Lack of effect of hormones and inducers of intracellular messengers on plasminogen activator production by bovine embryos *in vitro*

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Summary. Several hormones and inducers of intracellular messengers, known to affect plasminogen-activator (PA) production in other systems, were investigated for putative effects on bovine embryos. Day 8 embryos were cultured for 5 days in a humidified atmosphere of 5% CO₂ in air at 37°C in media containing different concentrations of progesterone, oestradiol, dexamethasone, retinoic acid, dibutyryl cyclic AMP (dbcAMP) and phorbol myristate acetate (PMA). At intervals of 24 h, the medium was recovered for PA analysis and overall embryonic diameter was measured. While none of the hormones and agents tested affected PA production ($P > 0.05$), dimethyl sulfoxide, which was used to dissolve PMA, inhibited PA production during the first 72 h of culture ($P < 0.05$). PA production was affected by duration of culture ($P < 0.05$). Concentrations of plasminogen activator in the media were low during the first 48 h, had increased after 72 and 96 h in culture, and either remained high or decreased slightly toward the end of the culture period. With the exceptions of dbcAMP and PMA, the hormones tested in this study did not affect embryonic size. Dibutyryl cAMP caused a progressive decrease in embryonic diameter. PMA resulted in embryo death at high concentrations but at lower concentrations it enhanced overall embryonic diameter throughout the time of culture ($P < 0.05$). These results suggest that cultured bovine embryos produce PA in a fixed, time-dependent manner, independent of exogenous hormonal regulation.

Keywords: embryo; plasminogen activator; hormones; cow

Introduction

Plasminogen activators (PA) are serine proteases that convert the zymogen plasminogen to the active protease plasmin by the cleavage of an Arg–Val peptide bond. Two types of PA have been identified by functional differences, relative molecular mass (M_r), and immunological activities: urokinase-type (uPA) and tissue-type (tPA) with M_r of $30\text{--}55 \times 10^3$ and 70×10^3 , respectively (Dano *et al.*, 1985). It has been suggested that uPA participates in localized proteolysis of the extracellular matrix accompanying tissue remodelling and cellular migration (Blasi *et al.*, 1987). Tissue-type PA displays a high affinity for fibrin and is therefore considered as the key enzyme in thrombolysis (Astrup, 1978).

As well as being present in many types of normal and malignant cells, PA are produced by the embryos of mice (Strickland *et al.*, 1976), rats (Liedholm & Astedt, 1975), pigs (Mullins *et al.*, 1980), cattle (Menino & Williams, 1987) and sheep (Menino *et al.*, 1989). Although the exact function of PA in early embryonic development is not known, it has been implicated in the migration of parietal endodermal cells and in the implantation in mouse embryos (Sherman *et al.*, 1976; Strickland *et al.*, 1976; Sherman, 1980; Axelrod, 1985; Sappino *et al.*, 1989), tissue

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remodelling and proliferation in pig embryos (Fazleabas *et al.*, 1983), and embryo hatching in cattle and sheep (Menino & Williams, 1987; Menino *et al.*, 1989).

The extracellular proteolytic activity of the PA system is regulated by a delicate balance between PA, plasminogen and their endogenous inhibitors. Several agents have been shown to modulate plasminogen activation in a variety of cells. Gonadotrophins stimulate PA activity in rat granulosa cells (Strickland & Beers, 1976; Reich *et al.*, 1985; Canipari & Strickland, 1986), in cultured human cytotrophoblasts (Queenan *et al.*, 1987), and in rat and bovine Sertoli cells (Lacroix *et al.*, 1981; Marzowski *et al.*, 1985; Hettler *et al.*, 1986; Coombs *et al.*, 1988). The effects of gonadotrophins are assumed to be mediated by cyclic adenosine monophosphate (cAMP). Oestrogen stimulates the production of PA in human breast carcinoma cell lines (Dickerman *et al.*, 1989) and enhances plasminogen uptake by the mouse uterus (Finlay *et al.*, 1983). Glucocorticoids, such as dexamethasone, inhibit PA activity in rat granulosa cells (Harlow *et al.*, 1987) and in bovine Sertoli cells (Coombs & Jenkins, 1988) by stimulating the production of PA-inhibitors (PAI). Similarly, progesterone was found to stimulate the production of protease inhibitors in mouse and pig uteri (Harpel *et al.*, 1966; Mullins *et al.*, 1980); thus inhibiting fibrinolytic activity within the uterus during specific stages of the oestrous cycle. Phorbol myristate acetate (PMA) and retinoic acid are also potent inducers of PA production in a number of different cells and tissues (Strickland & Mahdavi, 1978; Wilson & Reich, 1978; Opdenakker *et al.*, 1983; Santell & Levin, 1988).

Hormonal regulation of embryonic PA production has not been studied. This research evaluated the effects of progesterone, oestradiol, dexamethasone, retinoic acid, dibutyryl cAMP (dbcAMP) and PMA on PA production in cultured bovine embryos. Changes in embryonic size were also studied to assess embryo viability in response to the hormonal treatments.

Materials and Methods

Embryo collection and culture

Thirty-two crossbred beef cows from the Oregon State University beef herd were treated with prostaglandin $F_{2\alpha}$ (PGF_{2 α} ; Lutalyse: The Upjohn Co., Kalamazoo, MI) to synchronize oestrus and with porcine follicle-stimulating hormone (pFSH; Schering Corp., Kenilworth, NJ) to induce superovulation. Two 25 mg injections of PGF_{2 α} were administered i.m. 14 days apart (day 0 = first PGF_{2 α} injection) to all cows. Porcine follicle-stimulating hormone injections were administered twice a day i.m. at doses of 5, 4, 2 and 1 mg on days 12, 13, 14 and 15, respectively. Oestrus detection was initiated 24 h after the second PGF_{2 α} injection. Cows were either mated naturally at the onset of oestrus and at 12 h intervals thereafter, or artificially inseminated at 0, 12 and 24 h after onset of oestrus using one straw of frozen semen each time.

Embryos were collected 8 days after mating either nonsurgically or at death. In the nonsurgical collection, uteri were flushed with Dulbecco's phosphate-buffered saline (Dulbecco & Vogt, 1954) containing 0.5% bovine serum albumin (BSA; Sigma, St Louis, MO) and 10 ml l⁻¹ of an antibiotic-antimycotic solution (10 000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml in 0.9% sodium chloride; Sigma). Excised uteri removed at death were flushed with Ham's F-12 containing 10 ml l⁻¹ of the antibiotic-antimycotic solution (Sigma). Embryos were recovered from the flushings under a dissecting microscope, washed at least three times in microdrops of Ham's F-12 with either 15 or 1.5 mg BSA ml⁻¹, and morphologically evaluated using an inverted-stage, phase-contrast microscope. Only morphologically normal embryos were assigned to the various hormonal treatments (Lindner & Wright, 1983). Embryos were individually cultured in 35 µl microdrops under paraffin oil (Fisher Scientific Co., Tustin, CA) in a humidified atmosphere of 5% CO₂ in air at 37°C for 5 days. At the initiation of culture and at subsequent 24 h intervals, embryonic cell stage was recorded and overall embryonic diameter (OED) was measured using an ocular micrometer. At 24 h intervals, embryos were transferred to fresh microdrops and 15 µl of the culture medium were recovered and frozen at -20°C until assayed for PA activity. Another 15 µl of medium from a microdrop without an embryo was recovered for every treatment to correct for any spontaneous activation of plasminogen.

Effect of hormones and inducers of intracellular messengers on PA production and OED by cultured bovine embryos

In treatments where hormones and intracellular messengers were dissolved in absolute ethanol or dimethyl sulfoxide (DMSO), appropriate stocks and dilutions were formulated so that the percentages of solvent in the media were the same for all concentrations within a treatment. Appropriate vehicle controls were used for each treatment. When possible, for a given hormone or intracellular messenger, embryos from the same donor cows were allocated randomly to each treatment.

Progesterone

Progesterone (Sigma) was initially dissolved in absolute ethanol and diluted further to final concentrations of 10^{-8} , 10^{-7} and 10^{-6} g ml $^{-1}$ with Ham's F-12 containing 15 mg BSA ml $^{-1}$. The final concentration of absolute ethanol in progesterone-containing medium was 1%. Two controls were included in this experiment: Ham's F-12 with 15 mg BSA ml $^{-1}$ and this medium plus 1% absolute ethanol. Twelve morphologically normal embryos were randomly assigned to each treatment. All subsequent media formulations used Ham's F-12 with 1.5 mg BSA ml $^{-1}$ as the basal medium (HF-12).

Dexamethasone

Four concentrations (0 , 10^{-8} , 10^{-7} and 10^{-6} mol l $^{-1}$) of dexamethasone (Sigma) were prepared by diluting dexamethasone–absolute ethanol stock solutions with HF-12 and evaluated on 11–13 embryos per group.

Retinoic acid

Retinoic acid (Sigma) was dissolved in absolute ethanol and diluted to 0 , 10^{-8} , 10^{-7} and 10^{-6} mol l $^{-1}$ with HF-12. Twelve to 13 embryos were assigned to each treatment.

Oestradiol

Oestradiol (Sigma) was dissolved in absolute ethanol and diluted to four concentrations with HF-12 (0 , 10^{-8} , 10^{-7} and 10^{-6} g ml $^{-1}$) and evaluated on 12–14 embryos per group.

Phorbol myristate acetate and dbcAMP

Experiment 1. Phorbol 12-myristate 13-acetate (Sigma) was dissolved in DMSO (Sigma) and diluted with HF-12 to a final concentration of 10 μ g ml $^{-1}$. Medium with 1% DMSO served as the control. Dibutyryl cAMP (Sigma) was diluted with HF-12 to 5 mmol l $^{-1}$ and the corresponding control medium was HF-12. Seven to eight embryos were assigned to each of these four treatments.

Experiment 2. Phorbol 12-myristate 13-acetate was diluted to 100 ng ml $^{-1}$ and dbcAMP was diluted to 0.5 mmol l $^{-1}$. Two controls, HF-12 and HF-12 with 1% DMSO, were included in this experiment. Six to ten embryos were assigned to each treatment.

Experiment 3. Phorbol 12-myristate 13-acetate was further diluted to 1 ng ml $^{-1}$ in HF-12. Control medium contained 1% DMSO. Thirteen embryos were assigned to each treatment.

Assay for PA activity

Plasminogen-activator activities were determined by a caseinolytic assay as described by Kaaekuahiwi & Menino (1990). Briefly, a casein–agar solution was prepared by combining 2% agarose (Sigma) with an equal volume of warmed 2% nonfat dry milk (Carnation Co., Los Angeles, CA) in 0.038 mol Tris(hydroxymethyl)-aminomethane l $^{-1}$, 0.1 mol glycine l $^{-1}$ buffer containing 0.195 g CaCl $_2$ ·2H $_2$ O l $^{-1}$ and 0.325 g sodium azide l $^{-1}$. Fifteen millilitres of the warmed mixture were pipetted into 85 × 65 × 2 mm ^{3} plastic diffusion plates and allowed to solidify at room temperature. Plasminogen-activator activities were determined by combining 15 μ l of 150 μ g human plasminogen ml $^{-1}$ (Sigma) with 15 μ l of either conditioned medium or 0 , 0.001 , 0.005 , 0.01 , 0.05 and 0.10 milliunits urokinase ml $^{-1}$ (E.C. 3.4.21.7; Sigma) as the standard and incubated for 15 min at 37°C. Twenty-five microlitres of the incubated mixtures were aliquoted into 4 mm diameter wells cut in the casein–agar gel plate and incubated at room temperature for 24 h. Plates were fixed for 15 min with 3% acetic acid and rinsed in tap water. The resultant caseinolytic zones were measured with an electronic digital caliper. Concentrations of PA in the conditioned media were determined from equation of the line calculations for caseinolytic ring diameter by log urokinase concentration. The amount of embryonic PA was determined by deducting the amount of PA in the medium without an embryo from the amount in the medium with an embryo for each 24 h interval. Plasminogen activator activity was expressed as milliunits PA × 10 $^{-4}$ ml $^{-1}$ h $^{-1}$ per embryo.

Statistical analyses

With the exception of the PMA (10 μ g ml $^{-1}$ and 100 ng ml $^{-1}$) and the dbcAMP (5 mmol l $^{-1}$) treatments, statistical analyses were performed on data obtained from embryos that successfully hatched. Statistical comparisons were made by two-way analyses of variance where hormone treatment and time in culture were the main effects. Duncan's new multiple range test was used for multiple comparisons when appropriate. Analyses were performed using the NCSS statistical software program (Number Cruncher Statistical System, Version 4.1, 1984, J. L. Hintze, Kaysville, Utah).

Results

Embryo recovery

A total of 444 ova and embryos were collected from thirty-two cows of which 65% (287) were morphologically normal. Overall, 93.2% of these embryos completed hatching *in vitro*.

Effects of hormones and inducers of intracellular messengers on PA production by cultured bovine embryos and on OED

Progesterone

Hatching occurred in 54 of 59 embryos (92%). Because 1% absolute ethanol (Fig. 1a and b) had no effect ($P > 0.10$) on PA production or OED, this final concentration was maintained for all other treatments using absolute ethanol as vehicle. Progesterone concentrations of 10^{-8} , 10^{-7} and 10^{-6} g ml $^{-1}$ affected neither PA production nor OED ($P > 0.10$). However, duration of culture affected ($P < 0.05$) both PA production and OED. No interaction between time and concentration of hormone was observed ($P > 0.10$). At the beginning of the culture PA production was low. PA production increased ($P < 0.05$) after 72 h of culture, reaching a maximum after 96 h, and this rate of production was maintained thereafter. Overall embryo diameter increased with time in culture ($P < 0.05$), reached a maximum at 72 h and then declined ($P < 0.05$).

Fig. 1. (a) Plasminogen activator production and (b) overall embryonic diameter reached by bovine embryos cultured in Ham's F-12 with 15 mg bovine serum albumin per ml containing 0 (—), 0 plus ethanol (---), 10^{-8} (—), 10^{-7} (.....) or 10^{-6} (----) g progesterone ml $^{-1}$. Error mean squares for (a) and (b) are 0.222 and 8587, respectively.

Dexamethasone

Hatching occurred in 44 of 46 embryos (96%). Plasminogen-activator production and OED were not affected ($P > 0.10$) by dexamethasone treatments (Fig. 2a and b). Plasminogen-activator production increased progressively, reached a maximum at 96 h and then decreased ($P < 0.05$). Similarly, OED increased ($P < 0.05$) during the first 48 h, peaked at 72 h and then diminished. No interaction was detected ($P > 0.10$) between concentration of dexamethasone used and time in culture.

Retinoic acid

Hatching occurred in 41 of 43 embryos (93%). Retinoic acid did not affect ($P > 0.10$) PA production or OED (Fig. 3a and b). Plasminogen-activator production and OED changed with time ($P < 0.01$) but no interaction was observed between the main effects ($P > 0.10$). After 72 h in culture plasminogen-activator production increased ($P < 0.05$) and remained high thereafter.

Fig. 2. (a) Plasminogen activator production and (b) overall embryonic diameter reached by bovine embryos cultured in Ham's F-12 with 1.5 mg bovine serum albumin ml $^{-1}$ containing 0 (—), 10^{-8} (— — —), 10^{-7} (.....) or 10^{-6} (— · — · —) mol dexamethasone l $^{-1}$. Error mean squares for (a) and (b) are 0.046 and 6166, respectively.

Overall embryo diameter progressively increased ($P < 0.05$), reaching a maximum at 72 h, then declined toward the end of culture ($P < 0.05$).

Oestradiol

Hatching occurred in 45 of 48 embryos (94%). No effect was observed on PA production and OED ($P > 0.10$) by the oestradiol treatments (Fig. 4a and b). In contrast, PA production and OED were affected by time in culture ($P < 0.01$). The interaction between hormone treatment and time was not significant ($P > 0.10$). Plasminogen-activator production remained unchanged for the first 48 h ($P > 0.05$), then increased, reaching a plateau by 72 h ($P < 0.05$). Overall embryo diameter increased during the first 48 h ($P < 0.05$), reached a maximum between 72 and 96 h and then declined ($P < 0.05$).

Phorbol myristate acetate and dbcAMP

Experiment 1. The percentage of embryos hatching in HF-12 or HF-12 with 10 μ g PMA ml $^{-1}$, 5 mmol dbcAMP l $^{-1}$ or 1% DMSO was 100 (7 of 7), 0 (0 of 8), 63 (5 of 8), and 88 (7 of 8), respectively. Phorbol myristate acetate at 10 μ g ml $^{-1}$ caused embryos to degenerate and die within the first 24 h. Dibutyl cAMP also reduced the number of embryos hatching *in vitro* and caused embryo degeneration towards the end of culture ($P < 0.05$). Plasminogen-activator production was not affected ($P > 0.10$) by dbcAMP (Fig. 5a). Production of PA increased ($P < 0.01$) from 24 to 48 h, remained at a plateau until 96 h ($P > 0.05$) and then decreased ($P < 0.05$). No interaction was observed between the two main effects ($P > 0.10$). Overall embryo diameter decreased over time ($P < 0.10$) and was reduced ($P < 0.01$) in the medium containing 5 mmol dbcAMP l $^{-1}$ (Fig. 5b). The interaction between time and treatment was also significant ($P < 0.05$). Compared with HF-12, DMSO decreased PA production ($P < 0.01$) only during the first 72 h of culture (Fig. 6a). Overall embryo diameter was not affected ($P > 0.10$) by DMSO (Fig. 6b).

Fig. 3. (a) Plasminogen activator production and (b) overall embryonic diameter reached by bovine embryos cultured in Ham's F-12 with 1.5 mg bovine serum albumin ml $^{-1}$ containing 0 (—), 10^{-8} (— — —), 10^{-7} (.....) or 10^{-6} (— · — ·) mol retinoic acid l $^{-1}$. Error mean squares for (a) and (b) are 0.292 and 6377, respectively.

Fig. 4. (a) Plasminogen activator production and (b) overall embryonic diameter reached by bovine embryos cultured in Ham's F-12 with 1.5 mg bovine serum albumin ml $^{-1}$ containing 0 (—), 10^{-8} (— — —), 10^{-7} (.....) or 10^{-6} (— · — ·) g oestradiol ml $^{-1}$. Error mean squares for (a) and (b) are 0.015 and 6377, respectively.

Fig. 5. (a) Plasminogen activator production and (b) overall embryonic diameter reached by bovine embryos cultured in Ham's F-12 with 1.5 mg bovine serum albumin ml $^{-1}$ containing 0 (—) or 5 (·····) mmol dibutyryl cAMP l $^{-1}$. Error mean squares for (a) and (b) are 0.008 and 2583, respectively.

Fig. 6. (a) Plasminogen activator production and (b) overall embryonic diameter reached by bovine embryos cultured in Ham's F-12 with 1.5 mg bovine serum albumin ml $^{-1}$ containing 0 (—) or 1% (·····) dimethyl sulfoxide. Error mean squares for (a) and (b) are 0.004 and 3180, respectively.

Experiment 2. This experiment was designed to eliminate the deleterious effects of the high concentrations of PMA and dbcAMP observed in the first experiment. The percentage of embryos hatching in HF-12 or HF-12 with 100 ng PMA ml⁻¹, 0.5 mmol dbcAMP l⁻¹ or 1% DMSO was 80 (8 of 10), 17 (1 of 6), 90 (9 of 10) and 71 (5 of 7), respectively. As evidenced by the reduced incidence of hatching, embryo development in medium with 100 ng PMA ml⁻¹ was suppressed compared with medium with DMSO ($P < 0.01$). Plasminogen-activator production was not affected by 0.5 mmol dbcAMP l⁻¹ ($P > 0.10$), but was affected by time, increasing ($P < 0.05$) by 48 h, remaining unchanged until 96 h and then declining (Fig. 7a). In contrast, OED decreased ($P < 0.05$) in embryos cultured in 0.5 mmol dbcAMP l⁻¹, although duration of culture and the interaction were not significant (Fig. 7b). The results obtained for the effects of DMSO on PA production and OED were similar to those presented in Expt 1.

Fig. 7. (a) Plasminogen activator production and (b) overall embryonic diameter achieved by bovine embryos cultured in Ham's F-12 with 1.5 mg bovine serum albumin ml⁻¹ containing 0 (—) or 0.5 (·····) mmol dibutyryl cAMP l⁻¹. Error mean squares for (a) and (b) are 0.027 and 5802, respectively.

Experiment 3. The percentage of embryos hatching in HF-12 with 1 ng PMA ml⁻¹ and 1% DMSO, and HF-12 with 1% DMSO was 92 (12 of 13) and 92 (12 of 13), respectively. The toxic effect of PMA on embryos observed during the previous experiments were eliminated in this experiment. Phorbol myristate acetate did not ($P > 0.10$) affect PA activity (Fig. 8a), but did increase OED ($P < 0.05$) (Fig. 8b). Plasminogen-activator production did not increase ($P > 0.05$) during the first 72 h but then increased to a plateau at 96 h ($P > 0.05$) (Fig. 8a). Overall embryo diameter increased ($P < 0.05$) at 48 h, stabilized between 72 and 96 h, then decreased ($P < 0.05$) (Fig. 8b). No interaction between treatment and time in culture was observed with regard to PA production and OED.

Discussion

In this study, several known modulators of PA in other systems have been evaluated in cultured day 8 bovine embryos. Oestradiol increases PA activity in human breast carcinoma cell lines

Fig. 8. (a) Plasminogen activator production and (b) overall embryonic diameter achieved by bovine embryos cultured in Ham's F-12 with 1.5 mg bovine serum albumin ml $^{-1}$ containing 1% (—) dimethyl sulfoxide or 1% (.....) dimethyl sulfoxide and 1 ng phorbol 12-myristate 13-acetate ml $^{-1}$. Error mean squares for (a) and (b) are 0.013 and 5945, respectively.

MCF-7 (Dickerman *et al.*, 1989); retinoic acid stimulates PA production in chick embryo fibroblasts and muscle cells (Wilson & Reich, 1978; Miskin *et al.*, 1978); progesterone and dexamethasone have been shown to stimulate the production of an inhibitor that decreases plasminogen activation in several tissues including porcine endometrium (Fazleabas *et al.*, 1982), rat granulosa cells (Harlow *et al.*, 1987) and bovine Sertoli cells (Coombs & Jenkins, 1988). However, in the present study, neither progesterone, oestradiol, dexamethasone nor retinoic acid affected the production of PA by cultured bovine embryos. Furthermore, these modulators did not affect overall embryo diameter nor hatching rate. There are three possible explanations for the lack of effect of these hormones on preimplantation bovine embryos. The first is that hormones affect embryos indirectly through the stimulation of genital tract secretions; the second is that the embryo synthesizes its own hormones, which may act in autocrine fashion to regulate early developmental events (Paria & Dey, 1990); and the third is that the embryo has the ability to achieve normal development to the blastocyst stage *in vitro* or in the oviduct of other species regardless of their hormonal status, signifying a considerable autonomy in early embryonic development (Betteridge & Flechon, 1988). The normal growth of cultured bovine embryos observed in this study supports the existence of an endogenous control mechanism that contributes to the independence of the embryo from maternal hormones during *in vitro* development.

Although PA production by cultured bovine embryos was not affected by the hormone treatments, a substantial and consistent increase in this enzyme during culture was observed in all experiments. Plasminogen-activator production was relatively low during the first 48 h of culture, increasing at 72 h to reach a maximum by 96 h and then remaining at this rate. Some slight deviations from this pattern were noted with dexamethasone, where PA production decreased toward the end of culture, and with oestradiol, where maximum PA production was reached at 72 h. PA production by embryos cultured in the presence and absence of oestradiol was lower than

that observed in the other experiments. Kaaekuahiwi & Menino (1990) observed that the source of the embryos significantly affected OED and PA production by bovine embryos developing *in vitro*. As different cows were used as embryo donors for every experiment, it is possible that variation in PA production between experiments may be due to differences in embryo donors.

Dibutyl cAMP, a known inducer of cAMP-dependent protein kinase A, has been found to stimulate the production of PA in various types of cell (Beers *et al.*, 1975; Hettle *et al.*, 1986; Queenan *et al.*, 1987) by promoting the transcriptional activity of the PA gene (Nagamine *et al.*, 1983; Roesler *et al.*, 1988). In this study, although dbcAMP did not affect PA production, a progressive decrease in OED was observed. This inhibitory effect persisted, but to a lesser extent, when the dbcAMP concentration was reduced tenfold. Fisher & Gunaga (1975) have reported that the development of mouse blastocysts was retarded when cAMP accumulation was induced by theophylline, an inhibitor of intracellular breakdown of cAMP. High concentrations of cAMP are generally associated with inhibition of cellular proliferation (Bombik & Burger, 1973), presumably through inhibition of the rate of replication (Thomas *et al.*, 1973). In contrast, Manejawala *et al.* (1986) and Manejawala & Schultz (1989) demonstrated that cAMP analogues, which activate the cAMP-dependent protein kinase, stimulate the rate of blastocoel expansion and Na^+ uptake by mouse preimplantation embryos, suggesting a possible function for cAMP in blastocoel formation. The discrepancy between the observations of Manejawala *et al.* (1986) and Manejawala & Schultz (1989) and our results could be explained by differences in the duration of culture. Whereas embryos in this study were cultured for 5 days, Manejawala *et al.* (1986) and Manejawala & Schultz (1989) incubated embryos for only 8 h. Furthermore, our results with bovine embryos demonstrated that dbcAMP was without effect on OED during the first 24 h and that the inhibitory effect of dbcAMP did not appear until the later stages of culture.

Phorbol 12-myristate 13-acetate, a potent tumour promotor, has been found to enhance PA activity in many cell types (Wigler *et al.*, 1970; Vassalli *et al.*, 1977; Quigley, 1979), through the activation of phospholipid-dependent protein kinase C (Castagna *et al.*, 1982). The possibility that the embryo uses protein kinase C in regulating PA production was therefore investigated. Although PMA at a concentration not lethal to bovine embryos inhibited PA production during the first 72 h of culture, this inhibitory effect was due to DMSO which was used to dissolve the PMA in the culture medium. Results from the first and second experiments comparing the effect of DMSO to the control treatment suggests that this polar solvent inhibited PA production, particularly during the first 72 h of culture. Phorbol myristate acetate itself neither potentiated nor decreased PA production. Carlsen (1987), however, found that DMSO concentrations up to 2.5% greatly stimulated the production of PA in Chinese hamster ovary cells, and that new protein and RNA synthesis were required for this process. The inhibitory effect of DMSO on PA production observed in this study was not accompanied by changes in embryo viability. The reason for this inhibitory effect of DMSO requires further investigation. Embryos cultured in the presence of high concentrations of PMA failed to hatch, showed signs of degeneration within 24 h, and completely degenerated by 72 h of culture, whereas PMA at low concentrations significantly stimulated embryonic diameter. Phorbol esters have been shown to stimulate RNA synthesis (Sivak & Van Duuren, 1970), increase cell division (Driedger & Blumberg, 1977), enhance protein synthesis (Hiwasa *et al.*, 1982), and increase the specific activity of cell membrane-associated $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity (Sivak *et al.*, 1972). Ultimately, these processes would lead to an increase in embryonic diameter.

The physiological role of PA in early bovine embryogenesis is not known. Endodermal cells of the bovine embryo begin to migrate on day 8 and their migration involves the interaction with an extracellular matrix (Betteridge & Flechon, 1988). Plasminogen activator, particularly uPA, has been implicated in the migration of cultured cells (Ossowski *et al.*, 1975) as well as parietal endoderm in mouse embryos (Strickland *et al.*, 1976). Accordingly, PA released by bovine embryos may play a role in facilitating cellular migration during this preimplantation period. Hatching also occurs on day 10 and PA may be involved in inducing a sublysis of the zona pellucida to allow the blastocyst to escape.

In conclusion, bovine preimplantation embryos developing *in vitro* release an appreciable amount of PA, which is neither dependent upon nor influenced by hormonal regulation. With the exception of PMA and dbcAMP, the hormones tested here showed no effect on overall embryonic diameter. Further research is required to elucidate the function of PA during the early stages of bovine embryo development.

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