HAMMOND MEMORIAL LECTURE*

New concepts of the control of corpus luteum function

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Summary. Five new concepts concerning the control of corpus luteum function in the cow have been developed in recent years.

(1) Prostacyclin (PGI-2) plays a luteotrophic role. Conversely, products of the lipoxygenase pathway of arachidonic acid metabolism, particularly 5 hydroxy-eicosatetraenoic acid (5-HETE), play luteolytic roles.

(2) Luteal cells arise from two sources. The small luteal cells are all of theca cell origin; the large cells found early in the cycle (Days 2–6) are mainly of granulosa cell origin. However, a population of large cells found after Day 10 of the cycle are of theca cell origin.

(3) Oxytocin of luteal cell origin plays a role in development of the corpus luteum and possibly in its regression.

(4) The recently described Ca^{2+} -polyphosphoinositol-protein kinase C second messenger system, as well as the LH-cAMP system, is involved in control of progesterone synthesis in the bovine corpus luteum.

(5) Progesterone synthesis in the small theca-derived luteal cells is primarily controlled by the cAMP system. However, elevated intracellular calcium diminishes cAMP-mediated progesterone synthesis in these cells.

These findings modify our current concepts of the mechanisms of control of progesterone secretion by the corpus luteum and suggest several new lines of research.

Introduction

It seems most appropriate that the John Hammond Lecture for 1985 be devoted to a discussion of research on the control of functions of the corpus luteum—and in particular the bovine corpus luteum—for this subject was of great interest to Sir John. He (Hammond, 1927) recognized that removal of the corpus luteum (CL) from the ovary of the cow is followed within a few days by oestrus and ovulation, after which the rhythm of the cycle is re-set. Much of the research to be discussed in this lecture is based on this finding. Hammond was one of the first to be fully aware of the importance of the CL and progesterone secretion in normal fertilization, embryo transport and embryo survival (Hammond, 1953). Corpora lutea of sheep and cows have been subjected to intensive investigation during the past 25 years, as attested by a number of recent reviews (e.g. McCracken, Baird & Goding, 1971; Hansel, Concannon & Lukaszewska, 1973; Baird, 1978; Hansel & Convey, 1983; Inskeep, 1986). During the past 5 years some truly new ideas have begun to emerge, and these are the subject of this lecture.

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Current concepts

By 1980, when the experiments to be described were begun, the following facts concerning the control of luteal function in cows were generally accepted.

(1) Luteinizing hormone (LH) is the major (and probably the sole) pituitary luteotrophin (Hansel, 1967; Hoffman *et al.*, 1974).

(2) The uterus is essential for luteolysis and appears to exert its effects by a local mechanism (Armstrong & Hansel, 1959; Brunner, Donaldson & Hansel, 1969; Ginther, 1974).

(3) Prostaglandin (PG) F-2 α of uterine origin reaches the ipsilateral CL-bearing ovary by a veno-arterial transfer mechanism resulting in luteal regression (Hansel *et al.*, 1973; Ginther, 1974). However, veno-arterial transfer of PGF-2 α , a phenomenon that appears to be established for the ewe (Baird, 1978), remains questionable for the cow. Indeed, two publications on the subject from our own group have resulted in opposite conclusions (Hixon & Hansel, 1974; Milvae & Hansel, 1980a).

At the same time, however, there were a number of disconcerting facts for which we have no ready explanation. For example, numerous studies attested to the fact that the CL of the cow itself produces relatively large amounts of all of the prostanoids, including PGF-2 α , raising a question as to why the CL needs to rely on PGF-2 α of uterine origin for its regression. Unlike the ewe in which 99% of injected PGF-2 α is metabolized in a single passage through the lungs, substantial amounts of injected PGF-2 α pass through the lung of the cow without metabolic breakdown. Davis *et al.* (1984) found that $35.0 \pm 2.3\%$ of injected tritium-labelled PGF-2 α survived a first passage through the lungs and $15.7 \pm 6.9\%$ survived three circulations. Thus, PGF-2 can have a systemic effect in the cow.

Furthermore, administration of PGF-2 α to dispersed bovine luteal cells *in vitro* resulted in a clear-cut, although small, increase in progesterone production, rather than the expected decrease (Hixon & Hansel, 1979). Other observations that are difficult to reconcile with the above concepts were discussed by Hansel & Fortune (1978).

New concepts

At least five exciting new concepts concerning the control of CL function have been developed during the past 5 years. We plan to examine the evidence for each of these concepts and to attempt to integrate them into our current views of luteal function.

Concept 1: prostacyclin (PGI-2) plays a luteotrophic role, while products of the lipoxygenase pathway of arachidonic acid metabolism, such as 5-hydroxyeicosatetraenoic acid (5-HETE), play luteolytic roles

This concept, as recently outlined by Milvae (1986), is supported by considerable evidence. In our initial experiment with PGI-2 (Milvae & Hansel, 1980b), it was found that injections of this compound directly into the bovine corpus luteum on Day 10 of the oestrous cycle caused a remarkable increase in jugular vein progesterone concentrations within 5 min. The rise persisted for about 14 h, despite the relatively short biological half-life of the compound. Similar injections of the stable PGI-2 metabolite, 6-keto-PGF-1 α , or saline were without effect. Injections of PGI-2 had no effect on plasma LH concentrations, indicating that the luteotrophic effects were not mediated by increased LH secretion. Similarly, in *in-vitro* experiments, PGI-2 added to dispersed bovine luteal cells caused a dose-related increase in progesterone synthesis (Milvae & Hansel, 1980b).

Studies on the biosynthesis of PGI-2 by luteal tissue during the oestrous cycle (Milvae & Hansel, 1983) showed that CL collected early in the cycle (Day 5) have higher initial contents and synthesize more PGI-2 (as indicated by measurements of 6-keto-PGF-1 α) than do luteal tissues collected at all other stages of the cycle (Fig. 1). The ratio of 6-keto-PGF-1 α to PGF-2 α synthesis by the CL was 4.4,

Fig. 1. The biosynthesis of 6-keto-PGF-1 α by bovine luteal cells collected from heifers on different days of the oestrous cycle and after a variety of in-vivo treatments. (From Milvae, 1986.)

2.3, 1.3 and 1.1 on Days 5, 10, 15 and 18 of the cycle, respectively. Progesterone production *in vitro* declined as the CL aged and the concentration of PGI-2 declined. However, in pregnant animals the high levels of PGI-2 characteristic of the early cycle were maintained.

Studies with the prostaglandin synthetase inhibitor, indomethacin, which blocks the production of all products of the cyclooxygenase pathway including PGF-2 α , PGE-2, PGI-2 and PGA-2, also support the concept of a luteotrophic role for PGI-2. Intrauterine administration of indomethacin late in the oestrous cycle (Days 14–21) prolongs the lifespan of the CL, presumably by inhibiting the production of the luteolysin PGF-2 α (Lewis & Warren, 1977). However, intrauterine administration of this drug during early stages of the cycle (Days 4, 5, 6) inhibits luteal development, shortens the lifespan of the CL and decreases oestrous cycle length (Milvae & Hansel, 1985).

The data suggest that a product of the cyclooxygenase pathway is required for normal early development of the CL. The bovine CL has more PGI-2 synthetase activity than any other tissue that has been studied (Sun, Chapman & McGuire, 1977). This fact, along with the data cited above and the importance of PGI-2 in regulating cAMP levels, in causing vasodilatation and in regulating blood flow, has led us to suggest that PGI-2 plays a major regulatory role. PGE-1 has been reported to have similar effects, possibly because it acts through the same receptors, in some tissues (Abramowitz & Birnbaumer, 1979).

With the rapid evolution of knowledge concerning products of the lipoxygenase pathway of arachidonic acid metabolism (Fig. 2), it became apparent that some of these compounds might be involved in controlling CL function. Among the 5-, 9-, 10-, 11-, 12- and 15-hydroxy acid enzyme systems, the 5- and the 15- systems are of most interest. The 5-lipoxygenase pathway gives rise to the leukotrienes, while the 15-lipoxygenase pathway gives rise to lipoxins A and B, compounds whose physiological properties have not yet been well documented.

Since it is possible to form compounds of both the 5- and 15-series by reacting arachidonic acid with soybean lipoxidase (Goodman & Weigle, 1980), our first experiment in this area involved addition of these reaction products to dispersed bovine luteal cells to determine their effects on the biosynthesis of progesterone, PGI-2 and PGF-2 α . Somewhat surprisingly, the presence of these products of the lipoxygenase pathway caused a dose-related reduction ($P < 0.05$) in the synthesis of progesterone and PGI-2, but not of PGF-2 α (Milvae, Alila & Hansel, 1986).

In a second experiment, addition of 5-HETE to dispersed luteal cells produced the same effects. However, 5-HETE, even at very high levels, was less effective than the combined products of soybean lipoxidase in inhibiting PGI-2 and progesterone synthesis, suggesting that products of the 15-lipoxygenase pathway could also be involved in luteolysis.

Fig. 2. A simplified diagram of the oxidative metabolism of arachidonic acid. PG = prostaglandin; TX = thromboxane; EET = epoxy-eicosatrienoic acid; HPETE = hydroperoxyeicosatetraenoic acid; HETE = hydroxyeicosatetraenoic acid; LT = leukotriene.

As indicated previously, bovine luteal tissue possesses the ability to synthesize all of the major products of the cyclooxygenase pathway. However, luteal tissue may lack the ability to synthesize 5-HETE and other products of the 5-lipoxygenase pathway. In a preliminary experiment, three CL were collected, dispersed with collagenase and incubated for 2 h. Initial and final concentrations of 5-HETE in the medium were assayed. The initial content was high (39 ± 5 ng/ 10^6 luteal cells), but no measurable biosynthesis occurred during incubation.

In an attempt to examine the role of products of the lipoxygenase pathway *in vivo*, 7 normal Holstein heifers were given intrauterine infusions of nordihydroguaiaretic acid (NDGA), an inhibitor of the lipoxygenase pathways, twice daily on Days 14 through 18 of the oestrous cycle. Seven control heifers received intrauterine infusions of the vehicle during the same time. The results, shown in Fig. 3, indicated that NDGA delayed luteolysis and lengthened the oestrous cycle (27.2 ± 0.3 compared with 21.5 ± 1.0 days for controls, $P < 0.05$). NDGA appears to be a specific inhibitor of the lipoxygenase system; when added to dispersed bovine luteal cells it caused a slight increase, rather than a decrease, in products of the cyclooxygenase pathway (Milvae *et al.*, 1986).

In a follow-up experiment (Milvae, 1986), designed to determine whether this prolongation in luteal function was related to an increased ability of the CL of the treated animals to synthesize PGI-2, groups of 5 heifers each were given intrauterine infusions of NDGA or saline twice daily on Days 14, 15, 16 and 17 and once on Day 18 of the cycle. Luteal cells from CL collected 4 h after the last NDGA infusion and incubated *in vitro* contained more and produced 8 times more 6-keto-PGF-1 α during a 2-h incubation than did cells from CL collected from control heifers (Fig. 1).

These data strongly suggest a role for products of the lipoxygenase system in luteal function in normal animals. Taken together with the indications cited above that the CL contains, but may not be able to synthesize, 5-HETE, the results further suggest that lipoxygenase products such as 5-HETE may be important uterine luteolysins. Similar studies show that ovulation in the rat also requires lipoxygenase products and that lipoxygenase activity in the follicle is stimulated by gonadotrophins (Reich, Kohen, Slager & Tsafiriri, 1985).

Fig. 3. Plasma concentrations (mean $\pm$ s.e.) of progesterone in heifers (7/group) receiving intrauterine nordihydroguaiaretic acid (NDGA) ($\circ$ — $\circ$) or vehicle ($\bullet$ — $\bullet$) twice daily on Days 14–18 of the oestrous cycle. (From Milvae, Alila & Hansel, 1986.)

Concept 2: luteal cells arise from two sources; the small luteal cells are of thecal origin and the large cells found in early (Day 2–6) CL are of granulosa cell origin, but by Day 10 of the cycle nearly half of the large cells are of thecal origin

Donaldson & Hansel (1965) suggested, on the basis of histological studies, that bovine luteal cells are derived both from granulosa and theca cells and that theca-derived cells can become large cells. Later studies (Ursely & Leymarie, 1979; Koos & Hansel, 1981) based on separation of collagenase-dispersed luteal cells showed that the dissociated cells were of three size groups as follows: (1) large luteal cells ($>25\ \mu\text{m}$ in diam.), (2) small luteal cells ($10\text{--}20\ \mu\text{m}$ in diam. and (3) abundant smaller non-steroidogenic cells ($<10\ \mu\text{m}$ in diam.). The large luteal cells (Fig. 4) have round central nuclei with dispersed chromatin and a distinct nucleolus, two types of mitochondria, extensive smooth endoplasmic reticulum, highly convoluted cell surfaces and contain small ($0.3\ \mu\text{m}$) electron dense granules (Fig. 5).

The small luteal cells have acentric, cup-shaped nuclei with heterochromatin lining the nuclear envelope, both smooth and rough endoplasmic reticulum, pleiomorphic mitochondria which contain tubular cristae and which are arranged in an arc opposite the nucleus and a central region, usually containing a pair of centrioles and a large Golgi complex (Fig. 4). Clumps of small cells are held together by extensively interdigitated microvilli.

Although the large and small cells produce progesterone *in vitro*, the small cells are at least 6 times more sensitive to added LH than are the large cells (Koos & Hansel, 1981). Indeed, in the ewe, in which similar large and small luteal cells are found, Fitz, Mayan, Sawyer & Niswender (1982) showed that the small cells contain the majority of the LH receptors (33 260/small cell compared with 3074/large cell), while the large cells contain the majority of the prostaglandin F-2a receptors (68 143/large cell and 2115/small cell).

We have used highly specific labelled monoclonal antibodies to theca and granulosa cell surface antigens in an attempt to trace the contributions of the theca and granulosa to the bovine corpus luteum during the oestrous cycle and pregnancy (Alila & Hansel, 1984). Binding of each antibody to

Fig. 4. Bovine luteal cells on Day 5 of the cycle. The large cells (LL) contain many electron-dense granules and several mitochondria in their cytoplasm. The small cells (SL) have few mitochondria and lack granules. $\times 3610$.

Fig. 5. Electron-dense granules (arrows) at the periphery of a large luteal cell at Day 19 of the cycle. The plasma progesterone concentration in this animal had already declined to 1 ng/ml. The abundant smooth endoplasmic reticulum (SER) is intact in both cells shown. $\times 13490$.

collagenase-dispersed luteal cells from 18 cyclic and 14 pregnant animals was examined by indirect immunofluorescence. The results indicated that the large cells of early cyclic CL express an antigen present in granulosa cells, while the small cells at all stages of the cycle express an antigen of thecal origin. However, small (theca-derived) cells appear to develop into large cells as the age of the CL increases. When the granulosa cell antibody was used (Fig. 6a), the percentage binding of large cells

Fig. 6. Percentages of large and small bovine luteal cells binding (a) the granulosa cell antibody and (b) the theca cell antibody during the oestrous cycle and pregnancy. Values are mean $\pm$ s.e.m. for the no. of cows indicated. (From Alila & Hansel, 1984.)

decreased ($P < 0.01$) with increasing age of the CL, 77 ± 6 , 47 ± 3 , 30 ± 2 for Days 4-6, 10-12 and 16-18 respectively. The small cells were not bound by the granulosa antibody, except for a very small percentage on Days 4-6, and morphologically these cells appeared to be small granulosa cells. No large cells bound the granulosa cell antibody after the 100th day of pregnancy.

In contrast, when the thecal antibody was used (Fig. 6b) the proportions of large cells labelled increased ($P < 0.01$) between Days 4-6 ($10 \pm 1.3\%$) and 10-12 ($46 \pm 3\%$), and remained high during the remainder of the cycle. The percentage of large cells bound to the thecal antibody remained unchanged until mid-pregnancy, and then declined as pregnancy advanced.

It is unlikely that the surface antigens of granulosa-derived large cells changed to resemble thecal antigens during their development. Such an antigenic change should have been detected, at least in some cells, when theca antibodies and granulosa antibodies were used simultaneously. The antibodies did not recognize the same antigenic determinants, and if such a change occurred, at least

some cells in the process of such an antigenic conversion should have been labelled by both antibodies. An instantaneous antigenic change seems unlikely. Also, if such a conversion occurred, the decline in binding of granulosa antibodies to large cells would have been accompanied by a compensatory increase in the percentage of large cells bound to the theca antibody; this was not the case.

These observations may therefore be interpreted as indicating that small luteal cells of theca cell origin differentiate into large cells as the cycle progresses and, after the 100th day of pregnancy, all cells in the corpus luteum are of thecal origin. Collectively, these findings suggested to us that steroidogenesis in the theca-derived and the granulosa-derived cells may be controlled by different mechanisms, and a great deal of effort is currently being expended in studies designed to understand these mechanisms and their interrelationships.

Concept 3: oxytocin of luteal cell origin plays a role in CL development and regression

Interest in the role(s) of oxytocin in CL formation and function stems from the report of Armstrong & Hansel in 1959 that administration of pharmacological doses of oxytocin during a critical period early in the oestrous cycle (Days 2–6) resulted in inhibition of CL development and shortened oestrous cycles. These effects occurred only when oxytocin was administered during this critical period early in the cycle and the presence of the uterus was required for its manifestation. This report is sometimes quoted as being the first to show that oxytocin is luteolytic in the cow (Schallenberger, Schams, Bullermann & Walters, 1984). In fact, only an “anti-luteotropic” effect was claimed. Administration of oxytocin in this way has no effect on plasma LH concentrations (Wilks & Hansel, 1971), indicating that the oxytocin effect is not a result of decreased gonadotrophin secretion.

Recent research tends to support the idea that the ability of oxytocin given early in the cycle (Days 2–6 or 4–6) to interfere with normal CL development is due to its inhibition of luteotropic processes. Oxytocin is produced in the large granulosa-derived cells of the cow CL and luteinization appears to be an important step in increasing its production (Guldenaar, Wathes & Pickering, 1984; Kruip, Vullings, Schams, Jonis & Klarenbeek, 1985; Schams, Kruip & Koll, 1985a).

Interestingly, expression of the oxytocin gene in the bovine CL is first detected early in the oestrous cycle, at approximately the same time that administration of large amounts of exogenous oxytocin inhibit CL development and progesterone synthesis (Ivell, Brackett, Fields & Richter, 1985). Exogenous PGF-2 α is incapable of causing premature luteal regression during this same period. Plasma concentrations of oxytocin increase until mid-cycle (Days 8–13) and then decline. Pulses of oxytocin and progesterone occur concomitantly during this time (Schallenberger, Schams, Bullerman & Walters, 1984). Very low concentrations are found in pregnant animals (Schams *et al.*, 1985a). Collectively, these observations suggest that large amounts of oxytocin administered during the critical period (Days 4–6) may inhibit CL development and progesterone secretion by interfering with these normal mechanisms for oxytocin synthesis or release.

Further support for the concept derives from the fact that oxytocin treatment during the critical period depresses the ability of the CL to produce the potent luteotrophin, PGI-2, as indicated by the data shown in Table 1. In this experiment, 150 USP units of oxytocin (N = 5) or saline (N = 5) were injected subcutaneously twice daily on Days 4–6 inclusive and the CL were harvested on Day 7. The cells were dispersed with collagenase, incubated for 2 h and the biosynthesis of PGI-2 (expressed as 6-keto-PGF-1 α), progesterone and PGF-2 α was determined. Although the ability of the cells to produce progesterone had not yet been compromised, their ability to respond to LH was already diminished and their ability to synthesize PGI-2, but not PGF-2 α , was markedly impaired ($P < 0.05$). It therefore appears that the mechanism by which administration of these large amounts of oxytocin during Days 4–6 inclusive inhibits luteal development in the cow involves either an inhibition of the enzymes necessary for prostacyclin synthesis or excessive production of lipoxygenase products, such as 5-HETE, which inhibit prostacyclin synthesis.

Table 1. Net biosynthesis of progesterone, PGF₂ α and 6-keto-PGF₁ α (ng/10⁶ cells/2 h) by bovine luteal cells obtained from oxytocin- (150 USP units twice daily; N = 5) or saline- (N = 5) treated Holstein heifers

	Oxytocin	Control
Progesterone	176 $\pm$ 39	177 $\pm$ 28
Progesterone (+ 5 ng LH)	357 $\pm$ 49*	436 $\pm$ 64
6-keto-PGF-1 α	1.60 $\pm$ 0.11**	2.99 $\pm$ 0.18
PGF-2 α	0.26 $\pm$ 0.05	0.39 $\pm$ 0.06

Oxytocin was injected subcutaneously on Days 4–6 inclusive and CL were harvested on Day 7. Compared with control value: * $P < 0.10$; ** $P < 0.05$.

Table 2. The effects of oxytocin with or without LH on net progesterone production by dispersed bovine luteal cells (ng/10⁶ cells)

Oxytocin	No. of CL	– LH	+ LH (5 ng/ml)
None (control)	4	156 $\pm$ 40	480 $\pm$ 74
10 ng	4	96 $\pm$ 38	368 $\pm$ 81
100 ng	4	112 $\pm$ 86	389 $\pm$ 78
1000 ng	4	104 $\pm$ 42	278 $\pm$ 65
10 000 ng	4	98 $\pm$ 30	421 $\pm$ 43

Oxytocin, neurophysin and progesterone appear to be secreted simultaneously from the CL during the early and mid-luteal phases of the cycle (Schams *et al.*, 1985b). Oxytocin added to dispersed bovine luteal cells (Day 10) and incubated for 2 h, as described by Milvae & Hansel (1983), had little effect on progesterone synthesis (Table 2), suggesting that the hormone does not have a direct effect on the CL.

There is a widely held view that oxytocin released from the CL reaches the uterus where it causes the release of PGF-2 α which, in turn causes luteal regression (McCracken, Schramm & Okulicz, 1984; Schams *et al.*, 1985a). However, if this were the case, one would expect that exogenous oxytocin given at mid-cycle or later would cause premature luteal regression. This is not the case for the cow, since injections given at any time other than the critical period do not cause luteal regression (Hansel & Wagner, 1960). Furthermore, indomethacin (40 mg/day) infused into the uterine lumen was without effect on oxytocin-induced inhibition of luteal function (Milvae & Hansel, 1985).

However, exogenous oxytocin does cause the release of large amounts of PGF-2 α into the uterine vein in the cow. Milvae & Hansel (1980a) were unable to show that this release of PGF-2 α was reflected in concurrently collected ovarian arterial blood samples. The physiological significance of the uterine release of PGF-2 α in response to exogenous oxytocin in the cow therefore remains unclear.

Conversely, administration of luteolytic doses of PGF-2 α to the cow at Day 12 of the cycle causes an immediate release of oxytocin from the CL into the blood stream, followed by a marked decline in blood levels lasting for 3–5 h. It is not known whether a rise in PGF-2 α in blood reaching the ovary is a stimulus to oxytocin release at the time of luteolysis in normal animals (Schallenberger *et al.*, 1984).

In the ewe, elevated progesterone concentrations prevent induction of uterine oxytocin receptors, and the rise in uterine oxytocin receptors at oestrus is closely linked to declining levels of

progesterone (Sheldrick & Flint, 1985). This observation may explain the inability of exogenous oxytocin administered during the luteal phase to cause luteal regression but, by the same token, it makes it difficult to envisage oxytocin of either ovarian or hypothalamic origin as the initiator of luteolysis. In a recent study, Flint & Sheldrick (1985) found that continuous intravenous infusion of oxytocin (3 µg/h) into ewes between Days 13 and 21 of the cycle maintained luteal function and lengthened the cycle by 7 days. The low levels of uterine oxytocin receptors found in these ewes at Day 17 were either a reflection of the fact that progesterone levels were maintained, or of down regulation of oxytocin receptors.

In the ewe, both active and passive immunization against oxytocin cause a delay in luteal regression and prolong the length of the oestrous cycle (Sheldrick, Mitchell & Flint, 1980; Schams, Prokopp & Barth, 1983). Active immunization also delays luteolysis in the goat (Cooke & Homeida, 1985). To the best of our knowledge there is no report of a similar effect in the cow. These reports would appear to offer proof for a role of oxytocin in luteolysis in the ewe and goat. However, in the studies of Schams *et al.* (1983), oxytocin antibodies had no effect on cyclicity in some animals. In fact, only half of the ewes passively immunized with oxytocin had prolonged cycles; the remainder cycled normally despite free (unbound to the antibody) oxytocin concentrations in plasma similar to those of the animals that had prolonged cycles.

Results such as these focus attention on the need for more adequate controls in passive immunization experiments. Serum from normal animals is not the most appropriate vehicle for control animals since there are reports indicating that anti-Freunds' adjuvant (anti-FCA) serum can inhibit reproductive functions. For example, Reimers, Sluss & Seidel (1977) obtained significant reductions in both pregnancy rates and in fetuses per treated animal in pregnant mice treated with anti-FCA serum.

Concept 4: the Ca^{2+} -polyphosphoinositol-protein kinase C second messenger system, as well as the LH-cAMP system, is involved in control of progesterone synthesis in the CL of the cow

Nishizuka (1984), amongst others, recently described a new second messenger system by which hormones may pass information from the surface to the interior of target cells by two routes—protein kinase C activation and Ca^{2+} mobilization. Both routes are usually activated by the binding of the hormone to its receptor and subsequent phospholipase C hydrolysis of membrane-bound phosphatidylinositol 4,5-bisphosphate, yielding diacylglycerol and inositol 1,4,5-trisphosphate. The diacylglycerol activates protein kinase C in the presence of membrane phospholipid. The inositol 1,4,5-trisphosphate mobilizes intracellular Ca^{2+} . Both actions are usually necessary for a physiological response.

We (Shemesh, Hansel & Strauss, 1984) found that bovine placental cells (from fetal cotyledons and maternal caruncles) secrete progesterone by a calcium-dependent and cyclic nucleotide-independent mechanism. This finding prompted us to examine the possibility that a similar mechanism may exist in one or more of the bovine luteal cell types, and preliminary data suggest that such may be the case.

We have examined the role of calcium ionophores and agents specific for the C-kinase pathway in progesterone synthesis by total dispersed bovine luteal cell preparations from CL collected at three stages of the oestrous cycle. Corpora lutea were collected at Days 4–6, 9–10 or 16–17 of the cycle and dissociated with collagenase. Each treatment was performed on four replicate samples containing 5×10^5 luteal cells in 1 ml M199. Media were collected 2 h after the addition of treatments and assayed for progesterone. The data shown in Table 3 indicate that C-kinase is involved in progesterone synthesis. Addition of the phorbol ester, phorbol-12 myristate-13-acetate (TPA), an activator of C-kinase, at 50 and 100 nM (data for 100 nM not shown), to dispersed total luteal cell preparations resulted in an increased ($P < 0.05$) progesterone synthesis at Days 9–10 and 16–17, but had no effect on cells of CL at Day 4–6 of the cycle. LH added at levels of 1 and 5 ng (data for 5 ng not shown) was

Table 3. Effects of LH, phorbol-12-myristate-13-acetate (TPA), and the Ca^{2+} ionophore, A 23187, alone and in combination on net progesterone secretion by 5×10^5 luteal cells during 2-h incubations

	Treatments					
	LH (1 ng)	TPA (50 nM)	A 23187 (0.2 μM)	TPA + A 23187 (50 nM + 0.2 μM)	TPA + LH (50 nM + 1 ng)	A 23187 + LH (0.2 μM + 1 ng)
Day 4–6	100 ^a (0.15)	86 ^a (0.12)	61 ^a (0.06)	85 ^a (0.20)	107 ^a (0.21)	124 ^a (0.30)
Day 9–10	240 ^{a,b} (0.27)	171 ^{a,b} (0.26)	97 ^b (0.10)	199 ^{a,b} (0.47)	352 ^{c,d} (0.31)	374 ^c (0.45)
Day 16–17	292 ^{a,b,c} (0.62)	173 ^{a,b} (0.35)	97 ^b (0.13)	205 ^{a,b,c} (0.38)	348 ^{a,c} (1.09)	400 ^c (0.86)

Results are expressed as % $\pm$ s.e. (in parentheses) of control incubations. All values represent data from 4 or more corpora lutea. Data were analysed by one-way analysis of variance and Duncan's multiple range tests. Values identified by different subscripts are significantly different ($P < 0.05$) from one another.

also without effect in cells collected at Days 4–5, perhaps due to the high proportion of large cells present at this time, but was effective in increasing progesterone synthesis in cells collected at Day 6 or later. In general, the responses to TPA at Days 9–10 and 16–17 paralleled the C-kinase activities measured at these times (data not shown). Brunsuig *et al.* (1986) have recently found a similar stimulation of progesterone synthesis by dispersed bovine luteal cells by a phorbol ester.

The Ca^{2+} ionophore, A23187, had no effect on progesterone synthesis at any stage of the cycle. However, the ionophore enhanced the ability of 1 ng LH/ml to increase progesterone synthesis at Days 9–10 ($P < 0.05$).

The results suggested that C-kinase may be important in at least one type of luteal cell, while the Ca^{2+} ionophore may have a stimulatory effect by activating C-kinase, as well as an inhibitory effect by depressing cyclic AMP levels in one or more cell types.

Concept 5: steroidogenesis in the small theca-derived cells is primarily controlled by cAMP-dependent mechanisms, and elevated intracellular Ca^{2+} inhibits progesterone synthesis in these cells

Studies are underway to determine the roles of cyclic AMP and the Ca^{2+} -polyphosphoinositol-C-kinase second messenger systems in progesterone synthesis in each type of luteal cell. To date, limited data are available for the small, theca-derived cells, which are known to possess most of the LH receptors and few of the PFG-2a receptors found in the CL.

Corpora lutea obtained at Days 10–12 of the cycle were dissociated with collagenase and the small cell fraction was obtained by unit gravity sedimentation as described by Koos & Hansel (1981). Net progesterone synthesis was measured during 3-h incubations after addition of LH, methyl-isoxanthine (MIX), A23187 and combinations of these additives. Results obtained to date (Table 4) indicate that the small theca-derived cells at this stage of the cycle respond strongly to elements of the cyclic AMP system (LH), and that elevated intracellular Ca^{2+} inhibits LH-stimulated progesterone synthesis. MIX has a dual action in that it elevates intracellular Ca^{2+} and inhibits phosphodiesterase. Addition of MIX failed to cause a significant increase in progesterone biosynthesis over controls in this experiment, perhaps because of its ability to elevate intracellular Ca^{2+} .

It therefore appears that the small theca-derived cells which, because of their large numbers and great sensitivity to LH, contribute a large part of the total progesterone produced by the CL, respond to the cAMP system when intracellular Ca^{2+} concentrations are relatively low. However, factors that drastically elevate intracellular Ca^{2+} serve to turn off progesterone synthesis in these cells. These results are in general agreement with those of Gore & Behrman (1984) who showed that an influx of

Table 4. Effects of LH, methyl-iso-xanthine (MIX), and the Ca^{2+} ionophore, A 23187, on progesterone synthesis (mean $\pm$ s.e.m.) by small bovine luteal cells (Days 10–12) during 3-h incubations

Treatment	Without A 23187		With A 23187 (0.3 μM)		Significance
	No. of CL	Progesterone (ng/10 ⁶ cells)	No. of CL	Progesterone (ng/10 ⁶ cells)	
Control	7	122 $\pm$ 27	7	60 $\pm$ 16	NS
LH (5 ng)	7	555 $\pm$ 120	3	133 $\pm$ 58	$P < 0.01$
MIX (1 nM)	6	218 $\pm$ 51	4	77 $\pm$ 35	NS

Data were analysed by two-way analysis of variance. A 23187 decreased ($P < 0.01$) progesterone synthesis; LH increased ($P < 0.01$) progesterone synthesis over control and MIX treatment; MIX and control groups were not different ($P > 0.05$).

extracellular Ca^{2+} inhibits activation of adenylate cyclase in rat luteal cells and further suggested that luteal function in this species may occur, in part, by processes that maintain low intracellular Ca^{2+} levels.

At present we have little knowledge of the responses of the large luteal cells collected at various stages of the cycle to either the Ca^{2+} -polyphosphoinositol-C-kinase or the cAMP systems.

Some speculations

These concepts drastically alter our previous views of the control of CL function, but fall short of providing an integrated picture that fully explains all of the species differences and the sometimes contradictory results that have been reported concerning control of luteal function. Nevertheless, they do offer possible explanations for many of our previous results and clearly point the way for future research.

It seems clear that steroidogenesis is controlled by different mechanisms in the several luteal cell types and it is likely that products of one cell type can influence synthesis in other cell types. Our current working hypothesis is that progesterone synthesis in either the granulosa derived or the theca derived large luteal cells (or both) is controlled, at least in part, by the Ca^{2+} -polyphosphoinositol C-kinase system, while synthesis in the numerous small theca-derived cells is primarily controlled by LH and the cAMP system, which is 'turned off' in the presence of high intracellular Ca^{2+} . Protein kinase C may act by selectively activating cytochrome P-450_{sc}, which controls cholesterol side-chain cleavage in the inner mitochondrial membrane, as occurs in the adrenal cortex (Vilgrain, Defaye & Chambaz, 1984) and placentome (Shemesh, Strauss & Hansel, 1985) of cattle.

Within the framework of these general concepts, exogenous or endogenous PGF-2 α might produce its luteolytic effect by a combined action involving its ability to reduce blood flow and its ability to act as a Ca^{2+} ionophore (Campbell, 1983), thus elevating intracellular Ca^{2+} and inactivating cAMP to decrease progesterone synthesis in the small luteal cells.

Our results further suggest that luteolysis may require Ca^{2+} mobilization and activation of an arachidonic acid pool that preferentially supplies the lipoxygenase pathway. Oxytocin may indeed exert its effects at the level of the uterus by activating the Ca^{2+} -polyphosphoinositol-C-kinase system (as appears to be the case for vasopressin), resulting in the mobilization of arachidonic acid that is preferentially metabolized via the 5-lipoxygenase pathway, resulting in an increase in the luteolytic 5-lipoxygenase products and a decrease in synthesis of PGI-2. Elevated intracellular Ca^{2+} associated with exocytosis and the rapid release of oxytocin from the secretory granules in the large

granulosa-derived cells (Fig. 5) in response to exogenous PGF-2 α may represent yet another event in which a prostanoid (PGF-2 α) acts as a Ca²⁺ ionophore.

Clearly, these are all questions that are worthy of investigation and results of such investigations may soon lead to a completely integrated concept that will adequately explain control of corpus luteum function in all species.

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