STIMULATION OF BOVINE LUTEAL OXYTOCIN SECRETION IN VITRO BY A PHORBOL ESTER AND CALCIUM IONOPHORE

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ABSTRACT
Experiments were conducted to examine the in vitro effects of a phorbol ester and a calcium ionophore on bovine luteal oxytocin (OT) secretion and synthesis and progesterone secretion. Corpora lutea removed from beef heifers on d 8 of an estrous cycle were sliced and incubated for 2 h with .81 nM 12-O-tetradecanoylphorbl-13-acetate (TPA), 1.62 nM TPA or .3 μM calcium ionophore A23187. Both concentrations of TPA increased (P < .01) OT secretion (ng·g⁻¹·2 h⁻¹; control, 407.1; .81 nM TPA, 494.7; 1.62 nM TPA, 528.1; SE = 21.2). Increased secretion of OT was accompanied by a corresponding increase (P < .02) in synthesis of the hormone (ng·g⁻¹·2 h⁻¹; control, 368.5; .81 nM TPA, 427.6; 1.62 nM TPA, 492.1; SE = 25.7). Phorbol ester also induced (P < .025) progesterone secretion (ng·g⁻¹·2 h⁻¹; control, 1,056.2 vs A23187, 1,333.3; SE = 86.4). Calcium ionophore increased (P < .01) OT secretion (ng·g⁻¹·2 h⁻¹; control, 248.9 vs A23187, 327.4; SE = 16) and there was a trend (P = .09) toward increased synthesis of OT in response to the ionophore (control, 124.4 vs A23187, 165.6; SE = 16.4). Because TPA can activate protein kinase C and A23187 increases intracellular calcium, these intracellular constituents probably are involved in promoting secretion of OT and progesterone.
(Key Words: Bovidae, Corpus Luteum, Oxytocin, Calcium.)


Introduction
Prostaglandin F₂α (PGF₂α) induces oxytocin (OT) secretion from bovine corpora lutea in vivo (Schallenberger et al., 1984) and in vitro (Abdelgadir et al., 1987), but the mechanism of action of PGF₂α in provoking this response is unknown. In vitro treatment of bovine luteal cells with PGF₂α stimulated inositol phosphate accumulation, indicative of phosphoinositide hydrolysis (Davis et al., 1988). In general, hormonal stimulation of phosphoinositide hydrolysis causes intracellular calcium mobilization and diacylglycerol production, which ultimately participate in protein kinase C activation (Nishizuka et al., 1984). Thus PGF₂α-induced OT secretion may be due to changes in intracellular calcium concentrations (Alila et al., 1989) and/or protein kinase C activation. If this premise is true, then nonhormonal agents capable of affecting intracellular calcium levels or protein kinase C activity might mimic the effects of PGF₂α on OT secretion. Two compounds that fulfill the latter properties are the phorbol ester 12-O-tetradecanoylphorbl-13-acetate (TPA) and the calcium ionophore A23187. This phorbol ester mimics the action of diacylglycerol and directly activates protein kinase C (Castagna et al., 1982), whereas A23187 increases intracellular calcium concentrations.

These experiments were conducted to examine the effects of TPA and A23187 on secretion and tissue concentrations of OT in

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the bovine corpus luteum in vitro. A corollary objective was to determine whether TPA could evoke concomitant secretion of progesterone.

Materials and Methods

Eleven 18-mo-old crossbred beef heifers were checked twice daily for estrus using a vasectomized bull. After exhibiting at least two consecutive estrous cycles of normal duration (18 to 23 d), heifers were restrained on d 8 of the cycle (detected estrus = d 0 of the cycle), tranquilized by injection of 3 ml of acepromazine into the middle coccygeal vein and injected with 3 ml of 2% lidocaine to induce caudal epidural anesthesia. The corpus luteum was collected via a supravaginal incision and freed of connective tissue, weighed and sliced. Slices of tissue rather than dispersed cells were used in order to minimize damage to plasma membrane integrity and loss of OT during tissue processing. Tissue slices (.3 mm thickness) were washed four times with Ham’s F-12 (4°C), blotted on filter paper and divided into aliquots.

 Corpora lutea from six heifers were utilized to examine the in vitro effects of TPA on secretion and postincubation tissue concentrations of OT as well as the ability of one concentration of TPA to stimulate progesterone secretion. Tissue aliquots (255 ± 6 mg) were placed into siliconized incubation flasks (prepared in duplicate) containing 1.99 ml of Ham’s F-12 medium (4°C) to which the following were added: 1) vehicle (10 µl dimethylsulfoxide [DMSO] unincubated control); 2) vehicle (incubated control); 3) 1 ng/ml TPA (final concentration, .81 nM); or 4) 2 ng/ml TPA (final concentration, 1.62 nM). Concentrations of TPA and DMSO employed were less than those found to be toxic in porcine granulosa cells (Veldhuis and Demers, 1986) and bovine luteal cells (Brunswig et al., 1986), respectively. Further, these concentrations of TPA directly activated protein kinase C in rat brain cells (Castagna et al., 1982). All flasks were flushed with 95% O2-5% CO2 and stopped; appropriate flasks were placed in a Dubnoff incubator at 38°C for 2 h. Incubation was terminated by immersing flasks in an ice bath (4°C), after which the contents were transferred to plastic tubes and centrifuged at 3,000 × g. The tissue pellet was immediately separated from the supernatant and both were quick-frozen and stored at −20°C until they were assayed for OT and progesterone. Unincubated control flasks were processed similarly at the beginning of the incubation period.

Aliquots of luteal tissue (217 ± 5 mg) from the remaining five heifers were placed into flasks containing Ham’s F-12 medium (1.99 ml) as described above to examine the effect of calcium ionophore on secretion of OT and tissue concentrations of the hormone after incubation. Flasks and additions to the medium were as follows: 1) unincubated control (vehicle, 10 µl DMSO), 2) incubated control (vehicle) and 3) .31 µg/ml A23187 (final concentration, .3 µM). This concentration of ionophore was utilized because it stimulated protein kinase C activity in platelets (Kaibuchi et al., 1983). The protocol for incubation and postincubation processing of flask contents in preparation for assay of OT was identical to that described above.

Radioimmunoassays. Oxytocin released into the incubation medium was assayed directly without extraction. Tissue OT was extracted as described by Abdelgadir et al. (1987) with a mean extraction efficiency of 84.4 ± 1.7%.

Oxytocin in the media and tissue extracts was quantified by RIA as described by Abdelgadir et al. (1987) using an OT antibody provided by Dieter Schams, Technical University of Munich. Sensitivity of the assay was .25 pg/tube (P < .05, n = 32). Intra- and interassay coefficients of variation were 8.0 (n = 7) and 10.2% (n = 9), respectively.

Progesterone was extracted from medium and assayed as described previously (Koligian and Stormshak, 1977). Mean extraction efficiency was 90.3 ± 4.0% and the sensitivity of the RIA was 10 pg/tube (P < .05, n = 10). Intra- and interassay coefficients of variation were 9.0 (n = 6) and 4.3% (n = 5), respectively.

Data Analysis. Concentration of OT in the medium of each incubated sample minus the concentration of OT present in the medium of the appropriate unincubated control was considered to represent secreted hormone. An estimate of OT "synthesis" during incubation was derived by subtracting the initial tissue content of OT (unincubated control) from the sum of OT content in the medium (secreted hormone) plus the postincubation tissue con-
LUTEAL OXYTOCIN SECRETION

600

CT,  e

V 0  x

6

Unincubated

Tissue

Release

Incubated

Tissue

ng Oxytocin • g CL

Figure 1. Oxytocin concentrations in unincubated luteal tissue (mean ± SE) and in tissue and media (mean ± common SE) after a 2-h incubation with 12-O-tetradecanoylphorbol-13-acetate (TPA) on d 8 of an estrous cycle. *Different from control (P < .01).

tent of OT. “Synthesis” as used in the present context is assumed to represent conversion of prohormone to immunologically detectable OT and does not imply de novo production of the hormone from small molecule precursors. Postincubation tissue concentrations of OT were not adjusted for initial tissue concentrations of hormone if the quantity of secreted hormone exceeded the latter.

Data involving effects of two concentrations of TPA on OT synthesis and secretion as well as postincubation tissue concentrations of OT were analyzed statistically by analysis of variance for an experiment of randomized block design. Differences among means were tested for significance by orthogonal contrasts. Effects of TPA on progesterone secretion and of A23187 on synthesis and secretion of OT were analyzed by use of Student’s paired t-test.

Results

In vitro effects of TPA on luteal tissue concentrations of OT after incubation and on the quantity of OT released into the medium are depicted in Figure 1. Mean (± SE) concentration of OT in unincubated luteal tissue was 390.3 ± 60.2 ng/g; small quantities of the hormone (46.3 ± 12.0 ng/g) were released into the surrounding medium. Concentrations of OT in tissue after incubation alone or in the presence of the two concentrations of TPA were slightly less than that of the unincubated control tissue, but these differences were not significant. However, both concentrations of TPA increased secretion of OT relative to that of incubated control tissue (P < .01). Although exposure of tissue to the higher concentration of phorbol ester (1.62 nM) tended to cause greater secretion of OT, the effects of the two concentrations of TPA on this characteristic did not differ (P > .05). Synthesis of OT by luteal tissue incubated with either concentration of TPA was greater than that of incubated control tissue (P < .02; Figure 2). Synthesis of OT in response to TPA increased in a dose-dependent manner; however, the difference between the dose levels was not significant (P > .05).

In addition to enhancing luteal OT secretion, TPA (.81 nM) increased progesterone release (ng·g⁻¹·h⁻¹; 1,056.2 for control vs 1,333.3 for .81 nM TPA; SE = 86.4; P < .025). Concomitant secretion of progesterone and OT from the same tissue was correlated positively (r = .40).
Changes in in vitro luteal OT secretion and synthesis in response to calcium ionophore are presented in Table 1. Mean concentrations of OT in unincubated luteal tissue and the medium were 373.4 ± 35.5 and 18.7 ± 7.7 ng/g, respectively. Calcium ionophore increased luteal secretion of OT relative to secretion of the hormone by incubated control tissue (P < .01). There also was a trend for the ionophore to increase OT synthesis during incubation (P = .09).

Discussion

The phorbol ester TPA stimulated OT synthesis and secretion by bovine luteal tissue in vitro. This phorbol ester is able to intercalate into the plasma membrane and, because of its structural similarity to diacylglycerol, it directly activates protein kinase C (Castagna et al., 1982; Nishizuka et al., 1984). Thus, TPA-activated protein kinase C probably played a role in the secretion of OT; effects of this phorbol ester via other intracellular pathways remains a possibility. Support for a role of this kinase in luteal OT production has been provided by the observation that phospholipase C, an enzyme that causes diacylglycerol production and, thus, protein kinase C activation, increased OT secretion from ovine luteal slices (Hirst et al., 1988). Further, this phospholipase-induced increase in OT secretion was not detected in the presence of CoCl₂, which inhibits entry of calcium into cells. Because PGF₂α stimulates phosphoinositide hydrolysis in bovine luteal cells (Davis et al., 1988), activation of protein kinase C probably is involved in mediating the ability of this prostaglandin to induce luteal OT secretion in vivo and in vitro (Schallenberger et al., 1984; Abdelgadir et al., 1987). Precisely which protein(s) are phosphorylated by this enzyme to enhance exocytosis of the hormone is unknown but should be investigated. Activation of protein kinase C also could be accompanied by increased production of arachidonic acid, which in turn may enhance or facilitate luteal OT secretion (Hirst et al., 1988).

Secretion of OT during incubation of tissue alone or in the presence of TPA was accompanied by tissue replenishment of the hormone. Luteal concentrations of OT did not differ among unincubated control, incubated control...
or TPA-treated tissues. Quantity of OT secreted exceeded initial unincubated tissue content of the hormone, suggesting that the tissue content of OT after incubation represented newly "synthesized" hormone. Secretion of OT by bovine luteal tissue incubated for 2 h in the presence of the protein synthesis inhibitor cycloheximide failed to prevent tissue replenishment of the hormone (Abdelgadir, 1988). Thus, OT synthesis observed in our study apparently was not the result of OT mRNA translation but rather of post-translational conversion of the prohormone. This possibility also is supported in part by the report of Ivell et al. (1985), whose data indicate that bovine luteal OT mRNA concentration on d 8 of the estrous cycle is considerably lower than during earlier stages of the cycle. Estimates of OT synthesis in our study fail to account for any degradation of hormone that may have occurred. However, rate of degradation of hormone should not differ among the incubated samples of control and treated tissues.

Phorbol ester-induced secretion of OT was accompanied by an enhanced progesterone secretion. These results are in agreement with those of Brunswig et al. (1986) and Hansel and Dowd (1986), who found that TPA increased progesterone release from dispersed bovine luteal cells. Because the bovine corpus luteum consists of two steroidogenic cell types, large and small (Koos and Hansel, 1981), it is not known on which cell type the phorbol ester acted to promote the observed increase in progesterone secretion. However, Alila et al. (1988) reported that phorbol dibutyrate stimulated progesterone synthesis by dispersed small but not large luteal cells. Although large luteal cells that contain OT probably were acted on directly by TPA, the possibility that the stimulus for secretion of OT arose from small luteal cells affected by TPA cannot be excluded.

In contrast to the observed stimulatory effect of TPA on progesterone secretion by bovine luteal tissue, Conley and Ford (1989) reported that 10 to 1,000 nM of this phorbol ester inhibited progesterone synthesis by dispersed ovine luteal cells. These investigators suggested that the inhibitory effect of TPA may have been mediated by enhanced luteal synthesis of PGF2α. However, it was acknowledged that PGF2α could not be the sole cause for TPA-induced inhibition of luteal steroidogenesis.

Results of the current study also indicate that the calcium ionophore A23187 can enhance OT secretion by the bovine corpus luteum. Similar effects of A23187 on OT secretion by ovine luteal cells have been reported (Hirst et al., 1986). The response to this ionophore presumably is due to its ability to increase intracellular calcium levels. Activation of protein kinase C requires calcium mobilization; this ion has been shown to act synergistically with phorbol esters or diacylglycerol to increase activity of the enzyme (Nishizuka et al., 1984). Although not directly comparable, the quantity of OT synthesized by luteal tissue exposed to the ionophore was much less than that of tissue incubated with TPA. Because of the absence of a stimulus to promote increased intracellular concentrations of diacylglycerol or direct activation of protein kinase C, the reduced OT synthesis observed in response to A23187 may reflect basal enzyme activity.

In conclusion, the phorbol ester TPA or a calcium ionophore can stimulate in vitro OT secretion and synthesis by bovine luteal tissue slices. In addition, phorbol ester acts on luteal cells to increase secretion of progesterone.

**Implications**

Changes in intracellular concentrations of calcium and activation of protein kinase C are

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**TABLE 1. OXYTOCIN (OT) SECRETED AND SYNTHESIZED BY BOVINE LUTEAL SLICES IN RESPONSE TO INCUBATION WITH CALCIUM IONOPHORE A23187**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Secretiona</th>
<th>Synthesisb</th>
</tr>
</thead>
<tbody>
<tr>
<td>O (Control)</td>
<td>5</td>
<td>248.9</td>
<td>129.4</td>
</tr>
<tr>
<td>0.3 μM A23187</td>
<td>5</td>
<td>327.4b</td>
<td>165.6c</td>
</tr>
</tbody>
</table>

*aCommon estimate of the standard error = 16.0 (secretion); 16.4 (synthesis).

bp < .01 compared with control.

*cP = .09 compared with control.
involved in promoting secretion of oxytocin from the bovine corpus luteum. These data are supportive of the premise that prostaglandin F2α-induced secretion of oxytocin from the bovine corpus luteum in vivo likely occurs as a result of an increased intracellular concentration of calcium and activation of protein kinase C.

Literature Cited


