 10 min. After drying, the sheet was subjected to autoradiography. [^{35}S]GTP-γ-S binding was also checked after electrotransfer of the 23-kDa protein from an IEF gel to nitrocellulose. In this case, transfer was carried out for 15 h under 24 V, using 0.7% acetic acid; the nitrocellulose sheet was incubated with [^{35}S]GTP-γ-S, followed by autoradiography as described above.

Amino Acid Analysis. Protein samples were hydrolyzed with 6 N HCl, supplemented with 0.05% β-mercaptoethanol, for 18 h at 106 °C. Amino acid analysis was performed either on a Biotronik LC 6000 or on a Beckman 7300 amino acid analyzer.

Protein Assay. Protein was routinely determined by the method of Bradford (1976). Alternatively, the samples were treated with 10% (w/w) trichloroacetic acid; the precipitate was collected by centrifugation and solubilized in 10% SDS for protein determination by the method of Lowry et al. (1951). In both cases, bovine serum albumin was used as a standard.

Results

In Vivo Phosphorylation of a 23-kDa Protein in Bovine Neutrophils Activated by PMA. Upon addition of PMA to bovine neutrophils preincubated in an isotonic medium with glucose as a source of energy and $^{32}P_i$ for generation of [γ-^{32}P]ATP, predominant labeling of a 23-kDa protein occurs with maximal phosphorylation being attained in less than 1 min of contact with PMA. When longer periods of incubation were used, a number of other labeled bands appeared, particularly in the range of molecular masses of 35–70 kDa. In the absence of PMA, phosphorylation of the 23-kDa protein was negligible. As PKC is specifically activated by PMA, it appears likely that PKC plays a central role in the phosphorylation of the 23-kDa protein.

In the experiment illustrated in Figure 1, bovine neutrophils loaded with $^{32}P_i$ and activated with PMA were disrupted by sonication in buffer A, and the homogenate was subjected to centrifugation (cf. Materials and Methods). The membrane and cytosolic fractions were subjected to SDS–PAGE, and the gels were autoradiographed. More than 90% of the labeled 23-kDa protein was recovered in the cytosol (Figure 1, lane b), the remaining being bound to the membrane fraction (Figure 1, lane a). In neutrophils treated by Triton X-100, the membrane is solubilized and an insoluble fraction remains, which consists of the cellular cytoskeleton (Yassin et al., 1985). Roughly half of the labeled 23-kDa protein was associated with the Triton-insoluble fraction (Figure 1, lane c), the remaining being soluble. The double localization of the 23-kDa protein in soluble cytosol and the cytoskeleton fraction suggests either that the 23-kDa protein is anchored to cytoskeleton fibers and is readily released to the medium during homogenization of neutrophils or that, despite a cytosolic localization, the 23-kDa protein exhibits a strong propensity to bind to the cytoskeleton.

Purification of the 23-kDa Protein from Bovine Neutrophil Cytosol. The 23-kDa protein represents 4–6% of bovine neutrophil cytosolic proteins on the basis of the intensity of staining by Coomassie blue after SDS–PAGE. Cytosol was therefore chosen as starting material for the purification of the 23-kDa protein. The data in Figure 2a,b illustrate the fractionation of the 23-kDa protein from bovine neutrophil cytosol by DE-52 cellulose chromatography on the basis of the use of a linear 0–0.3 M NaCl gradient. As the absorbance of the 23-kDa protein at 280 nm is negligible, its presence in the eluted fractions was detected by Coomassie blue staining after SDS–PAGE (Figure 2b). The PKC activity of the eluates was also determined, as PKC comigrates with the 23-kDa protein in the first steps of the purification procedure. A small amount of the 23-kDa protein, devoid of PKC, was recovered in fractions 1–20 eluted between 0 and 0.08 M NaCl. These fractions were reassembled in pool 1 to be further processed. Fractions 21–46 (pool 2) eluted between 0.08 and 0.17 M NaCl and contained a large amount of the 23-kDa protein associated with the bulk of PKC and some protein contaminants, including an abundant 7-kDa protein (Figure 2b).

Cytosol was also prepared from ^{32}P-loaded neutrophils activated by PMA and further processed by DE-52 chromatography, as described above. An aliquot of pool was subjected to SDS–PAGE and autoradiographed (Figure 2c). Clearly, pool 2 contained a predominantly radiolabeled 23-kDa protein. In a control experiment in which the $^{32}P_i$-loaded neutrophils were not treated by PMA, much less $^{32}P_i$ was incorporated into the 23-kDa protein.

To check whether a stable association between PKC and the 23-kDa protein did exist, an aliquot of pool 2 was subjected to a 3–30% linear sucrose gradient centrifugation in buffer B supplemented with 100 mM NaCl by use of a SW41 Beckman rotor at 4 °C. After an 18-h centrifugation at 39000 rpm, PKC activity and the 23-kDa protein were located in two distinct regions of the gradient corresponding to 22% and 8% sucrose, respectively (data not shown). This result ruled out the possibility of a stable complex between PKC and the 23-kDa protein. In contrast, the 23- and the 7-kDa proteins migrated at the same place in the gradient.

The second step of the purification procedure consisted of Mono Q chromatography of pool 2 recovered from DE-52 cellulose. The original cytosolic material was issued from $^{32}P_i$-loaded and PMA-activated neutrophils to better visualize the radiolabeled protein species. The 23-kDa protein was eluted between 0 and 0.04 M NaCl in fractions 1–4, together with the 7-kDa protein as the only noticeable contaminant revealed by Coomassie blue staining after SDS–PAGE (Figure 3a,b). Fractions 1–4 were reassembled in pool 1′. The remainder of the 23-kDa protein was recovered between 0.04 and 0.1 M NaCl, together with PKC and a number of other

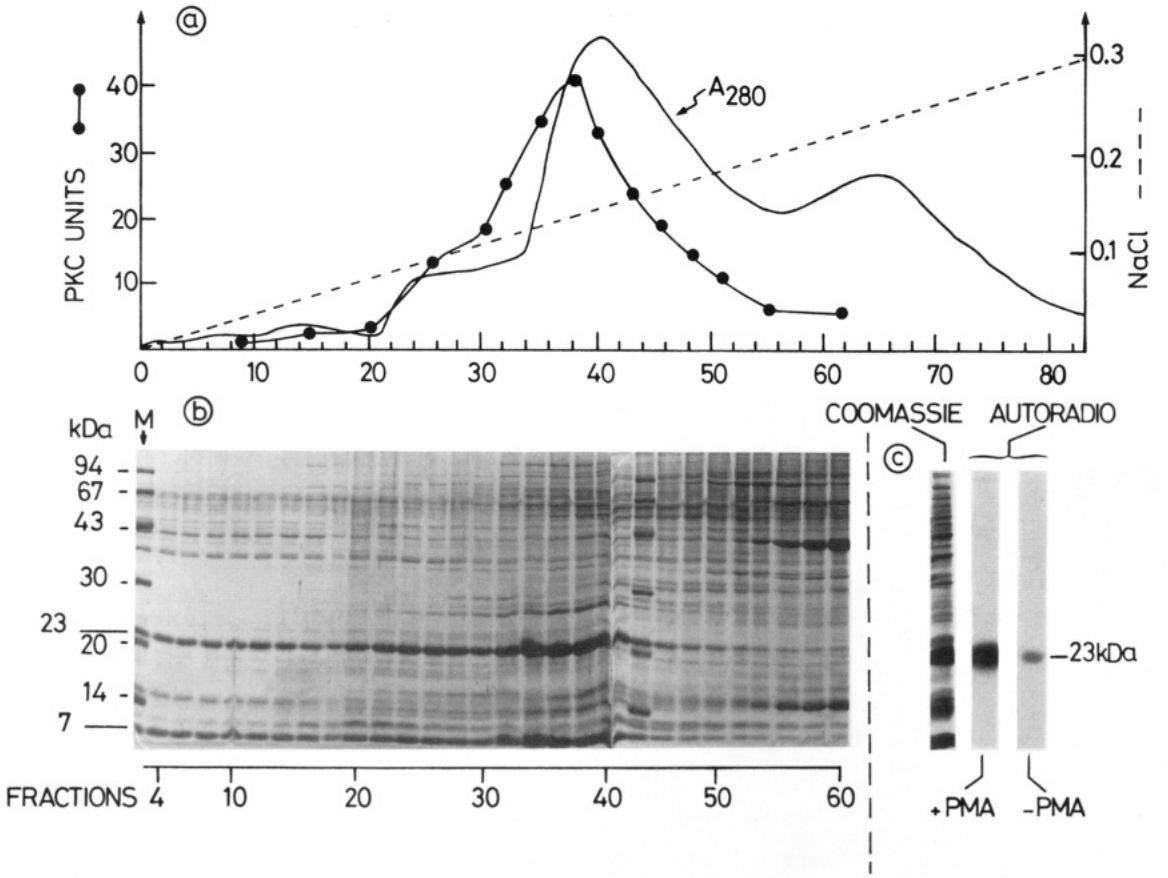

FIGURE 2: DE-52 chromatography of the 23-kDa protein. (a) Protein absorbancy and PKC activity of the eluted fractions. The high-speed supernatant of bovine neutrophil homogenate termed cytosol was loaded onto a DE-52 cellulose column. A linear gradient of NaCl was applied as detailed under Materials and Methods and Results. Absorbancy at 280 nm was continuously monitored. Five-milliliter fractions were collected. PKC activity was assayed in the presence of Ca^{2+}, phosphatidylserine, and diacylglycerol (●). Controls were included where Ca^{2+} and lipids were replaced by EGTA; their PKC activity was negligible. (b) SDS–PAGE of DE-52 cellulose eluates. Thirty-microliter aliquots were withdrawn from every other tube and subjected to SDS–PAGE (Laemmli & Favre, 1973) using a 4% polyacrylamide stacking gel and a 13% polyacrylamide resolving gel. Pool 1 corresponds to fractions 1–20 and pool 2 to fractions 21–46 (see Results). Molecular weight markers (M) were added in fractions 2 and 44. (c) Autoradiography of proteins corresponding to pool 2 after in vivo phosphorylation. $^{32}P_i$-loaded neutrophils were activated by addition of PMA, homogenized, and ultracentrifuged, and the supernatant was chromatographed on DE-52 cellulose as described above. Thirty-microliter of fractions corresponding to pool 2 were submitted to SDS–PAGE and stained with Coomassie blue R250, and the ^{32}P-phosphorylated proteins were revealed by autoradiography. A control assay was run in the absence of PMA.

proteins (pool 2′). Autoradiography of the SDS gel revealed that the 23-kDa protein was the predominantly labeled species among the eluted proteins (Figure 3c). Labeling was much lower when PMA had been omitted in the neutrophil incubation medium (Figure 3d).

Attempts to resolve the 23- and the 7-kDa proteins by chromatography on Superose 12, Bio-Gel P30, and Bio-Gel P60 in the presence or absence of mercaptoethanol were unsuccessful. Resolution of the two proteins was, however, achieved by treatment with urea as follows. Fractions 1–20 from DE-52 cellulose (pool 1) (Figure 2) and fractions 1–4 from Mono Q (pool 1′) (Figure 3) were mixed and concentrated on an Amicon PM10 filter. The concentrate was applied to a Bio-Gel P60 column equilibrated with 10 mM Mops, pH 7.4, 50 mM NaCl, 2 mM EGTA, 2 mM EDTA, 10 mM β-mercaptoethanol, and 6 M urea, and elution was performed with the same buffer at the rate of 6 mL/h. The eluted proteins were analyzed by SDS–PAGE, followed by Coomassie blue staining. The 23-kDa protein was eluted first, followed by the 7-kDa protein (data not shown). Urea was removed by extensive dialysis against 50 mM ammonium bicarbonate, pH 7.4. The 23-kDa protein free of urea could be stored at −20 °C for several months without detectable alteration. From 10 L of bovine blood, about 300 μg of the 23-kDa protein was purified to apparent homogeneity assessed by amino acid analysis.

Chemical Properties and in Vitro Phosphorylation of the 23-kDa Protein. The apparent molecular mass of the purified protein determined from the rate of migration on SDS–PAGE reference with standard proteins was found to depend on the percentage of polyacrylamide in the gel and the technique used. With the technique of Laemmli and Favre (1973) the rate of migration in a 13% polyacrylamide gel was that of a protein of 23 kDa. migration in a 20% polyacrylamide gel using the technique of Cabral and Schatz (1979) corresponded to an apparent molecular mass of 20 kDa. When the protein was supplemented with β-mercaptoethanol, only the monomeric form was visualized by Coomassie blue staining of the SDS gel. Omission of mercaptoethanol resulted in the accumulation of an apparent dimer (not shown).

The purified 23-kDa protein was also characterized by 2D-PAGE, i.e., IEF followed by SDS–PAGE (data not shown). Four discrete protein bands with p*I* ranging from 6.3 to 6.7 could be visualized in the Coomassie blue stained gel after migration in the first dimension. The four bands migrated in the second dimension as species of similar molecular mass, namely, 23 kDa.

The amino acid composition of the 23-kDa protein (Table I) showed a high percentage of serine and histidine. Pure 23-kDa protein obtained by electroelution from SDS–PAGE was subjected to Edman degradation on an automatic sequenator. No residue was released after 10 cycles, suggesting

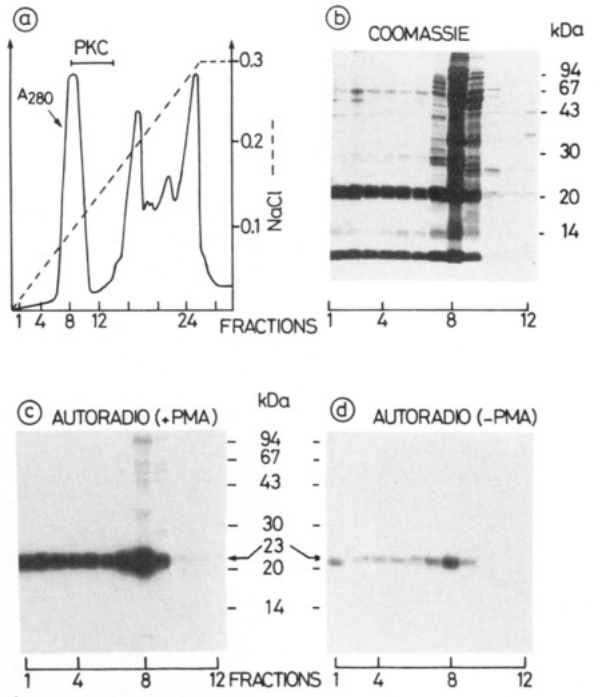

FIGURE 3: Mono Q chromatography of the 23-kDa protein from $^{32}P_i$-loaded bovine neutrophils activated by PMA. (a) Protein absorbancy and PKC activity of the eluted fractions. Cytosol from $^{32}P_i$-loaded neutrophils activated with PMA was loaded onto a DE-52 cellulose column as described in Figure 2a. Fractions from DE-52 cellulose column containing both PKC activity and the 23-kDa protein were pooled (pool 2 of Figure 2), desalted, concentrated on YM 30 membrane, and loaded onto a Mono Q column. A linear gradient of NaCl was applied to the Mono Q column as detailed under Materials and Methods. Absorption at 280 nm was continuously monitored. One-milliter fractions were collected, and PKC activity was measured on aliquots. (b) SDS–PAGE of fractions 1–12 recovered from Mono Q. Thirty-microliter fractions (1–12) were subjected to SDS–PAGE, and the gel was stained with Coomassie blue R250. Pool 1′ (fractions 1–4) contained the 23-kDa protein and the 7-kDa protein. Pool 2′ (fractions 5–8) contained the 23-kDa protein, the 7-kDa protein, and a fraction of PKC. (c) Autoradiography of the gel illustrated in panel b. (d) Autoradiography of a gel corresponding to a control experiment performed in the absence of PMA.

Table I: Amino Acid Composition of the 23-kDa Protein and MLC20 from Bovine Neutrophils

	23-kDa protein[a] (mol/mol)	MLC20[b] (mol/mol)		23-kDa protein[a] (mol/mol)	MLC20[b] (mol/mol)
Asp	15 ± 2	18–19	Met	5–6	4–5
Thr	6 ± 1	8–9	Ile	8 ± 1	6–7
Ser	18 ± 1	7–8	Leu	10 ± 1	10–11
Glu	24 ± 1	20–21	Tyr	4 ± 1	4–5
Pro	7 ± 1	5–6	Phe	7 ± 1	7–8
Gly	26 ± 1	13–15	His	10 ± 1	3–4
Ala	7–8	11	Lys	10 ± 1	9–10
Cys	nd	nd	Arg	5 ± 1	6–8
Val	7–8	4–5	Trp	0[c]	nd

[a] Mean values after analysis of five different preparations. [b] Analysis of two different preparations. [c] Determination by fluorescence spectrophotometry.

a blocked NH_2 terminus. No high-affinity Ca^{2+} binding sites for Ca^{2+} could be demonstrated by the technique of Maruyama et al. (1984). In contrast, Ca^{2+} was found to bind to the 7-kDa protein that comigrated with the 23-kDa protein during the first two steps of the purification procedure (data not shown).

Phosphorylation of the isolated 23-kDa protein by [γ-^{32}P]ATP required the presence of bovine neutrophil PKC and the appropriate cofactors, namely, Ca^{2+}, phosphatidylserine, and diacylglycerol. No phosphorylation occurred when these

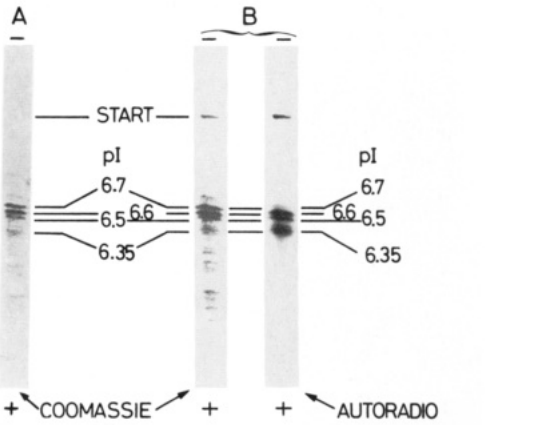

FIGURE 4: Isoelectric focusing of the ^{32}P-phosphorylated 23-kDa protein. The polyacrylamide gel used contained urea and ampholines (Materials and Methods). The 23-kDa protein (20 μg) was phosphorylated by neutrophil bovine PKC in the presence of [γ-^{32}P]ATP, Ca^{2+}, diacylglycerol, and phosphatidylserine and then subjected to IEF. The gel was stained by Coomassie blue and autoradiographed (B). Besides the major protein bands with p*I* in the range 6.35–6.7 corresponding to the 23-kDa protein, several minor bands were observed, possibly due to interaction with phospholipids used to activate PKC. A control IEF was carried out with 10 μg of the 23-kDa protein in the absence of PKC and [γ-^{32}P]ATP (A).

cofactors were omitted. Mercaptoethanol, which was always present in the reaction medium, facilitated the phosphorylation of the 23-kDa protein by PKC; in the absence of mercaptoethanol, not only was phosphorylation of the 23-kDa protein markedly depressed but an apparent dimer of the protein accumulated as mentioned above. It was checked, by use of histone type IIIS as a substrate, that without mercaptoethanol PKC was still fully active.

Analysis of the distribution of ^{32}P in different amino acid residues (cf. Materials and Methods) showed that only serine is phosphorylated. The kinetics of phosphorylation of the 23-kDa protein by PKC was adjusted so that the kinetics of phosphorylation were linear for at least 5 min. The double-reciprocal plots of the rate of phosphorylation of the 23-kDa protein vs the concentrations of [γ-^{32}P]ATP in the range 0.5–40 μM yielded a K_M value of 9 μM for ATP. The concentration of the 23-kDa protein was also varied in the range 0.2–10 μM, and its phosphorylation by PKC was studied by using a fixed concentration of [γ-^{32}P]ATP of 20 μM. The half-maximal rate of phosphorylation was attained with a concentration of the 23-kDa protein of 1 μM. The stoichiometry of phosphorylation of the 23-kDa protein was also studied under a number of conditions including the duration of the incubation with PKC and [γ-^{32}P]ATP and the variation of the [γ-^{32}P]ATP concentration. With 20 μM [γ-^{32}P]ATP, maximal phosphorylation was attained in 30 min, and the labeling stoichiometry was between 0.2 and 0.4 mol of ^{32}P incorporated/mol of 23-kDa protein. This moderate in vitro phosphorylation is discussed further.

The ^{32}P-phosphorylated 23-kDa protein was resolved by IEF into a few discrete bands that migrated in the gel between pH 6.3 and 6.7 at the same places as those revealed by Coomassie blue staining after phosphorylation (Figure 4B) or before phosphorylation (Figure 4A). It is noteworthy that the less acidic band strongly stained by Coomassie blue (p*I* = 6.7) was hardly radiolabeled, whereas the more acidic band with a p*I* of 6.35 faintly stained by Coomassie blue was revealed by autoradiography. Apparently, phosphorylation shifted protein to more acidic positions. Yet the preferential reactivity to phosphorylation of specific protein bands, for example, that with a p*I* of 6.35, is not excluded.

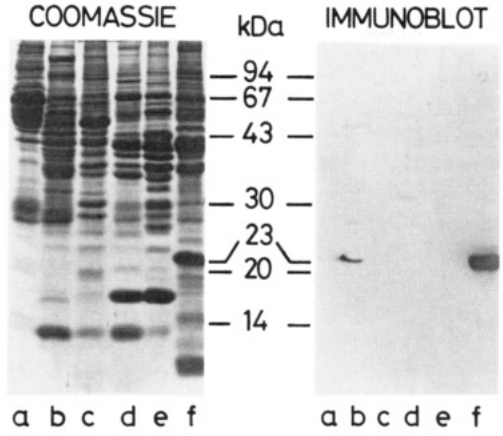

FIGURE 5: Immunodetection of 23-kDa protein in cytosols from bovine platelets (a), liver (b), brain (c), heart (d), skeletal muscle (e), and neutrophils (f). Cytosolic proteins (100–120 μg) from the different tissues were subjected to SDS–PAGE. The separated proteins were either stained by Coomassie blue or electrotransferred onto nitrocellulose and immunoreacted with the bovine neutrophil 23-kDa protein antiserum, as described under Materials and Methods.

Bovine brain PKC was also active as a catalyst for phosphorylation of the 23-kDa protein, though less than its neutrophil counterpart. Phosphorylation was also achieved with PKM, the proteolytic cleavage product of bovine neutrophil PKC that contains the catalytic center of the enzyme. No phosphorylation of the 23-kDa protein was detected with cAMP-dependent protein kinase. Furthermore, the addition of 5 mM cAMP in the phosphorylation reaction medium in the presence of cytosol did not have any significant effect on the phosphorylation of the 23-kDa protein.

Immunological Properties of the 23-kDa Protein. Western blot analysis was also used to explore the immunological specificity of cytosolic extracts prepared from different bovine tissues (Figure 5). Antibodies raised against the bovine neutrophil 23-kDa protein reacted with a protein of similar size present in a liver extract, probably due to the macrophagic Kupffer cells of liver. However, the reaction was faint compared to that observed with the bovine neutrophil 23-kDa protein. No immunoreactive protein was detected in brain, heart, and skeletal muscle extracts. A trace of an immunoreactive protein with an apparent mass of 23 kDa was occasionally detected in platelets. The 23-kDa protein antibodies prevented the PKC-dependent phosphorylation of the 23-kDa protein; by comparison, no inhibition was found on the PKC-dependent phosphorylation of histone type IIIS (data not shown).

Binding of [^{35}S]GTP-γ-S to the 23-kDa Protein. The purified 23-kDa protein electrotransferred from SDS–polyacrylamide gel onto nitrocellulose was found to bind efficiently [^{35}S]GTP-γ-S (Figure 6B, track b). When the same experiment was performed with crude cytosol, the 23-kDa protein was the most efficient protein to bind [^{35}S]GTP-γ-S (Figure 6B, track a). Competition for binding between [^{35}S]GTP-γ-S and other G nucleotides to the 23-kDa protein was analyzed by dot blot on a nitrocellulose sheet followed by extensive washing. Binding of [^{35}S]GTP-γ-S to the 23-kDa protein in the presence of a 1000-fold excess of GTP was decreased by about 50%; in contrast, GMP and ATP had no effect (data not shown), in accordance with the functioning of the 23-kDa protein as a GTP-binding protein. For further characterization, the 23-kDa protein was analyzed by IEF (Figure 6C). After electrotransfer onto a nitrocellulose sheet and incubation of the sheet with [^{35}S]GTP-γ-S, several labeled bands were revealed, with p*I* values similar to those stained with Coomassie blue in the absence of treatment with GTP-γ-S.

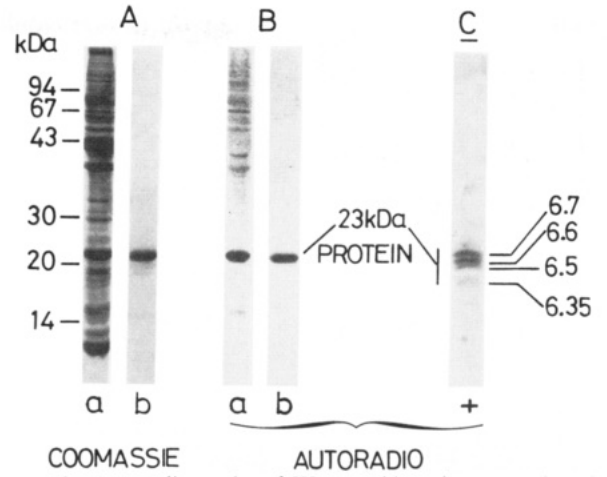

FIGURE 6: Autoradiography of Western blots demonstrating the binding of [^{35}S]GTP-γ-S to the 23-kDa protein in crude cytosol and the purified 23-kDa protein. After SDS–PAGE of crude cytosol (100 μg) and the purified 23-kDa protein (5 μg), the proteins on the gels were either stained by Coomassie blue (panel A, lanes a and b) or electrotransferred onto a nitrocellulose sheet, followed by incubation with [^{35}S]GTP-γ-S and autoradiography (panel B, lanes a and b). Components of the 23-kDa protein were resolved by IEF and electrotransferred onto nitrocellulose, followed by incubation with [^{35}S]GTP-γ-S and autoradiography (panel C).

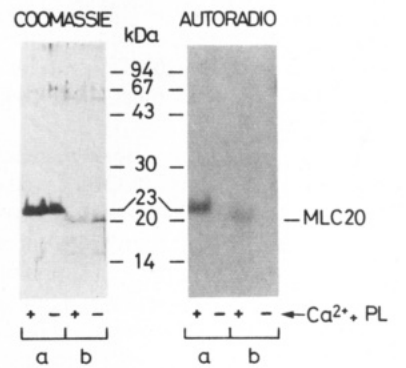

FIGURE 7: In vitro phosphorylation of the 23-kDa protein and regulatory myosin light chain (MLC20) purified from bovine neutrophils by PKC. The PKC-dependent phosphorylation of the 23-kDa protein (lanes a) and MLC20 (lanes b) purified from bovine neutrophils in the presence of [γ-^{32}P]ATP was performed in the presence of Ca^{2+}, diacylglycerol, and phosphatidylserine. A control was carried out in the absence of Ca^{2+} and lipids and in the presence of EGTA. The two proteins were submitted to SDS–PAGE and stained with Coomassie blue. ^{32}P-Phosphorylated proteins were revealed by autoradiography.

The 23-kDa Protein Is Different from the Regulatory Light Chain of Myosin (MLC20) Present in Neutrophils. It was recently reported that stimulation of human neutrophils by PMA results in the phosphorylation of a 20-kDa protein, tentatively identified with a myosin light chain (Pontremoli et al., 1987a,b). A protein of similar mass is present in bovine neutrophils. This protein, termed MLC20, was purified (cf. Materials and Methods) by following the procedure described for the purification of the regulatory light chain of smooth muscle myosin from chicken or turkey gizzards (Hathaway & Haeberle, 1983). Bovine neutrophil MLC20 was phosphorylated by [γ-^{32}P]ATP in the presence of neutrophil PKC, and its rate of migration in a 13% polyacrylamide gel was compared to that of phosphorylated 23-kDa protein (Figure 7). The apparent molecular mass of MLC20 assessed by SDS–PAGE was 20 kDa (Figure 7b), independent of the technique of migration used and the percentage of acrylamide in the gel. This is in contrast with the variation of the apparent

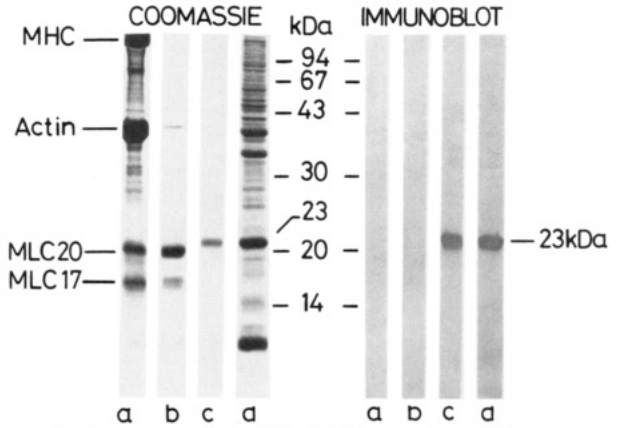

FIGURE 8: Immunoreactivity of the 23-kDa protein and the regulatory myosin light chain (MLC20) purified from bovine neutrophils. A preparation of actomyosin (50 μg) containing the myosin heavy chain (MHC), the two light chains (MLC20, MLC17), and actin (lane a), the partially purified myosin from chicken gizzard (8 μg) (lane b), the 23-kDa protein (2 μg) (lane c), and crude cytosol from bovine neutrophils (50 μg) (lane d) were subjected to SDS–PAGE. The proteins in the gel were either stained with Coomassie blue R250 or electrotransferred onto nitrocellulose to test their immunoreactivity against the 23-kDa protein antiserum.

molecular mass of the so-called 23-kDa protein, which was effectively 23 kDa in a 13% polyacrylamide gel (Figure 7a) and 20 kDa in a 20% polyacrylamide gel (data not shown). MLC20 also differed from the 23-kDa protein in the amino acid composition, which showed a much lower content of serine and histidine (Table I).

By immunoblotting, we observed no cross-reactivity of anti 23-kDa protein antibodies with MLC20 present in a crude bovine neutrophil actomyosin preparation and with a related myosin, namely the MLC20 isolated from chicken gizzard, whereas in the same conditions the 23-kDa protein was highly reactive (Figure 8). Finally, both serine and threonine residues of bovine neutrophil MLC20 were phosphorylated by [γ-^{32}P]ATP in the presence of bovine neutrophil PKC (data not shown), as in chicken and turkey gizzard muscle MLC20 (Bengur et al., 1987; Ikebe et al., 1987), whereas serine only was phosphorylated in the 23-kDa protein as previously mentioned. All these results taken together stressed the structural and functional differences between the 23-kDa protein and MLC20 in bovine neutrophils.

Discussion

The present work stems from the finding that during the course of bovine neutrophil PKC purification (Dianoux et al., 1989) a protein of molecular mass of 23 kDa comigrated with PKC and was phosphorylated by [γ-^{32}P]ATP, in the presence of the coeluted PKC and added Ca^{2+}, phosphatidylserine, and diacylglycerol, suggesting that it might be a natural substrate for PKC. This idea is reinforced by the present demonstration that a 23-kDa protein is predominantly phosphorylated in bovine neutrophils loaded with $^{32}P_i$ and supplemented with PMA, as PKC is a known target for PMA, and is activated by PMA. The function of the 23-kDa protein and the significance of its phosphorylation are not defined at present. However, the association of a fraction of the 23-kDa protein with cytoskeleton might have a physiological significance in terms of cell motility.

Comments on the Purification of the 23-kDa Protein and Its Distribution. In the course of the purification of the bovine neutrophil 23-kDa protein, attention was directed to the fact that the 23-kDa protein comigrated during the first two steps not only with PKC but also with a smaller protein of molecular mass of 7 kDa. Although the concomitant presence of the three proteins might be fortuitous, it is equally possible that transient association between the three proteins might occur in vivo. For example, the 7-kDa protein could function as a store for Ca ions directly available for PKC activation, and in turn the 23-kDa protein would be phosphorylated by the activated PKC. Association between PKC and the 23-kDa proteins was, however, rather loose as the two proteins were recovered at different places in a sucrose gradient after high-speed centrifugation. In contrast, urea was required to resolve the 23- and 7-kDa proteins by molecular sieve chromatography, indicating therefore strong interactions between the two proteins.

In the absence of enzymatic activity, the purity of the 23-kDa protein was assessed by SDS–PAGE, IEF, and 2D-PAGE. The similar pattern of labeling by [γ-^{32}P]ATP and [^{35}S]GTP-γ-S of the protein bands of the 23-kDa protein resolved by IEF strongly suggests that the preparation is free of contaminants. The distribution of the 23-kDa protein has been examined in different bovine tissues and in neutrophils from human blood and rabbit peritoneum. Upon addition of antibodies raised against the bovine neutrophil 23-kDa protein, besides the highly reactive cytosol from bovine neutrophils, small amounts of an immunoreactive 23-kDa protein were revealed in cytosolic extract from beef liver. This is consistent with the presence of Kupffer cells as resident macrophages of the liver, capable of eliciting a number of responses similar to those of neutrophils upon activation. No immunoreactive protein was found in human and rabbit neutrophil. On the other hand, cytosols from human and rabbit neutrophils were incubated with [γ-^{32}P]ATP and PKC for a search of a phosphorylated 23-kDa protein and other phosphorylated proteins. Comparison of the protein profiles after SDS–PAGE of cytosolic extracts from human, rabbit, and bovine neutrophils showed marked differences in the case of small proteins of molecular mass below 30 kDa. A faint radiolabeled band corresponding to a molecular mass of 23 kDa was detected in very low amounts in human and rabbit neutrophils (data not shown) compared to bovine neutrophils. It therefore appears that the 23-kDa protein is peculiar to or overexpressed in bovine neutrophils.

Significance of the Multiple Components of the 23-kDa Protein Resolved by IEF. High-resolution gel analysis by IEF indicated that purified 23-kDa protein consisted of at least four components with p*I* values ranging between 6.3 and 6.7, all of which were able to incorporate $^{32}P_i$ from [γ-^{32}P]ATP and to bind [^{35}S]GTP-γ-S. The same IEF pattern was found with at least 10 different preparations of the 23-kDa protein. These results can be interpreted in terms of different isoforms of the 23-kDa protein or a single 23-kDa protein with multiple phosphorylation states, the more acidic p*I* corresponding to the higher phosphorylation state. The latter hypothesis might explain the discrepancy between the rapid and extensive in vivo phosphorylation of the 23-kDa protein in intact bovine neutrophils treated by PMA and the limited in vitro phosphorylation of purified 23-kDa by PKC. In fact, in freshly prepared bovine neutrophils preincubated with $^{32}P_i$ and then treated by PMA, rapid renewal of the phosphate residues of the phosphorylated forms of the 23-kDa protein would be the result of two fast reactions, i.e., dephosphorylation by a phosphatase and rephosphorylation by PKC with incorporation of $^{32}P_i$ from newly made endogenous [γ-^{32}P]ATP. On the other hand, the in vitro phosphorylation of purified 23-kDa protein by PKC in the presence of [γ-^{32}P]ATP would be limited by the already present phosphorylated forms of the 23-kDa protein; under

these conditions, only the nonphosphorylated form of the 23-kDa protein would be readily labeled.

Comparison of the 23-kDa Protein with Proteins of Similar Size. The properties of the 23-kDa protein were compared to those of some neutrophil proteins with similar molecular mass. The most obvious candidate was the regulatory light chain of myosin termed MLC20. The two proteins exhibited a propensity to PKC-dependent phosphorylation by [γ-^{32}P]-ATP. Yet a scrutiny of other properties showed noticeable differences in the amino acid composition, the immunoreactivity, and the nature of phosphorylated amino acids. Other potential candidates among proteins of similar size present in neutrophils were calmodulin (Waterson et al., 1980) and the small subunit of the low-potential cytochrome *b* typical of neutrophils (Parkos et al., 1988). Yet these proteins showed marked structural and functional differences with the 23-kDa protein. Calmodulin is not a substrate for PKC, and it further differs from the 23-kDa protein by its amino acid composition and the presence of high-affinity Ca^{2+} binding sites (Waterson et al., 1980). The small subunit of neutrophil cytochrome *b* is highly hydrophobic and requires detergent for solubilization; in contrast to the 23-kDa protein, it is rich in tryptophanyl residues (Parkos et al., 1988).

Recent experiments in our group (Ligeti et al., 1989) have shown that after electrotransfer on nitrocellulose of bovine neutrophil cytosolic proteins separated by SDS–PAGE, a low molecular weight component exhibited a high propensity for binding [^{35}S]GTP-γ-S specifically. The purified 23-kDa protein described in the present paper is this component. It is indeed able to bind [^{35}S]GTP-γ-S in a specific manner, since GTP, but not GMP or ATP, compete against binding of [^{35}S]GTP-γ-S. The behavior of the 23-kDa protein of bovine neutrophils as a GTP-binding protein is all the more intriguing since Bokoch et al. (1988) recently reported that human neutrophils contain a membrane-bound GTP-binding protein with a molecular mass of 23 kDa that is ADP-ribosylated by [^{32}P]NAD in the presence of *Clostridium botulinum* toxin. We also found in bovine neutrophil cytosol a small molecular mass protein with high propensity for ADP-ribosylation by exoenzyme C3 from *C. botulinum* (data not shown). However, the rates of migration on SDS–PAGE of the 23-kDa protein and the ADP-ribosylated protein from bovine neutrophils were clearly different.

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Registry No. PKC, 9026-43-1; 5′-ATP, 56-65-5.

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