The 23-Kilodalton Protein, a Substrate of Protein Kinase C, in Bovine Neutrophil Cytosol Is a Member of the S100 Family†

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ABSTRACT: A bovine neutrophil protein termed p23 because of an apparent molecular mass of 23 kDa in SDS–PAGE is present in large amounts both in a soluble form in the cytosolic fraction of bovine neutrophil homogenates and associated to the cytoskeleton. P23 is accompanied during the first steps of the purification procedure by a smaller size protein termed p7 on the basis of a rate of migration in SDS–PAGE corresponding to a 7-kDa protein [Stasia, M. J., Dianoux, A. C., & Vignais, P. V. (1989) *Biochemistry 28*, 9659–9667]. The two proteins, p23 and p7, have been purified to homogeneity by an improved procedure consisting of two chromatographic steps. The electrospray mass spectrometry technique applied to p23 and p7 indicated molecular masses close to 17 and 10 kDa, respectively, significantly different from the masses derived by SDS–PAGE. Bovine neutrophil p23 and p7 presented large primary structure homologies with two human proteins, MRP14 and MRP8, which are expressed in large amounts in macrophages under conditions of chronic inflammation. In addition, p23 and p7 cross-reacted with monoclonal antibodies specific of MRP14 and MRP8. Bovine p23 and p7 bound Ca^{2+}, and their amino acid sequences contained two Ca^{2+}-binding domains per protein, largely identical to those of human MRP14 and MRP8. Bovine p23 and p7 associated together to form a heterodimeric complex, which largely escaped attack by trypsin, whereas the isolated p23 and p7 components were readily digested. These features are typical of Ca^{2+}-binding proteins belonging to the S100 family. Despite similarities, bovine p23 differed from human MRP14 by a more extended C-terminal region which most likely contained the PKC-specific site of phosphorylation of p23. By an immunofluorescent technique, the p23,p7 complex was found to form clusters evenly distributed in the cytoplasm of resting neutrophils and to concentrate under the plasma membrane in phorbol-activated neutrophils, suggesting possible association with cytoskeleton components. Whereas the p23 subunit in the p23,p7 complex is phosphorylated in bovine neutrophils activated by phorbol esters or opsonized zymosan, chemotaxis was hardly affected by added p23,p7 complex, and there was no significant effect of p23 antibodies on oxidase activation in a cell-free system.

A protein of 23 kDa (p23) abundantly present in bovine neutrophil cytosol has been shown to exhibit strong propensity to phosphorylation either in vivo, during the course of neutrophil activation by phorbol myristate acetate (PMA),[1] or in vitro, using the purified protein incubated in the presence of ATP and protein kinase C (PKC) with Ca^{2+}, phosphatidylserine, and diacylglycerol as activators or the proteolyzed form of PKC (PKM) without activators; cAMP-dependent kinase was ineffective (Stasia et al., 1989). During the course of the purification of p23, another abundant protein of 7 kDa (p7) also located in bovine neutrophil cytosol was found to coeluate with p23 during the first chromatographic step. Resolution of p23 and p7 could, however, be achieved by addition of a denaturating agent prior to molecular sieve chromatography, suggesting that p23 and p7 might form a tight complex in the cytosol.

In this paper, we describe a method of rapid and efficient purification of p23 and p7 from bovine neutrophil cytosol. We demonstrate by using electrospray spectrometry that the true molecular masses of p23 and p7 are 17.3 and 10.5 kDa, respectively, differing markedly from the masses of 23 and 7 kDa determined by SDS–PAGE with standard protein markers. Exploration of the amino acid sequence of p23 and p7 and antibodie's cross-reactivity led us to the unexpected conclusion that these two proteins in bovine neutrophils might be the counterparts of the two well-studied human Ca^{2+}-binding proteins of the S100 family, MRP14 of apparent mass 14 kDa and MRP8 of apparent mass 8 kDa, abundantly expressed in resident tissue macrophages under conditions of chronic inflammation (Odink et al., 1987; Lagasse & Clerc, 1988), and recently detected in human neutrophils (Edgeworth et al., 1991). Human MRP14 was found to be phosphorylated in myeloid cells incubated in the presence of Ca^{2+} and ionomycin (Edgeworth et al., 1989), but in contrast to bovine neutrophil p23, phosphorylation of MRP14 was not PKC-dependent. We therefore decided to characterize in detail the structural and functional properties of bovine neutrophil p23 and p7. Here we demonstrate that p23 and p7 are able to bind Ca^{2+} and

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[1] Abbreviations: 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; PBS, phosphate-buffered saline; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; DTT, dithiothreitol, ESMS, electrospray mass spectrometry; LTB4, leukotriene B4; TFA, trifluoroacetic acid, MRP14 and MRP8, macrophage-related proteins; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PVDF, poly(vinylidene difluoride); ZAS, zymosan-activated serum.

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to exhibit strong propensity to form a heterodimer complex in which some peptide bonds are hindered to the action of proteolytic enzymes like trypsin. We also show that, although p23 and p7 in bovine neutrophils and their counterparts in human neutrophils have in common some properties, they somewhat differ by their size, the specificity of their epitopes, and some specific features of p23 phosphorylation by PKC.

Materials and Methods

Materials. The following materials were supplied by the companies indicated: $H_3{}^{32}PO_4$ and $^{45}Ca^{2+}$, Amersham (U.K.); Mops, Tris, EGTA, CAPS, BSA, PMA, leupeptin, soybean trypsin inhibitor, zymosan, leukotriene B4, and A23187, Sigma Chemical Co.; TPCK, TLCK, β-mercaptoethanol, SDS, TFA, and Coomassie brilliant Blue R250 and G250, Serva; acrylamide, bis(acrylamide), aquamount, acetonitrile, Tween 20, and low molecular mass markers, BDH Biochemicals; RPMI 1640 medium, endopeptidase Arg-C, trypsin, and DTT, Boehringer; diaminobenzidine, EDTA, glucose, NaCl, methanol, and acetic acid, Prolabo; fluorescent $F(ab')_2$ fragments of goat IgG, BioArt; goat IgG with linked peroxidase, Miles; nitrocellulose membrane BA83, Schleicher & Schuell; Problott membrane, Applied Biosystem; DEAE-Sephacel and standard molecular mass markers, Pharmacia; HPLC chromatography system and C-18 RP, μBondapak column, Waters.

Biological Preparations. Between 5×10^9 and 1×10^{10} bovine neutrophils were isolated from 10 L of fresh blood collected in the presence of 5 mM EDTA, according to Morel et al. (1985). The cytosolic fraction of the cells was obtained as follows. Neutrophils [$(2–3) \times 10^8$ cells/mL] were suspended in 0.25 M sucrose, 10 mM Mops, pH 7.4, 2 mM EDTA, 10 mM EDTA, 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 cells were disrupted by ultrasonic irradiation for 4×15 s at 4 °C with a Branson sonifier at 60-W output. The homogenate was first centrifuged for 10 min at 10000*g* and then for 1 h at 140000*g*. The resulting supernatant was termed cytosol. The pellet contained the membranous fraction.

Purification of P23 and P7 from Bovine Neutrophil Cytosol. The cytosolic fractions (40–50 mL) containing 10 mg of protein/mL were loaded on a DEAE-Sephacel column (50 mL) equilibrated with 20 mM Mops, pH 7.4, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF. The proteins were eluted with 300 mL of a linear NaCl gradient (0–300 mM). To visualize the presence of p23 and p7, whose absorbance at 280 nm was too weak, aliquots of the collected fractions were submitted to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli & Favre, 1973). The fractions containing p23 and p7 as major components were injected directly on an HPLC reverse-phase (RP C-18) μBondapak column (4.6×250 mm) equilibrated with 0.1% aqueous TFA and 25% acetonitrile. Elution was monitored by the absorbance at 215 nm at a flow rate of 1 mL/min. Resolution of p23 and p7 was achieved by a gradient of 25–50% acetonitrile. The purity of the two proteins was controlled by SDS–PAGE followed by Coomassie Blue staining.

Molecular Mass Determination of P23 and P7 by Electrospray Mass Spectrometry (ESMS). The electrospray ionization method (Van Dorsselaer et al., 1990) was used to ascertain the apparent molecular masses of p23 and p7 determined by SDS–PAGE. ESMS was carried out with a VG Bio Q instrument. Samples of p23 and p7 (30–100 pmol/μL) in methanol/water (1/1) containing 1% acetic acid were injected into the ion source at a rate of 3 μL/min. The mass spectra were obtained from scans of mass to charge ratio values between 600 and 1600, over 10-s intervals. The instrument was calibrated with horse heart myoglobin.

Cyanogen Bromide Cleavage of P23. The p23 protein was cleaved, using 200 mM CNBr in 70% formic acid at room temperature, for 24 and 48 h to ensure completeness (Gross, 1967). After concentration under vacuum, the residue was taken up in 0.1% TFA and 10% acetonitrile, and the solution was injected into an HPLC RP C-18 μBondapak column (4.6×250 mm) equilibrated in the same medium. A 10–50% acetonitrile gradient was applied to the column with a flow rate of 1 mL/min. Elution was monitored by recording the absorbance at 215 nm. The CNBr cleavage products contained in the eluted fractions were separated by SDS–PAGE using a 20% acrylamide gel and silver-stained.

SDS–Polyacrylamide Gel Electrophoresis. Protein samples in 60 mM Tris-HCl, pH 7.2, containing 15% glycerol, 5% β-mercaptoethanol, 2.3% SDS, and 0.001% bromophenol were incubated for 30 min at 60 °C. The solubilized samples were submitted to slab SDS–PAGE at 30 mA for 3 h as described by Laemmli and Favre (1973), using a 4 or 10% polyacrylamide stacking gel and a 13 or 20% resolving gel. The standard protein markers were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa), and CNBr peptides from myoglobin with masses of 17, 14.5, 10.7, 8.2, and 6.3 kDa. Proteins and peptides were stained by Coomassie Blue R250 or silver (Wray et al., 1981). For autoradiography, gels were dried under vacuum and exposed to Fuji X-ray films. In some experiments, the radiolabeled proteins were excised from the gel, and the incorporated radioactivity was measured by liquid scintillation counting.

Isolation of P7 for Sequence Analysis. The proteins p23 and p7 obtained by DEAE-Sephacel chromatography were separated by slab SDS–PAGE and electrotransferred to a poly(vinylidene difluoride) (PVDF) membrane (Problott), using a transfer buffer consisting of 10 mM CAPS, pH 11, and 10% methanol for 30 min at 50 V in a blotting apparatus (Hoefer Scientific Instruments). The membrane was washed with water, stained with 0.1% Coomassie Blue in 40% methanol and 1% acetic acid for 1 min, destained in 50% methanol, and finally rinsed with water. The stained protein band was cut out and placed in the reaction chamber of an Applied Biosystem 477A protein sequencer directly coupled to an HPLC 120 A chromatography system to identify the phenylthiohydantoin amino acid derivatives. Samples of p7 purified by HPLC chromatography were also sequenced directly by Edman degradation.

Western Blot Analysis of P23. Immunodetection by Western blot was carried out with polyclonal antibodies raised in rabbit against the bovine neutrophil p23 (Stasia et al., 1989) and with two monoclonal antibodies, CF557 and CF145, directed against human MRP14 and MRP8, respectively (Hayward et al., 1986), and kindly provided by Dr. V. Van Heyningen (Edinburgh, U.K.). The monoclonal antibodies were used without dilution, whereas the p23 antiserum was diluted 500-fold. The immunoreaction was performed on the proteins electrotransferred on a 0.22-μm nitrocellulose membrane (Towbin et al., 1979) after they have been separated by SDS–PAGE. In the case of monoclonal antibodies, the transfer buffer was a sodium phosphate buffer, pH 9.4, instead of Tris/glycine buffer. Both transfer buffers contained 20% methanol and 0.1% SDS. The antigen–antibody complexes were revealed by goat anti-rabbit or anti-mouse IgG linked to peroxidase, the bound peroxidase activity being subsequently

revealed with 50 mM diaminobenzidine in 50 mM sodium phosphate, pH 7.2, in the presence of H_2O_2.

Binding of $^{45}Ca^{2+}$. Proteins resolved by SDS–PAGE were electrotransferred to a 0.22-μm nitrocellulose sheet (Towbin et al., 1979) in order to assay their Ca^{2+}-binding capacity under the conditions described by Maruyama et al. (1984). The nitrocellulose sheet was immerged in a buffered medium, pH 7, supplemented with 2 μmol of $^{45}Ca^{2+}$ (20 μCi) for 10 min. The nitrocellulose was washed with water and then with 50% ethanol and dried before exposure to a Fuji X-ray film for 1–2 days. The specificity of $^{45}Ca^{2+}$ binding was ascertained by a chase with 100 μM unlabeled Ca^{2+} for 1 h.

Protein Determination. The protein content of the samples was determined by the method of Bradford (1976), using bovine serum albumin (BSA) as a standard.

In Vivo Phosphorylation of P23. Isolated bovine neutrophils (10^7 cells) were loaded with [^{32}P]P_i by a 30-min incubation at 37 °C with neutralized $H_3{}^{32}PO_4$ (250 μCi) in 1.5 mL of 20 mM Tris-HCl, pH 7.4, and 0.9% NaCl. These neutrophils were then incubated at 37 °C with different activating agents, namely, PMA (4 μg) or opsonized zymosan (50 mg) or LTB4 (100 nM) for 5 or 20 min. Controls without activation were run in parallel. The incubation was terminated by dilution of the cell suspension with 10 volumes of ice-cold incubation buffer. The cells were centrifuged at 4 °C at 400*g* for 10 min, washed once with the ice-cold incubation medium, and then homogenized in 300 μL of buffer A, to prepare the cytosol and membrane fractions as described under Biological Preparations. Proteins were separated by SDS–PAGE. The labeled band corresponding to p23 was excised, and its radioactivity was determined by scintillation counting.

Intracellular Detection of P23 by Immunofluorescence. Bovine neutrophils (5×10^5 cells/mL) suspended in RPMI 1640 medium supplemented with 10% BSA were activated at 37 °C with PMA (0.5 μg/mL) for 1 min and left to sediment for 40 min on polylysin-coated coverslips. After a rapid wash with PBS, the cells were fixed with methanol at −20 °C for 6 min and washed again 3 times with PBS supplemented with 0.1% Tween 20 (PBS-T). They were then left in contact for 30 min at room temperature with the polyclonal antiserum directed against p23, diluted to 1/500. After three additional washings with PBS-T, the anti-p23 immuno complex was revealed with fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragment of goat anti-rabbit antibodies. This was followed by three washings with PBS-T and incubation with a 1/500-diluted solution of mouse monoclonal antibodies directed against actin. After three more washings with PBS-T, the anti-actin immuno complex was revealed with the rhodamine-conjugated $F(ab')_2$ fragment of anti-mouse antibodies. Nuclei were stained with 1% Hoescht solution. The cells were mounted with aquamount and examined with an epifluorescence microscope. Control assays in the absence of PMA and specific antibodies against p23 were performed in parallel.

Degranulation. Bovine neutrophils (2×10^7 cells/mL) were suspended in PBS supplemented with 20 mM glucose. The suspension was incubated at 37 °C for 30 min in the presence of either 1 μg of PMA/mL or 2 mg/mL opsonized zymosan or 5 μM A23187 plus 1 mM Ca^{2+}. Then the cells were sedimented by centrifugation at 400*g* for 10 min at 4 °C. The supernatants were withdrawn for assays of β-glycuronidase and myeloperoxidase activities (Dewald & Baggiolini, 1986), and an aliquot was subjected to SDS–PAGE to visualize the presence of p23 and p7 after Coomassie Blue staining. In addition, lactate dehydrogenase activity was determined to assess the extent of nonspecific release of cytosol. The total amount of glycuronidase and myeloperoxidase in bovine neutrophils was determined, using a lysate of the cells treated with 0.1% Triton X-100 for 5 min at 37 °C.

Chemotaxis. Spontaneous or ligand-directed migration of bovine neutrophils was measured by the agarose gel technique described by Nelson et al. (1975), using zymosan-activated bovine serum (ZAS) as a chemotactic factor. The cells were suspended in PBS supplemented with 20 mM glucose. Three-well series were cut in agarose. In each of them, the outer well received ZAS (10 μL), or ZAS (10 μL) plus 10 μg of p23 in 10 μL, or ZAS (10 μL) plus 10 μg of p7,p23 complex in 10 μL. The central well was filled with neutrophils (10^6 cells) and the inner well with PBS as a nonchemotactic control medium. The agarose plates were incubated in a humidified atmosphere containing 5% CO_2 for 2 h at 37 °C. This was followed by fixation of the cells. Agarose was then removed, and the cells were stained with the Giemsa solution. The distances of migration of cells from the margin of the center well toward the well containing ZAS and the well containing PBS were measured under the microscope and used as indexes of chemotaxis and spontaneous migration, respectively.

NADPH Oxidase in a Cell-Free System. Reduction of O_2 into O_2^- by the neutrophil oxidase was measured in a cell-free system of oxidase activation (Ligeti et al., 1988). This system contained membranes and cytosol fractions prepared from a bovine neutrophil homogenate, GTPγS, arachidonic acid, and Mg^{2+}. The amounts of arachidonic acid and proteins were adjusted to provide maximal oxidase activation (Pilloud et al., 1989). The rate of production of O_2^- was determined spectrophotometrically at 550 nm by the superoxide dismutase-inhibitable reduction of cytochrome *c* in the presence of NADPH. The role of p23 in oxidase activation was assayed by addition of p23 antiserum at the onset of the activation process.

Results

The Two Predominant Proteins of Bovine Neutrophil Cytosol, P23 and P7, Are Structurally Related to the Human Macrophage Proteins MRP14 and MRP8. P23 and p7 are two predominant proteins in bovine neutrophil cytosol (Figure 1B, lane a). These two proteins have the peculiarity to comigrate on column chromatography, whatever the type of column used, either ion exchangers or molecular sieves, using mild eluting buffers. They could, however, be resolved by treatment with urea (Stasia et al., 1979). An improved method of resolution and purification of p23 and p7 from bovine neutrophil cytosol in only two steps is described under Materials and Methods. In the first step consisting of chromatography on a DEAE-Sephacel column eluted by a linear gradient of NaCl, p23 and p7 were recovered as major components at about 150 mM NaCl, following the bulk of the other cytosolic proteins, as shown by SDS–PAGE (Figure 1B, lane b). These fractions were directly injected in an HPLC RP C-18 column, and a convex gradient of acetonitrile was applied (Figure 1A). The two proteins, p23 and p7, were recovered in two separate peaks at 18.50 min (p7) and 20.20 min (p23) (Figure 1A), and their purity was found to be satisfactory by SDS–PAGE (Figure 1B, lanes d and c). The amount of pure p23 and p7 recovered from 10^{10} neutrophils was between 15 and 20 mg. Taking losses inherent in fractionation procedures into account, the percentage of the two proteins in bovine neutrophil cytosol was estimated to amount to about 20% of the bulk of cytosolic proteins.

The recently introduced ESMS technique (see Materials and Methods) was applied to accurately determine the molecular masses of p23 and p7. The mass spectra obtained with

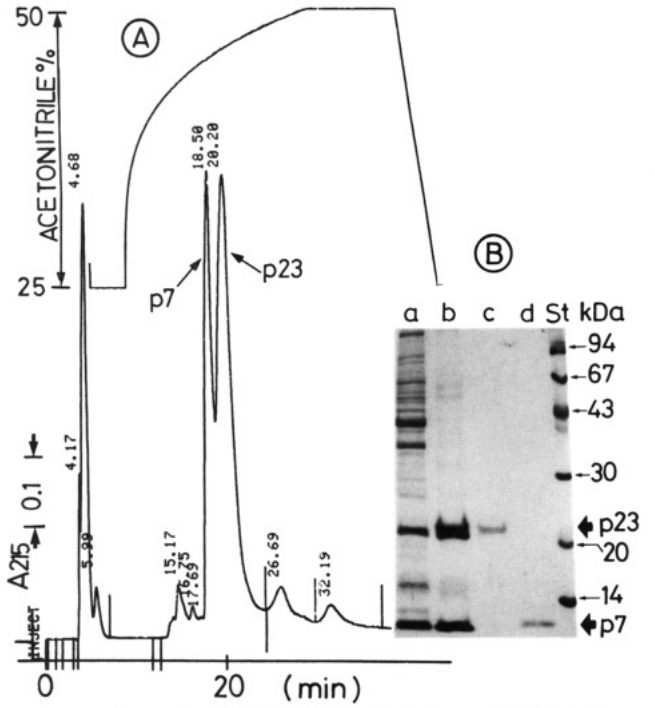

FIGURE 1: Separation of p23 and p7 by HPLC on a C-18 RP column. (A) Pattern of elution. Conditions are described under Materials and Methods. Absorbance was monitored at 215 nm as a function of time. The peak eluted at 18.5 min contained pure p7 and that eluted at 20.20 min pure p23. (B) SDS–PAGE of fractions from the different steps of purification of p23 and p7. Samples were separated in a 13% acrylamide gel and stained with Coomassie Blue. (St) Standard markers; (a) bovine cytosol; (b) DEAE-Sephacel column fraction eluted around 150 mM NaCl; (c) HPLC fraction eluted at 20.20 min (p23); (d) HPLC-fraction eluted at 18.50 min (p7); p23 and p7 are indicated by thick arrows.

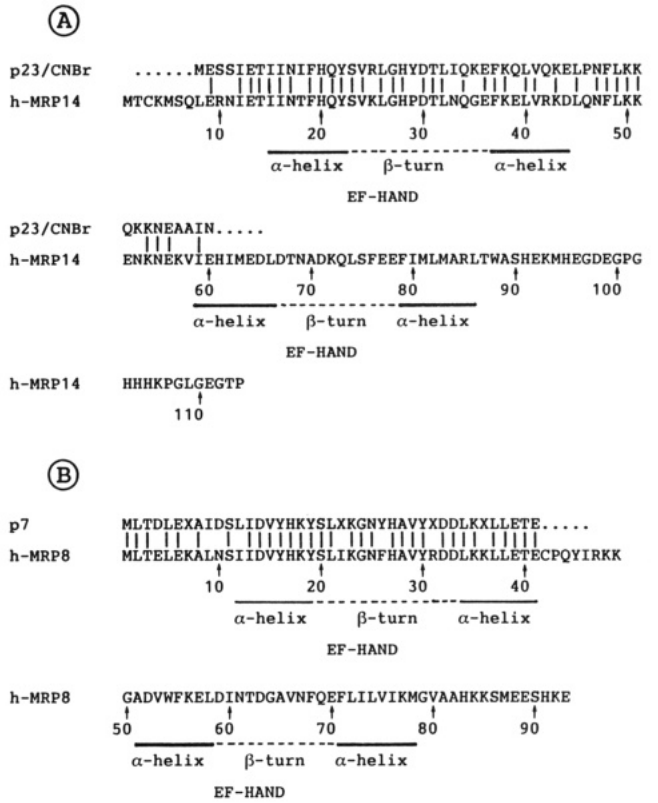

FIGURE 2: Comparison of amino acid sequences of bovine p23 and p7 with those of human MRP14 and MRP8. (A) A large cleavage product issued from bovine p23 by CNBr digestion was sequenced. The figure shows the sequence corresponding to the first 52 amino acids of this product. This sequence was aligned with that of human MRP14 (Odink et al., 1987), and the identical amino acids were indicated by a vertical bar. (B) The amino acid sequence of bovine p7 was obtained by Edman degradation directly from the N-terminus of this protein and aligned with that of human MRP8 (Odink et al., 1987), with vertical bars indicating identical residues. The Ca^{2+}-binding domains (EF-hands), comprising an α-helix followed by a β-turn and a second α-helix, are indicated by horizontal bars connected by dashed lines.

p23 showed the presence of two components of mass 17 288 ± 7 and 17 362 ± 7 Da in equal amounts. In the case of p7, two components of masses 10 460 ± 1 and 10 537 ± 1 Da were present in a stoichiometric ratio of 3 to 1. Although the molecular masses measured by ESMS markedly differ from those estimated by SDS–PAGE, we prefer for the sake of simplicity and reference to the previous work (Stasia et al., 1989) to continue to designate the two proteins as p23 and p7.

The primary structure of peptides from p23 and p7 was determined by Edman degradation. As p23 has a blocked N-terminal amino acid residue and contains four to five methionine residues (Stasia et al., 1989), a possible approach to amino acid sequence was CNBr cleavage. In two separate experiments, CNBr treatment in 70% formic acid for 24 and 48 h did not result in modification of the peptide composition of the digest. Further, no cleavage occurred when the protein was treated by 70% formic acid alone, suggesting that p23 does not contain Asp–Pro bonds.

A number of peptide fragments arising from CNBr cleavage of p23 could be resolved by HPLC (data not shown). The fraction corresponding to the top of the major peak eluted at 59.36 min was found by SDS–PAGE to contain a peptide of 7.3 kDa whose amino acid sequence was determined by Edman degradation. The first 52 residues of this peptide were identified (Figure 2A). Other peptides obtained from p23 by enzymatic digestion with endopeptidase Arg-C and with trypsin after succinylation (data not shown) were aligned with corresponding regions of p23 obtained by CNBr cleavage and found to have an identical amino acid sequence. Exploration of sequence homologies through the NBRF-PIR data bank using the Proscan program from DNASTAR (Madison, WI) (Lipman & Pearson, 1985) indicated the close structural relationship of the CNBr fragment with a sequence near the N-terminal region of human MRP14 (Odink et al., 1987); 69% of the amino acid residues were found to be identical, as shown in the aligned sequences of the two peptides in Figure 2A.

In contrast to p23, p7 possessed a freely available NH_2 in its N-terminal amino acid residue; it could therefore be directly sequenced by Edman degradation. For this purpose, the DEAE-Sephacel fraction enriched in p23 and p7 (Figure 1B, lane b) was subjected to SDS–PAGE. About 200 pmol of p7 resolved by gel electrophoresis was transferred to a PVDF membrane (see Materials and Methods). Samples of p7 purified by HPLC chromatography were also submitted to Edman degradation. The N-terminal amino acid sequence determined by Edman degradation is shown in Figure 2B. Seventy-eight percent of the amino acids of the analyzed sequence of p7 were identical to those of the N-terminal region of MRP8. In summary, bovine p23 and p7 have their structural counterparts in human MRP14 and MRP8, respectively.

Bovine Neutrophil P23 and P7 Are Associated in a Heterodimer Complex. The proteins p23 and p7 obtained by HPLC in a homogeneous form were extensively cleaved by trypsin into a number of peptide fragments, as it could be expected from their numerous lysine and arginine contents (not shown). Totally different results were obtained when the trypsin treatment was applied to the eluted fractions containing p7 and p23 recovered from the DEAE-Sephacel column. Upon

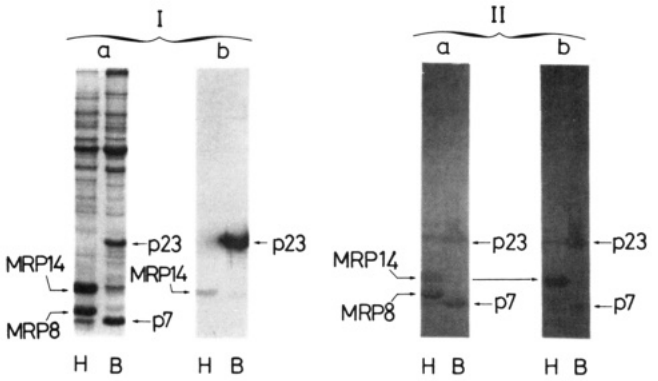

FIGURE 3: Cross-reactivity of human MRP14, MRP8 and bovine p23, p7. (I) Immunodetection by p23 polyclonal antibodies. Cytosolic proteins from human neutrophils (H) and bovine neutrophils (B) were subjected to SDS–PAGE (13% acrylamide). The separated proteins were stained with Coomassie Blue (a) or electrotransferred onto nitrocellulose and immunoreacted with p23 antiserum (b). (II) Immunodetection by monoclonal antibodies, CF145 directed against human MRP8 and CF557 directed against human MRP14. Cytosolic proteins from human neutrophils (H) and bovine neutrophils (B) were subjected to SDS–PAGE (20% acrylamide). The separated proteins were electrotransferred onto nitrocellulose and immunoreacted with CF145 (a) and CF557 (b).

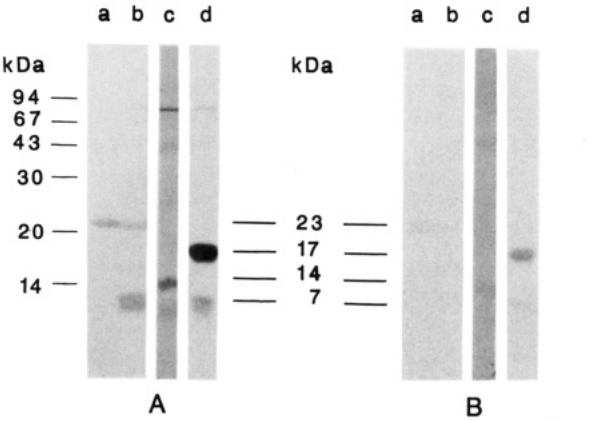

FIGURE 4: Autoradiograph illustrating the specific binding capacity of Ca^{2+} to bovine p23 and p7, human MRP14 and MRP8, and homologous proteins from rabbit. (A) Binding of $^{45}Ca^{2+}$ by the cytosolic fraction of neutrophils from different species. Cytosolic proteins were separated by SDS–PAGE and electrotransferred onto a nitrocellulose membrane. They were tested for $^{45}Ca^{2+}$ binding as described under Materials and Methods. The different tracks correspond to (a) pure bovine p23, (b) bovine neutrophil cytosol, (c) human neutrophil cytosol, and (d) rabbit neutrophil cytosol. The additional labeled band at about 80 kDa corresponds probably to $^{45}Ca^{2+}$-labeled PKC. (B) Chase of bound $^{45}Ca^{2+}$ by unlabeled Ca^{2+}. The chase was carried out on the same samples as those used in (A), with experimental conditions described under Materials and Methods.

incubation of these eluates with trypsin for 6 h at 37 °C, using an enzyme to substrate ratio of 1/50, no fragmentation of p7 could be detected, and digestion of p23 was only partial, resulting in a large fragment migrating in SDS–PAGE with a molecular mass of 13 kDa and a number of small peptides. The N-terminal amino acid residue of the 13-kDa fragment was shown to be blocked, indicating that trypsin had attacked p23 from its C-terminal region. These results taken together suggested that p23 and p7 are associated to form a complex, in which the whole sequence of p7 and a large portion of the N-terminal region of p23 escape the action of trypsin because the bonds involving lysine and arginine residues are hidden and not readily accessible to the enzyme.

Immunochemical Characterization of Bovine Neutrophil P23 and P7. The species specificity of polyclonal antibodies raised in rabbit against the bovine neutrophil p23 was analyzed by Western blot, using cytosols from human and bovine neutrophils (Figure 3-I). Bovine p23 antibodies were strongly immunoreactive against p23, but not against bovine p7. It should be added that the 13-kDa peptide obtained by tryptic digestion of the p23,p7 complex (see preceding section) did not react with the p23 polyclonal antibodies (data not shown). These polyclonal antibodies hardly reacted against the human MRP14 and not at all against the MRP8. On the other hand, the monoclonal antibodies CF145 and CF557, highly specific for human MRP8 and MRP14, did react with the bovine neutrophil proteins p7 and p23, respectively, with slight cross-reactivity (Figure 3-II).

Bovine Neutrophil P23 and P7 Are $^{45}Ca^{2+}$-Binding Proteins. As human MRP14 and MRP8 have the capacity to bind Ca^{2+}, it was interesting to test whether bovine p23 and p7 also possess this property. The Ca^{2+}-binding capacity of cytosolic proteins from human and bovine neutrophils was tested after protein transfer to a nitrocellulose membrane and reaction with $^{45}Ca^{2+}$ (see Materials and Methods). In the autoradiograph in Figure 4A, radioactive bands were localized at the level of the two predominant proteins of bovine neutrophil cytosol, p23 and p7 (lane b). The partially purified p23 and p7 obtained by DEAE-Sephacel chromatography (not shown) and pure p23 obtained by HPLC (lane a) displayed a similar binding capacity for $^{45}Ca^{2+}$. Pairs of Ca^{2+}-binding proteins of 14 and 8 kDa were found in human cytosol (lane c). The specificity of $^{45}Ca^{2+}$ binding to the bovine and human neutrophil proteins was ascertained by chase of bound $^{45}Ca^{2+}$ upon addition of a large excess of unlabeled Ca^{2+} (Figure 4B). In all cases, the extent of the chase amounted to more than 80–90%.

In summary, in human and bovine neutrophils, pairs of small-size Ca^{2+}-binding proteins were demonstrated. On nitrocellulose, after electrotransfer from an acrylamide gel, the immunoreactive bands revealed with the bovine neutrophil p23 antiserum and the human monoclonal antibodies CF557 and CF145 coincided with the radioactive bands containing $^{45}Ca^{2+}$. On the basis of a number of similarities between bovine p23 and human MRP14, on one hand, and bovine p7 and human MRP8, on the other, i.e., sequence homologies, strong affinity for Ca^{2+}, propensity to form heterodimeric complexes, and immunochemical cross-reactivities, it is inferred that the bovine Ca^{2+}-binding proteins p23 and p7 are the counterparts of the human Ca^{2+}-binding proteins MRP14 and MRP8. It should be added that the rabbit neutrophil cytosol also contains two predominant Ca^{2+}-binding proteins of 17 and 7 kDa (Figure 4A,B, lane d). The conclusions derived from bovine and human neutrophils apply to rabbit neutrophils and probably extend to phagocytic cells of mammalian species.

Localization of P23 in Bovine Neutrophils by Immunofluorescence. The term cytosol is an operational definition, cytosol being defined as the high-speed supernatant fluid from a cell homogenate. It is clear, however, that cell disruption, whatever the method used, i.e., ultrasonic irradiation or Potter–Elvehjem homogeneization, may break loose associations of soluble proteins with membrane components. It was therefore decided to explore in situ the localization of p23 in bovine neutrophils by an immunofluorescence technique based on the use of the p23 antiserum. Bovine neutrophils, either in the resting or in the PMA-activated states, were used. Further, a control was carried out with the same cells subsequently treated with actin antibodies. As illustrated in Figure 5, resting bovine neutrophils contained discrete fluorescent clusters evenly distributed within the cytoplasm (Figure 5A). The PMA-activated cells reproducibly showed a somewhat different pattern of p23 distribution (Figure 5C). In this case, the fluorescent p23 immuno complexes were essentially con-

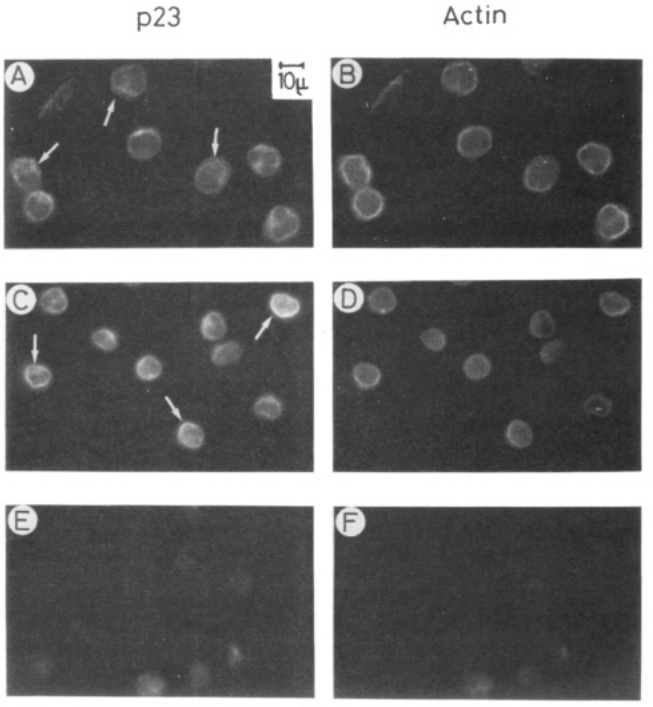

FIGURE 5: Immunochemical localization of p23 in bovine neutrophils. P23 was detected by FITC fluorescence in resting cells (A) and in PMA-activated cells (C). Typical discrete fluorescent clusters in (A) and fluorescent rings in (C) are indicated by arrows. The same neutrophil preparations were used for revelation of actin by rhodamine fluorescence in resting cells (B) and PMA-activated cells (D). (E) and (F) correspond to cells labeled only with the fluorescent secondary antibodies to evaluate the noise level of fluorescence.

centrated under the plasma membrane to form an intensely fluorescent ring. A control assay conducted with actin antibodies did not show any significant changes in the localization of actin, which was visualized as a thin edge on the border of the cell either in resting or in activated states (Figure 5B,D). The specific localization of p23 and actin described above was ascertained by control staining carried out with secondary fluorescent antibodies (Figure 5E,F).

Does P23 Play a Role in Phagocytosis? The bovine proteins p23 and p7, and their counterparts in man and rabbit, are found essentially in phagocytic cells, and they have been studied particularly in human myeloid cells (Hedgeworth et al., 1991). The question therefore arises whether these molecules play a role in some functions inherent in the mechanism of phagocytosis within or outside the cell. Another clue to test a possible implication of p23 in phagocytosis was its striking PKC-dependent phosphorylation (Stasia et al., 1989).

The first function to be explored was degranulation. MRP14 and MPR8 have been found in serum in pathological cases (Brüggen et al., 1988; Kuruto et al., 1990; Barthe et al., 1991), and it has been suggested that these proteins could be secreted from phagocytic cells (Hedgeworth et al., 1991). It was interesting to test whether they could be released from bovine neutrophils by exocytosis upon addition of stimulating ligands. Bovine neutrophils were incubated at 37 °C in the presence of PMA, opsonized zymosan, or the ionophore A23187 plus Ca^{2+}, all these agents being able to induce exocytosis of the content of neutrophil granules, as checked with β-glycuronidase and myeloperoxidase. Even after a period of time as long as 30 min at 37 °C, no release of p23 or p7 could be detected.

There is complete identity between the C-terminal sequence of MRP14 and the N-terminus of two small neutrophil-immobilizing factor proteins, NIF-1 and NIF-2 (Watt et al., 1983; Freemont et al., 1989). It was therefore interesting to determine whether p23 altered the cell motility. No effect of p23 or the p23,p7 complex on spontaneous or ligand-induced motility, using ZAS as chimioattractant, was detected.

Table I: In Vivo $[^{32}P]P_i$ Incorporation into P23 Using Bovine Neutrophils Stimulated by Various Activating Agents[a]

added ligand	$[^{32}P]P_i$ incorporation in p23 (dp 10 min)	
	in 5 min	in 20 min
phorbol myristate acetate	36785	35427
opsonized zymosan	1921	4109
leukotriene B4	<100	1533

[a] ^{32}P-loaded bovine neutrophils (10^7 cells) were activated by incubation for 5 and 20 min with the indicated ligands as described under Materials and Methods. Dp 10 min: disintegrations per 10 min.

The cell-free system for oxidase activation was used to test whether the p23 antiserum incubated with the components of this system could influence the elicited O_2^- production. Oxidase activation in the cell-free system was not modified by p23 antibodies.

Finally we addressed the property of p23 to be phosphorylated by PKC in bovine neutrophils activated by PMA (Stasia et al., 1989), and we extended this investigation to other types of stimuli, namely, opsonized zymosan and LTB4, which upon binding to specific receptors, initiate a cascade of reactions resulting in cell activation (Table I). A significant incorporation of $[^{32}P]P_i$ increasing with the period of incubation was found, when the applied stimulus was opsonized zymosan; the response to LTB4 was not conclusive. Contrary to p23 in bovine neutrophils, the counterpart proteins in human and rabbit neutrophils were not significantly phosphorylated by activated PKC (not shown). It is therefore possible that bovine p23, although significantly homologous to human MRP14, differs from it by an extra amino acid sequence containing one or several PKC-dependent phosphorylation sites, perhaps related to other functions.

Discussion

The results presented in this paper concerning some structural features of bovine neutrophil p23 and p7 extend those reported previously (Stasia et al., 1989) and can be discussed on the basis of the following experimental data. (1) Bovine p23 and p7 are Ca^{2+}-binding proteins which are largely homologous to two other Ca^{2+}-binding proteins, MRP14 and MRP8, first isolated from human phagocytic cells (Odink et al., 1987; Lagasse & Clerc, 1988). (2) Bovine p23, first characterized as a cytosolic protein, is probably loosely attached, directly or indirectly through other proteins, to the plasma membrane of PMA-activated bovine neutrophils.

Structural Similarities between Bovine P23 and P7 and Human MRP14 and MRP8. A large peptide segment obtained by CNBr cleavage of bovine neutrophil p23 and the sequence located in the N-terminal region of human macrophage MRP14 are structurally related with 69% of their amino acid residues being identical. It is noteworthy that the two Ca^{2+}-binding sites of MRP14 with a typical EF-hand motif are localized in the N-terminal region and in the middle region of MRP14 (Odink et al., 1987) (Figure 2A). The Ca^{2+}-binding domain present in the CNBr peptide fragment isolated from bovine p23 clearly corresponds to the first Ca^{2+}-binding domain of MRP14. MRP8 also contains two Ca^{2+}-binding domains. The N-terminal sequence of bovine p7 (Figure 2B) encompasses the first Ca^{2+}-binding domain of MRP8 (Odink et al., 1987). The predicted Ca^{2+}-binding sites of bovine p23

and p7 were corroborated by the demonstration of specific $^{45}Ca^{2+}$ binding to these proteins, ascertained by chase of bound $^{45}Ca^{2+}$ with unlabeled Ca^{2+} (see Results). The structural relationship of bovine p23 and p7 with human MRP14 and MRP8, respectively, was further corroborated by the immunochemical cross-reactivities of p23 and p7 with monoclonal antibodies directed against MRP14 (CF557) and MRP8 (CF145) (Figure 3-II). The cross-reactivity between p23 and p7 and between MRP14 and MRP8 may be explained by the presence of epitopes in the Ca^{2+}-binding domains of these proteins. Along this line, a puzzling observation is the absence of reactivity of MRP14 and MRP8 toward polyclonal antibodies directed against bovine p23. A simple explanation relies on the molecular masses of p23 and MRP14. The ESMS technique indicated a mass on the order of 17 kDa for bovine p23. Bovine p23 is therefore larger than human MRP14 for which a mass of about 13 kDa was calculated from the amino acid sequence deduced for the cloned cDNA (Odink et al., 1987). Since the N-terminal regions of p23 and MRP14 are largely homologous and can be readily aligned, the size difference between p23 and MRP14 might reside in the C-terminal region. The absence of reactivity of the p23 antiserum against MRP14 would therefore be explained by a localization of p23-specific epitopes in the C-terminal region, which has no counterpart in MRP14. In this context, it is noteworthy that, whereas p23 and MRP14 differ in size, their congeners p7 and MRP8 have similar masses, roughly 10.5 kDa for p7 as shown by the ESMS technique and 10.8 kDa for MRP8 as indicated by the amino acid sequence (Odink et al., 1987). Another puzzling observation is that bovine p23 and p7 fractionate together in chromatographic steps using mild elution buffers. The same observation was reported for human MRP14 and MRP8, and it was postulated that the two proteins are associated to form a heterodimer which could be stabilized by cross-linking (Edgeworth et al., 1991). The present study on bovine p23 and p7 corroborates this conclusion in showing that in cytosol or in the eluate from DEAE-Sephacel chromatography, p23 and p7 are not readily attacked by trypsin and that, in contrast p23 and p7 exposed to trypsin separately, after resolution by HPLC they are readily digested.

To summarize, bovine p23 and p7 belong to the S100 protein family, as human MRP14 and MRP8 do. In fact, they possess the basic attributes of the S100 proteins; namely, they form heterodimers, with each of the subunits containing Ca^{2+}-binding domains known as EF-hands, and they are particularly abundant in cytosol.

Despite many obvious similarities between the human MRP14,MRP8 pair and the bovine p23,p7 pair, a specific feature of p23 resides in its high propensity to be phosphorylated by PKC (Stasia et al., 1989). MRP14 was reported to be phosphorylated in human monocytes and neutrophils when these cells are supplemented with Ca^{2+} and the ionophore ionomycin; however, addition of PMA to activate PKC did not elicit phosphorylation of intracellular MRP14 (Edgeworth et al., 1989). One may wonder whether the phosphorylation site in p23 is not localized in the C-terminal region of this molecule. Two clues favor this interpretation: (1) the tryptic peptide of 13 kDa which contains the N-terminus of p23 does not react with the p23 antibodies; (2) the p23 antibodies, which react with the C-terminal region of p23 (see above), prevent the PKC-dependent phosphorylation of p23 (Stasia et al., 1989). The phosphorylation site in p23 would therefore reside apart from the Ca^{2+}-binding sites which are located close to the N-terminus and in the central region of p23.

Cellular Localization and Function of Bovine P23 and P7. By the criterium of subcellular fractionation of cell homogenates, p23 is predominantly a cytosolic protein. However, in a previous work (Stasia et al., 1989), it was shown that a significant fraction of p23 was associated with a Triton-insoluble fraction consisting of components of the cytoskeleton. The demonstration of an increased concentration of p23 immunofluorescent complexes close to the plasma membrane when cells are treated by PMA, therefore leading to p23 phosphorylation, might be relevant to an intracellular association of p23 with the cytoskeleton. The looseness of this association would explain why a large fraction of p23 is found in cytosol, when neutrophils are ruptured. Interaction of p23 with the cytoskeleton might be involved in coupling of cytoskeleton components with the plasma membrane. Despite this possibility, and the strong likelihood that in neutrophils p23 and p7 behave as efficient Ca^{2+} buffers due to their high concentration (20% of the cytosolic proteins), the function of these proteins in phagocytosis remains unclear. Bovine p23 and p7 and their human counterparts MRP14 and MRP8, as well as similar Ca^{2+}-binding proteins in rabbit neutrophils, belong to the S100 family, and they may share with members of this family some properties related to transduction of the Ca^{2+} signal [for a review, see Kligman and Hilt (1988) and Heizmann and Hunziker (1991)]. Some of the S100 proteins have their expression increased during cell differentiation (Murao et al., 1990) or in specific pathological events. This is the case for MRP14 and MRP8, whose expression is considerably enhanced during chronic inflammation response, such as is present in rheumatoid arthritis (Odink et al., 1987; Zwaldo et al., 1988) or cystic fibrosis (Brüggen et al., 1988). MRP14 and MRP8 have been reported to have a cytostatic activity toward fungi, yeast, bacteria, and human B-cell lines (Steinbakk et al., 1990; Sohnle & Collins-Lech, 1989). It is possible that p23 and p7 have a similar cytostatic function in association with the respiratory burst at a late stage of phagocytosis, when microorganisms have been engulfed by neutrophils.

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The Intrinsic ATPase Activity of Protein Kinase C Is Catalyzed at the Active Site of the Enzyme†

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ABSTRACT: We recently reported that autophosphorylated protein kinase C (PKC) has an intrinsic Ca^{2+}- and phospholipid-dependent ATPase activity and that the ATPase and histone kinase activities of PKC have similar metal-ion cofactor requirements and $K_{m,app}$(ATP) values. We hypothesized that the intrinsic ATPase activity of PKC may represent the bond-breaking step of its protein kinase activity. The rate of the ATPase reaction is several times slower than the histone kinase reaction rate. At subsaturating concentrations, various peptide and protein substrates stimulate the ATPase reaction by as much as 1.5-fold. In contrast, non-phosphorylatable substrate analogs are not stimulatory. These observations support a mechanism of PKC catalysis in which the productive binding of phosphoacceptor substrates enhances the rate of phosphodonor substrate (ATP) hydrolysis at the active site of PKC. However, this mechanism contains an assumption that the ATPase activity of PKC is catalyzed at the active site. In fact, sequence analysis indicates that PKC contains a potential second nucleotide binding site outside of its active site. In this report, we provide a detailed analysis of the relationship between the active site of PKC and the intrinsic ATPase activity of the enzyme. We show that the regulatory and catalytic properties of the ATPase reactions of three PKC isozymes are similar, despite critical differences among the isozymes in their consensus sequences for the potential non-active-site nucleotide binding site in their catalytic domains. We also show that the ATPase and histone kinase reactions of each isozyme have similar $K_{m,app}$(ATP) values. Furthermore, we demonstrate that an active-site-directed anti-PKC monoclonal antibody has parallel stimulatory effects on the ATPase and histone kinase activities of PKC and that monovalent salts have parallel inhibitory effects against these activities. Finally, we report that H7, which inhibits protein kinases but not other ATP-utilizing enzymes by competition with ATP, inhibits the ATPase activity of PKC with predominantly competitive kinetics. Taken together, the data presented in this report provide convincing evidence that the ATPase activity of PKC is catalyzed at the active site of the enzyme. Our results also indicate the usefulness of the ATPase reaction as a diagnostic tool in studies of the bond-breaking step of the protein kinase reaction of PKC.

Protein kinase C (PKC) is a family of closely related Ca^{2+}- and phospholipid-dependent protein kinases that are stimulated in vivo by the second-messenger *sn*-1,2-diacylglycerol (Bell & Burns, 1991; Bishop & Bell, 1988; O'Brian & Ward, 1992). While the regulation of PKC activity has been well characterized in recent years (Bell & Burns, 1991; Epand & Lester, 1990), much less is known regarding the active-site chemistry of the enzyme (O'Brian & Ward, 1990, 1992). The cAMP-

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