AN ABSTRACT OF THE THESIS OF

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Effects of phorbol myristate acetate (PMA), dibutyryl cyclic AMP (dbcAMP), 6-dimethylaminopurine (6-DMAP), okadaic acid (OA), cycloheximide (CHX), actinomycin D (AcD) and tunicamycin (TuM) on plasminogen activator (PA) activity and maturation rate in bovine and porcine oocyte-cumulus cell complexes (BOCC and POCC, respectively) in vitro were determined. Plasminogen activator activity was measured by SDS-PAGE, casein-agar zymography and densitometry. Three plasmingen-dependent lytic zones (92-95, 71-73 and 49-51 kD) and one PA inhibitor (52 kD) were observed in BOCC. Immunoprecipitation and amiloride sensitivity suggested that the 49-51 kD protease is a urokinase -type PA (uPA), the 71-73 and 92-95 kD proteases are a tissue -type PA (tPA) and tPA-PAI complex, respectively, and the PAI is PAI-1. In POCC, two plasminogen activators (71-78 and 93-96 KD) were observed. Lack of amiloride sensitivity suggested that the 71 -78 kD band is a tPA and the 93-96 kD band is possibly a tPA-PAI complex. Increasing dbcAMP in the culture medium increased activity in both BOCC and POCC in dose-dependent fashion ($P<0.05$).

In BOCC cultured with PMA, total PA activity increased, however high concentrations of PMA (10 and 100 ng/ml) decreased tPA activity in matured POCC. Plasminogen activator activity decreased in 6-DMAP, actinomycin D and cycloheximide and oocyte maturation was also inhibited in these treatments. When POCC were treated with 25 nM OA, uPA activity was observed. Plasminogen activator activity increased in either BOCC or POCC treated with up to 25 nM OA, however PA activity decreased at concentrations greater than 75 nM ($P<0.05$). Incubation of BOCC with tunicamycin reduced the molecular mass of tPA and tPA-PAI complex and PAI-1 by 5-10%, however PA activity was not inhibited. These data suggest that BOCC matured in vitro produce uPA, tPA and PAI-1 however POCC produce only tPA and PAI. The production of PA and PAI by either BOCC or POCC is associated with oocyte maturation and influenced by stimulators of the protein kinase A and C, modulators of intracellular phosphorylation and metabolic inhibitors.

FACTORS AFFECTING PLASMINOGEN ACTIVATOR ACTIVITY IN BOVINE AND PORCINE OOCYTE-CUMULUS CELL COMPLEXES MATURED IN VITRO

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INTRODUCTION

The plasminogen activator (PA) system is an important source of extracellular proteolysis accompanying cell migration and tissue remodelling (Dano et al., 1985; Mullins and Rohrlish, 1983). Two principal PA have been identified, tissue-type PA (tPA) and urokinase-type PA (uPA), and their activities are modulated physiologically by several different inhibitors including PA inhibitors-1 (PAI-1), -2 (PAI-2) and -3 (PAI-3) (Dano et al., 1985; Kruithof, 1988). Meiotic maturation of oocytes is associated with increased levels of tPA activity. In rodents, granulosa and cumulus cells secrete both tPA and uPA, however oocytes denuded of cumulus cells produce only tPA during spontaneous in vitro maturation (Huarte et al., 1985; Liu et al., 1986). Ny et al.(1987) have suggested that GnRH treatment increases PA activity in rat oocyte-cumulus cell complexes through the protein kinase C pathway. Conversely, FSH appears to mediate its effects through the protein kinase A pathway (Salustri et al. 1985). Although PA activity is increased in rat oocyte-cumulus cell complexes by stimulators of the protein kinase A and C systems, it has not been determined if oocyte-cumulus cell complexes from cattle and pigs produce PA and whether activity can be regulated by second messenger systems.

Cycles of cellular phosphorylation and dephosphorylation have important roles in maturation or M-phase promoting factor (MPF) activity involved in cell cycle control and oocyte maturation (Draetta and Beach, 1988; Moor and Crosby, 1986). Inhibition of protein phosphorylation by 6-dimethylaminopurine (6-DMAP) (Meijer and Pondaven, 1988; Neant and Guerrier, 1988) inhibits germinal vesicle breakdown (GVBD) in mouse (Rime et al., 1989) and bovine oocytes (Fulka et al., 1991). Okadaic acid (OA), a potent inhibitor of phosphatases 1 and 2A (Bialojan and Takai, 1988), induces meiotic maturation and MPF activity when microinjected into Xenopus and mouse oocytes (Jessus et al.,1991; Gavin et al., 1991). Okadaic acid is also a potent inducer of the uPA gene through a mechanism independent of the protein kinase A and C systems (Nagamine and Ziegler, 1991). Despite this information, little is known about the role of protein phosphorylation on PA activity in bovine and porcine oocytes matured in vitro. The regulatory mechanisms involved in meiotic maturation are quite different among species. In rodents GVBD occurs independently of protein synthesis (Schultz and Wassarman, 1977; Clarke and Masui, 1983), however both protein and mRNA synthesis are required for GVBD in cattle (Hunter and Moor, 1987; Sirard et al., 1989). Little is known about the role of mRNA and protein synthesis in PA activity in bovine and porcine oocytes matured in vitro.

Therefore the objectives of these studies were to 1) characterize the type of PA and PAI produced by bovine and

porcine oocyte-cumulus cell complexes (BOCC and POCC, respectively) 2) investigate the effects of dbcAMP, PMA, 6-DMAP and OA on PA activity and maturation rate in BOCC and POCC , and 3) investigate the effects of cycloheximide, actinomycin D and tunicamycin on PA activity and maturation rate in BOCC matured in vitro.

REVIEW OF LITERATURE

I. Meiotic Maturation of the Oocyte

Oogonia are mitotic cells of the germ line and are located in the primitive ovary. In response to an unknown signal, oogonia enter meiosis and become oocytes. In most mammals, the progression of meiosis in oocytes is initiated near the time of birth when oogonia cease proliferating and enter prophase I of meiosis. DNA synthesis and a number of cytoplasmic changes occur at this time during the transition from oogonium to primary oocyte. Primary oocytes are distinguished by a large nucleus, termed the germinal vesicle, containing decondensed chromatin. Oocytes then progress through the first meiotic prophase. Several reviews (Baker, 1982; Eppig, 1992; Schultz, 1992) have summarized the chromosomal morphology during this period. Meiotic prophase I is divided into five substages: leptotene, zygotene, pachytene and diplotene and diakinesis. During leptotene, chromosomal condensation occurs, and synaptonemal complexes consisting of two outer threads of chromosomal material separated by a fine association line are formed in zygotene. Pachytene, which is the stage of completion of synaptonemal complex formation, is characterized by genetic crossing over and recombination between chromatids. Oocytes then enter diplotene when loss of the synaptonemal complexes occurs. The prolonged diplotene stage in oocytes is referred to as the dictyate stage. Oocyte growth is generally correlated with granulosa cell proliferation. As the oocyte grows, the

number of layers of granulosa cells around the oocyte also increases. Anderson and Albertini (1976) and Gilula et al. (1978) reported that granulosa cells are involved in oocyte growth and gap junction development at points of apposition between the granulosa cell and oocyte plasma membranes. Gap junctions are membrane specializations that allow low molecular weight molecules to diffuse directly from one cell to another. These molecules can participate in the metabolism and regulation of oocyte development (Gilula et al., 1978).

The preovulatory surge in gonadotropins signals the release from arrest, and oocytes complete meiosis by undergoing two successive nuclear divisions. The nuclear membrane dissolves and commences germinal vesicle breakdown and chromosomes progress from prophase I thru metaphase I and anaphase I to telophase I. The completion of the first meiotic division is characterized by the extrusion of the first polar body. Shortly after the extrusion of the first polar body the secondary oocyte starts the second meiotic division. Spindle microtubules subsequently appear adjacent to the chromatin and the chromosomes arrange themselves on the metaphase plate. Metaphase II is a stage of arrest in the majority of species, and ovulation occurs at this stage.

It has been known for many years that oocytes present within an explanted preovulatory antral follicle do not resume meiosis, but do so in response to added gonadotropin or removal of the oocyte from the follicle (Pincus and Enzmann, 1935). A reasonable interpretation of these results is that the follicle

exerts an inhibitory effect on oocyte maturation. Cyclic adenosine 5'-monophosphate (cAMP), which is present in the oocyte, is the most likely candidate for maintenance of meiotic arrest based on the observation that membrane-permeable cAMP analogs inhibit oocyte maturation in vitro (Cho et al. 1974 ; Racowsky, 1986; Bornslaeger et al, 1986a). In addition phosphodiesterase inhibitors, which prevent degradation of intracellular cAMP, reversibly suppress GVBD in vitro (Eppig and Downs, 1988). Bornslaeger et al. (1986a) suggested that an inhibitory basal level of cAMP is present in the oocyte and this cAMP activates protein kinase A (PKA) which is a heterotetramer composed of two regulatory subunits and two catalytic subunits. Binding of cAMP to the regulatory subunits promotes the dissociation of the catalytic subunits, which are now active. The activated PKA phosphorylates an unknown protein, termed protein X, which is necessary for germinal vesicle breakdown (GVBD), and this results in maintenance of meiotic arrest. Resumption of meiosis is triggered by a decrease in the inhibitory levels of oocyte cAMP resulting in reassociation of active catalytic PKA subunits with regulatory subunits, and hence a decrease in PKA activity. A protein phosphatase is then speculated to convert inactive phosphorylated protein X to active dephosphorylated protein X, which is involved in resumption of meiosis.

Dekel and Beers (1980) have postulated a model for the mechanism of gonadotropin-induced meiotic resumption. The cAMP that is necessary to maintain meiotic arrest is derived

from the surrounding follicle cells, which possess an active adenylate cyclase, and enters the oocyte cytoplasm via gap junctions. This transfer of cAMP from follicle cells to the oocyte inhibits resumption of meiosis of follicle-enclosed oocytes. Gonadotropins induce resumption of meiosis by terminating gap junction-mediated intercellular communication between the cumulus cell and the oocyte. Without exogenous sources of cAMP, the oocyte is no longer able to maintain an inhibitory level of cAMP. The oocyte can not generate its own cAMP and the oocyte's phosphodiesterase activity induces a cAMP decrease that results in resumption of meiosis (Dekel and Beers, 1980). There is also indirect evidence that cAMP made in follicle cells can be transmitted to the oocyte. Oocytes obtained from cumulus cell-oocyte complexes treated with either follicle stimulating hormone (FSH) or cholera toxin contain more cAMP than denuded oocytes that are similarly treated (Bornslaeger and Schultz, 1985; Racowsky, 1984; Salustri et al., 1985). Bornslaeger and Schultz (1985) suggested that resumption of meiosis in vitro may be due to termination of follicle cell cAMP transfer to the oocytes. Gonadotropin-induced resumption of meiosis results in a rapid down-regulation of the number of homologous gap junction between cumulus cells and oocyte that correlates with GVBD in rat oocytes (Larsen et al., 1986). This reduction in the extent of communication between the cummulus cells and oocyte would efficiently isolate oocyte from the follicle cells.

The effects of protein kinase C (PKC), which is the calcium, phospholipid-dependent protein kinase activated by diacylglycerol or phorbol diesters, on oocyte maturation have been examined. Biologically active phorbol diesters inhibit GVBD in the mouse oocyte in a dose-dependent manner (Bornslaeger et al, 1986b). Retinoic acid, which is known to reverse the effects of phorbol diesters, overcomes the inhibitory effect of phorbol diesters on mouse oocyte maturation (Urner, 1984). Bornslaeger et al. (1986b) suggest the mechanism of these agents, which inhibit oocyte maturation, differ from that of cAMP. Elevation of oocyte cAMP levels after GVBD does not inhibit polar body emission, whereas addition of PKC activators after GVBD does inhibit polar body emission. Although the PKC activators do not inhibit the apparent dephosphorylation, their ability to inhibit GVBD correlates with inhibition of the apparent increase in phosphorylation. In addition, in contrast to the inhibitory effect of PKC activators on mammalian oocyte maturation, PKC activators induce maturation in Xenopus and Spisula oocytes (Stith and Maller, 1987; Eckholan et al.,1987).

Goren et al. (1990) reported that treatment of oocytes with calcium ionophore, A 23187, or increasing extracellular calcium, stimulated maturation in follicle-enclosed oocytes. In addition, LH stimulation of follicle-enclosed rat oocyte maturation was dependent upon external calcium concentration. Intracellular chelaters of calcium or inhibitors of calcium uptake have also been shown to prevent spontaneous maturation (Goren et al., 1990; Homa, 1991; De Felici et al., 1991). The release of calcium

from intracellular stores within the oocyte may positively influence the meiotic response (Homa et al., 1991). However, Flores et al. (1992) reported that FSH stimulated increase in intracellular calcium in isolated granulosa cells. This result suggests that calcium released by follicle cells in response to gonadotropin stimulation could flow down a concentration gradient, through the gap junctions, and into the oocyte to induce GVBD.

Purines such as hypoxanthine and adenosine have been also shown to inhibit oocyte maturation at concentrations present in follicular fluid. Hypoxanthine and adenosine have been shown to act synergistically to maintain meiotic arrest (Eppig et al., 1983). Eppig et al. (1983) hypothesized that the synergism of hypoxanthine and adenosine is due to suppression of phosphodiesterase by hypoxanthine combined with stimulation of cAMP production by adenosine. Purine-induced inhibition of GVBD is markedly enhanced when either dibutyryl cAMP (dbcAMP) or the phosphodiesterase inhibitor, isomethylxanthine (IBMX) is present at concentrations that by themselves do not result in significant inhibition of GVBD (Downs and Eppig, 1987). Thus, purines would potentiate dbcAMP-mediated inhibition of GVBD by raising the effective intracellular concentration of the cAMP analog or augment IBMX-induced inhibition of GVBD by further inhibiting endogenous phosphodiesterase activity (Downs et al. 1989). Steroid hormones such as progesterone, testosterone and 17 β-estradiol can inhibit GVBD in denuded mouse oocytes (Kaji

et al., 1987). These steroid hormones can also potentiate the maturation inhibition of denuded oocytes by low concentrations of dbcAMP (Eppig et al., 1983; Rice and McGaughey, 1981). Kaji et al. (1987) reported that the likely basis for this synergistic inhibition with dbcAMP is that steroid hormones inhibit oocyte phosphodiesterase activity. The activity of steroid hormones to inhibit oocyte phosphodiesterase thus accounts for their ability to potentiate inhibition of GVBD by low concentrations of dbcAMP.

A number of changes in the pattern of protein synthesis occur subsequent to GVBD, and these may be necessary for continued nuclear progression (Schultz and Wassarman, 1977; Schultz et al., 1979). However, addition of protein synthesis inhibitors shortly after GVBD permits the oocytes to emit a polar body and arrest at metaphase II (Schultz et al., 1979). In rodents, GVBD occurs when protein synthesis is inhibited (Eckholm and Magnusson, 1979; Wassarman et al., 1976), however protein synthesis is required for GVBD in porcine and ovine oocytes (Fulka et al., 1986; Moor and Crosby, 1986). A possible explanation for these species differences is that in rodents, GVBD starts to occur within 45 to 60 min following oocyte isolation, whereas in large animals, GVBD takes several hours following oocyte isolation (reviewed by Schultz, 1992).

There are several reports indicating that granulosa cells (Tsafriri and Channing, 1975), follicular fluid (Chang, 1955; Tsafriri et al., 1977) and granulosa cell-conditioned medium (Tsafriri et al., 1976) contain a substance(s) that can inhibit

GVBD in oocytes. This led to the idea that an oocyte maturation inhibitor (OMI) exits that is produced by somatic cells of the follicle, and causes maintenance of meiotic arrest in oocytes (reviewed by Leibfried-Rutledge et al., 1989). Oocyte maturation inhibitor, which is proposed to be a low molecular weight polypeptide, has been partially purified using a bioassay that quantifies the effect of follicular fluid and purified fractions on their ability to inhibit GVBD. The source of OMI has been shown to be the granulosa cells. Germinal vesicle breakdown can be suppressed when oocytes are cultured with granulosa cells or follicle walls (Tsafriri et al., 1975). Inhibition of GVBD was also observed if culture medium was conditioned by granulosa cells or contained extracts of granulosa cells (Anderson et al., 1985). The inhibitory activity separates into several components during purification, and thus it is not clear if multiple inhibitors that are chemically distinct are present or whether these differences reflect multiple forms of the same molecule. Also conflicting results are obtained following protease treatment of these partially purified fractions; sometimes the inhibitory activity is lost and sometimes it is not.

Mullerian inhibiting substance (MIS), which is related to transforming growth factor-β (TGF-β), is responsible for regression of the Mullerian duct. Takahashi et al (1986) reported that MIS is present in the ovary throughout fetal and adult life and is present in granulosa cells and follicular fluid. They observed that MIS inhibited GVBD in either denuded or cumulus

cell enclosed rat oocytes, and antibodies to MIS overcame MIS inhibition of GVBD. In contrast, results of another study immunopurified MIS could not demonstrate any inhibition of GVBD in cumulus cell-enclosed rat oocytes (Tsafriri et al., 1988). Although MIS may play a regulatory role in maintenance of meiotic arrest, further experimentation is obviously required to strengthen this proposal.

Maturation or M-phase promoting factor (MPF) which was first described in amphibian oocytes, controls the transition from G2 to M phase in both meiosis and mitosis (Masui and Market, 1971). Maturation promoting factor has recently been purified from Xenopus eggs, and one of its subunits has been identified as the frog homolog of the schizosaccaromyces prombe cdc protein (Dunphy et al., 1988; Gautier et al., 1988). Dunphy and Newport (1989) reported that the Xenopus cdc2 component of MPF is tyrosine phosphorylated in vivo, and this tyrosine phosphorylation oscillates with the cell cycle. Gautier et al. (1990) recently reported that highly purified MPF from Xenopus eggs contains cyclin. Cyclin synthesis is necessary for entry into mitosis and the destruction of cyclin is required for exit from mitosis (Murrary, 1989). Collectively mitosis and meiotic maturation are induced by the activation of MPF, a protein kinase whose principal subunits are the cdc protein, which has extensive homology to known protein kinases, and cyclin, a regulatory subunit whose abundunce fluctuates through the cell cycle (reviewed by Glotzer et al., 1991).

II. Plasminogen Activator

Biochemical Aspects

Plasminogen activators (PA) are serine proteases that convert the zymogen plasminogen to the serine protease plasmin. Plasminogen can be purified by a combination of affinity chromatography binding to agarose L-lysine columns and ion-exchange chromatography on carboxymethyl-cellulose or diethylamineethyl-sephadex. Human plasminogen has a molecular mass of 83-92 kD and is composed of a single chain glycoprotein consisting of 790 amino acids with a glutamic acid residue at the amino-terminus and an asparagine residue at the carboxy-terminus. Two major forms of plasminogen have been identified: Glu-plasminogen which has glutamic acid at the amino terminus, and Lys-plasminogen which has lysine at the amino terminus (Christman et al., 1977). Plasminogen activator can convert either Glu-plasminogen or Lys-plasminogen to Glu -plasmin or Lys-plasmin by cleavage of the Arg 560-Val 561 bond. Plasmin is a two chain molecule in which the polypeptide chains are held together by two disulfide bonds. The heavy (A) chain has a molecular mass of 48-68 kD and contains carbohydrate units as well as the lysine binding sites. The light (B) chain has a molecular mass of 20-25 kD and contains the serine protease portion of the molecule.

Plasminogen and plasmin have a high degree of homology among the different mammalian species in regard to terminal amino acid sequences and molecular mass (Robbins et al., 1973; Summaria et al., 1973; Christman et al., 1977). Summaria et al.

(1973) reported that the molecular masses and amino acid sequences of plasminogen from cat, dog, rabbit and bovine were similar with the human. The heavy (A) chain of plasmin is charaterized by five triple loop structures known as kringles. Kringle domains are also present in prothrombin, urokinase, and several other fibrinolytic proteases. Recent research suggested that kringles mediate the attachment of plasminogen or plasmin to the fibrin matrix of blood clots (Petros et al., 1988; Thewes et al., 1988). Receptors for plasminogen and plasmin have been identified in human tumor cell line SW 1116 (Burtin and Foundameche, 1988). These receptors are the same for both plasminogen and plasmin, but exhibit much higher affinity for plasmin. Plasmin bound to the receptor retains its enzymatic activity, and may activate prourokinase resulting in the formation of urokinase, which in turn increases plasmin. Ultimately, this process increases the concentration of plasmin at the surface of tumor cells, enabling them to degrade the component proteins in connective tissues and basement membranes. Plasma contains several protease inhibitors which regulate the fibrinolytic system. α2-antiplasmin and α2 -macroglobulin are the most important inhibitor of plasmin. α2 -antiplasmin is characterized by rapid inactivation of plasmin, while α2-macroglobulin exerts its action only when α2 -antiplasmin levels are depleted (Dano et al., 1985). The reactive site of α2-antiplasmin bind to the active site of plasmin, resulting in a covalent complex which is enzymatically

inactive.

Two major plasminogen activators have been identified by functional differences and by immunological reactivites: urokinase-type PA (uPA) and tissue-type (tPA). Urokinase-type PA exhibits a molecular mass between 30 and 55 kD, whereas tPA is approximately 72 kD (Dano et al., 1985). Both uPA and tPA are secreted as single-chain polypeptides (scuPA and sctPA). In its single-chain form, they are proenzymes with little or no activity (Eaton and Baker, 1984; Pannel and Gurewich, 1987). According to Pannell and Gurewich (1987), the enzyme activities of the single-chain form appear to be only 0.2-0.4% of the respective activities of the active enzymes. Lijnen et al. (1986) reported on the activity of scuPA. When scuPA and plasminogen were combined, two-chain uPA and plasmin were formed. Addition of plasmin inhibitors to this mixture abolishes the generation of uPA but not the activation of plasminogen to plasmin, suggesting that scuPA can activate plasminogen directly. ScuPA is activated to uPA by cleavage of a peptide bond, which results in the formation of the two chain form of uPA. This process takes place in the extracellular spaces and can be mediated by plasmin (Dano et al., 1985). Urokinase-type PA was originally isolated from human urine but has since been found in human plasma and in conditioned media of several types of cell lines (Dano et al., 1985). Urokinase-type PA is a glycoprotein that is synthesized as an one-chain proenzyme (55kD) and is converted to the active enzyme by a proteolytic cleavage yielding a disulfide-linked two -chain molecule. Gunzler et al. (1982) reported the complete

amino acid sequence of uPA from human urine. The high molecular mass form of uPA consists of a catalytic subunit (B -chain; 33 kD) and a non-catalytic subunit (A-chain; 25 kD). The B-chain is homologous to the catalytic subunit of other serine proteases. The A-chain or light chain contains one kringle domain and a domain required for interaction with a cellular receptor (Dano et al., 1985; Hart and Rehemtulla, 1988; Vassali et al., 1985).

Tissue-type PA is a serine protease also synthesized as a proenzyme and composed of 530 amino acids (Degen et al., 1986). Limited proteolysis by plasmin, kallikrein, or factor Xa cleaves the Arg-Ile bond of the proenzyme yielding two polypeptide chains linked by a disulfide bond. The catalytic site of tPA is located in the light chain (B-chain), which has a molecular mass of 30 kD, and is composed of His-322, Asp-371 and Ser-478 residues (Degen et al., 1986). The heavy chain (A-chain) consists of several structural domains and shares a high degree of homology with other hemostatic proteins. These domains are categorized as the fibronectin finger domain, the epidermal growth factor domain, and the two kringle domains (I and II) (Gerard et al., 1986). Van Zonneveld et al. (1986) reported that deletion of the kringle II domain reduced the activation of the enzyme by fibrin while deletion of both the finger and kringle domains abolished the fibrin-induced alteration in enzyme activity. Therefore, the finger and kringle domains are responsible for the interaction of tPA with fibrin. Tissue type plasminogen activator is a glycoprotein with approximately 7%

carbohydrate (Ranby et al., 1982). Little et al. (1984) reported that this carbohydrate does not likely influence the activity of the enzyme because removal of the carbohydrate by glycosidase did not alter enzyme activity. However they found that the enzyme was not secreted from a tissue culture cell line when cultured with the glycosylation inhibitor, tunicamycin. Therefore, glycosylation of the enzyme is required for transport of the protein out of cell.

Regulation of proteolysis is important for the maintenance of homeostasis, and is maintained in part by specific PA inhibitors. At least four inhibitors of PA have been identified: PA inhibitor-1 (PAI-1); PA inhibitor-2 (PAI-2); PA inhibitor-3 (PAI-3), and protease nexin. Plasminogen activator inhibitor-1 is the major PAI in plasma and is believed to be involved in the regulation of plasma tPA activity (Gerard et al., 1986; Kruithof et al., 1986). The primary structure of human PAI-1 has been determined by nucleotide sequencing of cloned cDNA and by amino acid sequencing (Andreasen et al., 1986; Ginsburg et al., 1986; Wun and Kretzmer, 1987). Plasminogen activator inhibitor-1 can form an SDS-stable complex with tPA yielding a proteinase-inhibitor complex of 110 kD (Kruithof et al., 1984; Thorsen and Philips, 1984; Wagner and Binder, 1986). Synthesis of PAI-1 is regulated by many of the same effectors that regulate the expression of PA. It is interesting that factors causing an increase in the expression of PA often also increase the secretion of the inhibitor (Saksela and Rifkin, 1988).

Plasminogen activator inhibitor-2 has been purified and

characterized from several tissues including placenta, monocytes, leukocytes and fibrosarcoma cells (Kruithof, 1988). Plasminogen activator inhibitor-2 exists in two different forms: a 47 kD, nonglycosylated form and a 60 kD, glycosylated form. The cDNA of PAI-2 has been cloned, and the amino acid sequence deduced from this cDNA confirms that this PAI belongs to the serpin serine protease inhibitor family. Plasminogen activator inhibitor-2 also can form SDS-stable complexes with PA yielding PA-inhibitor complexes detectable by zymography, and is thought to play a role in the regulation of extracellular PA activity involved in tissue remodelling.

Plasminogen activator inhibitor-3 has been purified and characterized from human urine and plasma (Stump et al., 1986). It is also a glycoprotein with a molecular mass of 50 kD, and forms complexes mainly with uPA. The physiological role of the human PAI-3 is unknown. Protease nexin is distinct from PAI 1 -3 in that it is not specific for PA. It can bind to and is activated by heparin and plasmin (Sprenger and Kluft, 1987). Protease nexin is also a glycoprotein (47 kD).

Functional Aspect

Plasminogen activators mediate the specific conversion of plasminogen into plasmin, a proteolytic enzyme of broad substrate specificity. The primary function of tPA is intravascular thrombolysis, whereas uPA is thought to be involved in the generation of proteolysis during cell migration and tissue remodelling (Dano et al., 1985; Saksela and Rifkin, 1988) The distribution of PA in reproductive organs is well

-documented and many studies have been conducted to investigate the role of PA in male and female reproduction.

Ovulation in mammals is a unique phenomenon involving both oocyte maturation and rupture of the follicle wall to release the egg. Plasminogen activators have been detected in large quantities during the ovulatory process indicating a close correlation between ovulation and PA. Beers et al. (1975) demonstrated increases in PA activities of in preovulatory follicles and proposed a causal relationship between PA increases and follicle rupture. Plasminogen, PA and PAI have been also detected in large amounts in the follicular fluid. The activation of follicular plasminogen to plasmin by PA decreases the tensile strength of the follicle wall (Beers, 1975). Beers et al. (1975) proposed that granulosa cells elaborate increasing amounts of PA in response to the increased levels of circulating LH. This enzyme acts on follicular plasminogen generating plasmin within the follicle wall. Plasmin, in turn, activates another protease, collagenase, which allows further degradation of collagen in the follicle wall.

Gonadotropins stimulate the release of PA from inactive ovarian granulosa cells in a time and dose-dependent fashion (Beers et al., 1975). Strickland and Beers (1976) reported that FSH was more effective in inducing PA activity than LH, and prostaglandin and cAMP stimulate the production of PA by granulosa cells. Both uPA and tPA have been demonstrated in granulosa cells. Liu et al. (1987) studied the activity of tPA and uPA in granulosa and theca-interstitial cells obtained from

gonadotropin-treated immature rats during the periovulatory period. They showed that tPA activity in both granulosa and theca-interstitial cells increased after pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin treatments and reached a maximum prior to ovulation. Liu and Hsueh (1987) also studied the molecular patterns and secretion of PA in rat cumulus-oocyte complexes. Both tPA and uPA were present in the cumulus-oocyte complexes, while denuded oocytes contained only tPA. Human chorionic gonadotropin (hCG) treatment induced a time dependent increase in tPA activity in both cumulus-oocyte complexes and denuded oocytes. Morphological signs of cumulus cell expansion and dispersion was observed after increases in oocyte tPA activity.

Tsafriri et al. (1989) examined the relationship between PA and ovulation using α2-antiplasmin and an anti-tPA. They reported that anti-tPA and α2-antiplasmin inhibited ovulation only when injected at the time of hCG treatment. Species differences were reported with regard to the types of PA secreted by granulosa cells. Rat granulosa cells secrete mainly tPA, while mouse cells secrete predominantly uPA (Canipari et al., 1987). Smokovitis et al. (1988) reported that the follicle wall and follicular fluid from sows have both tPA and uPA, and activities are greatest at ovulation. Ny et al. (1987) studied the effect of GnRH on tPA activity in rat granulosa cells and cumulus -oocyte complexes. Gonadotropin-releasing hormone increased the level of tPA activity in granulosa cells. Gonadotropin

-releasing hormone also increased tPA content in oocytes through a mechanism mediated by the adjacent granulosa cells. It has been suggested that GnRH may act on cumulus cells to increase unidentified messenger molecules which then may be transported to the oocyte via gap junctions to signal an increase in tPA level. Primary oocytes do not contain detectable amounts of tPA, but it is induced during meiotic maturation of the cell. Huarte et al. (1985) reported primary oocytes contain tPA mRNA, and its translation is triggered during the process of meiotic maturation. The induction of tPA activity in the oocytes is inhibited by cycloheximide suggesting that tPA mRNA is under translational control. The role of PA in the oocyte is unknown. It may prevent trapping of the released oocyte in the fibrin deposits observed in the oviduct during and after ovulation since most of it is secreted before or during fertilization.

Involution of the mammary glands is charaterized by considerable tissue degradation and remodeling. Ossowski et al. (1979) reported a strong correlation between mammary gland involution and the amount of uPA in mice. Only uPA was identified in extracts from involuting mammary gland. The importance of PA during involution of the mammary glands is believed to be necessary for preparing the udder for the next lactation (reviewed by Dano et al., 1985).

Sertoli cells are found along the basement membrane of the seminiferous tubules. Sertoli cells have been shown to secrete several proteins including PA in response to hormonal stimulation (Fritz et al., 1976; Lacroix et al., 1979). Lacroix et

al. (1981) observed PA activity in rat testis tubule segments at different stages of the cycle of the seminiferous epithelium. Plasminogen activators were observed to increase 100-fold at stages VII and VIII of the cycle. Stages VII and VIII of the cycle include the events of spermiation occurs and movement of Sertoli cell cytoplasmic processes around leptotene spermatocytes. Lacroix et al. (1981) concluded that PA is involved in the localized restructuring of the seminiferous epithelium during translocation of spermatocytes in meiosis into the adluminal compartment. Hettle et al. (1986) examined the type of PA synthesized and secreted by cultured rat Sertoli cells and seminiferous tubules. They observed that Sertoli cells maintained under basal conditions secreted uPA. In contrast, Sertoli cells cultured in FSH or dbcAMP produced mainly tPA. Immunohistochemical studies showed the presence of uPA exclusively at stages VII and VIII in Sertoli cells. On the other hand, tPA immunoreactivity was maximal during stage IX-XII (Vihko et al., 1988). These results suggest that PA secreted from Sertoli cells at stages VII and VIII facilitates the release of preleptotene spermatocytes and movement of cytoplasmic extensions allowing for maintenance of the tight junctional complexes in the basal region of the tubule.

Liedholm and Astedt (1975) provided the first report of the role of PA in the rat embryo. They demonstrated that PA activity of fertilized rat ova was high during tubal passage on days 1-4 but this activity decreased on day 5 and disappeared at implantation. Liedholm and Astedt (1975) suggested that the low PA activity

during implantation may be required for embryo attachment to the endometrium. Strickland et al. (1976) examined the production of PA during the early periods of mouse embryo development. Plasminogen activators were produced by cultured blastocysts in a complex pattern. In the first phase, PA activity increased to day 6 and reached a maximum at day 8 or 9, then decreased. In the second period, PA activity increased at day 11 and reached a peak at day 15, at which point the enzyme activity was 5-fold higher than on day 8.

Bode and Dziadek (1979) studied PA production in both embryonic and extraembryonic mouse tissues. Their results demonstrated that PA secretion was initiated progressively in different mouse embryo tissues during development from the 7th to 10th day of gestation. By day 10 of gestation, all tissues which were tested (parietal endoderm, sac endoderm, mesoderm, and amnion) secreted PA. They concluded that although PA may appear at different stages of development, the presence of this enzyme in various parts of the midgestation embryo indicates an important function in embryo growth. Sherman (1980) evaluated PA activity in empty zonae pellucidae and mouse embryos. He reported that PA activity was restricted to the zona pellucida, and that this enzyme was not involved in hatching from the zona pellucida. Because blastocysts produce PA at the same time trophoblast cells acquire their migratory properties, Sherman (1980) concluded that PA plays a pivotal role in trophoblast invasiveness during implantation. Mullins et al. (1980) examined whether the absence of invasive implantation within the porcine

uterus was due to a failure of the conceptus to produce PA or the presence of an inhibitor. Plasminogen activator activity in uterine flushings was high during the early and late stages of the estrous cycle. In contrast, the activity of the enzyme became low on day 12 of gestation. A time-dependent increase in PA activity was observed in conditioned medium from blastocysts, indicating that enzyme activity was inhibited in vivo. Plasminogen has been detected in uterine flushings from both the pig and mouse (Fazleabas et al., 1983; Finlay et al., 1983). Menino and O'Claray (1986) compared the development of mouse embryos in media supplemented with plasmin, plasminogen, trypsin or pronase. More embryos developed to blastocysts and hatched in medium with plasmin or plasminogen. Also, incidences of attachment to the substratum and trophoblastic growth were greater in the presence of plasmin and plasminogen. In our laboratory, several studies have been designed to charaterize PA production and to elucidate the function of this enzyme in early embryos from different species. Menino and Williams (1987) evaluated plasminogen activation by cultured bovine embryos. Plasminogen activator production was low for the first 48 h of culture, increased between 48-120 h and plateaued thereafter. They found that plasminogen had no effect on the hatching rate, but high concentrations of this zymogen accelerated hatching. Kaaekuahiwi and Menino (1989) demonstrated that PA activity was positively correlated with the viability of bovine embryos, suggesting that PA secretion may be used an indicator of embryo viability. Dyk and Menino (1991)

used SDS-PAGE and zymography to determine that day 12-14 bovine blastocysts secreted a light form (49kD) and heavy form (86kD) of PA. Subsequent results demonstrated that PA activity in bovine embryos could be completely suppressed by amiloride and antibodies to human uPA, whereas PA activity was unchanged with antibodies to human tPA suggesting the heavy form is a uPA -PAI complex (Berg and Menino, 1992). Menino et al. (1989) evaluated PA production by ovine embryos and the effects of plasminogen on ovine embryo development and zona pellucida integrity. They reported that more blastocysts hatched in medium containing 60 and 120 mg/ml plasminogen than in medium containing 0 mg/ml plasminogen. Plasminogen activator activity was low until the morula stage, increased during the morula-blastocyst transition and remained high throughout blastocoelic expansion and hatching. Unlike the cow, increasing levels of plasminogen in the culture medium increased both the hatching rate and the solubility of the zona pellucida.

Regulatory aspect

Total PA activity in a tissue is determined by the rate of biosynthesis, release from producer cells, conversion of proenzymes to active enzymes, and by the presence of stimulatory factors and inhibitors of enzyme activity (Dano et al., 1985). Several hormones, second messenger systems and other factors have been shown to modulate PA activity either directly or indirectly by affecting the availability of substrate or inhibitors.

Plasminogen activator activity has been found to be increased by LH, GnRH, FSH and hCG in granulosa cells of rat ovaries (Beers et al., 1975; Strickland and Beers, 1976; Liu et al., 1981; Ny et al., 1987) and rat cumulus-oocyte complexes (Liu and Hsueh, 1987, Liu et al., 1987; Ny et al., 1987). Follicle stimulating hormone also stimulates the activity of PA in rat Sertoli cells (Lacroix et al., 1977; Lacroix and Fritz, 1982). The generally accepted hypothesis is that cAMP mediates the response to this hormone. Other compounds known to increase cellular cAMP levels such as phosphodiesterase inhibitors and cholera toxin have also been found to affect PA activity (Dano et al., 1985).

The molecular mechanism of PA gene regulation by cAMP is not established. The gene regulatory effect of cAMP is usually mediated by cAMP-dependent protein kinase A. The binding of cAMP to the regulatory subunits results in the release and subsequent activation of the catalytic subunits. Nigg et al. (1988) showed subcellular distribution of protein kinase A by immunocytochemical analysis, indicating that cAMP regulates and induces the transcription of the PA gene.

Gonadotropin-releasing hormone (GnRH) induces the production of PA mRNA in granulosa cells in the rat. Ny et al. (1987) have suggested that GnRH treatment increases PA activity in rat oocyte-cumulus cell complexes through the protein kinase C pathway. Phorbol 12-myristate 13-acetate (PMA), a known stimulator of protein kinase C has been found to enhance PA activity (Vassalli et al., 1977; Quigley, 1979). Santell and Levin (1988) studied the effects of phorbol esters and cAMP on PA and

PAI secretion. Phorbol esters induced a time- and dose -dependent increase in PA release from endothelial cells, in which cAMP had no effect. However cAMP elevation can increase PAI secretion. Meyer et al. (1988) investigated the effect of phorbol esters on PA secretion in human rhabdomyosarcoma cells by using an enzyme-linked immunosorbent assay (ELISA). They reported that PMA increased the cellular level of PAI-1 mRNA and PAI-1 gene transcription rate. Opdenakker et al. (1983) reported that treatment with phorbol ester in human Bowe melanoma cells enhanced PA production and increased the cellular level of PA mRNA. This suggests that phorbol esters participate, either directly or indirectly, in inducing PA gene transcription.

Cycloheximide and actinomycin D have been reported to block cAMP effects on PA activity (Wilson and Reich, 1977; Granelli -Piperno and Reich, 1983). However cycloheximide treatment did not suppress PMA-stimulated increase of PAI-1 mRNA indicating that protein synthesis was not required for the PMA effect. Treatment with both cycloheximide and PMA resulted in an increase in PAI mRNA levels, which indicated that a short-lived protein negatively regulates the level of PAI-1 mRNA either by suppressing PAI-1 gene transcription or by destabilizing the mRNA.

Epidermal growth factor (EGF) enhances PA activity in cultured human foreskin fibroblasts (Eaton and Baker, 1983). Lee and Weinstein (1978) reported that EGF caused an increase in both intracellular and extracellular levels of PA activity in HeLa cells. Induction of PA activity in these cells was blocked by

actinomycin D and cycloheximide, suggesting that EGF induces de novo synthesis of PA in a manner dependent on the continued synthesis of mRNA (Dano et al., 1985).

Fibroblast growth factor (FGF) has been reported to increase PA production in cultured bovine capillary endothelial cells (Gross et al., 1982) and newborn rat astroglial cells (Rogister et al., 1988). In contrast, TGF-β inhibited PA activity in capillary endothelial cells (Saksela et al., 1987), and stimulated production of PAI-1 in human endometrial adenocarcinoma cells (Presta et al., 1988) and human tumor cell line HT-1080 (Laiho et al., 1987).

Estrogens have been reported to increase uPA activity in the rat uterus (Katz et al., 1976; Peltz et al., 1983) and in the human breast carcinoma cell line MCF-7 (Butler et al., 1979). Estrogen has been shown to induce the production of plasmin-inhibitor by pig endometrium during the period of implantation, thus preventing the proteolytic degradation of the uterine wall (Fazleabas et al., 1983). Glucocorticoids have been reported to suppress uPA activity in a variety of cultured cells (Dano et al., 1985). The response of tPA activity to glucocorticoids appears to be more variable. Glucocorticoids did not affect tPA activity in melanoma cell lines (Roblin and Young, 1980) and in cultured bovine aortic endothelial cells (Granelli-Piperno and Reich, 1983). These steroid hormones are generally believed to influence protein synthesis by changing the rate of gene transcription and translation. The protein synthesis inhibitor cycloheximide has been shown to block the estrogen induction of

PA activity in MCF-7 cells (Butler et al., 1979). It has also been found that RNA synthesis inhibitors such as actinomycin D, α -amanitin and bromotubercidin inhibit glucocorticoid and estrogen suppression of intracellular levels of uPA (Wilson et al., 1983).

FACTORS AFFECTING PLASMINOGEN ACTIVATOR ACTIVITY IN BOVINE OOCYTE-CUMULUS CELL COMPLEXES MATURED IN VITRO.

Absract

Effects of phorbol myristate acetate (PMA), dibutyryl cyclic AMP (dbcAMP), 6-dimethylaminopurine (6-DMAP) and okadaic acid (OA) on plasminogen activator (PA) activity and maturation rate in vitro in bovine oocyte-cumulus cell complexes (BOCC) were determined. Three plasminogen-dependent lytic zones (92-95 kD, 71-73 kD and 49-51 kD) were detected by SDS-PAGE and zymography in matured BOCC and reverse zymography revealed a PA inhibitor (PAI, 52 kD). Activity in the 49-51 kD band was reduced by amiloride suggesting a urokinase-type PA (uPA) and the 71-73 and 92-95 kD bands are a tissue-type PA (tPA) and tPA-PAI complex, respectively. Dibutyryl cyclic AMP increased ($P<0.05$) PA activity in BOCC in dose-dependent fashion. Phorbol myristate acetate increased ($P<0.05$) uPA activity, and at high concentration, tPA activity, in BOCC. Neither dbcAMP nor PMA inhibited ($P>0.05$) germinal vesicle breakdown (GVBD). Plasminogen activator activity decreased in 6-DMAP and oocyte maturation was inhibited in dose-dependent fashion ($P<0.05$). Plasminogen activator and PAI activities markedly increased ($P<0.05$) in BOCC treated with up to 25 nM OA, however activities decreased ($P<0.05$) at concentrations greater than 75 nM. These data suggest that PA and PAI production by BOCC is associated with GVBD and influenced by stimulators of the protein kinase A and C systems and modulators of intracellular phosphorylation.

Introduction

Plasminogen activators (PA) are serine proteases that convert the zymogen, plasminogen, into plasmin. Two principal PA have been identified, tissue-type (tPA) and urokinase-type (uPA), and their activities are modulated physiologically by several different inhibitors, including PA inhibitors-1 (PAI-1), -2 (PAI-2) and -3 (PAI-3) (Dano et al., 1985; Kruithof, 1988). Plasminogen activators and PAI are synthesized and secreted by numerous cell types, often under hormonal control, and are thought to play an important role in the extracellular proteolysis accompanying cell migration and tissue remodelling (Dano et al., 1985; Andreasen et al., 1990; Mullins et al., 1983) In the ovary, PA activity is markedly increased in granulosa cells obtained from follicles shortly before ovulation (Beers et al., 1975). Rat granulosa and cumulus cells secrete PA in response to gonadotrophins (Ny et al., 1985; Canipari et al., 1986) and it is believed that granulosa cell tPA contributes to follicle rupture (Liu et al., 1987). Rat and mouse oocytes denuded of cumulus cells produce only tPA during spontaneous in vitro meiotic maturation (Huarte et al.,1985), however cultured oocyte-cumulus cell complexes produce both tPA and uPA (Liu et al., 1986). Interestingly, in rats, FSH and GnRH stimulate tPA, but not uPA, activity in both cell types when cultured as oocyte-cumulus cell complexes (Liu et al., 1986; Ny et al., 1987). Ny et al. (1987) have suggested that GnRH treatment increases PA activity in rat oocyte-cumulus cell complexes through the protein kinase C pathway. On the other hand, FSH appears to mediate its effects through the cAMP-dependent protein kinase A pathway (Salustri et

al., 1985). Both phorbol myristate acetate (PMA), a stimulator of the protein kinase C pathway (Castagna et al., 1982), and dibutyryl cAMP (dbcAMP) can increase PA activity in a variety of cell types (Dano et al., 1985). Although PA activity is increased in rat oocyte-cumulus cell complexes in vitro by hormones, it has not been determined in oocyte-cumulus cell complexes from cattle if PA is present, and if produced, whether activity is regulated by second messenger systems.

Phosphorylation and dephosphorylation of proteins plays an important role in the cell cycle and oocyte maturation (Draetta et al., 1988; Moor and Crosby, 1986) The cdc protein, p34cdc2, is the key protein kinase component of maturation or M-phase promoting factor (MPF) that controls the transition from G2 to M phase in both meiosis and mitosis (Masui and Markert, 1971;Draetta et al., 1989). The activity of p34cdc2 is regulated by phosphorylation (Dunphy and Newport, 1988; Gautier et al., 1989). It has been shown recently that 6-dimethylaminopurine (6-DMAP), a serine/threonine protein kinase inhibitor (Meijer and Pondaven, 1988; Neant and Guerrier, 1988), inhibits germinal vesicle breakdown (GVBD) in mouse (Rime et al., 1989) and bovine oocytes (Fulka et al., 1991). In contrast, okadaic acid (OA), a potent inhibitor of phosphatases 1 and 2A (Bialojan and Takai, 1988), induces meiotic maturation and MPF activation when microinjected into Xenopus and mouse oocytes (Jessus et al., 1991; Gavin et al., 1991). Okadaic acid is also a potent inducer of the uPA gene through a mechanism independent of the protein kinase A and C systems (Nagamine and Ziegler, 1991). Despite this information in other species, nothing is known about

the role of protein phosphorylation on PA activity in bovine oocytes matured in vitro. Therefore, the objective of this study was to investigate the effects of dbcAMP, PMA, 6-DMAP and OA on PA activity and maturation rate in bovine oocyte-cumulus cell complexes (BOCC) matured in vitro.

Materials and Methods

Recovery and Culture of BOCC

Cattle ovaries were collected from a local slaughterhouse and transported to the laboratory in physiological saline (25-30 C) within 2 h. Oocytes were aspirated from 2- to 5-mm follicles and washed with Tissue Culture Medium 199 (TCM199, Sigma Chemical Co., St. Louis, MO, USA) supplemented with polyvinylpyrrolidone (PVP, 3 mg/ml, Sigma), sodium pyruvate (50 mg/ml), sodium bicarbonate (2.6 mg/ml) and 10 ml/l of an antibiotic-antimycotic solution (Sigma). Oocytes were pooled and carefully selected under a stereomicroscope and only those with compact cumulus were used. Oocytes were cultured in TCM199 modified as above at 39 C in a humidified 5% CO2 in air atmosphere (Saiki et al., 1991).

Experimental Treatments

Determination of the optimum number of BOCC and the time course for PA detection. Ten, twenty and thirty BOCC were initially used to evaluate the optimum number of BOCC required to produce satisfactory zymograms. The time course required for development of activity was determined by in vitro culture of twenty BOCC for 0, 6, 12 and 24 h. All subsequent experiments also used twenty BOCC for each treatment.

Dibutyryl cAMP. A stock solution of 25 µg/ml dbcAMP (Sigma) in TCM199 was used. This solution was diluted with culture medium to concentrations of 0.5, 1 and 2.5 mM.

Phorbol myristate acetate. Phorbol 12-myristate 13-acetate (Sigma) was used in concentrations of 1, 10 and 100 ng/ml and diluted from a 10 µg/ml stock solution dissolved in dimethylsulfoxide (DMSO, Sigma). Each final dilution of PMA contained 1% DMSO and medium with 1% DMSO served as a control.

6-Dimethylaminopurine. A stock solution of 100 mM 6-dimethylaminopurine (Sigma) in TCM199 was used in these experiments. This solution was diluted with culture medium to 0.2, 1 and 2 mM concentrations.

Okadaic acid. Okadaic acid (Moana Bioproducts, Honolulu, HI, USA) was diluted with TCM199 to a concentration of 25 µg/ml. This stock solution was diluted with culture medium to 2.5, 7.5, 25, 75 and 250 nM.

Electrophoresis and Zymography

Electrophoresis and zymography were performed using the procedures described by Granelli-Piperno and Reich (1978) and Vassalli et al. (1984) with modifications. Twenty oocytes with evenly enclosed cumulus cells were combined with 90 µl of sample buffer (5.0% SDS; 20% glycerol; 0.0025% bromophenol blue in 0.125M Tris HCl buffer). Each polyacrylamide gel included one lane containing the following molecular mass standards: phosphorylase b (97.4 kD), bovine serum albumin (66.2 kD), ovalbumin (45.0 kD), carbonic anhydrase (31.0 kD), soybean trypsin inhibitor (21.5 kD) and lysozyme (14.4 kD) (Bio-Rad Laboratories, Richmond, CA, USA)

and one lane with either human tPA (Sigma) or urokinase (American Diagnostica, Inc., Greenwich, CT, USA). One hundred microliters of BOCC samples, urokinase, tPA or MW standards were pipetted into castellated wells in a 4% stacking gel with a 12% separating gel. Electrophoresis was conducted at 20 mA through the stacking gel and 30 mA through the separating gel for 2 to 4 h.

Following electrophoresis, the polyacrylamide gels were gently shaken in 2.5% Triton X-100 (Sigma) in H_2O for 30 min, rinsed with distilled water and incubated in DPBS for 30 min. The gel was placed on plastic wrap and a casein-agar gel containing purified human plasminogen (Sigma) supported on a glass plate (the zymogram) was applied to the surface of the polyacrylamide gel. Zymograms were prepared by dissolving 4 g of nonfat dry milk (Carnation Co., Los Angeles, CA, USA) in 100 ml of buffer containing 0.0013 M CaCl2 2H2O, 0.1 M glycine, 0.038 M Tris and 0.005 M Na azide. Seven milliliters of the nonfat dry milk mixture were heated to 55 C and combined with 7.0 ml of 2% melted agarose (Sigma) with distilled water also maintained at 55 C. Purified human plasminogen was added to the warmed mixture of 4% nonfat dry milk and 2% agarose to yield a final concentration of 25-30 µg/ml and 10 ml of this mixture was cast onto a warmed 13 X 6 cm glass plate. Zymograms containing 0 µg/ml plasminogen were also used for detection of any nonspecific proteolytic activity. The gel sandwich (acrylamide gel and casein-agar gel) was incubated at 39 C for 24-48 h. Plasminogen activator migration was determined during the incubation period and after fixing the gel by measuring the distance from the edge of the separating gel to the center of the

lytic bands in each lane. Zymogram incubation was terminated by separating the gels, fixing the zymogram with 3% acetic acid for 10 min, and rinsing under tap water. Zymograms were dried and stained with 0.1% Amido Black (Bio-Rad) for permanent storage (Lowenstein and Inglid, 1976). Polyacrylamide gels were stained overnight with 0.05% Coomassie Brilliant Blue (Bio-Rad) in 65:25:10 water:isopropanol:acetic acid and destained with 80:10:10 water:isopropanol:acetic acid. Molecular masses of PA were determined from the regression calculations of log molecular mass and relative mobility.

Reverse zymograms were prepared according to Erickson et al. (1984) . Briefly, zymograms were prepared as regular zymograms except 500 ul of purified 5 IU/ml urokinase (American Diagnostica, Inc.) was added. The gel sandwich (acrylamide gel and casein-agar gel) was incubated at 39 C for 3-5 h until lysis occurred.

Zymograms containing amiloride (Vassalli and Belin, 1987) were prepared by combining 50:50 4.4% nonfat dry milk in buffer with 2.2% melted agarose in distilled water. Nine milliliters of this solution were combined with 1 ml of either 0 or 100 mM amiloride (Sigma) in phosphate buffered saline (PBS). Plasminogen was added to a final concentration of 25-30 µg/ml and the warmed mixture cast onto a 13 X 6 cm glass plate. The plate containing 0 mM amiloride was used as the control plate.

Assessment of In Vitro Maturation

At the termination of culture, thirty to fifty BOCC from each treatment were fixed and stained for assessment of meiotic stage. Cumulus cells were first removed from oocytes by vigorous

vortexing for 1-2 min. The denuded oocytes were washed twice in 2.0% sodium citrate, transferred to 0.7% sodium citrate for 3 min, fixed onto microscope slides with 25% acetic acid in ethanol, air dried (McGaughey and Chang, 1969) and stained with hematoxylin and eosin. Oocytes were evaluated for meiotic stage using bright-field microscopy.

Data Analysis

All experiments measuring PA and PAI activity were repeated at least three times. Protease activity was quantified by densitometric scanning of the zymogram using the Model 1650 reflectance/transmittance scanning densitometer (Bio-Rad) and computing software (Hoefer Scientific Instruments, San Francisco, CA, USA). A polar planimeter (model L-20-M; Lasico, Los Angeles, CA, USA) was used to trace the densitometric scans and determine areas of the lytic zones. Results are expressed relative to the control treatment where the lytic area is set to 1.0. Differences in PA activities in each experiment were determined by analysis of variance and Duncan's multiple range test. Differences in oocyte maturation rates were determined using Chi-square procedures. All analyses were conducted using the NCSS statistical software program (Number Cruncher Statistical System 4.01, 1984, Hintze, JL, Kaysville, UT).

Results

Plasminogen Activator Activities in BOCC During In Vitro Maturation

Three plasminogen-dependent lytic zones (92-95 kD, 71-73 kD and 49-51 kD) were observed in in vitro matured BOCC (Figure I-1). Addition of amiloride, a competitive inhibitor of uPA, to the zymogram eliminated the 49-51 kD band suggesting it is an uPA and the 71-73 and 92-95 kD bands are a tPA and tPA-PAI complex, respectively (FigureI-1). Ten, twenty and thirty BOCC were used to evaluate the optimum number of BOCC required to provide suitable caseinolysis. Tissue-type PA and uPA activity increased gradually but not significantly ($P>0.10$, Figure I-2) as BOCC number increased, however, tPA-PAI complex activity increased ($P<0.05$) between 10 and 20 BOCC. Plasminogen activator activity increased in a time-dependent fashion (Figures I-3 and 4), and as shown in figure I-4, a significant increase ($P<0.01$) was observed between 6-12 h of culture. In addition, tPA and tPA-PAI activity increased ($P<0.05$) more rapidly than uPA during in vitro maturation.

Effects of dbcAMP and PMA on PA Production by BOCC

The effects of dbcAMP on PA activity and maturation rate of BOCC were determined by in vitro culture with 0, 0.5, 1.0 and 2.5 mM dbcAMP for 24 h. As seen in Figures I-5 and 6, PA activities increased in a dose-dependent fashion ($P<0.05$). To determine whether an activator of the protein kinase C system can modulate PA production, BOCC were cultured with PMA for 24 h. One percent DMSO, which was used to dilute PMA, had no effect on PA production during maturation in vitro (Figures I-5 and 6). Increasing PMA in culture medium (1, 10 and 100 ng/ml) increased ($P<0.05$) uPA and tPA production by BOCC ($P<0.05$), but had no effect on tPA-PAI complex activity ($P>0.10$). Neither PMA nor dbcAMP inhibited

(P>0.10) GVBD (Figures I-8 and 9), however, progression to metaphase II was inhibited by high concentrations of these reagents (100 ng/ml PMA and 1 and 2.5 mM dbcAMP).

Effects of 6-DMAP and OA on PA Production by BOCC

In BOCC cultured with 0, 0.2, 1 and 2.0 mM 6-DMAP, the three forms of PA activity decreased (P<0.05) in dose-dependent fashion (Figures I-9 and 10). Oocyte maturation rate was also inhibited (P<0.01; Figure I-8A) by 6-DMAP and at a concentration of 2 mM, both GVBD and PA activity were almost completely suppressed.

When BOCC were treated with OA a new plasminogen-dependent lytic band (84-86 kD) appeared (Figure I-11). This novel band was amiloride-sensitive suggesting it may be a uPA-PAI complex (Figure I-12). The uPA-PAI and uPA activities increased (P<0.05) in BOCC treated with up to 25 nM OA, however at concentrations greater than 75 nM PA activity decreased (P<0.05, Figure I-13). The tPA and tPA-PAI band increased up to 7.5 nM OA, but decreased thereafter (P<0.05). Oocyte maturation was also inhibited by OA at concentrations greater than 75 nM (P<0.05, Figure 8d).

PA Inhibitor in BOCC

Reverse zymography revealed a plasminogen activator inhibitor (PAI, 52 kD) in 20 BOCC cultured for 24 h (Figure I-14). When BOCC were treated with 25 nM OA, PAI activity increased four to five-fold compared to 0 nM OA (P<0.01, Figure I-15). Plasminogen activator inhibitor activity was also increased in BOCC treated with 2.5 mM dbcAMP and 2.5 nM OA (P<0.05), however PAI activity was inhibited by 250 nM OA and 2 mM 6-DMAP (P<0.05).

Discussion

Zymographic analysis revealed that in vitro matured BOCC produced three plasminogen dependent proteases (49-51 kD, 71-73 kD and 92-95 kD) and one PAI (52 kD) during in vitro maturation. Low molecular PA activity (49-51 kD) is likely uPA because this PA activity is completely inhibited by amiloride, a specific and competitive inhibitor of uPA (Vassalli and Belin, 1987). The molecular mass of the 49-51 kD species also corresponds to the values reported by Dano et al. (1985) for uPA of 31-55 kD. The 71-73 kD and 92-95 kD species were resistant to amiloride treatment suggesting tPA and a tPA-PAI complex, respectively. Tissue-type PA is synthesized as a proenzyme with a MW of approximately 70 kD and composed of two polypeptide chains, a heavy chain (40 kD) and light chain (30 kD), linked by a single disulfie bond (Degen et al., 1986). Plasminogen activator inhibitors are a family of specific inhibitors of PA. The PAI can form complexes with either uPA or tPA that are resistant to breakdown by SDS (Degen et al., 1986). Hence, when analyzed by zymography, if a PAI is present with PA, PA-PAI complexes can be formed that will induce caseinolysis in a high molecular mass range (Degen et al., 1986). Typically complexes between serine proteases and serine protease inhibitors (serpins) are of lower molecular mass than would be mathematically expected, due to release of a cleavage fragment upon association (Wiman and Collen, 1979). Rehemtulla et al. (1990) reported a 92 kD PA in human K562 cells when they added excess urokinase (52 kD) to the culture medium, suggesting that the molecular mass of the binding protein was 40 kD. However, reverse

zymography showed a PAI with a molecular mass of 45 kD. Collectively, these findings suggest that when PA interacts with PAI, some portion of the PAI may be cleaved, thus leaving a complex of mathematically lower molecular mass than would be expected.

The function of PA in cumulus-oocytes complexes is unclear. However it has been suggested that PA is involved in several processes, including follicle rupture, oocyte maturation, cumulus cell expansion and dispersion, fertilization and the zona reaction (Huarte et al., 1987; Bicsok et al., 1989). In this study PA activity in BOCC increased in a time-dependent fashion during in vitro maturation with significant increases between 6 and 12 h of culture. In bovine oocytes GVBD occurs between 4 and 8 h of culture (Sirard et al., 1989). Our observations suggest that PA induction in BOCC occurred shortly after GVBD and it may be associated with the process of oocyte maturation.

The PAI observed in our study possesses several similarities with PAI-1. At least three classes of PAI have been identified on the basis of immunochemical reactivity and recombinant DNA probes (Hart and Rehemtulla, 1988). Plasminogen activator inhibitor-1 is a 52 kD glycoprotein which is synthesized by endothelial cells, hepatocytes and platelets, and is present in plasma. Plasminogen activator inhibitor-1 inhibits tPA, as well as uPA, by forming SDS-stable complexes. Plasminogen activator inhibitor-2 is synthesized by various tissues including the placenta and macrophages. Unlike PAI-1, PAI-2 exists in two different forms. Intracellularly, PAI-2 exists as a nonglycosylated molecule with a molecular mass of 47 kD, whereas the secreted form is

glycosylated and has a molecular mass of 60 kD. Plasminogen activator inhibitor-2 is a more selective inhibitor of uPA whereas PAI-1 has a higher binding affinity for tPA. Plasminogen activator inhibitor-3 is predominately found in urine and plasma and has a molecular mass of 51 kD. Plasminogen activator inhibitor-3 has recently been reported to be identical to the inhibitor of activated protein C.

Dibutyryl cAMP, a known inducer of cAMP-dependent protein kinase A, has been found to stimulate PA production by promoting the transcriptional activity of the PA gene (Nagamine et al., 1983). Rat granulosa cells secrete mainly tPA, whereas mouse cells secrete predominately uPA (Canipari et al., 1987). Mouse uPA and rat tPA in granulosa cells are stimulated by gonadotropins, prostaglandin E_2, and dbcAMP. In our study, dbcAMP increased both tPA and uPA activity in BOCC in a dose-dependent fashion.

Phorbol 12-myristate 13-acetate, a potent tumor promoter, has been found to enhance PA activity presumably through the activation of phospholipid-dependent protein kinase C (Vassalli et al., 1977; Quigley, 1979). In the present research, the possibility that BOCC utilize protein kinase C in regulating PA production was investigated. Phorbol myristate acetate increased uPA and tPA production by BOCC but had no effect on tPA-PAI complex and PAI activities when compared to the DMSO control. Phorbol esters have been shown to stimulate RNA synthesis (Sivak and Van Duurren, 1970) and enhance protein synthesis (Hiwasa et al., 1982). The effect of PMA on PA production in BOCC reported herein may be due to stimulation of protein synthesis in cumulus cells, however

further investigation is required to elucidate the mechanism by which PMA preferentially increased uPA activity. Although high concentrations of dbcAMP (1.0 and 2.5 mM) and PMA (100 ng/ml) inhibited progression to metaphase II, neither dbcAMP nor PMA inhibited GVBD in BOCC. Spontaneous meiotic maturation of denuded mouse oocytes is prevented by high concentrations of dbcAMP (Cho et al., 1974; Ecay and Powers, 1990).

As shown in our study, PA activity in BOCC was effectively inhibited and GVBD was also completely blocked when BOCC were exposed to 2 mM 6-DMAP. 6-Dimethylaminopurine blocks phosphorylation without affecting protein synthesis (Neant and Guerrier, 1988). Phosphorylation and dephosphorylation processes have been known to play an important role in MPF activity which controls the transition of meiotic or mitotic cells from G2 phase to M phase. Our results showed that the effective dose of this drug (2 mM) inhibited not only plasminogen-dependent protease activities but PAI activity as well. Previous research showed that PAI is an important modulator of PA activity. Politis et al. (1990) demonstrated that the increase in PA activity associated with follicular rupture in the sow ovary is due to an increase in follicular tPA-like content and a reduction in content of a PAI-1 type protein. Liu et al. (1991) reported a coordinated regulation of PAI-1 and tPA activities and mRNA levels in the ovary such that a peak of PA activity occurred just prior to ovulation. Our results demonstrate that the decrease of PA activity by 6-DMAP is not by modulating PAI activity.

Okadaic acid is a potent inhibitor of both protein phosphatases 1 and 2A. In this study OA induced new plasminogen-dependent protease activity (84-86 kD) in BOCC matured in vitro. Addition of amiloride to the zymograph eliminated this band suggesting it may be an uPA-PAI complex. A study from our laboratory reported that day 12-14 cattle embryos produced two plasminogen-dependent lytic zones, a light form of 41.5-47 kD and a heavy form of 86.1-92 kD (Dyk and Menino, 1991). Subsequent results demonstrated that PA activity could be completely suppressed by amiloride and antibodies to human uPA whereas PA activity was unchanged with antibodies to human tPA suggesting the heavy form may be a uPA-PAI complex (Berg and Menino, 1992). Our results demonstrated a four- to five-fold increase in PAI activity as well as a six- to seven-fold increase in uPA activity in BOCC treated with 25 nM OA compared to 0 nM OA. These results would explain the increased uPA-PAI complex activity observed in BOCC treated with 25 nM OA. Plasminogen activator activities were highest in tPA and tPA-PAI complex at 7.5 nM OA whereas uPA and uPA-PAI complex activities reached acmes at 25 nM OA. In cell-free extracts, OA inhibits protein phosphatase 2A at low concentrations (0.1 nM) and protein phosphatase 1 at high concentrations (10 nM) (MacKintosh and Cohen, 1989). Nagamine and Ziegler (1991) reported that a high concentration of OA (125 nM) induced uPA mRNA accumulation in LLC-PK cells, a cell line derived from porcine renal proximal tubule epithelia. The response of uPA, tPA and PAI activities to 25 nM OA may well be due to the differential dose effects of OA on phosphatases 1 and 2A. In BOCC kinases may be responsible for

inducing uPA and tPA activities whereas phosphatase 1 suppresses PAI and uPA and phosphatase 2A suppresses tPA expression. Maturation promoting factor activation can be induced by OA through inhibition of phosphatase 2A (Felix et al., 1990) and in Xenopus and mice, MPF activation has been characterized under OA microinjection (Jessus et al., 1991; Gavin et al., 1991). Despite 25 nM OA strikingly increasing PA activity, no effect was observed on bovine oocyte maturation rate in vitro. However, both oocyte maturation rate and PA activity were inhibited by OA at concentrations greater or equal to 75 nM.

In conclusion, these data suggest that production of PA and PAI by BOCC is temporally associated with in vitro maturation and influenced by stimulators of the protein kinase A and C systems and modulators of intracellular phosphorylation. The physiological roles of PA and PAI in the oocyte-cumulus cell complex remain unclear and additional work is warranted to elucidate the involvement of the PA-PAI system in oocyte maturation and fertilization.

Figure I-1. Zymographic analysis of in vitro matured BOCC. Zymogram with 0 mM (lane 1) and 10 mM amiloride (lane 2).

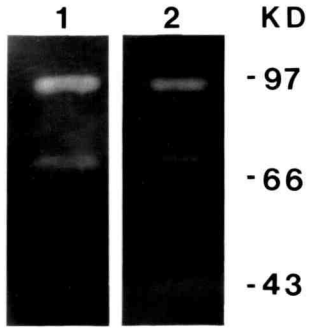


Figure I-2. Plasminogen activator activities in 10, 20 or 30 BOCC after 24 h of culture. Results are the means ± SE of three separate experiments and are expressed relative to the PA activity of 10 BOCC. Means without common superscripts are different ($P<0.05$).

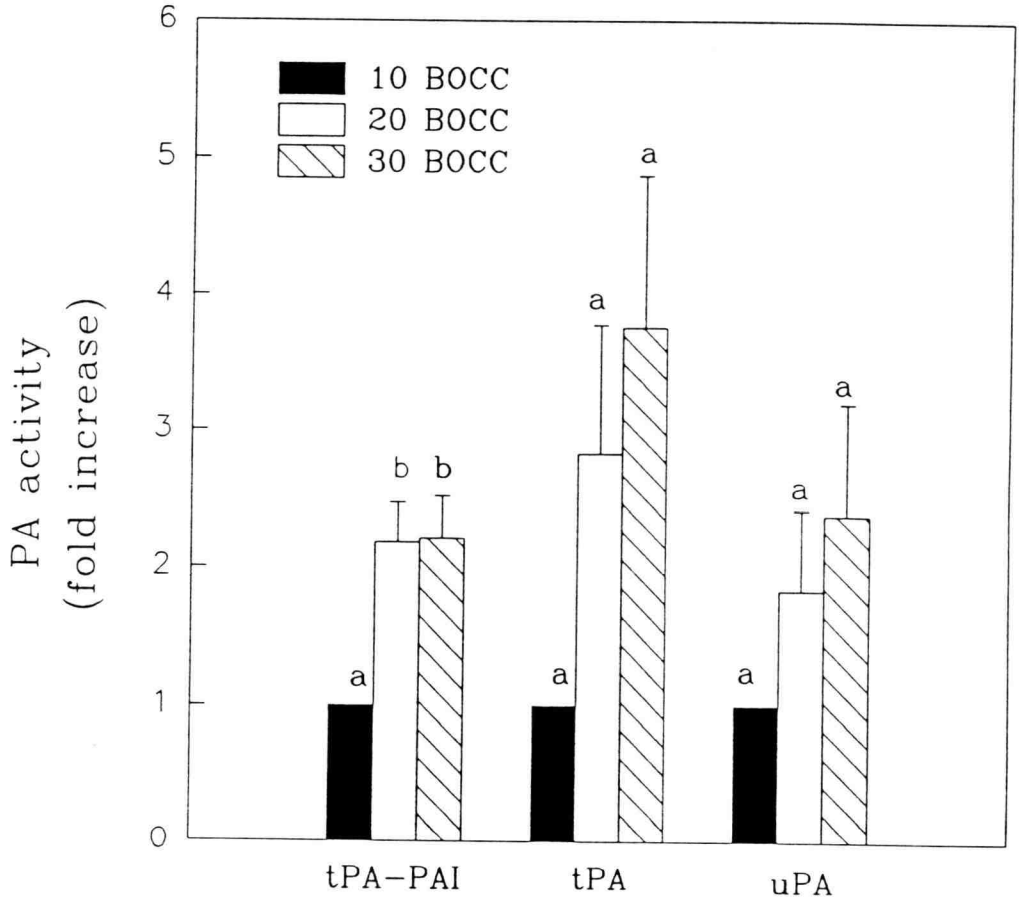


Figure I-3. Zymographic analysis of in vitro matured BOCC at 0 (lane 1), 6 (lane 2), 12 (lane 3) and 24 h (lane 4) of culture.

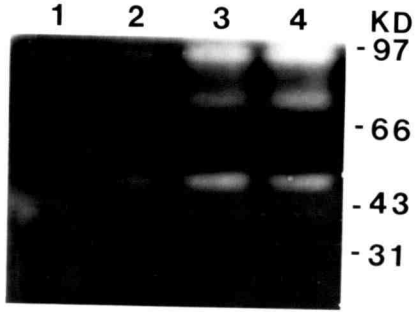


Figure I-4. Plasminogen activator activities associated with BOCC at 0, 6, 12 and 24 h of culture. Results are the means ± SE of three separate experiments and are expressed relative to the PA activity of BOCC at 0 h. Means without common superscripts are different ($P<0.05$).

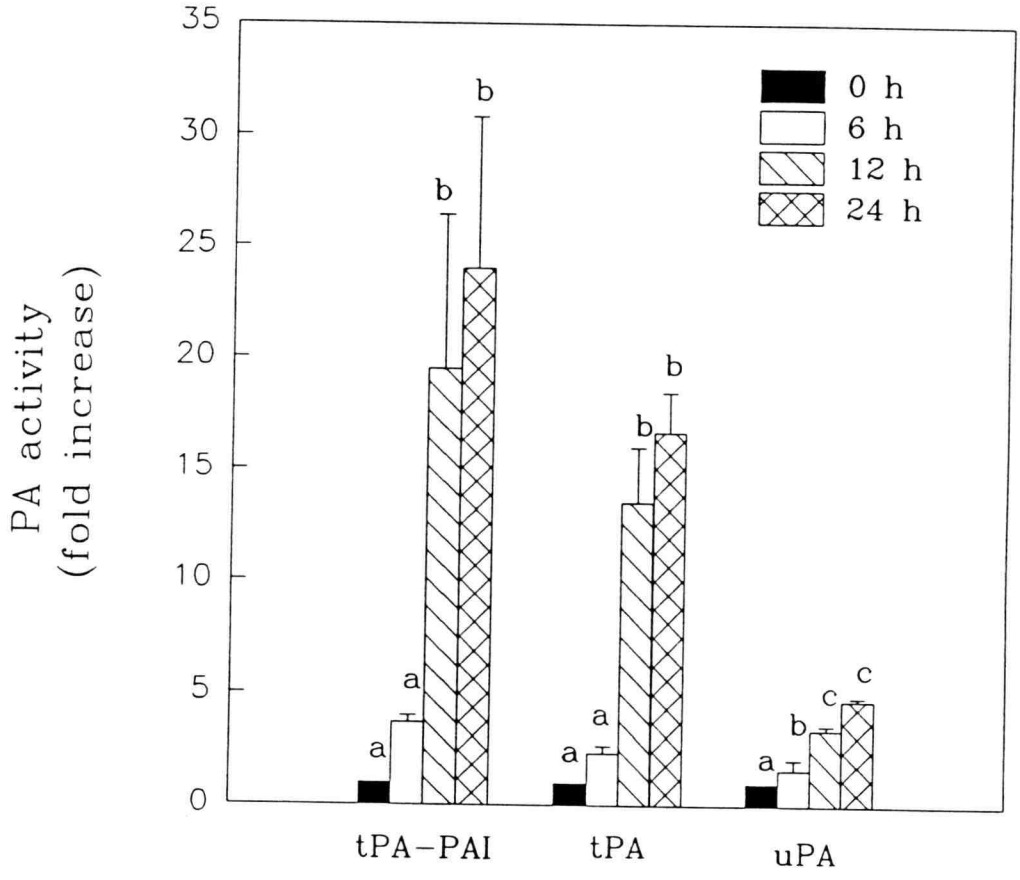


Figure I-5. Zymographic analysis of in vitro matured BOCC cultured in 2.5 (lane 1), 1.0 (lane 2), 0.5 (lane 3) and 0 (lane 4) mM dbcAMP and 100 (lane 5), 10 (lane 6) and 1 (lane 7) ng/ml PMA, 1% DMSO (lane 8) and 0% DMSO (lane 9).

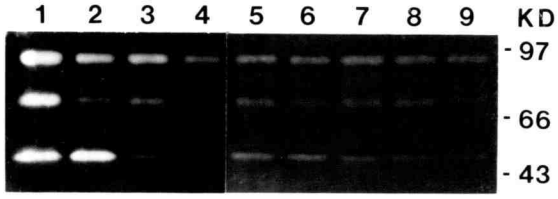


Figure I-6. Effects of dbcAMP on PA activities in in vitro matured BOCC. Results are the means ± SE of three separate experiments and are expressed relative to the PA activity of BOCC in O mM dbcAMP. Means without common superscripts are different (P<0.05).

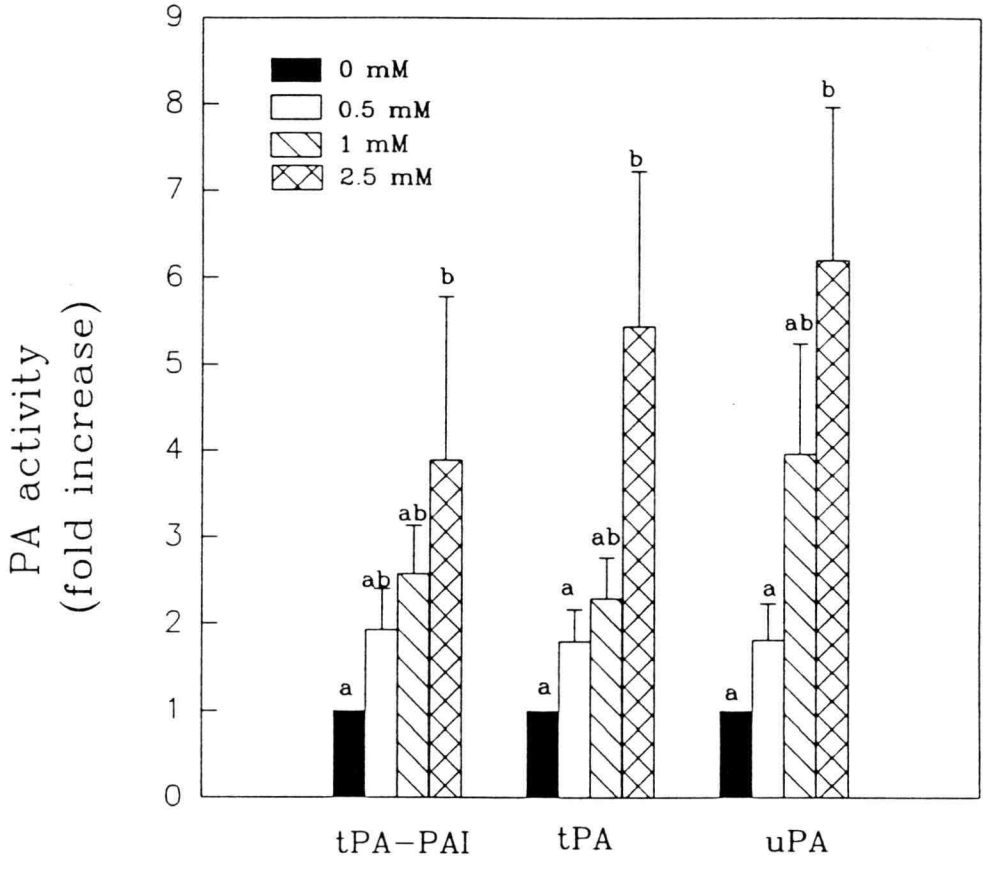


Figure I-7. Effects of PMA on PA activities in in vitro matured BOCC. Because PMA was dissolved in 1% DMSO, medium with 0 or 1% DMSO was used for comparison. Results are the means ± SE of three separate experiments expressed relative to the PA activity of BOCC in 0% DMSO. Means without common superscripts are different ($P<0.05$).

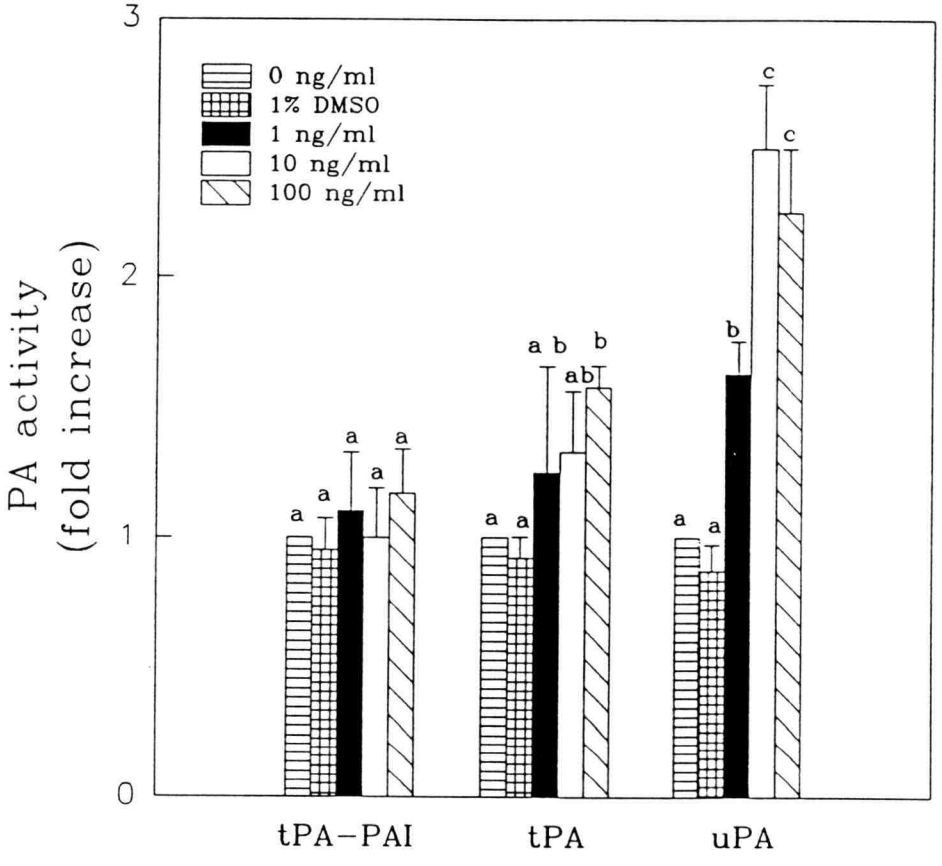


Figure I-8. Effects of dbcAMP, PMA, 6-DMAP and OA on germinal vesicle breakdown (GVBD) and development to metaphase II (MII). Bovine oocyte-cumulus cell complexes were cultured in a) 0, 0.5, 1 and 2.5 mM dbcAMP; b) 0 or 1% DMSO, 1, 10 or 100 ng/ml PMA, c) 0, 0.2, 1 and 2 mM 6-DMAP; d) 0, 2.5, 25 and 250 nM OA. Percentages without common superscripts are different ($P<0.05$).

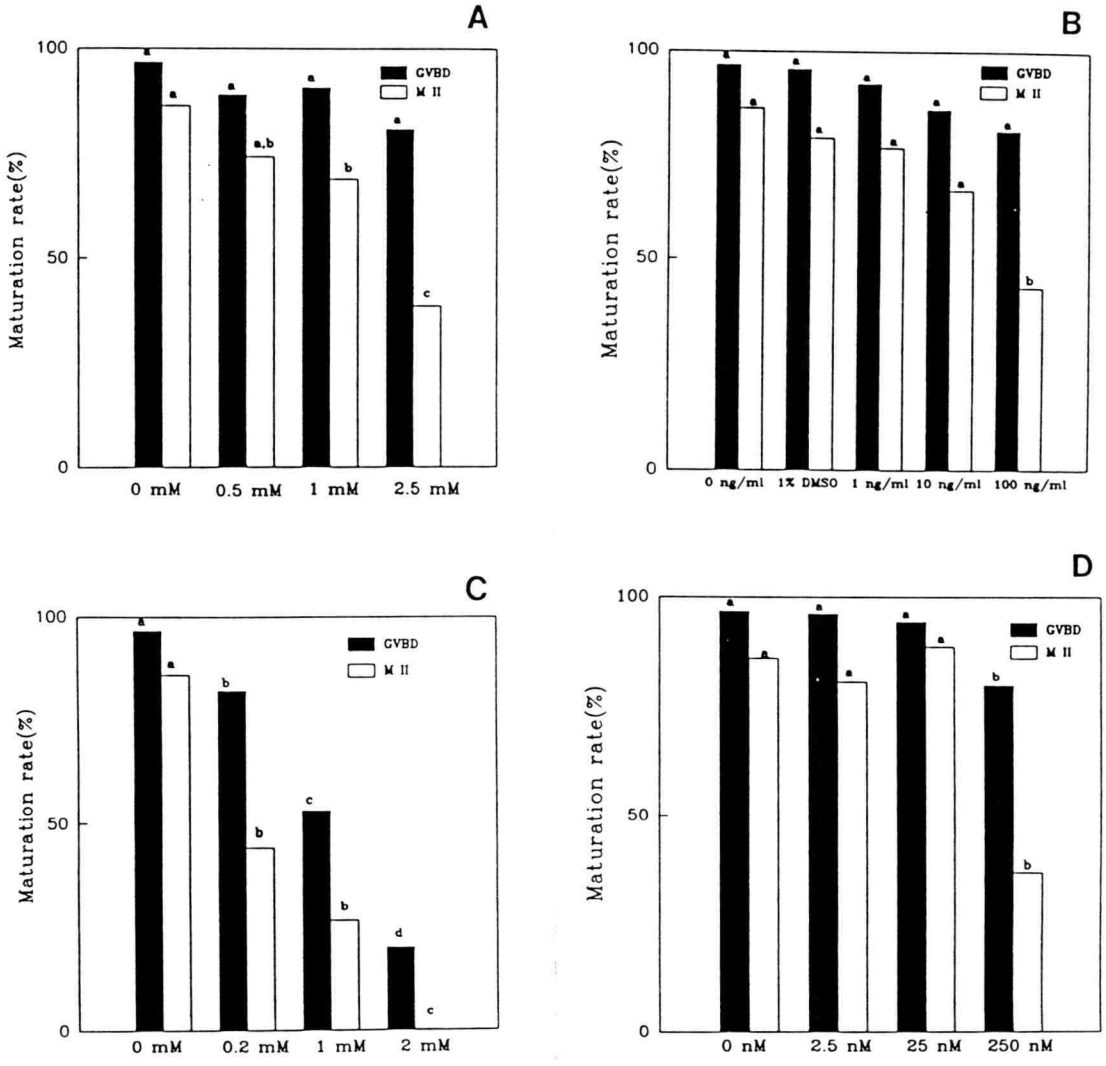


Figure I-9. Zymographic analysis of in vitro matured BOCC cultured in 2.0 (lane 1), 1.0 (lane 2), 0.2 (lane 3) and 0 (lane 4) mM 6-DMAP and BOCC at 0 h of culture (lane 5).

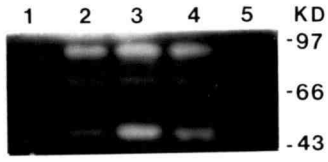


Figure I-10. Effects of 6-DMAP on PA activities in in vitro matured BOCC. Results are the means ± SE of three separate experiments and are expressed relative to the PA activity of BOCC in 0 mM 6-DMAP. Means without common superscripts are different ($P<0.05$).

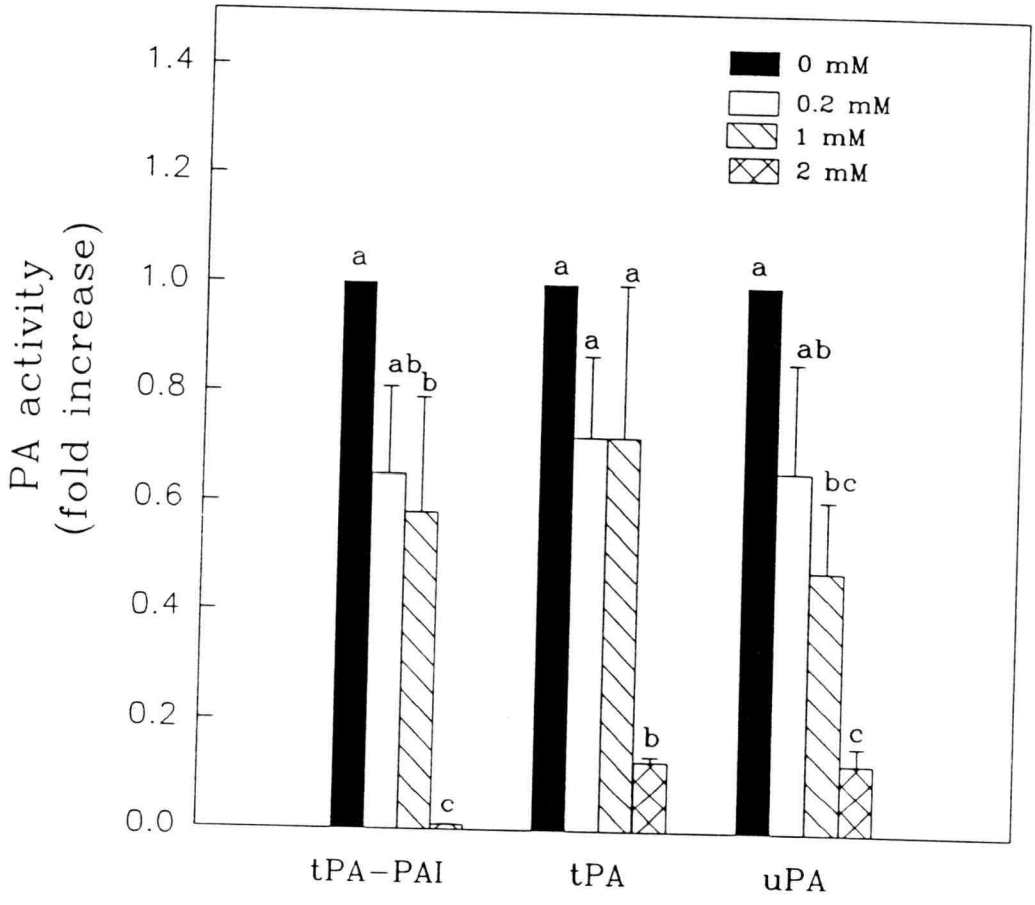


Figure I-11. Zymographic analysis of in vitro matured BOCC cultured in 0 (lane 1), 2.5 (lane 2), 7.5 (lane 3), 25 (lane 4), 75 (lane 5) and 250 nM OA (lane 6).

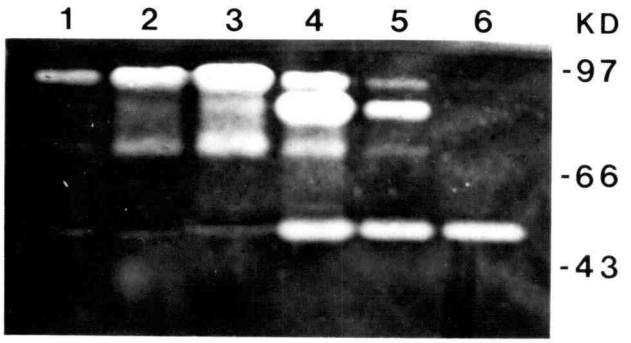


Figure I-12. Zymographic analysis of in vitro matured BOCC cultured in 25 nM OA (lanes 1 and 4). Lanes 2 and 5 contain 0.1 IU/ml human urokinase and lanes 3 and 6 contain 5 ng/ml human tPA. Lanes 1, 2 and 3 are in the zymogram with 0 mM amiloride, and lanes 4, 5 and 6 are in the zymogram with 10 mM amiloride.

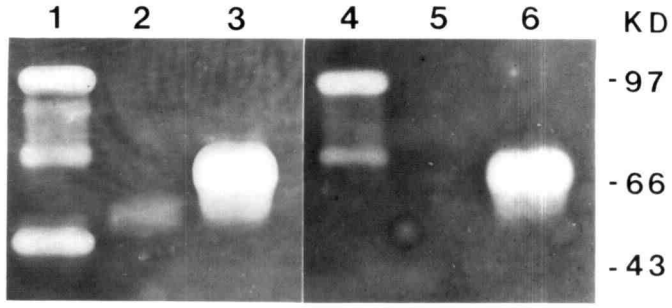


Figure I-13. Effects of OA on PA activities in in vitro matured BOCC. Results are the means ± SE of three separate experiments and are expressed relative to the PA activity of BOCC in O nM OA. Means without common superscripts are different ($P<0.05$).

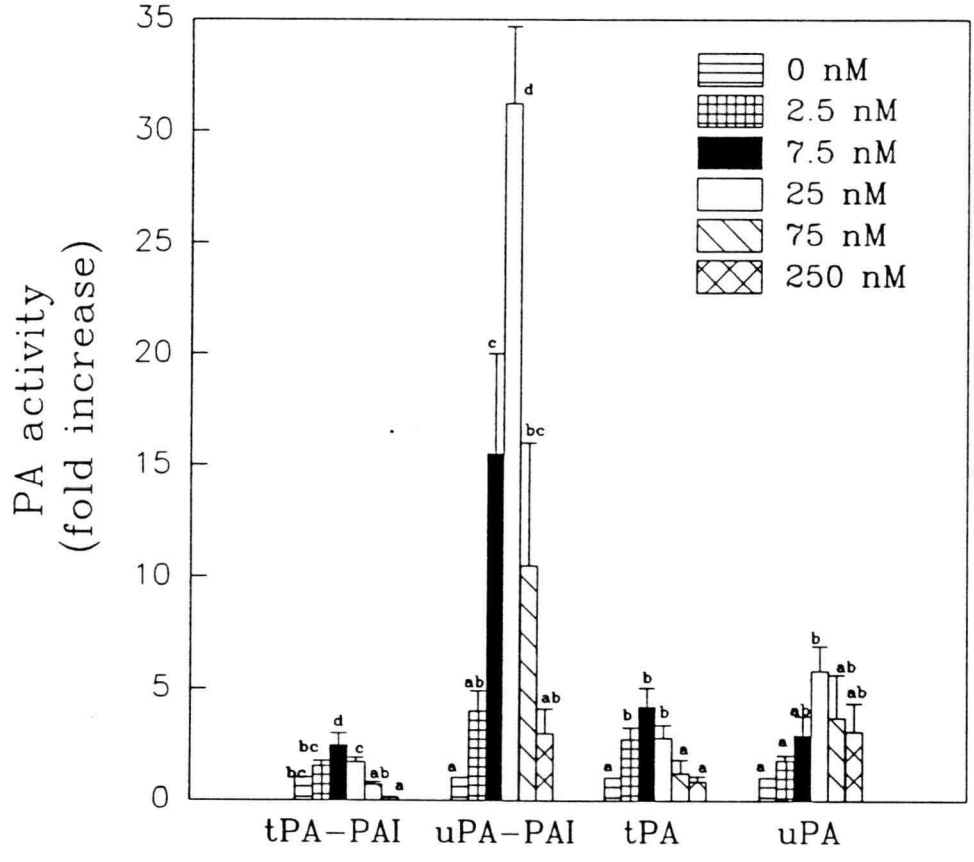


Figure I-14. Reverse zymographic analysis of in vitro matured BOCC. BOCC were cultured in medium only (lane 1), 1% DMSO (lane 2), 100 ng/ml PMA (lane 3), 2.5 mM dbcAMP (lane 4), 2 mM 6-DMAP (lane 5), 2.5 nM OA (lane 6), 25 nM OA (lane 7) and 250 nM OA (lane 8).

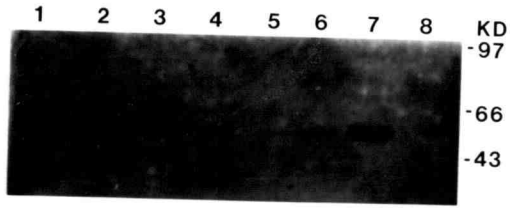
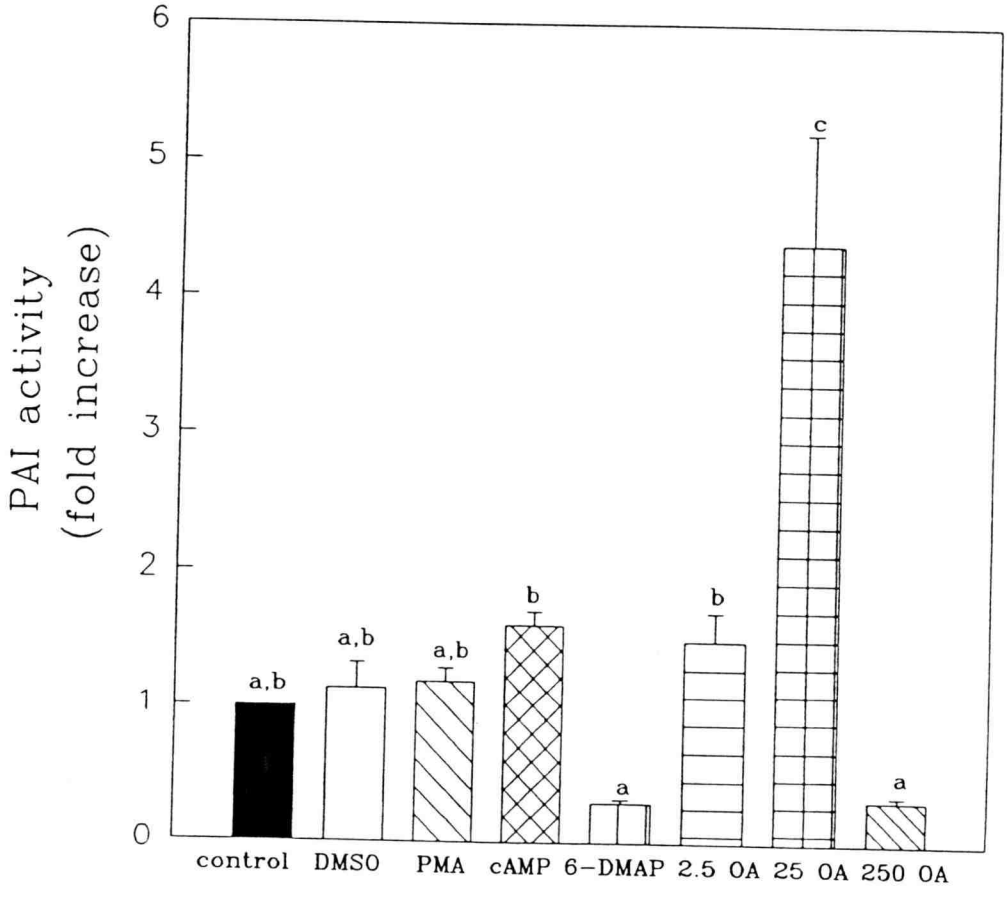


Figure I-15. Plasminogen activator inhibitor activities in in vitro matured BOCC. Results are the means ± SE of three separate experiments and are expressed relative to the PAI activity of BOCC in the control medium. Treatments included: control medium, 1% DMSO, 100 ng/ml PMA, 2.5 mM dbcAMP, 2 mM 6-DMAP, 2.5 nM OA, 25 nM OA and 250 nM OA. Means without common superscripts are different ($P<0.05$).



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EFFECTS OF STIMULATORS OF PROTEIN KINASE A AND C AND MODULATORS OF PHOSPHORYLATION ON PLASMINOGEN ACTIVATOR ACTIVITY IN PORCINE OOCYTE-CUMULUS CELL COMPLEXES MATURED IN VITRO.

Abstract

Effects of phorbol myristate acetate (PMA), dibutyryl cyclic AMP (dbcAMP), 6-dimethylaminopurine (6-DMAP) and okadaic acid (OA) on plasminogen activator (PA) activity and maturation rate in porcine oocyte-cumulus cell complexes (POCC) matured in vitro were determined. Cumulus cell-enclosed oocytes were collected from 1-4 mm antral follicles and cultured in TCM-199 with 0.3% polyvinylpyrrolidone for 48 h. Plasminogen activator activity in POCC was measured using SDS-PAGE, casein-agar zymography and densitometry. Two plasminogen-dependent lytic zones (93-96 kD and 71-78 kD) were observed in matured POCC. Addition of amiloride, a competitive inhibitor of uPA, to the zymograph failed to reduce activities in either zone suggesting the 71-78 kD band is a tPA and the 93-96 kD band is possibly a tPA-PAI complex. Changes in PA activity due to the various treatments were expressed relative to the PA activity in 40 POCC. Increasing dbcAMP in the culture medium (0.5, 1 and 2.5 mM) increased activity in a dose-dependent fashion ($P<0.05$). Concentration of 10 and 100 ng/ml PMA inhibited PA activity by POCC ($P<0.05$). Either PMA or dbcAMP inhibited oocyte maturation ($P<0.05$). Plasminogen activator activity decreased ($P<0.05$) in 6-DMAP and oocyte maturation was inhibited ($P<0.05$) in a dose-dependent fashion. When POCC were treated with 25 nM OA a new plasminogen -dependent lytic band (49-52 kD) appeared. This novel band was amiloride-sensitive suggesting it is an uPA. Plasminogen activator activity increased ($P<0.05$) in POCC treated with up to 25 nM OA, however PA activity decreased ($P<0.05$) at concentrations greater

than 75 nM. These data suggest that PA production by POCC is influenced by the protein kinase A and C systems and kinase inhibitors during oocyte maturation. Inhibition of intracellular phosphatases also induced novel PA production.

Introduction

Plasminogen activators (PA) are serine protease that convert the zymogen, plasminogen, into plasmin. Plasminogen activators are involved in a variety of physiologic processes including fibrinolysis, ovulation, mammary involution and implantation (Dano et al., 1985). Two types of PA have been characterized by molecular mass, tissue-type (tPA) and urokinase-type (uPA) PA. Rat and mouse oocytes produce only tPA during spontaneous in vitro meiotic maturation (Huarte et al.,1985), however cultured oocyte -cumulus cell complexes (OCC) produce both tPA and uPA (Liu et al.,1986). In rats, FSH and GnRH stimulate tPA, but not uPA, activity in both cell types when cultured as OCC (Liu et al., 1986; Ny et al. 1987). Ny et al. (1987) have suggested that GnRH treatment increases PA activity in rat OCC through the protein kinase C pathway, whereas FSH appears to mediate its effects through the protein kinase A pathway (Salustri et al. 1985). Although PA activity is increased in rat oocyte-cumulus cell complexes by stimulators of the protein kinase A and C systems, it has not been determined if porcine OCC (POCC) produce PA and if present, whether activity can be regulated by second messenger systems.

Cycles of cellular phosphorylation and dephosphorylation have important roles in maturation or M-phase promoting factor (MPF) activity involved in the cell cycle and oocyte maturation (Draetta and Beach, 1988; Moor and Crosby, 1986). Inhibition of protein phosphorylation by 6-dimethylaminopurine (6-DMAP) (Meijer and Pondaven, 1988; Neant and Guerrier, 1988) inhibits germinal vesicle breakdown (GVBD) in mouse (Rime et al., 1989) and bovine oocytes (Fulka et al., 1991). Okadaic acid (OA), a potent inhibitor of phosphatases 1 and 2A (Bialojan and Takai, 1988), induces meiotic maturation and MPF activity when microinjected into Xenopus and mouse oocytes (Jessus et al.,1991; Gavin et al., 1991). Okadaic acid is also a potent inducer of the uPA gene through a mechanism independent of the protein kinase A and C systems (Nagamine and Ziegler, 1991). Despite this information, little is known about the role of protein phosphorylation on PA activity in porcine oocytes matured in vitro. Therefore, the objective of this study was to investigate the effects of dbcAMP, PMA, 6-DMAP and OA on PA activity in POCC matured in vitro.

Materials and Methods

Recovery and Culture of POCC

Porcine ovaries were collected from a local slaughterhouse and transported to the laboratory in physiological saline (25-30 C) within 2 h. Oocytes were aspirated from 1- to 4-mm follicles and washed with Tissue Culture Medium 199 (TCM199, Sigma Chemical Co., St. Louis, MO, USA) supplemented with polyvinylpyrrolidone

(PVP, 3 mg/ml, Sigma), sodium pyruvate (50 μg/ml), sodium bicarbonate (2.6 mg/ml) and 10 ml/l of an antibiotic- antimycotic solution (Sigma). Oocytes were pooled and carefully selected under a stereomicroscope and only those with two or three layers of cumulus cells were used. Porcine oocyte-cumulus cell complexes were allocated randomly to the experimental treatments and those with distinct granulosa cells were not used. Oocytes were cultured in TCM199 modified as above at 39 C in a humidified 5% CO_2 in air atmosphere (Saeki et al., 1991).

Experimental Treatments

Determination of the optimum number of POCC and the time course for PA detection. Twenty, forty and sixty POCC were initially used to evalute the optimum number of POCC required to produce satisfactory zymographs. The time course required for development of activity was determined by in vitro culture of forty POCC for 0, 12, 24, 36 and 48 h. All subsequent experiments also used forty POCC for each treatment.

Dibutyryl cAMP. A stock solution of 25 μg/ml dbcAMP (Sigma) in TCM199 was used. This solution was diluted with culture medium to concentrations of 0.5, 1 and 2.5 mM.

Phorbol myristate acetate . Phorbol 12-myristate 13-acetate (Sigma) was used in concentrations of 1, 10 and 100 ng/ml and diluted from a 10 μg/ml stock solution dissolved in dimethylsulfoxide (DMSO, Sigma). Each final dilution of PMA contained 1% DMSO and medium with 1% DMSO served as the control.

6-Dimethylaminopurine. A stock solution of 100 mM 6-DMAP (Sigma) in TCM199 was used in these experiments. This solution was diluted with culture medium to 0.5, 1 and 2 mM concentrations.

Okadaic acid. Okadaic acid (Sigma) was diluted with TCM199 to a concentration of 25 µg/ml. This stock was diluted with culture medium to 2.5, 7.5, 25, 75 and 250 nM.

Electrophoresis and Zymography

Electrophoresis and zymography were performed using the procedures described by Dyk and Menino (1991) which were modified from Granelli-Piperno and Reich (1978) and Vassalli et al. (1984). Forty oocytes with evenly enclosed cumulus cells were combined with 90 ul of sample buffer (5.0% SDS; 20% glycerol; 0.0025% bromophenol blue in 0.125 M Tris HCl buffer). One hundred microliters of POCC samples were pipetted into castellated wells in a 4% stacking gel with a 12% separating gel. Electrophoresis was conducted at 20 mA through the stacking gel and 30 mA through the separating gel for 2 to 4 h. Zymographs were prepared by dissolving 4 g of nonfat dry milk (Carnation Co., Los Angeles, CA, USA) in 100 ml of buffer containing 0.0013 M $CaCl_2.2H_2O$, 0.1 M glycine, 0.038 M Tris and 0.05 M Na azide. Seven milliliters of the nonfat dry milk mixture were heated to 55 C and combined with 7.0 ml of 2% melted agarose dissolved in (Sigma) distilled water and maintained at 55 C. Purified human plasminogen was added to the warmed mixture of 4% nonfat dry milk and 2% agarose to yield a final concentration of 25-30 µg/ml and 10 ml of this mixture was cast onto a warmed 13X6 cm glass plate. Zymographs containing 0

μg/ml plasminogen were also used for detection of any nonspecific proteolytic activity.

Zymographs containing amiloride (Vassalli and Belin, 1987) were prepared by combining 50:50 4.4% nonfat dry milk in buffer with 2.2% melted agarose in distilled water. Nine milliliters of this solution were combined with 1 ml of either 0 or 100 mM amiloride (Sigma) in phosphate buffered saline (PBS). Plasminogen was added to a final concentration of 25-30 μg/ml and the warmed mixture cast onto 13X6 cm glass plate. The plate containing 0 mM amiloride was used as the control plate.

Assessment of In Vitro Maturation

At the termination of culture, thirty to fifty POCC from each treatment were fixed and stained for assessment of meiotic stage. Cumulus cells were first removed from oocytes by repeated pipetting. The denuded oocytes were washed twice in 2.0% sodium citrate, transferred to 0.7% sodium citrate for 3 min, fixed onto microscope slides with 25% acetic acid in ethanol, air dried (McGaughey and Chang, 1969) and stained with hematoxylin and eosin. Oocytes were evaluated for meiotic stage using bright-field microscopy.

Data Analysis

All experiments measuring PA activity were repeated at least three times. Protease activity was quantified by densitometric scanning of the zymograph using the Model 1650 reflectance/transmittance scanning densitometer (Bio-Rad) and computing software (Hoefer Scientific Instruments, San Francisco,

CA, USA). A polar planimeter (model L-20-M; Lasico, Los Angeles, CA, USA) was used to trace the densitometric scans and determine areas of the lytic zones. Results are expressed relative to the control treatment where the lytic area is set to 1.0. Differences in PA activities in each experiment were determined by analysis of variance and Fisher's least significant difference procedures. Differences in oocyte maturation rates were determined using Chi-square procedures. All analysis were conducted using the NCSS statistical softwatre program (Number Cruncher Statistical System 4.01, 1984, Hintze, JL, Kaysville, UT).

Results

Plasminogen Activator Activities in POCC During In Vitro maturation

Two plasminogen-dependent lytic zones (93-96 kD and 71-79 kD) were observed in in vitro matured BOCC (Figure II-1). Addition of amiloride, a competitive inhibitor of uPA, to the zymograph failed to eliminate activity in either zone suggesting the 71-79 kD band is a tPA and the 93-96 kD band is possibly a tPA-PAI complex (Figure II-1). Twenty, forty and sixty POCC were used to evaluate the optimum number of POCC required to provide suitable caseinolysis. Plasminogen activator activity increased ($P<0.05$) as POCC number increased (Figure II-2). Plasminogen activator activity also increased ($P<0.05$) in a time dependent fashion (Figures II-3 and 4) and as shown in Figure II-4, a significant induction of PA was observed between 12-24 and 24-36 h of culture.

Effects of dbcAMP and PMA on PA Production by POCC

The effects of dbcAMP on PA activity were determined by in vitro culture with 0, 0.5, 1.0 and 2.5 mM dbcAMP for 48 h. As seen in Figure II-5, PA activities increased in a dose-dependent fashion ($P<0.05$). To determine whether an activator of the protein kinase C system can modulate PA production, POCC were cultured with PMA for 48 h. One percent DMSO, which was used to dilute PMA, had no effect on PA production during in vitro maturation (Figure II -6). Ten and 100 ng/ml PMA inhibited ($P<0.05$) tPA production by POCC (Figure II-6). Dibutyryl cyclic AMP (2.5 mM) inhibited both GVBD and progression to metaphase II ($P<0.05$; Figure II-7). Phorbol myristate acetate (100 ng/ml) did not inhibit ($P> 0.10$) GVBD, however, progression to metaphase II was reduced (Figure II -7).

Effect of 6-DMAP and OA on PA Production by POCC

In POCC cultured with 0, 0.5, 1 and 2.0 mM 6-DMAP, PA activity decreased ($P<0.05$) in a dose-dependent fashion. At a concentration of 2 mM, oocyte maturation and PA activity were completely suppressed (Figures II-7 and 8).

When POCC were treated with OA a new plasminogen-dependent lytic band (52-55 kD) appeared (Figure II-9 lanes 4 & 5). This novel band was amiloride-sensitive suggesting it may be a uPA (Figure II-10). The tPA and tPA-PAI complex activities increased ($P<0.05$) in POCC treated with up to 25 nM OA, however at concentrations greater than 75 nM PA activity decreased ($P<0.05$, Figure II-11). The uPA activity found at a concentration of 25 nM OA did not change at the higher concentration of 75 nM. Oocyte

maturation was also inhibited by OA at concentration of 25 nM (Figure II-7).

Discussion

Zymographic analysis revealed that in vitro matured POCC produced two plasminogen dependent proteases (71-79 kD and 93 -96 kD). Both 71-79 kD and 93-96 kD species were resistant to amiloride treatment suggesting a tPA and a tPA-PAI complex, respectively. Tissue-type PA is synthesized as a proenzyme with a MW of approximately 70 kD and composed of two polypeptide chains, a heavy chain (40 kD) and light chain (30 kD), linked by a single disulfide bond (Degen et al., 1986). Plasminogen activator inhibitors are a family of specific inhibitors of PA. The PAI can form complexes with either uPA or tPA that are resistant to breakdown by SDS (Hart and Rehemtulla, 1988). Typically complexes between serine proteases and serine protease inhibitors (serpins) are of lower molecular mass than would be mathematically expected, due to release of a cleavage fragment upon association (Wiman and Collen, 1979; Rehemtulla et al., 1990)

The function of PA in cumulus-oocytes complexes is unclear. However it has been suggested that PA is involved in several processes, including follicle rupture, oocyte maturation, cumulus cell expansion and dispersion, fertilization and the zona reaction (Huarte et al.,1985; Liu et al., 1986; Huarte et al., 1987; Bicsok et al., 1989). In this study PA activity in POCC increased in a time-dependent fashion during in vitro maturation with significant increases between 24 and 36 h of culture. In pig oocytes GVBD

occurs between 22 and 24 h of culture (Motlik et al., 1976). Our observations suggest that PA induction in POCC occurred shortly after GVBD and it may be associated with the process of oocyte maturation.

Dibutyryl cAMP, a known inducer of cAMP-dependent protein kinase A, stimulates PA production by promoting the transcriptional activity of the uPA gene (Nagamine et al., 1983). In our study, dbcAMP increased tPA activity in POCC in a dose -dependent fashion. CyclicAMP has a dual effect on regulation of oocyte maturation (Dekel et al., 1988). According to this hypothesis, lower levels of cAMP act to maintain meiotic arrest, while transiently elevated levels of cAMP mediate gonadotropin action and induce meiotic resumption. The oocyte is coupled to the surrounding follicle cell through gap-junctional contacts. The increased cAMP concentration in response to gonadotropins can interrupt communication in the oocyte-cumulus cell complexes and reduce the supply of cAMP to the oocytes (Eppig, 1982). Our result demonstrated that a high concentration of dbcAMP (2.5 mM) inhibited oocyte maturation.

Phorbol 12-myristate 13-acetate, a potent tumor promoter, has been found to enhance PA activity presumably through the activation of phospholipid-dependent protein kinase C (Vassalli et al., 1977; Quigley, 1979). In the present research, the possibility that POCC utilize protein kinase C in regulating PA production was investigated. Phorbol esters have been shown to stimulate RNA synthesis (Sivak and Van Durren, 1970) and enhance protein synthesis (Hiwasa et al.,1982). Ny et al. (1987) reported that

GnRH or PMA increased tPA enzyme activity, but decreased uPA activity. However, our results showed that high concentrations of PMA (10 and 100 mg/ml) decreased tPA activity by POCC.

Plasminogen activator activity in POCC was effectively inhibited and GVBD was also completely blocked when POCC were exposed to 2 mM 6-DMAP. 6-DMAP blocks phosphorylation without affecting protein synthesis (Neant and Guerrier, 1988). The cdc protein, $P34^{cdc2}$, is the key protein kinase component of MPF that controls the transition from G2 to M phase in both meiosis and mitosis (Masui and Markert, 1971; Draetta et al., 1989). Germinal vesicle breakdown is inhibited by 6-DMAP in mouse (Rime et al., 1989) and bovine (Fulka et al., 1991) oocytes.

Okadaic acid is a potent inhibitor of both protein phosphatases 1 and 2A. In this study OA induced new plasminogen-dependent protease activity (49-59 kD) in POCC matured in vitro. Addition of amiloride to the zymograph eliminated this band suggesting it is in all likelihood uPA. In cell-free extracts, OA inhibits protein phosphatase 2A at low concentrations (0.1 nM) and protein phosphatase 1 at high concentrations (10 nM). Okadaic acid (125 nM) alone induced uPA mRNA accumulation in LLC-PK cells, a cell line derived from porcine renal proximal tubule epithelia. Despite 25 nM OA markedly increasing tPA activity and inducing uPA production, no effect was observed on porcine oocyte GVBD in vitro. However, progress to metaphase II was inhibited by 25 nM OA.

In conclusion, these data suggest that production of PA by POCC is temporally associated with in vitro maturation and influenced by stimulators of the protein kinase A and C system and modulators of intracellular phosphorylation. The physiological roles of PA in the oocyte-cumulus cell complex remain unclear and additional work is warranted to elucidate the involvement of the PA system in oocyte maturation and fertilization.

Figure II-1. Zymographic analysis of in vitro matured POCC. Zymographs contain 0 mM (lane 1) or 10 mM amiloride (lane 2).

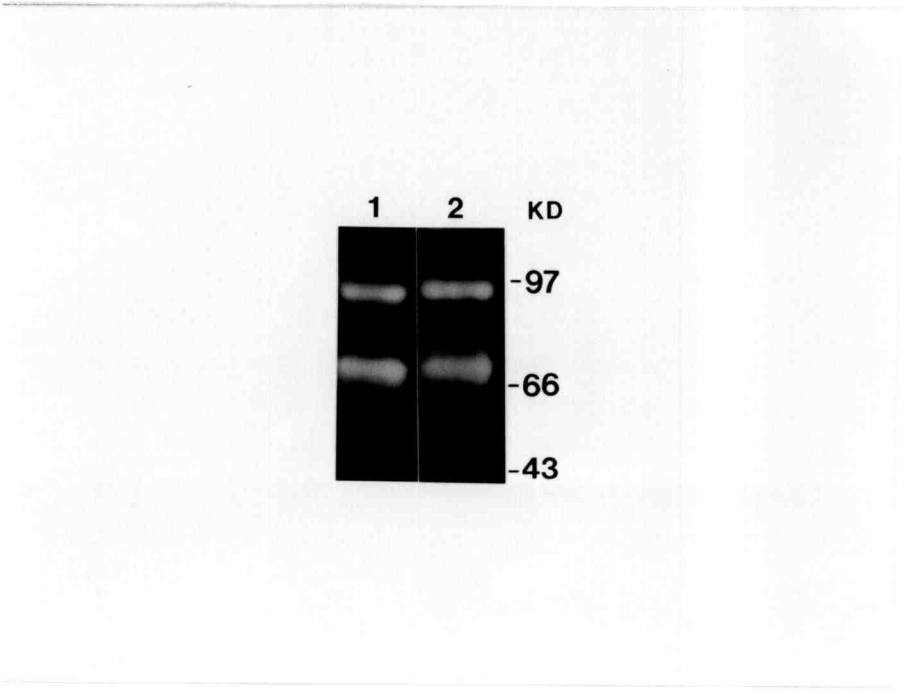


Figure II-2. Plasminogen activator activities in 20, 40, 60 POCC after 48 h of culture. Results are the means ± SE of three separate experiments and are expressed relative to the PA activity of 20 POCC. Means without common superscripts are different ($P<0.05$).

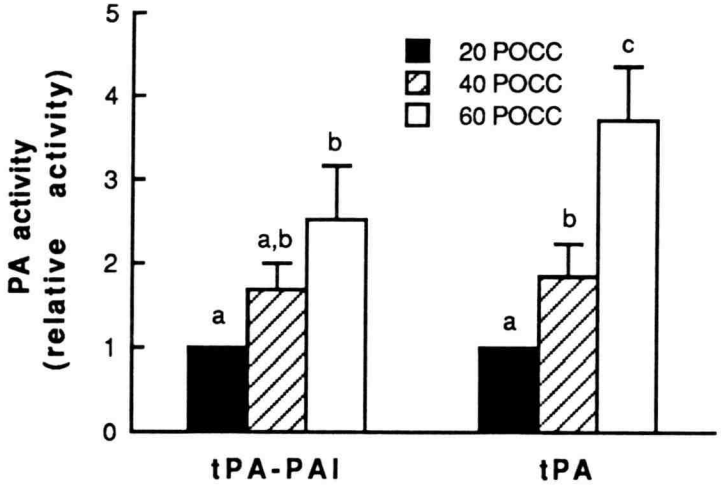


Figure II-3. Zymographic analysis of in vitro matured POCC at 0 (lane 1), 12 (lane 2), 24 (lane 3), 36 (lane 4) and 48 h (lane 5) of culture.

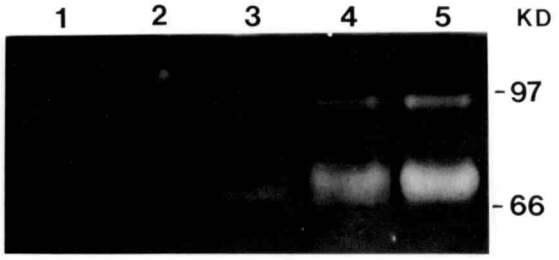


Figure II-4. Plasminogen activator activity associated with POCC at 0, 12, 24, 36 and 48 h of culture. Results are the means ± SE of three separate experiments and are expressed relative to the PA activity of 48 h of culture. Means without common superscripts are different ($P<0.05$).

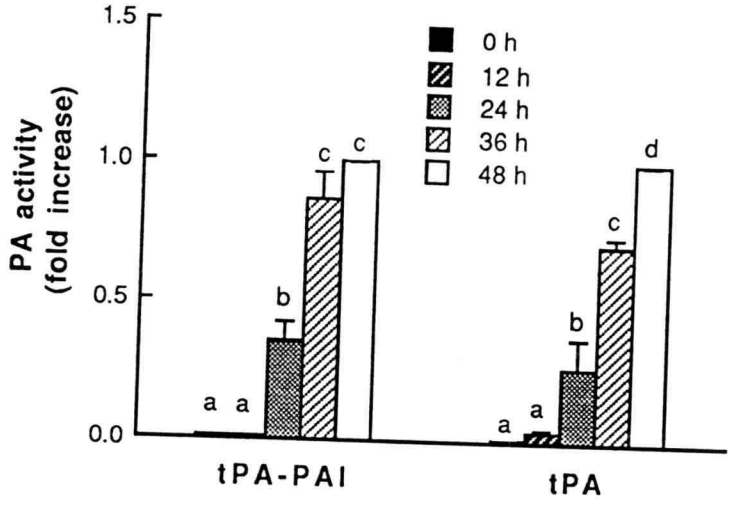


Figure II-5. Effects of dbcAMP on PA activities in in vitro matured POCC. Results are the means ± SE of three separate experiments and are expressed relative to the PA activity of 0 mM dbcAMP. Means without common superscripts are different ($P<0.05$).

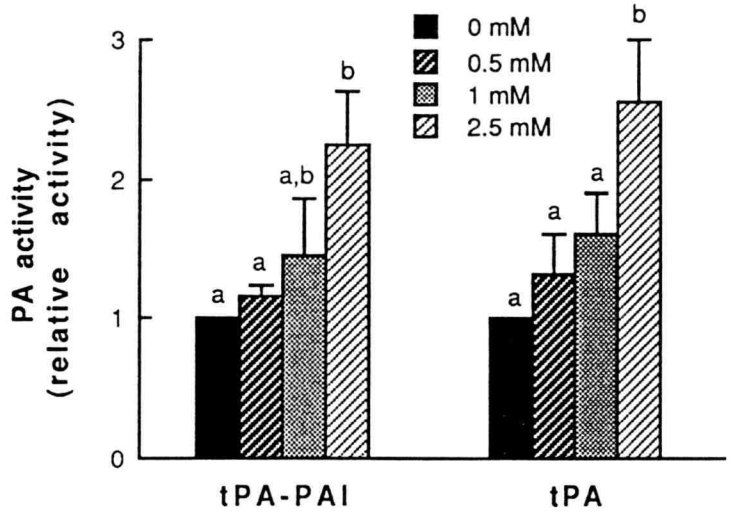


Figure II-6. Effects of PMA on PA activities in in vitro matured BOCC. Because PMA was dissolved in 1% DMSO, medium with 0 or 1% DMSO was used for comparison. Results are the means ± SE of three separate experiments and are expressed relative to the PA activity of 0 ng/ml PMA. Means without common superscripts are different ($P<0.05$).

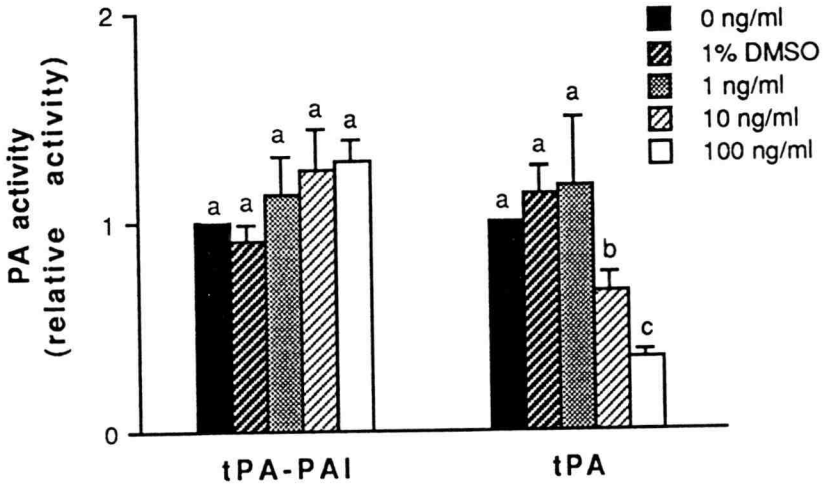


Figure II-7. Effects of dbcAMP, PMA, 6-DMAP and OA on germinal vesicle breakdown (GVBD) and development to metaphase II (M II). Porcine oocyte-cumulus cell complexes were cultured in control (C), 1% DMSO (DMSO), 100 ng/ml PMA (PMA), 2.5 mM dbcAMP (cAMP), 2 mM 6-DMAP (6-DMAP) and 25 nM OA (OA). Percentages without common superscripts for GVBD or M II are different ($P<0.05$).

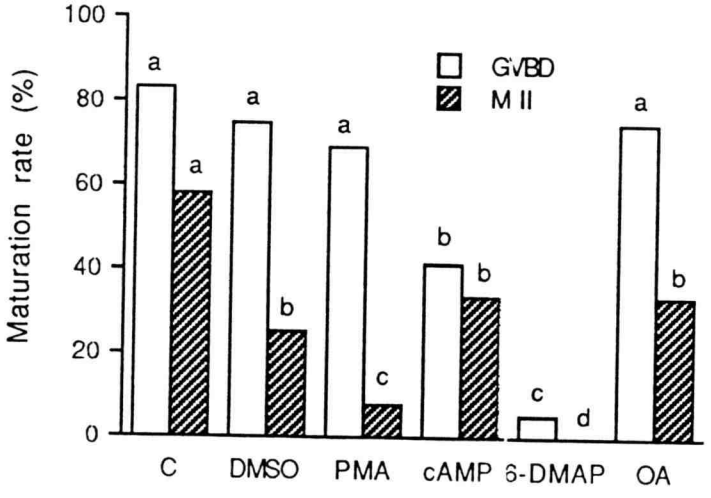


Figure II-8. Effects of 6-DMAP on PA activities in in vitro matured POCC. Results are the means ± SE of three separate experiments and are expressed relative to the PA activity of 0 mM 6-DMAP. Means without common superscripts are different ($P<0.05$).

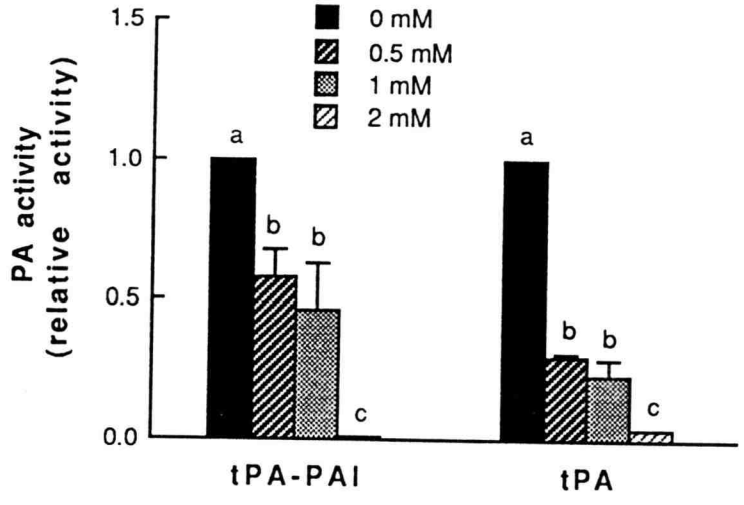


Figure II-9. Zymographic analysis of in vitro matured POCC cultured in 0 (lane 1), 2.5 (lane 2), 7.5 (lane 3), 25 (lane 4), 75 (lane 5) and 250 nM OA (lane 6). Lane 7 contains 0.1 IU/ml human urokinase.

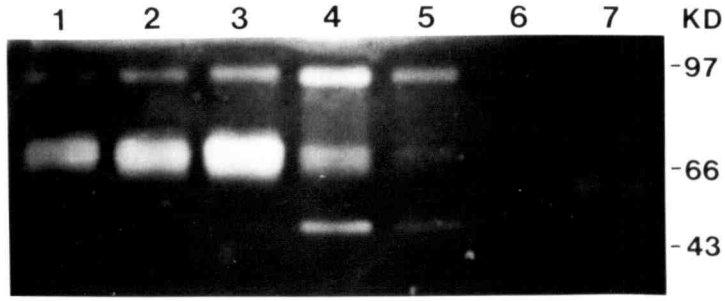


Figure II-10. Zymographic analysis of in vitro matured POCC cultured in 25 (lanes 3 and 7) and 0 nM OA (lanes 4 and 8). Lanes 1 and 5 contain 5 ng/ml human tPA and lanes 2 and 6 contain 0.1 IU/ml human urokinase. Lanes 1, 2, 3 and 4 are in the zymograph with 10 mM amiloride, and lanes 5, 6, 7 and 8 are in the zymograph with 0 mM amiloride.

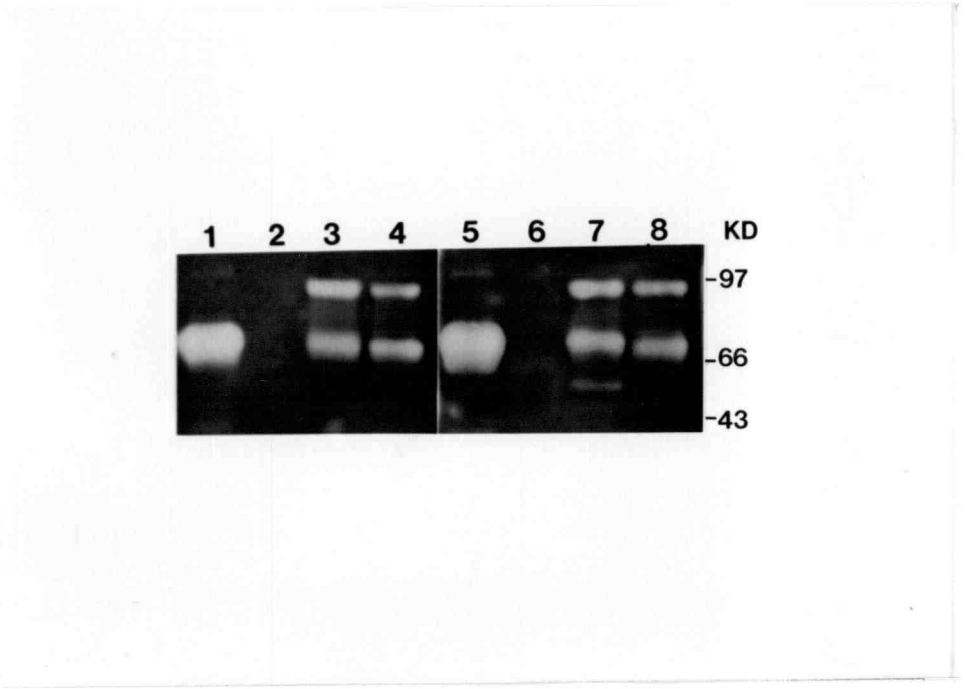
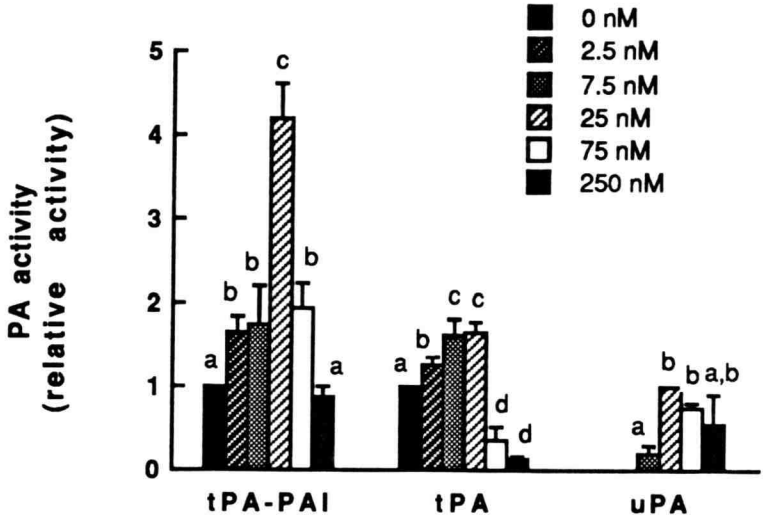


Figure II-11. Effects of OA on PA activities in in vitro matured POCC. Results are the means ± SE of three separate experiments. Tissue type PA and tPA-PAI complex activities are expressed relative to the PA activity of 0 nM OA, and uPA activities are expressed relative to the PA activity of 25 nM OA. Means without common superscripts are different ($P<0.05$).



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PARTIAL CHARACTERIZATION AND EVALUATION OF THE EFFECTS OF METABOLIC INHIBITORS ON PLASMINOGEN ACTIVATORS PRODUCED BY BOVINE OOCYTE-CUMULUS CELL COMPLEXES MATURED IN VITRO.

Abstract

Plasminogen activators (PA) were partially charaterized by immunoprecipitation and the effects of cycloheximide, actinomycin D and tunicamycin on PA actitvity and maturation rate in bovine oocyte-cumulus cell complexes (BOCC) matured in vitro were determined. Cumulus cell-enclosed oocytes were collected from 2 -5 mm antral follicles and cultured in TCM 199 with 0.3% polyvinylpyrrolidone for 24 h. Plasminogen activator activity in BOCC was measured using SDS-PAGE, casein agar zymography and densitometery. Three plasminogen-dependent protease activities (92-95 kD, 71-73 kD and 49-51 kD) and one PA inhibitor (PAI, 52 kD) were observed in matured BOCC. Immunoprecipitation experiments suggested that the 49-51 kD band is a urokinase-type PA, the 71-73 and 92-95 kD bands are a tissue-type PA (tPA) and tPA-PAI complex, respectively, and the PAI (52 kD) is PAI-1. Changes in PA activity due to the various treatments were expressed relative to the PA activity in 20 BOCC. In BOCC cultured in 1 and 10 μg/ml cycloheximide or 5 and 50 μM actinomycin D, PA activity decreased ($P<0.05$) and germinal vesicle breakdown and maturation rate were inhibited. Incubation of BOCC with 0.5 μg/ml tunicamycin reduced the molecular mass of tPA, tPA-PAI complex and PAI-1 by 5-10%, however PA activity was not inhibited by tunicamycin. These data suggest that BOCC matured in vitro produce uPA, tPA and PAI-1; the latter which complexes with tPA to form a high molecular mass species. Both mRNA and protein synthesis are necessary for PA production and germinal vesicle

breakdown in BOCC matured in vitro, however inhibition of glycosylation does not affect PA activity.

Introduction

The plasminogen activator (PA) system provides an important source of extracellular proteolysis accompanying cell migration and tissue remodelling (Dano et al., 1985). Two principal PA have been identified, tissue-type (tPA) and urokinase-type PA (uPA), and their activities are modulated physiologically by several different inhibitors including PA inhibitors-1 (PAI-1), -2 (PAI-2) and -3 (PAI-3) (Dano et al., 1985; Kruithof, 1988). The role of PA in ovulation has been suggested based on the observation that PA activity in the ovary is markedly increased in granulosa cells obtained from follicles shortly before ovulation (Beers et al., 1975). It has also been demonstrated that meiotic maturation of oocytes is associated with increased levels of tPA activity. In rats and mice, granulosa and cumulus cells secrete both tPA and uPA, however oocytes denuded of cumulus cells produce only tPA during spontaneous in vitro maturation (Huarte et al., 1985; Liu et al., 1986)

Most mammalian oocytes resume meiotic maturation when they are released from the follicle. The regulatory mechanisms involved in meiotic maturation are quite different among species. In mice and rats, germinal vesicle breakdown (GVBD) occurs independently of protein synthesis (Schultz and Wassarman, 1977; Clarke and Masui, 1983), however both protein synthesis and transcription are required for GVBD in cattle (Hunter and Moor,

1987; Kastrop et al., 1991). Actinomycin D, a specific transcriptional inhibitor, and cycloheximide, a blocker of protein synthesis, reduced PA activity in rat granulosa cells (Knecht, 1986) and osteoblasts (Hamilton et al., 1985). Although transcription and protein synthesis are required for the full expression of PA in rat granulosa cells, this has not been determined in oocyte-cumulus cell complexes from cattle. Tissue type PA, uPA and PA inhibitors are glycoproteins (Ranby et al., 1982; Hart and Rehemtulla, 1988). Tissue type PA is approximately 7% glycosylated (Dano et al., 1985). The degree of glycosylation and the effects of inhibiting glycosylation on PA activity in bovine oocyte-cumulus cell complexes (BOCC) also have not been determined. In a previous report, our laboratory demonstrated that on the basis of molecular mass, BOCC produced tPA, uPA and PAI. The identity of the PAI, however, was not definitively determined.

Therefore, the objective of this study was to investigate the effects of cycloheximide, actinomycin D and tunicamycin on PA activity and maturation rate and characterize the type of PA and PAI produced in BOCC matured in vitro.

Material and Methods

Recovery and culture of BOCC

Cattle ovaries were collected from a local slaughter house and transported to the laboratory in physiological saline (25-30 C) within 4 h. Oocytes were aspirated from 2- to 5-mm follicles and washed with Tissue Culture Medium 199 (TCM 199, Sigma Chemical

Co., St. Louis, MO, USA) supplemented with polyvinylpyrrolidone (PVP, 3 mg/ml, Sigma), sodium pyruvate (50 μg/ml), sodium bicarbonate (2.6 mg/ml) and 10 ml/l of an antibiotic antimycotic solution (Sigma). Oocytes were pooled and carefully selected under a stereomicroscope and only those with compact cumulus cells were cultured in TCM199 modified as above at 39 C in a humidified 5% CO_2 in air atmosphere (Saeki et al., 1990). Twenty oocytes were cultured for each treatment. Cycloheximide (Sigma) and actinomycin D (Sigma) were diluted with TCM 199. Tunicamycin was diluted from a 5 mg/ml stock solution dissolved in dimethylsulfoxide (DMSO, Sigma).

Electrophoresis and Zymography

Electrophoresis and zymography were performed using the procedures described by Dyk and Menino (1991) which were modified from Granelli-Piperno and Reich (1978) and Vassalli et al. (1984). Twenty oocytes with evenly enclosed cumulus cells were combined with 90 μl of sample buffer (5% SDS; 20% glycerol; 0.0025% bromophenol blue in 0.125 M Tris HCl buffer). One hundred microliters of BOCC samples were pipetted into castellated wells in a 4% stacking gel with a 12% separating gel. Electrophoresis was conducted at 20 mA through the stacking gel and 30 mA through the separating gel for 2 to 4 h. Zymographs were prepared by dissolving 4 g of nonfat dry milk (Carnation Co., Los Angeles, CA, USA) in 100 ml of buffer containing 0.0013 M $CaCl_2.2H_2O$, 0.1 M glycine, 0.038 M Tris and 0.05 M Na azide. Seven milliliters of the nonfat dry milk mixture were heated to 55 C and

combined with 7.0 ml of 2% melted agarose (Sigma) with distilled water also maintained at 55 C. Purified human plasminogen was added to the warmed mixture of 4% nonfat dry milk and 2% agarose to yield a final concentration of 25-30 µg/ml and 10 ml of this mixture was casted onto a warmed 13X6 cm glass plate. Zymographs containing 0 µg/ml plasminogen were also used for detection of any nonspecific proteolytic activity.

Reverse zymographs were prepared according to Erickson et al. (1984). Briefly, zymographs were prepared as regular zymographs except 500 ul of purified 5 IU/ml urokinase (American Diagnostica, Inc.) was added. The gel sandwich (acrylamide gel and casein-agar gel) was incubated at 39 C for 3-5 h until lysis occurred.

Immunoprecipitation

For the immunoprecipitation, 150 BOCC in SDS solubilization buffer (Huarte et al., 1985) were used at a final volume of 100 µl. Solubilized BOCC were divided among six treatments. Treatments consisted of the addition of 30 ul of PBS, 5 mg/ml non-specific goat immunoglobins (NSIgG, Sigma) or goat antibodies to human tPA (anti-tPA), uPA (anti-uPA), PAI-1 (anti-PAI-1) or PAI-2 (anti-PAI-2) (American Diagnostica Inc., Greenwich, CT, USA) and incubated overnight at 4 C. Thirty µl of protein G-bearing Streptococcus cells (Cal-Biochem; La Jolla, CA) were added to each sample and incubated for 2.5 h with shaking at room temperature. Samples were centrifuged with a Beckman microfuge and the supernatants were recovered. The pellets were rinsed three times with PBS by vortexing and centrifugation. Supernatants and pellets

were combined with SDS sample buffer and evaluated for PA using SDS-PAGE and zymography. Lytic zones (PA activity) and dark zones (PAI activity) in the zymograph were quantified densitometrically and were expressed relative to the PBS treatment.

Assessment of In Vitro Maturation

At the termination of culture, thirty to fifty BOCC from each treatment were fixed and stained for assessment of meiotic stage. Cumulus cells were first removed from oocytes by vigorous votexing. The denuded oocytes were washed twice in 2.0% sodium citrate, transferred to 0.7% sodium citrate for 3 min, fixed onto microscope slides with 25% acetic acid in ethanol, air dried (McGaughey and Chang, 1969) and stained with hematoxylin and eosin. Oocytes were evaluated for meiotic stage using bright-field microscopy.

Data Analysis

All experiments measuring PA activity were repeated at least three times. Protease activity was quantified by densitometric scanning of the zymograph using the Model 1650 reflectance/transmittance scanning densitometer (Bio-Rad) and computing software (Hoefer Scientific Instruments, San Francisco, CA, USA). A polar planimeter (Model L-20-M; Lasico, Los Angeles, CA, USA) was used to trace the densitometric scans and determine the lytic areas. Differences in PA activities in each experiment were determined by analysis of variance and Fisher's least significant difference procedures. Differences in oocyte maturation rates were determined using Chi-square procedures.

All analysis were conducted using the NCSS statistical software program (Number Cruncher Statistical System 4.01, 1984, Hintze, JL, Kaysville, UT; USA).

Results

Zymographic analysis of BOCC matured in vitro revealed the presence of three plasminogen dependent lytic zones (92-95 kD, 71 73 kD and 49-51 kD) and one PA inhibitor (PAI) activity (52 kD, Figure III-1). As shown in Figure III-2, anti-uPA and anti-tPA precipitated the 49-51 kD and 71-73 kD molecular mass species, repectively, suggesting the 49-51 kD band is uPA and the 71-73 kD band is tPA. When samples were treated with anti-tPA and anti-PAI-1, the high molecular mass species 92-95 kD was precipitated in either treatment, however neither anti-uPA nor anti-PAI-2 reduced this activity (Figure III-2). Plasminogen activator inhibitor activity was high in the pellet following treatment with anti-PAI-1 whereas no activity was observed in the pellet after treatment with anti-PAI-2, suggesting the PAI is PAI-1 (Figure III-3). In BOCC cultured with 1 and 10 ug/ml cycloheximide and 5 and 50 μM actinomycin D, all three forms of PA decreased ($P<0.05$, Figures III-4, 5 and 6). Oocyte maturation rate was also inhibited ($P<0.01$; Figure III-7). As shown in Figure III-8, when BOCC were treated with 0.5 and 5 $\mu g/ml$ tunicamycin, the molecular mass of tPA, tPA-PAI complex and PAI-1 were reduced by 5-10%, whereas the molecular mass of uPA did not change. The reduced molecular masses of tPA and tPA-PAI complex following treatment with the

glycosylation inhibitor were 64-67 and 86-89 kD, respectively, and the reduced form of PAI-1 is 48-49 kD. Total PA activity was not inhibited by tunicamycin (Figure III-9), however both GVBD and oocyte maturation rate were reduced (Figure III-7).

Discussion

Zymographic analysis revealed that in vitro matured BOCC produced three plasminogen dependent proteases (49-51, 71-73 and 92-95 kD) and one PAI (52 kD) during in vitro maturation. The 49-51 kD species is likely uPA because antiserum to human urokinase successfully precipitated this activity. The molecular mass of the 49-51 kD species also corresponds to the values (31-55 kD) reported by Dano et al. (1985) for uPA. Treatment with ant tPA reduced both the 71-73 and 92-95 kD forms in the supernatant and increased activities in the pellets whereas anti-huPA had little effect. Treatment with anti-PAI-1 reduced both the 71-73 and 92-95 kD activities and PAI activity in the supernatant and increased activities in the pellet suggest that BOCC matured in vitro produce uPA, tPA and PAI-1; the latter which complexes with tPA to form a high molecular mass species. Tissue-type PA is synthesized as a proenzyme with a molecular mass of approximately 70 kD and composed of two polypeptide chains, a heavy chain (40 kD) and light chain (30 kD), linked by a single disulfide bond (Dano et al., 1985; Degen et al., 1986). Plasminogen activator inhibitors are a family of specific inhibitors of PA. The PAI can form complexes with either uPA or tPA that are resistant to breakdown by SDS and PA-PAI complexes retain zymographic

activity (Hart and Rehemtulla, 1988; Andreasen et al., 1990). Typically complexes between serine proteases and serine protease inhibitors (serpins) are of lower molecular mass than would be mathmatically expected, due to release of a cleavage fragment upon association (Wilman and Collen, 1979; Bartlett and Menino, 1993). Our study showed that the PAI produced by BOCC matured in vitro is a PAI-1. At least three classes of PAI have been identified on the basis of immunochemical reactivity and recombinant DNA probes (Hart and Rehemtulla, 1988). Plasminogen activator inhibitor-1 is a 52 kD glycoprotein which is synthesized by endothelial cells, hepatocytes and platelets, and is present in plasma. Plasminogen activator inhibitor-1 inhibits tPA, as well as uPA, by forming SDS-stable complexes. Plasminogen activator-2 is synthesized by various tissues including the placenta and macrophages. Unlike PAI-1, PAI-2 exists in two different forms. Intracellularly, PAI-2 exists as a nonglycosylated molecule with a molecular mass of 47 kD, whereas the secreted form is glycosylated and has a molecular mass of 60 kD. Plasminogen activator inhibitor-2 is more selective inhibitor of uPA whereas PAI-1 has a higher binding affinity for tPA. Plasminogen activator inhibitor-3 is predominately found in urine and plasma and has a molecular mass of 51 kD. Plasminogen activator inhibitor-3 has recently been reported to be identical to the inhibitor of activated protein C.

Actinomycin D, a blocker of mRNA synthesis, and cycloheximide, a protein synthesis inhibitor, reduced PA production in BOCC. The present study demonstrated that both transcriptional and

translational activity are critical during in vitro maturation in BOCC for GVBD and for PA production. Total RNA and poly (A)-containing mRNA have been shown to accumulate during the growth of primary mouse oocytes (Bachvarova, 1985). Interestingly, the quantity of tPA mRNA remained constant in fully grown primary mouse oocytes cultured under conditions that prevented resumption of meiosis (Huarte et al., 1987). Huarte et al. (1987) reported that inhibition of RNA synthesis by actinomycin D or α-amanitin did not affect accumulation of tPA during maturation. In contrast, culture in the presence of protein synthesis inhibitors completely prevented enzyme production. During meiotic maturation in mammals, many intracellular changes occur related to the pattern of protein synthesis (Moor and Crosby, 1986; Kastrop et al., 1990; Gal et al., 1992). Protein synthesis plays an important role in the control of meiotic division. In large animals, including swine and cattle, resumption of meiosis requires active protein synthesis for GVBD (Fulka et al., 1986; Hunter and Moor, 1987), however in rodents, GVBD occurs independently of protein synthesis (Shultz and Wassarman, 1977; Fulka et al., 1986). Kastrop et al. (1991) have also reported that both mRNA and protein synthesis are necessary for GVBD and further development to metaphase II.

Tissue-type plasminogen activator is a glycoprotein with approximately 7% carbohydrate (Randy et al., 1982; Kruithof et al., 1988). Little et al. (1984) reported that the carbohydrate moeity dose not change enzyme activity because removal of the carbohydrate by glycosidase did not alter enzyme activity.

However glycosylation of tPA plays an integral role in transport of the protein out of the cell. Tunicamycin inhibits glycosylation of asparginyl residues during glycoprotein synthesis (Waecheter and Lennerz, 1976; Rothman et al., 1978). Our results showed that incubation BOCC with tunicamycin reduced the molecular mass of tPA, tPA-PAI complex and PAI by 5-10%, however PA activities were not changed by tunicamycin.

In conclusion, BOCC matured in vitro produce uPA and tPA and PAI-1 that complexes with tPA to form a high molecular mass species. Both mRNA and protein synthesis are necessary for PA production and germinal vesicle breakdown in BOCC matured in vitro, however inhibition of glycosylation does not affect PA activity.

Figure III-1. Zymographic (lane 1) and reverse zymographic (lane 2) analysis of in vitro matured BOCC.

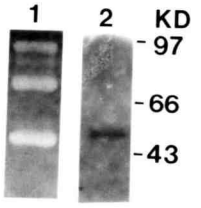


Figure III-2. Relative PA activities in the A) supernatant and B) pellet fractions of immunoprecipitated BOCC matured in vitro. Results are the means ± SE of three separate experiments and are expressed relative to the PA activity of the PBS treatment.

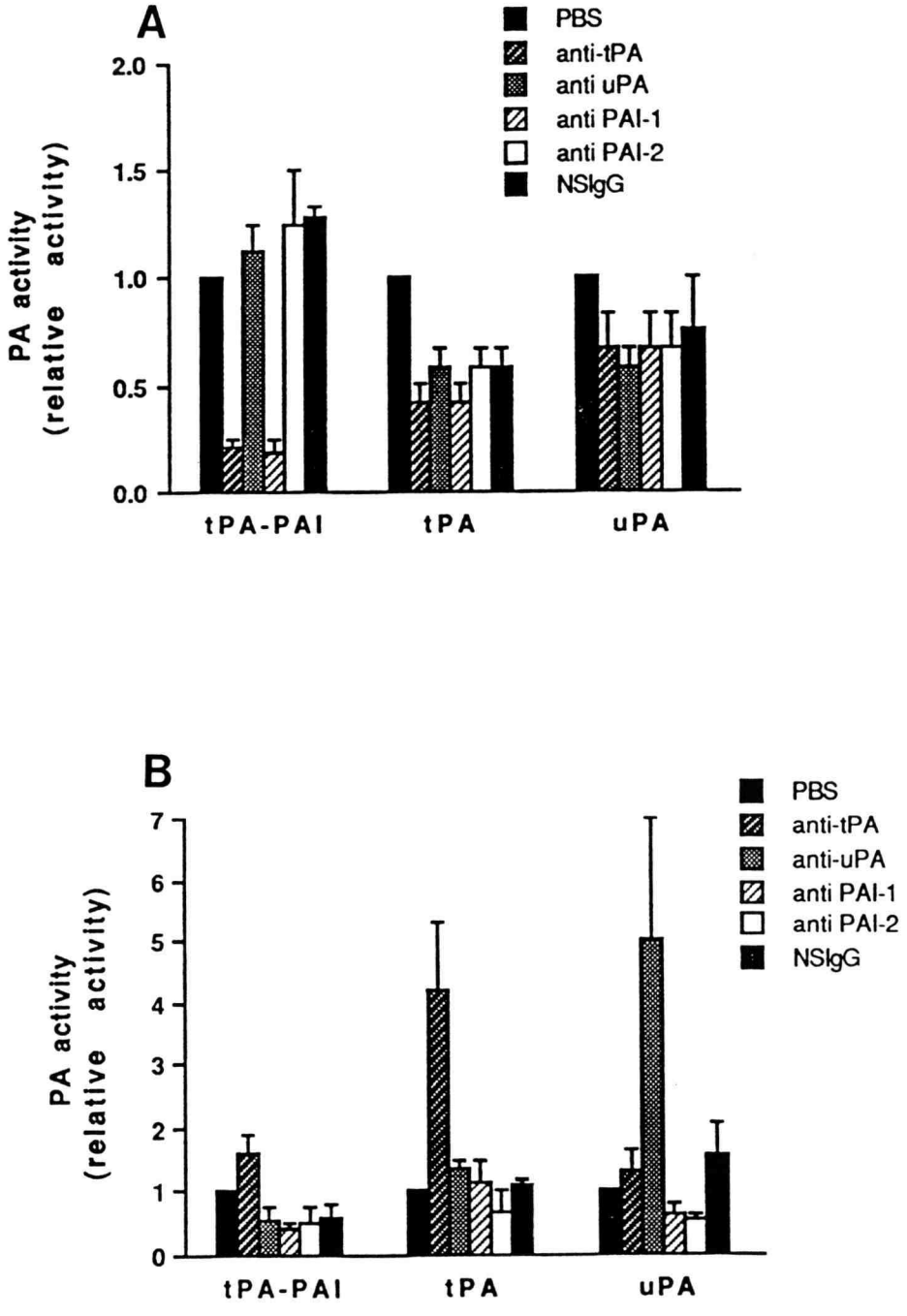
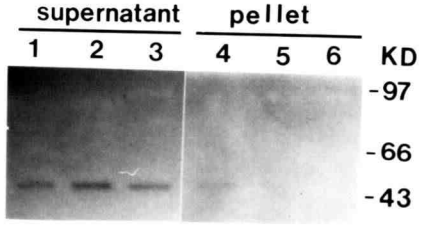


Figure III-3. Zymographic analysis of plasminogen activator inhibitor (A), anti-human PAI-1; lanes 1 & 4, anti-human PAI-2; lane 2 & 5, NSIgG; lane3 & 6, and relative activities (B) in immunoprecipitated BOCC matured in vitro. Results are the means ± SE of three separate experiments and are expressed relative to the PA activity of the PBS treatment. Means without common superscripts are different ($P<0.05$).



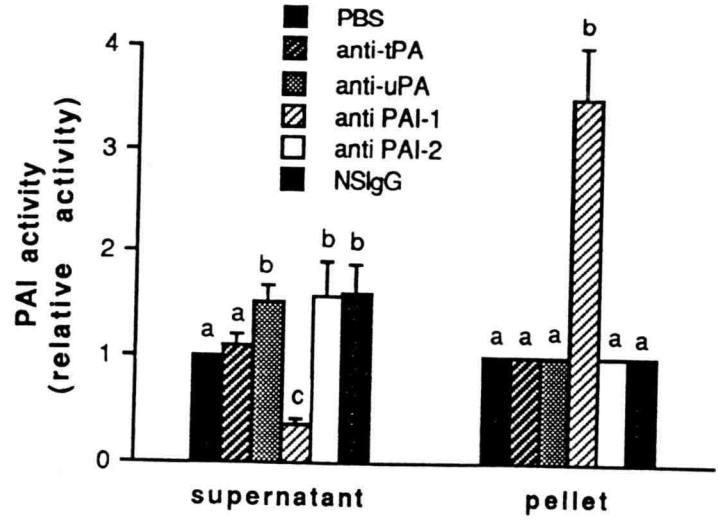


Figure III-4. Zymographic analysis of in vitro matured BOCC cultured in 0 (lane 1), 1 (lane 2) or 10 μg/ml (lane 3) cycloheximide or 5 μM actinomycinD (lane 4). Lanes 5 and 6 contain 0.1 IU/ml urokinase and 5 ng/ml human tPA, respectively.

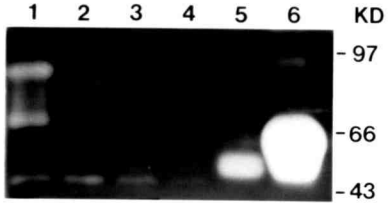


Figure III-5. Effects of cycloheximide on PA activities in in vitro matured BOCC. Results are the means ± SE of three separate experiments and are expressed relative to the PA activity of BOCC in 0 μg/ml cycloheximide. Means without common superscripts are different ($P<0.05$).

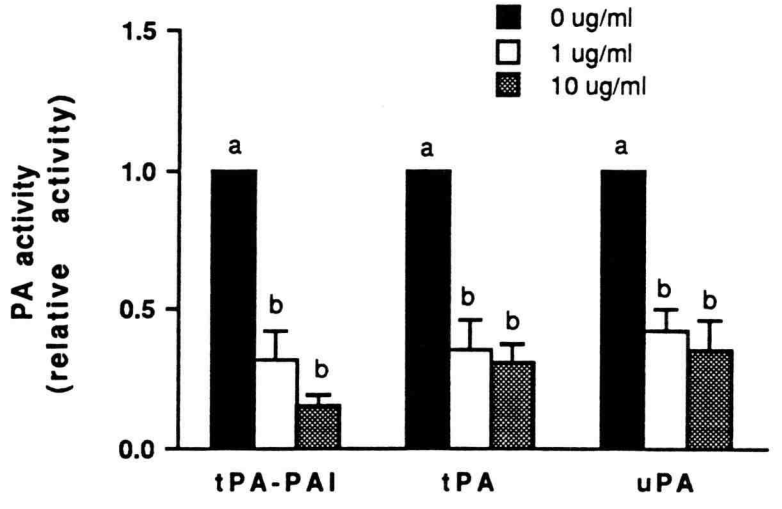


Figure III-6. Effects of actinomycin D on PA activities in in vitro matured BOCC. Results are the means ± SE of three separate experiments and are expressed relative to the PA activity of BOCC in 0 μM actinomycin D. Means without common superscripts are different ($P<0.05$).

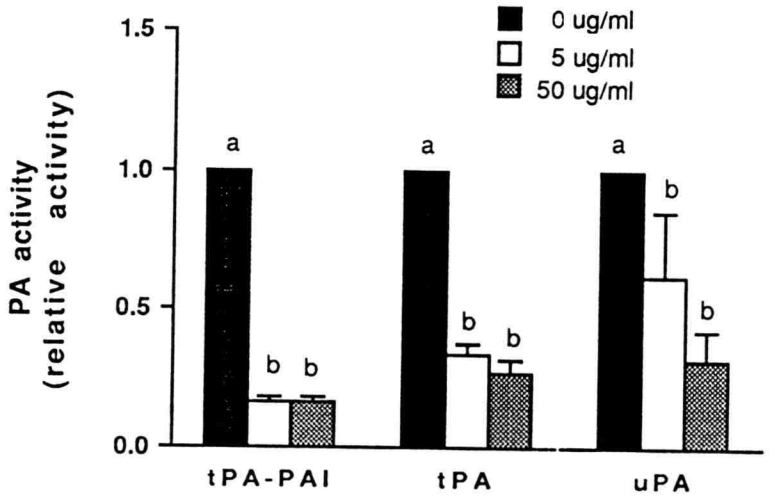


Figure III-7. Effects of cycloheximide, actinomycin D and tunicamycin on germinal vesicle breakdown (GVBD) and development to metaphase II (M II). Bovine oocyte-cumulus cell complexes were cultured in 0 (C) or 1 μg/ml cycloheximide (CHX), 5 μM actinomycin D, and 0.5 and 5 μg/ml tunicamycin (0.5 Tu and 5 Tu, respectively). Percentages without common superscripts for GVBD or M II are different ($P<0.05$).

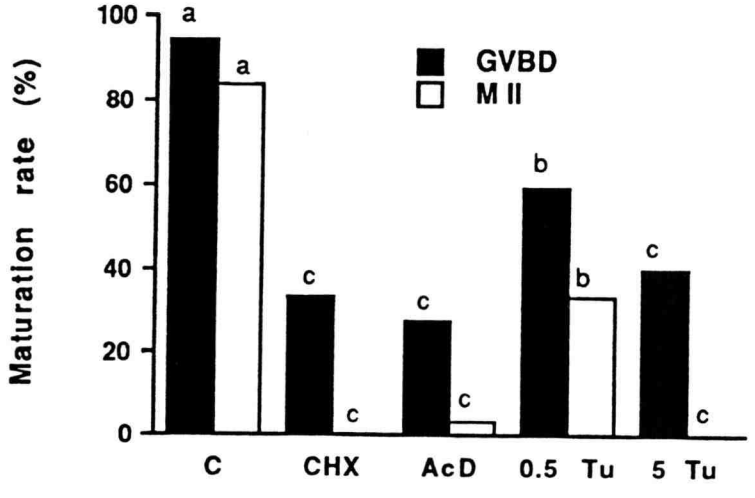


Figure III-8. Zymographic analysis of in vitro matured BOCC cultured in 0 (lane 1) or 1% DMSO (lane 2) and 0.5 (lane 3) or 5 μg/ml (lane 4) tunicamycin.

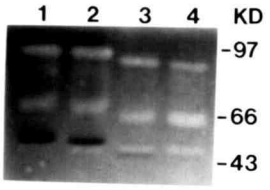
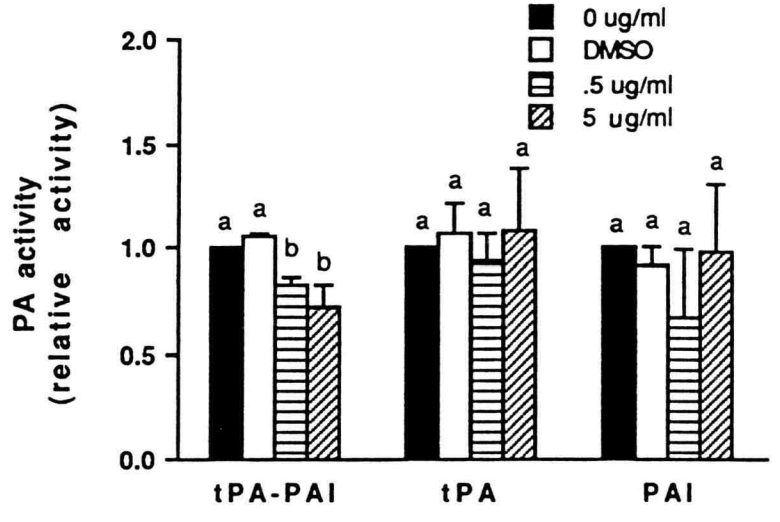


Figure III-9. Effects of tunicamycin on PA activities in in vitro matured BOCC. Results are the means ± SE of three separate experiments and are expressed relative to the PA activity of BOCC in 0 µg/ml tunicamycin. Means without common superscripts are different ($P<0.05$).



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SUMMARY

This study revealed that in vitro matured bovine oocyte-cumulus cell complexes (BOCC) produced uPA, tPA and PAI-1 whereas porcine oocyte-cumulus cell complexes (POCC) produce only tPA and PAI. Plasminogen activator activity in either BOCC or POCC increased in a dose-dependent fashion during in vitro maturation. Significant PA induction by either BOCC and POCC occurred shortly after germinal vesicle breakdown, suggesting PA may be associated with the process of oocyte maturation. Stimulators of the protein kinase A system increased PA activity in both BOCC and POCC in dose-dependent fashion. In BOCC cultured with activator of phospholipid-dependent protein kinase C, total PA activity increased, however high concentrations of PMA (10 and 100 ng/ml) decreased tPA activity in matured POCC. Inhibition of intracellular phosphorylation, transcription and translation decreased PA activity and inhibited oocyte maturation. Increasing intracellular phosphorylation using an inhibitor of phosphatase 1 and 2A caused induction and selective enhancement of uPA in POCC and uPA and PAI-1 in BOCC. Inhibition of glycosylation using tunicamycin reduced the molecular masses of tPA and PAI-1 by 5-10%, however PA activities were not changed.

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APPENDIX

EFFECTS OF EXTRACELLULAR POTASSIUM CONCENTRATION ON BLASTOCOEL FORMATION AND EXPANSION AND HATCHING IN PORCINE EMBRYOS DEVELOPING IN VITRO.

Abstract

Effects of varying the potassium concentration on blastocoel formation and expansion and hatching in porcine embryos were determined. One hundred seventeen porcine embryos were cultured individually in 50-µl microdrops of glucose-free Whitten's medium containing .4% BSA under paraffin oil in a humidified atmosphere of 5% CO_2 in air at 39 C. Two-cell to early blastocyst stage embryos were randomly allocated to medium containing 1.5, 3, 6 and 12 mM potassium. Osmolarity was maintained by adjusting the NaCl concentration. Overall embryo diameter (OD), blastocoel diameters (BD), if present, and zona pellucida thicknesses (ZPT) were measured every 24 h with an ocular micrometer. Incidences of blastocoel formation and expansion from two to four-cell embryos were not different ($P>.05$) in medium with 1.5, 3, 6 or 12 mM potassium. More two to four-cell embryos commenced hatching in 3 mM potassium (44%) than 1.5 mM (11%) ($P<.05$) and 12 mM (16%) potassium ($P<.10$). Percentages of morulae to early blastocysts undergoing blastocoel expansion and hatching were not different ($P>.05$) among the four treatments. Onset of blastocoel formation by cultured four-cell embryos was not different ($P>.05$) among 1.5, 3, 6 and 12 mM potassium concentration, however expansion was delayed ($P<.05$) in 1.5 mM (104 ± 5.7 h) compared to

6 mM (84 ± 6.0 h) and 12 mM (82.9 ± 5.2 h) potassium. No differences (P>.05) due to potassium concentration were observed in OD, BD and ZPT attained by either two to four-cell embryos or morulae to early blastocysts. These results suggest that a low concentration of potassium (1.5 mM) in the culture medium delays blastocoel expansion and reduces hatching. Although the porcine embryo appears to be relatively tolerant of higher concentrations of potassium, the potassium requirements at different cell stages may require further investigation.

Introduction

Blastocoel formation and expansion and hatching are important processes in early preimplantation embryo development that are dependent on the transepithelial ion transport properties of the trophectoderm. Two properties of the trophectoderm are responsible for generating transcellular ion transport: tight junctions between adjacent trophectodermal cells and the presence of the Na/K ATPase on the basolateral cell surfaces (Benos and Biggers, 1981). Hydrostatic pressure accumulation due to water influx in the blastocoel in part causes the force responsible for rupture of the zona pellucida and hatching.

Previous studies in the mouse have shown that embryo development is sensitive to the concentration of potassium in the culture medium. Wiley (1984) and Wiley et al. (1986) reported that reducing potassium (0.6 and 1.4 mM) in the culture medium accelerated development of two to four cell stage embryos and morulae into blastocysts compared to medium with the common

concentration (6 mM) of potassium (Biggers et al., 1971). Conflicting results have been presented by Roblero and Riffo (1986) who reported that high potassium (25 mM), similar to that found in the mouse oviduct, increased cell numbers of morulae and blastocysts developing from two-cell embryos compared to 4.7 mM. Studies in mice, swine and humans have reported a high potassium concentration in oviductal fluid compared to the bloodsteam (Roblero et al., 1976; Iritani et al., 1974; Borland et al., 1980).

Although culture of early cleavage stage porcine embryos to the blastocyst stage has been accomplished in simple medium with BSA or serum, few embryos commence and complete hatching in vitro (Reed et al., 1992). Porcine embryo development has been improved in mouse oviduct organ culture (Krisher et al., 1989; Reed et al., 1992), co-culture with oviductal epithelial cells (White et al., 1989) and in medium supplemented with oviductal fluid (Archibong et al., 1989). Differences in development under these various conditions may be due to ionic imbalances or the absence of macromolecular components, such as growth factors. Therefore the purpose of this study was to investigate effects of varying the potassium concentration on blastocoel formation and expansion and hatching in porcine embryos.

Materials and Methods

Eleven cycling gilts and sows and eight prepuberal gilts were used. Cycling gilts and sows were checked daily for estrus and handmated with boars of proven fertility. Prepuberal gilts were synchronized and superovulated with pregnant mare's serum

gonadotropin (PMSG) and human chorionic gonadotropin (HCG) and bred artificially 24 and 48 h after HCG with commercial semen (Baker and Coggins, 1968). Prepuberal gilts displaying estrus after hormone treatment were also hand-mated. Reproductive tracts were recovered at slaughter and oviducts and uteri were flushed with glucose-free Whitten's medium (WM) lacking BSA (Whitten and Biggers, 1968). Flushings were collected into embryological dishes and observed at 10 X with a dissecting microscope for the presence of embryos. Embryos were transferred to microdrops of WM with .4% BSA and evaluated for morphology. Embryos were randomly allocated to medium containing 1.5, 3, 6 and 12 mM potassium. Osmolarlity was maintained by adjusting the NaCl concentration. Embryos were cultured individually in 50 µl microdrops of WM containing .4% BSA under paraffin oil in a humidified atmosphere of 5% CO2 in air at 39 C. At 24-h interval embryos were morphologically evaluated at 100-200 X with an inverted-stage phase-contrast microscope and overall embryo diameters (OD), blastocoel diameters (BD), if present, and zona pellucida thicknesses(ZPD) were measured with an ocular micrometer.

Differences in the percentages of embryos developing to a particular cell stage due to treatment were determined by Chi-square procedures. Differences in OD, BD and ZPT due to concentration of potassium and time in culture were determined using two-way ANOVA and Duncan's new multiple range test. All analysis were performed using the NCSS statistical software

program (Number Cruncher Statistical System, Version 4.1, 1984, J.L. Hintze, Kaysville, UT, USA).

Results and Discussion

Seventy-four two- to four-cell embryos and 39 morulae to early blastocysts were recovered. Sufficient numbers of embryos were collected to allow allocation of embryos from a single female to all four treatments. Incidences of blastocoel formation and expansion from two to four-cell embryos were not different ($P > .05$) in medium with 1.5, 3, 6, or 12 mM potassium (Table 1). More two to four-cell embryos commenced hatching in 3 mM potassium (44%) than 1.5 mM (11%) ($P < .05$) and 12 mM (16%) potassium ($P < .10$). Fewer embryos commenced hatching in medium with 6 mM (28%) compared to 3 mM potassium (44%), however this difference was not significant. Percentages of morulae and early blastocysts undergoing blastocoel expansion and hatching were not different ($P > .05$) among the four treatments (Table 1).

Onset of blastocoel formation by cultured four cell embryos was not different ($P > .05$) among 1.5, 3, 6 or 12 mM potassium, however expansion was delayed ($P < .05$) in 1.5 mM compared to 6 and 12 mM potassium (Table 2). Formation and expansion of the blastocoel are due to passive entry of water caused by Na/K-ATPase translocation of Na into the blastocoel (Wiley, 1984; Manejwala et al., 1986). It has been observed in many mammals that in vitro embryo development is retarded compared with in vivo development. Different elemental and organic compositions or hormonal and growth factor deficiencies in culture medium,

compared with the natural oviductal and uterine environments, were suggested as potential causes of this developmental delay (Roblero and Riffo, 1986). Concentrations of potassium in the sow's oviduct and uterus are 12 and 16 mM, respectively (Iritani et al., 1974). The importance of this physiologically high potassium concentration in the oviductal and uterine microenvironments of the preimplantation embryo is not well understood. Previous work has suggested that the high potassium concentration influences sperm motility and sperm-egg fusion (Boldt et al., 1991). Roblero and Riffo (1986) observed a dose-related increase in the rate of cleavage of mouse embryos with 4.7, 10 and 25 mM potassium. Their results suggest that potassium concentrations similar to the mouse genital tract improved in vitro development. However conflicting results in the mouse embryo have been observed (Wiley, 1984; Wiley et al., 1986). Cavitation was accelerated when potassium was decreased from 6 to 0.5 mM and was delayed when potassium was increased from 6 to 25 mM (Wiley, 1984; Wiley et al., 1986). It has been suggested that decreasing the concentration of potassium may hyperpolarize the cell due to the difference between intra- and extracellular potassium concentrations. Conversely increasing potassium would depolarize the cell (Cohen et al., 1976; Wiley, 1984). It is also possible that potassium plays different roles in porcine embryos depending on developmental status. In early embryonic development, potassium may exert an effect only on embryonic cell division, whereas, during the period of blastocoel formation, potassium may also affect blastocoel expansion.

Figures IV-1 and 2 depict changes in OD, BD and ZPT of two-cell stage embryos to early blastocysts cultured in different concentrations of potassium. Although low potassium delayed onset of blastocoel expansion, no significant differences were observed in OD, BD and ZPT (Figures IV-1 and 2). Maximum expansion of blastocysts by cultured two- to four-cell embryos was observed on comparative gestational day 7 in medium with 3, 6 and 12 mM potassium, but on day 8 in medium with 1.5 mM potassium (Figure IV-1). In conclusion, our results suggest that a low concentration of potassium (1.5 mM) in the culture medium delayed blastocoel expansion and reduced hatching. Because superior development was not observed in medium with 6.0 mM potassium, the common concentration for most culture media, the potassium requirements for porcine embryos at different cell stages may require further investigation.

TABLE 1. DEVELOPMENT OF PORCINE EMBRYOS IN MEDIUM WITH 1.5, 3.6 OR 12 mM POTASSIUM

Initial cell stage	Potassium concentration	n	Number(%) of embryos developing to: Blastocyst	Expanded blastocyst	Hatching blastocyst	Hatched blastocyst
Two- to four-cell	1.5 mM	19	19(100)[a]	8(42.1)[a]	2(10.5)[b]	1(5.3)
	3 mM	18	16(88.9)[a]	11(61.1)[a]	8(44.4)[a]	--
	6 mM	18	15(83.3)[a]	9(50.0)[a]	5(27.8)[a,b]	--
	12 mM	19	18(94.7)[a]	11(57.9)[a]	3(15.8)[b]	--
Morula to early blastocyst	1.5 mM	10	10(100)	9(90)[a]	5(50)[a]	2(20)
	3 mM	10	10(100)	9(90)[a]	6(60)[a]	2(20)
	6 mM	9	9(100)	9(100)[a]	7(77.8)[a]	2(22.2)
	12 mM	10	10(100)	10(100)[a]	6(60)[a]	--

[a,b] Values in the same column for a specific initial cell stage without common superscripts are different (P<.10).

TABLE 2. TIME (h) TO BLASTOCOEL FORMATION AND EXPANSION AND ONSET OF HATCHING FOR FOUR-CELL PORCINE EMBRYOS CULTURED IN MEDIUM CONTAINING 1.5, 3, 6 OR 12 mM POTASSIUM

		Development to the:				
		Blastocoyst		Expanded blastocyst		Hatching blastocyst
Treatment	n	X ± SE	n	X ± SE	n	X ± SE
1.5 mM	15	73.6 ± 4.7[a]	11	104 ± 5.7[a]	4	120 ± 11.9[a]
3 mM	15	68.3 ± 4.7[a]	8	96 ± 5.7[a,b]	6	108 ± 11.9[a]
6 mM	13	62.8 ± 4.7[a]	9	84 ± 6.0[b]	4	100 ± 9.7[a]
12 mM	15	60.8 ± 4.3[a]	9	82.9 ± 5.2[b]	3	96 ± 16.9[a]

[a,b]Means in the same column without common superscripts are different ($P<.05$).

Figure IV-1. Changes in A) overall diameters, B) blastocoel diameters and C) zona pellucida (ZP) thicknesses for 2- to 4-cell porcine embryos cultured in 1.5, 3, 6 or 12 mM potassium.

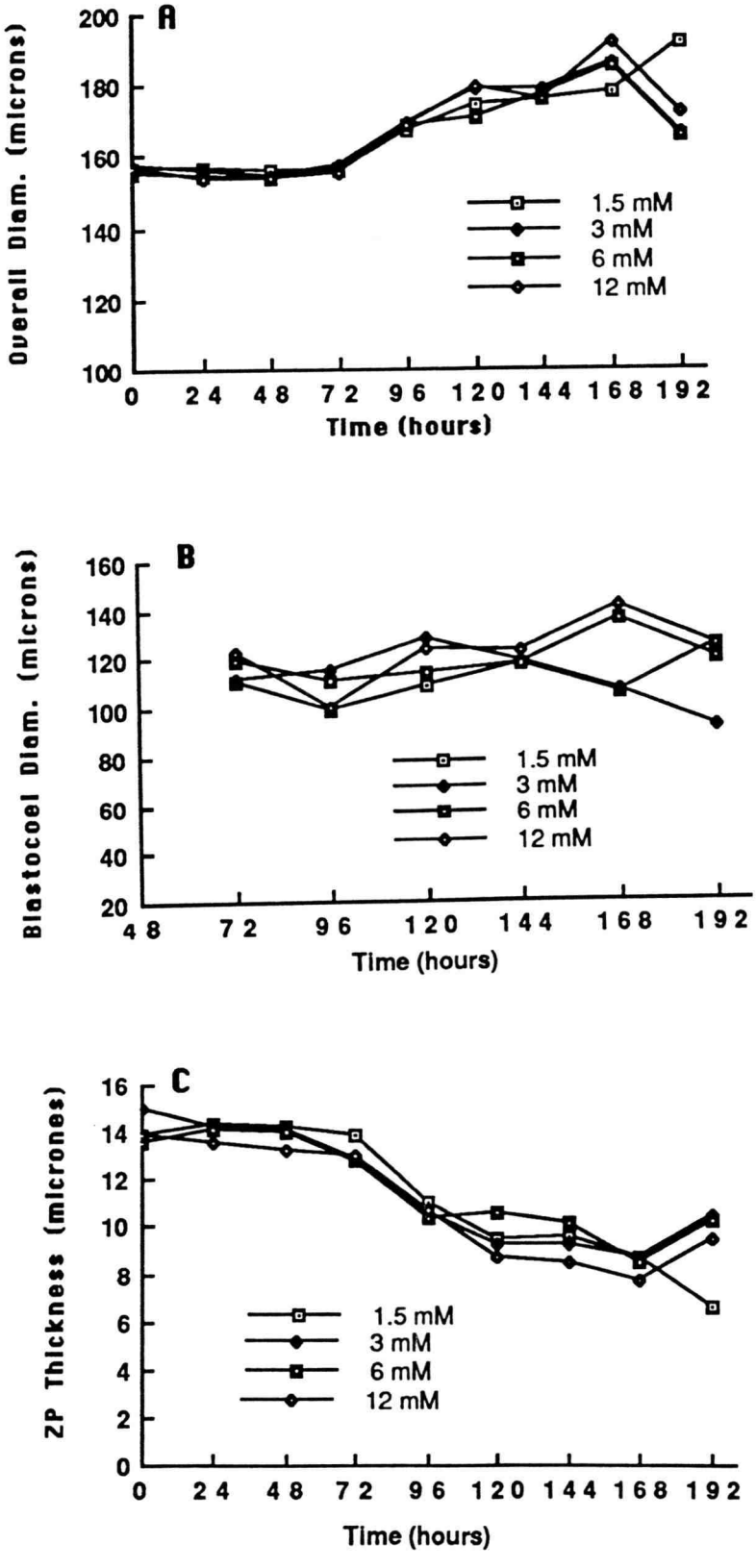
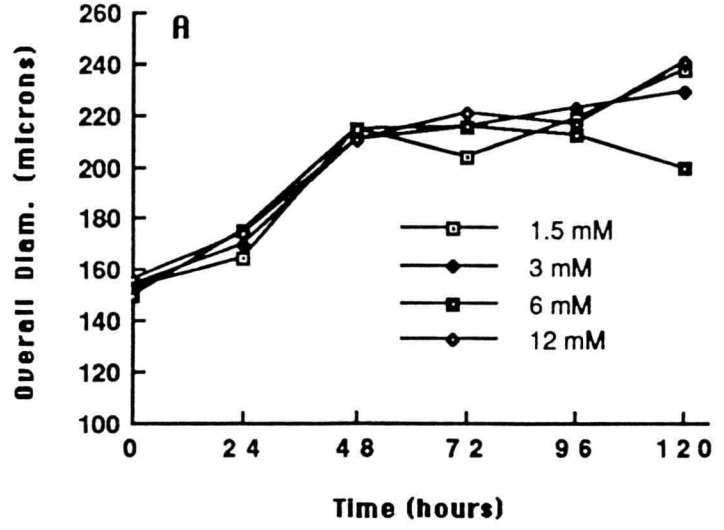
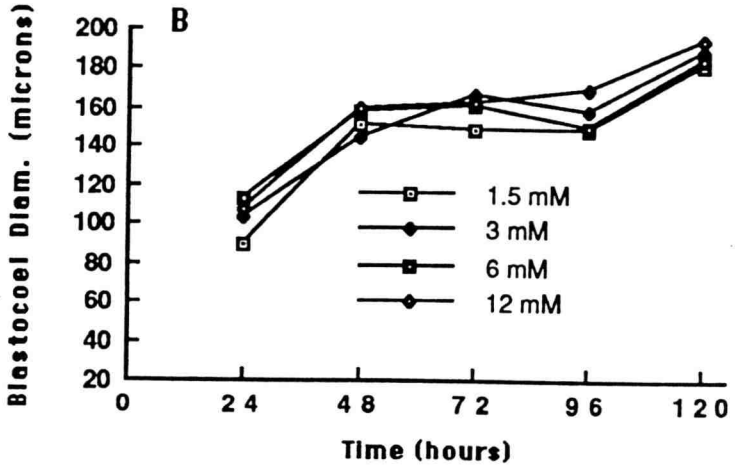
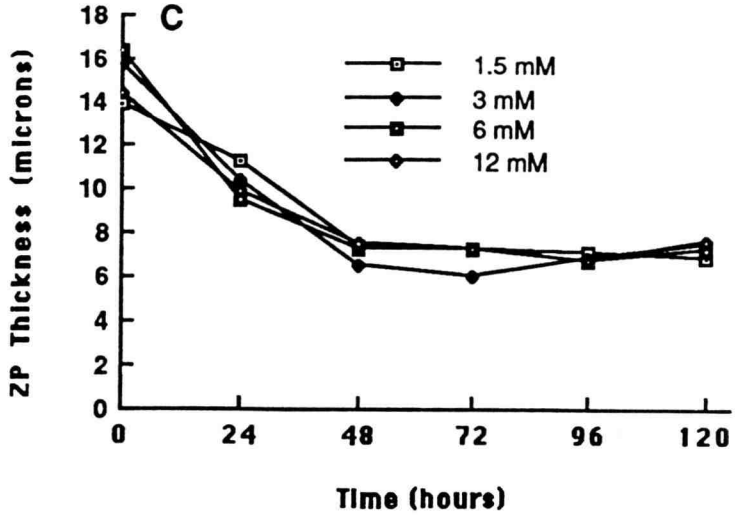


Figure IV-2. Changes in A) overall diameters, B) blastocoel diameters and C) zona pellucida (ZP) thicknesses for morulae and early blastocysts cultured in 1.5, 3, 6 or 12 mM potassium.







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