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Dynamics of molecular mechanisms underlying ovarian oxytocin secretion

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In the ruminant ovary, synthesis and secretion of oxytocin begin in the granulosa cells of the preovulatory follicle and are markedly stimulated by the surge of LH and FSH. Luteinization of the granulosa cells results in a further increase in oxytocin gene expression, but translation of mRNA appears to be retarded because the peak concentration of luteal oxytocin occurs later than the maximal accumulation of the message. Several hormones have been shown to stimulate oxytocin secretion from granulosa and luteal cells *in vivo* or *in vitro*. However, the role of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in regulating luteal oxytocin secretion has perhaps received more study than other hormones. The mechanism of action of $PGF_{2\alpha}$ has been shown to encompass a phosphoinositide cascade and activation of protein kinase C, events that are associated with luteal secretion of oxytocin. Protein kinase C phosphorylation of the actin-binding protein myristolated alanine-rich C kinase substrate (MARCKS) may be required for exocytosis of oxytocin.

Introduction

After the reports published during the early 1980s that the sheep ovary was an extrahypothalamic source of oxytocin (Sheldrick and Flint, 1981), a number of laboratories initiated research to determine whether ocytocin was also present in ovaries of other mammals. Indeed, subsequent reports revealed that oxytocin, and often the related peptide vasopressin, was present in the ovaries of women (Wathes *et al.*, 1982), cows (Fields *et al.*, 1983), monkeys (Khan-Dawood *et al.*, 1984), does (Homeida, 1986) and sows (Jarry *et al.*, 1990). Concomitantly, a plethora of literature has arisen detailing research conducted to define the role(s) that oxytocin plays in regulating utero–ovarian functional interrelationships, an area of research in which many are still actively engaged. A number of excellent reviews have been published that have addressed the characteristics of ovarian synthesis and secretion of oxytocin as well as physiological functions of this hormone (Schams, 1987; Flint *et al.*, 1990; Wathes and Denning-Kendall, 1992). With the exception of providing the reader with an updated background of information about concentrations of oxytocin in the ovary and some factors that may affect its synthesis and secretion, this report will have as its focus the luteal intracellular events triggered by the action of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) that are associated with the secretion of oxytocin in ruminants.

Characteristics of Synthesis and Secretion of Oxytocin in the Ovary

Follicular oxytocin

Initial evidence that the preovulatory follicles of cows and ewes were a source of oxytocin was provided by Wathes *et al.* (1984, 1986) and Schams *et al.* (1985a). Voss and Fortune (1991) measured

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oxytocin produced *in vitro* by granulosa cells isolated from bovine preovulatory follicles during the early, mid- and late follicular phase. Granulosa cells recovered from the preovulatory follicle during the early and mid-follicular phase secreted minimal quantities of oxytocin into the culture medium. However, large quantities of oxytocin were secreted by granulosa cells of the late stage preovulatory follicle that was removed approximately 20 h after the onset of oestrus. This latter response suggested that exposure of the follicle to the LH and FSH surges probably accounted for the enhanced production of oxytocin *in vitro*. This premise was supported by data demonstrating that exposure of granulosa cells isolated from preovulatory follicles to LH or FSH *in vitro* caused a marked increase in oxytocin secretion during culture for 5 days (Schams, 1987; Voss and Fortune, 1991). In a similar study conducted by Wathes *et al.* (1992), granulosa cells of the ovine preovulatory follicle exposed to the endogenous LH surge produced more oxytocin than did those recovered from follicles during the early follicular phase. Changes in the synthesis and secretion of oxytocin that occur in the preovulatory follicle after exposure to LH and(or) FSH are highly correlated with changes in mRNA for oxytocin–neurophysin-I. Messenger RNA for oxytocin was not detectable or barely detectable in the early or mid-follicular phase preovulatory follicle in cows as determined by northern blot analysis of granulosa cells (Voss and Fortune, 1992), but substantial quantities were present in the late follicular phase follicle subjected to the LH and FSH surges. Similarly, incubation of granulosa cells isolated from an early preovulatory follicle with LH for 72 h induced transcription of the gene encoding oxytocin–neurophysin-I. The results of these studies suggest that the preovulatory gonadotrophin surge stimulates the accumulation of oxytocin mRNA in the bovine granulosa cells and promotes the synthesis and secretion of this peptide hormone. In contrast to the asynchrony that characterizes oxytocin production by luteal cells (described below), the accumulation of mRNA and synthesis of oxytocin in granulosa cells are coincident events.

Although low concentrations of oxytocin have been detected in cultures of theca interna cells, the presence of the hormone has been attributed to contamination by granulosa cells (Voss and Fortune, 1991). Furthermore, no oxytocin mRNA was detected in theca cells even when stimulated with LH (Voss and Fortune, 1992).

A number of hormones have been shown to affect oxytocin synthesis and secretion by bovine and ovine granulosa cells. The ability of insulin, insulin-like growth factor I, noradrenaline and adrenaline to stimulate oxytocin production by granulosa cells has recently been reviewed by Wathes and Denning-Kendall (1992) and will not be discussed here.

The possibility that steroids produced by granulosa cells act in an autocrine manner to alter the synthesis and secretion of oxytocin is perhaps of greater interest and deserves to be mentioned in some detail. From the early to mid-follicular phase, granulosa cells synthesize primarily oestradiol, but after exposure of the follicle to the LH surge, oestrogen synthesis declines and progesterone production increases (Fortune and Hansel, 1985). Data on effects of oestradiol on oxytocin production by ovine preovulatory follicles *in vitro* are equivocal (Wathes *et al.*, 1992). In some follicles, oestradiol stimulated oxytocin production, while in others it was inhibitory. Low doses of oestradiol when cultured with bovine granulosa cells isolated from an early preovulatory follicle stimulated, while higher doses of the steroid inhibited, oxytocin secretion by the cells (Voss and Fortune, 1993). These authors hypothesized that the increased production of oestradiol by granulosa cells during the early to mid-follicular phase might serve to inhibit oxytocin production. Subsequently, the decline in oestrogen synthesis that occurs as the preovulatory follicle continues to develop would thus allow gonadotrophins to stimulate synthesis and secretion of oxytocin. Voss and Fortune (1993) also found that progesterone stimulated oxytocin secretion by bovine granulosa cells during the late stages of a 5 day culture. Hence, during the late follicular phase, the increased synthesis of progesterone by the follicle acting in concert with the gonadotrophins may enhance follicular oxytocin production. The mechanism of action of oestradiol in regulating the secretion of oxytocin from the granulosa cells is an enigma. When effective, does oestradiol enhance synthesis or only the secretion of oxytocin? An effect of oestradiol on synthesis seems remote at this time because only an imperfect response element for oestradiol is located proximal to the transcription start site of the bovine oxytocin gene (Walther *et al.*, 1991). However, there are response elements for oestrogen as well as for other steroids further upstream in the 3217 bp 5′ flanking region of the bovine gene encoding oxytocin–neurophysin-I. The functional significance of these response elements relative to the regulation of oxytocin synthesis is unknown.

Luteal oxytocin

It is now known that in cows and ewes, luteal concentrations of oxytocin increase from the early to the mid-luteal phase of the oestrous cycle and then decline (Sheldrick and Flint, 1983; Abdelgadir *et al.*, 1987; Parkinson *et al.*, 1992). In pregnant cows and ewes, the pattern of changes in luteal concentrations of oxytocin are similar to those found in the nonpregnant animal up to the time of normal luteal regression and remain low thereafter (Sheldrick and Flint, 1983; Parkinson *et al.*, 1992). During the oestrous cycle, the luteal tissue content of oxytocin is markedly reduced by the time of luteolysis (Wathes *et al.*, 1984). Within the bovine and ovine corpus luteum, oxytocin is sequestered in secretory granules found only within the large luteal cell (Fields and Fields, 1986; Theodosis *et al.*, 1986).

Luteal concentrations of mRNA encoding oxytocin–neurophysin-I in cows and ewes, as measured by northern blot analysis, increase early after luteinization of granulosa cells and attain maximal concentrations within approximately 3 days and then decline to low values as the luteal phase advances (Ivell *et al.*, 1985; Jones and Flint, 1988). Thus, in the ruminant corpus luteum, the biochemical events of transcription, translation and secretion do not occur concomitantly. Rather synthesis and secretion of oxytocin lags behind gene transcription. By measuring [^{35}S]cysteine incorporation, Swann *et al.* (1984) demonstrated that cultures of bovine and ovine luteal cells synthesized oxytocin. However, there is evidence that luteal cells of these species also produce intermediate forms of oxytocin or oxytocin-like peptides, depending upon the stage of the cycle (Camier *et al.*, 1991).

The pattern of secretion of oxytocin in ewes and cows is similar to that of progesterone (Webb *et al.*, 1981; Walters *et al.*, 1984). In both cows and ewes, onset of luteal regression is characterized by intermittent synchronous pulsatile secretion of oxytocin and $PGF_{2\alpha}$ (Flint and Sheldrick, 1983; Walters *et al.*, 1984). These latter data support in part the hypothesis, advanced by McCracken and Schramm (1983), that episodic secretions of luteal oxytocin and uterine $PGF_{2\alpha}$ are interrelated through a double positive feedback loop between the hormones, thereby promoting luteolysis. On the basis of the above data, it would be logical to assume that treatment of cows or ewes with $PGF_{2\alpha}$ should stimulate the secretion of luteal oxytocin. Flint and Sheldrick (1982) were the first to demonstrate that injection of ewes with a $PGF_{2\alpha}$ analogue (cloprostenol) caused an immediate increase in luteal oxytocin secretion. Similarly, administration of the $PGF_{2\alpha}$ analogue to cows during the mid-luteal phase of the oestrous cycle caused a rapid increase in luteal oxytocin secretion detectable in the vena cava blood within 5–15 min after injection (Schallenberger *et al.*, 1984). Likewise, studies with cultures of bovine luteal slices (Abdelgadir *et al.*, 1987) or cells (Jarry *et al.*, 1992) or with microdialysis of the bovine corpus luteum (Miyamoto *et al.*, 1993) showed that $PGF_{2\alpha}$ stimulates oxytocin secretion *in vitro*. Some laboratories have experienced difficulty in detecting a response of luteal tissue to $PGF_{2\alpha}$ *in vitro*, which may be related to how the corpora lutea are acquired and subsequently manipulated before slicing or cell dispersion. Prostaglandin E_2 failed to cause a significant release of oxytocin from slices of bovine corpora lutea collected at various stages of the oestrous cycle (Abdelgadir *et al.*, 1987), but was effective in stimulating oxytocin secretion in microdialysed corpora lutea (Miyamoto *et al.*, 1993). Kotwica *et al.* (1991) reported that infusing noradrenaline into cows during the mid-luteal phase of the cycle caused an immediate release of oxytocin. The physiological significance of noradrenaline, if any, in regulating oxytocin secretion is unknown, as is the mechanism by which this exogenous catecholamine can stimulate secretion of oxytocin.

Collectively, the above results from studies *in vitro* and *in vivo* strongly suggest that $PGF_{2\alpha}$ is a primary regulator of luteal oxytocin secretion in ruminants.

Role of Protein Kinase C in $PGF_{2\alpha}$-induced Luteal Oxytocin Secretion

Isoforms of protein kinase C

Protein kinase C (PKC) is a serine–threonine kinase that occurs as a family of multiple subspecies with closely related structures. At least ten isoforms of this enzyme (α, βI, βII, γ, δ, ε, ζ, η, θ and λ) have been identified (Nishizuka, 1992). These isozymes exhibit slightly different modes of activation, kinetic properties and tissue distribution. The α, βI, βII and γ isozymes have been designated conventional PKCs

(cPKC) and are activated by Ca^{2+}, phospholipids and diacylglycerol (DAG) (or phorbol ester). The δ, ε, η and θ isozymes, designated novel PKCs (nPKC), lack the calcium-binding domain found in the cPKCs; however, they are activated by phospholipids and DAG or phorbol ester. The λ and ζ isozymes are further atypical (aPKC) in that they are not affected by Ca^{2+}, DAG or phorbol ester, but are dependent on phosphatidylserine. In addition to different modes of activation, the various isozymes of PKC have different subcellular localization and substrate specificity, allowing the kinase to participate in many cellular events.

There is considerable variation among species with respect to the isoforms of PKC present in corpora lutea. In the rabbit corpus luteum, α, β and δ isoforms of PKC are present (Maizels *et al.*, 1992), while porcine corpora lutea appear to contain predominantly α but also β isozymes (DeManno *et al.*, 1992). Until recently the nature of the isoform(s) of PKC present in the bovine corpus luteum was unknown. Bovine corpora lutea collected on day 8 of the oestrous cycle were subjected to subcellular fractionation and the cytosol and membrane fractions analysed for PKC. Western blot analysis of the plasma membrane fractions, using affinity purified isozyme specific PKC antibodies, revealed that the bovine corpus luteum expressed α and ε isozymes of PKC but did not contain β, γ, δ or ζ (Orwig *et al.*, 1994a). The α and ε isozymes were present in both the cytosolic and plasma membrane fractions. The signal for PKCα was stronger in the cytosol than in the membrane fraction while the opposite situation prevailed for PKCε in unstimulated luteal cells (Fig. 1). Whether one or both of the isozymes of PKC are located within the large luteal cell is not yet known; however, both have been implicated in exocytotic events in other tissues.

Activation of PKC and calpains

Leung *et al.* (1986) demonstrated that binding of $PGF_{2\alpha}$ to its receptor on the luteal cell activates a phosphoinositide cascade which results in increases in DAG and intracellular Ca^{2+} concentrations. Because DAG and Ca^{2+} activate protein kinase C (PKC) it has been hypothesized that this enzyme when activated may regulate the secretion of oxytocin from large luteal cells. This premise is supported in part by data of Cosola-Smith *et al.* (1990), who found that treatment of bovine luteal slices *in vitro* with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate, which directly activates PKC, mimicked the effects of $PGF_{2\alpha}$ by inducing oxytocin secretion. Stimulation of oxytocin secretion by $PGF_{2\alpha}$ may also encompass activation of calpains, which are widely distributed calcium-activated neutral proteases that hydrolyse membrane-associated PKC molecules to a lower molecular weight form of the enzyme referred to as protein kinase C metabolite (PKM) (Melloni *et al.*, 1986). Protein kinase C metabolite is Ca^{2+} and phospholipid independent and can translocate to the cytosol where it may phosphorylate soluble proteins. However, it should be noted that PKM is subject to rapid degradation by other cellular proteases and may be the initial step in the downregulation of this enzyme (Kikkawa and Nishizuka, 1986). Calpains, which are cysteine proteases, exist in two forms, μ-calpain and m-calpain, and are activated by micromolar and millimolar concentrations of intracellular Ca^{2+}, respectively (Murachi, 1983). Because calpains are intracellular proteases that possess an absolute requirement for Ca^{2+}, they may be as important as PKC and calmodulin in Ca^{2+}-mediated regulation of cellular function (Murachi, 1983). Limited hydrolysis is a typical feature of the action of calpains. They hydrolyse protein substrates only to large fragments, not to small peptides or amino acids (Suzuki *et al.*, 1987).

Activities of calpains are specifically suppressed by an endogenous and widely distributed protein inhibitor referred to as calpastatin (Murachi, 1983). Like calpains, calpastatin is activated by Ca^{2+}, but the inhibitor does not affect the nature of the calcium dependence of calpain or sequester Ca^{2+}, making it unavailable to the protease. Rather, in the presence of calcium, calpain and calpastatin form a large aggregate that does not have proteolytic activity. Aggregate formation is reversible depending upon the intracellular Ca^{2+} concentration (Murachi, 1983).

We examined changes in PKC, calpain and calpastatin activities in relation to $PGF_{2\alpha}$-induced oxytocin secretion by bovine corpora lutea (Orwig *et al.*, 1994b). On day 8 of the oestrous cycle, beef heifers were given an i.v. injection of 500 μg cloprostenol (a $PGF_{2\alpha}$ analogue) and corpora lutea were surgically removed at 0, 2, 7.5 or 30 min after injection. Jugular blood samples were collected before injection and at frequent intervals after injection to monitor changes in the pattern of oxytocin secretion.

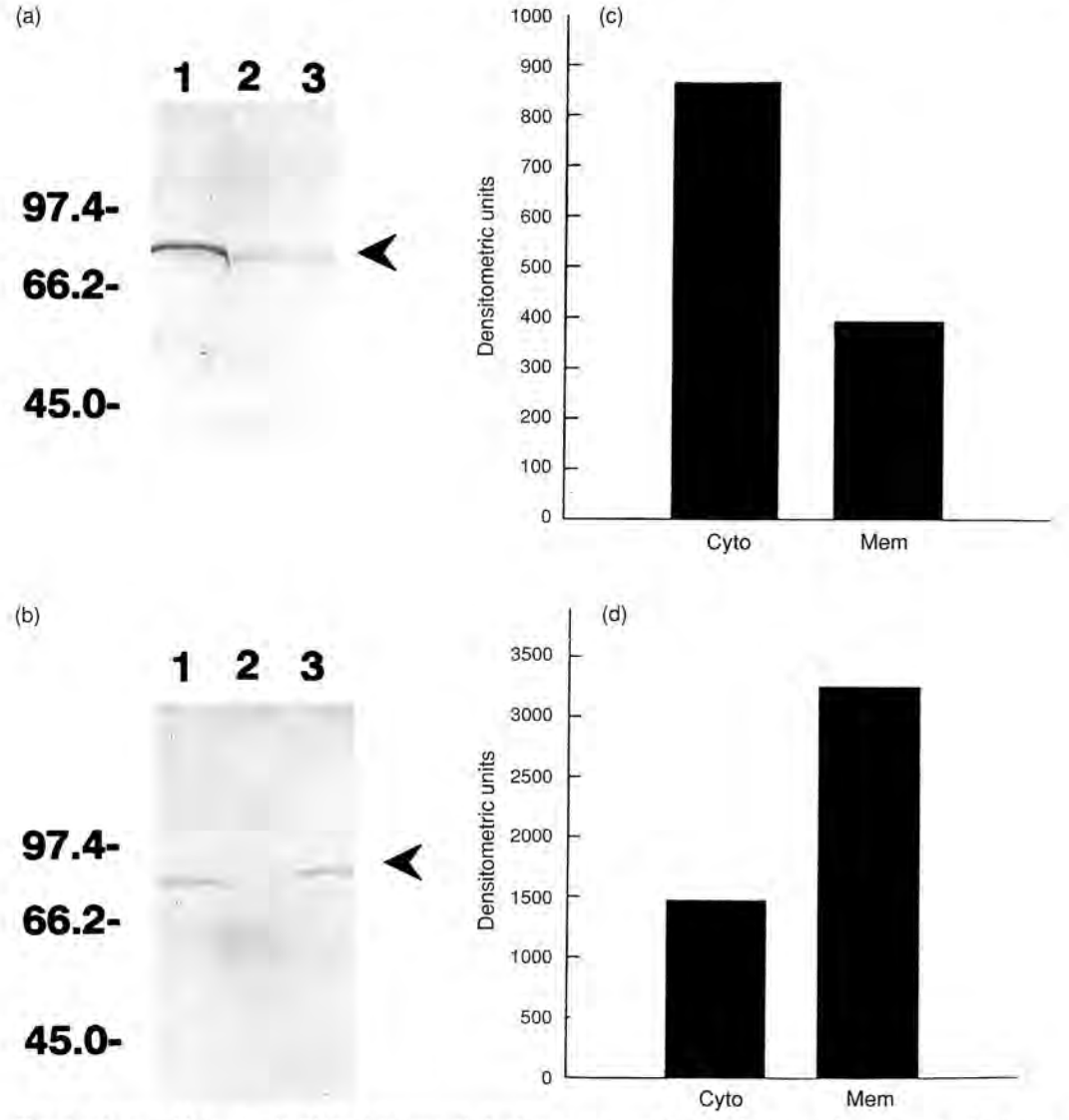

Fig. 1. Western blots of subcellular fractions of the bovine corpus luteum depicting the presence of protein kinase Cα (PKCα) (a) and PKCε (b). Lane 1, whole corpus luteum; lane 2, cytosol; lane 3, membrane. Corresponding densitometric analyses are depicted in (c) and (d). Cyto: cytosol; Mem: membrane. Reprinted from *Comparative Biochemistry and Physiology*, Volume 108B, Orwig *et al.* (1994a), "Immunochemical characterization and cellular distribution of protein kinase C isozymes in the bovine corpus luteum," pp. 53–57, Copyright 1994, with kind permission from Elsevier Science Ltd, Oxford.

Protein kinase C activity in luteal cytosol and membrane fractions and activities of μ- and m-calpains as well as calpastatin were determined. Administration of the $PGF_{2\alpha}$ analogue stimulated oxytocin secretion and peak plasma concentrations were observed between 1.5 and 10 min after injection. This time-course change in luteal oxytocin secretion in response to $PGF_{2\alpha}$ is similar to that reported by Schams *et al.* (1985b). Protein kinase C activity in the luteal plasma membrane tended to increase concomitant with the increase in oxytocin secretion but the differences in activities of the enzyme over the times of corpus luteum removal were not significant (Fig. 2). However, membrane PKC activity was positively correlated

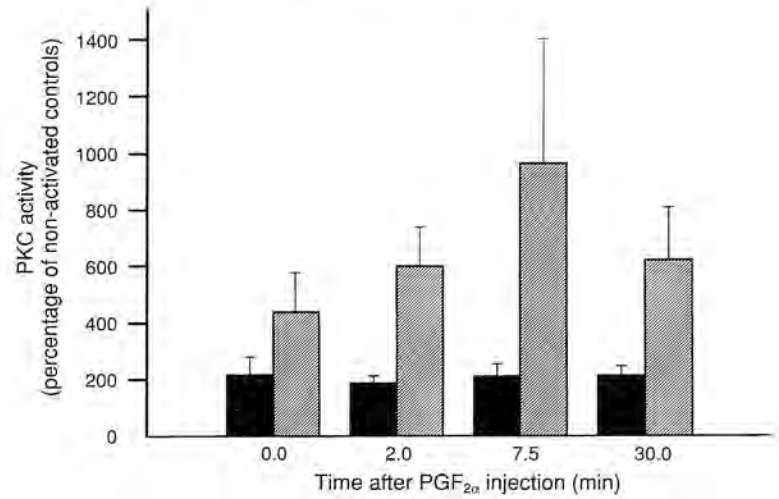

Fig. 2. PKC activity in the cytosol (■) and membrane (▨) fractions of the bovine corpus luteum expressed as a percentage of nonactivated control values at various times after injection of $PGF_{2\alpha}$. Values are the means ± SEM. Reproduced from Orwig *et al.* (1994b) with permission from the Endocrine Society, Copyright 1994.

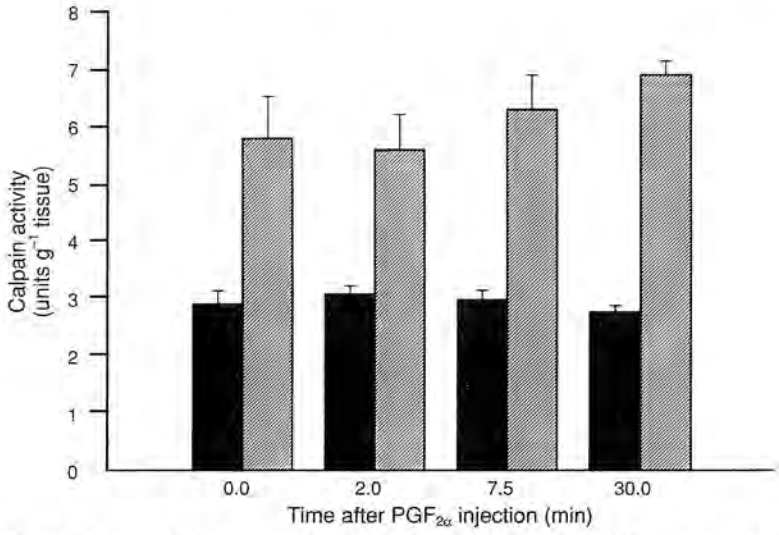

Fig. 3. Activities of μ-calpain (■) and m-calpain (▨) (units g^{-1} tissue) in the bovine corpus luteum at various times after injection of $PGF_{2\alpha}$. Values are means ± SEM. Reproduced from Orwig *et al.* (1994b), with permission from the Endocrine Society, Copyright 1994.

with plasma concentration of oxytocin when the corpus luteum was removed ($r = 0.82$; $P < 0.0025$). Protein kinase C activity in the cytosol did not change over time.

Activities of μ- and m-calpains in the corpus luteum did not differ with time after injection of $PGF_{2\alpha}$ (Fig. 3). However, the activity of m-calpain was significantly greater than that of μ-calpain at each time point. Luteal calpastatin activity was significantly greater 30 min after injection than at 0 min or 2 and 7.5 min after injection (Fig. 4).

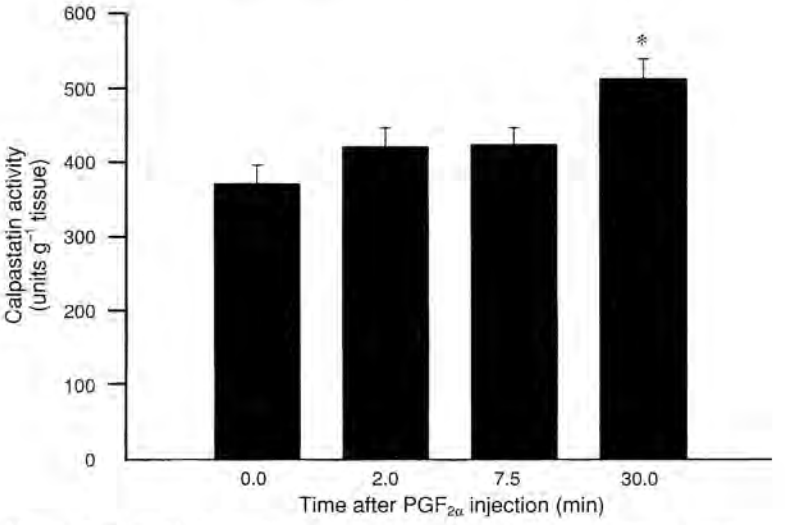

Fig. 4. Activity of calpastatin (units g^{-1} tissue) in the bovine corpus luteum at various times after injection of $PGF_{2\alpha}$. Values are means ± SEM. Reproduced from Orwig *et al.* (1994b), with permission from the Endocrine Society, Copyright 1994.

Collectively, the results of this study demonstrate that changes in luteal PKC activity are associated with oxytocin secretion by this endocrine gland. Although we could not detect changes in μ- or m-calpain activities with time after injection of $PGF_{2\alpha}$, this should not be taken as evidence that these calpains are not essential in regulating PKC activity. It is conceivable that our assay of these calpains was not sensitive enough to detect small changes in their activity. However, m-calpain is the predominant form of these proteases found in the bovine corpus luteum. Calpastatin activity was markedly increased with time after injection of $PGF_{2\alpha}$. It is conceivable that the increase in calpastatin activity would result in inhibition of the proteolytic action of calpains, thus increasing the pool of activated PKC holoenzyme at the membrane and thereby prolonging the time available for PKC to phosphorylate proteins essential for the process of exocytosis of oxytocin.

The cell cortex

The sequence of events that characterizes the intracellular translation, packaging and transport of oxytocin and neurophysin eventually culminating in exocytosis has been reviewed by Wathes and Denning-Kendall (1992). Transport of secretory granules across the cytoplasm is made possible by the presence of a cytoskeleton, consisting of a complex array of actin filaments and microtubles. However, for secretory granules to undergo the process of exocytosis they must first traverse a network of cross-linked monomeric actin filaments lying just beneath the plasma membrane (Alberts *et al.*, 1994). These filaments consisting of β or α actin in cells other than muscle cells are crosslinked or interconnected by various actin-binding proteins, which serve to modulate the geometrical configurations of the actin filaments and their functions. It is noteworthy that this cortical meshwork of actin filaments rearranges rapidly in response to signals from outside the cell that impinge on the plasma membrane (Alberts *et al.*, 1994). Evidence that the cell cortex can actually impede exocytosis was provided by Orci *et al.* (1972), who demonstrated that disruption of the cortex in pancreatic β cells with cytochalasin B enhanced glucose-stimulated secretion of insulin.

Although a number of actin-binding proteins have been identified, one particular protein referred to as myristoylated alanine-rich C kinase substrate (MARCKS) may become particularly important in delineating the mechanism of action of $PGF_{2\alpha}$ in stimulating secretion of oxytocin from luteal cells.

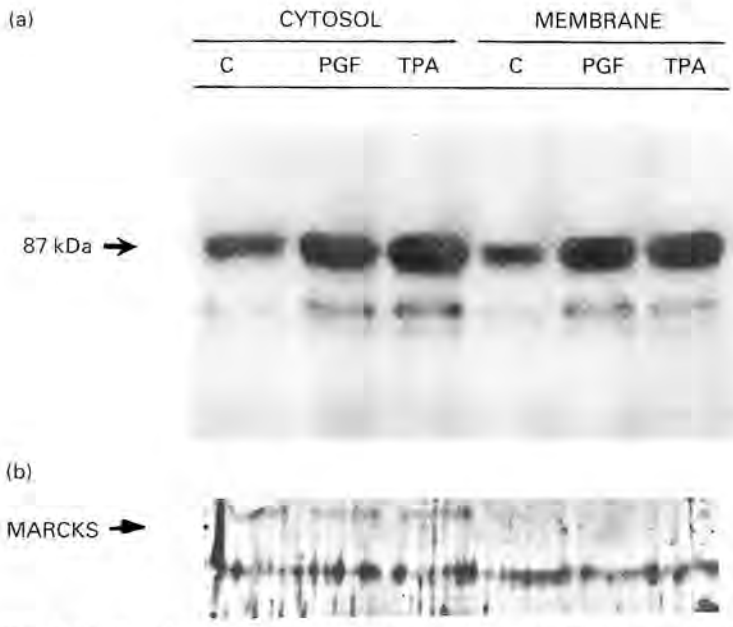

Fig. 5. (a) Phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS) in cytosolic and membrane fractions of bovine luteal slices treated *in vitro* with $PGF_{2\alpha}$ or the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA). C: control; PGF: $PGF_{2\alpha}$; (b) Western blot showing the amount of MARCKS protein in each corresponding lane from the phosphorylation experiment. The lower band visible in all lanes is nonspecific binding.

Myristoylated alanine-rich C kinase substrate

It has been established that $PGF_{2\alpha}$ acting upon the bovine large luteal cell results in activation of PKC and ultimately secretion of oxytocin. However, no information is available concerning the substrate(s) of PKC that may be connected to the exocytosis of oxytocin. Identification of a single protein phosphorylated in response to activation of PKC would perhaps provide an initial step in further delineating the intracellular mechanisms involved in $PGF_{2\alpha}$-induced secretion of oxytocin. In this regard, one such protein, the MARCKS protein, has come to light (Stumpo *et al.*, 1989). This protein is a direct substrate of PKC and cannot be phosphorylated by other protein kinases including cAMP- and cGMP-dependent protein kinases or calmodulin-dependent protein kinases I, II and III (Blackshear *et al.*, 1987). The bovine MARCKS is a 31 kDa protein but it migrates with a high M_r of 87 kDa on SDS gels (Blackshear, 1993). A conserved 25 amino acid basic domain contains the known sites of PKC-catalysed phosphorylation. Whether MARCKS protein is myristoylated post-translationally or co-translationally has not been resolved. Nevertheless, myristoylation of the protein is essential for its ability to associate with the plasma membrane (George and Blackshear, 1992). As mentioned above, the MARCKS protein may also play a role in maintaining the integrity of the cytoskeleton. Hartwig *et al.* (1992) demonstrated that dephosphorylated MARCKS could bind and cross-link filamentous actin *in vitro*. In macrophages, activation of PKC has been shown to release the MARCKS protein from the plasma membrane and elements of the cytoskeleton, changes in protein location that were associated with major changes in the cytoskeleton (Rosen *et al.*, 1990). MARCKS also binds calmodulin in a Ca^{2+}-dependent manner, but phosphorylation inhibits calmodulin binding (Blackshear, 1993). Thus, MARCKS may serve to anchor calmodulin to cellular membranes in reservoir form until calmodulin is released by phosphorylation of the protein by PKC.

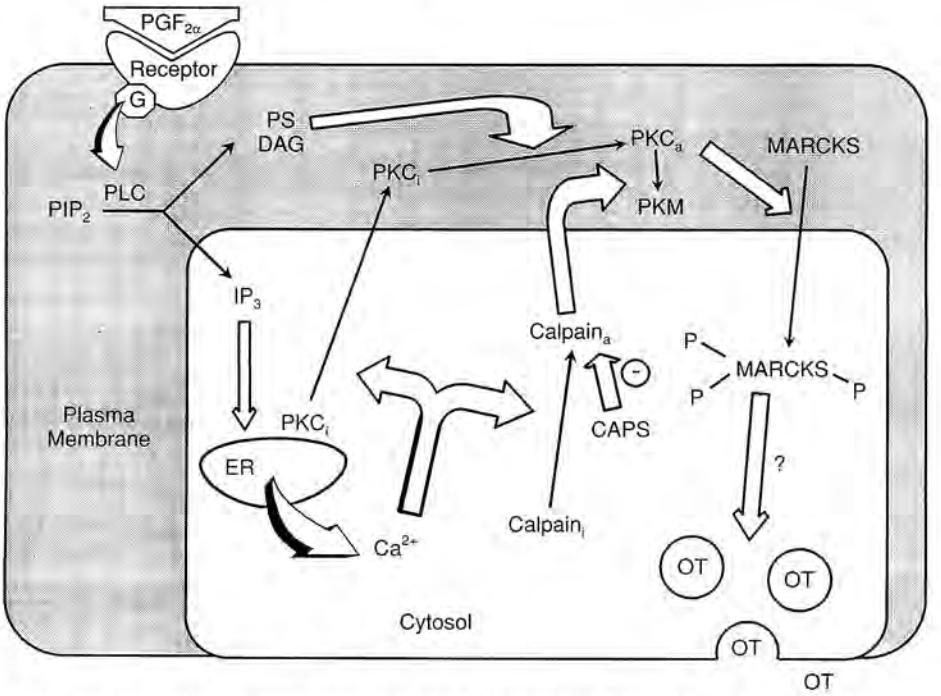

Fig. 6. Proposed model depicting the mechanism of action by which $PGF_{2\alpha}$ induces the exocytosis of oxytocin (OT) from large luteal cells in ruminants. The large arrows denote key biochemical events in the pathway that results in exocytosis of OT. Binding of $PGF_{2\alpha}$ to its plasma membrane receptor promotes interaction of the ligand–receptor complex with a G-protein (G) to signal activation of phospholipase C (PLC). This enzyme catalyses the conversion of phosphatidylinositol-4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol trisphosphate (IP_3). Inositol trisphosphate activates the mobilization of calcium (Ca^{2+}) from the endoplasmic reticulum (ER). The increased intracellular concentration of Ca^{2+} causes translocation of cytosolic inactive protein kinase C (PKC_i) to the plasma membrane where the holoenzyme interacts with DAG and phosphatidylserine (PS) to become fully active (PKC_a). Activated PKC is believed to phosphorylate the actin-binding protein, myristoylated alanine-rich C kinase substrate (MARCKS), consequently resulting in disruption of the cytoskeleton and thus permitting exocytosis of OT granules. The increase in intracellular Ca^{2+} also causes translocation and activation of cytosolic inactive calcium-activated neutral protease ($calpain_i$) to the plasma membrane where the activated protease ($calpain_a$) hydrolyses PKC_a resulting in the liberation of a catalytic subunit referred to as protein kinase C metabolite (PKM). The PKM is released into the cytosol where it retains the ability to phosphorylate cytosolic proteins. Hence, PKM may be the active form of PKC that phosphorylates MARCKS. Ultimately the activity of calpain is suppressed by an inhibitor of these proteases called calpastatin (CAPS) thus ensuring maintenance of a basal level of PKC activity necessary for cell viability. Adapted from Orwig *et al.* (1994b), with permission of the Endocrine Society, Copyright 1994.

On the basis of the characteristics of MARCKS, it is conceivable that it is somehow involved in $PGF_{2\alpha}$-induced secretion of oxytocin. It is noteworthy that $PGF_{2\alpha}$ induces phosphorylation of the MARCKS protein in cultured osteoblasts (MC-3T3-E1) (Quarles *et al.*, 1993). We have conducted studies to determine whether MARCKS protein is present in the bovine corpus luteum and whether it is phosphorylated in response to $PGF_{2\alpha}$. Using western blot analysis with bovine brain as a positive control, we found that the bovine corpus luteum on day 8 of the oestrous cycle does contain the MARCKS protein (Orwig and Stormshak, 1994). In a subsequent study, we demonstrated that incubation of bovine luteal slices with [^{32}P]orthophosphate in the presence of $PGF_{2\alpha}$ or the phorbol ester markedly increased phosphorylation of MARCKS in the cytosolic and membrane fractions compared with controls (Fig. 5; Orwig and Stormshak, 1994). Because the level of phosphorylation of MARCKS

in both the cytosol and membrane was approximately equal, western blot analysis was performed to ascertain whether the observed phosphorylation of cytosolic MARCKS was due to a translocation of the protein from the membrane. No noticeable change in the quantity of MARCKS in the cytosol in response to treatment was evident; however, the concentration of MARCKS in the membrane was so low that it was undetectable by western blot. These data suggest that most of the membrane bound MARCKS is phosphorylated.

Clearly, the fact that MARCKS is present in the bovine corpus luteum, is phosphorylated in response to $PGF_{2\alpha}$ and is associated with the plasma membrane provides the potential for probing deeper into the molecular aspects of oxytocin secretion.

Conclusion

In conclusion, an attempt has been made in this report to identify some of the molecular events associated with secretion of luteal oxytocin. An integrative model is proposed (Fig. 6) showing our current concept of the functional interrelationships among PKC, calpains, calpastatin and MARCKS in promoting oxytocin secretion from $PGF_{2\alpha}$-stimulated large luteal cells in ruminants. In this model, $PGF_{2\alpha}$ binds to its receptor in the plasma membrane of the large luteal cell to trigger a phosphoinositide cascade, resulting in the generation of DAG and inositol trisphosphate (IP_3). Inositol trisphosphate mobilizes Ca^{2+} from intracellular stores in the endoplasmic reticulum, which is involved in the activation of PKC, μ- and m-calpains and calpastatin. Membrane bound PKC is activated by Ca^{2+}, DAG and phosphatidylserine and phosphorylates MARCKS associated with the cytoskeleton and (or) plasma membrane to facilitate exocytosis of oxytocin. Alternatively, activated PKC may be hydrolysed by calpains to PKM, which is intrinsically active, and may be the biologically active form of the enzyme that phosphorylates MARCKS.

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