Modulation by Sphingosine of Phosphorylation of Substrate Proteins by Protein Kinase C in Nuclei from Cow Mammary Gland

Norio KATOH

1) National Institute of Animal Health, 3–1–5 Kannondai, Tsukuba, Ibaraki 305–0856, Japan

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ABSTRACT. Protein kinase C (PKC) is an enzyme activated by diacylglycerols such as 1-oleoyl-2-acetyl-sn-glycerol (OAG), phospholipids (in particular phosphatidylserine; PS) and Ca\textsuperscript{2+}, which regulate a wide variety of intracellular functions by phosphorylating multiple substrate proteins and enzymes. The effect of sphingosine, the backbone moiety of sphingolipids, on PKC activity and phosphorylation of endogenous proteins catalyzed by PKC was investigated in nuclei of cow mammary gland. Sphingosine inhibited nuclear PKC activity when lysine-rich histone was used as the substrate. The sphingosine inhibition of the PKC activity was reversed by the excess addition of PS, but not by OAG or Ca\textsuperscript{2+}. Several nuclear proteins, including 56-kDa, 43-kDa, 38-kDa and 36-kDa proteins, were shown to be substrates for PKC. Of the substrate proteins, the 38-kDa and 56-kDa proteins were identified as annexin I, the Ca\textsuperscript{2+}-phospholipid-binding protein; the 56-kDa and 43-kDa proteins have not yet been identified. Sphingosine inhibited phosphorylation of the 56-kDa protein and the 36-kDa annexin I, whereas it enhanced that of the 43-kDa protein. As with the PKC activity, inhibition by sphingosine of phosphorylation of the 56-kDa protein and 36-kDa annexin I was reversed by the excess addition of PS, but not by OAG or Ca\textsuperscript{2+}. In addition, by the excess addition of PS and not by OAG or Ca\textsuperscript{2+}, the sphingosine-enhanced phosphorylation of the 43-kDa protein was reversed and returned to near the level in the absence of sphingosine. It is suggested that sphingosine is involved in the regulation of PKC-dependent phosphorylation in the nucleus by modulating the association of PKC or its substrates, particularly annexin I, with membrane phospholipids in cow mammary gland.

KEY WORDS: annexin I, cow mammary gland, phosphorylation, protein kinase C, sphingosine.

Protein kinase C (PKC) is a phosphotransferase activated by diacylglycerols, phosphatidylserine (PS) and Ca\textsuperscript{2+}, and plays an essential role in transmembrane signal transduction [28, 36]. Responding to extracellular signals such as hormones, diacylglycerols are transiently produced from phosphatidylinositol-4,5-bisphosphate in the plasma membrane. The diacylglycerols produced increase the affinity of PKC for Ca\textsuperscript{2+} as well as for PS, thereby activating PKC. The activated PKC phosphorylates substrate proteins particularly in the plasma membrane, and the phosphorylated proteins regulate diverse biological functions.

Other than in the plasma membrane, it has recently been found that PKC is resident in the cell nucleus as well as in other intracellular organelles, or the enzyme is translocated from the cytosol to the nucleus [4, 7, 20, 31, 32, 42]. In addition, accumulating evidence suggests that a wide variety of lipids, including diacylglycerols, phospholipids, cholesterol, gangliosides and sphingolipids, are distributed in the nucleus and act as second messengers for PKC-dependent phosphorylation of nuclear substrates [1, 11, 29, 30, 34]. The substrates identified in the nucleus include DNA methyltransferase [3], DNA topoisomerase II [39], and chromosomal high mobility group I protein [26].

Annexins (also known as calpain inhibitors, endonexins and lipocortins and so on) are a family of structurally related proteins that are characterized by their ability to bind membrane phospholipids in a Ca\textsuperscript{2+}-dependent manner [10, 35]. Annexins are suggested to be involved in the regulation of various physiologic and pathologic processes, including suppression of inflammation. Of the annexins, annexin I is induced by glucocorticoids and mediates the anti-inflammatory effects of glucocorticoids by inhibiting phospholipase A\textsubscript{2} activity [6, 24]. Annexin I is mainly localized in the cytoplasm beneath the plasma membrane, but some annexin I is also resident in the nucleus [37] or is translocated from the cytosol to the nucleus in response to an increase in the intracellular Ca\textsuperscript{2+} concentration [25]. Annexin I is phosphorylated by PKC [10, 25, 35, 38]. We have previously shown that PKC is present in the cow mammary gland cytosol and particulate fraction [12] and in nuclei [20], and it phosphorylates annexin I that is distributed in cytosol [18], particulate [21] and nuclei [22] of the cow mammary gland. PKC-dependent phosphorylation of substrate proteins in the cytosol is modulated (inhibited or enhanced) by sphingosine [13], gangliosides [16] and sulfatide [19]. It is of interest to determine whether sphingosine modulates phosphorylation by PKC of annexin I and other substrate proteins in the nucleus. The purpose of the present study was to evaluate PKC activity and to explore PKC substrates in the nuclei of the cow mammary gland, and to examine whether sphingosine modulates PKC activity and substrate phosphorylation.

MATERIALS AND METHODS

Materials: Sphingosine, 1-oleoyl-2-acetyl-sn-glycerol (OAG), PS and lysine-rich histone (type III-S) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CM-cellulose (CM-52) and DEAE-cellulose (DE-52) were from Whatman (Maidstone, Kent, Great Britain), and

DEAE-Sepharose, Mono-S, Sephacryl S-200 and phenyl-Sepharose were from Pharmacia Biotech (Upsala, Sweden), respectively. [γ-32P]ATP was obtained from New England Nuclear-DuPont (Wilmington, DE, U.S.A.). Cow mammary glands in the midlactating stage (150 to 200 days after parturition) were collected from a local slaughterhouse.

**Nuclear phosphorylation:** Mammary nuclei were prepared as described previously [20]. In brief, fresh *pars glandularis* (100 g) was cut into small pieces, minced with a meat grinder, and then homogenized in 9 volumes of 0.25 M sucrose containing 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 2.5 mM ethyleneglycol bis(2-aminoethyl ether)-tetraacetic acid (EGTA), 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 µg/ml leupeptin, in a Polytron PTA-205. After filtration through two layers of surgical gauze, the homogenate was centrifuged at 800 × g for 10 min. The crude pelleted nuclei were suspended in 2 M sucrose containing 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, and 10 mM 2-mercaptoethanol and then centrifuged at 38,000 × g for 30 min to form pelleted nuclei. The nuclei were again suspended in the 2 M sucrose solution, and the centrifugation was repeated once more. The nuclei were finally washed three times with the 0.25 M sucrose solution by centrifugation at 800 × g for 10 min. The purity of the nuclei was assessed by evaluating Na⁺K⁺-ATPase activity and the DNA concentration, and their values were close to those previously reported, showing the high purity of the nuclear preparation. The purified nuclei, suspended in 0.25 M sucrose containing 20 mM Tris-HCl (pH 7.5), 2.5 mM EGTA and 10 mM 2-mercaptoethanol, were treated with 0.1% Triton X-100 for 60 min on ice and then centrifuged at 105,000 × g for 60 min. The resulting supernatant was used as the nuclear extract.

PKC was assayed as described previously [20].Briefly, the reaction mixture (0.2 ml) contained 25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 5 mM 2-mercaptoethanol, 50 µM [γ-32P]ATP, 40 µg of lysine-rich histone, 20 µl of nuclear extract (containing endogenous PKC), with or without 1 µg of OAG, 5 µg of PS, 1 µM Ca²⁺, or both. Sphingosine (20 µl) was added to the reaction mixture after sonication in 20 mM Tris-HCl (pH 7.5). The reaction was initiated by the addition of ATP and proceeded for 5 min at 30°C, which was in the linear phase of the time course.

The reaction conditions for endogenous protein phosphorylation were the same as those described for the PKC assay except that histone was deleted from the mixture. The reaction was allowed to continue for 5 min at 30°C, then stopped by adding 0.1 ml of Laemmli’s buffer, and boiling for 3 min. Phosphorylated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), in a 12% constant gel, then visualized by autoradiography.

**Annexin I purification:** The 36-kDa annexin I from cow mammary gland was purified as described previously [21]. Briefly, the tissue (100 g), minced by means of a meat grinder, was mixed with 300 ml of 0.25 M sucrose containing 20 mM Tris-HCl (pH 7.5), 10 mM EGTA, 1 mM PMSF, 10 µg/ml leupeptin and 1% Triton X-100, and stood at 4°C for 30 min with occasional stirring. To the supernatant separated by centrifugation at 9,600 × g for 20 min, solid ammonium sulfate was added to achieve 60% saturation. The pellet obtained by centrifugation at 9,600 × g for 20 min was dissolved in a minimum volume of 20 mM 2-(N-morpholino)ethanesulfonic acid-NaOH buffer (pH 6.0) and dialyzed against the same buffer, and thereafter applied to a CM-52 column (2.5 × 18 cm). The flow-through fractions containing annexin I were collected. Annexin I was further purified by sequential chromatographies with DE-52, DEAE-Sepharose, Sephacryl S-200 and Mono S.

**Other methods:** Cytosol and the particulate other than nuclei were prepared by centrifugation at 105,000 × g for 60 min of the postnuclear fraction (the supernatant obtained by centrifugation of the homogenate at 800 × g for 10 min) [20]. PKC from cow mammary gland cytosol was purified by ammonium sulfate fractionation, DE-52, Sephacryl S-200, protamine-Sepharose and phenyl-Sepharose chromatography procedures [21]. Phosphorylation by PKC of annexin I was performed as described previously [18]. Anti-bovine annexin I was prepared in rabbits and immunoblot analysis was done as described elsewhere [17]. Protamine-Sepharose 4B was made as in [27]. Protein was determined by the method of Bradford [2].

**RESULTS**

When lysine-rich histone was used as the substrate, sphingosine inhibited nuclear PKC activity (Table 1), as in the cytosolic and total particulate PKC activity [13]. The inhibition of nuclear PKC activity by sphingosine was partially overcome by the addition of a higher concentration of PS, but not or only slightly by OAG or Ca²⁺.

Phosphorylation of nuclear proteins in the absence or presence of the PKC cofactors and subsequent autoradiography revealed that 56-kDa, 43-kDa, 38-kDa (above the 36 kDa) and 36-kDa proteins were major substrates for PKC because of their enhanced phosphorylation by the cofactors (Fig. 1). It was unclear whether the 120-kDa, 20-kDa and 19-kDa proteins were PKC substrates, because their differences in the absence (lane 1) and presence of the cofactors (lanes 2 to 4) were not distinct. The 120-kDa protein has not yet been identified. The 20-kDa protein was inferred to be the cytosolic 21-kDa smooth muscle myosin light chain (MLC20) [19]. The 19-kDa protein seemed to be the substrate for sphingosine- [13] or melittin-activated protein kinase [14, 15]. Other than the PKC substrates, the 97-kDa protein appeared to be the substrate for phosphorylase b kinase [13]. The 30-kDa casein was the substrate for casein kinase [5]. During nuclear preparation, casein is suggested to comigrate with nuclei [20]. Phosphorylation of 88-kDa, 48-kDa and 24-kDa proteins, as indicated by arrowheads on the right, was not found in lanes 1 to 4, but was detected in lane 5.

Of the four major PKC substrates, the 38-kDa and 36-kDa proteins were identified as annexin I, as revealed by
immunoblot analysis (figure not shown); the 38-kDa annexin I was detected only in nuclei, whereas the 36-kDa species was distributed in both cytosolic and nuclear fractions [22]. Phosphorylation of the 38-kDa annexin I was increased by OAG (lane 2), OAG and PS (lane 3) and OAG, PS and Ca\textsuperscript{2+} (lane 4), when compared with lane 1. Lanes 2 to 4 were not different. In contrast, phosphorylation of the 36-kDa annexin I was not detected by OAG alone and OAG plus PS, but it was remarkably increased by the simultaneous addition of the three cofactors. Inclusion of Ca\textsuperscript{2+} alone did not increase the 36-kDa annexin I phosphorylation (figure not shown), indicating that the annexin I was phosphorylated by PKC but not by calmodulin-sensitive Ca\textsuperscript{2+}-dependent protein kinases such as myosin light chain kinase.

Phosphorylation of 56-kDa and 43-kDa proteins, like the 36-kDa annexin I, was increased by the presence of the three cofactors together, not by OAG alone and OAG plus PS, although these two proteins have not yet been identified.

Sphingosine inhibited phosphorylation of the 56-kDa protein and 36-kDa annexin I, whereas it enhanced phosphorylation of 88-kDa, 48-kDa, 24-kDa, 20-kDa and 19-kDa proteins (Fig. 1, lane 5). As described before, the 88-kDa, 48-kDa and 24-kDa proteins were not found in lanes 1 to 4, but were observed in lanes 5 to 8. The 38-kDa annexin I was unaffected by sphingosine. Inhibition by sphingosine of the 56-kDa protein and 36-kDa annexin I was reversed, at least in part, by the excess addition of PS (indicated by the arrowheads in lane 7). The enhanced phosphorylation by sphingosine of the 56-kDa and 36-kDa annexin I was reversed, at least in part, by the excess addition of PS (indicated by the arrowheads in lane 7). The enhanced phosphorylation by sphingosine of 48-kDa, 43-kDa, 24-kDa, 20-kDa and 19-kDa proteins was reversed by the addition of PS, not OAG or Ca\textsuperscript{2+}, and returned to near the levels in lane 4.

The 36-kDa annexin I was purified from whole tissue of cow mammary gland [21], and an experiment was conducted to examine whether phosphorylation of the 36-kDa annexin I by PKC was inhibited by sphingosine, and whether the inhibition was reversed by the excess addition of PKC cofactors (Fig. 2). Using the isolated substrate and enzyme, it was found that PKC-dependent phosphorylation of annexin I was inhibited by sphingosine and that the inhibition was reversed nearly completely by PS, but not or only slightly by OAG or Ca\textsuperscript{2+}. The nucleus-specific 38-kDa annexin I has not yet been purified.

DISCUSSION

The present study indicated that PKC activity in cow

Table 1. Effect of sphingosine on cow mammary gland nuclear PKC activity and the reversal of the activity by excess concentrations of PKC cofactors

<table>
<thead>
<tr>
<th>OAG (µg/ml)</th>
<th>PS (µg/ml)</th>
<th>Ca\textsuperscript{2+} (µM)</th>
<th>Sphingosine (µM)</th>
<th>Protein kinase activity (pmol/min/mg protein)</th>
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<td>250</td>
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An aliquot (20 µl containing 17.4 µg of protein) of the nuclear fraction was assayed in the absence or presence of the PKC cofactors OAG, PS and/or Ca\textsuperscript{2+}. All values are means of two separate experiments and each assay was done in duplicate, with assay error being less than 10%.
mammary gland nuclei was inhibited by sphingosine when lysine-rich histone was used as the substrate. Nuclear proteins were phosphorylated to explore endogenous substrates for PKC. As in histone, phosphorylation of the 56-kDa protein and the 36-kDa annexin I was inhibited by sphingosine. In contrast, phosphorylation of the 43-kDa protein was enhanced by sphingosine. The 38-kDa annexin I phosphorylation was unaffected by sphingosine. The inhibition by sphingosine of phosphorylation of 56-kDa protein and the 36-kDa annexin I and the enhancement of that of the 43-kDa protein were reversed by PS, but not by OAG or Ca2+.

Sphingosine is the backbone moiety of the complex sphingolipids, including sphingomyelin, gangliosides and sulfatides [41]. Sphingosine is formed from the breakdown of sphingomyelin. By the action of a number of extracellular signals, sphingomyelin is hydrolyzed by sphingomyelinases to ceramides (a mixture of fatty acids and sphingosine) which is subsequently degraded by ceramidases to sphingosine. The intermediary metabolites of sphingomyelin catabolism, including ceramides, sphingosine and sphingosine-1-phosphate (the phosphorylated form of sphingosine), are suggested to function as second messengers for PKC-dependent phosphorylation [8, 33]. Sphingosine inhibits [9] or modulates [13, 33, 40] PKC activity. More recently, it has been reported that metabolites such as sphingosine are resident within the nucleus, or they translocate from the endoplasmic reticulum, the Golgi apparatus or the plasma membrane to the nucleus [1, 11, 20, 23, 29, 30].

PKC is classified into three isoforms: conventional (or classical) (α, β, βI and γ), novel (δ, η, θ, ι and µ) and atypical (ζ and λ) isoforms [28, 36]. cPKCs require PS, Ca2+ and diacylglycerols (or phorbol esters); nPKCs are dependent on PS and diacylglycerols but insensitive to Ca2+; aPKCs are dependent on PS but insensitive to both Ca2+ and diacylglycerols. Most, but not all, of the isoforms, for example, PKC-α [32], -δ [4] and -e [7] are translocated from cytosol to the nucleus by treating various cells with phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA).

Annexin I is a PKC substrate in cytosol as well as in nuclei, and has been previously shown to be phosphorylated by cPKCs, but not by nPKCs or aPKCs [38]. Nevertheless, it has recently been reported that annexin I, together with nPKC-δ, is translocated by TPA from the cytosol to the nucleus [25]. Phosphorylation of the 36-kDa annexin I was activated by the three cofactors OAG, PS and Ca2+. In particular, Ca2+ is required for full activation (Fig. 1, lane 4), suggesting that the 36-kDa annexin I is preferentially phosphorylated by cPKCs, rather than by nPKCs or aPKCs. By comparison, the 38-kDa annexin I phosphorylation was essential for the presence of OAG and PS, but not for Ca2+ (lane 3). Although the structural relatedness of the two species of annexin I is unknown, the 38-kDa annexin I is detected only in nuclei, whereas the 36-kDa annexin I is distributed in both cytosolic and nuclear fractions [22]. It is
conceivable that the 38-kDa annexin I resides in the nucleus and is phosphorylated by nPKCs or aPKCs which are present in or translocated from the cytosol, whereas the 36-kDa annexin I is translocated together with cPKCs from the cytosol to the nucleus.

Phosphorylation of lysine-rich histone, the 36-kDa annexin I and unidentified 56-kDa protein was inhibited by sphingosine and their inhibition was reversed by PS; that of the 43-kDa protein (also unidentified) was in contrast enhanced by sphingosine whereas its enhancement was reversed by PS; and the 38-kDa annexin I phosphorylation was unaffected. Whereas the mechanism of the modulation by sphingosine on PKC phosphorylation is unknown and awaits further characterization, sphingosine appears to more preferentially bind PKC substrates, rather than PKC enzymes, because the modes of sphingosine modulation are different among the three types of substrates. Sphingosine, synthesized within the nucleus or translocated from other subcellular sites such as the endoplasmic reticulum, may initially interact with the substrate, and modulate the complex of cofactors OAG, PS and/or Ca\(^{2+}\), PKC and the substrate in the nuclei.

In conclusion, sphingosine is suggested to be involved in the regulation of PKC-dependent phosphorylation by modulating the association of PKC or its substrates, in particular annexin I, with phospholipids in the nuclei of the cow mammary gland.

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REFERENCES


