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Original Article

NYD-SP27, a novel intrinsic decapacitation factor in sperm

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Abstract:

Prior to fertilization sperm has to undergo an activation process known as capaciation, leading to the acrosome reaction. Till now, little is known about the mechanism for preventing premature capacitation in sperm although decapacitation factors from various sources have been thought to be involved. In this study, we report that NYD-SP27, an isoform of phospholipase C Zeta 1 (PLCZ1), is localized to the sperm acrosome in mouse and human spermatozoa by immunofluorescence using a specific antibody. Western blot and double staining analyses show NYD-SP27 becomes detached from sperm, as they undergo capacitation and acrosome reaction. The absence of HCO_3^-, a key factor in activating capacitation, from the capacitation-inducing medium prevents the loss of NYD-SP27 from sperm. The anti-NYD-SP27 antibody also prevents the loss of NYD-SP27 from sperm, reduced the number of capacitated sperm, inhibited the acrosome reaction induced by ATP and progesterone, and inhibited agonist-induced PLC-coupled Ca^{2+} mobilization in sperm, which can be mimicked by the PLC inhibitor, U73122. These data strongly suggest that NYD-SP27 is a physiological inhibitor of PLC that acts as an intrinsic decapacitation factor in sperm to prevent premature capacitation and acrosome reaction.

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Keywords: acrosome reaction, capacitation, decapacitation factor, NYD-SP27, phospholipase C

1 Introduction

Mammalian spermatozoa cannot undergo fertilization without proper activation, termed ‘capacitation’, in the female tract [1]. During capacitation, extensive changes occur in various sperm compartments. These include the loss or redistribution of factors acquired during the epididymal transient or from seminal plasma, as well as reorganization of membrane proteins and lipids, initiation of calcium ion fluxes, leading to biochemical changes in the sperm and hyperactive sperm motility [2]. These changes lead to the acrosome reaction, which is essential for the sperm to penetrate the ovum [3].

For natural fertilization to take place, timely capacitation and induction of the acrosome reaction are important, and maintenance of spermatozoa in an uncapacitated state prior to ejaculation is thus essential.

The loss of decapacitation factors, such as seminal plasma glycoproteins, from the sperm and the presence of capacitation-inducing molecules, such as HCO_3^- and serum albumin, in the female tract are important for

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sperm capacitation and acrosome reaction. Although a number of surface decapacitation factors have been reported, most of these factors are derived from seminal plasma [2]. To date, no decapacitation factors intrinsic to sperm have been reported.

Phospholipase C (PLC) plays a critical role in capacitation and acrosome reaction [3, 4]. When activated, PLC and the phosphatidylinositol-PLC (PI-PLC) pathways mediate intracellular Ca^{2+} release and hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate, resulting in sperm membrane fusion and acrosome reaction [5, 6]. We hypothesized that inhibition of PLC activation or PLC-coupled signalling could thus prevent the premature capacitation and acrosome reaction.

We have identified a new human PLC isoform, NYD-SP27 (accession number AY035866), which belongs to the PLCζ family, but lacks the EF domains in its N terminus. The structural difference between NYD-SP27 and other PLCζ isoforms suggests differences in the mode of action. Indeed, our earlier studies have showed an inhibitory effect of NYD-SP27 on PLC-coupled mobilization of calcium ions and the activity of protein kinase C (PKC) in the pancreas [7]. These results suggest that NYD-SP27 may act as a negative modulator for PLC signalling. As NYD-SP27 is also highly expressed in adult testis [7], we hypothesized that NYD-SP27 may be an intrinsic decapacitation factor that maintains the uncapacitated state of sperm. Through searches of the National Center for Biotechnology Information (NCBI) database, we found a homologous sequence in mouse, designated mNYD-SP27, which has a similar structure to NYD-SP27 (accession number AK006672, named mNYD-SP27 to distinguish it from the full length of mouse Plcz1). We were therefore able to make use of mouse as an animal model to study the function of NYD-SP27 in this study.

2 Materials and methods

2.1 Human and mouse samples

This study was approved by the Ethics Committee of Nanjing Medical University (Nanjing, China), and informed written consent was obtained from the adult human volunteer participants (30–50 years old). Testicular tissue was obtained by biopsy, and ejaculate was obtained by masturbation after 3–6 days of abstinence. Normal semen samples were used in this study after routine semen analysis, according to the World Health Organization (WHO) criteria (1999) [8]. Normal sperm met the following criteria: > 20 × 10^6 spermatozoa mL^{-1} with more than 50% motile sperm, over 25% of the sperm moving forcefully in one direction (rapid and linearly progressive) and < 1 lymphocyte per high-power field (× 40). For mouse samples, male mice (3–6 months old) were killed to obtain the testes and mature sperm were obtained by making small incisions throughout the cauda epididymis, followed by extrusion and suspension in phosphate-buffered saline (PBS) or culture medium (human tubal fluid [HTF] media, *In Vitro* Care, Frederick, MD, USA).

2.2 Expression of recombinant proteins and antibody production

The full-length coding sequence of NYD-SP27 was subcloned into the pET28a expression vector (GE Healthcare, San Francisco, CA, USA) coding for six N-terminally located histamine residues to obtain recombinant NYD-SP27 protein. The construct was subsequently used to transform the competent BL21 (DE3) pLysS cells. The transformed cells were grown in Luria-Bertani medium (10 g of tryptone, 10 g of yeast extract, 5 g of NaCl) containing kanamycin (50 μg mL^{-1}). When the cell concentration reached around 1.7 × 10^8 cells mL^{-1} (as determined by the optical density [OD] reading at 600 nm, which reached 0.6), isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mmol L^{-1} to induce the expression of the NYD-SP27 recombinant protein. After 6 h of induction at 37ºC, cells were collected by centrifugation at 1 500 × *g* and suspended in buffer containing 8 mol L^{-1} urea. The cells were sonicated for 10 min on ice and then centrifuged at 10 000 × *g* at 4ºC for 30 min. The recombinant protein in the supernatant was purified by high performance liquid chromatography (AKTA Basic, Amersham Biosciences, Piscataway, NJ, USA) under denaturing conditions according to the manufacturer's protocol (HiTrap™ Chelating HP 1 mL column), and the purified His-NYD-SP27 was refolded by dialysis against a decreasing linear gradient of the denaturant buffer.

An antibody against NYD-SP27 was produced by immunization of Balb/c female mice with the purified recombinant NYD-SP27, and the titre of the antisera was determined by enzyme-linked immunosorbent assay.

2.3 Protein extraction and immunoblot analysis

Spermatozoa were separated by centrifugation (1 500 × *g*, for 10 min, at room temperature) and washed thrice in chilled PBS. They were then treated with lysis buffer containing 7 mol L^{-1} urea, 2 mol L^{-1} thiourea, 4% (w/v) 3-([3-cholamidopropyl]-dimethylammonio)-1-propane sulphonate (Chaps) and 2% (w/v) dithiothreitol in the presence of 1% (v/w) Protease Inhibitor Cocktail kit (Pierce Biotechnology, Rockford, IL, USA). Testicular tissue was homogenized (Ultra–Turrax®; IKA, Stanfen, Germany) and then treated with the same lysis buffer. The concentration of the extracted protein was determined with a Bio-Rad DC protein assay [9] kit (Bio-Rad Laboratories Inc., Mississauga, ON, Canada) using bovine serum albumin as a protein standard. The capacitating medium was collected after capacitation culture and then submitted to ultrafiltration (Amicon Ultra-15 centrifugal filter unit with Ultracel-50 membrane; Millipore, Billerica, MA, USA) to isolate proteins from the medium.

Protein extracted from the testis or sperm (60 μg per 10 μL) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (12% (w/v) polyacrylamide gel) and the resolved proteins were transferred to a nitrocellulose membrane. The membranes were blocked with 1% skim milk in Tris-buffered saline, and immunoblotted with the anti-NYD-SP27 serum (1:1 000), pre-immune serum (1:1 000) and antigen pre-absorbed anti-NYD-SP27 serum (1:1 000) or polyclonal rabbit anti-β-tubulin (1:2 000; Abcam, Cambridge, MA, USA) and a horseradish peroxidase-labelled secondary antibody (1:1 000; Beijing Zhongshan Biotechnology Co., Beijing, China). Immunoreactivity was detected using an Enhanced Chemiluminescence Reaction kit (Amersham Biosciences) and the images were captured by a FluorChem® 5500 imaging system (Alpha Innotech, San Leandro, CA, USA). The molecular mass of the proteins was deduced by comparison with molecular mass standards (New England BioLabs, Ipswich, MA, USA).

The intensity of the protein band was scanned with Adobe PhotoShop software (Adobe Systems Inc, San Jose, CA, USA) and normalized against that of tubulin.

2.4 Chlortetracycline fluorescence assay

A chlortetracycline (CTC) stock solution, containing 750 μmol L^{-1} CTC–HCl (Sigma), 130 mmol L^{-1} NaCl, 5 mmol L^{-1} L-cysteine and 20 mmol L^{-1} Tris-HCl (pH 7.8), was prepared daily and stored at 4ºC in the dark [10]. A 50-μL sample of sperm suspension was mixed with 50 μL of CTC stock solution in a 1.5-mL Eppendorf tube at room temperature and then 17.5 μL of 10% formalin in 2.5 mol L^{-1} Tris base was added as a fixative. The samples were kept at 37ºC for 1 h and then at 4ºC overnight for sedimentation of the sperm. Microscope slides were prepared and 200 sperm per slide were observed under a Zeiss Axioskop2 plus fluorescence microscope at an excitation wavelength of 430 nm. Using CTC staining, our earlier experiments have revealed three distinct staining patterns after sperm were incubated in capacitating medium [11]. The F pattern is characterized as uncapacitated, acrosome-intact sperm; the B pattern represents capacitated but acrosome-intact sperm; and the AR pattern corresponds to sperm that had undergone both capacitation and acrosome reaction.

2.5 Antibody and PLC inhibitor treatment

Mouse sperm (3×10^7 mL^{-1}) were suspended in culture medium (HTF; *In Vitro* Care) containing different dilutions of anti-NYD-SP27 serum (final dilutions, 1:10, 1:20, 1:40 and 1:80) or 10 μmol L^{-1} of the PLC inhibitor U73122 (diluted in $CHCl_3$; Sigma) and then incubated for 100 min to allow capacitation. To induce the acrosome reaction, progesterone (prepared in dimethylsulphoxide; Sigma) or extracellular ATP (prepared in normal saline) was added directly to the culture medium containing either the antiserum or the PLC inhibitor to a final concentration of 15 μmol L^{-1} [12] or 2.5 mmol L^{-1} [13], and incubated for an additional 15 min to induce the acrosome reaction. At different time points, spermatozoa suspensions were collected for CTC staining. Controls included spermatozoa that were incubated with the pre-immune serum, antigen pre-absorbed anti-NYD-SP27 serum (final dilution 1:10) or the solvents alone.

2.6 Indirect immunofluorescence and double-labelled fluorescence

Sperm samples were fixed with 4% paraformaldehyde in PBS for 1 h, permeabilized with 0.2% Triton X-100 in PBS for 20 min at 37ºC (this step was omitted from the indirect Immunofluorescence experiments with non-permeabilized sperm), and then blocked with goat serum (1%, Beijing Zhongshan Biotechnology Co.) for 2 h at room temperature. Following incubation with anti-NYD-SP27 (1:500) overnight at 4ºC, sperm were incubated with fluorescein isothiocyanate (FITC; Beijing Zhongshan Biotechnology Co.)-conjugated

anti-mouse IgG at a dilution of 1:100 for 1 h at room temperature and observed by fluorescence microscopy at an excitation wavelength of 470 nm. For double-labelled fluorescence, mouse sperm were first stained with CTC and then smeared onto slides. Sperm were observed by fluorescence microscopy at an excitation wavelength of 430 nm and recorded with a digital camera. The positions of the detected sperm were marked, and indirect immunofluorescence was performed as described above using tetraethyl rhodamine isothiocyanate (TRITC)-labelled secondary antibody. The slides were examined by fluorescence microscopy at an excitation wavelength of 560 nm to find the earlier recorded visual fields with CTC staining to identify the same sperm. Negative controls were prepared by replacing the first antiserum with pre-immune serum.

2.7 Fluorescence recordings of $[Ca^{2+}]_i$ of mouse sperm

Cauda sperm were incubated in 3 μmol L^{-1} fluo-4-acetoxymethyl ester and 1.6 μmol L^{-1} pluronic F127 (Molecular Probes, Eugene, OR, USA) for 30 min at 37ºC. Then the PLC inhibitor U73122 (10 μmol L^{-1}) or anti-s-PLZ1 serum (1:50) was added to the medium and incubated for an additional 30 min at 37ºC before being loaded into the image chamber. Imaging chambers were prepared by coating coverslips with 50 μg mL^{-1} poly-D-lysine, shaking off the excess and air-drying. Loaded sperm were then centrifuged for 5 min at 1 000 × *g* and suspended in the original volume of medium.

Labelled sperm were diluted 1:2 in the medium, placed into the chamber immediately and left for 1–3 min, after which the unattached sperm were removed by washing with sperm medium. After being washed, the sperm were incubated in a chamber containing 1 mL of sperm washing medium. After allowing a few seconds to establish stable recording, 100 μmol L^{-1} ATP or progesterone was added to the chamber. The temperature-controlled chamber (37ºC) was mounted on a microscope (IX70, Olympus, Tokyo, Japan) with a × 40 fluorescence objective. Changes in fluorescence were recorded using a charge coupled device (CCD) camera in continuous acquisition mode and processed using MetaFluor software from Universal Imaging Corp. (Washington D.C., USA). The fluorescence intensity at 490 nm was recorded throughout the experiment.

2.8 Statistical analysis

To assess the possible effects of the anti-NYD-SP27 serum, we calculated the percentage of sperm with three different CTC staining patterns. The data were expressed as means ± SEM. We used one-way analysis of variance (ANOVA) to analyse the data and the least significant difference *post hoc* test to examine any significant results. The results were considered statistically significant at $P < 0.05$. All statistical analysis was done with SPSS for Windows (version 11.0; SPSS Inc., Chicago, IL, USA).

3 Results

3.1 Expression and cellular localization of NYD-SP27

Figure 1A shows a single band with the expected molecular mass for NYD-SP27 of approximately 58 kDa. The same antibody also detected the 62 kDa mouse homologue. The specificity of this antiserum was verified by the absence of the protein band when pre-immune serum and antigen pre-absorbed serum were used. β-Tubulin was used as a loading control (Figure 1B).

Cellular localization of NYD-SP27 in the sperm was further examined by indirect immunofluorescence using the anti-NYD-SP27 serum. Typical immunofluorescence patterns of human and mouse spermatozoa are shown in Figure 2A. Bright fluorescence signals were invariably observed in the acrosome of both human and mouse sperm, and in the equatorial segment of human sperm. Immunofluorescence was not seen when pre-immune or pre-absorbed sera were used (Figure 2). These results suggest that NYD-SP27 (or mNYD-SP27) is a cytoplasmic protein, which is associated with the acrosome in sperm.

3.2 Detachment of mNYD-SP27 from sperm during capacitation and acrosome reaction

Decapacitation factors are known to be detached from sperm during capacitation and acrosome reaction. As shown in Figure 3, there was a time-dependent decrease in NYD-SP27 protein in the sperm when incubated in capacitating medium. This result suggests detachment of this protein during capacitation and acrosome reaction. To further test this hypothesis, western blot analysis was used to measure mNYD-SP27 protein in sperm during capacitation.

Figure 4 shows that the abundance of mNYD-SP27 was greatly reduced when incubated in the presence of a known capacitation agent, HCO_3^- [1], when compared with the mNYD-SP27 that were

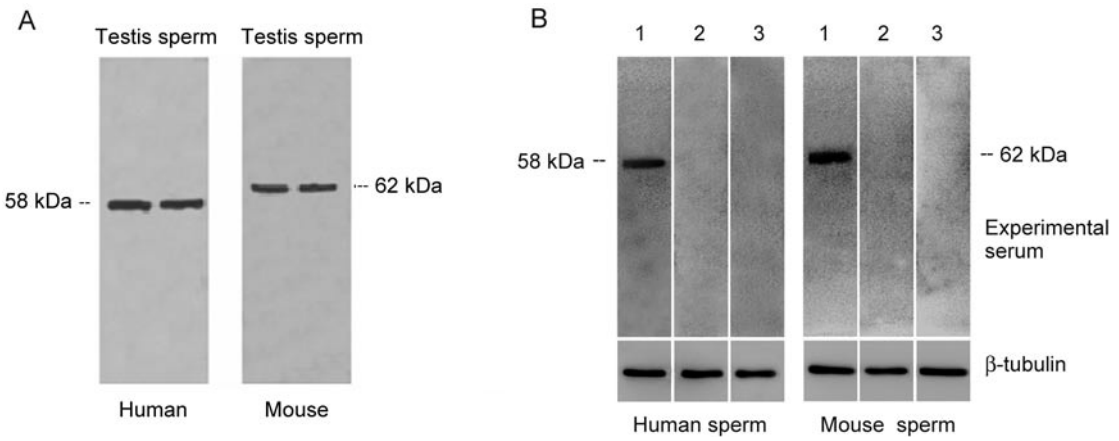

Figure 1. (A): The expression of NYD-SP27 and its mouse homologue. Western blots showed the expression of NYD-SP27 and its mouse homologue, mNYD-SP27, in the testes and spermatozoa with expected molecular weights. (B): Verification of the specificity of anti-NYD-SP27 serum in human and mouse spermatozoa samples. Upper panels: western blots done with experimental sera with the same dilution, lane 1: anti-NYD-SP27 serum, lane 2: pre-immune serum; lane 3: recombinant protein pre-absorbed anti-NYD-SP27 serum. Lower panels: western blots done with beta-tubulin antibody as a loading control.

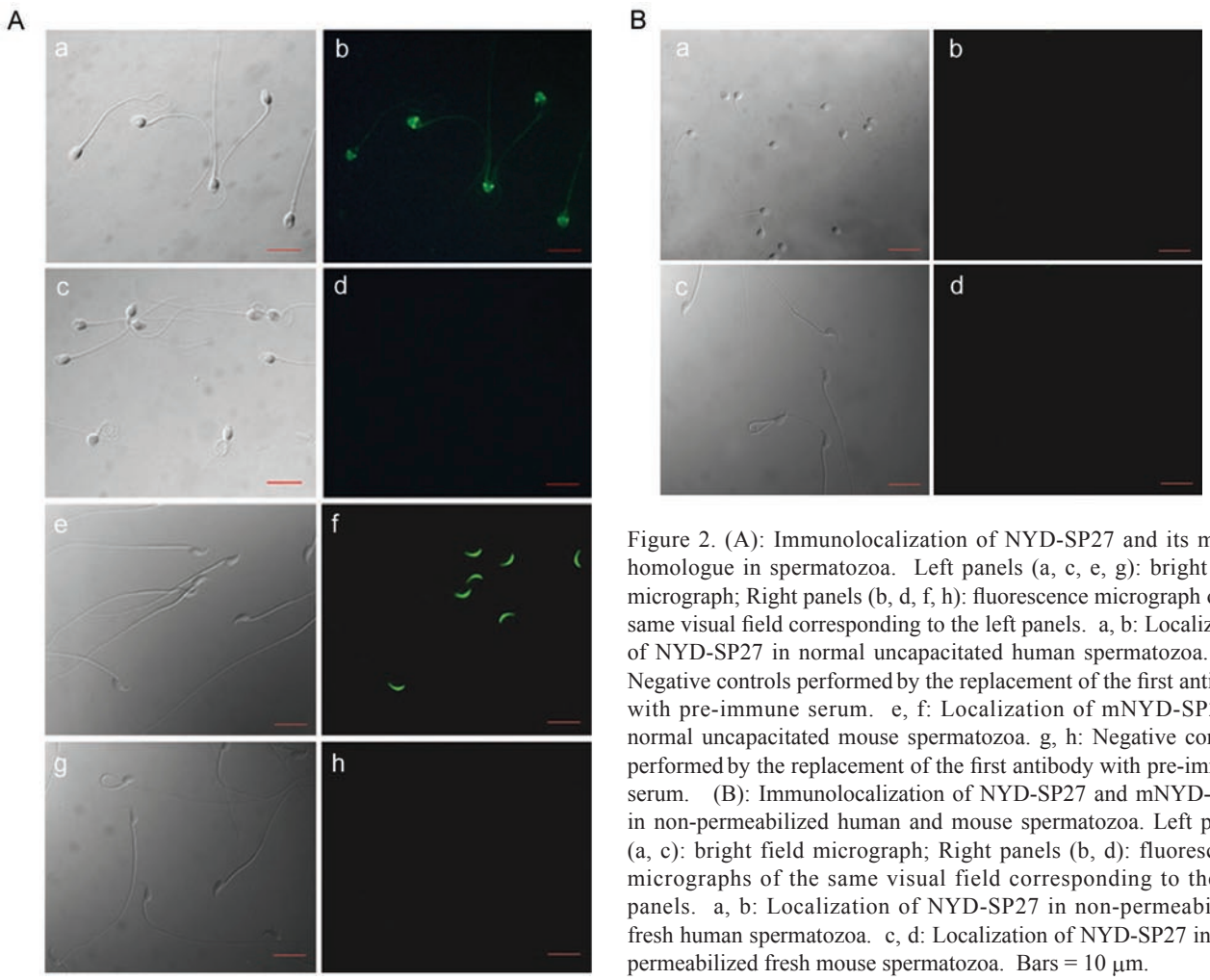

Figure 2. (A): Immunolocalization of NYD-SP27 and its mouse homologue in spermatozoa. Left panels (a, c, e, g): bright field micrograph; Right panels (b, d, f, h): fluorescence micrograph of the same visual field corresponding to the left panels. a, b: Localization of NYD-SP27 in normal uncapacitated human spermatozoa. c, d: Negative controls performed by the replacement of the first antibody with pre-immune serum. e, f: Localization of mNYD-SP27 in normal uncapacitated mouse spermatozoa. g, h: Negative controls performed by the replacement of the first antibody with pre-immune serum. (B): Immunolocalization of NYD-SP27 and mNYD-SP27 in non-permeabilized human and mouse spermatozoa. Left panels (a, c): bright field micrograph; Right panels (b, d): fluorescence micrographs of the same visual field corresponding to the left panels. a, b: Localization of NYD-SP27 in non-permeabilized fresh human spermatozoa. c, d: Localization of NYD-SP27 in non-permeabilized fresh mouse spermatozoa. Bars = 10 μm.

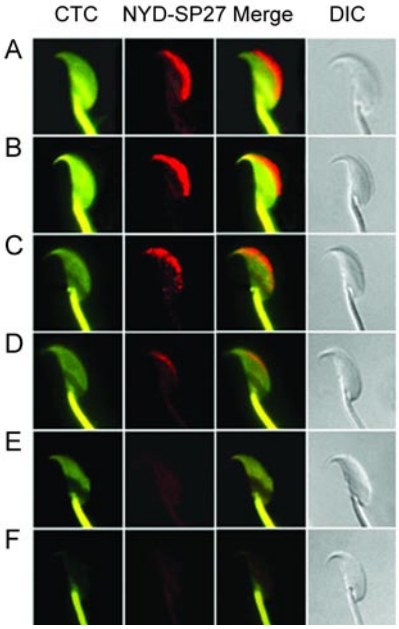

Figure 3. Demonstration of detachment of mNYD-SP27 from mouse sperm. Demonstration of the detachment of mNYD-SP27 from mouse sperm during the capacitation and the acrosome reaction by chlortetracycline (CTC) and mNYD-SP27 immunofluorescence double staining. CTC staining was used to show the different conditions of the sperm, from uncapacitated to capacitated to acrosome-reacted (a to f). The amount of mNYD-SP27 in sperm was determined by immunofluorescence using a secondary antibody labelled with tetraethyl rhodamine isothiocyanate (TRITC). CTC and mNYD-SP27 merged micrographs were used to show the change of mNYD-SP27 in sperm at different stages. The bright field micrographs were taken through differential interference contrast and show the morphology of the sperm. The merged images show that the amount of mNYD-SP27 in sperm decreased as the sperm underwent capacitation and the acrosome reaction, and almost disappeared after the acrosome reaction. DIC, differential interference contrast.

exposed to HCO_3^- free medium. These results confirm the association of mNYD-SP27 detachment with capacitation. Interestingly, detachment of NYD-SP27 from sperm was apparently attenuated by the presence of NYD-SP27 antiserum, because the pre-immune serum showed no effect on detachment (Figure 4). The presence of NYD-SP27 in the medium, after the sperm were incubated with capacitation agents, is consistent with detachment of this protein during capacitation (Figure 4C).

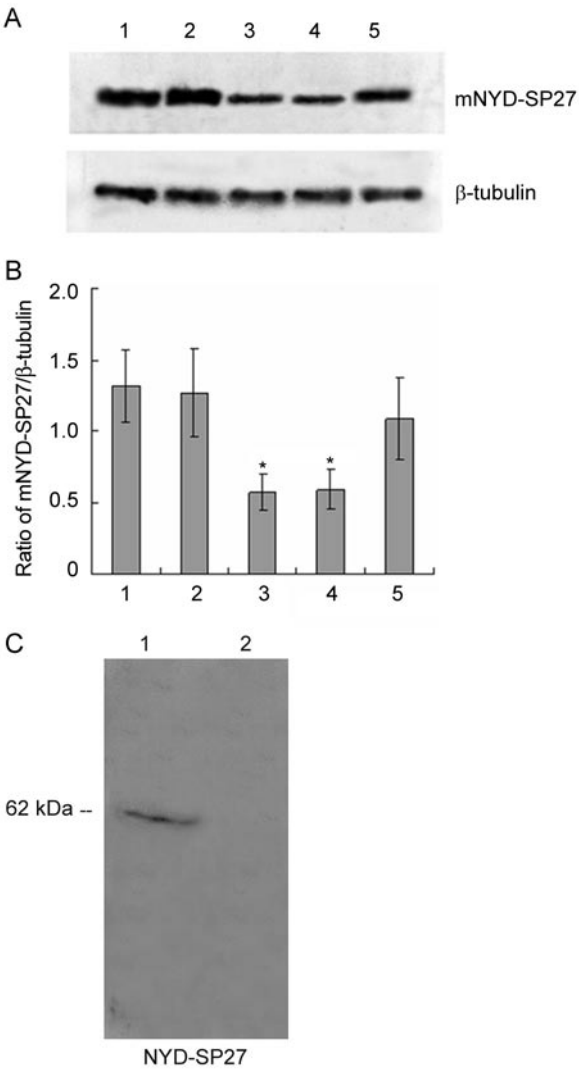

Figure 4. Dependence of detachment of mNYD-SP27 from sperm on medium conditions. (A): Western blots of mNYD-SP27 (upper band) in sperm incubated under different medium conditions: lane 1, uncapacitated sperm freshly obtained from the cauda epididymis; lane 2, sperm incubated in HCO_3^- free capacitating medium for 100 min; lane 3, sperm incubated in HTF medium for capacitation for 100 min; lane 4, sperm incubated in HTF medium containing pre-immune serum (1:10 dilution); lane 5, sperm incubated in HTF medium containing anti-NYD-SP27 serum (1:10 dilution). β-tubulin (lower band) was used as a protein loading control. (B): Corresponding ratio of mNYD-SP27 to β-tubulin in each group indicated in (A). The data representing the mean ± SEM were obtained from at least three independent experiments. $^*P < 0.05$, compared with the other lane groups. (C): Detection of mNYD-SP27 in capacitating medium after capacitation culture. Western blots detected mNYD-SP27 in the capacitating medium after capacitation culture (lane 1) and fresh culture medium was used as a control (lane 2).

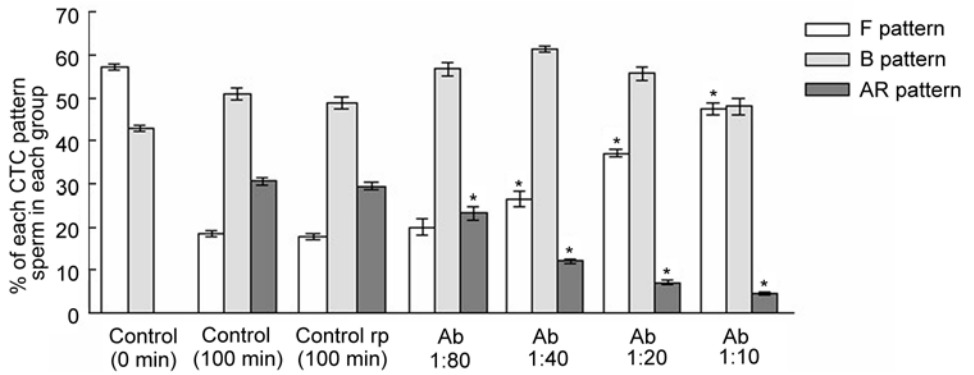

Figure 5. Demonstration of the effect of anti-NYD-SP27 serum on sperm capacitation and the acrosome reaction. Summary of chlortetracycline (CTC) staining results showing the percentages of spermatozoa assigned to the F pattern (uncapacitated), B pattern (capacitated) and AR pattern (acrosome-reacted). A total of 200 spermatozoa were counted to calculate the percentages of the three patterns. Control (0 min and 100 min) represents spermatozoa dispersed into a suspension medium immediately before incubation in capacitating medium (0 min) and in capacitating culture (100 min), together with a 1:10 dilution of the pre-immune serum, respectively. Control rp (100 min) represents spermatozoa incubated in the capacitating medium for 100 min together with recombinant protein pre-absorbed with anti-NYD-SP27 serum (dilution 1:10). The control at 100 min represents spermatozoa incubated in the capacitating medium for 90 min, followed by the addition of pre-immune serum and an additional 10 min of culture. Other groups were incubated for 100 min in the capacitating medium containing anti-NYD-SP27 serum (Antibody) at the indicated dilutions. Data representing the mean ± SEM were obtained from at least three independent experiments. $^{*}P < 0.01$, compared with control (100 min).

3.3 Inhibitory effects of NYD-SP27 and PLC inhibition on capacitation and acrosome reaction

As detachment of mNYD-SP27 from sperm during capacitation suggests that it plays a role as an intrinsic decapacitation factor, we further examine the effect of mNYD-SP27 in capacitation and acrosome reaction when detachment was prevented by antiserum. Mouse sperm were incubated in capacitating medium in the presence of pre-immune serum (control), recombinant protein pre-absorbed with anti-NYD-SP27 serum (control rp) or anti-NYD-SP27 serum at different dilutions for 100 min. As shown in Figure 5, the percentages of uncapacitated sperm (F pattern) were correlated with concentration of the antiserum, and the number of capacitated sperm (B + AR patterns) was reduced significantly as the concentration of the antiserum increased.

NYD-SP27 exerted inhibitory effects on PLC-coupled pancreatic anion secretion [7]. To determine whether the effects of NYD-SP27 on sperm capacitation and acrosome reaction involve PLC-coupled pathways, we examined the effects of a PLC inhibitor, U73122, on sperm capacitation and acrosome reaction. As shown in Figure 6, U73122 (10 μmol L^{-1}) mimicked the effect of anti-NYD-SP27 serum because it increased the percentage of uncapacitated sperm (F pattern) and

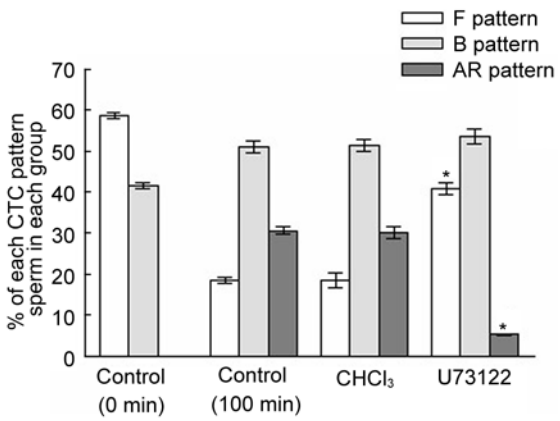

Figure 6. The inhibitory action of U73122 on capacitation and the acrosome reaction. Summary of chlortetracycline (CTC) staining results showing the percentages of spermatozoa assigned to the F pattern (uncapacitated), B pattern (capacitated) and AR pattern (acrosome-reacted). A total of 200 spermatozoa were counted to calculate the percentages of the three patterns. Control (0 min and 100 min) represents spermatozoa dispersed into a suspension medium immediately before incubation in the capacitating medium (0 min) and after 100 min, respectively. $CHCl_3$ was used as a solvent control for U73122 (both were incubated for 100 min in the capacitating medium). Data representing the mean ± SEM were obtained from at least three independent experiments. $^{*}P < 0.01$ compared with the $CHCl_3$ control. Note that the $CHCl_3$ group was not significantly different from the normal control (100 min) in capacitating medium.

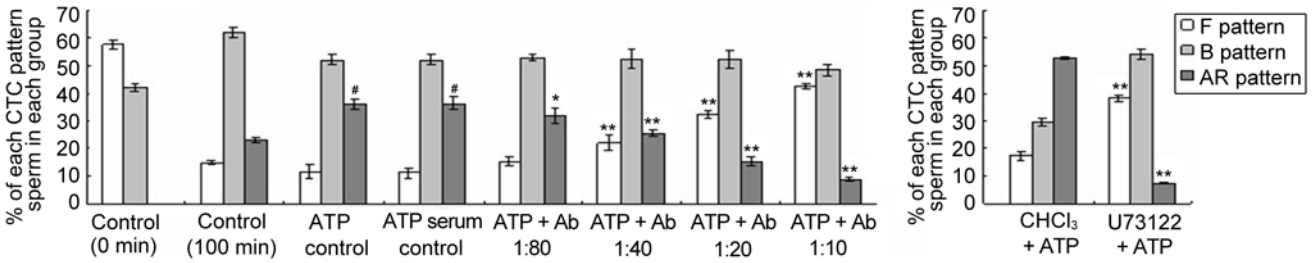

Figure 7. Comparison of the effect of anti-NYD-SP27 serum and phospholipase C (PLC) inhibitor, U73122, on ATP-induced acrosome reaction. Control (0 min and 100 min) represents spermatozoa dispersed into a suspension medium immediately before incubation in capacitating medium (0 min) and after 100 min, respectively. After 100 min of incubation in human tubal fluid (HTF) medium (ATP control) or HTF medium containing pre-immune serum (1:10; ATP serum control), anti-NYD-SP27 serum at different dilutions or U73122 (10 μmol L^{-1}) and ATP (2.5 mmol L^{-1}) was added to the medium for another 15 min. The data represent the mean ± SEM and were obtained from at least three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$, compared with the respective control (ATP) or $CHCl_3$ (ATP), $^{\#}P < 0.05$, compared with control (100 min).

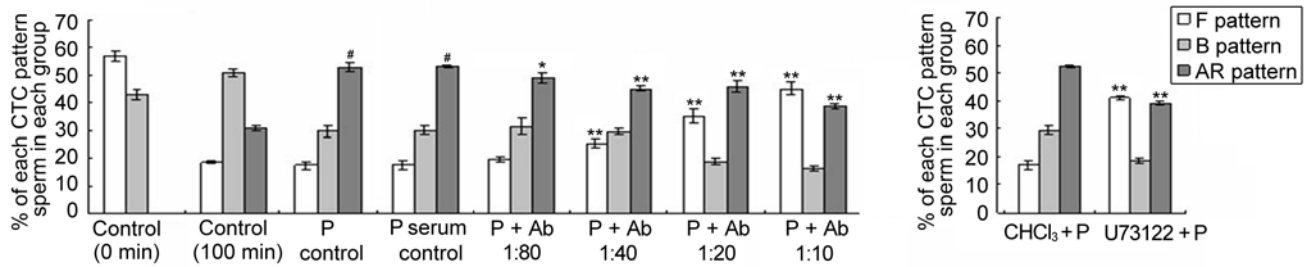

Figure 8. Comparison of the effect of anti-NYD-SP27 serum and the phospholipase C (PLC) inhibitor, U73122, on progesterone-induced acrosome reaction. Control (0 min and 100 min) represents spermatozoa dispersed into a suspension medium immediately before incubation in capacitating medium (0 min) and after 100 min, respectively. After 100 min of incubation in HTF medium (P control) or human tubal fluid (HTF) medium containing pre-immune serum (1:10; P serum control), anti-NYD-SP27 serum at different dilutions or U73122 (10 μmol L^{-1}), progesterone (P) (15 μmol L^{-1}) were added to the medium for another 15 min. The data represent the mean ± SEM and were obtained from at least three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$, compared with the respective control (P) or $CHCl_3$ (P). $^{\#}P < 0.05$, compared with control (100 min).

reduced the percentage of capacitated sperm (B + AR patterns).

Interestingly, anti-NYD-SP27 serum significantly inhibited the acrosome reaction induced by extracellular ATP (Figure 7). Extracellular ATP can stimulate the acrosome reaction in bovine spermatozoa through the P_2 purinoceptor, and its coupling to the PLC pathway is well known [13]. On the other hand, the anti-NYD-SP27 serum had less of an effect on the progesterone-induced acrosome reaction (Figure 8), which is mediated by both the PLC and the phospholipase A_2 (PLA_2) signalling pathways. However, the increased number of uncapacitated sperm in the presence of different dilutions of anti-NYD-SP27 serum was similar in both cases, which could be mimicked by the PLC inhibitor, U73122 (Figures 7 and 8; right-hand panels). These results indicate that mNYD-SP27 in sperm act similar to the PLC inhibitor, as shown in pancreatic duct cells [7], exerting an inhibitory effect primarily on capacitation.

3.4 The inhibitory effect on agonist-induced, PLC-coupled $[Ca^{2+}]_i$ increase in spermatozoa

As PLC-coupled Ca^{2+} signalling is essential to sperm capacitation and acrosome reaction, we examined the effect of mNYD-SP27 and the PLC inhibitor on agonist-induced, PLC-coupled intracellular $[Ca^{2+}]$ increase. As shown in Figure 9, incubation of the sperm with anti-NYD-SP27 serum reduced the basal Ca^{2+} level significantly, as well as the ATP- and progesterone-induced $[Ca^{2+}]_i$ increases, whereas, the control pre-immune serum had no effect. The inhibitory effect

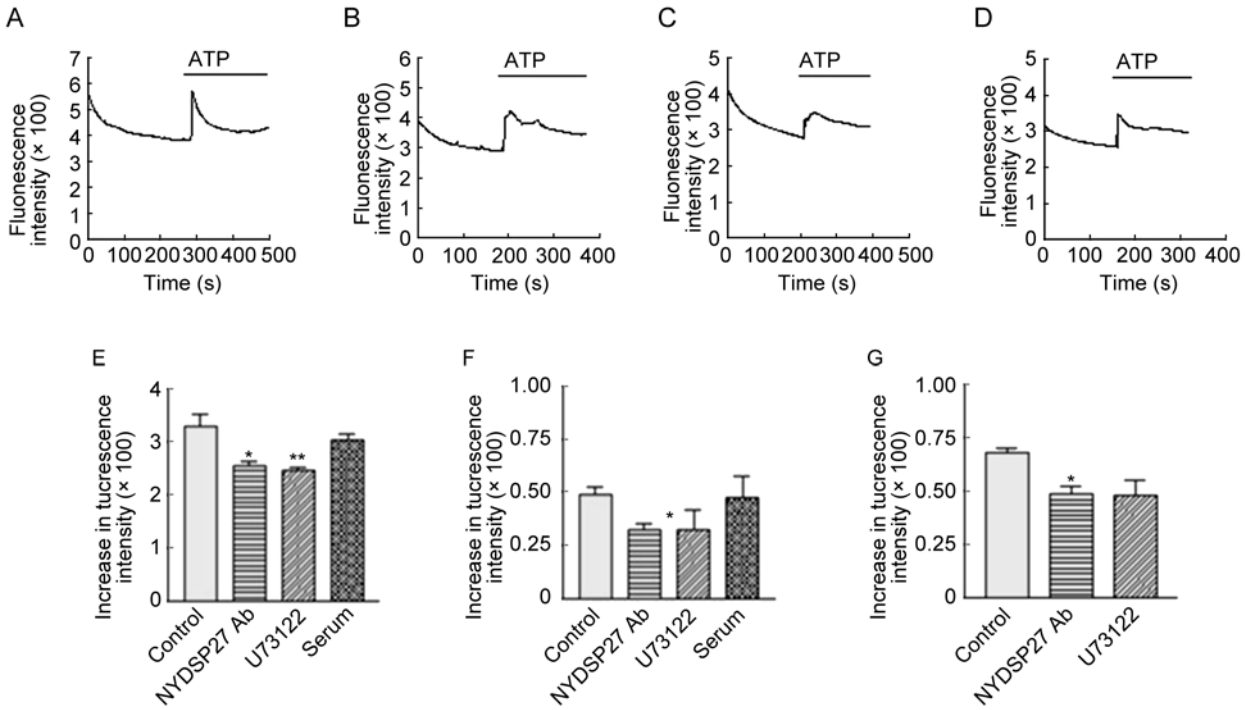

Figure 9. Comparison of the effect of anti-NYD-SP27 serum and the phospholipase C (PLC) inhibitor, U73122, on agonist-induced PLC-coupled Ca^{2+} mobilization in mouse sperm. Representative time-dependent changes in intracellular Ca^{2+} concentration, reflected by the ratio of Fura-2 fluorescence in sperm upon the addition of ATP (100 μmol L^{-1}) without pre-treatment (A) or pre-treated with control serum (B), anti-NYD-SP27 serum (1:50) (C) or PLC inhibitor U73122 (10 μmol L^{-1}) (D). (E): Mean level of changes in basal Ca^{2+} concentration under different conditions. (F): Mean level of ATP (100 μmol L^{-1})-induced Ca^{2+} concentration changes under different conditions. (G): Mean level of progesterone (100 μmol L^{-1})-induced Ca^{2+} concentration changes under different conditions. The data represent the mean ± SEM obtained from at least three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$, compared with the control.

could be mimicked by the PLC inhibitor, confirming an inhibitory role of mNYD-SP27 in the PLC-coupled Ca^{2+} pathway. Together, these results suggest that retention of mNYD-SP27 in sperm, as a sperm-specific PLC isoform, inhibited coupling of extracellular signals to intracellular [Ca^{2+}], thus having an important role in regulating sperm capacitation and acrosome reaction.

4 Discussion

Fertilization involves many complex regulatory mechanisms and processes, including timely capacitation and acrosome reaction, which enable the sperm to fertilize ova properly. A number of capacitation factors (such as HCO_3^-) and decapacitation factors [1, 2] have been implicated in these processes. Although the presence of decapacitation factors in the seminal plasma has been reported, no such factor intrinsic to the sperm has been identified. To our knowledge, NYD-SP27 described in this report is the first such factor.

Localization of NYD-SP27 and its mouse homologue (mNYD-SP27) to the acrosome indicate a close relationship between these proteins and capacitation, supporting the possibility that it might serve as an intrinsic decapacitation factor. To test this postulate, we further examined whether sperm capacitation and acrosome reaction can be prevented by NYD-SP27 antiserum. Indeed, the total number of uncapacitated sperm was observed to be increased significantly as the concentration of antiserum increased. The results suggest that retention of mNYD-SP27 in sperm inhibits the process of capacitation, which appears to be consistent with the roles proposed for other proteins that are recognized as decapacitation factors and whose main function is to prevent the onset of capacitation of sperm [2, 14]. It should be noted, however, that the antibody must enter the cell to act on intracellular mNYD-SP27. Although the ability of antibodies to get

into cells has been documented, as exemplified by the anti-actin monoclonal antibody [15], the details of this process require further investigation.

How does NYD-SP27 work as a decapacitation factor? Our earlier study [7] showed that in pancreatic cells NYD-SP27 acts as an inhibitor of PLC, which is also involved in the initiation of capacitation and acrosome reaction [3, 4]. Therefore, it is logical to hypothesize that NYD-SP27 also acts as an inhibitor to the onset of capacitation and acrosome reaction. In other words, the detachment of NYD-SP27 from sperm is a necessary step for the initiation of capacitation and acrosome reaction. This notion is supported by the present results obtained using U73122, a known inhibitor of PLC. In fact, the PLC inhibitor increases the number of uncapacitated sperm to an extent similar to that observed when mNYD-SP27 is retained in sperm by anti-serum.

Bioinformatics analysis shows that NYD-SP27 has a short N terminus and lacks EF domains [7]. It has been shown that the EF domain is the binding site for Ca^{2+} and the activation site for hydrolysis activity in other PLC isoforms [16]. Similarly, the mouse homologous protein mNYD-SP27 also lacks EF-hand domains in the N terminal. A study investigating the function of the domains in mouse Plcz1 has confirmed that the N-terminal EF-hand domains, especially EF-1 and EF-3, are essential for the function of Plcz1. The lack of the three EF-hand domains has been shown to result in a low level of Ca^{2+} sensitivity [17] and loss of the Ca^{2+} oscillations associated with fertilization in mouse eggs injected with EF domain RNA. This is in contrast to the activity of the full-length Plcz1, which can trigger Ca^{2+} oscillations in mouse eggs [18–20]. In fact, our earlier study in pancreatic duct cells has showed that NYD-SP27 acts as a physical PLC inhibitor [7]. In this study, Ca^{2+} mobilization in sperm induced by either extracellular ATP or progesterone, whose receptors are known to be coupled to PLC [13, 21–23], was inhibited by both PLC inhibitor and retention of mNYD-SP27 in sperm by its anti-serum. Taken together, these observations show that capacitation is made possible only if NYD-SP27, an intrinsic decapacitation factor, is removed from sperm.

Further evidence supporting a primary inhibitory role of NYD-SP27 in the PLC-coupled pathway comes from the observation of a similar effect of anti-NYD-SP27 serum and the PLC inhibitor on the ATP- and progesterone-induced acrosome reaction. ATP and progesterone have been considered physiological agonists of the PI-PLC pathway [13, 21, 22] and can induce the acrosome reaction *in vitro* through different pathways and mechanisms. Our study indeed shows that although NYD-SP27 was retained in sperm, ATP and progesterone show differential effects on the number of acrosome-reacted sperm (Figures 7 and 8). This difference was due to varying degrees of PLC involvement in the two cases. Furthermore, ATP and progesterone also show a similar differential effect on PLC inhibitor-treated sperm (Figures 7 and 8). ATP activates PLC through binding to the P_{2y} receptor on the sperm membrane, which is coupled to a G protein. This leads to increased intracellular Ca^{2+} and activation of PKC, and finally, the acrosome reaction [13]. On the other hand, progesterone induces the acrosome reaction by binding to its receptor, leading to activation of PLC and PLA_2 simultaneously [22, 23], and the progesterone-induced acrosome reaction can be prevented only when both PLC and PLA_2 inhibitors are present. The present results indeed show that both PLC inhibitor and mNYD-SP27 retention almost completely abolished the ATP-induced acrosome reaction, but with less effect on the progesterone-induced acrosome reaction. This is consistent with a primary inhibitory role of NYD-SP27 on the PLC-coupled pathway. As NYD-SP27 and PLC inhibitor could block the onset of capacitation and ATP or progesterone could only induce the acrosome reaction in capacitated sperm, they showed no significant effect on uncapacitated sperm. These results suggest that NYD-SP27 has an inhibitory role in the PLC-coupled pathway, which is primarily linked to the process of capacitation. After capacitation, NYD-SP27 may or may not influence the acrosome reaction, depending on the type of activators and signalling pathways involved.

Taken together, the results of this study suggest that as an inhibitor of PLC, NYD-SP27 serves as an intrinsic decapacitation factor in sperm and has an important role in preventing the onset of capacitation. The detachment of NYD-SP27 from sperm in the presence of capacitation activator may ensure the correct priming of capacitation and thus subsequent acrosome reaction. Although the details of how the detachment of NYD-SP27 occurs and how the anti-serum in the culture medium could block the detachment of NYD-SP27 remain to be elucidated, the involvement of NYD-SP27 currently shown in the process of capacitation indicates that it may be indispensable for fertilization. Defects

in NYD-SP27 function, such as defective inhibition of PLC or its abnormal detachment from sperm, may lead to disruption of the precise priming of capacitation, resulting in infertility.

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