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Control of Steroidogenesis in Small and Large Bovine Luteal Cells*

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Abstract

Evidence was cited to show that: (1) prostacyclin (PGI_2) plays a luteotrophic role in the bovine corpus luteum and that products of the lipoxygenase pathway of arachidonic acid metabolism, especially 5-hydroxyeicosatetraenoic acid play luteolytic roles; (2) oxytocin of luteal cell origin plays a role in development, and possibly in regression, of the bovine corpus luteum; and (3) luteal cells arise from two sources; the characteristic small luteal cells at all stages of the oestrous cycle and pregnancy are of theca cell origin; the large cells are of granulosa cell origin early in the cycle, but a population of theca-derived large cells appears later in the cycle. Results of *in vitro* studies with total dispersed cells and essentially pure preparations of large and small luteal cells indicate that : (1) the recently described Ca^{2+}–polyphosphoinositol–protein kinase C second messenger system is involved in progesterone synthesis in the bovine corpus luteum; (2) activation of protein kinase C is stimulatory to progesterone synthesis in the small luteal cells; (3) activation of protein kinase C has no effect on progesterone synthesis in the large luteal cells; and (4) protein kinase C exerts its luteotrophic effect in total cell preparations, in part at least, by stimulating the production of prostacyclin. The protein kinase C system may cause down regulation of LH receptors in the large cells.

Introduction and Background

It seems entirely fitting that the James R. Goding Lecture for 1986 be devoted to new knowledge of the control of corpus luteum (CL) function, for it was in this area that he made his major scientific contributions. By about 1980, when the work to be described was begun, the following salient facts concerning the control of corpus luteum function in ruminant animals were generally accepted:

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

(2) The uterus is essential for luteolysis to occur in the normal way and exerts its effect by a local mechanism (Wiltbank and Casida 1956; Armstrong and Hansel 1959).

(3) Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) of uterine origin reaches the ipsilateral CL by a veno-arterial transfer mechanism resulting in luteal regression. This mechanism was established for the sheep by Goding and his co-workers by 1971 (see McCracken *et al.* 1971; Ginther 1974).

However, a number of differences between the mechanisms for the ewe and the cow soon became apparent. In *in vitro* experiments, $PGF_{2\alpha}$ proved to increase progesterone synthesis, rather than decrease it, when added to bovine luteal cells; $PGF_{2\alpha}$ also enhanced

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the progesterone synthesis stimulated by submaximal levels of added LH (Hixon and Hansel 1979). In contrast, $PGF_{2\alpha}$ inhibits adenylate cyclase activity and LH-stimulated P_4 synthesis when added to ovine luteal cells (Fletcher and Niswender 1982). Furthermore, the bovine CL itself produces relatively large amounts of all of the prostanoids, including $PGF_{2\alpha}$, raising a question as to why it would need to rely on $PGF_{2\alpha}$ of uterine origin for its regression. The need for a veno-arterial transfer system for $PGF_{2\alpha}$ of uterine origin in the ewe is thought to be a result of the fact that little or no systemic $PGF_{2\alpha}$ escapes metabolism in the lungs. Indeed, >99% of injected $PGF_{2\alpha}$ is metabolized in a single passage through the lungs of the ewe. However, in the cow Davis *et al.* (1984) found that $35{\cdot}0 \pm 2{\cdot}3\%$ of injected tritium-labelled $PGF_{2\alpha}$ survived a first passage through the lungs and $15{\cdot}7 \pm 6{\cdot}9\%$ survived three circulations. This finding indicates that $PGF_{2\alpha}$ can have a systemic effect in the cow. Results of a recent experiment, in which 7 of 23 cows underwent two or more normal oestrous cycles within 60 days after removal of the horn of the uterus ipsilateral to the functioning ovary, as well as the contralateral ovary (Hansel, unpublished observations), are in agreement with this idea. Other observations difficult to reconcile with the above concepts were discussed by Hansel and Fortune (1978).

Recently, Hansel and Dowd (1986) summarized data supporting five new concepts concerning bovine CL function that have been developed during the past 5 years, as follows:

(1) Prostacyclin (PGI_2) plays a luteotrophic role, while products of the lipoxygenase pathway of arachidonic acid metabolism, in particular 5-hydroxyeicosatetraenoic acid (5-HETE), play luteolytic roles.

(2) Luteal cells arise from two sources; the small luteal cells are all of theca cell origin and large luteal cells found early in the cycle are of granulosa cell origin. However, a population of large cells appears later in the cycle that appears to be of thecal origin.

(3) Oxytocin of luteal cell origin plays a role in development and, possibly, in regression of the CL.

(4) The recently described Ca^{2+}–polyphosphoinositol–protein kinase C second messenger system is involved in control of progesterone synthesis in the CL.

(5) Progesterone synthesis in the small theca-derived cells is primarily controlled by the LH–cAMP system, but elevated intracellular calcium decreases cAMP-mediated progesterone synthesis in these cells.

These findings have greatly modified our concepts of the mechanisms of control of luteal cell function and explain some of the apparently contradictory data found in the literature. However, a completely integrated concept of the control mechanisms will clearly require a better understanding of the nature of the factors regulating steroidogenesis in the individual luteal cell types and the interactions among the cells; these are the subjects of this communication.

Characterization and Derivation of Luteal Cell Types

Three main cell types are found in collagenase-dispersed preparations (Ursely and Leymaire 1979; Koos and Hansel 1981) of the bovine CL: (*a*) small (<10 μm in diameter) non-steroidogenic cells which consist mainly of vascular cells (endothelial cells, erythocytes and leukocytes) and connective tissue cells such as fibrocytes; (*b*) the small steroidogenic cells (10–20 μm diameter); and (*c*) the large steroidogenic cells (>25 μm diameter). Other intermediate cells have been described in early CL tissues (McNutt 1924; Hofliger 1948; Foley and Greenstein 1958; Gier and Marion 1961; Priedkalns and Weber 1968). These intermediate cell types have not been observed in sheep (O'Shea *et al.* 1979).

Both small and large steroidogenic cells of the bovine CL contain abundant smooth and occasional rough endoplasmic reticulum, mitochondria, Golgi and lipid droplets. The small cells have peripherally located and deeply lobulated (cup-shaped) nuclei with densely staining

nucleoplasm (Koos and Hansel 1981). Their mitochondria are arranged in an arc opposite the nucleus. A Golgi complex and, occasionally, pairs of centrioles are present in the central region of the cytoplasm. These cells usually occur in clumps held together by interdigitating microvilli or junctional complexes.

The large cells of bovine CL of the oestrous cycle have large central nuclei with a distinct nucleolus and dispersed chromatin. Mitochondria, more abundant than in the small cells, surround the nucleus, and are almost absent from the cell periphery. Their membrane

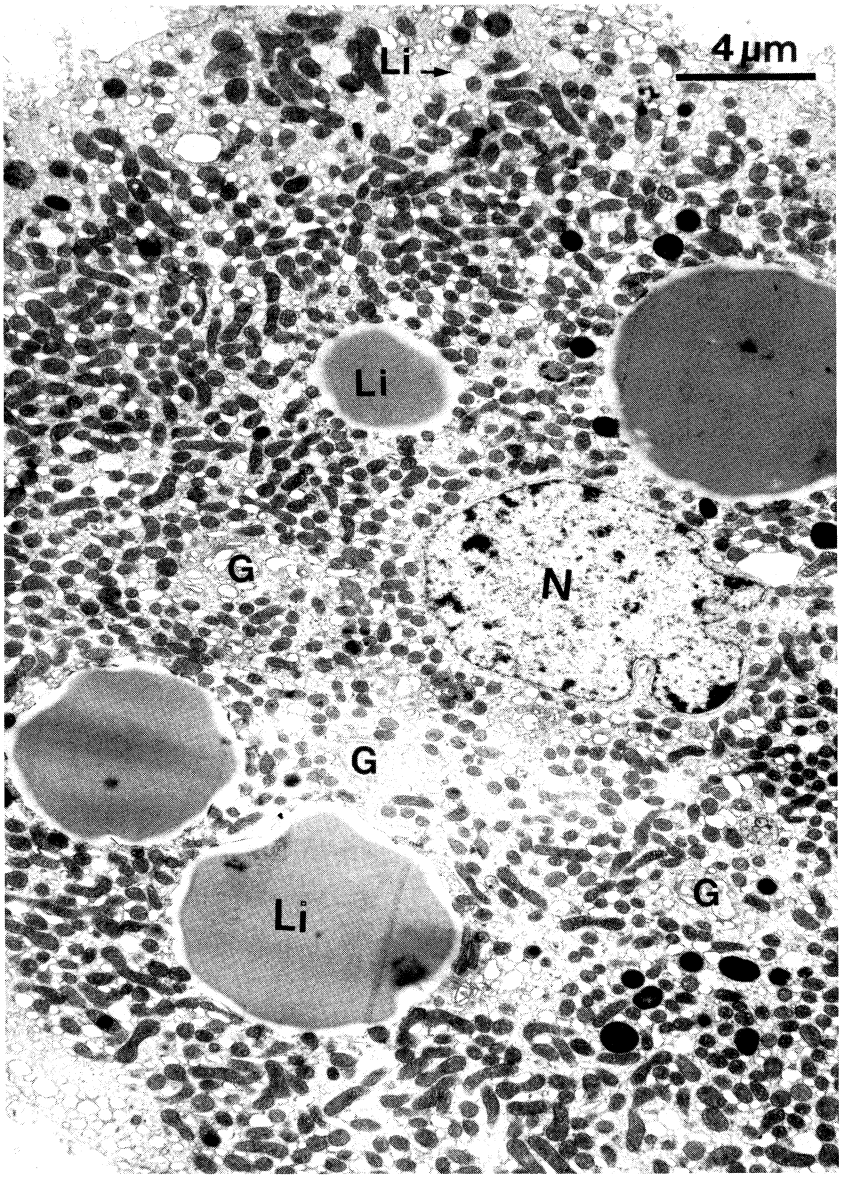

Fig. 1. Large luteal cell from a dispersed cell preparation from a corpus luteum removed at 150 days of gestation. The nucleus (*N*) is lobulated and the cytoplasm contains large lipid droplets (*Li*) of various sizes. Golgi (*G*) and electron-dense granules are also present.

surfaces are highly convoluted and contain extensive microvillous projections. The main distinguishing feature between the large and small luteal cells is the presence of electron-dense granules of various sizes in the cytoplasm of some large cells; the cytoplasm of all small cells is devoid of these granules (Koos and Hansel 1981).

Luteal Cells in CL of Pregnancy

During pregnancy, the nuclei of several large cells show lobulations and the mitochondrial content of the cytoplasm, as well as the size of lipid droplets, increases (Fig. 1). Some of the large cells have the typical round nuclei and contain granules which are uniformly distributed in the cytoplasm, similar to the large cells of the oestrous cycle. However, some large cells of pregnancy contain granules enclosed by a common membrane (Singh 1975), while in other large cells these granules occur in clusters in the paranuclear area (Fields *et al.* 1985). The granules enclosed by a common membrane and the intramitochondrial dense bodies are absent from the large cells of the oestrous cycle. As pregnancy progresses, intramitochondrial electron-dense bodies, as well as cytoplasmic granules, are seen (Sorensen and Singh 1973; Singh 1975; Fields *et al.* 1985). The presence of these intramitochondrial dense bodies is probably an indication of degenerative changes.

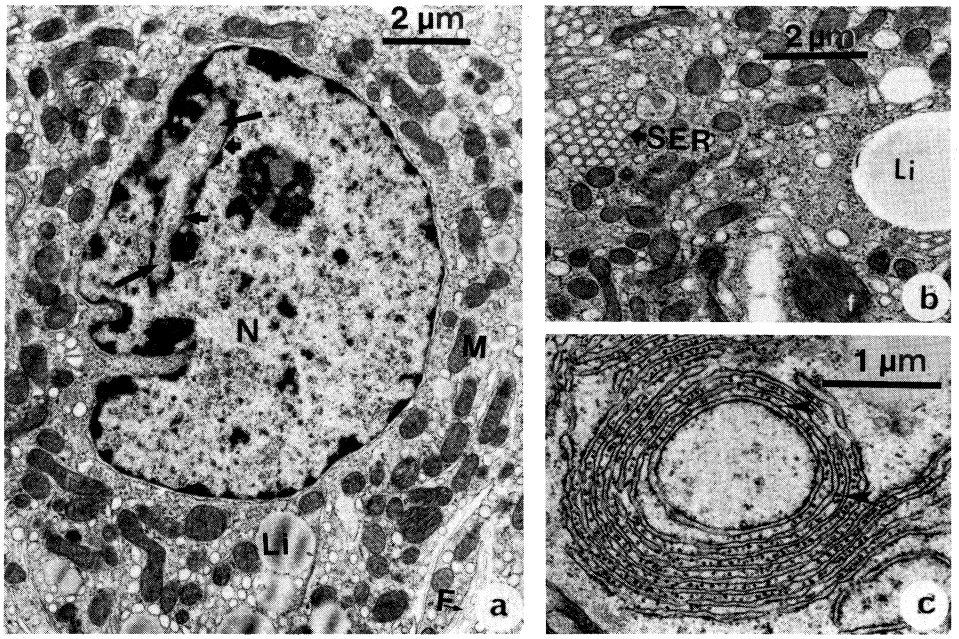

Fig. 2. (*a*) A small luteal cell from a corpus luteum removed at 150 days of gestation. The cytoplasm contains several mitochondria (*M*) and lipid droplets (*Li*), smooth endoplasmic reticulum, and filamentous material (*F*) resembling cytoskeleton. The nucleus (*N*) is highly lobulated and gives an appearance of a cytoplasmic inclusion (arrows). (*b*) Cytoplasm of the same type of cell showing stacks of smooth endoplasmic reticulum (*SER*), *Li*, and a portion of intramitochondrial inclusion containing a series of evenly arranged longitudinal filaments (*f*). (*c*) Glycogen granules (arrowheads) occurring between adjoining cisternae of smooth endoplasmic reticulum in a small luteal cell to form a glycogen body.

The small cells of pregnancy are generally larger (20–25 µm in diameter) than those found during the oestrous cycle; they have highly lobulated nuclei, several stacks and whorls of smooth endoplasmic reticulum and occasional glycogen bodies (Figs 2*a*, 2*b*, 2*c*). The presence of glycogen bodies is a unique feature of the small luteal cells of the bovine species. In the

sheep ovary they occur only in granulosa cells and disappear after the formation of the CL (O'Shea *et al.* 1978, 1979). During pregnancy, some small luteal cells have also been observed to contain secretory granules (Fields *et al.* 1985) and some also show extensive crystalline-like inclusions, which may also represent degenerative processes.

Results of immunocytochemical studies showed that the granules (0·3 μm) contain oxytocin and neurophysin (Guldenaar *et al.* 1984; Kruip *et al.* 1985; Fields *et al.* 1986). The presence of relaxin in corpora lutea of late pregnant cows has also been demonstrated (Fields *et al.* 1980). It is not known whether each granule contains several hormones, or whether each hormone is contained in a different granule. Neither is it known whether all large cells (theca- or granulosa-derived) produce oxytocin.

Table 1. Number (mean ± s.e.) and percentage of luteal cells (<10 μm) observed in dispersed bovine luteal cells during the oestrous cycle and pregnancy

Means in the same column with different superscripts differ ($P < 0·05$)

Reprod. state (days)	*N*	10^{-6}×No. of cells/g tissue Large cells	Small cells	Large cells (%)	Small cells (%)
		Oestrous cycle			
3–5	6	$0·6 \pm 0·2^{a}$	$28·6 \pm 6·0^{a}$	$1·6 \pm 0·5^{a}$	$98·3 \pm 0·5^{a}$
10–12	13	$1·13 \pm 0·2^{b}$	$19·3 \pm 3·0^{b}$	$5·1 \pm 0·8^{b}$	$95 \pm 0·8^{b}$
15–18	6	$0·9 \pm 0·3^{ab}$	$18 \pm 0·6^{b}$	$4·5 \pm 0·5^{b}$	$95·4 \pm 0·5^{b}$
19–20	3	$0·19 \pm 0·1^{c}$	$8·3 \pm 3·0^{c}$	$2·2 \pm 0·8^{a}$	$97·7 \pm 2·0^{ab}$
		Pregnancy			
25–80	10	$1·6 \pm 0·3^{b}$	$11·6 \pm 2·0^{c}$	$12·3 \pm 1·5^{c}$	$86·9 \pm 1·5^{c}$
100–180	9	$2·5 \pm 0·7^{d}$	$8·5 \pm 2·0^{c}$	$24·1 \pm 4·0^{d}$	$76·0 \pm 4·0^{d}$
214–272	6	$0·1 \pm 0·05^{c}$	$3·2 \pm 1·5^{d}$	$1·8 \pm 0·5^{a}$	$98·1 \pm 0·6^{a}$

Dynamic Changes in Cell Populations

In the dissociated cell preparations, the quantities of the small and large luteal cells vary with stage of the reproductive cycle (Table 1). Although they did not regard large and small cells as different types, Parry *et al.* (1980) stated that the large luteal cells occupy approximately 70% of the area of the CL at the mid-cycle. However, large cells account for less than 10% of the steroidogenic cells and the small cells are 20–40 times more numerous than the large cells during the oestrous cycle. Few large cells are found in the CL early in the cycle but they increase as the age of the CL increases. Generally there are fewer large cells per gram of tissue during the oestrous cycle ($P < 0·05$) than during gestation (Table 1; and Ursely and Leymarie 1979), when they amount to 15–30% of the steroidogenic cells. Conversely, there are fewer small cells during pregnancy than during the oestrous cycle. During the onset of luteolysis, the reduction in the number of large cells precedes the reduction of small cells, suggesting that one of the first events during luteolysis involves the large cells. Similar observations have been made for the sheep CL during $PGF_{2\alpha}$-induced luteolysis (Braden and Niswender 1985).

The dynamic changes in the proportions of the small and large luteal cells suggest that the cells in the CL undergo continuous differentiation and that the large cells in the bovine CL represent the final stage of differentiation of the small cells. Both theca and granulosa cells contribute to the formation of the CL. In the preovulatory follicle, the cells of the theca interna that are incorporated into the CL are less differentiated than the granulosa cells (Priedkalns and Weber 1968; Mossman and Duke 1973). Thus, the theca-derived cells in the CL are less differentiated than the granulosa-derived cells, at least during the initial stages of CL development.

On the basis of morphological studies, Donaldson and Hansel (1965) suggested that the small cells in the cow CL differentiate into large cells. This opinion was based on the fact that mitotic activity in granulosa-derived cells ceased early during CL formation, whereas small cells continued to divide (Moss *et al.* 1954; Donaldson and Hansel 1965; Priedkalns *et al.* 1968).

In an attempt to understand the contributions of the theca and granulosa cells to the formation of the bovine CL, we used specific monoclonal antibodies to surface antigens of the preovulatory theca and granulosa cells to trace the origins of the cells in the corpus luteum (Alila and Hansel 1984). This study showed that all the small cells are of thecal origin and, until the sixth day of the oestrous cycle, nearly all the large cells are of granulosa origin. After this time, large cells of thecal origin (derived from the small cells) appear. The granulosa-derived large cells disappear during early pregnancy, while the cells of thecal origin persist throughout pregnancy. Therefore, the large cells are comprised of cells of both granulosa and thecal origin.

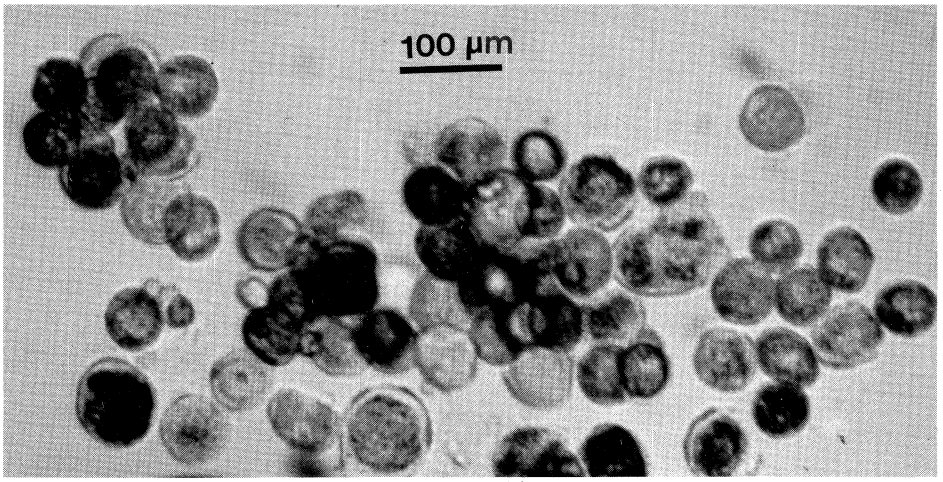

Fig. 3. Large luteal cells obtained by micromanipulation from a corpus luteum removed at day 11 of the oestrous cycle. Note that some cells appear dark while others are light.

There was no major morphological distinction between the large cells labelled by theca antibody and by granulosa antibody (Alila and Hansel 1984). However, at the level of the light microscope (Fig. 3) many of the granulosa antibody-bound cells appear darker and more granular. When these cells are examined by electron microscopy (Fig. 4), the darker cells (upper) contain more mitochondria and electron-dense granules in the cytoplasm. The mitochondria in these dark cells are small, elongated, and diffusely distributed throughout the cytoplasm; these cells also contain numerous secretory granules. The remaining large cells (light cells, Fig. 4, lower), which did not stain, had larger, rounded mitochondria and fewer granules. Similar cells are also seen in tissue sections (Fig. 5). Most of the small cells in the younger CL (days 4–6) that were labelled by the granulosa antibody resembled the large cells in both nuclear and cytoplasmic features (Koos and Hansel 1981), indicating that, in the young CL, some granulosa-derived cells have not yet enlarged.

Interestingly, the pattern of binding of granulosa antibody observed (Alila and Hansel 1984) closely resembled the profile of oxytocin secretion described by Walters *et al.* (1984). The disappearance of granulosa cells during pregnancy coincides with the period when luteal oxytocin concentration is low, suggesting again that granulosa-derived large cells are the source of oxytocin in the bovine CL.

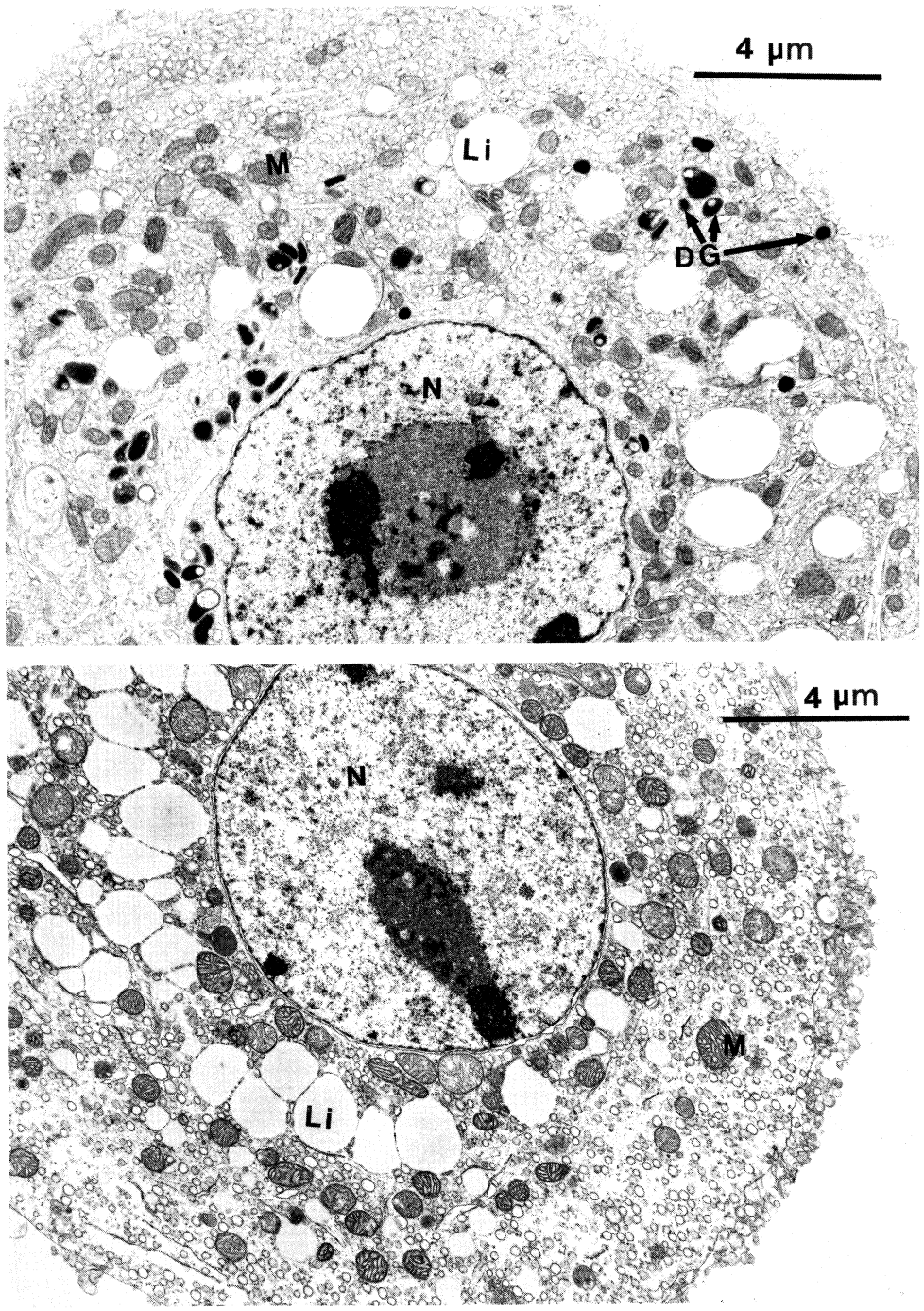

Fig. 4. Large luteal cells from a corpus luteum of day 11 of the oestrous cycle. *Upper:* this cell appears as a dark cell under light microscopy shown in Fig. 3, due to numerous electron-dense granules (*DG*), several mitochondria (*M*) and lipid droplets in the cytoplasm. *Lower:* this cell appears as a light cell when examined under a light microscope. Note that many mitochondria (*M*) are larger and more rounded, but fewer than in the upper figure. There are also very few electron-dense granules.

Steroidogenic Capabilities of Large and Small Cells

Differences in the *in vitro* steroidogenic capabilities of the small and large cells of the cow CL have been demonstrated (Ursely and Leymarie 1979; Koos and Hansel 1981). The small cells produce less basal progesterone than the large cells. However, the small cells are at least six times more responsive to added LH than the large cells.

A major problem in studies on the large cells has been the lack of an effective method to separate them. Existing methods, such as unit gravity sedimentation or elutriation yield large cell fractions that are usually contaminated by clumps of small cells. Steroid production by the large-cell fraction is often calculated by subtracting steroid assumed to be produced by the contaminating small cells from the total production. The calculated result is based

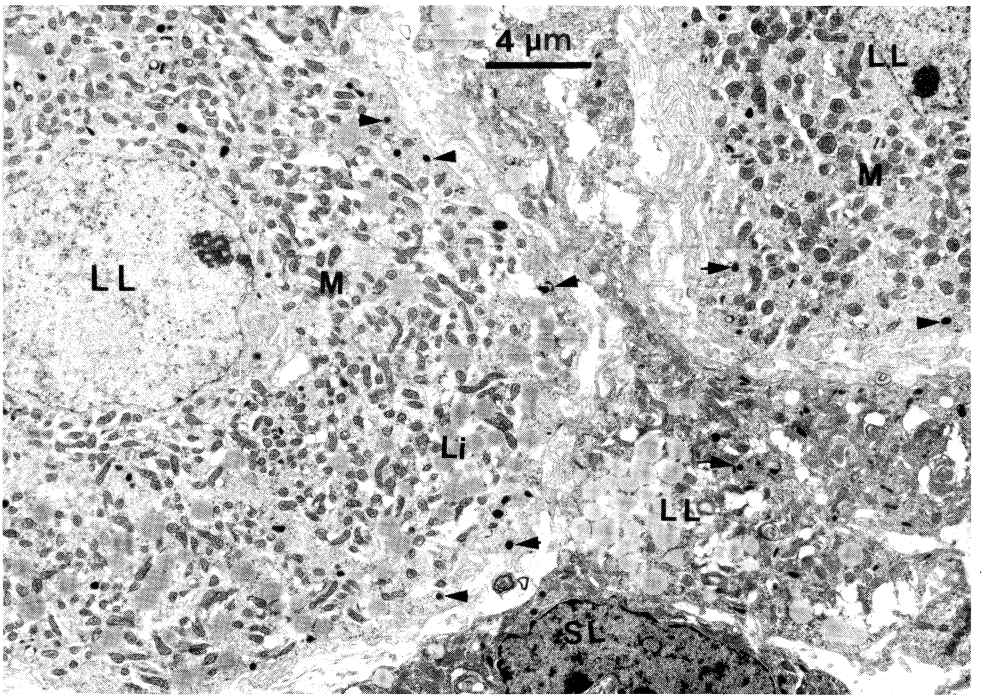

Fig. 5. Tissue sections of a bovine corpus luteum removed at day 10 of the oestrous cycle. The large luteal cell (*LL*) on the left has several lipid droplets (*Li*), electron-dense granules (arrowheads) and mitochondria (*M*) throughout the cytoplasm. The large cell at top right has larger mitochondria (*M*), fewer lipid droplets, electron-dense (arrowheads) granules and extensive surface microvillous projections. The small luteal cell (*SL*) has a lobulated nucleus which has a densely staining nucleoplasm.

on the assumption that no interactions occur between these cells, which is unlikely. Attempts have been made to separate the cells by use of cell flow cytometry, but the method is hampered by lack of suitable circuitry to remove clumps of small cells. Yet another consideration is the fact that there are two antigenically (and perhaps functionally) distinct large-cell populations derived from theca and granulosa cells.

Recently, we have developed an improved separation technique for large cells by use of micromanipulation techniques. Although the method is tedious, >95% pure large cell preparations can be obtained (Fig. 3) and as few as 50 incubated cells produce measurable amounts of progesterone during incubation.

Control of Steroidogenesis in Small Theca-derived Luteal Cells

Following the demonstrations of Ursely and Leymarie (1979) and Koos and Hansel (1981) that the small bovine luteal cells are six or more times more responsive to added LH than the large cells, Fitz *et al.* (1982) showed that the small luteal cells in the sheep CL contain the majority of LH receptors (33 260 per small cell compared with 3074 per large cell), while the large cells contain the majority of the $PGF_{2\alpha}$ receptors (68 143 per large cell, and 2115 per small cell).

In 1984 we (Shemesh *et al.* 1984) found that bovine placental cells secrete progesterone by a calcium-dependent and cyclic-nucleotide-independent mechanism. In the same year, Nishizuka (1984) summarized information concerning a new second messenger system by which hormones pass information from the surface to the interior of target cells. The system involves two steps—intracellular Ca^{2+} mobilization and protein kinase C activation. Both steps are activated by binding of the hormone to its receptor and subsequent phospholipase C hydrolysis of membrane-bound phosphatidylinositol-4,5-bisphosphate, yielding diacylglycerol (DG) and inositol-1,4,5-triphosphate (IP_3). The DG activates protein kinase C in the presence of membrane phospholipid. The IP_3 mobilizes intracellular Ca^{2+}. Both the Ca^{2+} and the DG signals are transient and the two pathways are essential and often synergistic in evoking cellular responses (Nishizuka 1986). The rapid disappearance of DG is due, in part, to its rapid conversion to arachidonic acid which is the precursor of all of the prostanoids; these compounds are known to have both luteotrophic and luteolytic effects in the bovine CL. A major function of protein kinase C in a number of tissues and cells appears to be in the feedback control of surface receptors resulting in the phenomenon known as down regulation.

Table 2. Effects of LH, phorbol-12-myristate-13-acetate (TPA), and the Ca^{2+} ionophore, A23187, alone and in combination, on net progesterone secretion by 5×10^5 luteal cells during 2-h incubations
Results are expressed as percentage ± s.e. (in parentheses) of control incubations. Data were analysed by one-way analysis of variance and Duncan's multiple-range tests. Values identified by different superscripts are significantly different ($P < 0{\cdot}05$) from one another. LH and TPA increased progesterone synthesis ($P < 0{\cdot}05$) over controls at days 9–10 and 16–17. All values represent data from four or more corpora lutea. (From Hansel and Dowd 1986.)

Day of cycle	LH (1 ng)	TPA (50 nM)	A23187 (0·2 μM)	TPA + A23187 (50 nM + 0·2 μM)	TPA + LH (50 nM + 1 ng)	A23187 + LH (0·2 μM + 1 ng)
4–6	100 (0·15)[a]	86 (0·12)[a]	61 (0·06)[a]	85 (0·20)[a]	107 (0·21)[a]	124 (0·30)[a]
9–10	240 (0·27)[a,d]	171 (0·26)[a,b]	97 (0·10)[b]	199 (0·47)[a,b]	352 (0·31)[c,d]	374 (0·45)[c]
16–17	292 (0·62)[a,b,c]	173 (0·35)[a,b]	97 (0·13)[b]	205 (0·38)[a,b,c]	348 (1·09)[a,c]	400 (0·86)[c]

In our first experiment, designed to test whether this system is involved in progesterone synthesis in the bovine CL (Hansel and Dowd 1986) we examined the effects of Ca^{2+} ionophores and agents, such as phorbol esters, that activate protein kinase C, on progesterone synthesis by total dispersed bovine luteal cell preparations from CL collected at three stages of the oestrous cycle. The corpora lutea were collected at days 4–6, 9–10 and 16–17 of the cycle, dispersed with collagenase, and 5×10^5 cells were incubated for 2 h in 1 ml medium 199 and assayed for progesterone. The data (Table 2) clearly indicate that protein kinase C is involved in progesterone synthesis. Addition of a phorbol ester, phorbol-12-myristate-13-acetate (TPA), an activator of protein kinase C, at both 50 and 100 nM (data for 100 nM not shown) to the dispersed luteal cells increased ($P < 0{\cdot}05$) progesterone synthesis in cells from CL collected at days 9–10 and 16–17, but had no effect on cells of days 4–6 CL. Brunswig *et al.* (1986) also observed increased progesterone synthesis in mid-luteal phase bovine luteal cells after addition of a phorbol ester. LH was also effective in increasing progesterone synthesis in cells from days 9–10 and 16–17 CL and ineffective in cells collected

at days 4–5; however, it was effective in cells of CL collected at day 6. The Ca^{2+} ionophore A23187 given alone had no effect on progesterone synthesis at any stage of the cycle, but enhanced the ability of a submaximal (1 ng/ml) level of LH to increase progesterone synthesis in cells of mid-cycle (days 9–10) CL ($P < 0{\cdot}05$).

We tentatively interpreted these results to suggest that the Ca^{2+}–polyphosphoinositol–protein kinase C second messenger system was operative in one of the luteal cell types, presumably the large granulosa-derived cells, while the calcium ionophore possibly had a dual effect, stimulating protein kinase C and inhibiting cAMP-mediated steroidogenesis in the small theca-derived cells. In order to test this hypothesis, highly purified small cell preparations (5×10^5) prepared by unit gravity sedimentation (Koos and Hansel 1981) from mid-cycle (days 9–10) CL were incubated in medium 199 for 2 h, as in the previous experiment, after additions of a phorbol ester, phorbol dibutyrate (PBt_2, 20 nM) and a Ca^{2+} ionophore (A23187, 0·2 μM) alone and in combination. Cells from four CL were tested and there were four replicates per CL.

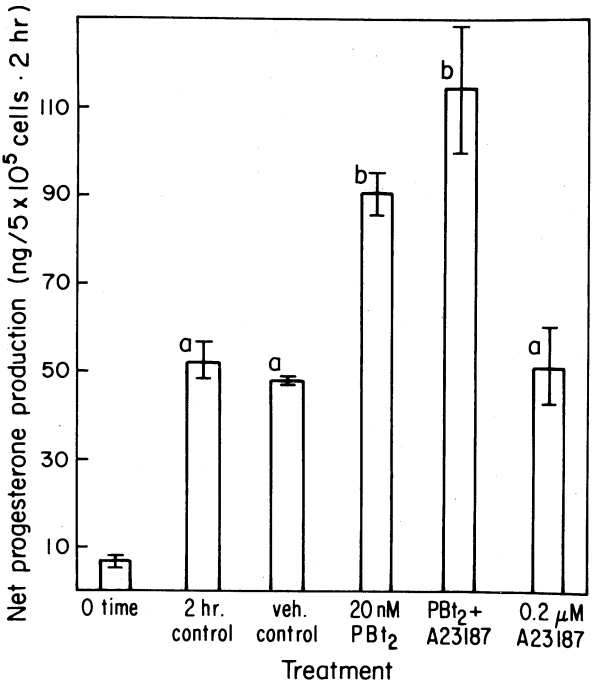

Fig. 6. Net progesterone production by small bovine luteal cells treated with a phorbol ester (phorbol dibutyrate, PBt_2), a Ca^{2+} ionophore (A23187) or A23187 plus PBt_2. CL were obtained at midcycle (days 9 or 10). Small luteal cells were purified on a 1–3% (v/v) BSA gradient. Cells (1×10^5) were incubated for 2 h in M199 alone or with the indicated treatments. Data represent the means of four separate experiments ($n = 4$). Treatments were performed on quadruplicate samples. Bars with different superscripts are significantly different ($P < 0{\cdot}01$), Duncan's multiple-range test.

Contrary to our hypothesis, the stimulatory effect of activating protein kinase C, noted in the previous experiment with total dispersed cells, appears to be a function of the small, rather than the large, luteal cells (Fig. 6). Addition of the phorbol ester PBt_2 and PBt_2 plus the Ca^{2+} ionophore A23187 each resulted in an approximate doubling of progesterone synthesis, a result quite similar to that previously obtained with total dispersed cells (Table 2). As in the previous experiment, the calcium ionophore (0·2 μM) alone had no effect on progesterone synthesis.

Interactions between LH and the ionophore A23187 in the small luteal cells are not clear-cut. In a previous preliminary experiment (Hansel and Dowd 1986) it was found that addition of A23187 inhibited LH-stimulated progesterone secretion in small luteal cells and, as a result, it was suggested that elevated intracellular Ca^{2+} may be inhibitory to cAMP-mediated steroidogenesis. However, in the current experiment (Fig. 7), addition of A23187

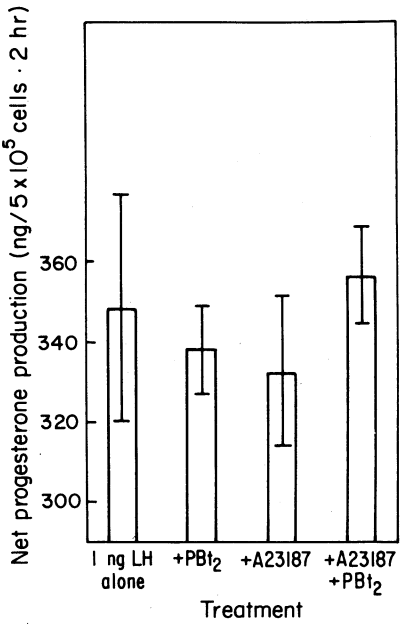

Fig. 7. Net progesterone production in 2 h by small bovine cells in response to LH, LH plus a phorbol ester (PBt_2), LH plus the Ca^{2+} ionophore A23187 and LH plus A23187 and PBt_2. All experimental conditions were as described for Fig. 6. None of the treatments caused significant differences from LH alone.

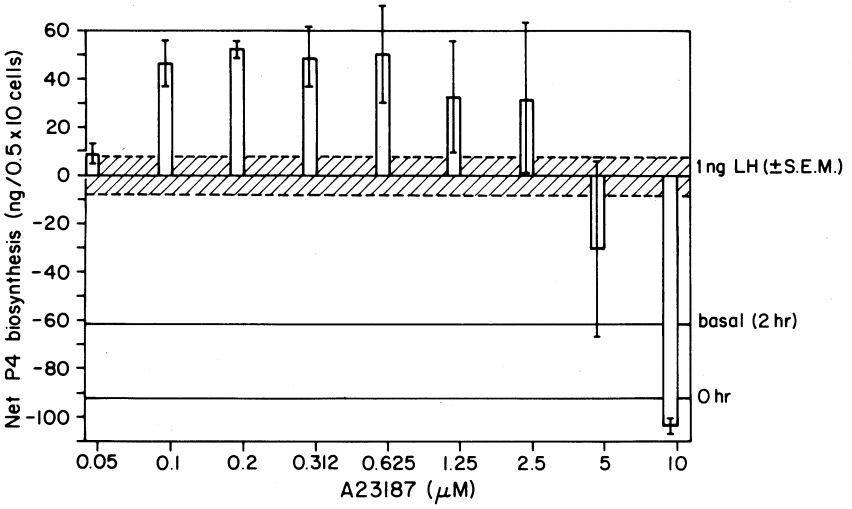

Fig. 8. Effects of adding graded doses of the Ca^{2+} ionophore A23187 on net progesterone (P_4) production in a 2-h period by total dispersed bovine luteal cells ($0{\cdot}5 \times 10^6$ cells) treated with a submaximal stimulatory dose of LH (1 ng/ml).

($0{\cdot}2$ μM) failed to cause a significant reduction of LH-stimulated progesterone secretion ($P > 0{\cdot}05$). The difference in results between the two experiments may be related to differences in the level of ionophore added. In any case, it seems clear that addition of the Ca^{2+} ionophore does not cause an increase in LH-stimulated synthesis in the small luteal cells.

In contrast, addition of the calcium ionophore at concentrations of 0·1–2·5 μM to total dispersed cells consistently results in a stimulation of LH-mediated progesterone synthesis (Table 2; Fig. 8). These results suggest that the stimulatory effect of increased intracellular Ca^{2+} on LH-mediated progesterone synthesis in total dispersed cells is attributed to either the small non-luteal (endothelial) cells or to the large luteal cells.

There is a marked similarity in the results we have obtained with small bovine luteal cells and those reported for purified rat Leydig cells by Lin (1985). Calcium and/or phospholipid-dependent protein kinase is involved, in addition to the classical cAMP-dependent protein kinase pathway, in steroid production in each case. In the Leydig cell, phorbol ester-induced testosterone formation was shown to be dependent on extracellular Ca^{2+}, since the response was blocked by a channel-blocking agent, nifedipene. Since the Leydig cell in the male is homologous to the theca cell in the female, and the small luteal cells are derived from theca cells, this result might be expected.

Control of Steroidogenesis in Large Luteal Cells

Little is known of the mechanisms controlling steroidogenesis in the large luteal cell. As previously mentioned, these cells are unresponsive to LH added in concentrations that maximally stimulate steroidogenesis in small cells. In ovine CL, these cells contain few LH receptors (Fitz *et al.* 1982). Work with these cells, at least in the cow, has been hampered by lack of a good separation system.

Recently, we succeeded in separating nearly pure fractions of large cells from dispersed cell preparations by micromanipulation. Cells were dispersed, the small luteal cell fraction and the small (< 10 μm) vascular cell fraction were removed by unit gravity sedimentation, as described previously (Koos and Hansel 1981). Most of the small cells remaining in the crude large cell fraction were then removed by micromanipulation through a pipette having an internal diameter less than 20 μm. The 20-μm pipette was then replaced by one having a diameter of 25 μm or larger and the large cell fractions were collected.

The purity of the large cell fractions obtained in this way is illustrated in Fig. 3; clearly, two kinds of large cells—dark and light cells—are present in these unstained preparations. The dark cells contain secretory granules and are possibly derived from the granulosa cells. As such, they would be expected to contain oxytocin (and possibly relaxin) in the secretory granules. The light cells have larger, rounded mitochondria and fewer lipid droplets (Fig. 4). These cells may be of thecal origin, although the possibility that these are also granulosa-derived cells in a different stage of differention cannot be excluded.

Aliquots of the large luteal cells obtained in this way from five CL were allocated (50 cells per treatment) to the following treatment groups: (1) 0-h controls; (2) 14-h incubated controls; (3) LH (1 ng/ml); (4) the Ca^{2+} ionophore, A23187 (0·2 μm/ml); (5) the phorbol ester, PBt_2 (20 nM); (6) PBt_2 plus LH; (7) PBt_2 + A23187; and (8) LH + A23187. Cells were incubated in an atmosphere of air (95%) and CO_2 (5%) for 14 h in Hams F-12 medium supplemented with 5% (v/v) bovine fetal serum, after which the incubation was terminated and progesterone in cells plus medium was measured, as in previous experiments. The large cells did not divide during these incubations. The results, expressed as picograms of progesterone produced per cell, are shown in Table 3.

Although the separated large cells produced surprisingly large and variable amounts of progesterone under these conditions, it is clear that, contrary to our hypothesis, none of the treatments exerted a stimulatory effect on progesterone synthesis. The phorbol ester, PBt_2 failed to cause a stimulation in progesterone biosynthesis indicating that activation of protein kinase C does not stimulate progesterone synthesis, as is the case for small cells. LH, at the concentration used, also failed to stimulate progesterone synthesis in the large cells, and addition of PBt_2 and LH did not result in a significant depression of synthesis when compared to LH alone (24·7 ± 12·2 *v.* 6·2 ± 1·9, $P > 0·05$).

Table 3. Progesterone synthesis by dispersed large bovine luteal cells in response to added LH, phorbol ester (PBt_2), calcium ionophore (A23187), and combinations of these agents

Incubations were carried out for 14 h in Hams F12 medium plus bovine fetal serum. Values are means ± 1 s.e.

Treatment	*N*	Progesterone (pg/cell)	Treatment	*N*	Progesterone (pg/cell)
0 h (control)	5	0·1 ± 0·1	PBt_2 (20 nM)	5	26·7 ± 16·0
14 h (control)	5	29·9 ± 18·1	PBt_2 + LH	3	6·2 ± 1·9
LH (5 ng/ml)	5	24·7 ± 12·2	PBt_2 + A23187	4	30·6 ± 14·4
A23187 (0·2 μM)	5	25·5 ± 14·8	LH + A23187	4	33·0 ± 18·8

Mechanism(s) of Action of the Protein Kinase C Second Messenger System

In another study (Dowd *et al.*, unpublished data 1986), we were able to purify partially the phorbol ester receptor, show that it is protein kinase C, and establish the dissociation constant (K_d) for phorbol (PBt_2) binding to intact luteal cells at 8 nM.

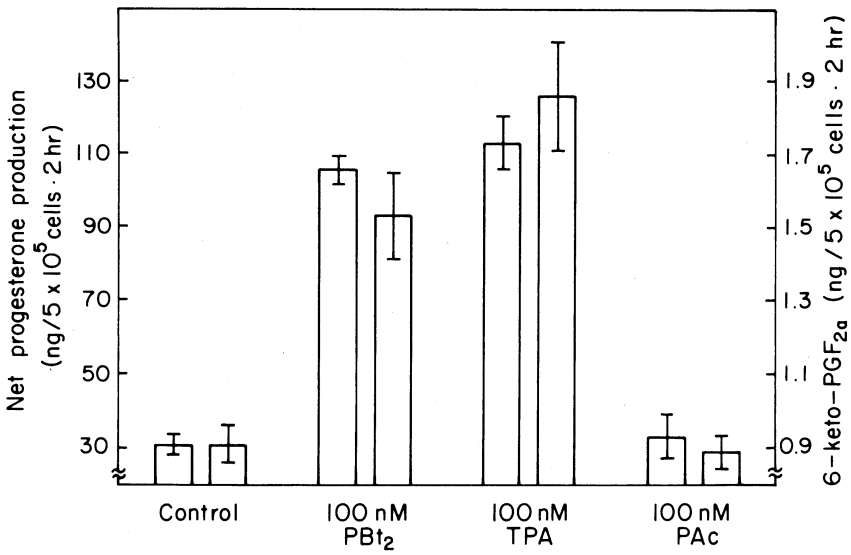

Fig. 9. Progesterone and prostacyclin (6-keto-$PGF_{1\alpha}$) biosynthesis by total dispersed bovine luteal cells ($0·5 \times 10^6$) during a 2-h incubation in response to addition of two phorbol esters that activate protein kinase C (PBt_2 and TPA) and one that does not (PAC).

Since we had previously shown that prostacyclin (PGI_2) is a potent luteotrophin and that its production in luteal cells is highly correlated with progesterone biosynthesis (see Hansel and Dowd 1986), it became of interest to determine the relationship between luteal PGI_2 synthesis and protein kinase C activation. Total dispersed cells ($0·5 \times 10^5$) from CL collected at day 10 were incubated, as previously described, with two phorbol esters known to activate protein kinase C (PBt_2 and TPA) and one that does not, phorbol-13-acetate (PAc), after which net progesterone synthesis was measured. Four replicates per CL were carried out. Data for a single CL are shown in Fig. 9, from which it may be seen that the two active phorbol esters caused remarkable increases in both progesterone and PGI_2 (measured as the inactive stable metabolite, 6-keto-$PGF_{1\alpha}$) biosynthesis. In additional studies (data not shown) it was found that PBt_2 increased both progesterone and PGI_2 synthesis in total dispersed luteal cells in a dose-dependent fashion over a range of 10–

100 nM. Thus, it appears that at least one of the mechanisms by which protein C activation increases progesterone synthesis may involve the production of PGI_2, a known luteotrophic prostanoid.

Discussion

The results cited above indicate that the Ca^{2+}–polyphosphoinositol–protein kinase C second messenger system is stimulatory to progesterone synthesis by small, theca-derived bovine luteal cells, and has no significant effect in the large luteal cells. Although addition of phorbol ester resulted in an approximate doubling of net progesterone production in the small luteal cells, activation of protein kinase C in this way failed to increase progesterone production mediated by a submaximal level of added LH.

Nishizuka (1986) described two classes of cells having either 'monodirectional' or 'bidirectional' control systems, in which the two classes of receptors (those that are cAMP-generating and those involving Ca^{2+} mobilization, protein kinase C activation, arachidonate release and cGMP formation) either potentiate one another or have opposing effects. Pinealocytes, pituitary cells and S-49 lymphoma cells are examples of monodirectional cells of the first type, in which protein kinase C appears to potentiate cAMP production. In contrast, in bidirectional cells, the two classes of receptor counteract each other, and protein kinase C inhibits and densensitizes the adenylate cyclase system. Cells of this type are exemplified by erythrocytes and ovarian granulosa cells.

Small luteal cells behave as monodirectional cells, insofar as both second messenger systems stimulate progesterone synthesis. However, there is no evidence that the protein kinase C system potentiates cAMP production in these cells and activation of this system does not synergize with a submaximal dose of LH in promoting progesterone synthesis (Fig. 7). West *et al.* (1986) demonstrated that $PGF_{2\alpha}$ stimulates phospholipase C and increases phosphoinositide-derived second messengers in total dispersed bovine luteal cells. $PGF_{2\alpha}$ was luteotrophic in these experiments, as previously shown by Hixon and Hansel (1979). These results probably reflect activities of the protein kinase C system in the small luteal cells.

The large cells may behave as 'bidirectional' cells although no clear inhibiting effect of activating protein kinase C on LH-stimulated progesterone synthesis has been demonstrated (Table 3). Since LH, at the levels added, was not stimulatory in these experiments (Table 3), there is a possibility that LH receptors may already have been down regulated in these mid-cycle large cells. A major function of protein kinase C appears to be the down regulation of cell surface receptors. This feedback control of down regulation by protein kinase C extends to the receptors of a number of other signalling systems (Nishizuka 1986) and could prove to be involved in down regulation of LH receptors in the large granulosa-derived luteal cells.

The complete amino acid sequence of protein kinase C has now been established (Parker *et al.* 1986) and a family of protein kinase C-related genes has been identified in bovine, human and rat genomes (Coussens *et al.* 1986). Three members of this family (protein kinase C-α, -β and -γ) have been characterized and extensive similarity among them exists. The existence of a family of protein kinase C molecules suggests that cellular responses may be affected by activation of one or more of these polypeptides. Individual cell lines do show different patterns of protein kinase C-α, -β and -γ mRNA and protein expression. These observations may well serve as a basis for explaining the differences we have observed in responses of the large and small luteal cells to activation of protein kinase C by phorbol esters and Ca^{2+} ionophores.

One of the most interesting findings of these experiments is that activation of protein kinase C by phorbol esters in total cell preparations results in an increase in PGI_2 synthesis and that this increased PGI_2 synthesis is highly correlated with progesterone synthesis. The ability of PGI_2 to stimulate progesterone synthesis *in vivo* and *in vitro* was previously

described by Milvae and Hansel (1980, 1983). Since this experiment was carried out with total dispersed cell preparations, the cell type(s) involved in prostacyclin production is unknown, but the very small (<10 μm) non-luteal cell fraction contains many endothelial cells, which are known to produce PGI_2, and these cells could have been responsible. PGI_2 production by these very small non-luteal cells may also be responsible for the increased progesterone synthesis observed as a result of addition of the Ca^{2+} ionophore A23187 to LH-stimulated total luteal cell preparations (Fig. 8), since the response to the ionophore was not obtained in either LH-treated small cells (Fig. 7) or LH-treated large cells (Table 3).

In these experiments the untreated small cells produced only about 0·1 pg of progesterone per cell, compared to about 25 pg per cell by the untreated large cells. Although these values are not strictly comparable, because of the differences in culture media used and the incubation times, they may indicate the relative capacities of the two cells to produce progesterone. After LH stimulation, the large cells, although unresponsive to the added LH, still produced 35 times as much progesterone per cell as the small luteal cell. Obviously, calculations such as these, although they might indicate potential production by the large and small cells, may bear little relationship to production *in vivo*, because of interactions between these two cell types and other cells in the CL.

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