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Mechanism of LHRH-Stimulated Steroidogenesis in Rat Leydig Cells: Lipoxygenase Products of Arachidonic Acid May Not Be Involved

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Luteinizing hormone releasing hormone agonist, [(imBzl)-DHis6,Pro9,NEt]-LHRH (LHRH-A), caused a two to threefold increase in *in vitro* testosterone (T) secretion by rat Leydig cells. This LHRH-A-induced T secretion was completely blocked by quinacrine and chloroquine, inhibitors of phospholipase A_2. Addition of phospholipase A_2, however, was ineffective in stimulating basal or LHRH-A-induced T secretion. Phospholipase C, on the other hand, significantly stimulated both basal and LHRH-A-induced T secretion. Exogenously added arachidonic acid stimulated basal T secretion in a dose dependent manner, the maximum increase being about 100% over basal at a dose of 100 μM. Higher doses of arachidonic acid had no stimulatory effect. In the presence of LHRH-A, the stimulatory effect of arachidonic acid was additive up to a concentration of 100 μM; but higher concentrations of arachidonic acid (200 μM) were inhibitory. LHRH-A-induced steroidogenesis was inhibited by 5, 8, 11, 14 Eicosatetraynoic acid (ETYA), an inhibitor of all the three known pathways of arachidonic acid metabolism, and by nordihydroguaiaretic acid, an inhibitor of the lipoxygenase pathway of arachidonic acid metabolism. LHRH-A-stimulated T secretion was not inhibited by indomethacin, an inhibitor of the cyclo-oxygenase pathway of arachidonic acid metabolism. ETYA inhibited arachidonic acid-induced T secretion. Nordihydroguaiaretic acid, on the other hand, augmented basal, arachidonic acid–, phospholipase C–, or phorbol 12, myristate 13 acetate-induced testosterone secretion. These results suggest that arachidonic acid, whose release is influenced by phospholipase C, is involved in LHRH-A-induced T secretion by rat Leydig cells. Furthermore, LHRH-A-induced steroidogenic responses in Leydig cells seem to be mediated by arachidonic acid metabolites other than cyclo-oxygenase or lipoxygenase products.

Key words: Phospholipases, arachidonic acid, Leydig cell steroidogenesis

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LHRH agonists have been shown to have direct inhibitory effects on gonadal function (Cao et al, 1982). It has recently been shown that over a short period of time (up to 8 h), LHRH agonists augment T secretion *in vitro* (Sharpe and Cooper, 1982; Hunter et al, 1982). The mechanism by which these peptides stimulate T secretion in Leydig cells is not clear. It has been shown that these stimulatory effects

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are not mediated by cyclic AMP but are calcium dependent (Lin, 1984b; Sullivan and Cooke, 1984).

It is fairly well-established that hormones using calcium as a second messenger also enhance phospholipid turnover (Michell, 1975; Berridge, 1984; Nishizuka, 1984). The earliest event after the coupling of a hormone to its receptor is hydrolysis of phosphatidylinositol by a specific phospholipase C, resulting in the formation of inositol triphosphate and diacylglycerol, which is rich in arachidonic acid. The diacylglycerol is phosphorylated to phosphatidic acid, which constitutes the so-called primary response to stimulation with hormones and appears to precede the liberation of arachidonic acid by the action of phospholipase A_2 (Lapetina, 1982). Alternatively, arachidonic acid can be released directly from diacylglycerol by the action of specific diglyceride lipase (Bell et al, 1979).

In the pituitary, LHRH-induced LH release was shown to be associated with phospholipid turnover and arachidonic acid release (Naor and Catt, 1981). Arachidonic acid metabolites have also been shown to be involved in LHRH-induced LH release (Snyder et al, 1983). Recently LHRH agonists have been shown to increase phospholipid labeling in Leydig cells (Molcho et al, 1984a; Lin, 1984a), indicating the occurrence of phospholipid turnover during LHRH stimulated T secretion. Recently, arachidonic acid metabolites have been shown to be involved in LH/hCG-induced steroidogenesis in Leydig cells (Dix et al, 1984; Didolkar and Sundaram, 1987). Furthermore, arachidonic acid has been implicated to have an intermediary role in LHRH-A-induced T secretion. There is controversy, however, over whether arachidonic acid itself or its lipoxygenase metabolites are involved in LHRH-induced T secretion (Lin, 1985a; Sullivan and Cooke, 1985a, 1985b; Didolkar and Sundaram, 1986). In the present study, we investigated the involvement of phospholipases A_2 and C, and arachidonic acid in LHRH-A-stimulated steroidogenesis in rat Leydig cells. We have also studied the effects of a relatively specific inhibitor of arachidonic acid metabolism by the lipoxygenase pathway to resolve the controversy over whether lipoxygenase products are involved.

Materials and Methods

Medium 199 (with Earle's salts and glutamine) was purchased from Gibco Laboratories, NY, [(imBzl)-DHis6,Pro9,NEt]-LHRH was obtained from Dr. Jean Rivier (Salk Institute, San Diego, CA). Collagenase (Type I) bovine albumin, arachidonic acid (99% pure), phospholipase A_2, phospholipase C, chloroquine, quinacrine, nordihydroguaiaretic acid, and indomethacin were purchased from Sigma Chemical Company (St. Louis, MO). ETYA was supplied by Dr. Peter Sorter (Hoffman LaRoche, Nutley, NJ). Adult male Sprague Dawley rats were purchased from Charles River Laboratories Inc., NY, and maintained under standard conditions.

Four to five rats were killed by ether or carbon dioxide asphyxiation for each experiment. The testes were removed, decapsulated, and the interstitial cells prepared by collagenase treatment as previously described (Wang et al, 1983). The viability of cells was checked by trypan blue exclusion. Cells that stained positively for 3 β-hydroxysteroid dehydrogenase (3BSDH) activity were counted. One to 2×10^6 3BSDH positive cells were used for incubation in 1 ml medium 199 containing 0.1% bovine albumin.

To rule out the possibility that the effects of LHRH-A or arachidonic acid were not due to secondary effects of contaminating cells present in the crude Leydig cell preparation, we purified Leydig cells using a linear density gradient of Percoll. A 35 ml linear gradient (0–90%) of Percoll was generated by high speed centrifugation. A 2 ml crude Leydig cell preparation was layered on gradient and centrifuged at 800 × g for 20 min. Twenty-five ml from the top of the gradient were discarded. Next, an 8 ml fraction containing mostly Leydig cells was collected, washed twice with M-199, and finally suspended in M-199. This preparation contained over 70% Leydig cells.

Cells suspended in 1 ml medium were incubated with various concentrations of LHRH-A for 3 h at 34C in a shaking water-bath (120 strokes/min) under an atmosphere of 95% oxygen and 5% carbon dioxide. For studying the effects of arachidonic acid and various inhibitors on LHRH-A-stimulated T production, the cells were preincubated with different compounds for 30 min before adding LHRH-A. The effects of phospholipase A_2, phospholipase C, and arachidonic acid on Leydig cells were studied over a 3.5 h incubation period. At the end of incubation, tubes were centrifuged at 1500 × g, and the supernatant stored at −20C until analysis for T. T was determined by radioimmunoassay (Wang et al, 1983). The inter- and intra-assay coefficients of variation were 7.3% and 5.3%, respectively. All samples from one experiment were run in duplicate in the same assay. All experiments were carried out at least twice, and results of representative experiments are presented.

Statistical analysis of the data was done by multivariate analysis of variance followed by Contrasts test using BMDP software (Dixon, 1983).

Results

The viability of Leydig cells was over 90% and did not change during incubation. 3BSDH positive cells constituted between 40 and 60% of the cells in different experiments. Results are expressed as nanograms (ng) of T produced per 1×10^6 3BSDH positive cells except when purified Leydig cells were used, for which results are expressed as per 1×10^6

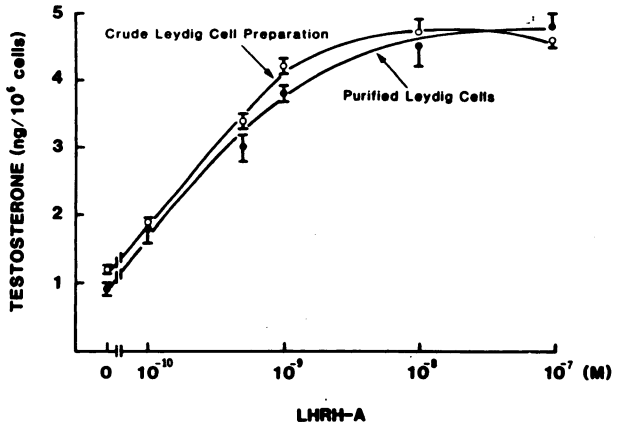

Fig. 1. Effect of LHRH-A on testosterone secretion by rat crude Leydig cell preparation and Percoll purified Leydig cells during 3 h incubation. Leydig cells were incubated with different concentrations of LHRH-A or Medium M-199. Values are mean ± S.E.M. of triplicate incubations.

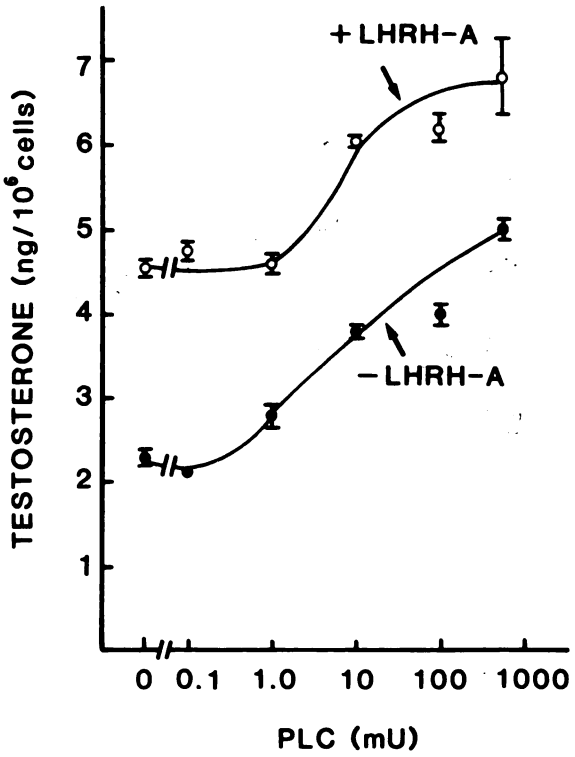

Fig. 2. Effect of phospholipase C on testosterone secretion by rat crude Leydig cell preparation. Leydig cells were incubated with different concentrations of phospholipase C for 30 min and then with or without LHRH-A for an additional 3 h. Values are mean ± S.E.M. of six determinations.

total cells. Ten μl of ethanol, used to introduce various compounds in the medium, had no significant effect on basal T production.

LHRH-A produced a dose dependent increase in T production up to a dose of 1 nM; higher doses did not further increase T output signficantly. Crude Leydig cell preparations and purified Leydig cells responded equally well to LHRH-A (Fig. 1). In subsequent experiments, therefore, 1.0 nM of LHRH-A was used as the stimulatory concentration. Crude Leydig cell preparations were used for other experiments since they showed similar effects compared with purified cells. Chloroquine and quinacrine inhibited LHRH-A-induced T secretion (LHRH-A: 100%; 100 μM chloroquine + LHRH-A: 42.5 ± 1%; 100 μM quinacrine ± LHRH-A: 29.4 ± 0.5%). Phospholipase A_2 (purified from different tissue sources) had no stimulatory effect on either basal or LHRH-A-stimulated T secretion. Phospholipase C, on the other hand, stimulated T secretion in a dose dependent manner (Fig. 2), the maximum being over twofold. Phospholipase C was also able to augment LHRH-A-induced T secretion.

The addition of arachidonic acid also stimulated T secretion in a dose dependent manner up to a concentration of 100 μM arachidonic acid. The maximum stimulation was about 200% of basal. Arachidonic acid produced essentially similar effects in crude and purified Leydig cell preparations. Higher concentrations of arachidonic acid (above 100 μM) were not stimulatory (Fig. 3). When arachidonic acid was added with LHRH-A, up to a concentration of

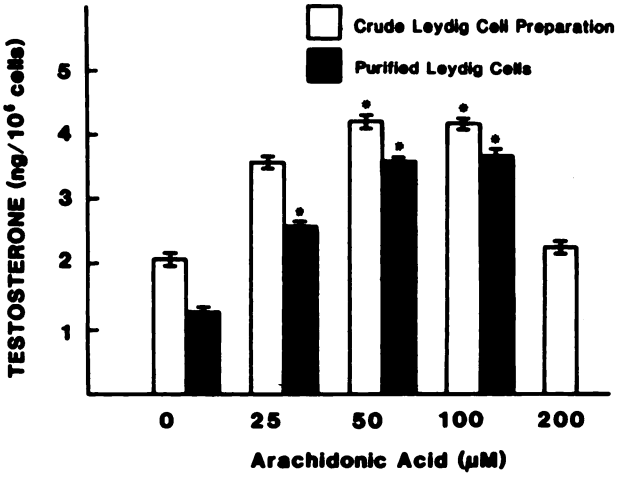

Fig. 3. Effect of arachidonic acid on testosterone secretion by crude Leydig cell preparation and Percoll purified rat Leydig cells during 3.5 h incubation. Arachidonic acid was introduced into the incubation medium in a volume of 10 μl as ethanolic solution. Control incubations had equivalent amount of ethanol. Each value is mean ± S.E.M. of six determinations. $P < 0.001$

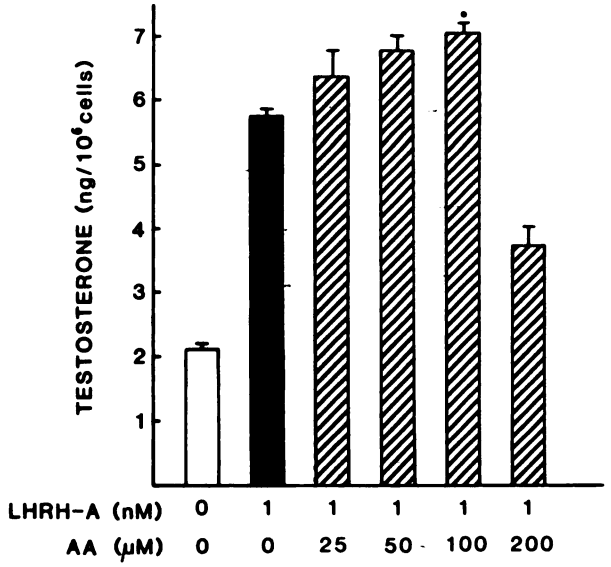

Fig. 4. Effect of LHRH-A with and without exogenous arachidonic acid on testosterone secretion by rat crude Leydig cell preparation. Cells were preincubated with different concentrations of arachidonic acid for 30 min. LHRH-A was then added and incubation continued for another 3 h. Each bar represents mean ± S.E.M. of six values. $P < 0.05$ compared to LHRH-A stimulated testosterone secretion in absence of AA.

100 μM, arachidonic acid further stimulated LHRH-A-induced T secretion. But 200 μM of arachidonic acid was inhibitory (Fig. 4).

To investigate whether steroidogenic effects of arachidonic acid are brought about by arachidonic acid itself or its metabolites, we employed inhibitors of various pathways of arachidonic acid metabolism. ETYA, an inhibitor of arachidonic acid metabolism by all the three known pathways, significantly inhibited LHRH-A-stimulated steroidogenesis (Table 1). It also completely blocked arachidonic acid-induced T secretion (Table 2). Nordihydroguaiaretic acid, an inhibitor of lipoxygenase pathway, also completely blocked LHRH-A-induced T secretion (Table 1). Indomethacin, an inhibitor of cyclo-oxygenase pathway, however, did not have any inhibitory effect on LHRH-A-stimulated T secretion (Table 1). It did have a slight but significant ($p < 0.05$) stimulatory effect. To rule out the possibility that inhibitory effects of ETYA and nordihydroguaiaretic acid were due to cytotoxicity, we checked the viabilty of Leydig cells before and after incubation with these compounds. The initial viability was 94%. After incubation, it was 92, 93, and 85% for LHRH-A, LHRH-A + nordihydroguaiaretic acid, and LHRH-A + ETYA, respectively. Basal T secretion was enhanced by ETYA and nordihydroguaiaretic acid, whereas, indomethacin had no effects. Nordihydroguaiaretic acid augmented arachidonic acid—, phospholipase C—, or phorbol 12, myristate 13 acetate-induced T secretion (Table 3).

TABLE 1. Effect of ETYA, Nordihydroguaiaretic Acid (NDGA), or Indomethacin on LHRH-A-Stimulated Testosterone Secretion by Rat Leydig Cells

Treatment	Testosterone (% of Basal)
Basal	100.0
ETYA (100 μM)	129.96 ± 3.25*
NDGA (100 μM)	166.14 ± 11.67*
Indomethacin (100 μM)	101.71 ± 2.5
LHRH-A (1 nM)	245.51 ± 10.0
LHRH-A + ETYA (100 μM)	167.27 ± 2.07†
LHRH-A + NDGA (100 μM)	96.8 ± 4.8†
LHRH-A + Indomethacin (100 μM)	305.76 ± 12.7‡

Leydig cells were incubated with the different inhibitors of arachidonic acid metabolic pathways for 30 min. LHRH-A was added and incubation continued for another 3 h. Each value is mean ± S.E.M. of triplicate incubations. All values are representative of three experiments.

*$P < 0.01$ for differences from basal.

†$P < 0.01$ for differences from 1 nM LHRH-A.

‡$P < 0.05$ for differences from 1 nM LHRH-A.

TABLE 2. Effect of ETYA, on Arachidonic Acid (AA)-Stimulated Testosterone Production by Rat Leydig Cells

Treatment	Testosterone (% of Basal)
Basal	100
AA (100 μM)	172 ± 5
AA (100 μM) + ETYA (50 μM)	84 ± 4*
AA (100 μM) + ETYA (100 μM)	88 ± 2*
ETYA (50 μM)	207 ± 4†
ETYA (100 μM)	130 ± 4†

Cells were incubated with ETYA for 30 min and indicated concentrations of AA were added and incubated further for another 3 h. Each value is mean ± S.E.M. of six incubations. Values are representative of two experiments.

*$P < 0.001$ for differences from 100 μM AA.

†$P < 0.001$ for differences from control.

Discussion

This study has provided evidence for the possible involvement of phospholipase C and arachidonic acid in LHRH-A-stimulated steroidogenesis in rat Leydig cells. Both phospholipase C and arachidonic acid were shown to stimulate T secretion. However, phospholipase A_2, an enzyme which has been shown

to be associated with the release of arachidonic acid from phospholipids (Lapetina, 1982), was unable to stimulate T secretion from Leydig cells. A stimulatory effect of phospholipase C but not of phospholipase A_2 on prolactin release from a pituitary clonal cell line (GH3) has also been reported (Martin and Kowalchyk, 1984). In other systems, such as pituitary lactotrophs (Grandison, 1984), pancreatic B cells (Yamamoto et al, 1983), and adrenal glomerulosa cells (Kojima et al, 1985), phospholipase A_2 has been shown to stimulate prolactin, insulin, and aldosterone secretion. Recently, Mukhopadhyay et al, (1985) have shown that although phospholipase A_2, by itself, was without effect on Leydig cells, it enhanced the steroidogenic actions of hCG, LHRH-A, and cyclic AMP. In our studies chloroquine and quinacrine had an inhibitory effect on basal, LHRH-A-induced, and arachidonic acid-stimulated T secretion. This indicates that the inhibitory effects of chloroquine and quinacrine on LHRH-A-induced T secretion involve mechanisms other than, or in addition to, the specific inhibition of phospholipase A_2.

At present there are two known mechanisms by which arachidonic acid can be released from phospholipids: (1) by the action of phospholipase A_2 on phospholipids as mentioned above and (2) by the initial hydrolysis of phosphatidylinositol by a specific phospholipase C to produce inositol tri-phosphate and diacylglycerol. This diacylglycerol, which is rich in arachidonic acid, can subsequently be hydrolysed by diglyceride lipase to release arachidonic acid (Bell et al, 1979). It seems that the latter mechanism is in operation in Leydig cells, since exogenous phospholipase C could stimulate steroidogenesis as effectively as arachidonic acid.

Exogenously added arachidonic acid had a biphasic effect on steroidogenesis in Leydig cells. Up to 100 μM arachidonic acid could stimulate T secretion. At higher concentrations, arachidonic acid did not stimulate T secretion. Since exogenous arachidonic acid could enhance T secretion above the maximum obtainable with LHRH-A, it would seem that the intracellular concentrations of arachidonic acid may be a limiting factor during LHRH-A-stimulated T secretion. These results, and the fact that LHRH-A-induced T secretion is associated with accumulation of prostaglandin E2, a metabolite of arachidonic acid (Molcho et al, 1984a, 1984b), indicate that arachidonic acid is released during LHRH-A action and may have an intermediary role in the steroidogenic action of LHRH-A.

TABLE 3. Effect of Nordihydroguaiaretic Acid (NDGA), on Arachidonic Acid (AA)-, Phorbol 12, Myristate 13, Acetate (PMA)-, or Phospholipase C (PLC)-Stimulated Testosterone Secretion

Treatment	Testosterone (% of Basal)
Basal	100
NDGA (100 μM)	207.8 ± 3.8
AA (100 μM)	175.7 ± 5.5
NDGA + AA	213.4 ± 3.4
PLC (500 mU)	186.1 ± 4.5
NDGA + PLC	242.0 ± 16.2
PMA (500 nM)	270.5 ± 12.7
NDGA + PMA	326.6 ± 18.7

Leydig cells were preincubated with or without 100 μM NDGA for 30 min and then appropriate concentrations of stimulators were added and incubation continued for an additional 3 hours. All the values are mean ± S.E.M. of six determinations. Values for incubations containing NDGA are significantly different from the corresponding values that did not include NDGA. All values are representative of three different experiments.

Since arachidonic acid could stimulate steroidogenesis in Leydig cells, and since LHRH-A-stimulated T secretion is associated with prostaglandin E_2 secretion (Molcho et al, 1984a, 1984b), we were prompted to investigate whether arachidonic acid needs to be metabolized before it can exert its steroidogenic action. To accomplish this, we used ETYA, an inhibitor of arachidonic acid metabolism via lipoxygenase, cyclo-oxygenase, and epoxygenase, the three known pathways (Snyder et al, 1983); nordihydroguaiaretic acid, an inhibitor of lipoxygenase pathway (Hamberg, 1976); and indomethacin, an inhibitor of cyclo-oxygenase pathway (Ferreira et al, 1971). Both ETYA and nordihydroguaiaretic acid abolished LHRH-A-induced T secretion. Indomethacin was ineffective in this respect. To confirm the possibility that lipoxygenase products are involved, we studied the effects of nordihydroguaiaretic acid, which has been shown to inhibit formation of lipoxygenase products but not cyclo-oxygenase products of arachidonic acid (Dix et al, 1984), on arachidonic acid—, phospholipase C—, or phorbol 12, myristate 13 acetate-induced T secretion from Leydig cells. Surprisingly, nordihydroguaiaretic acid could further stimulate T secretion in addition to the stimulatory effect of these agents alone. These results suggest that lipoxygenase products are not involved. In fact, leukotriene B-4, a lipoxygenase product, was unable to stimulate steroidogenesis in Leydig cells (Sullivan

and Cooke, 1985b). There are two possible explanations for the stimulatory action of nordihydroguaiaretic acid on arachidonic acid-induced T secretion. The first possibility is that arachidonic acid need not be metabolized to exert its effects (Lin, 1985a). This is unlikely since the steroidogenic effect of arachidonic acid can be blocked if its metabolic conversion is inhibited by ETYA (Table 2), which competitively inhibits arachidonic acid metabolism by all three known pathways. ETYA also completely blocked phospholipase C- or phorbol 12, myristate 13 acetate-induced T secretion (Didolkar and Sundaram, 1987). The second possibility is that metabolites of arachidonic acid other than lipoxygenase products are involved. We and other authors (Dix et al, 1984) have observed that cyclo-oxygenase products are not involved. Therefore, it can be hypothesized that when the lipoxygenase pathway is blocked by noridhydroguaiaretic acid, more arachidonic acid is available for metabolism by the epoxygenase pathway. At this time we are not aware of the formation/secretion of any epoxygenase products in Leydig cells and, therefore, further work will be needed before we reach any conclusions. It is also not clear how noridhydroguaiaretic acid inhibits LHRH-A-induced T secretion, but our data suggest that noridhydroguaiaretic acid may act at the steps prior to release of arachidonic acid during LHRH-A stimulation. Similar inhibitory effects of NDGA have been observed in the action of LH on Leydig cells (Dix et al, 1984).

As with LHRH-A, arachidonic acid did not stimulate cyclic AMP accumulation (Didolkar and Sundaram, 1987). Thus, its role in T production must be independent of cyclic AMP action. How arachidonic acid metabolites stimulate T secretion is not known. Based on the recent observations that protein kinase C in neutrophils can be activated by arachidonic acid (McPhail et al, 1984) and the demonstrated presence of protein kinase C in rat Leydig cells (Lin, 1985b), it can be postulated that arachidonic acid metabolites stimulate synthesis of specific protein(s) and their phosphorylation by protein kinase C, which in turn leads to steroidogenesis. Clearly, more work is required to understand this mechanism, since phorbol 12, myristate 13 acetate, a protein kinase C activator, and LHRH-A have differential effects on protein phosphorylation and protein synthesis in Leydig cells (Themmen et al, 1986). In addition to protein kinase C stimulation, arachidonic acid metabolites have been implicated in the modulation of activity of some of the enzymes involved in steroidogenic pathway (Dix et al, 1984). We are now exploring these possibilities.

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VI International Congress on Spermatology

The VI International Congress on Spermatology will be held August 30 to September 5, 1990, at the Nuovo Complesso Didattico, University of Siena, Siena, Italy. The scientific program will include symposia and workshops. A limited number of contributed papers pertinent to the subjects of the symposia will be selected by the Program Committee for presentation as posters.

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