80 kDa Mouse Sperm Protein as a Substrate of Protein Kinase C

Setsuko KOMATSU* and Hisashi HIRANO

Department of Molecular Biology, National Institute of Agrobiological Resources, Kannondai 2-1-2, Tsukuba, Ibaraki 305, Japan.
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Calcium and phospholipid-dependent protein kinase (PKC) activity was detected mainly in the cytosol of the mouse sperm. The PKC in the cytosol fraction was partially purified by ion-exchange chromatography. Using the partially purified PKC, the phosphorylation of PKC substrates was examined *in vitro*. The phosphorylation of the 80 kDa protein was enhanced by phorbol ester treatment *in vitro* as well as *in vivo*. The partial amino acid sequence of this protein was homologous with that of guanosine 5′-cyclic monophosphate (cGMP)-dependent protein kinase and myosin light chain kinase, both of which are related to ligand–receptor-transduction. The present data suggest that the activation of PKC and subsequent specific protein phosphorylation might be involved in the regulation of the zona pellucida-induced acrosome reaction.

Keywords mouse sperm; protein kinase C; protein phosphorylation; acrosome reaction

Introduction

The signal transduction mechanism involved in the mouse sperm acrosome reaction is a special type of exocytosis.[1-3] Since the calcium and phospholipid-dependent protein kinase (protein kinase C, PKC) plays some roles in the regulation of exocytosis in somatic cell systems,[4] it is possible that sperm PKC may be involved in the regulation of the acrosome reaction. Lee *et al.*[5] reported that treatment of mouse sperm with a phorbol ester (12-*O*-tetradecanoyl-13-acetate; TPA) during the binding of the sperm to the zona resulted in the acceleration of the early stage of the acrosome reaction (B to S transition in the chlortetracycline assay) and in the inhibition of the late stage (S to AR transition). Komatsu *et al.*[6] reported that mouse sperm had PKC activity and that phosphorylation of the 215, 80 and 35 kDa proteins was enhanced by both TPA and calcium ionophore A23187 treatment. In addition, the phosphorylation of the 45 kDa protein was also augmented during the acrosome reaction induced by dibutyryl adenosine cyclic monophosphate (dbcAMP) or dibutyryl guanosine cyclic monophosphate (dbcGMP).[7] However, the possible functional roles of these phosphorylated proteins during the mouse sperm acrosome reaction have not yet been demonstrated. In this study, we analyzed the *in vitro* phosphorylation of these proteins and have determined the amino acid sequence of the 80 kDa protein.

Materials and Methods

Preparation of Sperm Suspension Ten- to 12-week-old male mice of DDY strain were obtained from Shizuoka Animal Center (Hamamatsu, Japan). Mice were sacrificed by cervical dislocation to obtain epididymides, which were scratched by a fine needle to remove the sperm mass. The sperm mass was then introduced into a modified Krebs Ringer bicarbonate buffer (m-KRB)[8] containing 4 mg/ml polyvinylpyrrolidene (PVP, Sigma, St. Louis, U.S.A.) and 10 mM HEPES, pH 7.4.

Preparation of Subcellular Fractions The following procedures were carried out at 4 °C. The sperm was suspended in a homogenization buffer [20 mM Tris–HCl, pH 7.5, 0.25 M saccharose, 10 mM ethylene glycol-bis (β-amino ethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA), 2 mM ethylenediaminetetraacetic acid (EDTA), 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 1 mM dithiothreitol, 5 mM *p*-nitrophenylphosphate, 5 mM benzamidine][9,10] and homogenized in a glass homogenizer. The sperm homogenates were centrifuged at 3000 rpm for 5 min and then the collected supernatants (sperm extract) were centrifuged at 100000 rpm for 15 min in a Beckman TLA100.2 rotor (CA, U.S.A.). The supernatant and the pellet was used as a cytosolic and membrane fractions, respectively.

Assay for Protein Kinase C Cytosolic and membrane fractions of sperm were obtained as described above. PKC was partially purified from both fractions by DEAE cellulose chromatography (DE52, Whatman, Maidstone, U.K.).[11] PKC activity was assayed in a reaction mixture (100 μl) containing 20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.4 mg/ml histone III-S, 0.2 mM $CaCl_2$, 25 μg/ml phosphatidylserine (PS), 4 μg/ml TPA (Sigma), 1 μCi [γ-^{32}P]ATP (1.1 Ci/mmol, Amersham, Buckinghamshire, U.K.) and 10 μl of PKC preparation. The basal level of histone III-S phosphorylation was measured in the presence of 4 mM EGTA instead of $CaCl_2$, PS and TPA. The reaction mixture was incubated for 15 min at 30 °C and stopped by immersion in an ice-water bath, and then transferred onto a P-81 filter (Whatman). Radioactivity was determined by Cherenkov counting.

***In Vitro* Phosphorylation of Sperm Extract by Purified PKC from Cytosolic Fraction of Mouse Sperm** The *in vitro* phosphorylation assay was carried out in the reaction mixture (40 μl) described above, except that histone III-S was not used and 5 μl of purified partially PKC from cytosolic fraction of mouse sperm and 5 μl of sperm extract were used. After phosphorylation, the sample was added to lysis buffer,[12] and subjected to two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).[13] The gels were dried and exposed for 3 d at −80 °C to Kodak X-ray film.

Amino Acid Sequence Analysis After separation by 2D-PAGE, proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (ProBlott, Applied Biosystems, Foster City, U.S.A.) and were detected by Ponceau 3R staining.[14] PVDF membrane pieces containing the major proteins were cut out and applied to the upper glass block of the reaction chamber of a gas-phase protein sequencer (477A, Applied Biosystems). Edman degradation was performed according to the standard program supplied by Applied Biosystems and the released phenylthiohydantoin derivaties of amino acid residues were identified by the on-line, high performance liquid chromatography system (120A, Applied Biosystems). Amino acid sequences obtained were compared with sequences of SEQDB data base (Protein Research Foundation, Osaka, Japan).

Results and Discussion

Mouse sperm proteins were fractionated into cytosolic and membrane fractions by ultracentrifugation, PKC was partially purified from both fractions by DE52 chromatography and the PKC activity of each fraction was examined. Since no significant PKC activity (50 pmol/min/10^8 cells) was detected in the cytosolic fraction obtained from crude sperm homogenates, PKC was partially purified by DE52. The PKC activities of the cytosolic and membrane fractions were 850 and 190 pmol/min/10^8 cells, respectively. On the other hand, protein kinase A (PKA) and protein kinase G (PKG) activities were not detected in these fractions (data not shown). This indicates that the majority

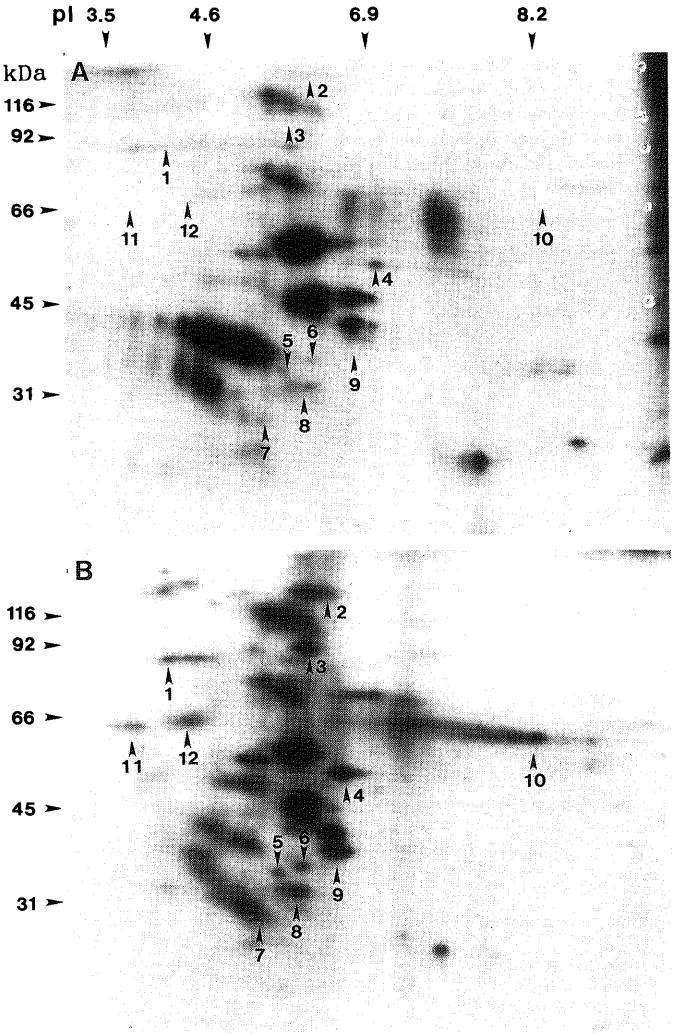

Fig. 1. *In Vitro* Protein Phosphorylation in Mouse Sperm Extract by Exogenous PKC

Right to left, isoelectric focusing for first dimension; top to bottom, sodium dodecyl sulfate (SDS)-PAGE for second dimension. No. 1 of arrowhead shows the position of 80 kDa protein. Twelve proteins were phosphorylated by TPA, PS and Ca^{2+}. PKC was purified from the cytosolic fractions of mouse sperm. *In vitro* protein phosphorylation was carried out in the presence of EGTA (A) of PS, $CaCl_2$ and TPA (B) by addition to the mouse purified cytosolic fraction and the sperm extract. The reaction was terminated by the addition of lysis buffer and the samples were subjected to two-dimensional gel electrophoresis. The gels were dried and exposed for 3 d at −80 °C to Kodak X-ray film.

of PKC activity is located in the cytosolic fraction, a finding similar to that previously published for somatic cells.[4]

Using the partially purified PKC from the cytosolic fraction, the phosphorylation of PKC substrates was examined *in vitro*. The mouse sperm extracts were incubated with the cytosolic PKC in the presence of EGTA, or PS, $CaCl_2$ and TPA. After the enzymatic reaction, the proteins were separated by 2D-PAGE (Fig. 1). When the sperm extract was incubated, proteins were not phosphorylated in the presence of PS, $CaCl_2$ and TPA, compared with EGTA. However, when the sperm extract was incubated with the purified PKC from cytosolic fraction, proteins were phosphorylated. Twelve spots detected in the presence of PS, $CaCl_2$ and TPA were absent following EGTA treatment. These 12 spots, which included the 80 kDa protein with an isoelectric point of

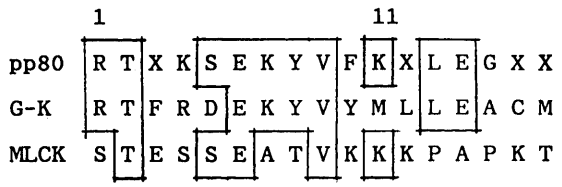

Fig. 2. Amino Acid Sequence Homology among pp80, P-G and MLCK

pp80, 80 kDa protein; G-K cGMP-dependent kinase [14]; MLCK, myosin light chain kinase [15]. The amino acid sequence of pp80 shows the N-terminal sequence, and the amino acid sequences of G-K and MLCK show the internal sequences.

4.0, were considered to be substrates of PKC *in vitro*. However, an acidic 215 kDa and basic 35 kDa proteins were not phosphorylated. We think that we could not recognize the phosphorylation *in vitro* of the acidic 215 kDa and basic 35 kDa proteins because the phosphorylation *in vivo* of these two proteins was very weak.[6] It is known that the 80 kDa protein with an isoelectric point of 4.0 is phosphorylated in intact cells.[6] This was confirmed by an *in vitro* phosphorylation experiment using PKC purified from mouse in this study. It was concluded that the 80 kDa protein is considered to be a substrate of PKC in mouse sperm.

The 80 kDa protein was transferred onto PVDF membrane and the 15 amino acid N-terminal sequence was determined using a gas-phase protein sequencer (Fig. 2). A structural homology search indicated that this N-terminal amino acid sequence of the 80 kDa protein was highly homologous with those of cGMP-dependent protein kinase[15] and myosin light chain kinase[16] proteins, which are known to be related to ligand–receptor-transduction. Thus, PKC-induced protein phosphorylation may be involved in the regulation of the zona pellucida-induced mouse sperm acrosome reaction.

Ligand–receptor-transduction mechanisms have previously been implicated in the regulation of sperm–egg interactions.[1,2] The mouse sperm acrosome reaction may serve as a model to study this regulation, since sperm binds to the sperm receptor, ZP3, and a portion of ZP3 subsequently induces the acrosome reaction,[3] which is a form of exocytosis. Further, it has been pointed out that cyclic nucleotides and calcium play a role in the acrosome reaction.[5,7] It has previously been reported that the 215, 80, 35 and 45 kDa proteins were phosphorylated when intact sperm was incubated in the presence of the calcium ionophore A23187 and TPA or cyclic nucleotides, all of which induce the mouse sperm to acrosome reaction. However, we did not know whether or not these proteins are catalyzed with PKC and PKA. The results of *in vitro* experiment confirm that at least the 80 kDa protein among these four proteins is a substrate of PKC.

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