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Protein phosphorylation in bovine oocytes following fertilisation and parthenogenetic activation *in vitro*

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Summary

This study examined the event of protein phosphorylation in bovine oocytes in response to sperm penetration and parthenogenetic activation. *In vitro* matured oocytes were labelled with [^{32}P]orthophosphate at 3 h intervals from 3 h to 18 h or from 0 h to 12 h following *in vitro* fertilisation and parthenogenetic activation, respectively. The level of protein dephosphorylation, at approximately 43 kDa, was similar in fertilised and parthenogenetically activated bovine oocytes. However, the level of protein phosphorylation at 40 kDa, 23 kDa and 18 kDa was different between these two samples. There were no such changes of protein phosphorylation and dephosphorylation in the control oocytes. Further, by two-dimensional gel electrophoresis there is a difference in the level of protein phosphorylation at 18 kDa between the fertilised and activated oocytes. These results suggest that this protein phosphorylation may be related to the formation of the male pronucleus in bovine oocytes.

Keywords: Activation, Fertilisation, Oocyte, Phosphorylation, Sperm

Introduction

Following fertilisation, cytoplasmic factors in the oocyte trigger major changes in the sperm nucleus. Upon sperm entry at fertilisation, the oocyte undergoes an M/G_1 transition, and subsequently enters interphase to synthesise DNA. At the same time, the sperm nucleus undergoes a dramatic transformation to form the male pronucleus, and becomes capable of DNA synthesis. The mature oocyte can begin embryonic development after fertilisation or parthenogenetic activation. Parthenogenesis has been defined as the production of an embryo from a female gamete without any contribution of a male gamete (Beatty, 1957). Activation of the mature oocyte is characterised by cortical granule exocytosis, release from metaphase II arrest, and extrusion of the second polar body, followed by pronucleur formation. Although it is believed that the maturational age of bovine oocytes is the main determinant in oocyte activation (Ware *et al.*, 1989), the molecular events controlling parthenogenetic activation are still poorly understood.

Events associated with pronucleur formation appear to be related to the meiotic cycle of the oocytes. Protein phosphorylation plays a major role in the regulation of cell growth and division. It is known that phosphorylation of protein complexes occurs at the time of pronucleus formation in mouse (Howlett & Bolton, 1985; Howlett, 1986) and pig (Ding *et al.*, 1992*a*, *b*) oocytes. Recently, it has been suggested that the male pronucleur formation is associated with protein phosphorylation and that the formation of the male and the female pronuclei may involve different factors in bovine oocytes (Chian *et al.*, 1998). However, it is unclear whether oocyte activation by sperm or parthenogenesis is induced by different cytoplasmic responses in protein phosphorylation. The objective of the present study was to examine the cytoplasmic responses of protein phosphorylation in oocytes activated by sperm penetration or parthenogenetic activation in order to determine which protein(s) are related to the activation of

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the cell cycle and which protein(s) respond to male pronucleur formation.

Materials and methods

Maturation of oocytes *in vitro*

Ovaries from cycling or pregnant heifers or cows were removed within 30 min of slaughter and transported to the laboratory at approximately 35 °C in 0.9% NaCl aqueous solution containing 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. The cumulus–oocyte complexes (COC) were aspirated from 2–5 mm follicles with an 18 G needle, pooled and selected as described previously (Sirard *et al.*, 1988). The COC were rapidly washed four times in HEPES-buffered Tyrode's medium (TLH; Bavister *et al.*, 1983) supplemented with 10% heated (56 °C, 30 min) fetal bovine serum (FBS), 0.25 mM pyruvic acid and 50 µg/ml gentamycin. After washing, 10 oocytes were cultured in 50 µl droplets of TC-199 medium under mineral oil. The maturation medium, TC-199 medium, was supplemented with 10% FBS, 1 µg/ml oestradiol-17β (Sigma), 0.5 µg/ml FSH (oFSH; NIADDK-oFSH-17) and 5.0 µg/ml LH (bLH; NIADDK-LH-B9, generously donated by the National Hormone and Pituitary Program, MD, USA). The oocytes were incubated at 38.5 °C under an atmosphere of 5% CO_2 and 95% air with high humidity. After maturation for 24 h or 33 h, the oocytes were used for *in vitro* fertilisation or parthenogenetic activation *in vitro*, respectively.

Sperm preparation and *in vitro* fertilisation

Frozen semen, pooled from five bulls, was donated by the Centre d'Insemination Artificielle du Quebec (CIA, St-Hyacinthe, Quebec). Straws of semen were thawed in a water bath (35 °C) for 30 s and processed by swim-up as described Parish *et al.* (1986). The sperm were then washed twice in modified Tyrode's albumin lactate pyruvate medium (Sp-TALP) used for sperm culture containing 6 mg/ml fatty acid-free bovine serum albumin (BSA) (Sigma), 10 mM pyruvic acid, and 50 µg/ml gentamycin. Sperm/oocytes were incubated in 50 µl drops of the fertilisation medium, modified Tyrode's medium (mTALP; Parrish *et al.*, 1988) containing 2 µg/ml heparin and 2 µl PHE (20 µM/ml penicillamine/ 10 µM/ml hypotaurine/1 µM/ml adrenaline; Leibfried & Bavister, 1982) under mineral oil at 38.5 °C in 5% CO_2 and 95% air with high humidity. The final sperm concentration of 1×10^6 spermatozoa/ml was used for insemination and five oocytes were used for each 50 µl drop as described by Sirard *et al.* (1988). To examine a time course of sperm penetration and pronucleus formation, oocytes were fixed at 1 h intervals from 0 h to 18 h after insemination.

Parthenogenetic activation *in vitro*

After 33 h of culture in the maturation medium, COC were completely denuded of the cumulus cells by treatment with 0.1% hyaluronidase (H-3506; Sigma) in PBS and by repeated pipetting. Oocytes with a visible first polar body and an evenly granulated ooplasm as observed under a stereomicroscope were exposed to 20 µM calcium ionophore A23187 in PBS for 5 min at room temperature. After activation, the oocytes were washed and returned to culture for 12 h in the fertilisation medium containing 6 mg/ml BSA, 0.25 mM pyruvic acid and 50 µg/ml gentamycin. To examine a time course of female pronucleus formation, the oocytes were fixed at 1 h intervals from 0 h to 12 h after activation.

Radiolabelling of oocytes

To determine the pattern of protein phosphorylation after fertilisation, the oocytes were transferred into mTALP + 0.3% polyvinylpyrrolidone (PVP-40; Sigma) containing 400 µCi/ml [^{32}P]orthophosphate (Amersham, Arlington Heights, IL, USA) at 3 h, 6 h, 9 h, 12 h and 15 h after insemination and at 0 h, 3 h, 6 h and 9 h after parthenogenetic activation, and then labelled for 3 h respectively. The oocytes were then washed twice in a TLH containing 0.3% PVP and were lysed with 50 µl sodium dodecyl sulphate (SDS) buffer. The samples were precipitated with acetone (99%), dried and resuspended with SDS buffer, and then frozen at –80 °C until further use. One-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 5% stacking gel and a 12% separating gel according to the method of Laemmli (1970). For the two-dimensional gels, the procedure was as described by Levesque and Sirard (1995). Briefly, the first dimension of the two-dimensional gel was performed in acryl gel with Nonidet P40 and urea. The second dimension was performed on 12% SDS-PAGE. Twenty-five oocytes were put into each well for electrophoresis. All gels were soaked for 30 min in fixation solution of 5% glycerol/25% isopropanol/10% acetic acid and then dried under vacuum at 80 °C. Autoradiography was carried out at –80 °C with intensifying screens.

Fixation of oocytes

At the end of each culture period, the oocytes were fixed as described previously (Chian & Sirard, 1995). Oocytes that had been penetrated were identified when a sperm head or male pronucleus with its accompanying sperm tail was present in the cytoplasm. Oocytes with two

pronuclei and a clear second polar body but without a sperm tail were also considered penetrated. Only the oocytes that had pronucleur formation (including multiple pronuclei) were considered to be activated.

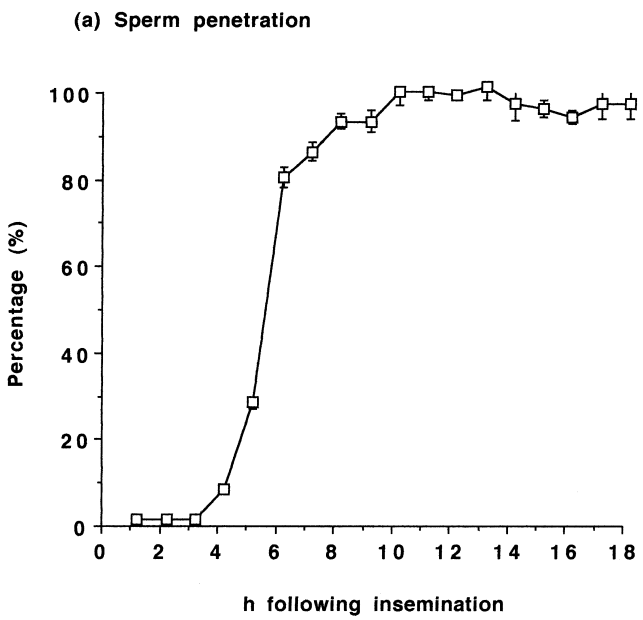
Statistical analysis

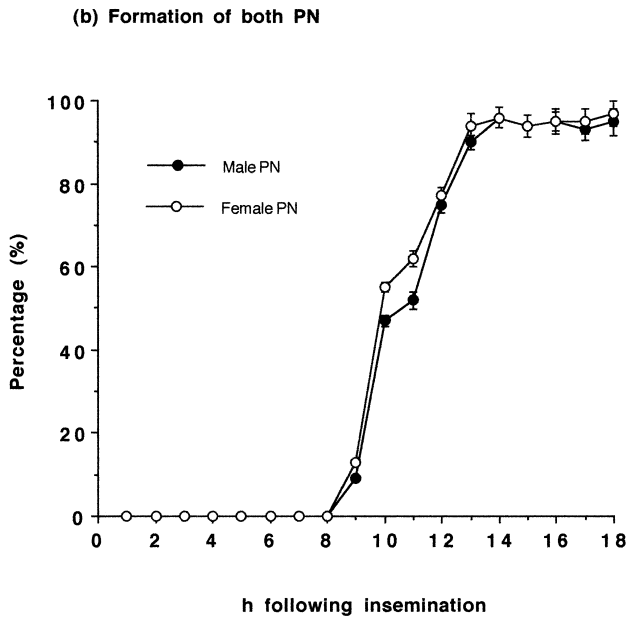
The percentages of penetrated oocytes and pronuclear formation were analysed by one-way ANOVA. When ANOVA revealed a significant treatment effect, the treatments were compared by the Student–Newman–Keuls' test (Steel & Torrie, 1980).

Results

The first evidence of sperm penetration (7% ± 0.9%) was observed 4 h following insemination (Fig. 1*a*). The penetration rate was significantly increased (79% ± 4.7%) by 6 h post-insemination and a maximum penetration rate (92% ± 1.1%) was reached at 8 h following insemination. The first evidence of male and female pronuclear formation (12% ± 1.6% vs 16% ± 2.4%) was observed 9 h post-insemination and the maximum rates (91% ± 0.9% vs 95% ± 1.2%) were reached 13 h following insemination (Fig. 1*b*). The development of male and female pronuclei was observed concurrently from 10 h to 14 h post-insemination. As shown in Fig. 1*c*, the first female pronucleus (4% ± 0.9%) was observed 5 h following parthenogenetic activation. Formation of female pronuclei significantly increased (72% ± 2.3%) 8 h post-activation, with a maximum rate (91% ± 2.5%) being reached 10 h following parthenogenetic activation.

Changes in protein phosphorylation in oocytes occur following fertilisation (Fig. 2). The levels of protein phosphorylation at approximately 23 kDa and 18 kDa increased gradually from 3 h to 18 h following insemination (lanes 1′–5′). The level of protein phosphorylation at approximately 43 kDa decreased gradually from 9 h to 18 h post-insemination (lanes 3′–5′). In contrast, phosphorylation of protein at approximate 40 kDa was observed in the oocytes from 15 h to 18h following insemination (lane 5′). There were no such differences in protein phosphorylation in the control oocytes (control; lanes 1–5).





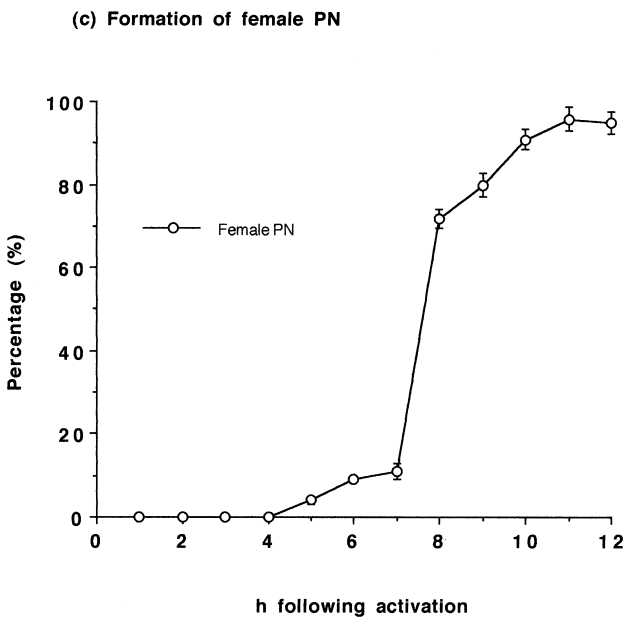


Figure 1 Time courses of sperm penetration (*a*), formation of male and female pronuclei (*b*) and development of female pronucleus (*c*) following *in vitro* fertilisation or parthenogenetic activation with 20 μM calcium ionorphore A23187 (total of 2004 oocytes; three replicates). PN, pronucleus.

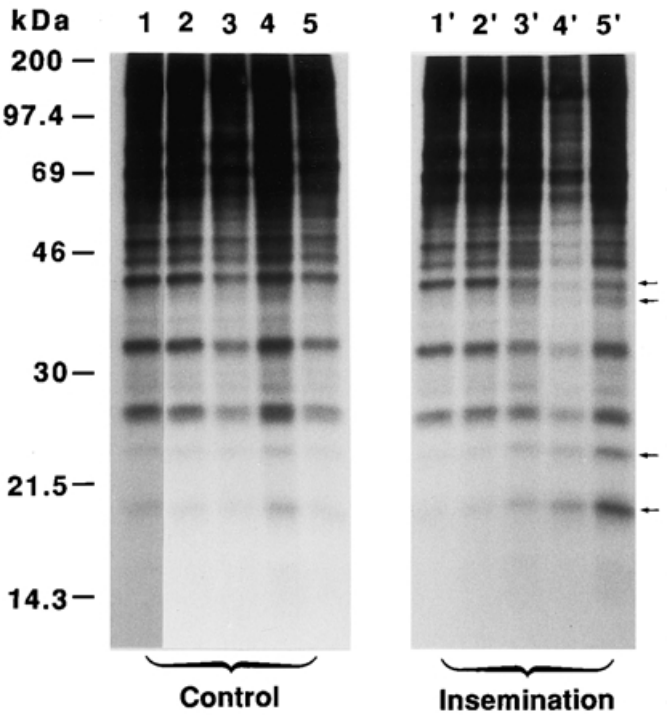


Figure 2 One-dimensional SDS–12% polyacrylamide gel electrophoresis of [^{32}P]orthophosphate-labelled proteins. Molecular weight (range 14 300–200 000) is migration of [^{14}C]methylated proteins. The protein phosphorylation of the oocytes after fertilisation was labelled at 3 h intervals from 3 h to 18 h following insemination. Control: lane 1, 3–6 h post-incubation (hpi); lane 2, 6–9 hpi; lane 3, 9–12 hpi; lane 4, 12–15 hpi; lane 5, 15–18 hpi. Insemination: lane 1′, 3–6 h post-insemination; lane 2′, 6–9 h post-insemination; lane 3′, 9–12 h post-insemination; lane 4′, 12–15 h post-insemination; lane 5′, 15–18 h post-insemination. Arrows point to differences described in the text. Each lane represents 25 oocytes. The experiment was performed twice with similar results.

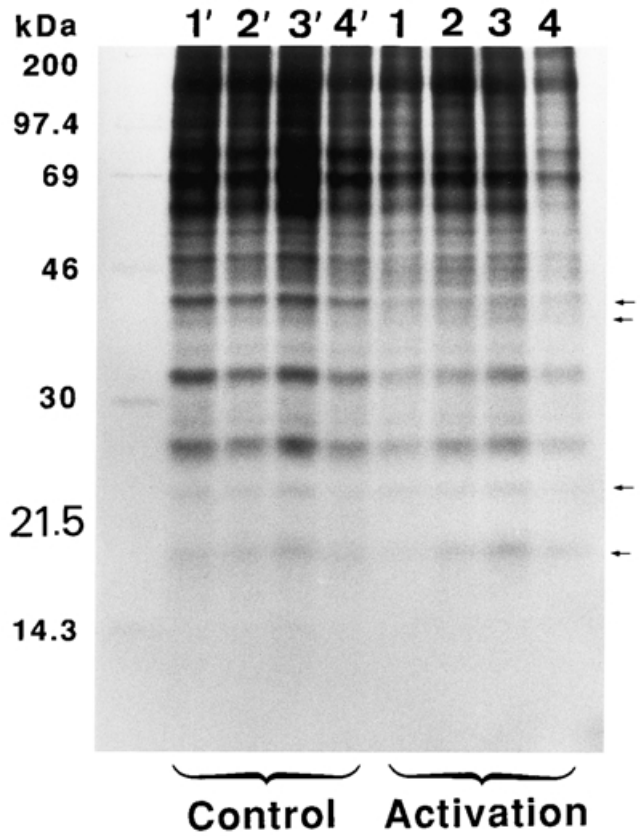


Figure 3 One-dimensional SDS–12% polyacrylamide gel electrophoresis of [^{32}P]orthophosphate-labelled proteins. Molecular weight (range 14 300–200 000) is migration of [^{14}C]methylated proteins. The protein phosphorylation of the oocytes following parthenogenetic activation *in vitro* was labelled at 3 h intervals from 0 h to 12 h following activation. Control: lane 1′, 0–3 h post-incubation (hpi); lane 2′, 3–6 hpi; lane 3′, 6–9 hpi; lane 4′, 9–12 hpi. Activation: lane 1, 0–3 h post-activation (hpa); lane 2, 3–6 hpa; lane 3, 6–9 hpa; lane 4, 9–12 hpa. Arrows point to differences described in the text. Each lane represents 25 oocytes. The experiment was performed twice with similar results.

Fig. 3 shows the profile of protein phosphorylation in oocytes following parthenogenetic activation. The level of protein phosphorylation at approximately 43 kDa decreased from 0 h to 12 h post-activation (lanes 1–4). However, the levels of protein phosphorylation at approximately 40 kDa, 23 kDa and 18 kDa were not increased prominently in the oocytes following activation compared with the control (lanes 1′–4′).

Figs. 4 and 5 show the two-dimensional pattern of phosphorylated proteins following fertilisation and parthenogenetic activation, respectively. Referring to Figs 1 and 2, some points of protein phosphorylation were clearly comparable in the fertilised and parthenogenetically activated oocytes. As shown in Fig. 4*d*, *e*, although the point of protein phosphorylation at approximately 18 kDa (circles) was increased prominently in the fertilised oocytes, this point of protein phosphorylation were not obvious in the parthenogenetic activated oocytes (Fig. 5*c*).

Discussion

The results of the present study demonstrate that protein phosphorylation and dephosphorylation occur in bovine oocytes following fertilisation and parthenogenetic activation. Following fertilisation, the sperm-specific protamines are replaced by histone and non-histone proteins during pronuclear formation (Zirkin *et al.*, 1989). There are changes in the pattern of oocyte protein synthesis initiated by sperm penetration in the mouse (Howlett & Bolton, 1985; Endo et al., 1986), rabbit (Van Blerkom, 1979), sheep (Osborn & Moor, 1983; Moor & Gandolfi, 1987) and pig (Ding *et al.*, 1992*a*, *b*). However, oocytes activated by sperm and by parthenogenetic activation induce different cytoplasmic responses for protein synthesis, with newly syn-

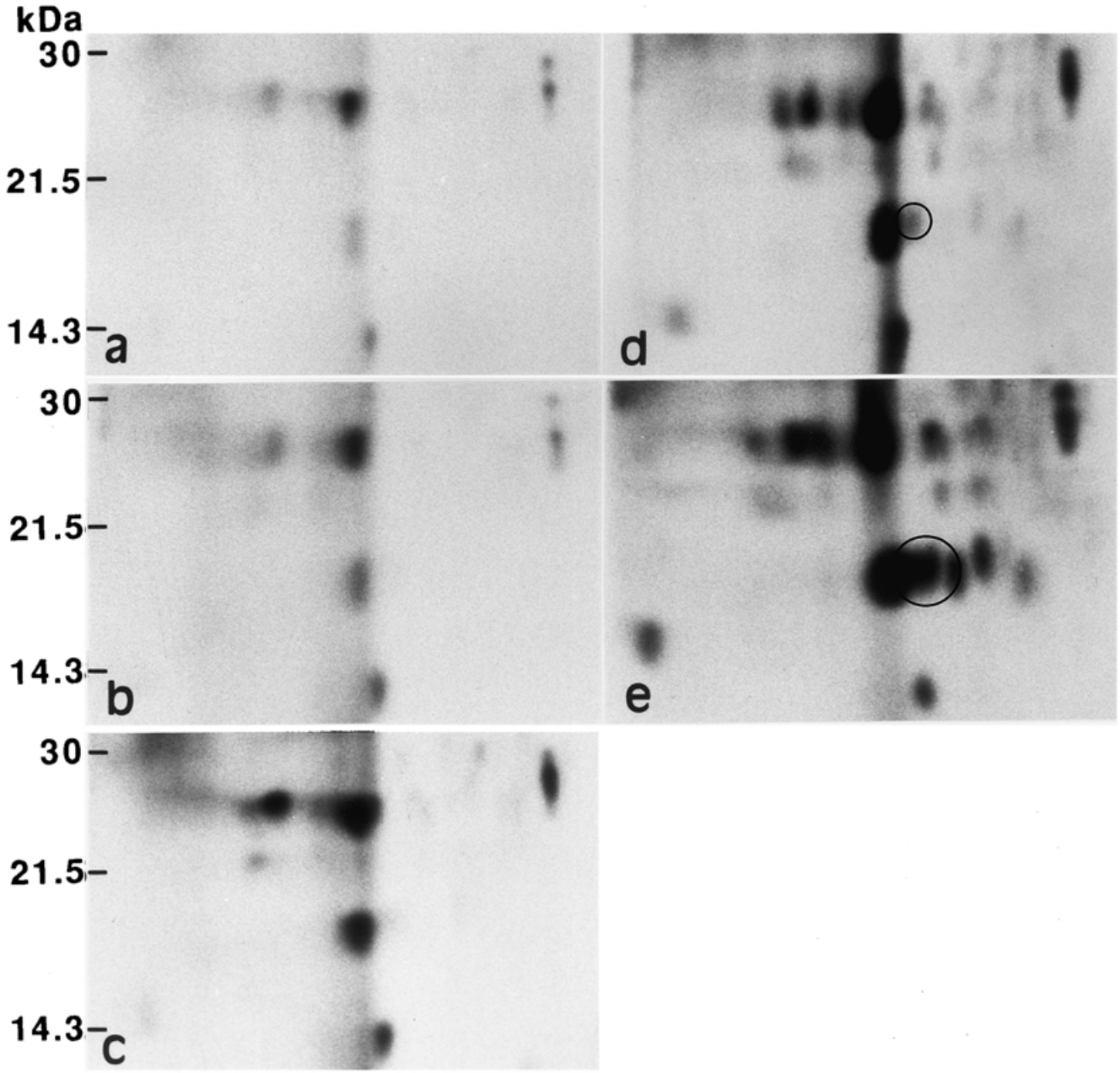


Figure 4 Two-dimensional gel electrophoresis of [^{32}P]orthophosphate-labelled proteins. Molecular weight (range 14 300–200 000) is migration of [^{14}C]methylated proteins. The phosphorylated proteins of the oocytes were first separated on IEF acryl gel with an ampholine range of 3–10, and secondly separated on 12% SDS-PAGE. The proteins phosphorylated in the oocytes were labelled at 3 h intervals from 3 h to 18 h following insemination: (*a*) 3–6 h post-insemination (hpi); (*b*) 6–9 hpi; (*c*) 9–12 hpi; (*d*) 12–15 hpi; (*e*) 15–18 hpi. The circle indicates differences described in the text. Each lane represents 25 oocytes. The experiment was performed twice with similar results.

thesised proteins not required for male pronuclear formation in bovine oocytes (Chian & Sirard, 1996). Two categories of enzymes control the phosphorylated and non-phosphorylated forms of protein substrates: protein kinases and protein phosphatases. The maturation-promoting factor (MPF) drives the G_2/M transition in the oocytes and in the mitotic cells. The serine/threonine protein kinase p34^{cdc2} and cyclin B have been shown to be the catalytic subunit of MPF (see review by Maller, 1994). The regulation of kinase activity of p34^{cdc2} is controlled by the phosphorylation of tyrosine, threonine and serine residues (Morla *et al.*, 1989). A tyrosine phosphatase is also required for MPF activation during the G_2/M transition (Gould & Nurse, 1989). Therefore, phosphorylation and dephosphorylation by MPF-specific tyrosine phosphatase and

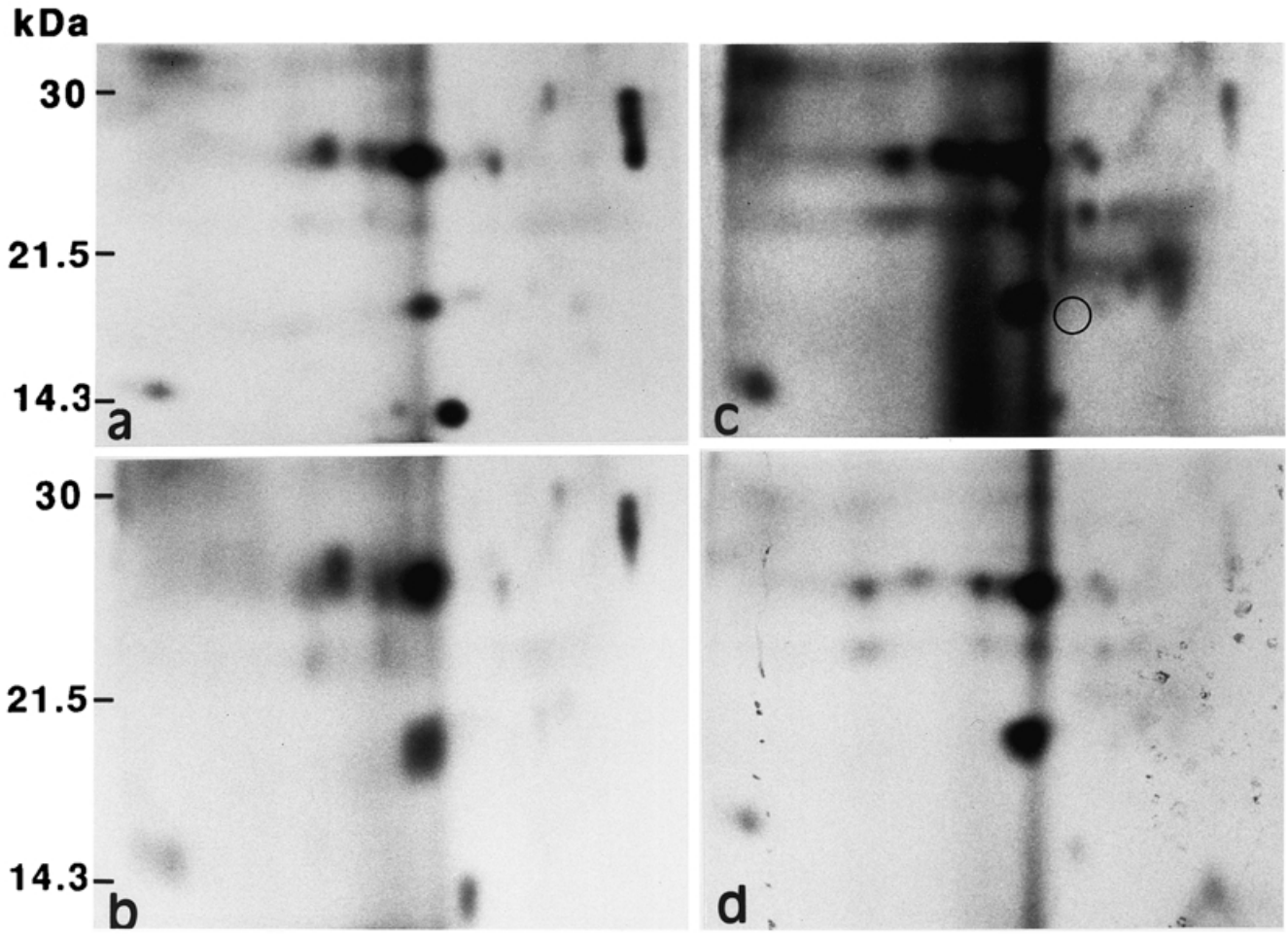


Figure 5 Two-dimensional gel electrophoresis of [^{32}P]orthophosphate-labelled proteins. Molecular weight (range 14 300–200 000) is migration of [^{14}C]methylated proteins. The phosphorylated proteins of the oocytes were first separated on IEF acryl gel with an ampholine range of 3–10, and secondly separated on 12% SDS-PAGE. The proteins phosphorylated in the oocytes following parthenogenetic activation were labelled at 3 h intervals from 0 h to 12 h following activation: (*a*) 0–3 h post-activation (hpa); (*b*) 3–6 hpa; (*c*) 6–9 hpa; (*d*) 9–12 hpa. The circle indicates differences described in the text. Each lane represents 25 oocytes. The experiment was performed twice with similar results.

tyrosine kinase may play a key role in the control of the cell cycle. The results from studies with amphibian eggs indicate that protein phosphorylation plays an important role in controlling nuclear envelope assembly–breakdown and chromosome condensation and decondensation in cell-free extracts (Lohka & Maller, 1987). In the mouse (Howlett & Bolton, 1985; Howlett, 1986) and pig (Ding *et al.*, 1992*b*), it has been suggested that protein phosphorylation, not protein synthesis, controls the formation of pronuclei following fertilisation. The results of the present study show that the development of male and female pronuclei was concurrent from 9 h to 14 h post-insemination (Fig. 1*b*). Accordingly, protein complexes at approximately 43 kDa, 40 kDa, 23 kDa and 18 kDa were phosphorylated and dephosphorylated in bovine oocytes during this period (Fig. 2). Therefore, apparently, the formation of pronuclei is accompanied by the phosphorylation and dephosphorylation of these protein complexes.

Oocytes can be activated artificially by mechanical and chemical treatments that cause an increase in the concentration of intracellular calcium. These treatments are routinely employed to investigate the mechanism of oocyte activation. Although it has been shown that electrostimulation induces both cortical granule exocytosis and protein reprogramming in porcine oocytes, it does not reproduce the pattern of Ca^{2+} changes induced by sperm entry at fertilisation (Sun *et al.*, 1992). Zernicka-Goetz *et al.* (1995) reported that although metaphase II oocytes fused with fertilised oocytes undergo activation and pass into interphase, an oocyte fused with a parthenogenetic oocyte remains arrested in metaphase. We have demonstrated that protein synthesis is different between bovine oocytes activated by sperm or by parthenogenetic stimulation

(Chian & Sirard, 1996). The results of the present study show that formation of the female pronucleus was observed from 4 h following parthenogenetic activation (Fig. 1*c*). Accordingly, the patterns of proteins at approximately 43 kDa, 40 kDa, 23 kDa and 18 kDa were phosphorylated and dephosphorylated in a similar manner in oocytes following parthenogenetic activation or fertilisation (Figs. 2–5). However, the results of two-dimensional gel electrophoresis indicate that the point of protein phosphorylation at approximate 18 kDa was increased prominently in fertilised oocytes (Fig. 4*d*, *e*) and absent in parthenogenetically activated oocytes (Fig. 5*c*). It has been indicated that the formation of male and female pronuclei may involve different factors in bovine oocytes (Chian *et al.*, 1998). Taken together, these data indicate that this point of protein phosphorylation (at approximately 18 kDa) may be involved in male pronucleus formation, and that this phosphorylated protein is different with the cell cycle regulator. In conclusion, these results suggest that protein phosphorylation at 18 kDa may be related to formation of the male pronucleus in bovine oocytes.

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