# Immunocharacterization of β- and ζ-subspecies of protein kinase C in bovine neutrophils

M.J. Stasia[1], B. Strulovici[3], S. Daniel-Issakani[3], J.M. Pelosin[2], A.C. Dianoux[1], E. Chambaz[2] and P.V. Vignais[1]

[1]*Biochimie et* [2]*Biochimie des Régulations Cellulaires Endocrines, Département de Biologie Moléculaire et Structurale, Centre d'Etudes Nucléaires, 85 X, 38041 Grenoble Cedex, France* and [3]*Institute of Cancer and Developmental Biology, Syntex Research, Palo Alto, CA 94304, USA*

Received 31 August 1990

The isoforms present in a crude preparation of bovine neutrophil protein kinase (PKC) were identified by immunodetection with antibodies directed against specific sequences of bovine and rat brain PKC isozymes. The major isoform of bovine neutrophil PKC was identified as β-PKC and the minor one as ζ-PKC.

Protein kinase C; Isoform; Neutrophil

## 1. INTRODUCTION

Cloning and sequence analysis of cDNA encoding protein kinase C (PKC) have established the molecular diversity of this enzyme family. In fact, PKC consists of several subspecies with a high degree of homology. Initially, the presence of three PKC subspecies designated as α, β (βI plus βII) and γ, was deduced from the sequence of cloned cDNAs obtained from bovine, rat, rabbit and human brain, and later human spleen cDNA libraries. More recently, three additional members of this family, termed δ, ε and ζ, have been identified, which lack one of the four conserved regions found in the first three mentioned subspecies [1]. Different cellular localizations have been found for the PKC subspecies, suggesting specific functions for each subspecies in the cell economy [2]. A purified preparation of PKC was recently isolated from bovine neutrophils in which the enzyme is abundant [3]. Its PKC isozyme pattern was, in a first approach, deduced from the profile of elution from an hydroxyapatite (HTP) column by a gradient of potassium phosphate and from the nature of the phosphorylated amino acid residues in autophosphorylated PKC. Compared to bovine brain PKC, whose activity was recovered from HTP in 3 main peaks, termed I, II and III in the order of elution, corresponding to the γ-, β- and α-isoforms, bovine neutrophil PKC was eluted in a single peak located in the phosphate gradient at the same position as peak I of bovine brain PKC. On the other hand, autophosphorylation of bovine neutrophil PKC in the presence of [γ-$^{32}$P]ATP, $MgCl_2$, $CaCl_2$, phosphatidylserine and diacylglycerol resulted in the labelling of serine and threonine residues, which was typical of the β-isoform of PKC [4]. This discrepancy warranted a direct determination of the isotype pattern of bovine neutrophil PKC by an immunochemical approach. In this report, we demonstrate through the use of antipeptide antibodies directed against the α-, β-, δ-, ε- and ζ-isoforms of brain PKC that bovine neutrophil PKC exists as two isoenzymes, namely β-PKC and ζ-PKC.

*Correspondence address:* M.J. Stasia, Laboratoire de Biochimie, Département de Biologie Moléculaire et Structurale, Centre d'Etudes Nucléaires de Grenoble, 85X, 38041 Grenoble Cedex, France

*Abbreviations:* PKC, protein kinase C; HTP, hydroxyapatite; PMN, polymorphonuclear neutrophils

## 2. MATERIALS AND METHODS

### 2.1. *Chemicals*

The following materials were supplied by the companies indicated: [$^{125}$I]Protein A and [γ-$^{32}$P]ATP, Amersham; nitrocellulose BA83, Schleicher and Schuell; acrylamide and bis(acrylamide), BDH; DE52 cellulose, Whatman; hydroxylapatite HPLC column (10 × 0.76 cm), Mitsui Toatso Chemicals (Tokyo); MonoQ column (HR 5/5), phenyl-Sepharose CL-4B, Percoll and molecular weight markers, Pharmacia-LKB; all other chemicals, Sigma. Extract from COS-7 transfected with PKC-ζ-cDNA was kindly provided by J. Knopf and Ron Kritz (Genetics Institute, Cambridge, MA, USA).

### 2.2. *Purification of bovine neutrophil PKC, and rat and bovine brain PKC*

Bovine neutrophil PKC was purified to homogeneity [3], all steps being conducted at 2-4°C. Briefly, $5 \times 10^9$-$10^{10}$ neutrophils obtained from 10 liters of fresh cow's blood were disrupted by sonication in a medium consisting of 0.25 M sucrose, 10 mM Mops, pH 7.4, 2 mM EGTA, 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. The supernatant recovered after 1 h of centrifuga-

*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/90/$3.50 

tion at 150 000 × g in a Ti 45 rotor (Beckman) was loaded onto a DE52 cellulose column and a linear 0–0.3 M NaCl gradient was applied. Active fractions eluted between 0.08 and 0.12 M NaCl were desalted and chromatographed on a MonoQ column in an FPLC system. Two peaks of PKC activity, both $Ca^{2+}$ and phospholipid dependent, were recovered with 0.05 M NaCl (peak 1) and 0.12 M NaCl (peak 2), respectively. The material of peak 2 from the MonoQ could be readily purified to homogeneity by phenyl-Sepharose chromatography. The phenyl-Sapharose column was eluted with a decreasing linear (1.5–0 M) gradient NaCl and the active fractions were recovered at low salt concentration. Purified PKC was stored in 0.05% Tween 20. PKC active fractions were dialysed overnight against 10 mM phosphate, pH 7.5, 15% glycerol, 10 mM β-mercaptoethanol, 0.05% Tween 20 prior to chromatography on HTP.

Rat brain PKC and bovine brain PKC were purified as described in [5] and [3], respectively. Brains were homogenized with a Potter-Elvejhem homogenizer in the same buffer as for bovine neutrophils. The supernatant recovered after a 60 min centrifugation at 100 000 × g was loaded onto a DE52 column and a linear (0–0.4 M NaCl) gradient was applied. Active PKC fractions were eluted with about 0.08 M NaCl and pooled. Powdered ammonium sulfate was added to 80% saturation. After 4 h at 2–4°C, the precipitate was collected by centrifugation. The pellet was solubilized in 4 ml of a medium containing 20 mM Tris, pH 7.5, 2 mM EDTA, 2 mM EGTA, 2 mM DTT and 2% glycerol. The next two steps were gel filtration on AcA 44, followed by chromatography on phenyl-Sepharose similar to that used for bovine neutrophil PKC purification. The active fractions were pooled and dialysed overnight against 10 mM phosphate, pH 7.5, 15% glycerol and 1 mM DTT prior to HTP chromatography.

### 2.3. *Separation of PKC isoforms from bovine neutrophils and rat or bovine brains by HTP chromatography*

The enzyme solution was applied to an HTP column (10 × 0.76 cm). Elution was carried out with a concave 0.010–0.175 M potassium phosphate gradient, pH 7.4, programmed in an HPLC system [3,5]. The three main isoforms, numbered I, II and III according to the order of elution from HTP corresponded to the γ-, β- and α-PKC gene products, respectively.

### 2.4. *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 [6].

### 2.5. *Western blot*

PKC partially purified at the stage of the MonoQ chromatography was subjected to SDS-PAGE using a 8% acrylamide gel. The proteins were transferred onto a nitrocellulose membrane [7,8]. Non-specific sites were blocked by incubating the membrane with 5% instant milk (Carnation, USA) and 0.5% Tween 20 for 1 h at 41°C. The immunoreaction complex was detected with [$^{125}$I]protein A (Amersham) (0.1 μCi/ml) exactly as described [9,10]. The nitrocellulose membrane was exposed to Kodak XA5 film with intensifying screens at −70°C overnight for β-PKC and two days for all other isoenzymes.

### 2.6. *Synthesis of peptide and preparation of antipeptide antibodies*

Peptides representing selected amino acid clusters of PKC were prepared from the cDNA sequences related to different PKC isoenzymes from bovine brain. They were coupled either to keyhole limpet hemocyanin [10] or to tuberculin PPD [11], and injected into rabbits. The specificity of the antisera was previously documented [9,11]. Another set of peptides corresponding to selected sequences of α-, β- and γ-PKC isoform from rat brain was prepared and coupled to BSA before injection into rabbits (Pelosin et al., unpublished).

## 3. RESULTS

The isoforms of bovine neutrophil PKC and bovine brain PKC were resolved in parallel by HTP chromatography using a linear 0.01–0.175 M gadient of potassium phosphate, pH 7.4, 15% glycerol, 1 mM DTT [3]. The elution profiles are illustrated in Fig. 1. Bovine neutrophil PKC was eluted in a single peak with 0.03 M potassium phosphate, i.e. with the same phosphate concentration as that required for the elution of peak I of bovine brain PKC corresponding to the γ-subspecies. The same pattern of elution was obtained with enzyme preparations at earlier stages of the purification procedure.

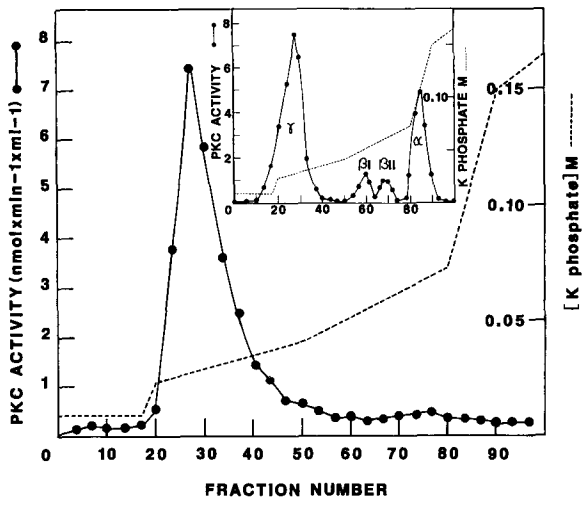


Fig. 1. Separation of bovine and neutrophil PKC isoforms by HTP chromatography. PKC-containing fractions eluted from phenyl-Sepharose column were loaded on HTP column. The column was eluted with a linear gradient (0.010–0.175 M) of potassium phosphate. Fig. 1 represents the HTP elution profile of purified bovine neutrophil PKC. The inset represents the HTP elution profile of purified bovine brain PKC. PKC activity was measured in the presence of 1 mM $CaCl_2$, 20 μg/ml phosphatidylserine, 2 μg/ml diolein as described in [3]. No kinase activity was detected in the absence of 1 mM $Ca^{2+}$, 20 μg/ml phosphatidylserine, 2 μg/ml diolein and in the presence of 1 mM EGTA.

The PKC isoforms of bovine neutrophils were analyzed by immunoblotting experiments, using polyclonal antipeptide antibodies. In the case of the isoforms α, β and γ, two series of antibodies were used, which were obtained against peptides chosen in one of the five variable domains of bovine brain and rat brain PKC referred to as V3, whose accessibility is ascertained by its ready attack by calpain [12]. For the δ-, γ- and ζ-isoforms, the antibodies were generated from peptides corresponding to specific sequences of bovine brain PKC.

In the case of bovine brain PKC, 6 peptides were synthesized, which corresponded to the following residues: 318–331 (ISPSEDRRQPSNNL) for α-PKC [9], 319–332 (EKTTNTISKFDNNG) for β-PKC [9], 316–332 (VRTGPSSSPIPSPSPT) for γ-PKC [9], 318–334 (KTAVSGNDIPDNNGTYG) for δ-PKC [10,11], 836–853 (NQEEFKGFSYFGEDLMP) for ε-PKC [10,11] and 128–142 (SEDLKPVIDGDDGIK) for ζ-PKC [10,11]. Each antipeptide antibody reacted effi-

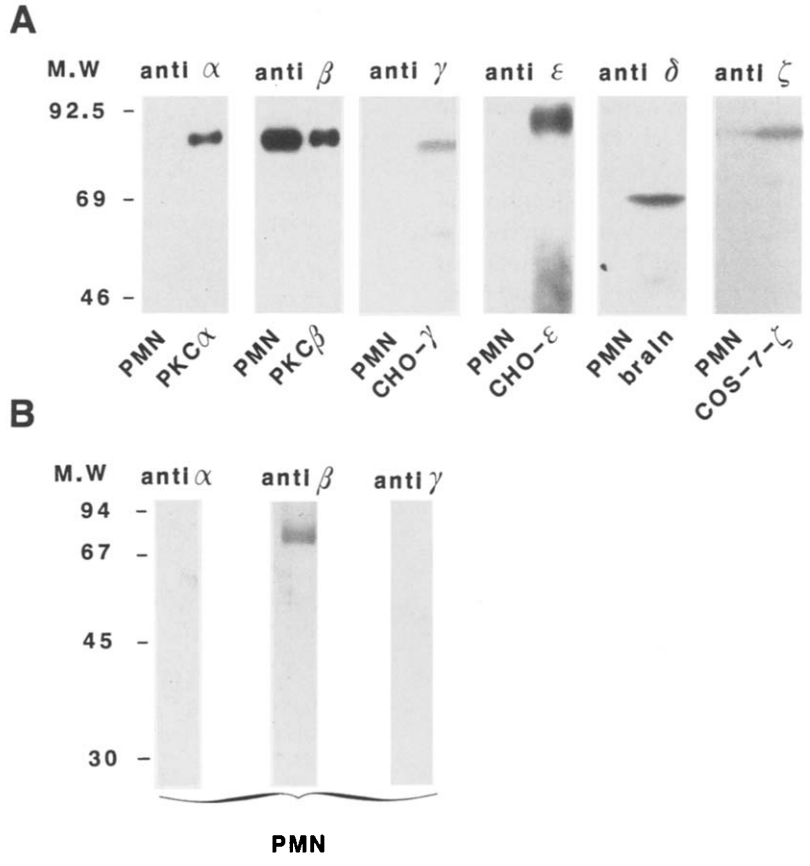


Fig. 2. Immunoreactivity of neutrophil PKC isoforms with antipeptide antibodies directed against α-, β-, γ-, ε-, δ- and ζ-PKC. (A) PKC from bovine neutrophils (PMN) purified by MonoQ chromatography was subjected to 8% SDS-PAGE and electrotransferred onto a nitrocellulose sheet. The transferred PKC bands were detected with antisera directed against α-PKC (sequence 318–331), β-PKC (sequence 319–332), γ-PKC (sequence 316–332) and specific sequences from ε-, δ- and ζ-PKC as described in sections 2 and 3. Positive controls were obtained using purified α-PKC and β-PKC from rat brain, extracts from transfected CHO cells with cDNA derived from γ- and ε-sequences [9], extracts from COS-7 cells transfected with cDNA derived from ζ-sequences and brain extract from δ-isoform. (B) A parallel immunodetection experiment was performed with bovine neutrophil PKC recovered from phenyl-Sepharose and antibodies directed against the following sequences: α-PKC (320–335), β-PKC (319–335), γ-PKC (683–697).

ciently with its own antigen [9–11]. Concerning the α-, β- and γ-isoforms of rat brain PKC, the peptides used corresponded to the following residues: 320–335 (PSEDRKQPSLNLDR) for α-PKC, 319–335 (EKTALTISKFPNNGNR) for β-PKC and the C-terminus peptide 683–697 (PDARSPTSPVPVPVM) for γ-PKC (Pelosin et al., unpublished).

Western blot analysis of bovine neutrophil PKC partially purified by chromatography on MonoQ (Fig. 2A) or purified to homogeneity using phenyl-Sepharose chromatography (Fig. 2B) is shown in Fig. 2. Among the antibodies raised against chosen peptide sequences from bovine brain and rat brain, only those raised against sequences of β-PKC and ζ-PKC were found to be immunoreactive (Fig. 2A,B).

## 4. DISCUSSION

The immunochemical demonstration that one of the two isozymes of bovine neutrophil PKC is a β-subspecies agrees with the conclusion drawn from the nature of the phosphorylated amino acid residues in autophosphorylated PKC [3,4]. The other PKC isozyme of bovine neutrophil reacts with the anti-ζ-antiserum and is present in relatively low amounts as compared to the β-isoenzyme. So far, the ζ-PKC isozyme has been identified in expression experiments using the corresponding specific cDNA [13]. Only recently, ζ-PKC was described in nuclei of nerve cells [14]. Bovine neutrophils are another example of cells where the ζ-isoform of PKC is expressed.

The use of HTP chromatography as a means to resolve PKC isoforms deserves some comment. In fact, bovine neutrophil PKC is eluted from the HTP as a single peak which coincides with that of the isoform I or γ of brain PKC. In the present immunochemical investigation, using antibodies directed against two distant peptide sequences of the γ-isoform of brain PKC, no immunochemical reaction could be detected between bovine neutrophil PKC and the anti-PKC γ-antibodies.

These results indicate that the HTP elution pattern of PKC cannot be used as a reliable criterion to characterize the PKC isozymes in a tissue extract.

Like bovine neutrophils, human neutrophils contain a major β-isoform of PKC; however, they differ from bovine neutrophils by the presence of a minor α-isoform of PKC and that of a $Ca^{2+}$, phospholipid dependent kinase not recognized by the antibodies against the α-, β- and γ-isotypes [15]. The presence of the ζ-isoform in human neutrophils has not been reported. In short, not only PKC isoforms have a different tissue distribution [16], but they appear to exhibit species specificity for a given cell type. Yet the presence of a common subspecies, namely the β-isoform, in both bovine and human neutrophils suggests that the β-isoform may play a central role in specific functions of phagocytic cells.

*Acknowledgements:* This work was supported by grants from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Association pour la Recherche sur le Cancer et la Fondation pour la Recherche Médicale. We thank Jacqueline Boyer for secretarial assistance.

## REFERENCES

[1] Kikkawa, U., Kishimoto, A. and Nishizuka, Y. (1989) Annu. Rev. Biochem. 58, 31-44.
[2] Nishizuka, Y. (1988) Nature 334, 661-665.
[3] Dianoux, A.C., Stasia, M.J. and Vignais, P.V. (1989) Biochemistry 28, 424-431.
[4] Huang, K.P., Chan, K.F.P., Singh, T.J., Nakabayashi, H. and Huang, F.L. (1986) J. Biol. Chem. 261, 12134-12140.
[5] Pelosin, J.M., Vilgrain, I. and Chambaz, E. (1987) Biochem. Biophys. Res. Commun. 147, 382-391.
[6] Stasia, M.J., Dianoux, A.C. and Vignais, P.V. (1987) Biochem. Biophys. Res. Commun. 147, 428-436.
[7] Towbin, O.H., Rosebrought, J.C., Favor, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
[8] Burnette, W.N. (1981) Anal. Biochem. 112, 195-199.
[9] Strulovici, B., Daniel-Issakani, S., Oto, E., Nestor, J., Chan, H. and Tsou, A.P. (1989) Biochemistry 28, 3569-3576.
[10] Strulovici, B., Daniel-Issakani, S., Baxter, G., Kurpf, J., Sultzman, L., Cherwisuki, H., Webb, D.R., Nestor, Jr J. and Ransom, J. (1990) J. Biol. Chem. (in press).
[11] Heidenreich, K.A., Toledo, S.P., Brunton, L.L., Daniel-Issakani, S. and Strulovici, B. (1990) J. Biol. Chem. (in press).
[12] Kishimoto, A., Mikawa, K., Hashimoto, K., Yasuda, I., Tanaka, S., Tominaga, M., Kuroda, T. and Nishizuka, Y. (1989) J. Biol. Chem. 264, 4088-4092.
[13] Ono, Y., Fugi, T., Igarashi, K., Kuno, T., Tanaka, C., Kikkawa, U. and Mishizuka, Y. (1989) Proc. Natl. Acad. Sci. USA 86, 4868-4871.
[14] Hagiwara, M., Uchida, C., Usuda, N., Nagata, T. and Hidaka, H. (1990) Biochem. Biophys. Res. Commun. 168, 161-168.
[15] Pontremoli, S., Melloni, E., Sparatore, B., Michetti, M., Salamino, F. and Horecker, B.L. (1990) J. Biol. Chem. 265, 706-712.
[16] Yoshida, Y., Huang, F.L., Nakabayashi, H. and Huang, K.P. (1988) J. Biol. Chem. 263, 9868-9873.