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Lipocortin I is not accessible for protein kinase C bound to the cytoplasmic surface of the plasma membrane in streptolysin-O-permeabilized pig granulocytes

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We previously observed a 38 kDa protein that was a major protein component of the cytosolic extract of pig granulocytes and the dominant substrate of protein kinase C at supra-physiological Ca^{2+} concentrations. The purified 38 kDa protein itself required Ca^{2+} to be phosphorylated by protein kinase C. Now we demonstrate that this protein, which is also present in human granulocytes, is identical to lipocortin I. The identification is based on the chromatographic properties and immunoblot of the purified protein which is also a good substrate for tissue transglutaminase. Phosphorylation of lipocortin I by protein kinase C was investigated in granulocytes permeabilized with streptolysin-O. At physiological intracellular Ca^{2+} concentrations lipocortin I was not phosphorylated at all. At supra-physiological Ca^{2+} concentrations (0.5 mM), lipocortin I was also not phosphorylated when protein kinase C was translocated to the membrane by treatment of the cells with phorbol myristate acetate. Its phosphorylation was detectable only in control experiments when protein kinase C was activated in the cytosol by the addition of dioleoylglycerol and phosphatidylserine to the permeabilized cells. The data presented show that, in permeabilized granulocytes, Ca^{2+} -lipocortin is not formed at physiological Ca^{2+} concentrations, and at supra-physiological Ca^{2+} concentrations the Ca^{2+} -lipocortin I is not accessible to protein kinase C bound to the cytoplasmic surface of the plasma membrane.

Introduction

Lipocortin I (annexin I) belongs to a family of highly homologous proteins, named annexins, which are able to bind reversibly to cellular membranes or particulate fractions in the presence of Ca^{2+} . Several members of the family are involved in Ca^{2+} -dependent membrane events including exocytosis and attachment of cytoskeletal elements to plasma membrane [1–6]. Lipocortin I was originally defined as a mediator of the antiinflammatory actions of glucocorticoids [7,8]. Recent findings have confirmed this role of lipocortin I [9], though the molecular basis of the antiinflammatory actions is not exactly known. Inhibition of the synthesis of cellular lipid mediators is considered to be one of the possible ways of lipocortin I action but the mechanism of this inhibition is an open question. The long-

held belief that lipocortin inhibits the release of arachidonic acid via the direct inhibition of phospholipase A_2 has not been confirmed by recent investigations [10–12].

The binding of calcium is considered to be a key feature of the biology of annexins [1]. However, the Ca^{2+} affinity of lipocortin I is very low. Since the possibility exists that the Ca^{2+} concentration required for the formation of Ca^{2+} -lipocortin is far lower inside the cell than in vitro, our aim was to investigate the Ca^{2+} concentration needed for the appearance of the Ca^{2+} -lipocortin I in granulocytes. This problem was approached with the aid of phosphorylation catalyzed by protein kinase C. In vitro, at supra-physiological Ca^{2+} concentrations, lipocortin I is known to be a good substrate for protein kinase C [13–17]; however, its intracellular phosphorylation stimulated by the selective activation of this enzyme has been hardly detected. We observed that in vitro protein kinase C was able to phosphorylate only the Ca^{2+} -lipocortin I but not the free lipocortin I. On the basis of this finding we investigated the phosphorylation of lipocortin I by protein kinase C in permeabilized pig granulocytes. We

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demonstrate that in permeabilized granulocytes the formation of Ca^{2+} -lipocortin needs a supra-physiological Ca^{2+} concentration and even when it is formed it is not accessible for endogenous protein kinase C translocated to the cytoplasmic surface of the plasma membrane by phorbol ester.

Materials and Methods

Purification and immunoblot of the 38 kDa Ca^{2+} /membrane-binding protein of granulocytes

Neutrophil granulocytes were isolated from blood, the cellular extract was prepared and the 38 kDa protein was partially purified from the DEAE-cellulose flow-through fraction by hydroxyapatite chromatography, as described previously [18,19]. Preparation of the cellular extract and the purification procedure were performed in the presence of the protease inhibitors phenylmethylsulphonyl fluoride (2 mM) and benzamide (1 mM). Purification was checked by the SDS-PAGE of the purified samples followed by the densitometric analysis of the stained protein pattern. The protein content of the stained 38 kDa band was determined according to the method described [20]. For Western analysis samples containing 4 μg of the 38 kDa protein each were subjected to SDS-PAGE and the proteins were transferred to nitrocellulose membranes [21] in a transfer buffer containing 25 mM Tris, 0.192 M glycine, 0.037% (w/v) SDS and 20% (v/v) methanol. Membranes were soaked in a 20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 0.5% (v/v) Tween 20, 0.1% bovine serum albumin blocking buffer for 1 h at room temperature. After overnight incubation with a 1:200 dilution of rabbit anti-lipocortin I or II antibodies (a kind gift of Dr. R. Blake Pepinsky, Biogen, Cambridge, MA, USA; [22,23]) at 4°C the immunocomplexes were visualized by means of peroxidase conjugated anti-rabbit antibodies (Sigma) and subsequent treatment with 4-chloro-1-naphthol and H_2O_2 .

Protein kinase C type II isoenzyme was purified from rabbit thymus cells, as described [24] and the *in vitro* phosphorylation of the 38 kDa protein was carried out as reported in our previous work [19].

Assay of the tissue transglutaminase reaction

Samples containing 50 μg /ml of the 38 kDa protein each were incubated in a reaction mixture comprising 50 mM Tris-HCl (pH 7.5), 5 mM NaCl, 6 mM CaCl_2 , 0.5 mM EDTA, 4 mM dithioerythritol, 100 μg /ml phosphatidylserine (Sigma P 8518) and 12 or 24 μg /ml tissue transglutaminase (Sigma). Incubation was carried out at 37°C for 60 min and the reaction was stopped by the addition of trichloroacetic acid (10%, w/v final concentration). After centrifugation the pellet was subjected to SDS-PAGE on a 10% polyacrylamide gel.

Permeabilization of pig granulocytes and the assay of protein kinase C activity in streptolysin-O-permeabilized cells

Pig granulocytes were permeabilized according to the method described by Alexander et al. [25] with minor modifications. The final concentrations of components in the medium used for permeabilization were: 8 mM MgCl_2 , 120 mM KCl, 12 mM Hepes (pH 7.4), 10 mM EGTA, various concentrations of free Ca^{2+} and 0.4 U/ml streptolysin-O freshly reconstituted from freeze-dried powder. Cells were incubated in the streptolysin-O-containing medium ($5 \cdot 10^6$ – 10^7 cells in 0.4 ml medium) for 10 min at 37°C. At the end of the incubation about 90% of the cells seemed to be permeabilized on the basis of staining with trypan blue. (Not more than 5% of the cells were stained before treatment with streptolysin-O.)

The activity of protein kinase C was measured with the aid of the oligopeptide substrate Ala-Ala-Ala Ser-Phe-Lys-Ala-Lys-Lys-amide [26–28]. In some experiments the oligopeptide substrate (0.7 mg/ml) and [^{32}P]ATP (0.1 mM, about 100 000 cpm per nmol) were added to the medium used for permeabilization and the incubation was stopped by the addition of ice-cold glacial acetic acid (0.4 ml). After centrifugation ($10\,000 \times g$ for 2 min) aliquots were taken from the supernatant and the radioactivity of [^{32}P]phosphate incorporated into the oligopeptide was measured as described previously [26–28]. In other experiments permeabilized granulocytes were centrifuged ($1500 \times g$ for 1 min), and the pellet was resuspended in the same medium without streptolysin-O. The medium was completed with the oligopeptide (0.7 mg/ml), the reaction was started by the addition of [^{32}P]ATP (0.1 mM) and stopped by the addition of glacial acetic acid as above. Protein kinase C activity was defined as the difference between the phosphotransferase activities of cells treated or not with 40 ng/ml phorbol myristate acetate (PMA) (Sigma). The assays were performed in triplicate.

Phosphorylation of endogenous proteins was carried out in the medium used for permeabilization. In these experiments [^{32}P]ATP (0.1 mM, about 100 000 cpm per nmol) was added to the medium and the incubation was stopped by the addition of trichloroacetic acid (10%, w/v final concentration). The samples were centrifuged and the resulting pellet was washed with acetone, dissolved in Laemmli buffer [29] and subjected to SDS-PAGE on 12% polyacrylamide gels.

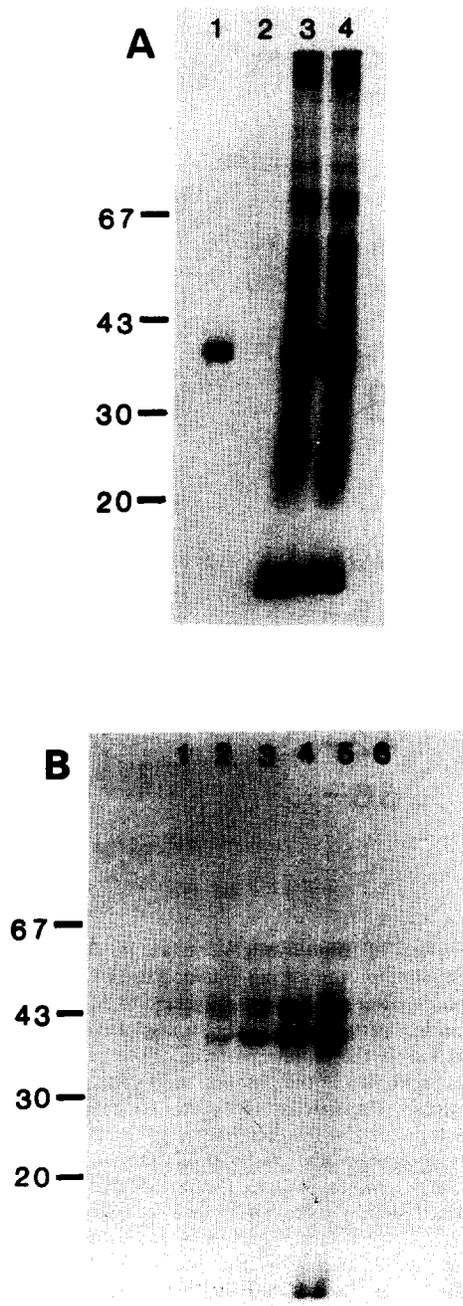
Results

Identification of lipocortin I in neutrophil granulocytes

In our earlier studies we detected a 38 kDa Ca^{2+} /membrane-binding protein that was the dominant substrate of protein kinase C in the cytosolic extract of pig

neutrophil granulocytes at supra-physiological Ca^{2+} concentrations [18]. The detailed analysis of the phosphorylation has shown that this protein itself requires Ca^{2+} for phosphorylation by protein kinase C. While protein kinase C was found to be fully active at 10^{-6} M Ca^{2+} , or a Ca^{2+} -independent form of protein kinase C was used, well detectable phosphorylation of the purified protein was observed only at Ca^{2+} concentrations in the region of 10^{-4} M [19].

A 38 kDa protein with the same properties was found in the cytosolic extract of human neutrophils as



well, although present in a smaller amount than in pig granulocytes and it was not the single dominant substrate of protein kinase C at high Ca^{2+} concentrations (Fig. 1).

On the basis of densitometric analysis of one-dimensional SDS gels the 38 kDa protein fraction accounted for about 3% of the total protein content of the cytosolic extract prepared from pig neutrophils and the significant part of the 38 kDa protein fraction was bound to the membrane when the cells were sonicated in the presence of 5 mM Ca^{2+} (data not shown). The 38 kDa protein was partially purified according to the method described for the separation of different lipocortins [30]. The partial purification from the cytosolic extract of pig granulocytes yielded preparations in which the 38 kDa protein represented 65–85% of the total protein. Western analysis of the 38 kDa protein purified from pig granulocytes was performed with the aid of antisera against lipocortin I and II. Relatively large amounts of the 38 kDa proteins were analyzed in order to demonstrate the presence of any cross-reacting proteins. In spite of this, while strong reactions were found with lipocortin I antiserum, hardly detectable traces of immunoreactivity were observed only with lipocortin II antiserum (Fig. 2). Immunoprecipitation of the phosphorylated form of the protein was observed exclusively when lipocortin I antiserum was applied (data not shown). Though some other members of the annexin family may also cross-react with lipocortin I antibody, the chromatographic behaviour of the 38 kDa protein on DEAE-cellulose (it was found in the flow-through fraction) and failure to react with lipocortin II antiserum showed that it was lipocortin I [30].

The 38 kDa Ca^{2+} -dependent substrate of protein kinase C purified partially from human granulocytes was also analyzed by immunoblotting and proved to be identical to lipocortin I (Fig. 2B). This finding shows

Fig. 1. Phosphorylation of the 38 kDa Ca^{2+} /membrane-binding protein of human neutrophil granulocytes by protein kinase C and the Ca^{2+} requirement of this phosphorylation. Samples phosphorylated in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ were subjected to SDS-PAGE followed by autoradiography. (A) Cytosolic proteins of granulocytes phosphorylated by protein kinase C at 1 mM Ca^{2+} . Samples of the cytosolic extract were incubated in the absence (lane 2) and presence (lanes 3, 4 identical samples) of phosphatidylserine (25 $\mu\text{g}/\text{ml}$) + dioleoylglycerol (2.5 $\mu\text{g}/\text{ml}$). Lane 1 shows a sample of the 38 kDa Ca^{2+} /membrane-binding protein purified from pig granulocytes and phosphorylated by purified protein kinase C type II. (B) Ca^{2+} requirement of the phosphorylation of the 38 kDa protein purified from human granulocytes. Partially purified samples of the protein were incubated with protein kinase C (type II) in the presence of phosphatidylserine (25 $\mu\text{g}/\text{ml}$) and dioleoylglycerol (2.5 $\mu\text{g}/\text{ml}$) at various Ca^{2+} concentrations: $5 \cdot 10^{-6}$ M, 10^{-5} M, $5 \cdot 10^{-5}$ M, 10^{-4} M, $5 \cdot 10^{-4}$ M, in lanes 1–5, respectively. Lane 6 shows the result of the incubation in the absence of activators of protein kinase C.

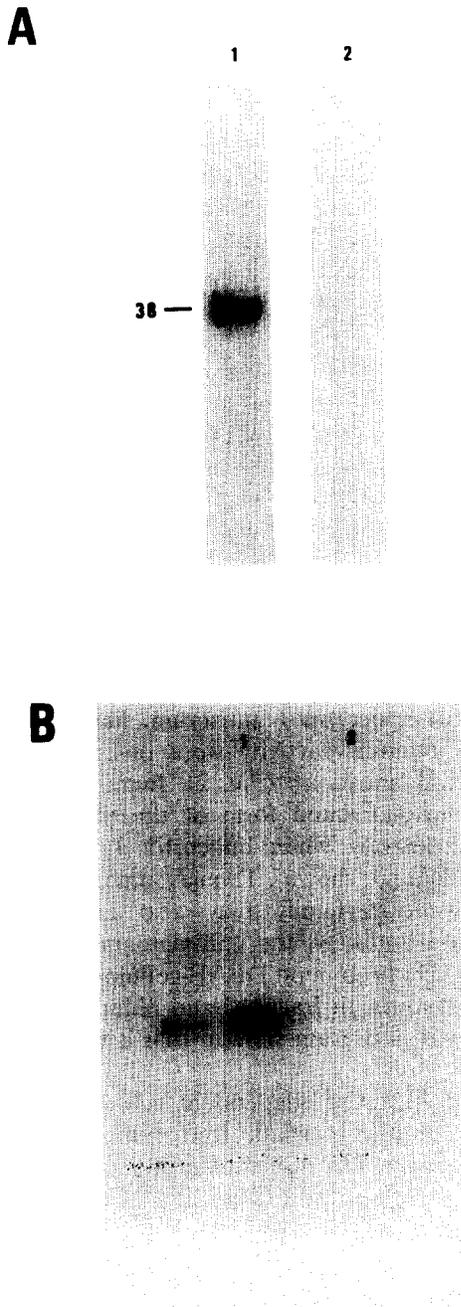


Fig. 2. Western analysis of the 38 kDa Ca^{2+} /membrane-binding protein of granulocytes. Results obtained with protein samples purified from pig (A) and human (B) granulocytes. Lane 1, anti-lipocortin I; lane 2, anti-lipocortin II.

that porcine lipocortin I and human lipocortin I both are rather particular substrates of protein kinase C, since they can be phosphorylated only when they bind Ca^{2+} and this occurs at supra-physiological Ca^{2+} concentrations, at least in vitro.

Our partially purified lipocortin I preparation may be contaminated by other 38 kDa proteins, and was therefore also checked by another method. Lipocortin I is known to be a good substrate for tissue transglu-

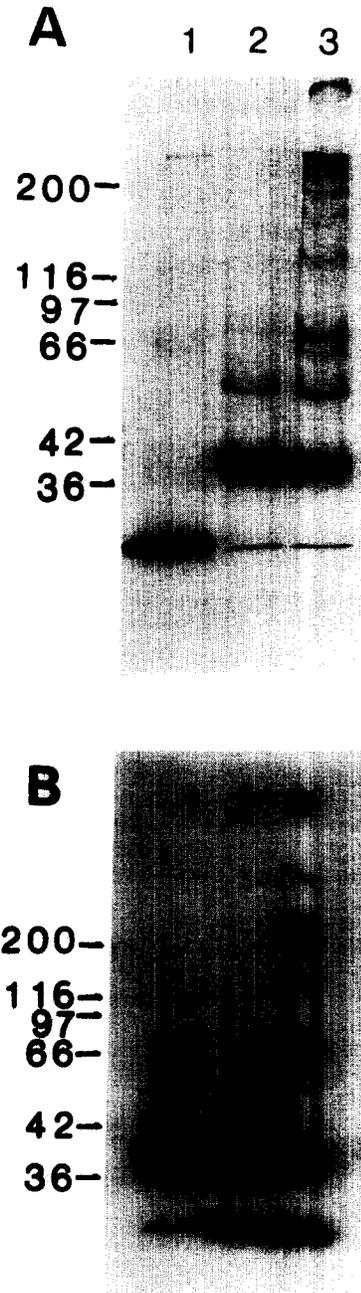


Fig. 3. The effect of tissue transglutaminase on the 38 kDa Ca^{2+} /membrane-binding protein of pig granulocytes. (A) Coomassie-blue-stained protein pattern of samples subjected to SDS-PAGE. Lane 1 shows the protein pattern of the tissue transglutaminase preparation, lanes 2 and 3 show the protein patterns of the partially purified 38 kDa protein incubated in the absence and in the presence of tissue transglutaminase, respectively. (B) Autoradiogram of ^{32}P -labelled protein bands yielded by the incubation of the phosphorylated form of the 38 kDa protein with tissue transglutaminase: the phosphorylated 38 kDa protein without further incubation (lane 1); the phosphorylated 38 kDa protein incubated for 60 min in the reaction mixture of the tissue transglutaminase reaction without tissue-transglutaminase (lane 2); phosphorylated sample of the 38 kDa protein was incubated with tissue transglutaminase for 60 min (lane 3). A and B show results obtained from two independent experiments.

TABLE I

The activity of protein kinase C measured with the oligopeptide Ala-Ala-Ala-Ser-Phe-Lys-Ala-Lys-Lys-amide as a substrate in permeabilized pig granulocytes

Treatment 1	Treatment 2	Phosphate incorporated (cpm)
Permeabilization + phosphorylation without PMA, $5 \cdot 10^{-7}$ M Ca^{2+}	-	8 800
Permeabilization + phosphorylation with PMA, $5 \cdot 10^{-7}$ M Ca^{2+}	-	91 400
Permeabilization without PMA $5 \cdot 10^{-5}$ M Ca^{2+}	phosphorylation without PMA	9 800
Permeabilization without PMA $5 \cdot 10^{-5}$ M Ca^{2+}	phosphorylation with PMA	27 500
Permeabilization with PMA $5 \cdot 10^{-7}$ M Ca^{2+}	phosphorylation without addition	137 500
Permeabilization with PMA $5 \cdot 10^{-7}$ M Ca^{2+}	phosphorylation with PMA added	127 200

Samples containing $2.5 \cdot 10^6$ cells each in $200 \mu\text{l}$ medium were incubated for 10 min for phosphorylation. The specific radioactivity of [^{32}P]ATP was 115 000 cpm/nmol.

taminase [31,32]. Incubation of the 38 kDa porcine protein with tissue transglutaminase yielded different oligomeric forms accompanied by a reduction in the amount of the 38 kDa form (Fig. 3A). The effect of transglutaminase was well detectable even when the phosphorylated form of the 38 kDa protein was incubated with the enzyme (Fig. 3B), indicating that the phosphorylated 38 kDa protein was lipocortin I.

Phosphorylation of lipocortin I by protein kinase C in permeabilized pig neutrophils

Since lipocortin I is a major protein fraction in pig granulocytes its phosphorylation was investigated in permeabilized pig granulocytes. The activity of protein kinase C was measured in the permeabilized cells with the aid of the nonapeptide Ala-Ala-Ala-Ser-Phe-Lys-Ala-Lys-Lys-amide, which is a selective substrate for

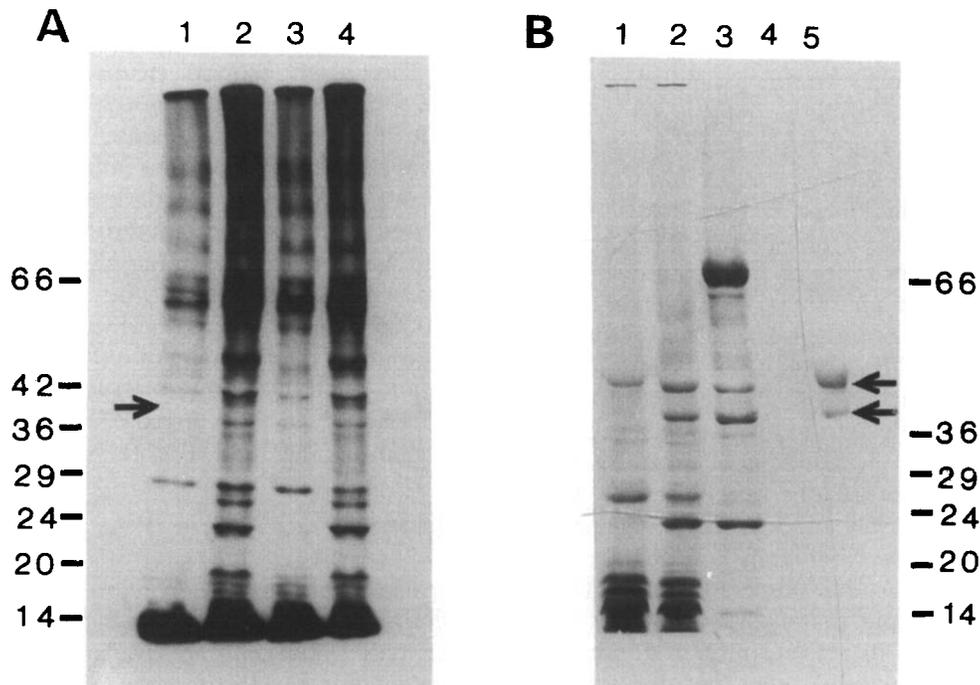


Fig. 4. The phosphoprotein pattern and subcellular distribution of proteins in streptolysin-O-permeabilized pig granulocytes. (A) ^{32}P -labelled proteins of granulocytes permeabilized in the presence of [γ - ^{32}P]ATP. Samples of the granulocytes were subjected to SDS-PAGE followed by autoradiography. Cells were incubated in the absence (lanes 1, 3) or presence (lanes 2, 4) of 40 ng/ml phorbol myristate acetate, the medium served for permeabilization contained $5 \cdot 10^{-7}$ M (lanes 1, 2) or 10^{-4} M (lanes 3, 4) free calcium. The arrow indicates the position of lipocortin I on the gel. (B) Coomassie-blue-stained bands of proteins found in the particulate fraction (lanes 1, 2) or in the supernatant of cells (lanes 3, 4) permeabilized by streptolysin-O in the presence of 10^{-4} M free calcium (lanes 1, 3) or incubated in the absence of permeabilizing agent (lanes 2, 4). Lane 5 shows the bands of purified actin and purified lipocortin I.

protein kinase C [26–28]. In cells treated with phorbol myristate acetate in the course of permeabilization with streptolysin O, the phosphotransferase activity was about 40 pmol phosphate/min per 10^6 cells. This value agrees well with that measured previously in the extracts of sonicated cells [28]. As in the case of intact cells, treatment of cells with PMA induced the translocation of protein kinase C to the membrane and the activated enzyme was retained by the permeabilized cells. In the absence of PMA only a part of the protein kinase C activity was retained by the cells after permeabilization (Table I).

The activity of protein kinase C in permeabilized granulocytes was demonstrated also by the appearance of phosphorylated proteins in PMA treated cells (Fig. 4A). PMA stimulated the phosphorylation of several proteins, including a 47 kDa protein which is a characteristic substrate of protein kinase C [33], but the phosphorylation of lipocortin I was not detectable. Since purified lipocortin I is not phosphorylated by protein kinase C at physiological Ca^{2+} concentrations [19], we also investigated the phosphorylation at high Ca^{2+} concentrations. We did not observe any difference between the phosphoprotein patterns of permeabilized cells treated with PMA at 0.5 μM or 0.1 mM Ca^{2+} . Lipocortin I was not phosphorylated at all in PMA-treated permeabilized cells; instead of this it leaked out from the cells. After 10 min permeabilization at 0.1 mM Ca^{2+} , granulocytes practically lost the total amount of their lipocortin I (Fig. 4B).

At 0.5 mM Ca^{2+} concentration a significant part of lipocortin was retained by the permeabilized cells, but the phosphorylation of lipocortin I could not be detected in PMA-treated cells even at 0.5 mM Ca^{2+} . This indicated that protein kinase C translocated to the membrane was unable to phosphorylate lipocortin I (Fig. 5, lanes 1, 2). In order to demonstrate that the absence of lipocortin phosphorylation is not due to nonspecific inhibition of protein kinase C or to proteolysis of phosphorylated lipocortin, several control experiments were performed. As a first test we activated protein kinase C in the cytosol of permeabilized cells; this was achieved by the addition of dioleoylglycerol and phosphatidylserine. In this case lipocortin I was phosphorylated intensively and was the dominant substrate of protein kinase C (Fig. 5, lane 3). The phenomenon was similar to that observed in the cytosolic extract of sonicated pig granulocytes [18]. Phosphatidylserine was added to activate protein kinase C in the cytosol of permeabilized cells, though we cannot exclude the possibility that it contributed to the presentation of Ca^{2+} -lipocortin I as a substrate. However, diacylglycerol and phosphatidylserine did not cause detectable phosphorylation of lipocortin I in permeabilized cells at Ca^{2+} concentrations lower than 0.1 mM (data not shown).

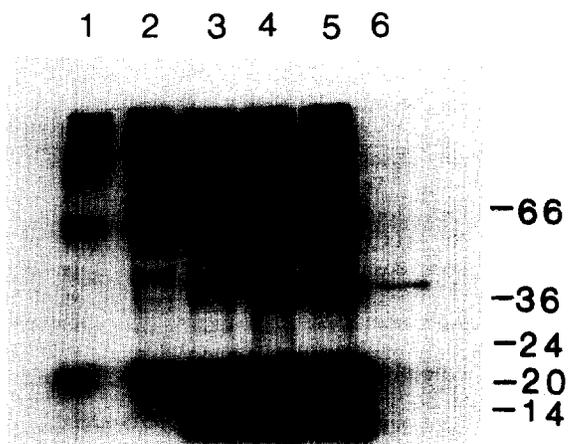


Fig. 5. The absence of phosphorylation of lipocortin I in phorbol ester-treated pig granulocytes and its phosphorylation by protein kinase C activated in the cytosol of permeabilized granulocytes. Cells were permeabilized in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and subjected to SDS-PAGE followed by autoradiography. Permeabilization was carried out at $5 \cdot 10^{-4}$ M Ca^{2+} without any activator of protein kinase C (lane 1), or in the presence of 40 ng/ml phorbol myristate acetate (lane 2), or in the presence of 25 $\mu\text{g}/\text{ml}$ phosphatidylserine + 2.5 $\mu\text{g}/\text{ml}$ dioleoylglycerol (lanes 3–5). In lanes 4 and 5 the system was supplemented with exogenous protein kinase C and with exogenous lipocortin I + exogenous protein kinase C, respectively. Lane 6 shows a sample of purified lipocortin I phosphorylated *in vitro*.

This result showed clearly that the absence of lipocortin phosphorylation was not caused by nonspecific inhibition of protein kinase C, and when lipocortin I was phosphorylated it was not eliminated by proteolysis due to the increased susceptibility of the phosphorylated form. Nevertheless, further control experiments were also performed. In the presence of phosphatidylserine and diacylglycerol the addition of exogenous protein kinase C to the permeabilized cells increased the rate of phosphorylation of lipocortin (Fig. 5, lane 4), and when this system was supplemented with exogenous lipocortin I, an increase was observed in the ^{32}P labelling of the 38 kDa protein band (Fig. 5, lane 5).

Discussion

The *in vitro* phosphorylation of lipocortin I by protein kinase C has been investigated in detail [13–17], but only a few data are available which demonstrate undoubtedly that the phosphorylation state of lipocortin I is affected by the selective activation of protein kinase C in cellular systems. Phosphorylation of a membrane bound, Triton-X-100-extractable form of lipocortin I in rat liver cells has been detected in response to activation of protein kinase C by phorbol ester [34]. Phosphorylation of lipocortin I in stimulated

chromaffin cells has been reported; however, the role of additional kinases besides protein kinase C has also been suggested [37].

Our experiments clearly demonstrate that, in pig granulocytes, where lipocortin I is a major protein component of the cytosol and probably has an important function: selective activation and translocation of protein kinase C to the membrane cannot lead to the phosphorylation of lipocortin I even at supra-physiological Ca^{2+} concentrations when Ca^{2+} -lipocortin I is formed. (We use the term ' Ca^{2+} -lipocortin I' to represent that form of lipocortin which can be phosphorylated by protein kinase C.) This finding was unexpected because in our previous experiments lipocortin I attached to the particulate fraction of granulocytes sonicated in the presence of Ca^{2+} was well phosphorylated by protein kinase C found in the same subcellular fraction. In our previous experiments the membrane was disintegrated and the orientation of the N-terminal tail of the lipocortin molecule containing the phosphorylation sites had no significance, while in permeabilized cells this orientation may be important from the point of view of accessibility for membrane-bound protein kinase C.

Protein kinase C is a family of structurally related but individual proteins with different regulatory properties and localization (reviewed in Refs. 35,36). In pig neutrophil granulocytes type II (β) protein kinase C is the dominant species [19], while in other cell types other members of the protein kinase C family are also present. It is conceivable that in other cell types the accessibility of the phosphorylation site of Ca^{2+} -lipocortin I is better for some other members of the family. However, our data indicate also that when protein kinase C is selectively activated the phosphorylation of cytosolic lipocortin I requires so high a Ca^{2+} concentration that those signals which produce the activation of protein kinase C and the normal increase of cytosolic Ca^{2+} cannot lead to the phosphorylation of cytosolic lipocortin I.

We investigated the phosphorylation of lipocortin in pig granulocytes and the amino acid sequence of porcine lipocortin is known to differ slightly from that of the human protein [38]. In the case of human lipocortin I the target sites of phosphorylation by protein kinase C are Thr-24, Ser-27 and Ser-28 [17]. One of these target sites is not present in porcine lipocortin that has a glycine residue in the position analogous to Ser-27 while its Thr-12 and Ser-16 are in positions analogous to Thr-24 and Ser-28 of human lipocortin I. We found that the porcine lipocortin was phosphorylated on Thr and Ser residues in an approximate molar ratio of 1:1. Nevertheless, the calcium requirement of phosphorylation by protein kinase C was in the same supra-physiological concentration range for both the human and porcine proteins.

If lipocortin I is phosphorylated at all by protein kinase C inside the cell this process requires an additional signal-transducing mechanism increasing dramatically the Ca^{2+} sensitivity of this protein. Stimulus-dependent liberation of arachidonic acid may be involved in the modulation of the calcium sensitivity of annexins [1], but in our experiments the addition of arachidonic acid to the permeabilized cells did not stimulate the phosphorylation of lipocortin I. On the other hand, lipocortin I is a good substrate for growth factor receptor tyrosine kinases [16,39]. Although this phosphorylation also seems to require the presence of Ca^{2+} , it proceeds at a Ca^{2+} concentration of about one order of magnitude lower than that catalyzed by protein kinase C, and phosphorylation of lipocortin I on tyrosine has been reported to increase the affinity of this protein for Ca^{2+} [40]. Epidermal growth factor has been found to stimulate the phosphorylation of lipocortin I at threonine as well as at tyrosine residues [41]. The integration of two signalling pathways plays a crucial role in the regulation of various biological processes [42] and may also lead to protein-kinase-C-catalyzed phosphorylation of lipocortin I.

Nevertheless, when in vitro experimental systems are investigated the phosphorylation of lipocortin I by protein kinase C may serve as a tool to detect the appearance and orientation of Ca^{2+} -lipocortin.

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