THE ROLE OF Ca^{2+} AND cAMP IN GnRH-STIMULATED LH RELEASE

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by

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DEDICATION

This thesis is dedicated to my parents, Gerald and Grace Wakefield, who have supported me in all my endeavours.

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ABBREVIATIONS

AF	Ammonium formate
8-Br-cAMP	8-Bromo-cAMP
dBu-cAMP	Dibutyryl cAMP
CAM	Calmodulin
cAMP	Cyclic-3',5'-monophosphate
CRF	Corticotrophin-releasing hormone
D600	Methoxyverapamil
DAG	1,2 Diacylglycerol
DHP	Dihydropyridine
EGTA	Ethyleneglycol-bis-(ß-aminoethyl ether) N,N,N,N-tetraacetic acid
FCS	Foetal calf serum
GnRH	Gonadotropin-releasing hormone
HEPES	N-2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid
IBMX	1-Methyl-3-isobutyl-xanthine
IP	Inositol phosphate
IP_3	Inositol-1,4,5-trisphosphate
LH	Luteinising hormone
MTT	Maitotoxin
PA	Phosphatidic acid
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol-4,5-bisphosphate
RR	Ruthenium red
ROCC	Receptor-operated calcium channel
TPA	12-0-Tetradecanoyl phorbol acetate
TRH	Thyrotropin-releasing hormone
VSCC	Voltage-sensitive calcium channel

SUMMARY

In this thesis a detailed study of the kinetics of GnRH-stimulated LH release was made. GnRH stimulated LH release in a biphasic manner. During the first 3 minutes of stimulation, there was a transient spike phase of release followed by plateau phase of lower amplitude.

Both phases of release are largely dependent on extracellular Ca^{2+}. The spike phase of release is dependent on Ca^{2+} entry via a receptor-operated Ca^{2+} channel (ROCC) (about 90%) and on the mobilization of intracellular Ca^{2+} stores. The role of ROCC were examined by using ruthenium red which inhibits both ROCC and voltage-sensitive Ca^{2+} channels (VSCC). VSCC are not involved in the spike phase of GnRH-stimulated LH release since D600 and nifedipine, inhibitors of VSCC, have no effect on the spike phase. The plateau phase of release is dependent on Ca^{2+} entry via VSCC (about 50%) and ROCC (about 50%).

Forskolin, an activator of adenylate cyclase, was used to investigate the role of cAMP in LH release. Forskolin stimulated an increase in both LH release and cellular cAMP levels. GnRH was also able to elevate the cellular cAMP concentration. GnRH interacted synergistically with forskolin to stimulate LH release. The synergism between GnRH and forskolin was not due to an interaction at (1) the GnRH receptor, (2) the level of intracellular Ca^{2+} mobilization, or (3) inositol phosphate metabolism. However, forskolin was able to synergistically interact with secretagogues that increase the cytosolic Ca^{2+} concentration and activators of protein kinase C. This suggested that forskolin was interacting with GnRH at a site distal to

the activation of the Ca^{2+} second messenger system and protein kinase C.

The data suggest that the initial response to GnRH is largely Ca^{2+}-dependent and that other second messengers, if active, play a minor role. cAMP is thought to play a modulatory role and may be involved in the maintenance of secretion.

1 INTRODUCTION

Gonadotropin-releasing hormone (GnRH), a decapeptide released by the hypothalamus, stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. The mammalian form of GnRH with the sequence pyro-Glu^1-His^2-Trp^3-Ser^4-Tyr^5-Gly^6-Leu^7-Arg^8-Pro^9-Gly^{10}-NH_2 was isolated and purified by Schally et al., 1971. Different molecular forms of GnRH have been characterised in many species, including porcine (Matsuo et al., 1971), ovine (Amoss et al., 1971), avian (King and Millar, 1982), salmon Sherwood et al., 1983) and lamprey (Sherwood et al., 1986).

Two molecular forms of GnRH have been isolated from the chicken hypothalamus: chicken GnRH I ([Gln^8]GnRH) and chicken GnRH II ([His^5, Trp^7, Tyr^8] GnRH). Both forms of chicken GnRH stimulate the release of both LH and FSH (Millar and King, 1988; Millar et al., 1986), acting through a single GnRH receptor type in the chicken pituitary (King et al., 1988). It can therefore be assumed that both activate the same signal transduction mechanism. Chicken GnRH II is five to six times more potent than chicken GnRH I in stimulating LH release from chicken pituitary cells. Chicken GnRH I appears to be the prime regulator of gonadotropin release since its concentration is 180-fold greater than that of chicken GnRH II in the median eminence of the hypothalamus of chicken brain (Katz et al., 1990). The function of chicken GnRH II has not been clearly elucidated, but it may act as a neurotransmitter since it is predominantly present in the extra hypothalamic areas of the brain (Katz et al., 1990).

Chicken GnRH I was used in this study because (a) I wanted to compare

this study to previous data obtained in our laboratory, and (b) unlimited stocks of chicken GnRH I was available to me.

In this thesis the signal transduction mechanism by which chicken GnRH I stimulates LH release from chicken pituitary cells was investigated. In the literature available, it is clear that the signal transduction mechanism has been extensively studied in rat anterior pituitary cells. The signal transduction mechanism had not been well probed in chicken pituitary cells. This thesis addresses the questions:(1) What are the signal transduction mechanisms for GnRH in the chicken pituitary cell ? and (2) How do these differ from those in the rat pituitary ? Aspects of GnRH-stimulated LH release which were studied are (1) the dependence on extracelluar Ca^{2+}, (2) Ca^{2+} entry routes, and (3) the interaction between cAMP, protein kinase C and Ca^{2+}. The literature review focuses on the molecular events involved in the mechanism of GnRH-stimulated LH release. All the studies using in which chicken anterior pituitary cells were used, used chicken GnRH I.

1.1 The GnRH receptor

GnRH binds to the GnRH receptor on the plasma membrane of piuitary gonadotrophs activating intracellular events that lead to the release of LH. The synthesis of high affinity metabolically stable GnRH agonist, which could easily be iodinated, facillitated the identification of a single class of GnRH receptors on pituitary plasma membranes with a K_a =1-3x10^{-10}M (Marian et al., 1981). The apparent MW of the binding component of the GnRH receptor as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is 60 kd (Hazum 1981, Eidne et al., 1985). However, using target size analysis, the

functional MW of the receptor was determined to be 136 kd (Conn and Venter, 1985). This suggests that the 60 kd protein seen on SDS-PAGE could be a sub-unit of the receptor.

Using flouresent analogues of GnRH, Naor et al. (1981) found that GnRH was initially evenly distribute over the cell surface of gonadotrophs but was localised at one pole of the cell after 20 min of incubation. Internalisation of the the flouresent GnRH was evident after 30 min. Studies using ferritin-GnRH also showed thet polar aggregation of the GnRH-receptor complex was observed prior to internalisation (Hopkins and Gregory, 1977). Following internalisation, the lysosomal and golgi compartments are the major sites of accumulisation of the internalised receptor complex (Jennes et al.,1983; Pelletier et al., 1982).

Is internalisation of the receptor complex essential for LH release ? The use of an immobilized form of GnRH which was unable to be internalised but nevertheless still able to evoke LH release (Conn, Smith And Rogers, 1981) indicated that this was not the case. Also, vinblastin, at a dose which inhibits receptor capping and internalisation, did not inhibit GnRH-stimulated LH release (Gorospe and Conn, 1987). These results indicate that the internalisation of the GnRH-receptor complex can be uncoupled from the LH secretory response.

1.2 GnRH analogues

A large number of GnRH analogues have been made giving investigators much information about the structure of GnRH needed for it to be functionally active (Karten and Rivier, 1986). Modification of the C- and N-teminal amino acid residues of GnRH decreases the

affinity of the peptide for the GnRH receptor (Coy et al., 1979). Substitutions of the first, second, and third amino acid residues by D-amino acids result in antagonists with good receptor binding activity and reduced biological activity (Conn et al., 1986)

When substitutions are made that enhance the net hydrophobicity of the peptide, it enhances its biological potency. Replacement of the sixth amino acid residue with D-amino acids containing bulky, hydrophobic substituents yield agonist resistant to enzymatic cleavage (Coy et al., 1976). Substitution of the sixth amino acid with a D-amino combined with the Gly^{10} residue being changed for an ethylamide further enhances the affinity and potency of the GnRH agonists (Fujimo et al., 1974). The available knowledge about how amino acid sustitutions affect the affinity and biological potency of GnRH, has facillitated the design of antagonists and agonists of GnRH.

1.3 Ca^{2+} as a second messenger in LH secretion

Three types of observations provide evidence that Ca^{2+} acts as an intracellular messenger to stimulate LH release : (1) GnRH-stimulated LH release is inhibited by the removal of extracellular Ca^{2+} and by Ca^{2+} channel blockers; (2) elevation of the cytosolic Ca^{2+} concentration is sufficient to stimulate LH release; and (3) GnRH causes measurable changes in Ca^{2+} fluxes and in the cytosolic Ca^{2+} concentration.

1.3.1 The dependence of GnRH-stimulated LH release on extracellular Ca^{2+}

In 1968, Samli and Geshwind showed that the stimulation of LH

release from rat pituitaries by hypothalamic extract was dependent on extracellular Ca^{2+}. In the two decades since this finding, the role of Ca^{2+} in the mechanism of GnRH-stimulated LH release has been extensively investigated, and the dependence of LH release on extracellular Ca^{2+} has been repeatedly confirmed (Wakabayashi et al., 1969; Hopkins and Walker, 1978; Marian and Conn, 1979; Bourne and Baldwin, 1980; Conn and Rogers, 1980; Borges et al., 1983; Bates and Conn, 1984; Jinnah and Conn, 1986; Conn et al., 1986; Hanson et al., 1987; Tasaka et al., 1988; Chang et al., 1988).

1.3.2 Kinetic phases of LH release

In kinetic studies, various investigators have demonstrated that GnRH-stimulated LH release occurs in a biphasic manner. Four reports describe a first phase of 40 - 60 minutes followed by a longer second phase of 3 - 4 hours (Bourne and Baldwin, 1980; Stern and Conn, 1981; Eddie et al., 1982; Borges et al., 1983). The protein synthesis inhibitor, cycloheximide, inhibited GnRH-stimulated LH release with its inhibitory effect seen only in the second phase of release (Bourne and Baldwin, 1980; Eddie et al., 1982).

Bourne and Baldwin (1980) and Stern and Conn (1981) reported that the first phase was completely abolished by the removal of extracellular Ca^{2+} and that the second phase was only partially Ca^{2+}-dependent. However, Borges et al. (1983), using dispersed cells and shorter collection periods, found that Ca^{2+} was an essential requirement for both phases of release. These studies suggest that extracellular Ca^{2+} is important in the first phase of release and for the maintenance of LH release. Other kinetic studies which investigated the first phase (0-60 minutes) of LH release more closely, have shown that this phase

of LH release is itself composed of two phases, a spike phase (0 - 5 minutes) followed by a plateau phase (Hopkins and Walker, 1978; Hopkins and Gregory, 1981; Naor et al., 1982; Bates and Conn, 1984; Chang et al., 1986).

The spike phase peaks within the first few minutes following the addition of GnRH. It occurs both when the cells are continuously exposed to GnRH (Hopkins and Walker, 1978; Naor et al., 1982; Borges et al., 1983) and when they are pulsed with GnRH (Chang et al., 1986). These authors presented conflicting data concerning the dependence of the spike phase on extracellular Ca^{2+}. The plateau phase was shown to be abolished by the removal of extracellular Ca^{2+}. Hopkins and Walker (1978) reported that the spike phase was unaffected by "Ca^{2+}-free" medium but markedly inhibited by EGTA. Naor et al. (1982) and Borges et al. (1983) reported that the spike phase was totally dependent on extracellular Ca^{2+}. This lack of consensus concerning the effect of the removal of extracellular Ca^{2+} on the spike phase indicates that the Ca^{2+}-dependence of this phase of LH release requires further investigation.

1.3.1 The effects of Ca^{2+} channel blockers on LH release

Organic voltage-sensitive calcium channel (VSCC) blockers have been used as tools to study the Ca^{2+} entry routes which are activated by GnRH. It has been demonstrated that D600 (methoxyverapamil) inhibited GnRH-stimulated LH release (Hopkins and Walker, 1978; Conn and Rogers, 1980; Borges et al., 1983). In 1983, Conn et al. investigated the effects of a range of blockers of VSCC on LH release from rat gonadotropes. They reported that the phenylalkylamines (e.g.

verapamil and D600), were the most potent inhibitors of GnRH-stimulated LH release, with diltiazem being less effective. In that study, the dihydropyridines (DHP) used did not inhibit LH release, and the authors concluded, since DHP are potent blockers of VSCC in muscle tissue, that there may be differences between the Ca^{2+} ion channels in muscle tissue and those in gonadotropes. However, it has subsequently been demonstrated that DHP that are blockers of VSCC are able to inhibit LH release from both rat (Chang et al., 1984, 1986, 1988; Tasaka et al., 1988) and chicken (Davidson et al., 1987, 1988) gonadotropes.

Borges et al. (1983) reported that both the spike and plateau phases were partially inhibited by D600. In contrast, Bates and Conn (1984) reported that the spike phase was unaffected, with only the plateau phase being inhibited. Chang et al. (1986) concluded that the spike phase was inhibited by 40% in the presence of the DHP nitrendipine. Less specific inorganic Ca^{2+} channel blockers have also been used to study Ca^{2+} entry routes. Lanthanum (La^{3+}) inhibited both GnRH- and K^{+}-stimulated LH release (Hopkins and Walker, 1978). This suggests that the VSCC that are activated by high K^{+} can be blocked by La^{3+}. An analysis of the effects of various Ca^{2+} channel blockers on the spike and plateau phases of LH release is presented in this thesis.

1.4 Effects of the manipulation of the cytosolic calcium concentration on LH release

1.4.1 Depolarization

In order to further assess the role of Ca^{2+} as a second messenger in LH release, it has been useful to manipulate the Ca^{2+}

concentrations independently of GnRH. The treatment of cells with high concentrations of extracellular K^+ has been shown by several investigators to stimulate LH release (Samli and Geshwind, 1968; Wakabayashi et al., 1969; Hopkins and Walker, 1978; Conn et al, 1980; Davidson et al., 1987; Meier et al., 1988; Stojilkovic et al., 1988). This secretagogue effect of high K^+ suggests that in gonadotropes as in other cell types, membrane potential is maintained by K^+ efflux through K^+ channels. This secretagogue effect of high K^+ suggests that in gonadotropes, as in other cell types, membrane potential is maintaned by K^+ efflux through K^++ channels. High concentrations of K^+ depolarise the plasma membrane, activating VSCC to allow Ca^{2+} entry (Meier et al., 1988). The hormone-releasing action of K^+ is inhibited by Ca^{2+} removal and the organic blockers of VSCC , including DHP.

Veratridine, a sodium channel agonist, depolarizes the cell membrane by allowing sodium (Na^+) entry (Leabowitz et al., 1986; Sutro, 1986). This results in Ca^{2+} entry via either VSCC or the Na^+/Ca^{2+} exchange, thereby stimulating LH release (Conn et al., 1980; Conn et al., 1983; Davidson et al., 1987). The ability of veratridine to stimulate LH release is inhibited by the specific Na^+ channel antagonist tetrodotoxin and the Ca^{2+} channel antagonist D600 (Conn et al., 1980; Conn et al., 1983). The removal of extracellular Ca^{2+} also inhibits veratridine-stimulated LH release (Conn et al., 1980; Davidson et al., 1987).

Since both veratridine- and high K^+-stimulated LH release are mediated via VSCC (Conn et al., 1980), these stimuli can be used to monitor the efficacy of VSCC blockers.

1.4.2 Ionophores

Ionophores have the ability to transport ions across membranes independently of ion transport systems. Ca^{2+} ionophores are able to transport Ca^{2+} across the plasma membrane from an extracellular source and to release Ca^{2+} from intracellular stores. The Ca^{2+} ionophores A23187, X537A and ionomycin have been used to probe the mechanism of LH secretion. A23187 and ionomycin stimulated LH release from gonadotropes in a manner which was largely dependent on extracellular Ca^{2+} (Hopkins and Walker, 1978; Conn et al., 1979; Kiesel and Catt, 1984; Naor and Eli, 1985; Davidson et al., 1987). The ionophore X537A stimulated LH release independently of extracellular Ca^{2+} (Conn et al., 1979).

1.4.3 Ca^{2+} channel agonists

Although the DHP are commonly used as VSCC blockers, the DHP derivative Bay K8644 is a Ca^{2+} channel agonist. Bay K8644 is thought to act by shifting the equilibrium between the open and closed states of the channels in favour of the open form, thereby increasing the mean channel opening time (Schramm et al., 1983; Towart and Schramm, 1984). Bay K8644 alone stimulated LH release (Chang et al., 1986; Stojilkovic et al., 1988) and enhanced submaximal GnRH-stimulated LH release (Chang et al., 1986). Bay K8644 did not enhance LH release at high concentrations of GnRH. This suggests that the extent to which VSCCs are activated at the high concentrations of GnRH is sufficient to achieve maximal LH release.

Maitotoxin (MTT), a dinoflagellate toxin, also stimulates LH release by allowing Ca^{2+} entry via a Ca^{2+} channel (Conn et al., 1987). MTT-

stimulated LH release was inhibited by both Ca^{2+} removal and D600. MTT is thought to act by activating a Ca^{2+} channel that is involved in GnRH-stimulated LH release.

1.5 $^{45}Ca^{2+}$ fluxes

The availability of $^{45}Ca^{2+}$ has made it possible to study Ca^{2+} fluxes across the plasma membrane. By means of $^{45}Ca^{2+}$ influx studies, in which La^{3+} was used to displace surface bound $^{45}Ca^{2+}$, Hopkins and Walker (1978) reported that GnRH caused an increase in $^{45}Ca^{2+}$ influx during a 3-minute stimulation period. However, although LH release during this period was not inhibited by La^{3+}, $^{45}Ca^{2+}$ influx was abolished. This suggested that the influx of Ca^{2+} was not essential for the spike phase of GnRH-stimulated LH release.

Two studies have reported that GnRH stimulates $^{45}Ca^{2+}$ efflux from cells preloaded with $^{45}Ca^{2+}$ (Williams, 1976; Hopkins and Walker, 1978). This stimulated efflux was maximal within the first 1-2 minutes of stimulation and rapidly returned to basal levels. However, unlike $^{45}Ca^{2+}$ influx, this increase in $^{45}Ca^{2+}$ efflux was shown to be independent of extracellular Ca^{2+}. Since secretion by GnRH was partially inhibited by the removal of extracellular Ca^{2+} by EGTA (Hopkins and Walker, 1978) and $^{45}Ca^{2+}$ efflux was not inhibited, it suggests that $^{45}Ca^{2+}$ efflux may be important for the residual portion of the spike phase not inhibited by extracellular Ca^{2+} removal.

1.6 Measurement of cytosolic Ca^{2+} levels

Since gonadotropes comprise only a small fraction of the cells in the anterior pituitary, the investigation of changes in the Ca^{2+}

concentration in the cytosol of gonadotropes has required the development of methods for obtaining gonadotrope-enriched cell preparations. The use of fluorescent Ca^{2+} chelators (quin-2 and fura-2) which can be used as Ca^{2+} indicators has facilitated the measurement of changes in cytosolic Ca^{2+} concentrations in cells (Tsien et al., 1982; Grynkiewiz et al., 1985).

Clapper and Conn (1985), using a gonadotrope-enriched cell population prepared by density elutriation and loaded with quin-2, showed that GnRH elevates the cytosolic Ca^{2+} concentration. Similar results were obtained by Chang et al. (1986) and Limor et al. (1987) using a gonadotrope-enriched cell population prepared by centrifugal elutriation, also measuring changes in the cytosolic Ca^{2+} concentration with quin-2. They showed that GnRH caused a rapid rise (6-8 seconds) in the cytosolic Ca^{2+} concentration followed by a prolonged plateau phase of elevated Ca^{2+} concentration which lasted for 15 minutes. The initial rise in Ca^{2+} concentration was not affected by the removal of extracellular Ca^{2+} whereas the plateau phase was decreased in the absence of Ca^{2+}. These workers suggested that the first phase of increased cytosolic Ca^{2+} was due to the release of Ca^{2+} from intracellular stores and that the influx of Ca^{2+} across the plasma membrane sustains the elevated cytosolic Ca^{2+} concentration during the plateau phase.

They found that elevation of cytosolic Ca^{2+} was dampened by prior stimulation with GnRH, a desensitization effect which is similar to the desensitization seen at the level of LH release. GnRH antagonist did not elevate Ca^{2+} levels indicating that receptor occupation was not sufficient to elicit a Ca^{2+} response, and that receptor activation

was also required.

This rapid increase in the cytosolic Ca^{2+} concentration due to GnRH coincides with the spike phase of GnRH-stimulated LH release. It was therefore concluded that this release of Ca^{2+} from intracellular stores is important to the spike phase of LH release.

1.7 Calmodulin (CAM)

A potential role for CAM in GnRH-stimulated LH release was suggested by the finding that GnRH caused the redistribution of CAM from the cytoplasm to the plasma membrane where it associated with GnRH receptor patches (Conn et al., 1981; Jennes et al., 1985; Naor et al., 1985). Studies using CAM antagonists have also suggested a possible role for CAM in LH release. Conn et al. (1981) reported that the CAM antagonist pimozide inhibited GnRH-stimulated LH release. Stimulation of LH release due to other secretagogues that elevate the cytosolic Ca^{2+} concentration is also inhibited by CAM antagonists. Davidson et al. (1987) reported that trifluoperazine, W7, and chlorpromazine inhibited A23187- and veratridine-stimulated LH release. These results suggest that the CAM antagonists act at a site distal to the mobilization of Ca^{2+} and possibly at the level of activation of CAM.

However, the interpretation of studies using CAM antagonists is not straightforward, since these drugs can inhibit protein kinase C (Mori et al., 1981). In addition, recent studies suggest that the inhibition of Ca^{2+}-triggered exocytosis by CAM antagonists can be explained by an effect on membrane surface charge density, due to an association of these lipophilic cations with the membrane (Whitaker,

1987). Thus the observed inhibition of secretion by these drugs may not be due to CAM inhibition, and the role of CAM in LH release remains unclear.

1.8 Inositol Phosphate Metabolism

As early as 1953, Hokin and Hokin demonstrated a possible role for phosphatidylinositol (PI) turnover in signal transduction. Subsequently, it was proposed that the increase in PI turnover could be linked to the mechanism of action of Ca^{2+}-mobilizing hormones (Mitchell et al., 1976). Since then, the metabolism of inositol phospholipids and their relation to Ca^{2+} mobilization have been extensively investigated, and recently reviewed (Sekor and Hokin, 1986; Berridge, 1987; Majerus et al., 1988). In brief, receptor activation of phospholipase C (PLC) leads to the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to 1,2 diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3). Both of these compounds act as second messengers, IP_3 causing intracelluar Ca^{2+} mobilization (Berridge 1984, 1987) and DAG activating protein kinase C (Nishizuka 1984, 1986).

Recently it has been shown that GnRH induces rapid phosphodiester hydrolysis of phosphoinositides in pituitary gonadotropes (Kiesel and Catt, 1984; Andrews and Conn, 1986; Naor et al., 1986). Kiesel and Catt (1984), using an enriched population of gonadotropes, demonstrated that GnRH stimulated the incorporation of ^{32}P into total phospholipids, phosphatidylinositol (PI) and phosphatidic acid (PA). PA stimulated both LH release and cyclic guanosine 3',5'-monophosphate formation in a calcium-dependent manner. This suggested that the PA formed in response to GnRH receptor activation might have an important

function in the mechanism of LH release.

Similarly, Andrews and Conn (1986), also looking at short-term ^{32}P labelling, showed that GnRH rapidly stimulated ^{32}P incorporation into PA, PI, PIP and PIP_2. Data obtained from short-term ^{32}P labelling reflect changes in the turnover of phospholipids rather than mass changes. Mass changes in phospholipids were investigated in cells labelled to isotopic equilibrium with ^{32}P (Andrews and Conn, 1986). In these experiments GnRH stimulated a rapid loss of labelled PI, PIP and PIP_2. These data suggest that GnRH activates PLC leading to changes in the turnover rates of these metabolites. Morgan et al. (1987) showed that PIP and PIP_2 are the principal targets of the PLC activated by GnRH rather than PI.

In gonadotropes pre-labelled with ^{3}H-myo-inositol, GnRH stimulated the rapid formation of IP_3, IP_2 and IP_1 (Morgan et al., 1986; Naor et al., 1986). The effect on IP_3 was biphasic and the rapid increase in IP_3 was followed by a nadir and a second rise between 5 and 20 minutes (Naor et al., 1986). The initial rapid increase in IP_3 corresponds to a rapid loss of PI and PIP_2 when gonadotropes are stimulated with GnRH. The increases in IP_2 and IP_1 occurred with a slower time-course than that of IP_3, increased levels of these products being observed 90 minutes after stimulation (Huckle and Conn, 1987). The stimulatory effect of GnRH on IP_3, IP_2 and IP accumulation is abolished in the presence of GnRH antagonist (Naor et al., 1986), indicating that the increase in inositol phosphate production is a receptor-mediated event.

Using HPLC to resolve isomers of the inositol phosphates, Morgan et

al. (1987) showed that more than one isomer of IP_3 was produced by the gonadotropes. These workers showed that GnRH stimulated the production of $I(1,4,5)P_3$ rather than the $I(1,3,4)P_3$ isomer. This was followed by progressive increases in $I(1,4)P_2$. GnRH also stimulated the elevation of IP_4, IP_5 and IP_6; however, the roles of these metabolites are not known.

In contrast to LH release, Huckle and Conn (1987) reported that IP production was not inhibited in Ca^{2+}-free medium. In addition, the Ca^{2+} channel blocker D600, at concentrations which inhibited GnRH-stimulated LH release, did not inhibit IP production. At high concentrations, D600 inhibited both IP production and LH release. Pimozide, a CaM antagonist, inhibited GnRH-stimulated LH release but not IP production. From these results the authors concluded that GnRH-stimulated IP production was independent of extracellular Ca^{2+} and the Ca^{2+}-CaM system.

1.9 Protein Kinase C

A second messenger role for DAG, a product of the hydrolysis of phospholipids, was first recognized by Nishizuka (1984). He observed that DAG increased the affinity of protein kinase C for Ca^{2+} and phosphatidylserine (PS), thereby causing activation of the enzyme at resting intracellular Ca^{2+} concentrations. DAG interacts with protein kinase C at the same site as phorbol esters which are potent activators of the enzyme (Sharkey **et al**, 1984). Phorbol esters are not only more potent than DAG in activating protein kinase C, but they have a prolonged action since they are not readily metabolized. GnRH has been shown to stimulate an immediate and transient (5 minute)

increase in DAG in cells labelled to isotopic equilibrium with ^{3}H-arachidonic acid (Andrews and Conn, 1986).

Phorbol esters have been shown to stimulate LH release in several studies (Smith and Vale, 1980; Naor and Catt, 1981; Smith and Vale, 1981; Conn et al., 1985; Naor et al., 1985; Davidson et al., 1987). Phorbol ester-stimulated LH release is Ca^{2+}-independent and not inhibited by Ca^{2+} removal and Ca^{2+} channel antagonists. More recently, the synthetic DAG, Sn-1-oleoyl-2-acetylglycerol (OAG) and Sn-1,2-dioctanocylglycerol (diC_8) have been reported to stimulate LH release (Conn et al., 1985). The 3' hydroxyl group of these diacylglycerols was found to be important for LH release.

In the unstimulated pituitary, 70% of the protein kinase C activity is in a soluble, cytosolic form and 30% is found in the particulate fraction, i.e. bound to the membranes (Naor et al., 1985). In in vitro studies using ovariectomized rats, GnRH was reported to stimulate the redistribution of protein kinase C from the soluble to the particulate fraction (Naor et al., 1985; McArdle et al., 1986). GnRH-stimulated redistribution of protein kinase C was inhibited by D600 at concentrations that inhibit LH release (McArdle and Conn, 1986), suggesting that protein kinase C redistribution occurs as a result of entry of extracellular Ca^{2+}.

The phorbol ester, 12-O-tetradecanoyl phorbol acetate, interacts synergistically with the Ca^{2+} ionophore A23187 to stimulate LH release (Harris et al., 1985; Naor et al., 1985; Davidson et al., 1987). Synergism has also been observed between phorbol esters and stimuli which elevate Ca^{2+} by activating VSCC (high K^{+} and veratridine) (Davidson et al., 1987). This synergistic interaction was inhibited

by extracellular Ca^{2+} removal, D600, and CaM antagonists trifluoperazine and chlorpromazine (Davidson et al., 1987). This was interpreted as suggesting that the synergistic interaction was due to an event that occurs distal to the activation of CaM However, other interpretations are possible in light of the action of these drugs on Ca^{2+} binding to membranes (Whitaker et al., 1983) as discussed above in section 1.5.

Pretreatment of pituitary cells with phorbol esters decreases protein kinase C activity and inhibits the response of the gonadotropes to subsequent challenges with protein kinase C activators (Huckle and Conn, 1987; McArdle et al., 1987). However, this depletion of protein kinase C activity had no effect on GnRH-stimulated LH release, and the authors concluded that protein kinase C may not be involved in GnRH-stimulated LH release (McArdle et al., 1987). In contrast to this, retinal, a putative inhibitor of protein kinase C, at a dose which inhibited TPA stimulation of LH release, reduced the LH release due to GnRH by 40%. The spike phase of release was unaffected with only the plateau phase being partially inhibited, leading the authors to conclude that protein kinase C-dependent mechanisms participated in the maintenance of the LH response.

Although numerous compounds have been shown to be protein kinase C inhibitors **in vitro**, they all either (1) show toxic effects on whole cells and/or (2) lack specificity in that they affect other cellular processes. The absence of a specific and non-toxic inhibitor of protein kinase C makes it difficult to determine to what extent GnRH stimulation of LH release is dependent on the activation of protein kinase C.

Pre-treatment with the protein kinase C activator TPA inhibited GnRH-stimulated IP production but not LH release (McArdle et al., 1987). This effect is analagous to the inhibitory effect of protein kinase C activators on agonist-induced IP production in other cell types (Ballester and Rosen, 1985; Melloni et al., 1988). This is a possible mechanism by which protein kinase C activators could uncouple the GnRH receptor from IP production.

In summary, a role for protein kinase C in GnRH-stimulated LH release is suggested by the fact that (1) protein kinase C activators stimulate LH secretion; (2) GnRH stimulates inositol lipid hydrolysis; and (3) protein kinase C translocates to the membrane on GnRH stimulation.

1.10 Cyclic AMP (cAMP) as a second messenger in LH release

The role of cAMP as a mediator of LH release is controversial. In early studies it was reported that GnRH was able to stimulate LH release and increase cellular cAMP concentrations (Borgeat et al., 1972; Makino, 1973; Naor et al., 1975; Bonney and Cunningham, 1977; Sen and Menon, 1977; Bourne and Baldwin, 1987). Other studies were unable to demonstrate that GnRH increased cellular cAMP concentrations (Sundberg et al., 1976). Conflicting data have made it difficult to assess the role of cAMP as a second messenger in the mechanism by which GnRH stimulates LH release.

Cholera enterotoxin stimulated an increase in the concentration of cellular cAMP without stimulating LH release (Naor et al., 1975; Sen and Menon, 1979). These studies had shown that GnRH increased cellular cAMP concentrations. These authors concluded that cAMP did

not play a role in GnRH-stimulated LH release since an increase in cAMP did not necessarily coincide with the release of LH. Naor et al. (1975) reported that the cAMP phosphodiesterase inhibitors 1-methyl-3-isobutyl-xanthine (IBMX) and theophylline raised cellular cAMP levels but did not stimulate LH release. Sundberg et al. (1976) were also unable to show any effect with theophylline and cholera enterotoxin on LH release. However, in other studies theophylline (Makino, 1975; Bonney and Cunningham, 1977) and IBMX (Wun et al., 1988) have been reported to stimulate LH release.

Membrane-permeant cyclic AMP analogues have also been used to investigate the role of cAMP in LH release. Dibutyryl cAMP (dBu-cAMP) has been shown to stimulate LH release (Makino, 1973; Bonney and Cunningham, 1977; Kerchet et al., 1977). Similarly, 8-Br-cAMP has also been shown to stimulate LH release (Wun et al., 1988). This was confirmed by Smith et al. (1982), who showed that these cAMP analogues stimulated LH release but inhibited the binding of GnRH to its receptor.

In contrast to these data, Liu et al. (1981) and Turgeon and Waring (1986) reported that 8-Br-cAMP and dBu-cAMP did not stimulate LH release on their own but did potentiate GnRH-stimulated LH release. Thus, conflicting data about the effects of these cAMP analogues make it difficult to assess the role of cAMP in LH release.

More recently, forskolin, an activator of adenylate cyclase (Seamon et al., 1981), has been used as a tool to investigate the role of cAMP in GnRH-stimulated LH release. Forskolin increased cellular cAMP concentrations (Cronin et al., 1984), stimulated LH release on its

own, and enhanced GnRH-stimulated LH release (Cronin et al., 1983 and 1984; Evans et al., 1986). Turgeon and Waring (1986) demonstrated that forskolin enhanced GnRH-stimulated LH release but had no effect on its own. Recently, Bourne and Baldwin (1987) demonstrated that sodium flufenamate, an inhibitor of cAMP production, inhibited both the release of LH by GnRH and its ability to increase the cellular cAMP concentration. This suggested that cAMP may have a direct role in the mechanism by which GnRH stimulates LH release.

In conclusion, the available evidence suggests that cAMP may play a role in GnRH-stimulated LH release. However, the relationships and interactions between cAMP and other second messengers (Ca^{2+} and diacylglycerol) in GnRH action require clarification.

2. METHODS

2.1 Preparation of primary cultures of chicken anterior pituitary cells

Anterior pituitaries were removed within 2 hours of death, from chicken heads (Golden Grove Poultry Company, Cape Town, South Africa) that had been kept on ice and washed with disinfectant (Hibitane: 0.5% chlorhexidine gluconate in 80% methanol). The pituitaries were collected into Dulbecco's Modified Eagles Medium (DMEM) (Gibco, Grand Island, NY) buffered with 20 mM HEPES (pH 7.4) at room temperature. The pituitaries were then washed twice in buffer A which comprised (millimolar): NaCl, 140; KCl, 4; $MgCl_2$, 1; Na_2HPO_4, 1.4; glucose, 8.3; HEPES, 20 (pH 7.4); and phenol red, 6 mg/liter.

The pituitaries were minced with a razor blade, and digested with collagenase for 1 hour at 37°C with continuous agitation by a slowly rotating stirrer. The collagenase solution consisted of collagenase 0.9% (wt/vol) (155 U/mg, Worthington Biochemical Corporation Freehold, NJ) and 1.8 mg/liter deoxyribonuclease (Miles Laboratories, Elkhardt) in a buffer of (millimolar) NaCl, 137; KCl, 5; Na_2HPO_4, 0.7; HEPES, 25 (pH 7.2); Ca_2Cl_2, 0.36; glucose, 10 and 1% (wt/vol) bovine albumin (BSA) (fatty acid free, Pentex fraction V, Miles Laboratories). The suspension was passed up and down a 5 ml pipette at 20 minute intervals during the incubation and finally centrifuged for 5 minutes at 500 x g. The pellet was resuspended in Mg^{2+}-free buffer A containing 0.5 mM EDTA and 0.3% (wt/vol) BSA at room temperature, and recentrifuged. This step was repeated, and the pellet resuspended in DMEM. The cell suspension was filtered through nylon gauze and diluted into DMEM with 10% fetal calf serum (FCS)

(Gibco), penicillin (60 mg/liter), streptomycin (100 mg/liter), and amphotericin B (20 mg/liter). The suspension was dispensed into plastic tissue culture wells (Falcon) at a density of 1.5 or 3 pituitary equivalents per well (6-well plates) or 0.7 pituitary equivalents per well (24-well plates). One pituitary equivalent represents 5 - 6.7 x 10^5 cells as determined by coulter counter. The cells were cultured at 37°C in 8% CO_2 for 24 hours, after which the medium was replaced with fresh medium without amphotericin B. The cells were used for experiments after a further 24 hours in culture.

2.2 Cell stimulation

The cells were washed once briefly , followed by two five-minute washes with buffer A containing 1 mM $CaCl_2$ and 2% FCS. The cells were washed with 2ml of washing buffer and placed on a rotary shaker (60 rpm) at 37°C during the wash periods. The cells were stimulated at 37°C in the same buffer cotaining the test secretagogue, except where indicated. At the end of the stimulation period, the medium was collected, and centrifuged at 4°C for 5 minutes at 500 x g to remove cells that had detached during the stimulation period. The supernatants were stored at -20°C prior to radioimmunoassay (RIA) for chicken LH. For the measurement of total cellular LH, cells were lysed with 0.5 ml 1% nonidet-P40. cAMP was extracted from the cells using 0.1 M HCl which was subsequently neutralised with 0.1 M NaOH, and stored at -20°C prior to assay for cAMP using a RPA 509 cAMP assay kit (Amersham, England).

Where detailed time-course studies were performed, the total volume of medium was collected at the end of each time point and replaced with

fresh medium. In experiments where LH release was divided into spike, intermediate and plateau phases, the total volume of medium was collected and replaced at 3, 9 and 30 minutes after the start of stimulation. In studies in which gadolinium (Gd^{3+}) was used, phosphate was omitted from the stimulation buffer to avoid precipitation.

2.3 $^{45}Ca^{2+}$ efflux

Pituitary cells which had been cultured for 48 hours in 6-well tissue culture plates (3 pituitary equivalents per well) were pre-incubated for 1.5 hours with $^{45}Ca^{2+}$ (10 μCi/ml) in 0.5 ml buffer A containing 0.2 mM unlabelled Ca^{2+} and 5% FCS at 37°C. The labelled cells were then washed 6 times with 1ml buffer A containing 1 mM $CaCl_2$. The cells were placed on a rotary shaker (60 rpm at 37°C) for 5 minutes during each of the last three washes. This extensive washing procedure allows exchange of $^{45}Ca^{2+}$ bound to extracellular sites and located in rapidly exchanging intracellular compartments. The basal fractional efflux rate of the remaining $^{45}Ca^{2+}$ was 2-3%/min (see results section 3.5). This corresponds to a kinetic compartment with an average half life of 35-70 minutes. $^{45}Ca^{2+}$ efflux was measured by replacing the efflux buffer at 3 minute intervals. The composition of the efflux buffer was varied as indicated. At the end of the efflux period the cells were solubilized in 0.5% sodium dodecyl sulphate to determine the remaining cellular $^{45}Ca^{2+}$. Radioactivity was determined by scintillation counting. $^{45}Ca^{2+}$ fractional efflux was calculated as $^{45}Ca^{2+}$ released during each 3-minute interval as a percentage of the calculated total cellular $^{45}Ca^{2+}$ at the start of the 3-minute interval.

2.4 Inositol phosphate production

Pituitary cells in 6-well tissue culture plates (3 pituitary equivalents per well) were prelabelled for 20 hours in 2 ml/well of Medium 199 (Gibco, Grand Island, NY) containing 5 μCi/ml of ^{3}H-myo-inositol (Amersham). Cells were washed for two five-minute periods and then pre-incubated for 10 minutes in buffer A containing 1 mM Ca^{2+}, 2% FCS, and 10 mM LiCl, before incubation with GnRH in the same buffer. At the end of the stimulation period, the medium was removed and replaced with boiling water, after which the plates were left for 10 minutes on a slowly rotating shaker, at room temperature. Supernatants containing the water-soluble inositol phosphates were stored at -20°C prior to being chromatographed on dowex anion exchange columns (Martin, 1986).

The columns were made using Gilson P5000 pipette tips plugged with glass wool, containing 0.5 ml bed of anion exchange resin (Dowex-1, 1x8-200, Sigma). The dowex was originally in the chloride form and was equilibrated with 3 M ammonium formate (AF) for 1 hour to convert the resin to the formate form. The dowex was washed with 3 M AF prior to the addition of the samples to the column. After the samples had been added, the columns were washed with 10 ml water to elute the excess ^{3}H-myo-inositol that had not been incorporated into the inositol phosphates. Glycerophosphorylinositol was eluted with 2 x 3 ml of 25 mM sodium tetraborate/60 mM sodium formate. IP_1, IP_2 and IP_3 were eluted with 2 x 3 ml of 0.2 M AF, 0.6 M AF and 1 M AF in 0.1 formic acid. It was confirmed that the putative IP_1 fraction coeluted with authentic myo-[2-^{3}H] inositol 1-phosphate (The Radiochemical Centre, Amersham) and that ^{3}H-inositol was completely eluted by the

washing procedure used. Radioactivity recovered was determined by scintillation counting in a Beckman LS3801 scintillation counter using the scintillant Packard Insta-gel (Illinois, USA).

3.5 Radioimmunoassay of LH

RIA of chicken LH was performed as previously described (Follett et al., 1972). All samples from an experiment were assayed in duplicate in a single assay. Intrassay and interassay coefficients of variation were 7% and 16%, respectively. The inorganic ions used in the stimulation experiment did not interfere with the RIA (see Table below). A control value in the RIA was assayed in the presence of Co^{2+} and Gd^{3+} (see Table below).

EFFECT OF Co^{2+} AND Gd^{3+} ON THE RIA

SAMPLE	% CONTROL
Control	100
Control + Co^{2+} 0.3 mM	104
Control + Co^{2+} 1 mM	98
Control + Gd^{3+} 0.3 mM	106
Control + Gd^{3+} 1 mM	105

2.6 Reagents

[Gln^{8}]GnRH was synthesized by R.C.del.Milton,Department of Chemical Pathology, University of Cape Town. D600 (methoxyverapamil)(Knoll AG, Ludwigshafen, FRG) was dissolved in dimethylsulphoxide, and nifedipine (Bayer Pharmaceuticals, Johannesburg, South Africa) in ethanol. Nifedipine is light-sensitive and was handled accordingly. TPA and forskolin were bought from Sigma Chemical Co. (St. Louis, MO), and were dissolved in ethanol. IBMX was

obtained from the same source, but was dissolved in dimethylsulphoxide. Final solvent concentrations were 0.1% (vol/vol) or less and did not affect LH release. Ω-Conotoxin was synthesized by J.E. Rivier, The Salk Institute (La Jolla, CA). Gadolinium chloride was purchased from ICN Biochemicals (CA). Ruthenium red was purchased from Sigma Chemical CO. (St.Louis, MO), and was assumed to be 35% pure ,as stated by the supplier. $^{45}Ca^{2+}$ and myo-[2-^{3}H]inositol were from Du Pont de Nemours International (Zurich, Switzerland). ^{125}I was obtained from Amersham, England.

2.7 Data presentation

Each figure shows results obtained from a single experiment which was representative of two to six experiments. Points represent the mean ± SEM of triplicate wells. Where indicated, some figures contain data which were combined from more than one experiment.

RESULTS

Part 1: INVESTIGATION OF THE Ca^{2+} DEPENDENCE OF GnRH-STIMULATED LH RELEASE.

3.1 Kinetics of GnRH-stimulated LH release

GnRH stimulated LH release from primary cultures of chicken pituitary cells in a biphasic manner (Fig.1). GnRH consistently induced a rapid initial spike of LH release within the first 2 minutes of stimulation. This spike phase was followed by a sustained plateau phase of LH release. This biphasic LH response to stimulation with GnRH was closely similar in 5 detailed time-course experiments of the type shown in Fig.1. The 3 intervals chosen for further study were the spike phase (0-3 min), intermediate phase (3-9 min) and the plateau phase (9-30 min). Table 1 shows the results from 20 experiments in which LH release in these 3 intervals was measured. In subsequent data presented, results for the spike and plateau phases only are shown for clarity, since the intermediate phase was shown to possess characteristics intermediate between these two phases.

The EC_{50} for GnRH stimulation of the spike and plateau phases of LH release were similar being $2x10^{-9}$ M and $8x10^{-10}$ M respectively (Fig.2).

3.1.1 Dependence of GnRH-stimulated LH release on protein synthesis

To determine whether or not the LH released during the spike and plateau phases was dependent on protein synthesis, the kinetics of GnRH-stimulated LH release was investigated in the absence and presence of cycloheximide. Cycloheximide at a concentration of 20 µg/ml inhibited the incorporation of ^{3}H-phenylalanine by 95.8% (Table 2) (i.e. inhibited protein synthesis effectively), but had no effect

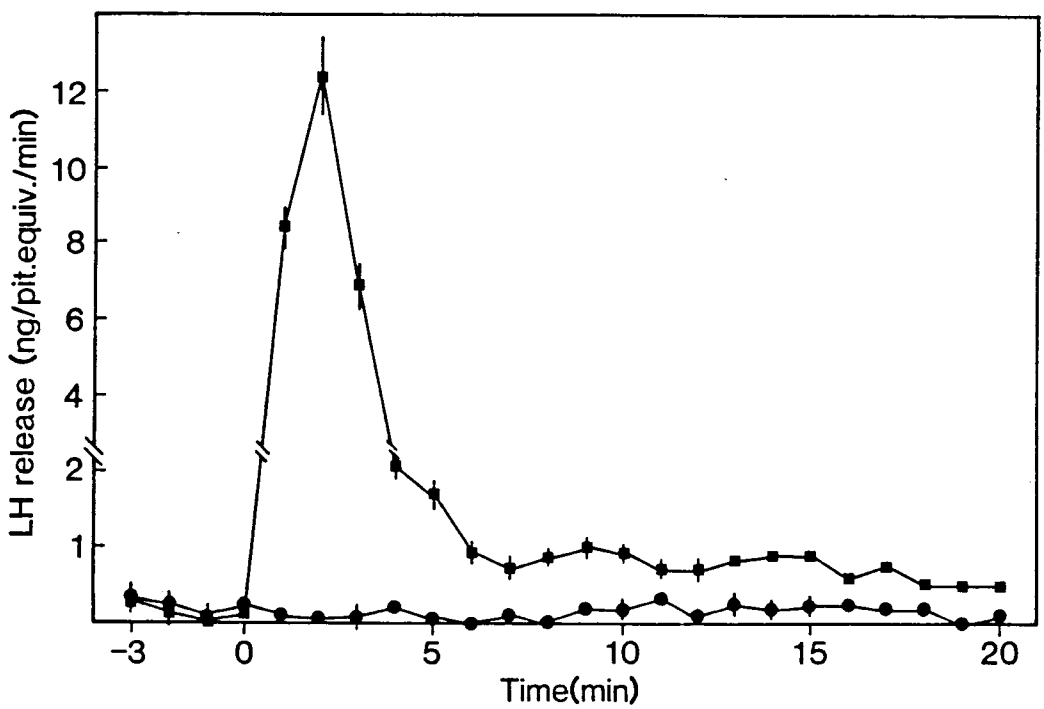

Fig.1 Kinetics of GnRH-stimulated LH release in cultured chicken pituitary cells.

(●) Basal, (■) 10^{-6} M GnRH present continuously from t = 0 minutes. The medium was changed at 1 min intervals. This experiment is representative of 5 similar experiments.

Table 1 : The three phases of GnRH-stimulated LH release (results from 20 independent experiments).

	LH RELEASED ng/pituitary equivalent					
EX NO	SPIKE PHASE		INTERMEDIATE PHASE		PLATEAU PHASE	
	BASAL	GnRH	BASAL	GnRH	BASAL	GnRH
108	0.48	9.02	0.62	5.82	1.63	8.00
109	1.88	18.00	0.48	14.54	2.20	21.53
110	1.71	21.43	4.01	31.24	3.70	17.31
111	1.51	17.48	0.85	5.46	1.73	9.18
113	1.12	11.22	1.18	9.38	3.83	12.63
114	5.38	21.57	2.91	10.63	2.13	15.33
116	3.93	47.43	1.17	11.13	7.60	32.23
117	2.67	23.14	0.88	7.41	1.90	10.17
118	1.32	65.53	1.69	31.30	6.33	36.58
119	1.05	27.68	1.08	14.65	2.36	24.19
121	1.93	49.06	2.16	19.07	5.94	34.77
122	1.77	20.18	1.69	23.28	4.99	37.92
123	1.27	22.62	1.46	20.41	4.53	29.00
127	0.16	24.25	0.83	26.88	1.56	40.57
128	1.02	27.70	1.00	21.25	4.99	35.05
131	0.45	10.51	0.31	7.10	3.20	13.55
133	0.35	29.63	0.53	14.47	3.33	31.25
135	0.80	12.52	1.46	8.94	2.21	25.33
138	1.44	20.24	0.03	16.74	2.44	34.72
141	1.40	40.08	3.13	15.58	4.59	21.77
MEAN	1.59	25.96	1.37	14.89	3.56	24.56
± SEM	±0.28	± 3.23	±0.23	± 1.60	±0.39	± 2.38

Cells were stimulated with a maximal concentration of GnRH (10^{-7} or 10^{-6} M) at time t = 0. Three phases of GnRH-stimulated LH release were identified from kinetic experiments: the spike phase (0-3 min), intermediate phase (3-9 min) and the plateau phase (9-30 min). The medium was replaced with fresh medium at the start of each period. At the end of each period (3, 9 and 30 minutes) the medium was collected for LH determination.

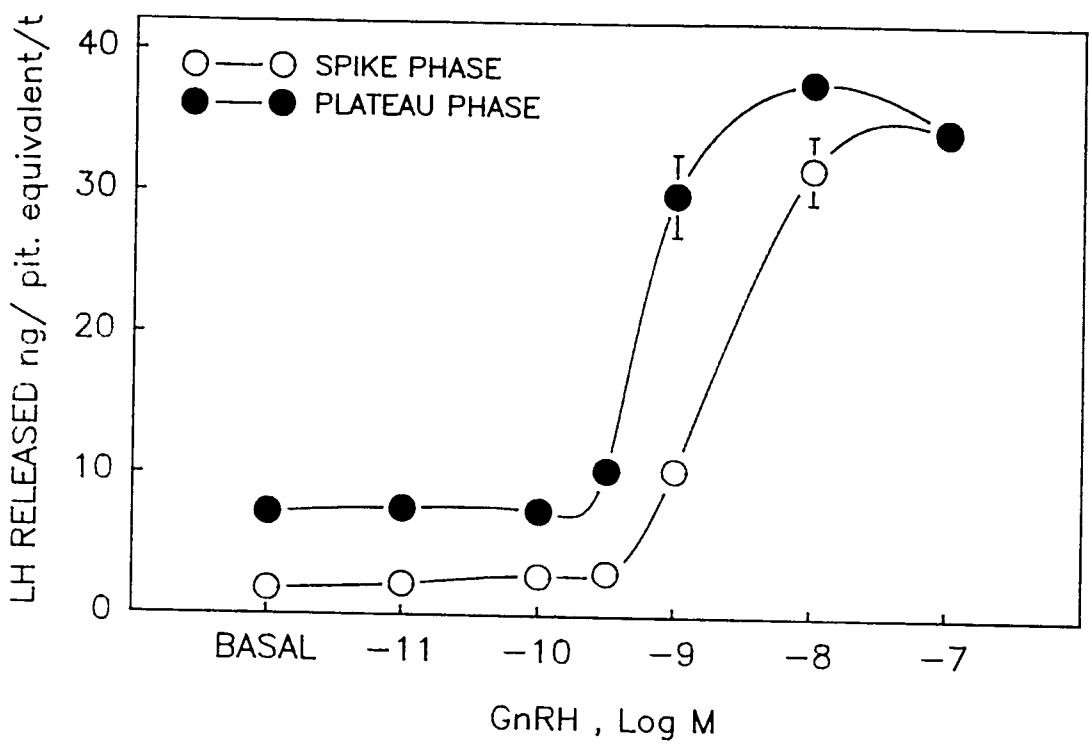

Fig.2 GnRH dose response curves for the spike (0-3 min) and plateau (9-30 min) phases of LH release. This experiment is representative of 3 similar experiments.

Table 2: Inhibition of ^{3}H-Phenylalanine incorporation into TCA-insoluble material by cycloheximide over a 60-min period. The data were obtained from triplicate wells of the same batch of cells as used in the experiment shown in Fig. 3 . The data represented are data obtained in a single experiment. This data is similar to data obtained in two similar experiments.

	CPM (^{3}H-Phe)	Mean ± SEM	%
No cycloheximide	14607 14949 12592	14049 ± 735	100
20 µg/ml cycloheximide	805 476 498	626 ± 92	4.15

on GnRH-stimulated LH release for up to 1 hour (Fig.3). During this 1 hour period, GnRH does not deplete the cell of LH, since the amount of LH released by the Ca^{2+} ionophore A23187 plus the phorbol ester TPA is much greater than that released by GnRH (Fig.3). GnRH typically released $3.0^{\pm}$ 0.37% (n=3 experiments) of the total immunoreactive cellular LH during a 20-minute stimulation period. The large amount of LH release induced by A23187 plus TPA was also not significantly inhibited by cycloheximide (Fig.3).

3.2 The effect of extracellular Ca^{2+} removal and intracellular Ca^{2+} depletion on GnRH-stimulated LH release.

In nominally Ca^{2+}-free medium, without a Ca^{2+} chelator, both the spike and plateau phases of GnRH-stimulated LH release were inhibited (Figs.4 & 5). LH release in the absence of extracellular Ca^{2+} during the spike and plateau phases was 39.0 ± 3.8 (n=3) and 4.8 ± 2.9 (n=3) percent respectively of release in media containing 1 mM $CaCl_2$ (Fig.4). Thus both phases of release are dependent on extracellular Ca^{2+}, with the spike phase being partially inhibited by Ca^{2+} removal and the plateau phase being totally abolished. The degree of inhibition of the spike phase was dependent on the pre-incubation period in Ca^{2+}-free medium prior to stimulation with GnRH (Fig.6). Unlike the plateau phase, the spike phase was never totally abolished even after 10 minute pre-incubation in the absence of extracellular Ca^{2+}. Similarly, in the presence of the Ca^{2+}-specific chelator EGTA, a portion of the spike phase persisted, while the plateau phase was abolished (Fig.4).

To determine whether or not this extracellular Ca^{2+} - independent portion of the spike phase was dependent on the mobilization of stored

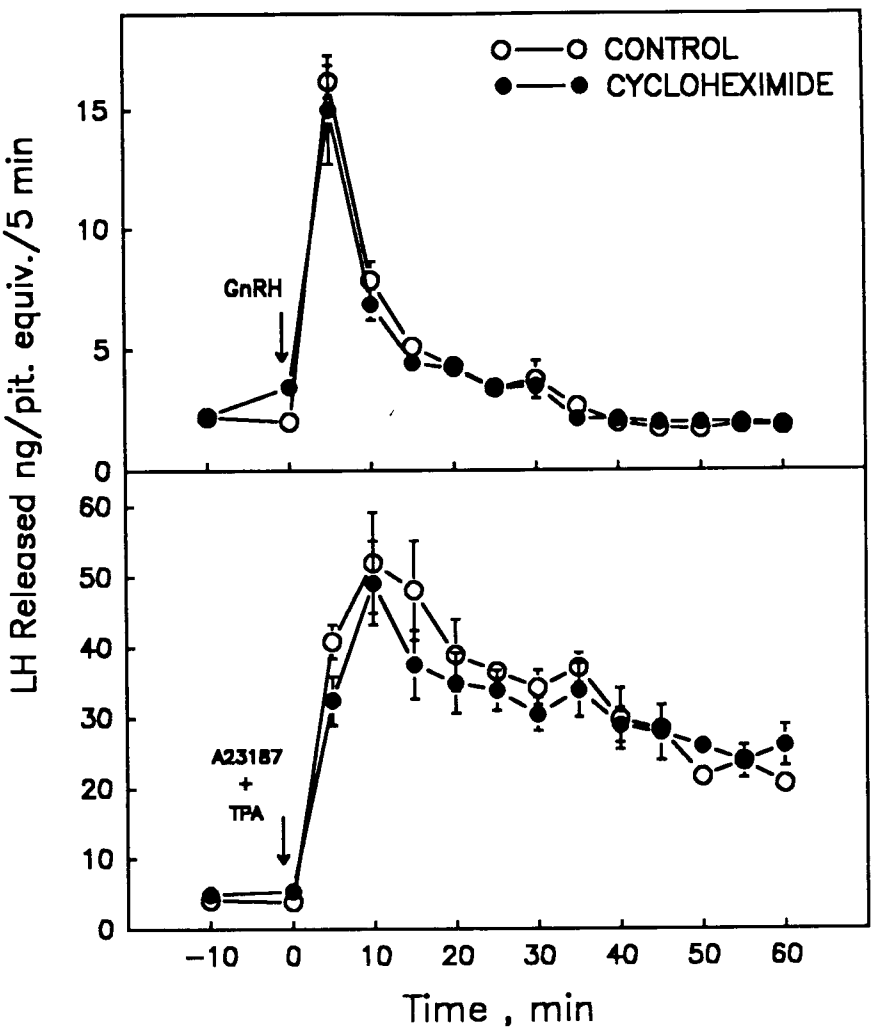

Fig.3 Dependence of GnRH-stimulated LH release on protein synthesis.

Top: Cells stimulated with GnRH (10^{-6} M) Bottom: Cells stimulated with A23187 (1 μM) and TPA (50 nM). These secretagogues were present continuously from t = 0 minutes. Cycloheximide (20 μg/ml) was present continuously from t = -10 minutes. This experiment is representative of 3 similar experiments.

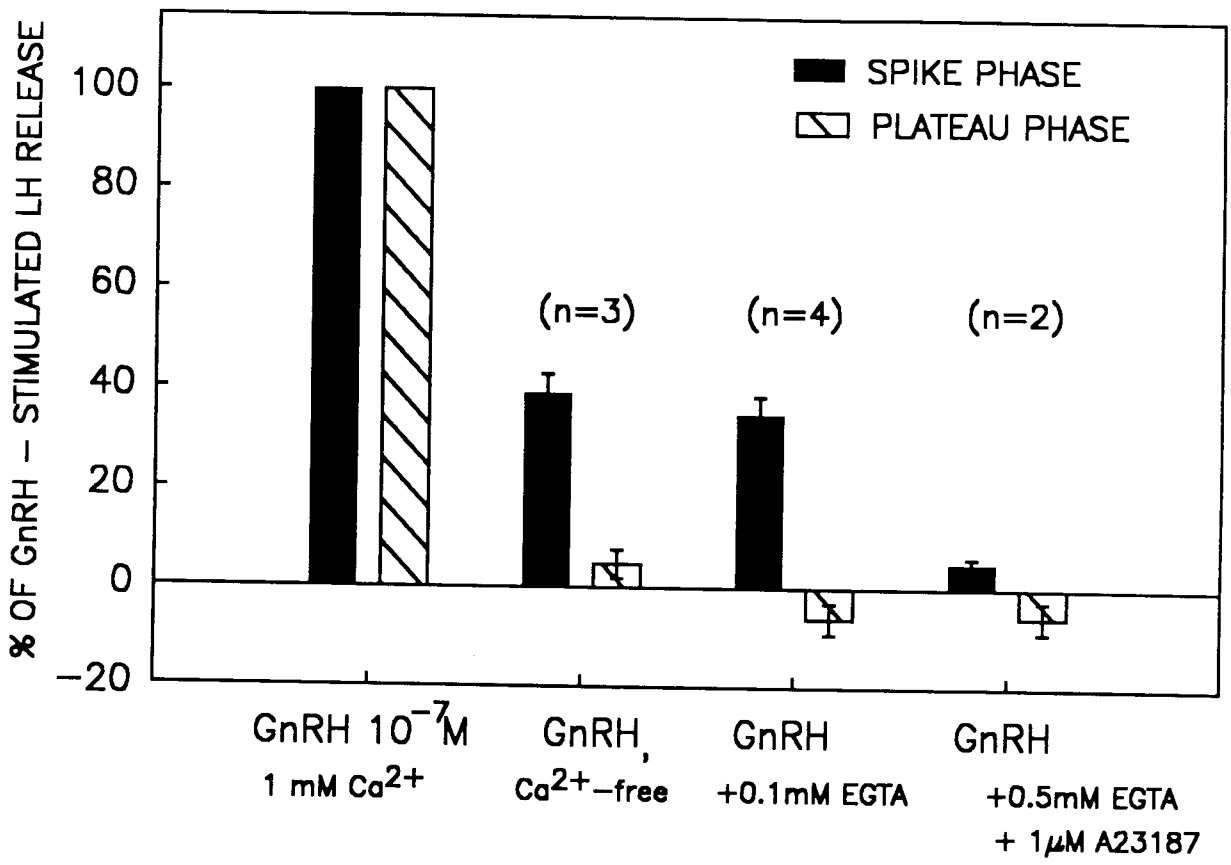

Fig.4 Effect of extracellular Ca^{2+} removal and intracellular Ca^{2+} depletion on the spike (0-3 min) and plateau (9-30 min) phases of GnRH-stimulated LH release.

The cells were pre-incubated with 1 mM Ca^{2+}, Ca^{2+}-free medium (no chelator), 0.1 mM EGTA, or 0.5 mM EGTA plus 1 μM A23187 for 10 minutes prior to stimulation with GnRH. Data from 2 to 4 experiments has been normalized to percent of control (1 mM Ca^{2+}).

Basal release (in the presence of 1mM Ca^{2+}) was subtracted and negative values reflect LH release below basal.

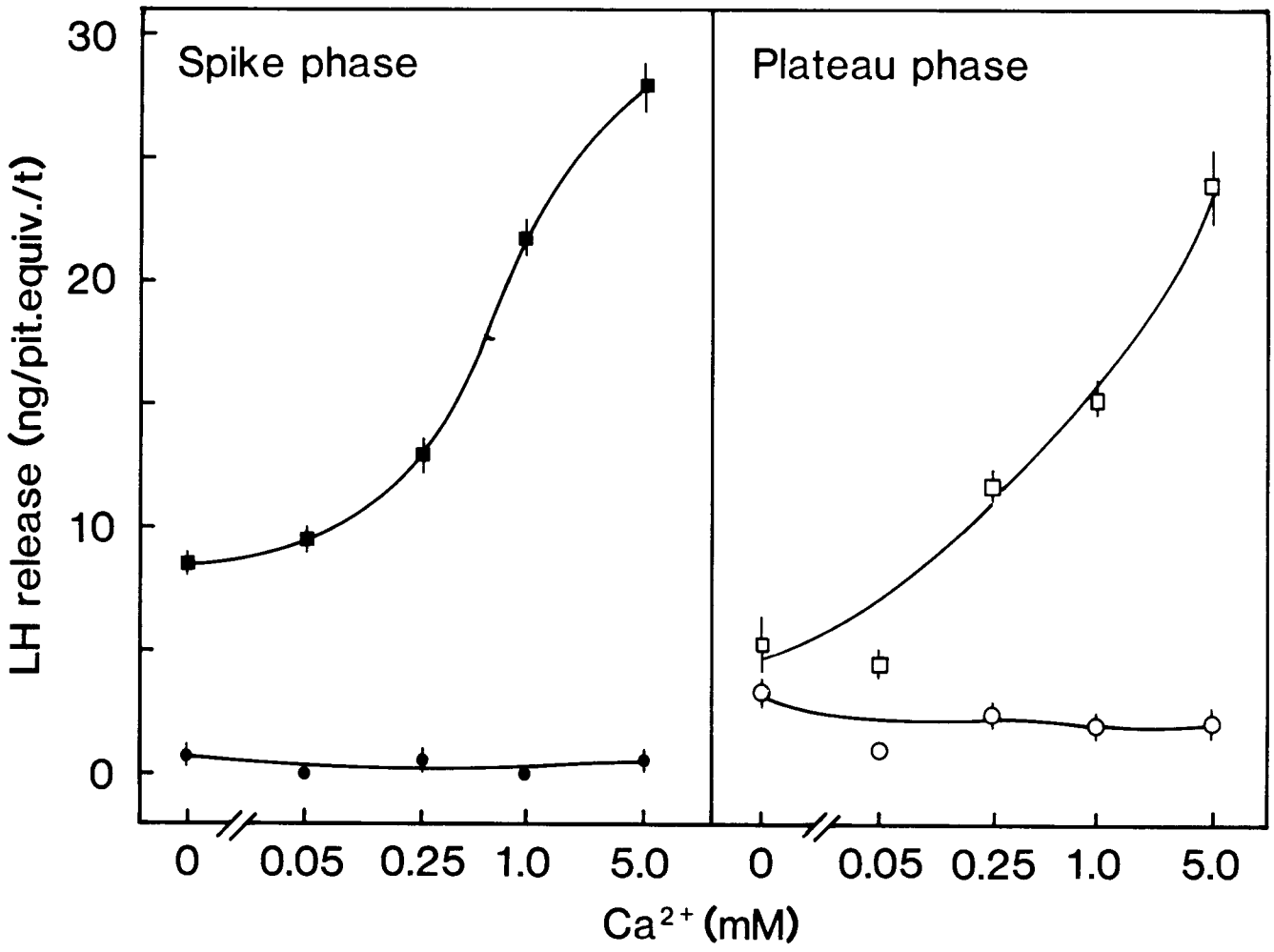

Fig.5 Dependence of the spike and plateau phases of GnRH-stimulated LH release on the extracellular Ca^{2+} concentration. (●,○) Basal, (■,□) 10^{-7} M GnRH. The cells were pre-incubated with the indicated Ca^{2+} concentrations for 5 minutes prior to stimulation with GnRH. No chelators were present at the Ca^{2+} concentration of "0 mM" Ca^{2+}. This experiment is representative of 3 similar experiments

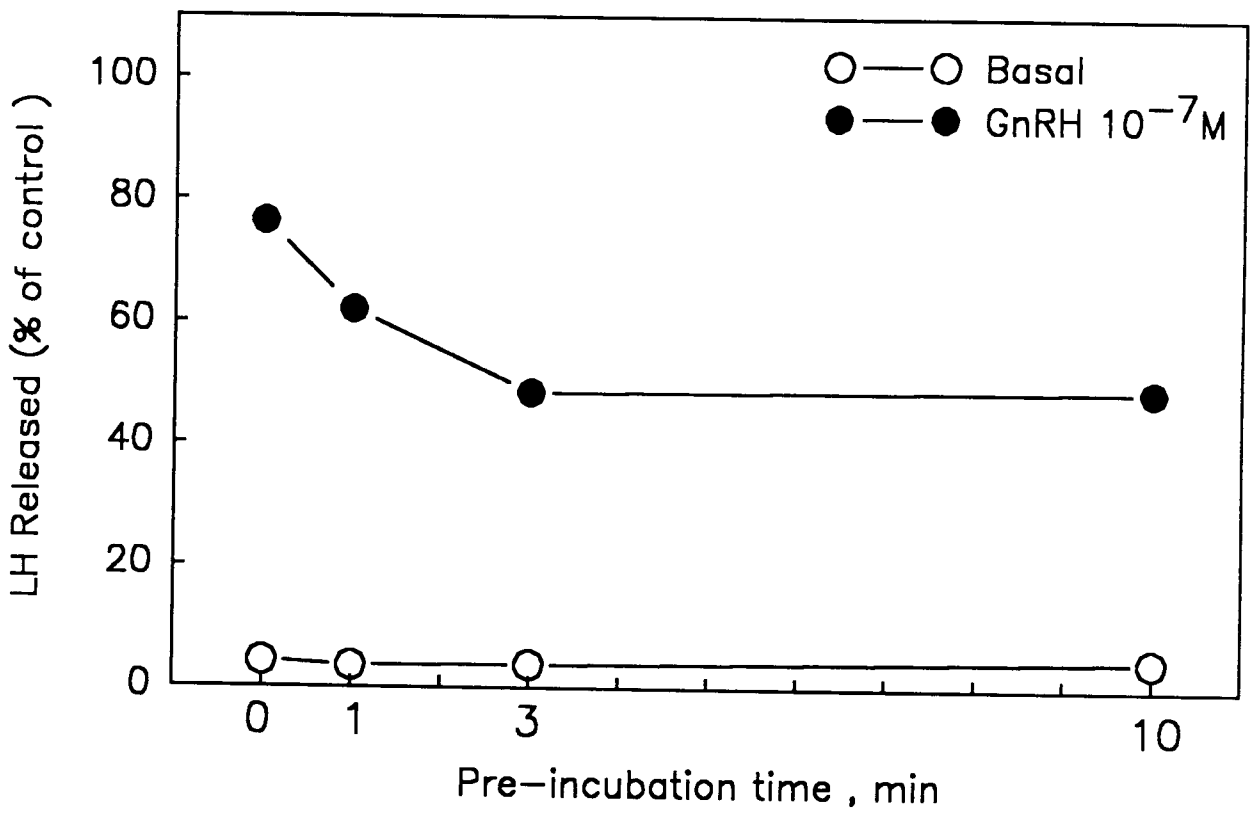

Fig.6 Effect of the length of the pre-incubation time in Ca^{2+}-free medium (without EGTA) on GnRH-stimulated spike phase. LH secretion is expressed as a percentage of LH release in presence of 1 mM Ca^{2+}. Representative of 3 similar experiments.

intracellular Ca^{2+}, the cells were pretreated in medium containing EGTA and the Ca^{2+} ionophore A23187. This treatment was designed to deplete intracellular stores of Ca^{2+}. The spike phase was completely abolished in cells pre-incubated in medium containing 0.5 mM EGTA and 1 uM A23187 for 10 minutes prior to stimulation with GnRH in Ca^{2+}-free medium plus 0.5 mM EGTA (Fig.4). This indicates that the portion of the spike phase which is independent of extracellular Ca^{2+} is dependent on the mobilization of stored intracellular Ca^{2+} which can be depleted by this pre-treatment. In contrast the plateau phase of release is entirely dependent on extacellular Ca^{2+}, since this phase of release is abolished in Ca^{2+}-free medium.

3.2.1 The effect of the removal of extracellular Ca^{2+} on basal LH release

It was noted that in experiments in which the cells were exposed to Ca^{2+}-free medium, the basal LH release levels were raised (Table 3). This increase in basal LH release was enhanced even further in the presence of the Ca^{2+} chelator EGTA (Table 3). The mechanism by which this increase in basal release occurs is unclear.

3.3 The effect of organic voltage-sensitive calcium channel (VSCC) blockers on GnRH-stimulated LH release

Since both the spike and plateau phases of LH release were dependent on extracelllular Ca^{2+}, the routes of Ca^{2+} entry (e.g. VSCC) in these two phases of release were investigated using various Ca^{2+} channel blockers.

Table 3: The effect of extracellular Ca^{2+} removal on basal LH release

Ca^{2+} (mM)	EGTA (mM)	LH release (ng/pit. equiv./20 min	n
1	0	2.7 ± 0.4	6
0	0	7.0 ± 1.1	3
0	0.1	13.3 ± 1.4	3

(Mean ± SEM)

The cells were pre-incubated for 10 minutes in the indicated medium, then basal LH release during the subsequent 20 minutes was determined. Results are mean ± SEM of n independent experiments.

3.3.1 Inhibition of GnRH-stimulated LH release by D600

The effect of methoxyverapamil (D600), a specific VSCC blocker, was both qualitatively and quantitatively different from the effects of Ca^{2+} removal on GnRH-stimulated LH release. Unlike the removal of extracellular Ca^{2+}, D600 caused only a minor inhibition of the spike phase (Fig. 7 & 8). A time course examining the kinetics of GnRH-stimulated LH released by D600 was done once using 2 min time intervals an was repeated with 3 min and 5 min intervals.

The efficacy of D600 in blocking VSCC was monitored by its ability to inhibit K^+-stimulated LH release. K^+ (50 mM) depolarizes the cell membrane, opening VSCC, to allow Ca^{2+} entry which results in LH release (Meier et al., 1988). Similar to GnRH, K^+ elicited a rapid spike phase of LH release followed by a sustained plateau phase of LH release (Fig.7). In 4 experiments D600 inhibited the spike and plateau phases of K^+-stimulated LH release by 81.0 ± 3.8% and 93.2 ± 3.3% (Table 4) respectively. These control experiments indicate that the dose and pre-incubation period of D600 used was adequate to block VSCC's.

To determine if increasing the extracellular Ca^{2+} concentration would be able to overcome the inhibition of LH release by D600, an experiment was done at two Ca^{2+} concentrations. The degree of inhibition of the spike and plateau phases of GnRH-stimulated LH release by D600 was similar at both 1 mM and 5 mM Ca^{2+} (Table 5) .

Dose response curves for the effects of D600 were determined. The plateau phase of GnRH-stimulated LH release was inhibited by D600 with IC_{50} = 0.2 μM (Fig.8). The plateau phase of K^+-stimulated LH release

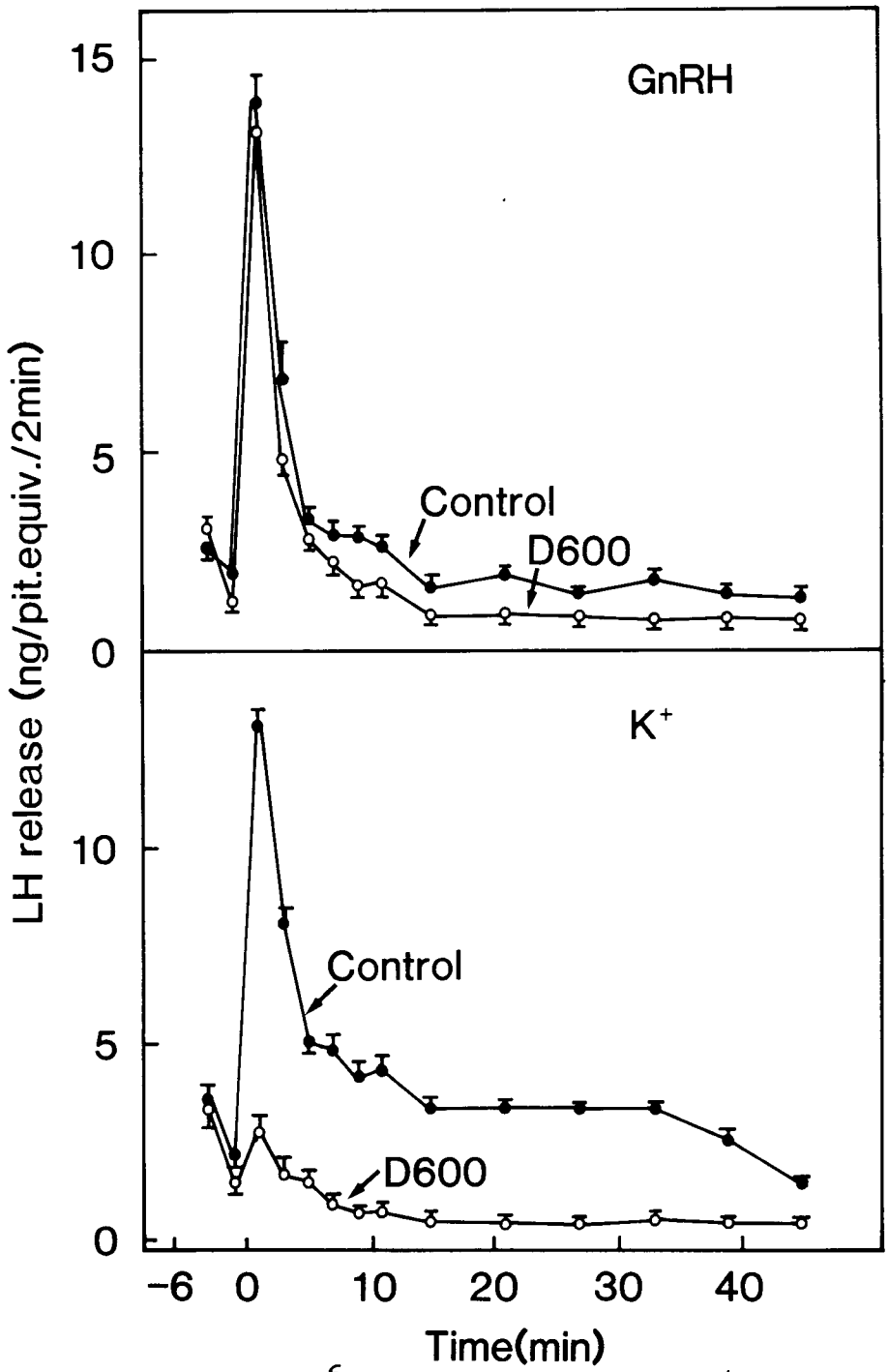

Fig.7 Effect of D600 on 10^{-6} M GnRH- and 50 mM K^+-stimulated LH release.

( ) No inhibitor, (O) 20 µM D600 present from t = -10 to t = + 48 minutes. The cells were pre-incubated for 10 min with D600 and then stimulated with GnRH and high K^+ at t=0 min. The medium was replaced at 2 min time intervals.

Extracellular Ca^{2+} was 1 mM. This experiment is representative of 3 similar experiments

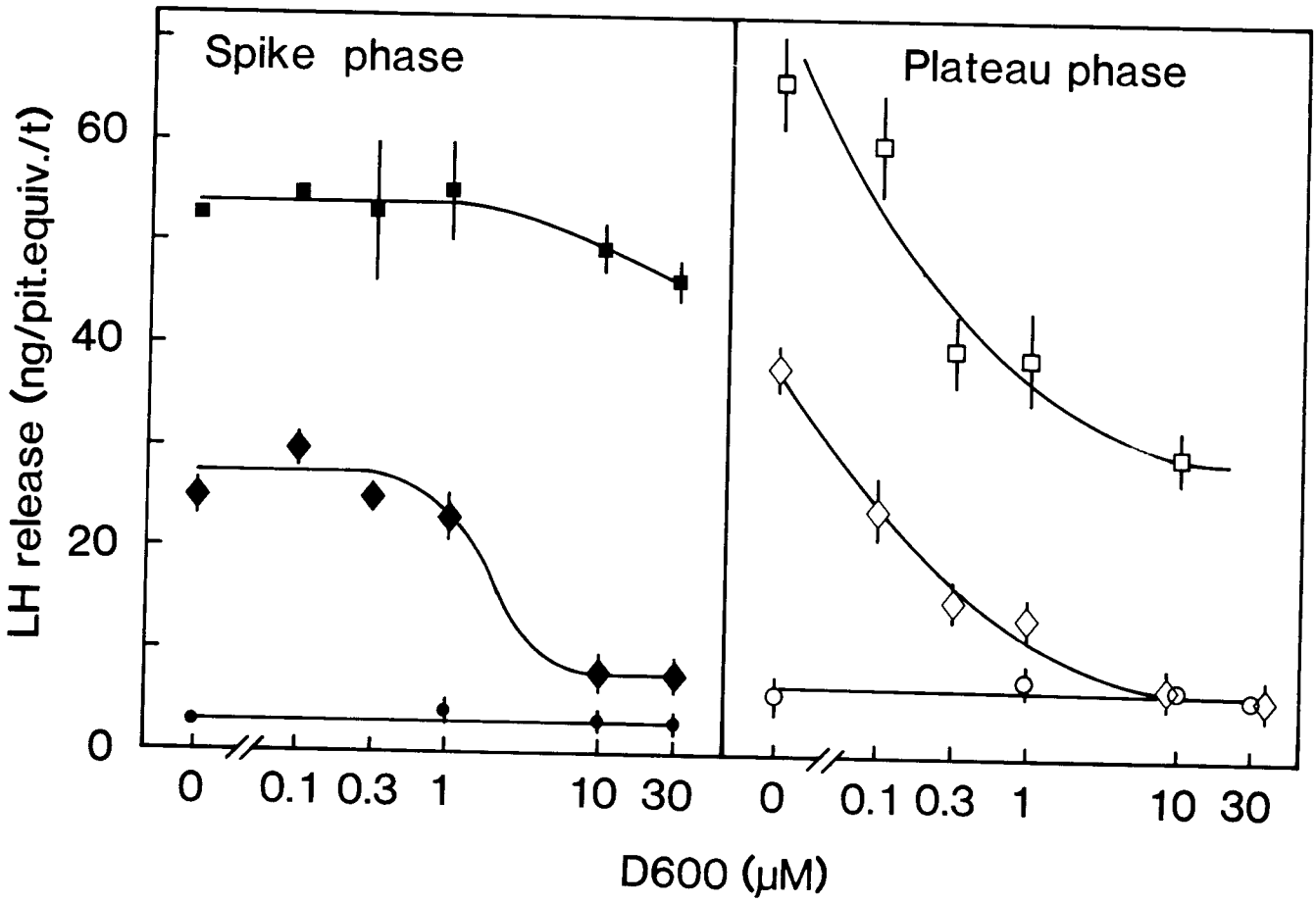

Fig.8 Dose-inhibition curves of the effect of D600 on spike (0-3 minutes) and plateau (9-30 minutes) phases of GnRH- and K^+-stimulated LH release.

D600 was present 10 minutes before the start of stimulation. (●,○) Basal, (■,□) 10^{-6} GnRH, (◆,◇) 50 mM K^+. Extracellular Ca^{2+} was 1 mM. This experiment is representative of 3 similar experiments.

Table 4: Percentage inhibition of GnRH and K^+-stimulated LH release by the Ca^{2+} channel blocker D600 (20 μM)

	GnRH 10^{-7} M		K^+ 50 mM	
EXPT NO.	SPIKE 0-3 min	PLATEAU 9-30 min	SPIKE 0-3 min	PLATEAU 9-30 min
76	5.1	54.3	78.8	84.6
79	11	58		
83	21	76		
84	23.1	51.1	81.2	99.6
94	8	62	73	97
91	13	50		
100	7	60	91	92
MEAN	12.6	58.7	81.0	93.2
± SEM	2.6	3.3	3.8	3.3
n	7	7	4	4

Data from n independent experiments. Data shown are the percentage inhibition by 20 μM D600 of GnRH- and K^+-stimulated LH release in the absence of D600. The cells were pre-incubated with D600 for 10 min prior to stimulation. Basal release has been subtracted.

Table 5: Inhibition of GnRH-stimulated LH release by D600 at 2 different Ca^{2+} concentrations.

D600 (μM)	Ca^{2+} (mM)	LH Release (% of control)	
		SPIKE Phase (0-3 min)	Plateau Phase (9-30 min)
50	1	96.6	68.4
50	5	100.0	62.4

Prior to stimulation, the cells were pre-incubated with 50 μM D600 for 10 minutes. The data shown is LH release stimulated by 10^{-7} M GnRH in the presence of D600, expressed as a percentage of GnRH-stimulated release in the absence of channel blocker. Basal release was subtracted.

was inhibited with a similar potency (IC_{50} = 0.2 μM). However, the spike phase of K^+-stimulated LH release required higher concentrations of D600 for inhibition (IC_{50} = 2μM) (Fig.8).

The ability of D600 to inhibit the plateau phase of GnRH-stimulated LH release suggests that VSCC are involved in this phase. However, the D600-sensitive portion of the GnRH plateau phase is less than the D600-sensitive portion of the K^+-stimulated plateau phase . This indicates that GnRH does not activate all the D600-sensitive VSCCs which are activated by K^+.

3.3.2 Inhibition of GnRH-stimulated LH release by nifedipine

The effects of the VSCC blocker, nifedipine, were both qualitatively and quantitatively similar to that of D600. Nifedipine had a very small effect on the spike phase, but inhibited the plateau phase of GnRH-stimulated LH release more markedly (Fig.9 & 10). The doses of nifedipine used and the pre-incubation period were adequate since both the spike and plateau phases of K^+-stimulated LH release were essentially abolished (Fig.10). The potency of nifedipine for inhibition of the plateau phase of GnRH-stimulated LH release (IC_{50} = 10^{-8}M) was similar to the drug's potency for inhibition of both phases of K^+-stimulated release (Fig.10).

3.4 The effect of inorganic ions and ruthenium red

The results presented in the previous sections showed that in the absence of extracellular Ca^{2+}, a residual portion of the spike phase persisted, while the plateau phase was abolished. In contrast, the organic VSCC blockers had little effect on the spike phase while the

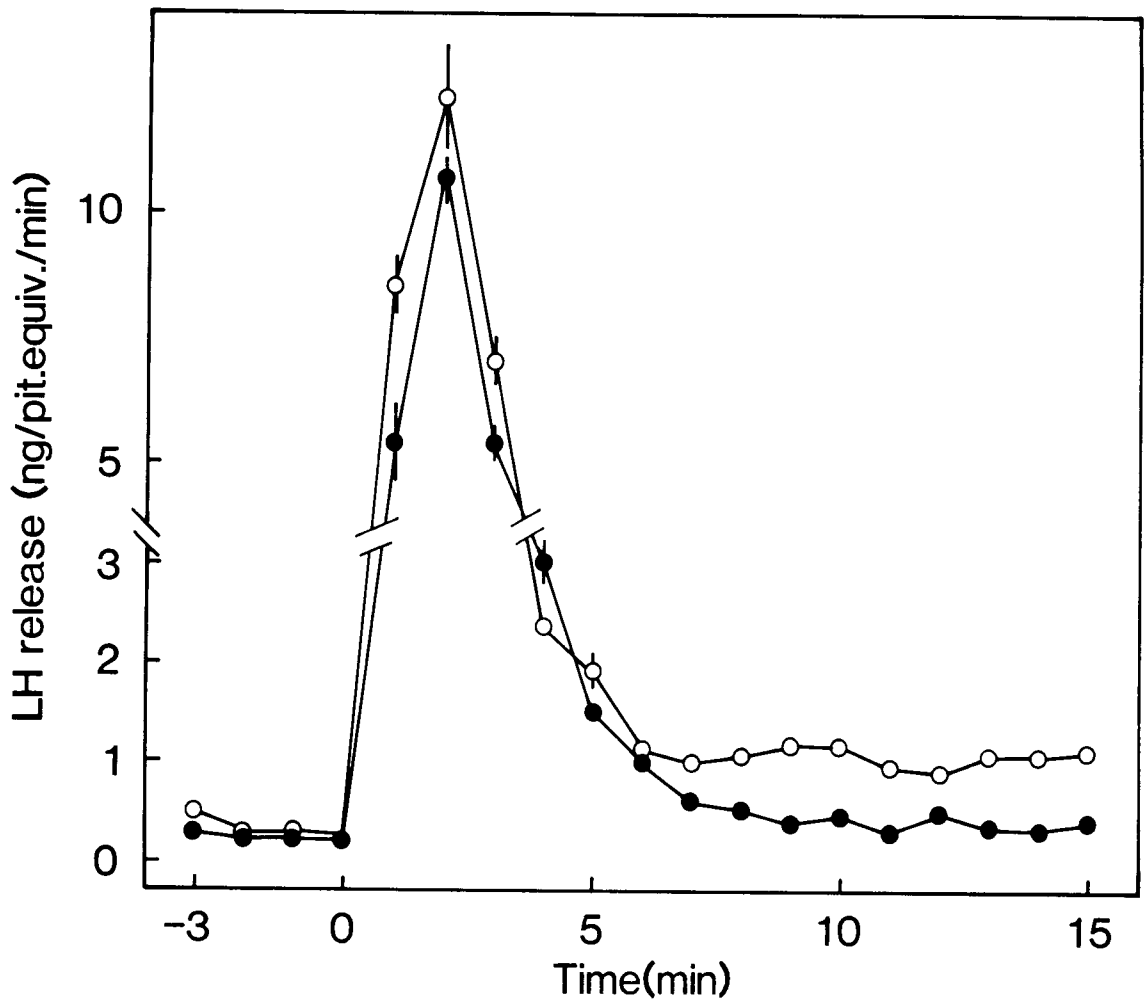

Fig.9 Effect of Nifedipine on the spike and plateau phases of GnRH-stimulated LH Release.

Cells were stimulated with 10^{-6} M GnRH in the presence (●) or absence (○) of 10^{-5} M nifedipine. In nifedipine-treated cells the drug was present 10 minutes before the addition of 10^{-6} M GnRH.

Extracellular Ca^{2+} was 1 mM. This experiment is representative of 3 similar experiments.

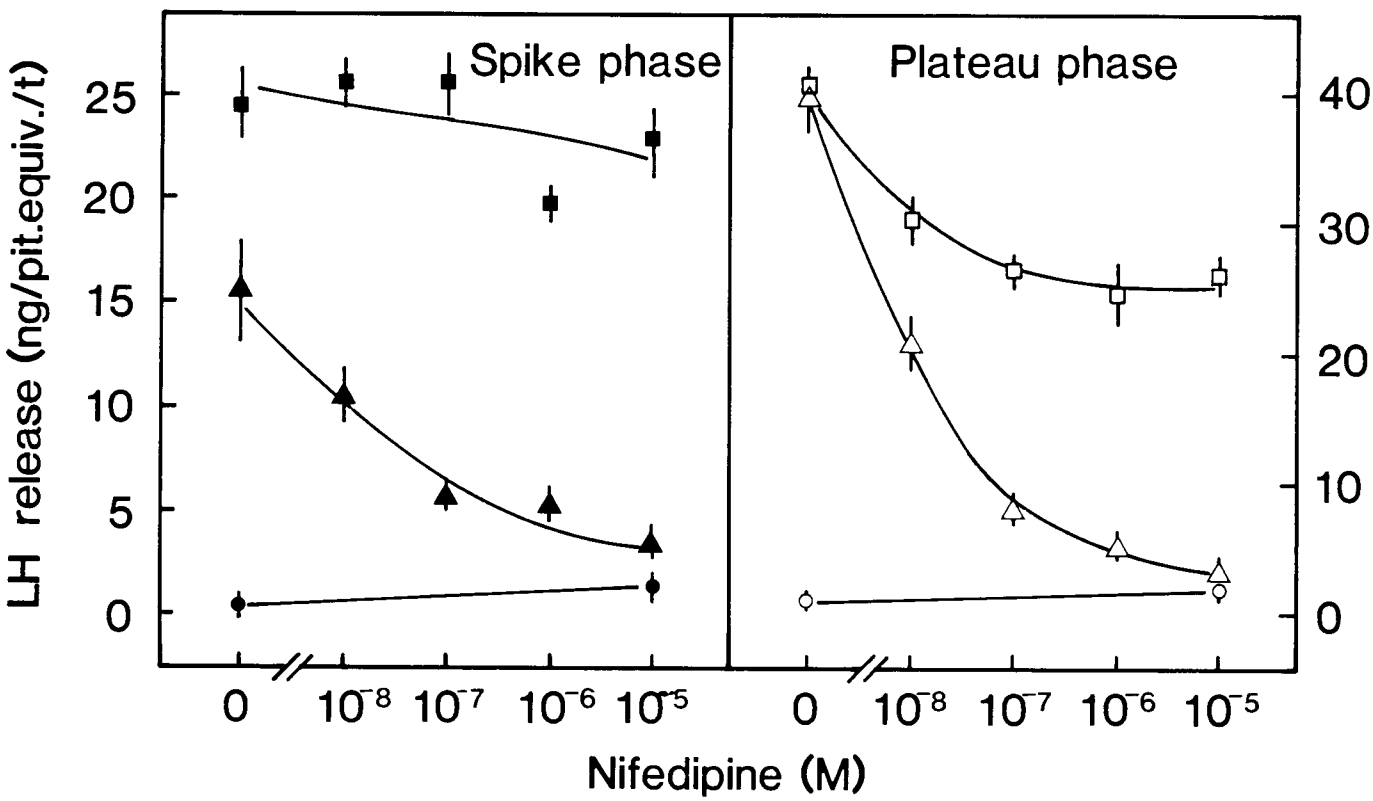

Fig.10 Dose-inhibition curves of the effect of nifedipine on the spike (0-3 minutes) and plateau (9-30 minutes) phases of GnRH- and K^+-stimulated LH release.

K^+ was present from 10 minutes before the start of stimulation period. (●, ○) Basal; (■, □) 10^{-6} M GnRH; (▲, △) 50 mM K^+. Extracellular Ca^{2+} was 1 mM. This experiment is representative of two similar experiments.

plateau phase was partially inhibited. This suggested that GnRH caused Ca^{2+} entry via route(s) additional to D600/nifedipine-sensitive VSCC. To characterize the other possible modes of Ca^{2+} entry, the effects of a range of divalent cations and other possible Ca^{2+} channel blockers were investigated.Thie effects on both the spike and plateau phases of GnRH-stimulated LH release were investigated.

3.4.1 The effect of Ni^{2+}, Zn^{2+}, La^{3+}, and Cd^{2+}

Initially, the effect of an array of inorganic ions of GnRH-stimulated LH release was investigated. Experiments with some of the ions were done once only. Only those those ions which yielded interesting data were used in further experiments. The chloride salts were used. Nickel, zinc and cadmium inhibited both phases of LH release (Table 6b). A detailed time course of the effect of Cd^{2+} is shown to illustrate its effects (Fig.11). However, these ions also non-specifically enhanced basal LH release (Table 6a). La^{3+} increased basal LH release (Table 6a) and also enhanced the spike phase of GnRH-stimulated LH release, while it inhibited the plateau phase (Table 6b). This increase in basal LH release indicates some action of these ions other than simply Ca^{2+} channel blockade. For this reason further studies with these particular inorganic ions were not pursued.

3.4.2 The effects of Co^{2+}

The cation Co^{2+} inhibited both phases of GnRH-stimulated LH release, without increasing basal LH release (Fig.12). The IC_{50} of Co^{2+} for inhibition of both the spike and plateau phase was 300 μM

(Fig.12). To characterize the action of Co^{2+} further, its effects on LH release stimulated by phorbol ester, forskolin, K^+ and Ba^{2+} were determined. Phorbol esters are analogues of diacylglycerol which activate protein kinase C (Nishhizuka, 1984). Phorbol esters have previously been shown to stimulate LH release from chicken pituitary cells (Davidson et al., 1987). Forskolin is an activator of adenylate cyclase that act by activating the catylytic subunit of adenylate cyclase (Seamon and Daly,1981). Ba^{2+} ions are a powerful LH secretagogue in chicken pituitary cells. The action of Ba^{2+} has previously been characterised by Davidson et al. (1987).

Co^{2+} inhibited LH release evoked by high K^+ and by Ba^{2+} (Fig.13). The actions of both these stimuli require the involvement of voltage-sensitive calcium channels (Davidson et al., 1987). In contast Co^{2+} had no effect on secretion evoked by either the phorbol ester TPA or forskolin (Fig.13).

Table 6a : Effect of Ni^{2+}, Zn^{2+}, La^{3+} and Cd^{2+} on basal LH release in the spike and plateau phases.

	Fold increase of basal release		
	(0-3 min)	(9-30 min)	n
Nickel 1 mM	2.22	0.79	1
Zinc 1 mM	1.43	2.21	1
Lanthanum 1 mM	1.21	2.73	1
Cadmium 1 mM	2.17 ± 0.61	1.84 ± 0.66	2

The cells were pre-incubated with the chloride salts of the ions for 10 minutes prior to the release period.

Table 6b : Effect of Ni^{+}, Zn^{2+}, La^{3+} and Cd^{2+} of GnRH-stimulated LH release

	% of Control		
	SPIKE(0-3min)	PLATEAU(9-30min)	n
Nickel 1 mM	59.57	23.25	1
Zinc 1 mM	9.05	0.09	1
Lanthanum 1 mM	126.96	71.78	1
Cadmium 1 mM	16.75 ± 10.99	-7.32 ± 8.95	2

The cells were pre-incubated with the indicated ions for 10 minutes prior to stimulation with 10^{-7}M GnRH. The data are presented as a percentage of the LH released by GnRH in the absence of the ions. Basal release was subtracted. Negative values indicate release below basal levels.

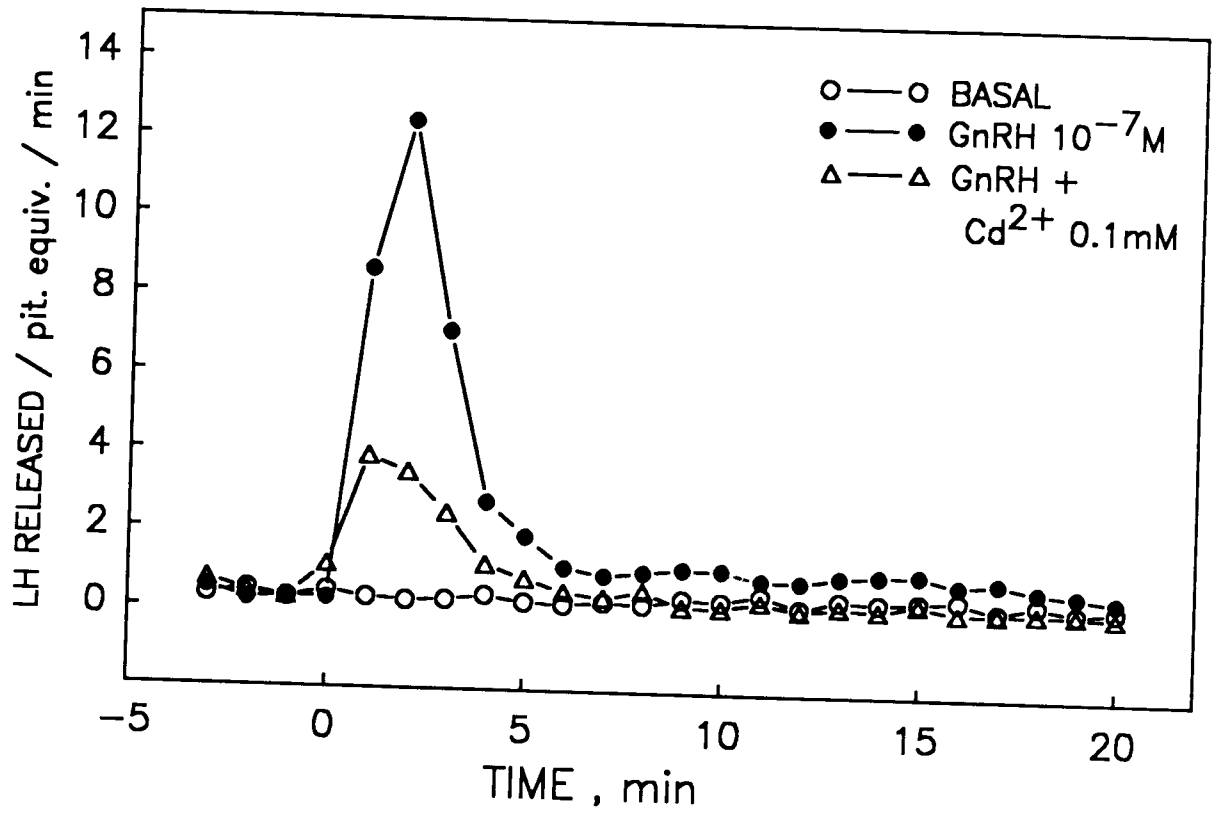

Fig.11 Kinetics of Cd^{2+} inhibition of GnRH-stimulated LH release. Cd^{2+} was added at t = -1 min.
Extracellular Ca^{2+} was 1 mM. The medium was changed at 1 min intervals.

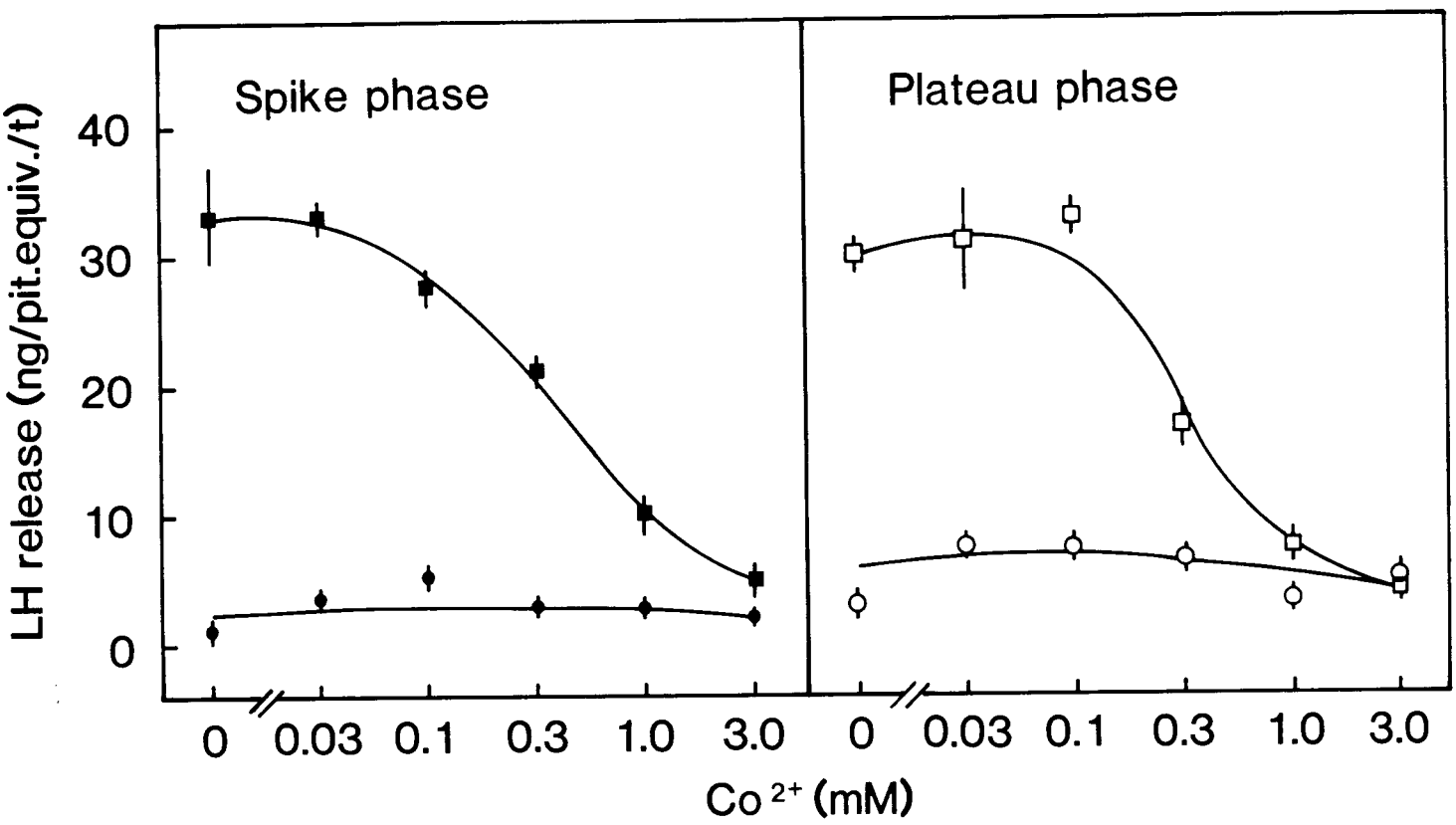

Fig.12 Inhibition of Spike (0-30 minutes) and Plateau (9-30 minutes) phases of GnRH stimulated LH release by Co^{2+}.
Co^{2+} was present from 10 minutes prior to stimulation.
(●, ○) Basal, (■, □)10^{-7}M GnRH. The extracellular Ca^{2+} was 1mM. This experiment is representative of 3 similar experiments.

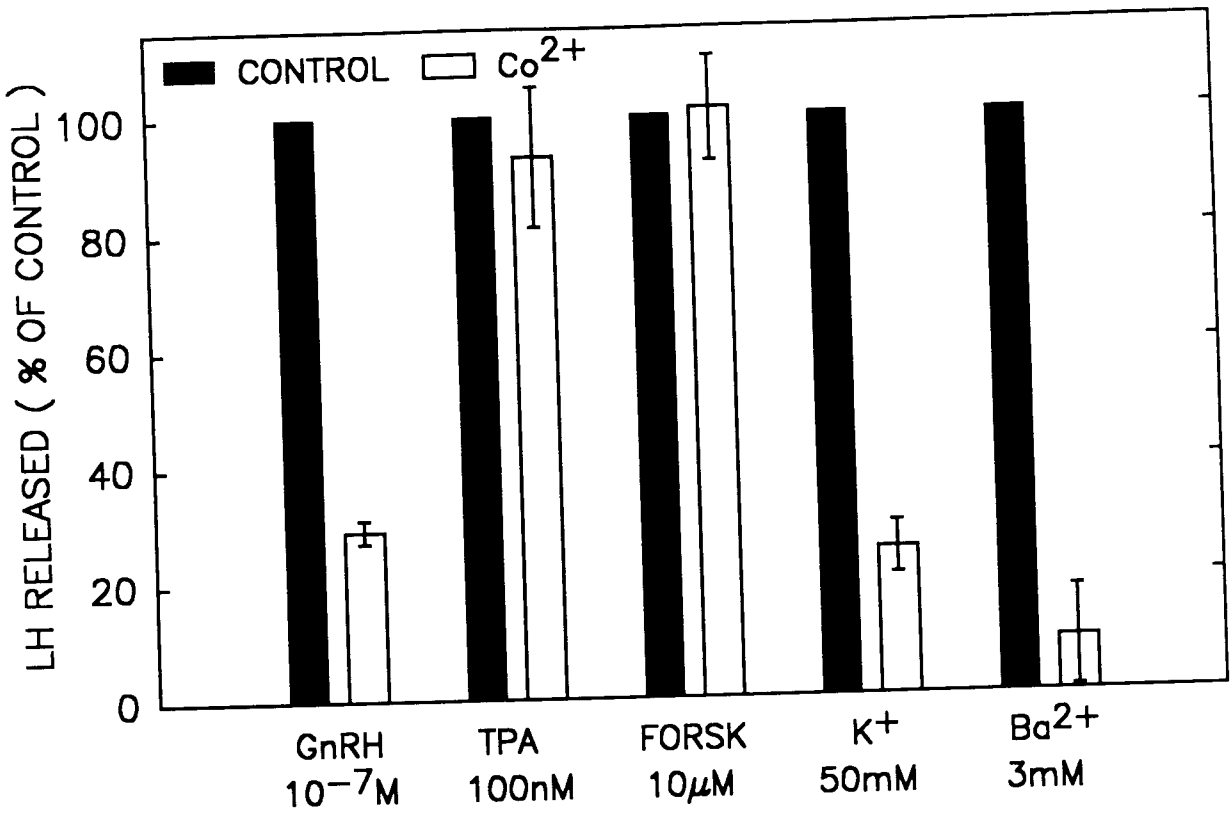

Fig.13 Effect of Co^{2+} on LH release stimulated by various secretagogues.

LH release was measured during a 20 minute stimulation period in the presence or absence of 1mM Co^{2+}, after a 10-minute pre-incubation period with 1 mM Co^{2+}. LH release is expressed as a percentage of LH release stimulated by the secretagogues in the absence of Co^{2+}. Basal release was subtracted. Representative of 2 to 3 similar experiments. The data are the mean of a combination of experiments.

3.4.3 Effects of Gd^{3+}

Gd^{3+} inhibited both the spike and plateau phases (IC_{50} = 3 μM and 1 μM respectively) of GnRH-stimulated LH release (Fig.14). It did not completely block by K^+-stimulated LH release, but it did abolish Ba^{2+}-stimulated LH release (Fig. 15). Unlike Co^{2+}, Gd^{3+} interacted synergistically with the protein kinase C activator TPA, enhancing the LH releasing ability of (Fig.15).

3.4.4 Ruthenium Red (RR)

The polycationic dye, ruthenium red (RR) was investigated as a possible Ca^{2+} channel blocker because of its known inhibitory effects on Ca^{2+} transport in mitochondria (Carofoli and Crompton, 1978). RR caused a marked inhibition of both phases of GnRH-stimulated LH release (Fig.16). A residual portion of the spike phase remained in the presence of 100 μM RR, while the plateau phase was abolished by this concentration of the compound.

The inhibitory effect of RR on the spike phase of GnRH-stimulated LH release was dependent on the pre-incubation period with RR prior to stimulation with GnRH (Fig.17).

The ability of TPA and forskolin to stimulate LH release were only slightly inhibited in the presence of RR (Fig.18). However, RR virtually abolished LH release in response to K^+, Ba^{2+} and veratridine, all of which depend on D600-sensitive VSCC for their activity (Fig.18).

3.4.5 Effect of Ω-conotoxin

The effect of Ω-conotoxin GVIA a specific peptide blocker of a class of neuronal VSCC (Rivier et al.,1987) was also investigated.

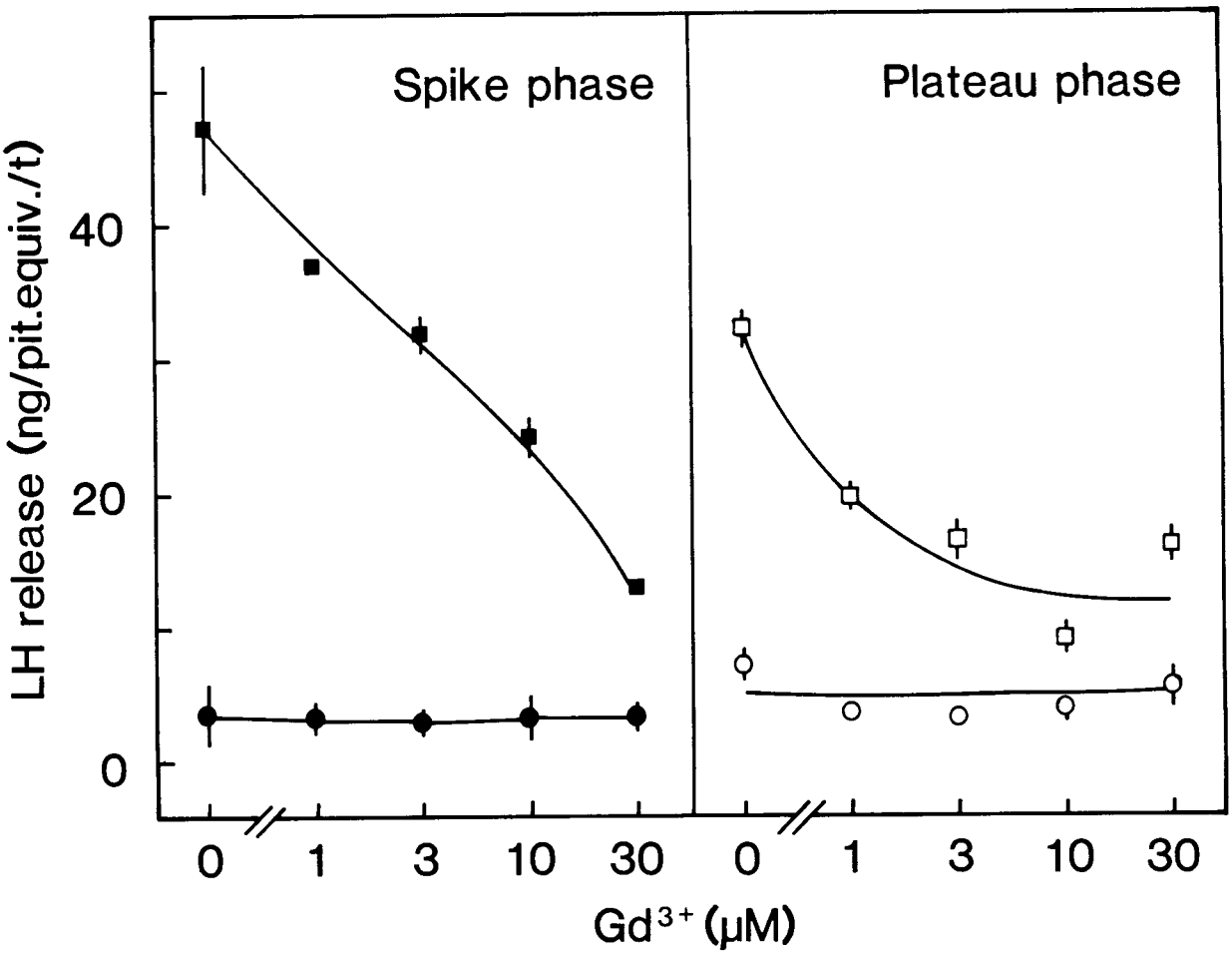

Fig.14 Inhibition of spike (0-3 minutes) and plateau (9-30 minutes) phases of GnRH-stimulated LH by Gd^{3+}.

Gd^{3+} was present from 10 minutes prior to stimulation with GnRH. The cells were then stimulated for the indicated time periods. (●, ○) Basal, (■, □) 10^{-7} M GnRH. Extracellular Ca^{2+} was 1 mM. This experiment is representative of 4 similar experiments.

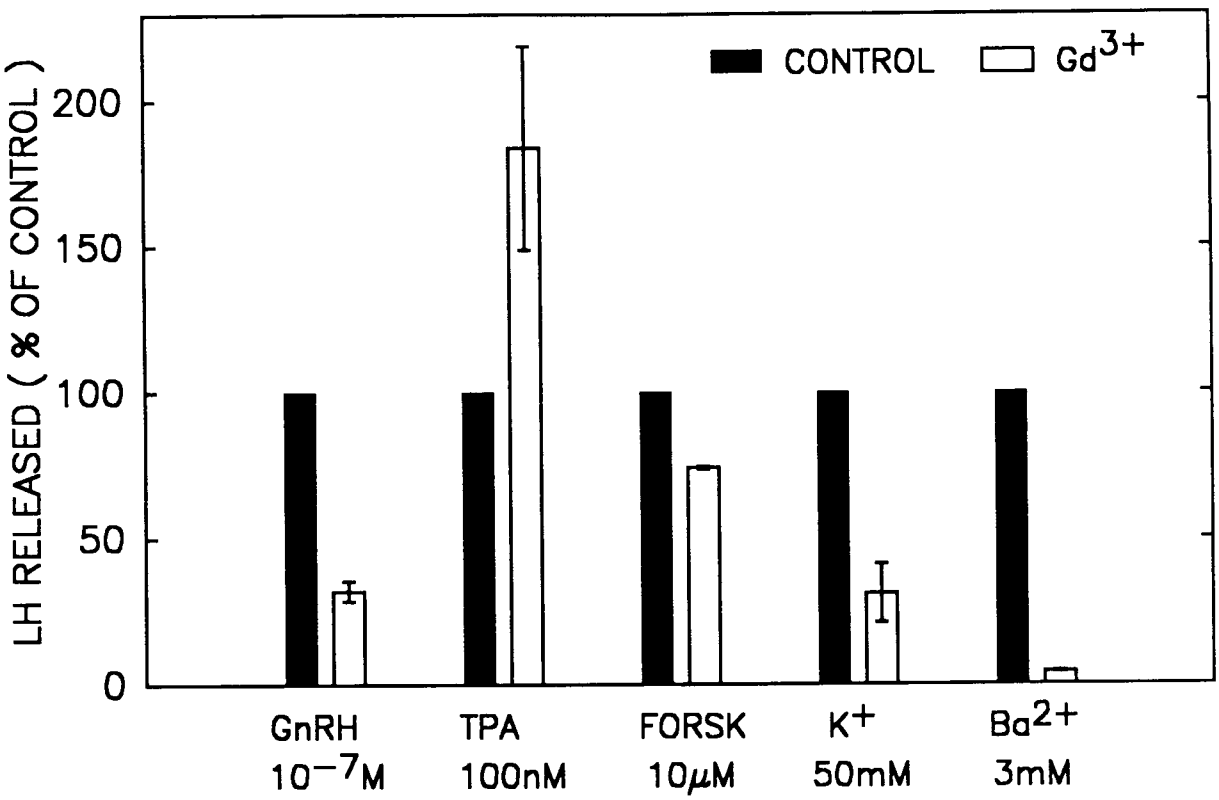

Fig.15 Effect of Gd^{3+} on LH release stimulated by various secretagogues.

LH release was measured during a 20 minute stimulation period in the presence or absence of 10 μM Gd^{3+}. The stimulation period was preceded by a 10-minute pre-incubation period with or without 10 μM Gd^{3+}. LH release is expressed as a percentage of LH release stimulated by the secretagogues in the absence of Gd^{3+}. Basal release was subtracted. Representative of 3 similar experiments.

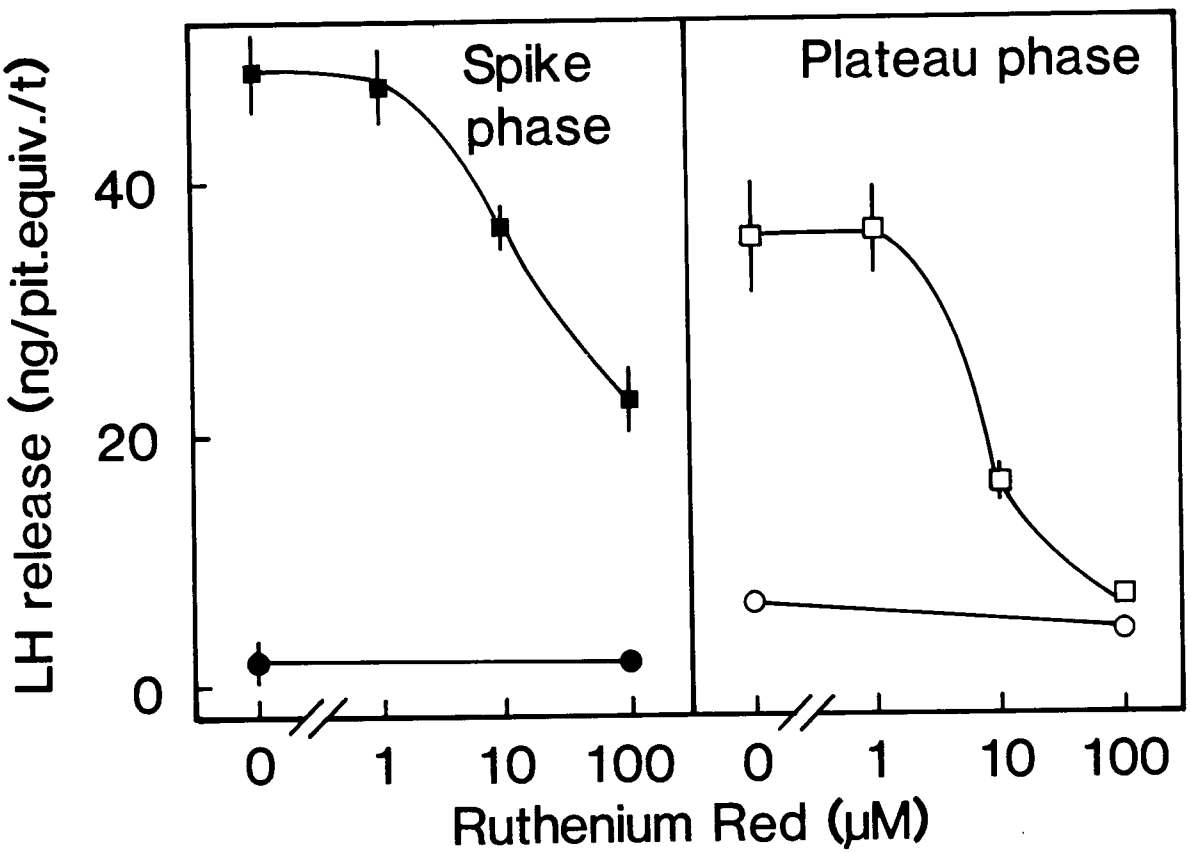

Fig.16 Inhibition of spike (0-3 minutes) and plateau (9-30 minutes) phases of GnRH-stimulated LH release by ruthenium red (RR). RR was present from 10 minutes prior to stimulation with GnRH. The cells were then stimulated for the indicated time periods with GnRH.(●,○) Basal, (■,□) 10^{-7} M GnRH. Representative of 3 similar experiments

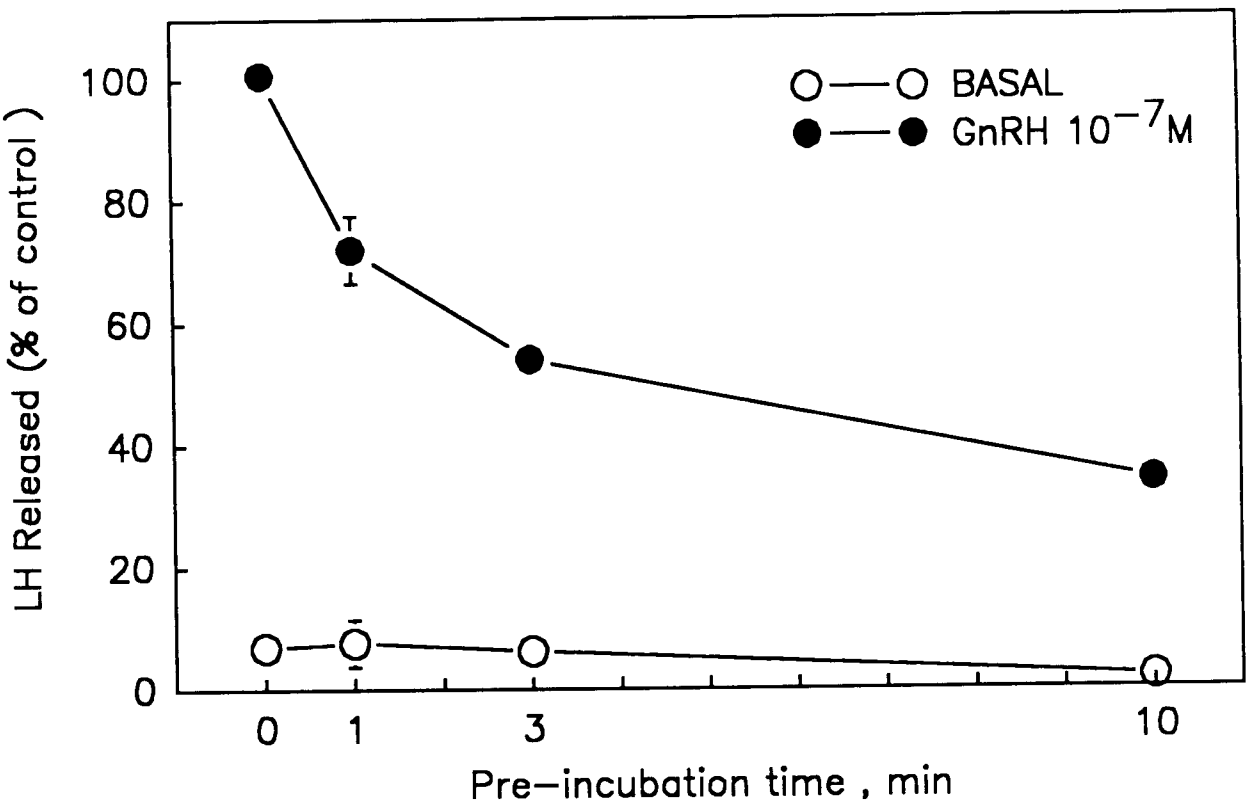

Fig.17 Effect of the length of the pre-incubation time with ruthenium red on the spike phase (0-3 min) of GnRH-stimulated LH release.

The cells were pre-incubated for the indicated time periods with RR. This pre-incubation period was followed by a 3 min stimulation period with GnRH. LH secretion is expressed as a percentage of LH release stimulated by 10^{-7} M GnRH in the absence of ruthenium red. This experiment is representative of 3 similar experiments.

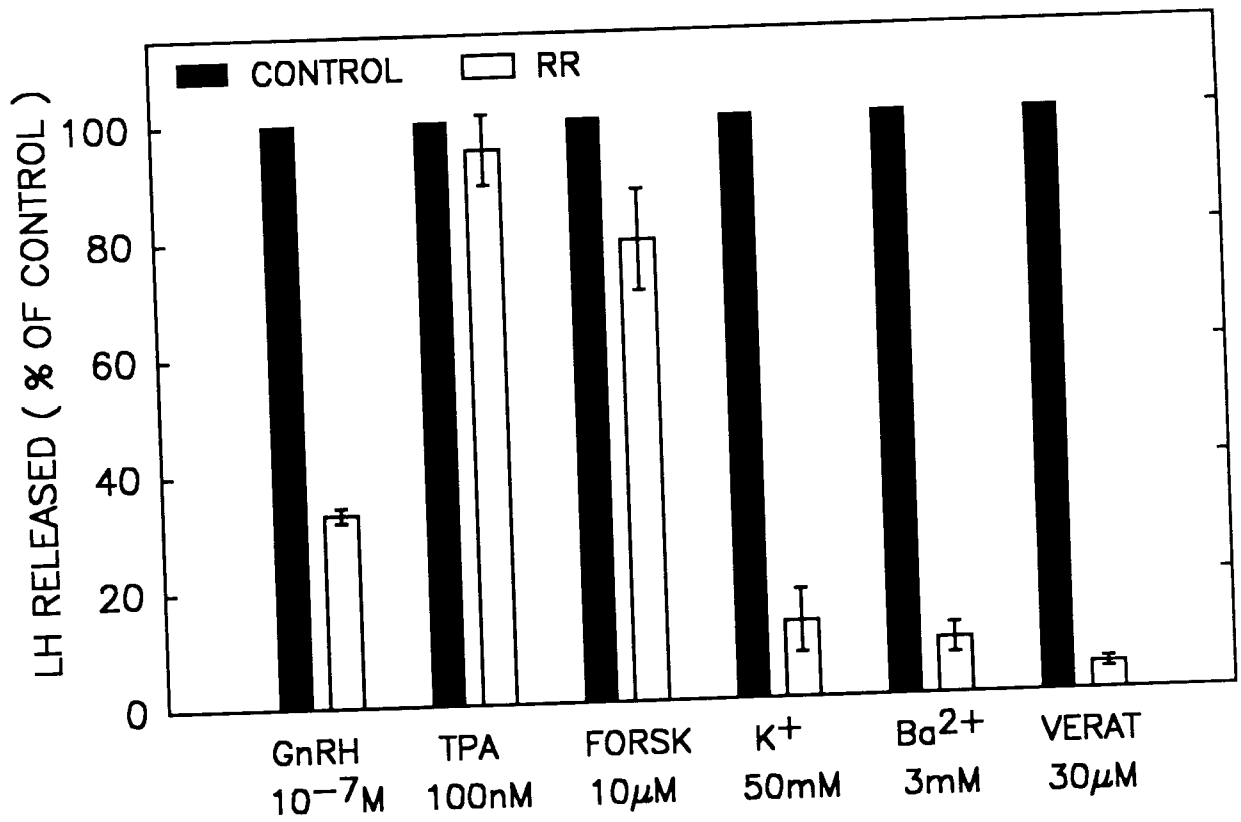

Fig.18 Effect of ruthenium red (RR) on LH release stimulated by various secretagogues.

LH release was measured during a 20-minute stimulation period in the presence or absence of 100 μM RR. This stimulation period was preceded by a 10 minute pre-incubation period with or without 100 μM RR. LH release is expressed as a percentage of LH release stimulated by the secretagogues in the absence of RR. Basal release was subtracted. Representative of 3 similar experiments.

Ω-Conotoxin had no effect on either the spike or plateau phases of GnRH-stimulated LH release (Fig. 19).

3.5 $^{45}Ca^{2+}$ efflux studies

Many hormones are known to mobilize Ca^{2+} from intracellular stores (Berridge , 1984). It was therefore important to characterize the effects of of Ca^{2+} channel blockers on intracellular Ca^{2+} mobilization by GnRH. $^{45}Ca^{2+}$ efflux was used to monitor this phenomenon.

The cells were preloaded with $^{45}Ca^{2+}$ for 2 hours, after which they were vigorously washed for 20 minutes (see Methods). During this washing period $^{45}Ca^{2+}$ is released from the rapidly- exchanging intracellular Ca^{2+} pools. The efflux of $^{45}Ca^{2+}$ from slowly-exchanging labelled pools in the cells was then measured during consecutive 3 minute intervals. Basal $^{45}Ca^{2+}$ efflux, i.e. the release of $^{45}Ca^{2+}$ from unstimulated cells, reflects the release of $^{45}Ca^{2+}$ from a slowly exchanging kinetic compartment with half-life = 35-70 minutes (Fig.20).

In Table 7, the results obtained in a typical $^{45}Ca^{2+}$ efflux experiment are shown. These results are typical of 19 experiments comparing basal efflux to GnRH-stimulated $^{45}Ca^{2+}$ efflux. There are 6 efflux periods. The counts remaining inside the cells at the end of the experiment are marked "cells". The fractional efflux was calculated for each well seperately in a set of triplicate wells. The mean ± SEM fractional efflux of triplicate wells were calculated for each efflux period. These results were plotted as fractional $^{45}Ca^{2+}$ efflux versus time. The data in Table 7 are shown to illustrate typical values obtained and how the fractional efflux values were calculated.

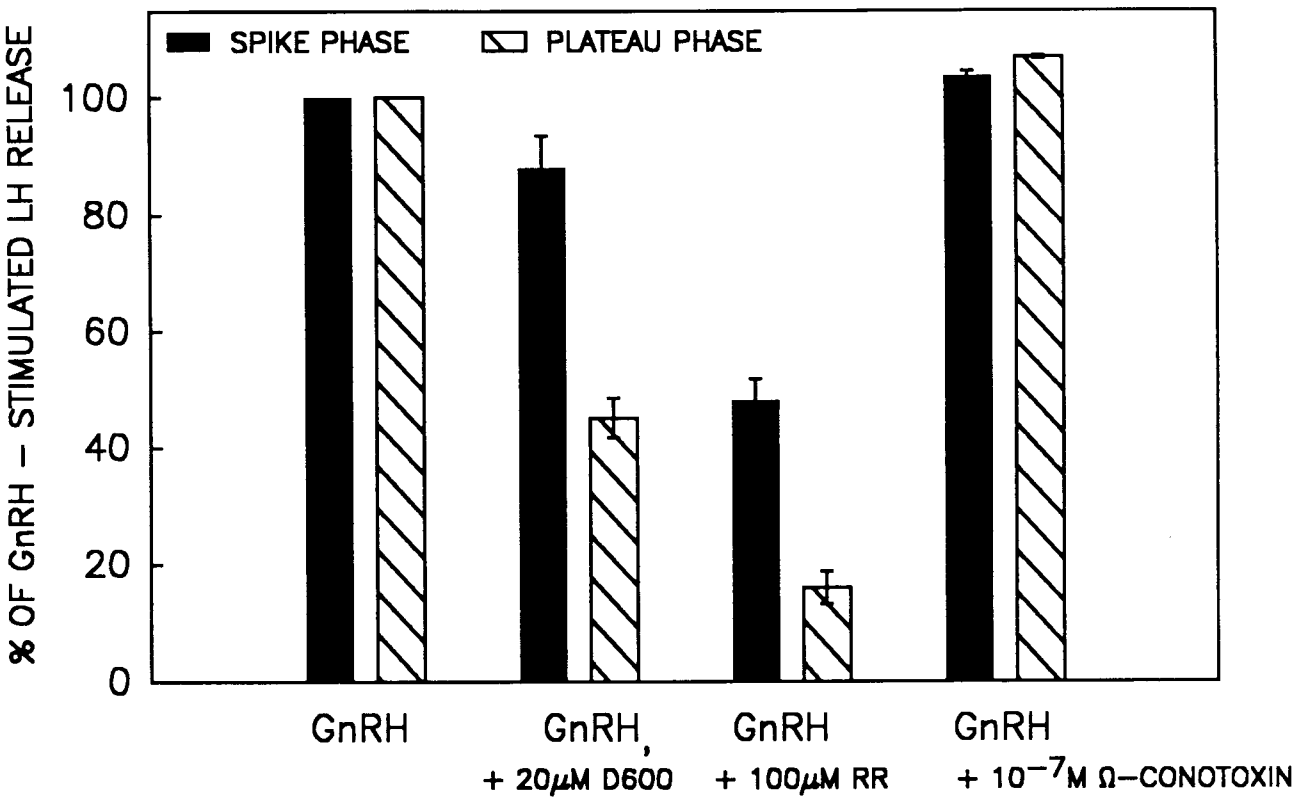

Fig.19 The effects of Ω- conotoxin on the spike and plateau phases of GnRH-stimulated LH release. The effects of D600 and ruthenium red in the same experiment are shown for comparison. D600 (20 μM), RR (100 μM) and Ω-conotoxin (0.1 μM) were present from 10 minutes prior to stimulation with 10^{-7} M GnRH.

This experiment was only done once because of the lack of availability of Ω-conotoxin.

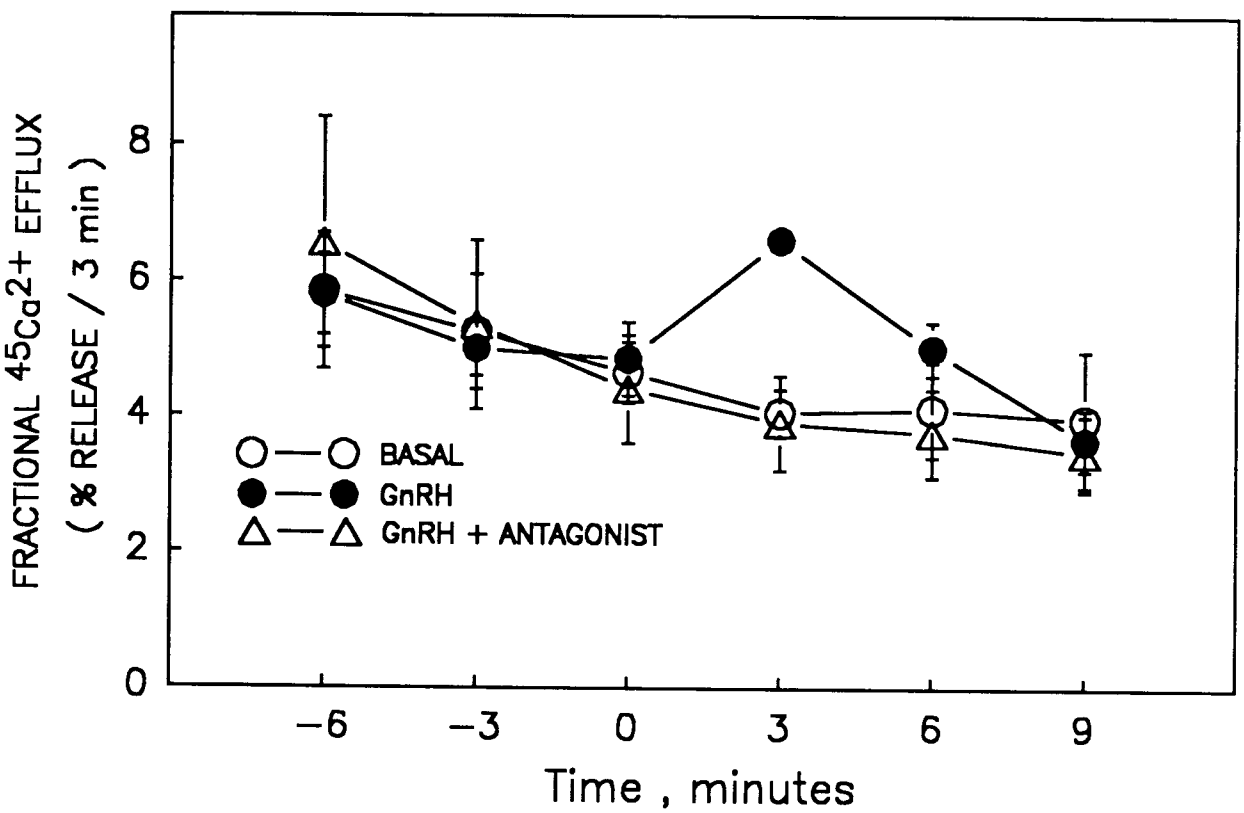

Fig.20 Effects of a GnRH antagonist on GnRH-stimulated $^{45}Ca^{2+}$ efflux. 10^{-5} M GnRH antagonist (Ac-D-Nal-Me-4-CIPHE-D-Trp-Ser-N-Isopropyl Lys-D-Tyr-Leu-Arg-Pro-D-AlaNH$_3$) was present from t = -6 min and 10^{-7}M GnRH was added at t = 0 min. This experiment is representative of 3 similar experiments.

Table 7: Data from a typical $^{45}Ca^{2+}$ efflux experiment (PTO for legend)

Efflux Period (min)	CPM	F.E	F.E. (mean ± S.E.M.)
Basal			
0-3	4374	5.15	5.38 ± 0.12
	4591	5.47	
	4429	5.54	
3-6	3384	4.17	4.40 ± 0.20
	3370	4.21	
	3647	4.80	
6-9	3101	3.98	3.93 ± 0.03
	2999	3.90	
	2836	3.90	
9-12	2449	3.26	3.44 ± 0.22
	2362	3.19	
	2712	3.88	
12-15	2357	3.24	3.31 ± 0.10
	2286	3.18	
	2368	3.51	
15-18	2081	2.95	2.94 ± 0.03
	2011	2.89	
	1940	2.97	
Cells	69398		
	68643		
	64294		

Efflux Period (min)	CPM	F.E	F.E. (mean ± S.E.M)
GnRH			
0-3	4475	5.42	5.66 ± 0.28
	4446	5.33	
	5217	6.22	
3-6	3556	4.53	4.34 ± 0.27
	3026	3.80	
	3715	4.68	
6-9	2995	3.98	3.83 ± 0.12
	2763	3.61	
	2961	3.90	
9-12	5170	7.27	7.02 ± 0.16
	4907	6.73	
	5087	7.07	
12-15	4272	6.43	6.15 ± 0.16
	4187	6.12	
	3968	5.88	
15-18	2917	4.64	4.82 ± 0.09
	3178	4.91	
	3135	4.90	
Cells	61378		
	63107		
	62347		

Table 7: The data displayed in this table are data obtained in a typical $^{45}Ca^{2+}$ efflux experiment. After the cells were loaded with $^{45}Ca^{2+}$, they were rigorously washed so that only $^{45}Ca^{2+}$ effluxing from a slowly turning-over pool remained. This wash period was followed by six efflux periods. At the end of the experiment the cells were lysed to determine the radioactivity remaining in the cells. Each experiment was done in triplicate. The radioactivity effluxed during the six efflux periods and the radio activity present in the cells at the of the efflux periods are shown after subtraction of the machine blank (40 CPM). The fractional efflux (F.E) for each well was calculated as described in section 2.5.

GnRH stimulated a 1.74 ± 0.05 (mean ± S.E.M.) fold increase in $^{45}Ca^{2+}$ efflux during the first 3 minutes after its addition, in 10 independent experiments (Table 8). This increase in $^{45}Ca^{2+}$ efflux is a GnRH-receptor mediated event, since it was inhibited by a GnRH-antagonist (Fig.20). Thyrotropin releasing hormone (TRH) also stimulated $^{45}Ca^{2+}$ efflux (Fig.21). Corticotropin releasing factor (CRF), vasopressin and dopamine did not produce this effect (Fig.21)

GnRH-stimulated $^{45}Ca^{2+}$ efflux was not dependent on extracellular Ca^{2+} since it was not inhibited when the cells were stimulated in Ca^{2+}-free medium (no chelator) (Fig.22). Similarly, Gd^{3+} had no effect on GnRH-stimulated $^{45}Ca^{2+}$ (Fig.23). RR caused a slight inhibition of GnRH stimulated $^{45}Ca^{2+}$ efflux (Fig.24).

Table 8: GnRH-stimulated $^{45}Ca^{2+}$ efflux: results from 10 independent experiments.

EX NO	BASAL	GnRH	FOLD STIMULATION	
E 1	4.05	6.60	1.63	
E 3	4.05	7.70	1.90	
E 4	4.00	6.93	1.73	
E 5	4.00	6.73	1.66	
E 8	6.00	11.60	1.93	
E 7	4.70	7.73	1.64	
E 9	4.57	9.13	2.00	
E 12	4.80	8.45	1.76	
E 20	3.73	5.74	1.54	
E 21	3.44	5.11	1.49	
MEAN ± SEM	4.33 ± 0.23	7.57 ± 0.59	1.74 ± 0.05	n=10

Basal and GnRH-stimulated fractional $^{45}Ca^{2+}$ efflux (% per 3 min) for the first 3 minutes after the addition of GnRH (10^{-7} M) are presented.

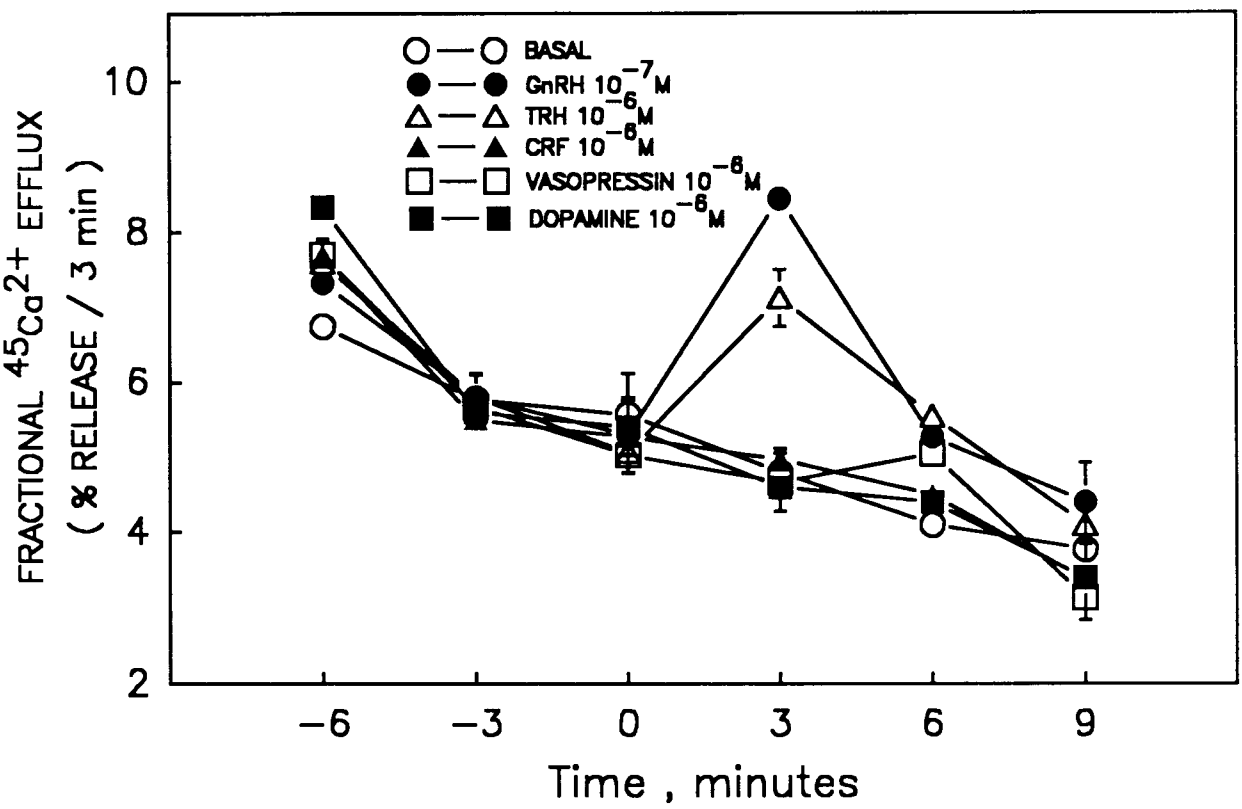

Fig.21 $^{45}Ca^{2+}$ efflux from cultured chicken pituitary cells. Effects of GnRH, TRH, CRF, vaspressin, and dopamine

The test substances were added at t = 0 min. This experiment is representative of 3 similar experiments.

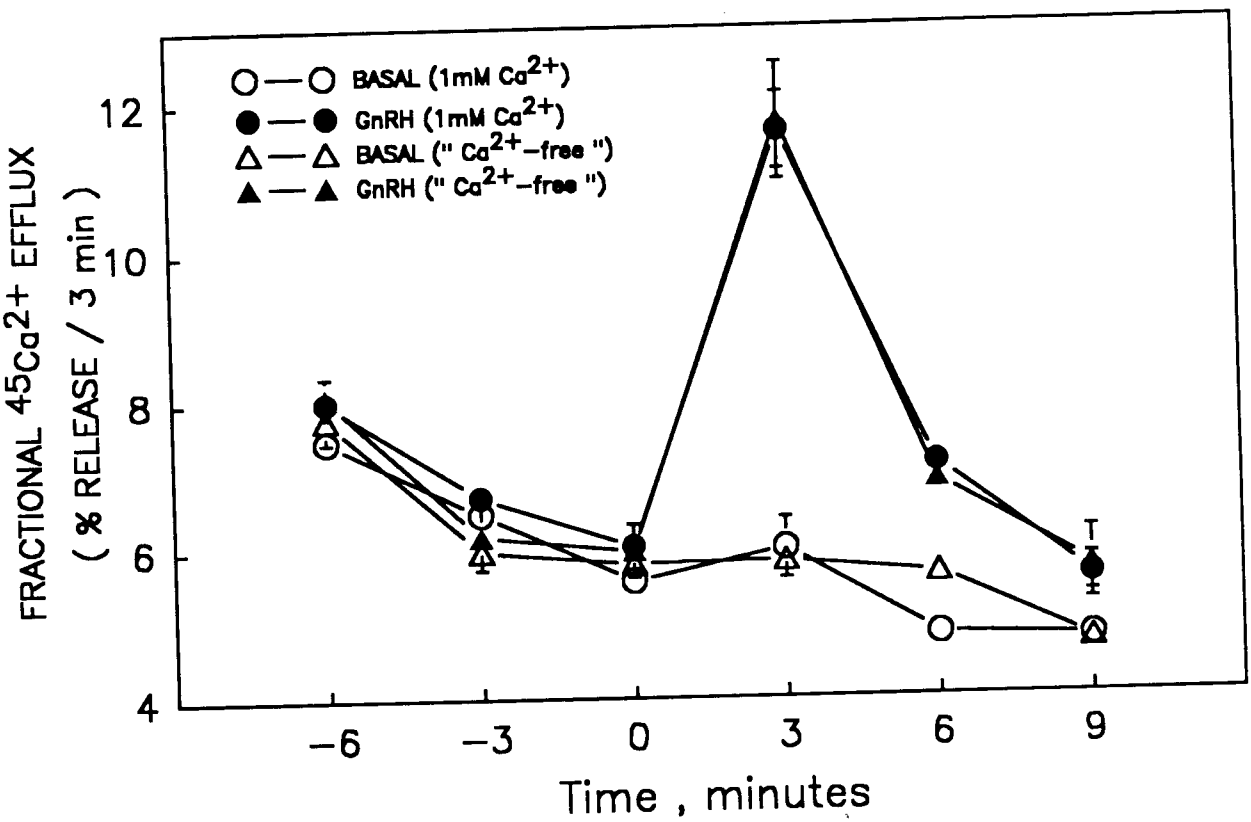

Fig.22 The effect of Ca^{2+} removal on GnRH-stimulated $^{45}Ca^{2+}$ efflux. The medium was replaced with Ca^{2+}-free medium (without EGTA), at t = -6 min and 10^{-7} GnRH was added at t = 0 min. This experiment is representative of 3 similar experiments.

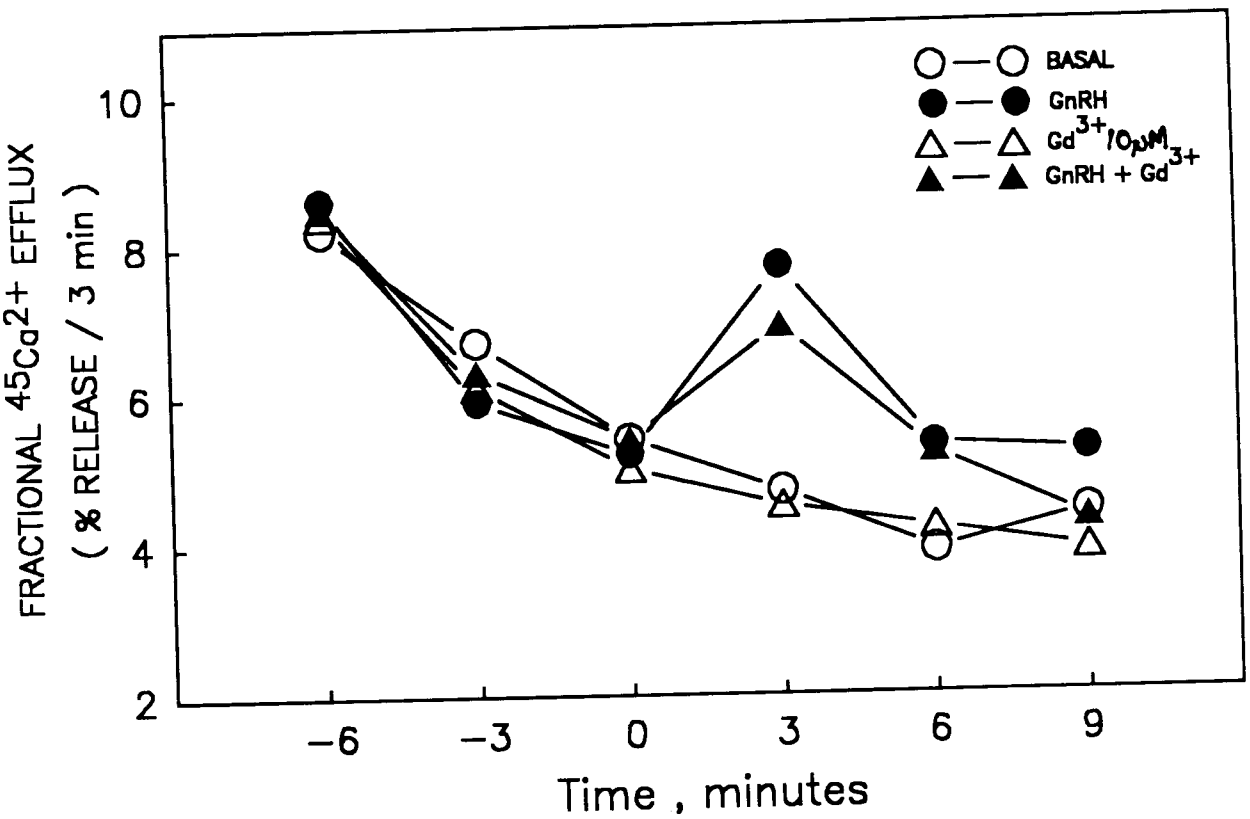

Fig.23 The effect of Gd^{3+} on GnRH stimulated $^{45}Ca^{2+}$ efflux. Gd^{3+} was present from t = -6 min and 10^{-7} M GnRH was added at t = 0 min. Representative of 3 similar experiments.

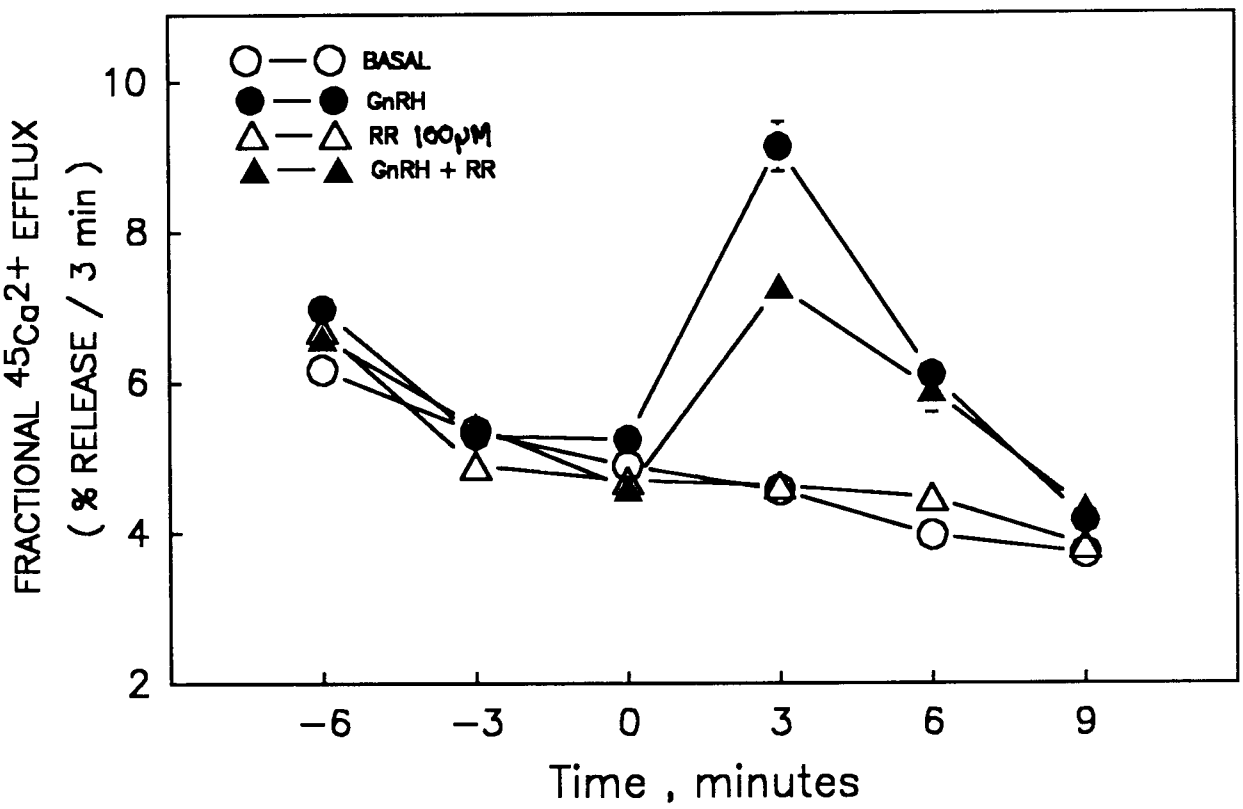

Fig.24 The effect of ruthenium red on GnRH stimulated $^{45}Ca^{2+}$ efflux. Ruthenium Red was present from t = -6 min and 10^{-7} M GnRH was added at t = 0 min. Representative of 3 similar experiments.

Part 2: INVESTIGATION OF THE ROLE OF CYCLIC AMP IN GnRH-STIMULATED LH RELEASE

4.1 The effects of forskolin, IBMX and dibutyryl cAMP on LH release

Forskolin, an activator of adenylate cyclase (Seamon et al., 1981), stimulated LH release from chicken pituitary cells (Fig.25). In 3 independent experiments, the concentration of forskolin giving half - maximal stimulation varied between 2.5 and 10 μM. 1-Methyl-3-isobutyl-xanthine (IBMX), a cAMP phosphodiesterase inhibitor, stimulated a small increase in LH release on its own and enhanced forskolin-stimulated LH release (Fig.25). At higher concentrations than required to stimulate LH release, forskolin elevated the cellular cAMP concentration (Fig.25). Similar to its effect on forskolin-stimulated LH release, IBMX enhanced the ability of forskolin to elevate the concentration of cellular cAMP (Fig.25). The stimulatory effect of the cAMP analogue dibutyryl-cAMP (dBu-cAMP) on LH release was poor in comparison to forskolin- and IBMX-stimulated LH release (Fig.26).

4.2 The kinetics forskolin-stimulated LH release

The onset of forskolin-stimulated LH release was rapid, with maximal secretion occuring within the first 20 minutes of exposure to the drug (Fig.27). The stimulatory effect of forskolin was prolonged, lasting up to 100 minutes which was the longest period tested (Fig.27). An analysis of the very early time course of forskolin stimulation was undertaken in 3 experiments, which are summarized in Table 9. The stimulatory effect of forskolin was detectable within the first 3 minutes of exposure. However forskolin did not show the initial transient spike phase of release which is characteristic of

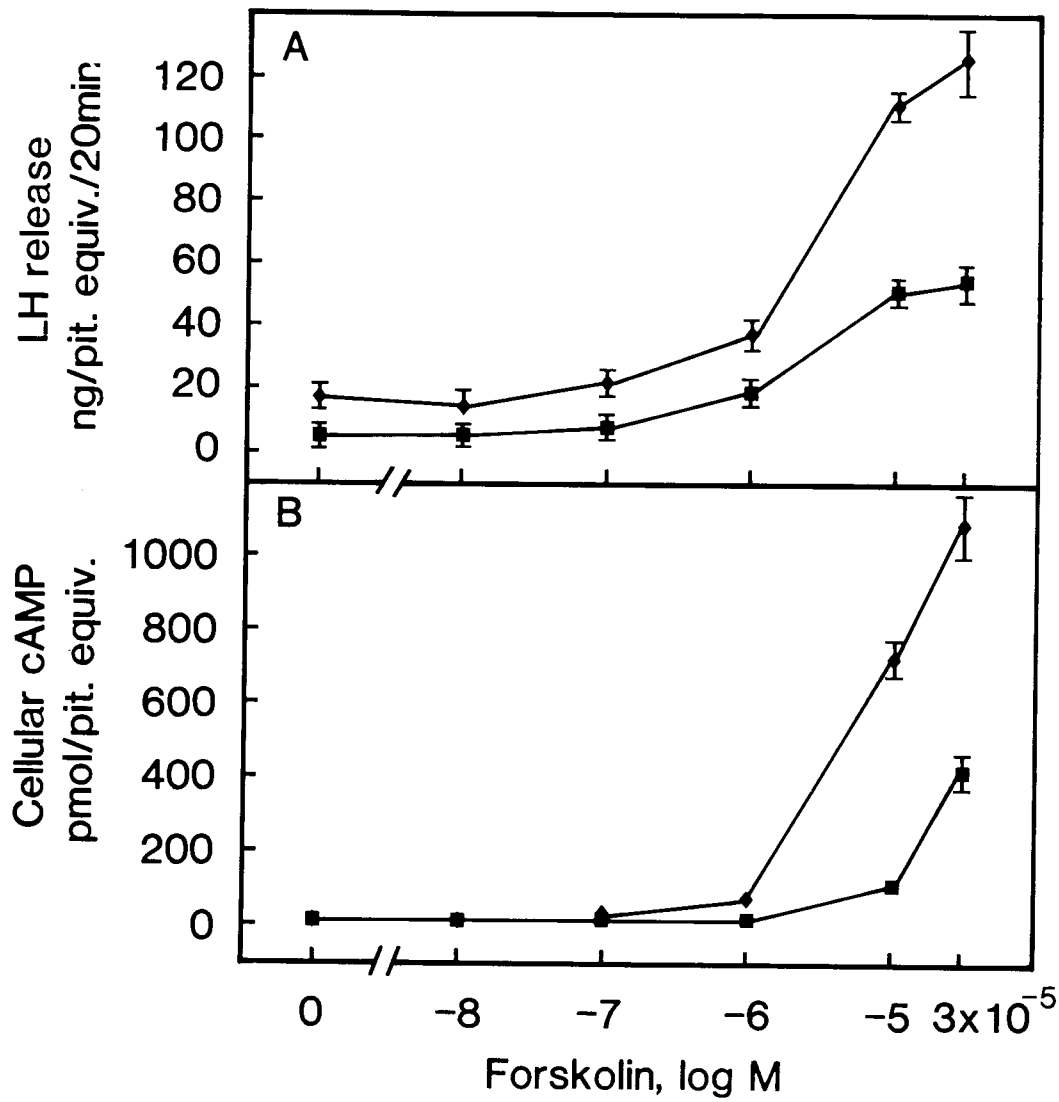

Fig.25 Forskolin dose-response curve: effects on LH release and cellular cAMP, in presence and absence of IBMX.

The cells were stimulated for 20 min. The LH released during the 20-min period is shown in A. The cAMP present in the cells at the end of the stimulation period is shown in B.

(■) No IBMX, (◆) IBMX (0.5 mM) present during stimulation period. This is representative of 2 similar experiments.

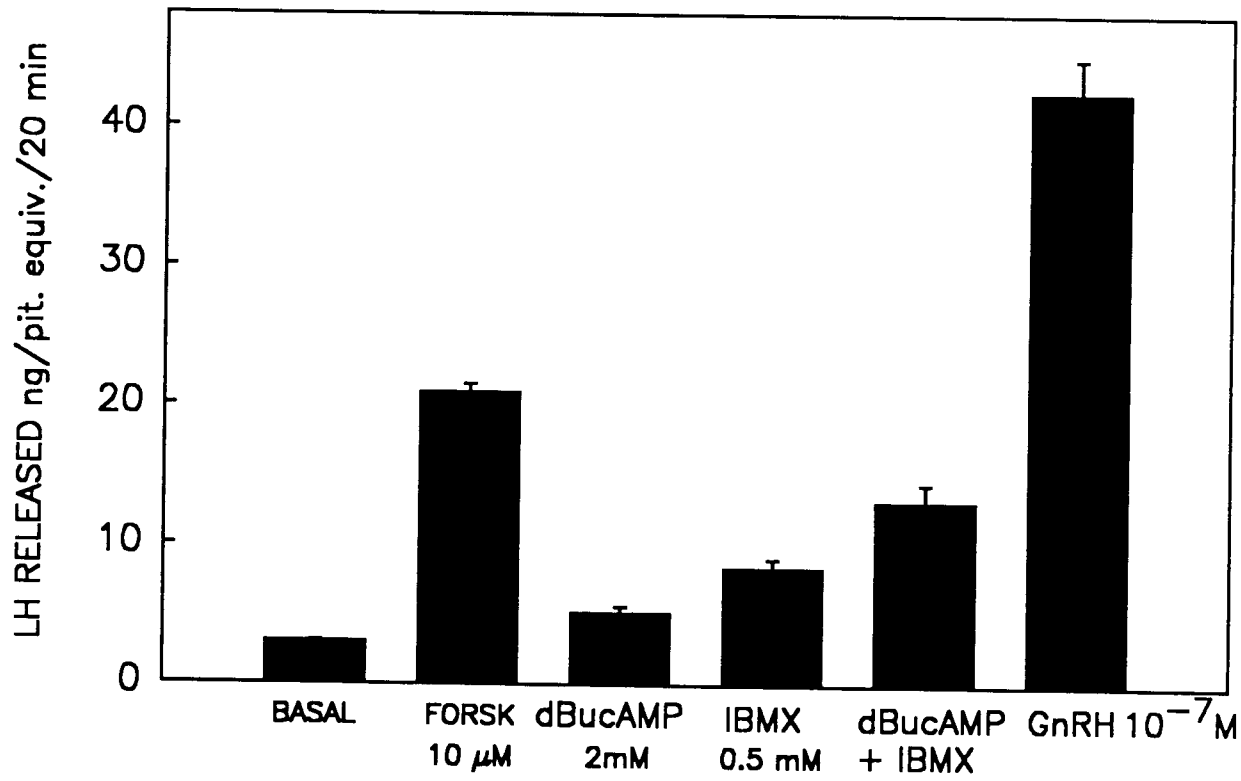

Fig.26 Comparison of the effects of forskolin, dibutyryl cAMP, IBMX, and GnRH on LH release.

The cells were stimulated with the secretagogues for 20 minutes. This data is representative of 2 similar experiments.

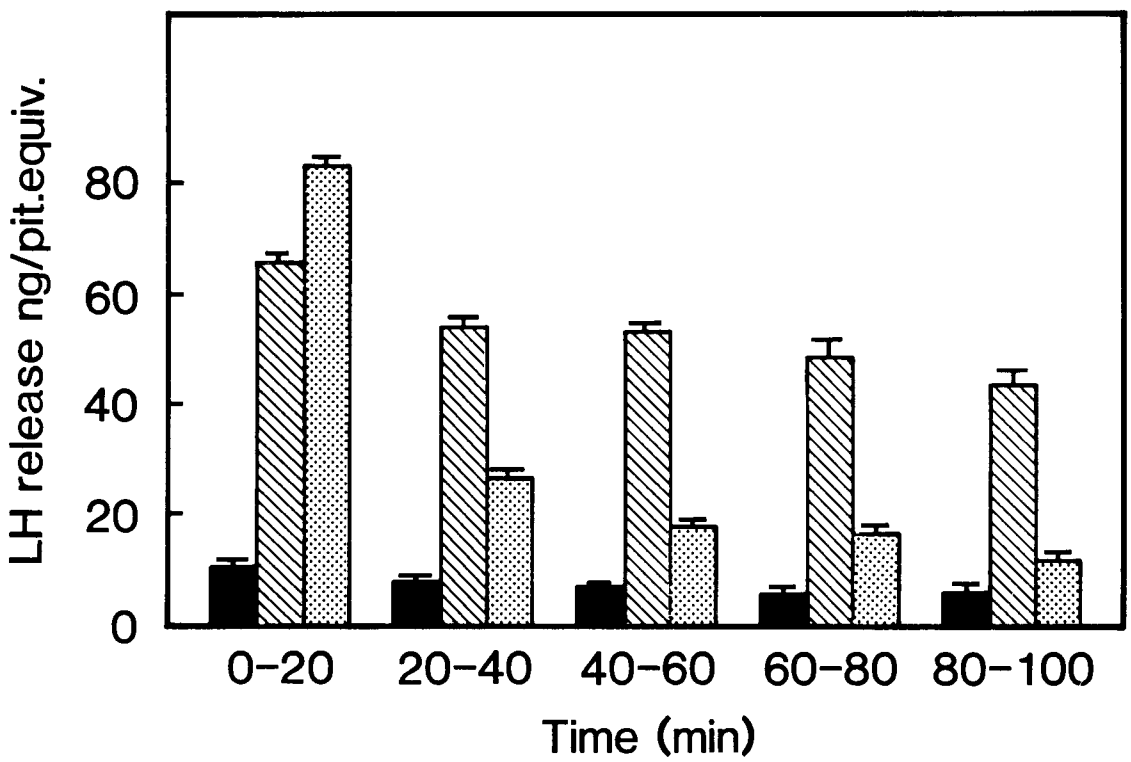

Fig.27 Kinetics of forskolin and K^+-stimulated LH release. Solid bars, basal; hatched bars, forskolin (10 μM); dotted bars, K^+ (60 mM). Forskolin and K^+ were present continuously from t = 0 to t = 100 minutes. The medium was replaced with fresh medium at 20-minute intervals.

Table 9: Time-course of Forskolin-stimulated LH release: comparison with GnRH and phorbol ester

EX NO	Time Period	Basal	GnRH	Forskolin	TPA
98	0-3 min	2.49	34.4	5.54	
116		3.93	47.4	6.91	2.95
121		1.93	49.1	2.63	7.20
MRR		2.78	43.6	5.02	5.08
± SEM		± 0.84	± 6.6	± 1.78	±2.1
98	3-9 min	3.40	25.9	14.56	
116		1.17	11.3	8.72	9.02
121		2.16	19.1	10.9	33.2
MRR		1.12	9.4	5.7	10.6
± SEM		± 0.32	± 2.14	± 1.01	± 7.46
98	9-30 min	9.08	51.8	60.0	
116		7.60	32.2	31.7	38.5
121		5.94	34.8	63.4	141.0
MRR		1.08	5.66	7.39	12.8
± SEM		± 0.13	± 1.08	± 0.58	± 7.3

MRR = Mean release rate (ng/pituitary equivalent/3 min).

The cells were stimulated for the time-periods indicated. The medium was replaced with fresh medium at the start of each time-period

GnRH stimulation (Table 9). The time course of forskolin stimulation was similar to that produced by phorbol ester (Table 9). In comparison to the kinetics of forskolin-stimulated LH release, K^+-stimulated LH release showed a more transient spike phase, followed by a slow decline (Fig.27).

4.3 Ca^{2+} dependence of forskolin-stimulated LH release

Some of the effects of cAMP are known to be mediated by the cAMP-dependent phosphorylation of calcium channels with a subsequent increase in cytosolic Ca^{2+} (Tsien, 1983; Catterall, 1988). Effects mediated via this mechanism should be entirely dependent on the presence of extracellular Ca^{2+}. The Ca^{2+} dependence of forskolin-stimulated LH release was therefore investigated. LH release due to forskolin was largely independent of extracellular Ca^{2+} since it was only slightly inhibited in Ca^{2+}-free medium with or without the Ca^{2+} chelator EGTA (Fig.28). This was in contrast to K^+-stimulated LH release which was abolished, as expected, in the presence of Ca^{2+}-free medium (Fig.28).

4.4 Effect of GnRH on cellular cAMP concentration

Cellular levels of cAMP were found to be highly variable between experiments (Table 10). However in each of the 4 experiments , GnRH consistently stimulated an increase in cellular cAMP (Table 10). In cells stimulated for 1 hour, the mean increase in cAMP levels was 1.33 ± 0.20 fold.

4.5 Synergistic reaction of GnRH with forskolin

In addition to activating adenylate cyclase directly, forskolin has also been reported to interact synergistically with hormones which

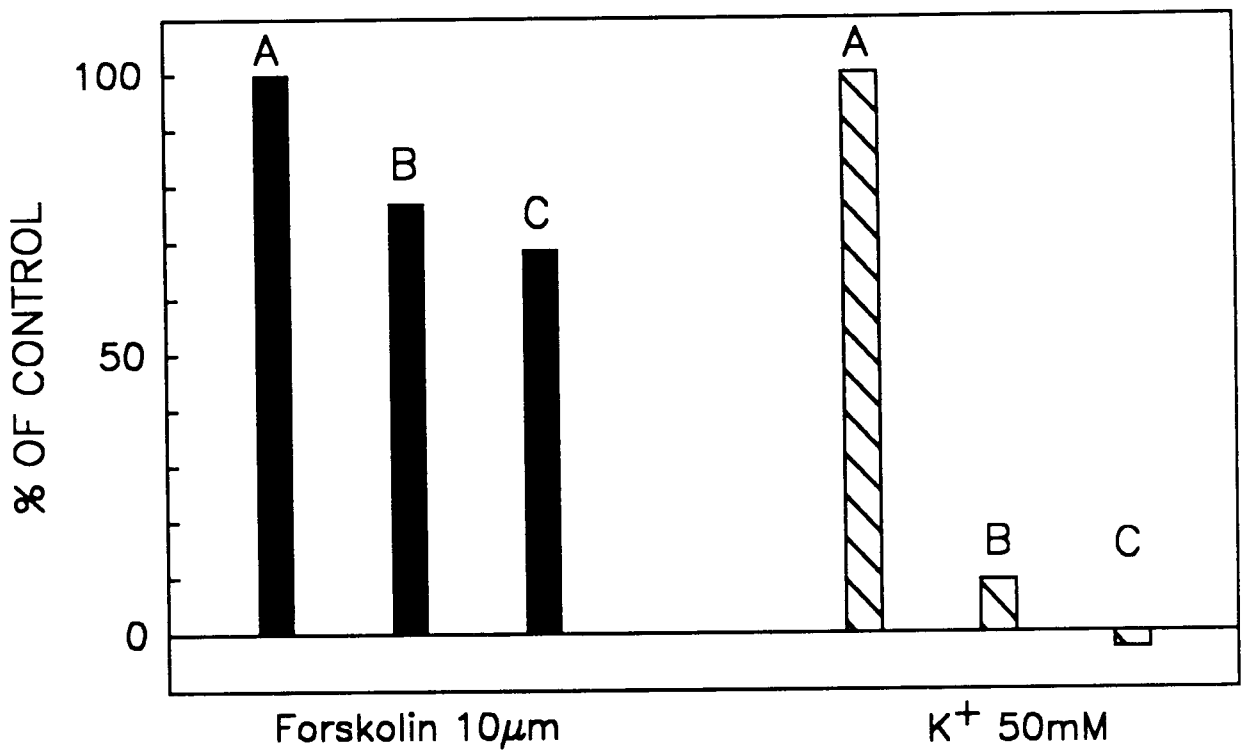

Fig.28 Ca^{2+} dependence of forskolin- and K^+-stimulated LH release. Cells were stimulated with forskolin (10 μM) or K^+ (50 mM) in the presence of (A) 1 mM Ca^{2+} (B) Ca^{2+} free medium (no added Ca^{2+}) and (C) 0.5 mM EGTA in Ca^{2+}-free medium. The cells were pre-incubated in these media for 10 minutes, then stimulated for 20 min. Release in Ca^{2+}-free media is expressed as a percentage of that in medium containing 1 mM Ca^{2+}. Representative of 3 similar experiments.

Table 10: The effect of GnRH on the cellular cAMP concentration.

Results of 4 experiments.

EX NO	STIMULATION PERIOD	BASAL (cAMP pmol/pit. equiv.)	GnRH 10^{-7}M	FOLD STIM
156	1 hr	11.80	19.4	1.64
	3 hr	4.25	11.5	2.71
	6 hr	4.42	8.4	1.56
172	10 min	1.27	2.06	1.62
	1 hr	1.52	1.96	1.29
179	1 hr	1.98	2.16	1.09
180	1 hr	1.50	1.96	1.31

The cells were stimulated for the indicated time periods and, at the end of the stimulation period the cAMP in the cells was extracted, and the cAMP concentration of the samples was determined by radioimmunoassay (see section 2.2).

activate adenylate cyclase (Nelson and Seamon, 1986). This latter effect occurs at very much lower concentrations of forskolin (10 to 100 nM) than required for direct activation (10 μM) of adenylate cyclase (Nelson and Seamon, 1986). Therefore the effect of forskolin at concentrations between 10^{-10} M and 10^{-7} M on GnRH -stimulated LH release was investigated.

In a previous section, it was shown that GnRH stimulates LH release in a biphasic manner with a distinct spike phase (0-30 min) followed by a sustained plateau phase (9-30 min) (Fig.1). The spike phase of GnRH-stimulated LH release was not affected by co-stimulation with low doses of forskolin (Fig.29). However, the plateau phase was enhanced in the presence of these low doses of forskolin (Fig.29). The absence of any notable effect of forskolin on the spike phase could be because the cells were exposed to forskolin for too short a period of time (3 minutes). Forskolin at higher concentrations (10 μM) also interacted synergistically with GnRH to stimulate LH release (Fig.30). However, although forskolin augmented GnRH-stimulated LH release, it did not alter the EC_{50} of GnRH (Fig.30).

4.6 The effect of forskolin on GnRH-stimulated $^{45}Ca^{2+}$ efflux

As demonstrated earlier in Part 1, GnRH stimulates $^{45}Ca^{2+}$ efflux from a slowly turning over intracellular Ca^{2+} pool. In order to gain insight into the mechanism of the synergism between GnRH and forskolin, the effect of forskolin on GnRH-stimulated $^{45}Ca^{2+}$ was examined. In the presence of forskolin, the basal rate of $^{45}Ca^{2+}$ efflux was raised (Fig.31). Forskolin had no effect on GnRH-stimulated $^{45}Ca^{2+}$ efflux (Fig.31). This indicates that forskolin does not augment GnRH-stimulated LH release by enhancing its ability to

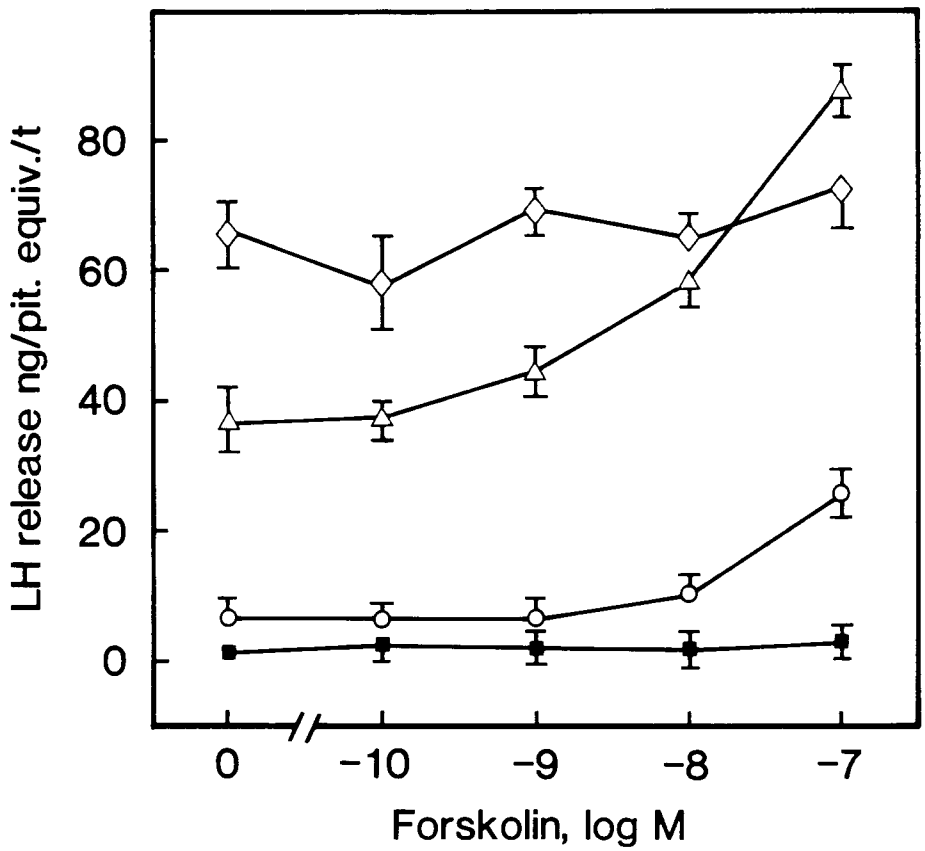

Fig.29 Effect of low concentrations of forskolin on the spike phase (0-3 min) and plateau phase (9-30 min) of GnRH stimulated LH release, and on basal release.

(■) Basal (0-3 min), (◇) 10^{-7}M GnRH (0-3 min)

(○) Basal (9-30 min), (△) 10^{-7}M GnRH (9-30 min).

The cells were pre-incubated with forskolin for 3 mins prior to the stimulation period.

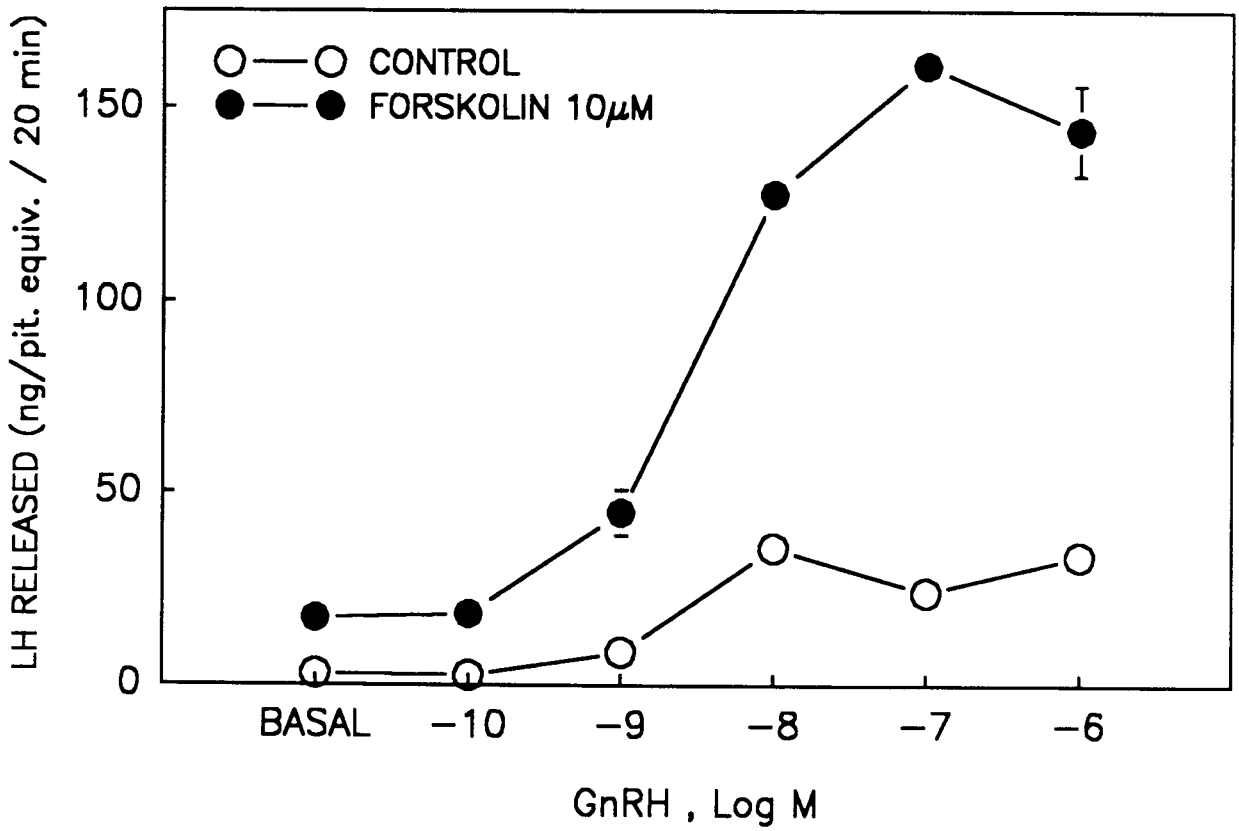

Fig.30 Effect of forskolin on GnRH dose-response curve. The cells were stimulated with GnRH alone (○) and in combination with forskolin 10 μM (●) at the indicated doses. The experiment is representative of 2 identical experiments.

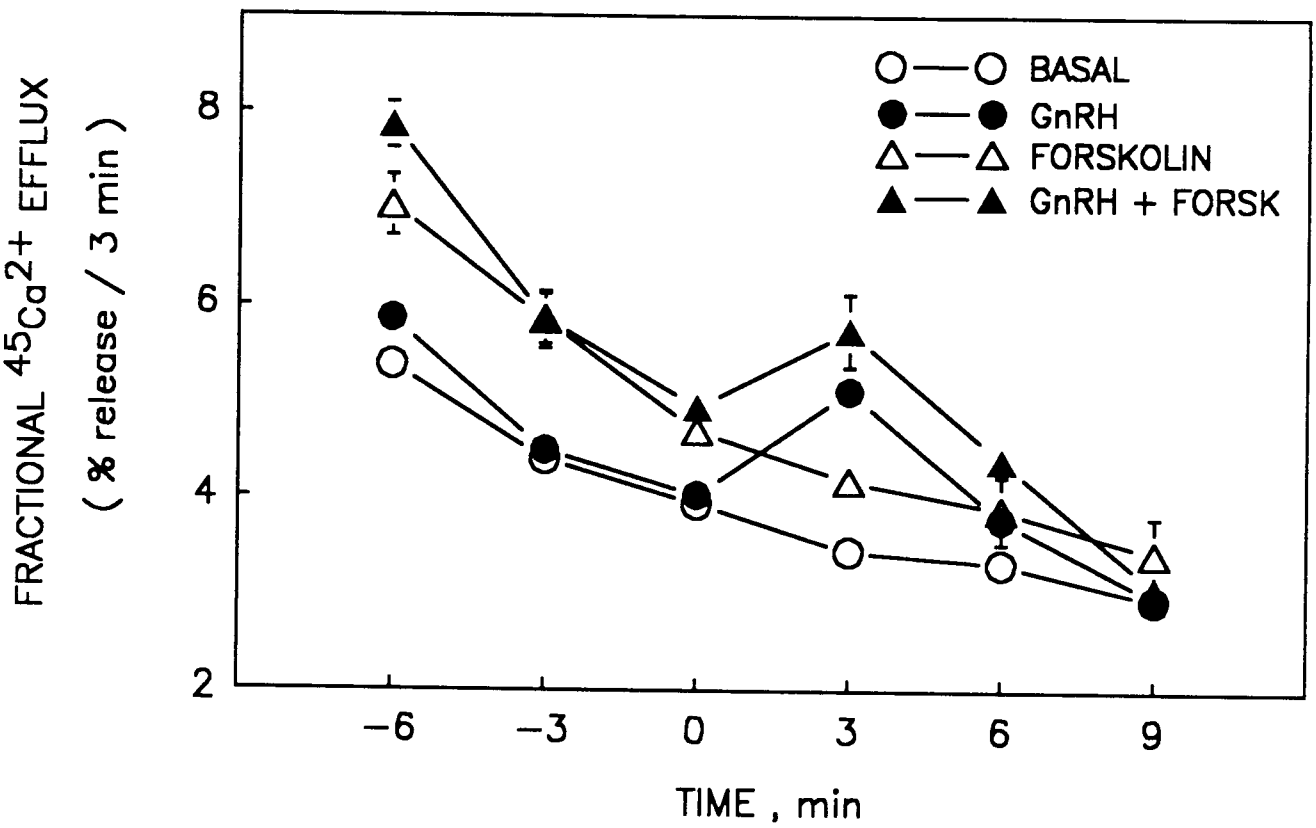

Fig.31 The effect of forskolin on GnRH stimulated $^{45}Ca^{2+}$ efflux. Forskolin (10 μM) was present from t = -6 min and GnRH (10^{-7}M) was added at t = 0 min. This experiment is representative of 2 similar experiments.

mobilise stored intracellular Ca^{2+}.

4.7 The effect of forskolin on GnRH-stimulated inositol phosphate production

One of the signalling systems activated by GnRH is inositol phospholipid breakdown. A possible mechanism by which forskolin could enhance GnRH-stimulated LH release (Section 4.5) might be via enhancement of this signal. The effect of forskolin on GnRH-stimulated inositol phosphate (IP) production was therefore investigated. Total IP accumulation in Li^{+}-treated cells after 30 min of GnRH stimulation was determined, as this provides an easily-measured index of inositol phospholipid breakdown.

GnRH stimulated a 1.49 ± 0.16 fold increase in total inositol phosphate production in 3 independent experiments (Fig.32). In the presence of forskolin, the increase in inositol phosphate production due to GnRH was decreased slightly to 82.6 ± 24.5 % (n=3) of the increase in the absence of forskolin (Fig.32). Forskolin did not significantly alter unstimulated inositol phosphate levels (Fig.32). Therefore it appears that the synergism between GnRH and forskolin seen at the level of LH release is not due to an enhancement of the effect of GnRH on inositol phosphate production.

4.7 The interactions between cAMP, Ca^{2+} and protein kinase C

The results from the previous sections indicated that the observed synergism between forskolin and GnRH could not be accounted for by enhanced inositol phospholipid breakdown or Ca^{2+} mobilisation. It seemed possible that forskolin might exert its synergistic action at a more distal step in the signalling cascade leading to LH release.

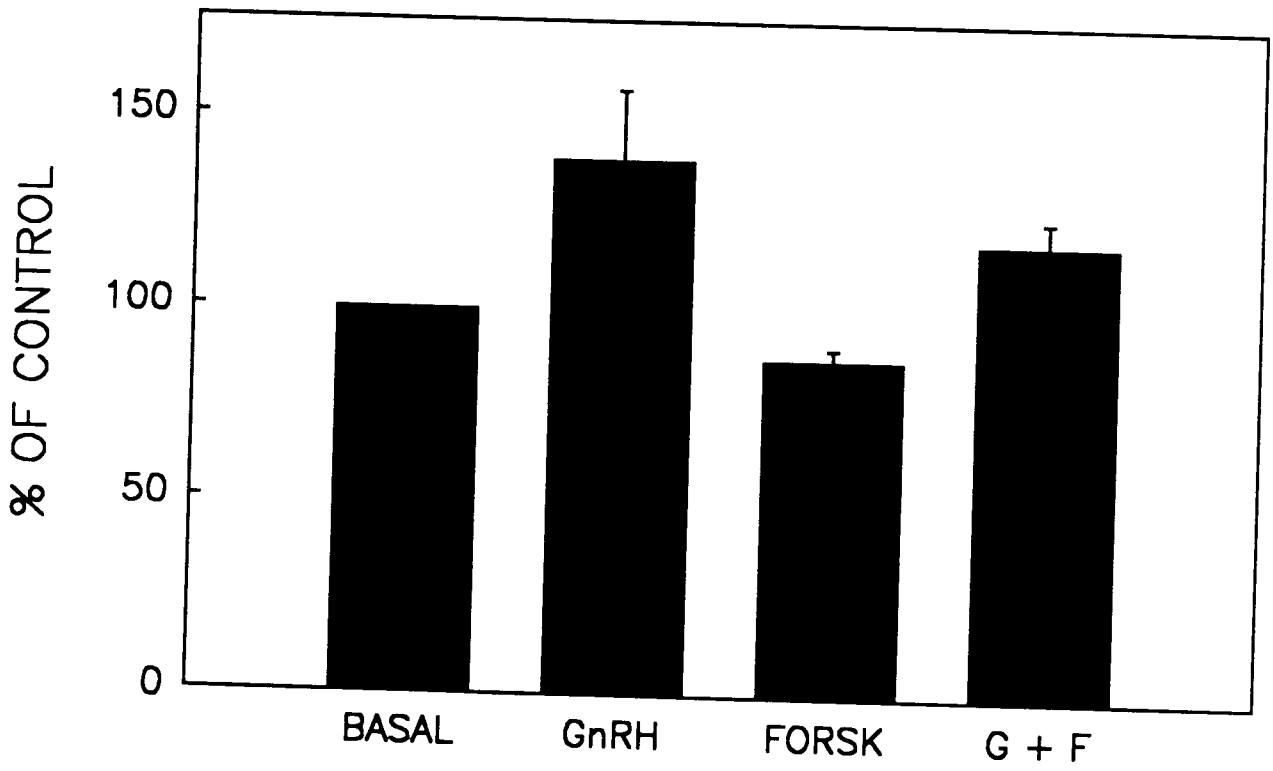

Fig.32 The effect of forskolin on unstimulated and on GnRH-stimulated inositol phosphate production. The data are the mean of 3 experiments and are expressed as a percentage of basal inositol phosphate production.

The interactions between forskolin and two pathways that are known to be activated by GnRH, namely Ca^{2+} and protein kinase C, were therefore investigated. Forskolin was tested in combination with three secretagogues that increase the cytsolic Ca^{2+} concentration : Ca^{2+} ionophore A23187, veratridine, and 50 mM K^{+}. Forskolin interacted synergistically with all 3 these stimuli, as shown in Fig 33 which depicts 2 representative experiments. Synergism between forskolin and A23187 was consistently observed in 4 experiments, and between forskolin and veratridine in 3 experiments. In addition, forskolin interacted synergistically with Ba^{2+} (Fig.30), which enters the pituitary cells via Ca^{2+} channels and "substitutes" for Ca^{2+} in stimulating LH release (Davidson et al., 1987).

The phorbol ester TPA, an activator of protein kinase C, also interacted synergistically with forskolin to stimulate LH release (Fig.33). Synergism between forskolin and TPA was observed in 7 experiments. A "three-way" synergism resulted when forskolin, veratridine and TPA were used simultaneously to stimulate LH release (Fig.34). These results suggest that cAMP interacts with GnRH at a site distal to the activation of the Ca^{2+} and protein kinase C pathways.

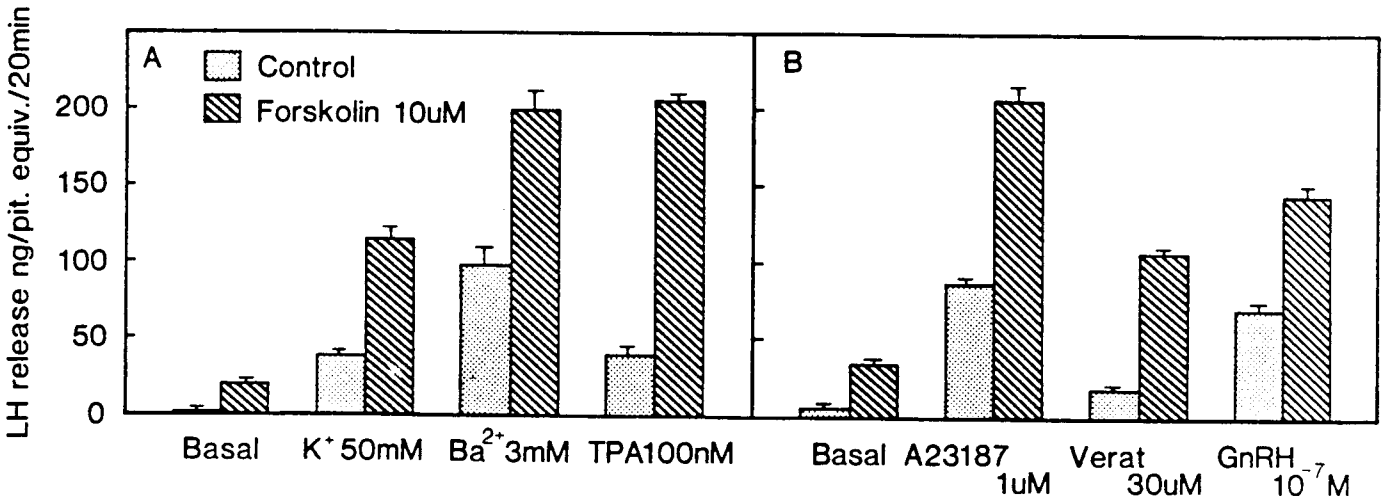

Fig.33 The effect of forskolin in combination with various secretagogues.

Dotted bars secretagogue; hatched bars forskolin (10 μM) plus secretagogue. The cells were stimulated for 20 min. The data are representative of 2 to 7 experiments, as discussed in text.

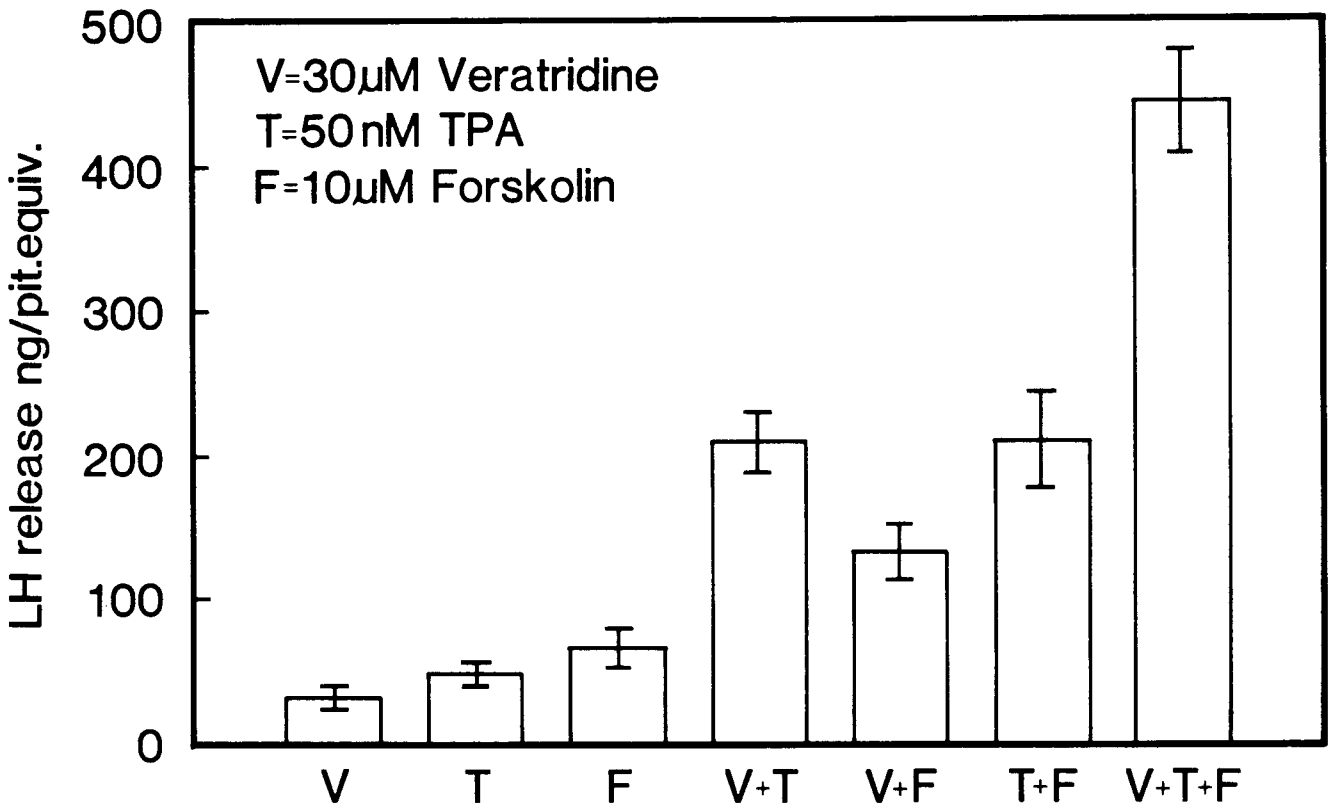

Fig.34 "Three-way" synergism between cAMP, Ca^{2+} and protein kinase C. The cells were stimulated with each secretagogue or combinations of the different secretagogues for 20 minutes. Similar data were obtained in an identical experiment.

5. DISCUSSION

Ca^{2+} is known to play an important role in the mechanism by which GnRH stimulates LH release. An increase in cytosolic Ca^{2+} levels activates a secretory response in a variety of cell types. The activation of a secretory response by Ca^{2+}-dependent hormones has been shown to involve the mobilization of intracellular Ca^{2+} and/or the entry of extracellular Ca^{2+}. The relative importance of these two sources of Ca^{2+} is thought to vary in different cell types and for different exocytotic processes. Also, the routes by which extracellular Ca^{2+} enters cells, may vary depending on the cell type.

Kinetic studies in this thesis demonstrated that GnRH stimulates LH release from chicken gonadotropes in a biphasic manner. The spike phase of LH release was shown to be largely dependent on the entry of extracellular Ca^{2+} (± 90%). The plateau phase of LH release was totally dependent on extracellular Ca^{2+}. Early experiments investigating the mechanism by which GnRH stimulated the entry of extracellular Ca^{2+}, reported that GnRH stimulated Ca^{2+} entry via D600-sensitive Ca^{2+} channels (Hopkins and Walker, 1978; Borges et al., 1980; Conn and Rogers, 1980). In contrast to this, the VSCC blockers that belonged to the class, dihydropyridines (DHP), were reported to be ineffective in inhibiting GnRH-stimulated LH release (Conn et al., 1983). More recently, Chang et al. (1986), reported that nitrendipine, a DHP, inhibited GnRH-stimulated LH release. This thesis demonstrates that the plateau phase of GnRH-stimulated LH release in the chicken gonadotrope can be partially inhibited by the DHP nifedipine. This suggested that the VSCC in rat and chicken gonadotropes played a partial role in LH release during the plateau

phase.

In this study the role of VSCCs in the chicken gonodotrope was determined by examining the effects of the VSCC blockers D600 and nifedipine on the spike and plateau phases of GnRH-stimulated LH release. The spike phase of LH release was found to be independent of the activation of VSCCs that could be inhibited by D600 and nifedipine whereas approximately 50% of the plateau phase was dependent on Ca^{2+} entry via these VSCCs.

Since the spike phase is not dependent on Ca^{2+} entry via VSCCs ,the question is posed as to the nature of extracellular Ca^{2+} entry and the role of Ca^{2+} and the other known second messengers in this phase of GnRH-stimulated LH release. GnRH activates the hydrolysis of PIP_2 in the chicken (Davidson et al., 1987; and Fig. 32) and in the rat (Kiesel and Catt, 1984; Andrews et al., 1986; Noar et al., 1986) gonadotrope. IP_3 and DAG, the resulting products, are responsible for the mobilization of stored intracellular Ca^{2+} and the activation of protein kinase C respectively (Berridge et al., 1984). This appears to be one of the initial events that occurs once GnRH has bound to its receptor since an increase in IP_3 is seen within seconds (Kiesel and Catt, 1984).

It is, therefore, feasible that the production of IP_3 which leads to the mobilization of stored intracellular Ca^{2+} may give rise to the residual LH release during the spike phase apparent in the absence of extracellular Ca^{2+}. The experiments conducted suggest that this residual release is due to the mobilization of intracellular Ca^{2+} stores since : (1)depletion of the intracellular Ca^{2+} by pretreatment with EGTA and A23187 abolishes all of the spike phase of release;

(2)GnRH- stimulated $^{45}Ca^{2+}$ efflux from slowly turning over Ca^{2+} pools is not inhibited by the removal of extracellular Ca^{2+}; and (2) GnRH is able to stimulate an increase in inositol phosphate production.

Since the removal of extracellular partially Ca^{2+} inhibited the spike phase, the mechanism by which extracellular Ca^{2+} enters the gonadotrope was futher investigated by examining the effects of inhibitors of extracellular Ca^{2+} entry on GnRH-stimulated LH release. A range of inorganic ions was tested in an attempt to find an inhibitor of the route via which Ca^{2+} entered the gonadotrope which was insensitive to blockage by D600 and nifedipine. Several of these cations tested e.g. Cd^{2+}, Ni^{2+}, Zn^{2+} and La^{3+} inhibited both the spike and plateau phases. However, they also increased basal LH release. The mechanism by which these ions induced LH release on their own is not known ,but it might be similar to that of Cu^{2+} ions which are thought to stimulate LH release by interacting with the GnRH receptor (Schwartz and Hazum, 1986). Two other ions tested, Co^{2+} and Gd^{3+}, did not increase LH release on their own but appeared to have non-specific effects besides the inhibition of Ca^{2+} entry. They interacted with secretagogues that stimulated LH release by a Ca^{2+}-independent manner and were inconsistent in their ability to inhibit Ca^{2+} entry via VSCC that were activated by K^{+}. Therefore, an inhibitor that only blocked the entry of extracellular Ca^{2+} and did not interact with secretagogues that activated other intracellular events was sought.

The polycationic dye ruthenium red (RR) has been reported to inhibit several Ca^{2+} transport processes including mitochondrial Ca^{2+} uptake

(Carafoli et al., 1978) and the Ca^{2+} release channel of the sarcoplasmic reticulum (Smith et al., 1985). In the present study, RR inhibited both the spike and plateau phases of GnRH-stimulated LH release and did not increase basal LH release. The pattern of RR inhibition on the spike and plateau phases was similar to the effect of the removal of extracellular Ca^{2+} on GnRH-stimulated LH release. Also, unlike the cations tested, RR did not significantly affect stimulation of LH release by the protein kinase C activator, TPA, and the adenylate cyclase activator, forskolin. This was expected since both these secretagogues are capable of stimulating LH release independently of extracellular Ca^{2+}. In additon, RR did not inhibit GnRH-stimulated $^{45}Ca^{2+}$ efflux indicating that it did not affect immediate post-receptor events. This, together with the fact that RR did not appear to affect the whole exocytotic process, since it did not affect TPA- and forskolin-stimulated LH release, suggested that RR was acting at an extracellular site.

RR blocked the entry of Ca^{2+} via VSCC, since it inhibited both K^{+}- and veratridine-stimulated LH release. Ba^{2+} is known to elicit LH release by entry through VSCC (Smith et al., 1987). Ba^{2+}-stimulated release was also inhibited by RR. These three secretagogues require the entry of either Ca^{2+} or Ba^{2+} via VSCC which can be blocked by D600 and nifedipine to stimulate LH release from gonadotropes. Therefore, it is evident that RR inhibits Ca^{2+} entry via VSCC. However, RR appears to inhibit an additional Ca^{2+} entry mechanism, because unlike D600 and nifedipine it inhibits the spike phase of secretion. This pattern of inhibition is similar to the removal of extracellular Ca^{2+}. Since RR has a large molecular size and is hydrophilic because of its

polycationic structure, it is unlikely that it crosses the plasma membrane to act at an intracellular site. This view was supported by observations that it did not inhibit the effects of secretagogues that activate Ca^{2+}-independent intracellular events, e.g. TPA and forskolin. This suggested that the inhibition of the spike phase by RR was due to the inhibition of Ca^{2+} entry through the non-VSCC mechanism.

Both organic VSCC blockers tested, D600 and nifedipine, inhibited the plateau phase of secretion by approximately 50%. However, the removal of extracellular Ca^{2+} or treatment with RR completely inhibited the plateau phase. Some of the inorganic ions also inhibited the plateau phase to a similar extent. These findings indicate that in addition to Ca^{2+} entry via VSCC during the plateau phase, Ca^{2+} also enters through a non-VSCC. This route of Ca^{2+} entry can be blocked by RR and is active in both the spike and plateau phases of GnRH-stimulated LH release whereas VSCC are only active in the plateau phase.

The mechanism by which this additional route of Ca^{2+} entry is activated is not known. What is apparent is that this route of Ca^{2+} entry is insensitive to inhibition by organic VSCC blockers. This would suggest that this route of Ca^{2+} entry is not activated by membrane depolarisation, since K^{+}-stimulated LH release was completely inhibited by these organic VSCC blockers. Three types of neuronal VSCC have been characterized (Nowycky et al. 1986; Miller, 1987). Two of these classes of VSCC are insensitive to inhibition by organic VSCC blockers with the other being sensitive to inhibition by the organic VSCC blockers. It is, therefore, possible that the additional route of Ca^{2+} entry present in the chicken gonadotrope might be one of

the VSCC described in neurones that is insensitive to organic VSCC blockers. However, this appears to be unlikely since K^+-stimulated LH release, which is assumed to activate all VSCC in the gonadotrope, is completely abolished in the presence of organic VSCC blockers.

Another possibility for the additional route of Ca^{2+} entry is that of a "receptor-operated" Ca^{2+} channel (ROCC). ROCC have been postulated in many cell types and several different types of ROCC have been identified electrophysiologically. ROCCs activated by IP_3 have been identified in T lymphocytes (Kuno and Gardner, 1987). In addition inositol-(1,3,4,5)-tetrakisphosphate has been demonstrated to activate ROCC in sea urchin eggs (Irving and Moor, 1986). Furthermore, a Ca^{2+}-activated Ca^{2+} channel has been described in neutrophils (von Tchanner et al., 1986), and in smooth muscle cells a Ca^{2+} channel that is activated by an ATP receptor without requiring any intracellular second messenger has been reported (Benham and Tsien, 1987).

Another model to explain the stimulation of Ca^{2+} influx by Ca^{2+}-dependent agonists is the capacitative model proposed by Putney (1986). In this model, an IP_3-sensitive pool exists within the cell. This IP_3-sensitive pool is assumed to be a portion of the endoplasmic reticulum which is close to the plasma membrane and which supplies the Ca^{2+} for exocytosis. Because of its proximity to the plasma membrane, this pool is replenished with Ca^{2+} directly from the extracellular space. Therefore, inhibition of secretion occurs in the absence of extracellular Ca^{2+}. Since this Ca^{2+} pool requires replenishment from the extracellular Ca^{2+} pool, maximal inhibition due to the removal of extracellular Ca^{2+} is not immediate and is dependent on the length of

time the cells have been pre-incubated in Ca^{2+}-free medium prior to stimulation with the agonist. RR, Co^{2+} and Gd^{3+} could act by blocking replenishment of this pool, and similarly the degree of inhibition by these inhibitors would also be dependent on the length of pre-incubation time. The data presented for GnRH effects in this thesis in relation to the dependence of pre-incubation time of the effects of Ca^{2+} removal and RR are consistent with this model and suggest that this pool has a $t_{\frac{1}{2}}$ = 0.5 to 1 minute. Due to its short half-life, this pool cannot be studied using the $^{45}Ca^{2+}$ technique, but it does illustrate that Ca^{2+} pool is different from the Ca^{2+} pool ($t_{\frac{1}{2}}$ = 35-70 minutes) studied using the $^{45}Ca^{2+}$ technique. However, even though the data presented are compatible with this capacitative model, it is not possible to exclude the possibility that the effects seen could be due to the extracellular Ca^{2+} trapped in the glycocalyx or in an unstirred layer adjacent to the plasma membrane. This would be compatible with Ca^{2+} entry into the cytosol directly via a channel.

In non-muscle cells, e.g. gonadotropes, in which receptor activation is coupled to the generation of IP_3, the intracellular target of IP_3 has been suggested to be the endoplasmic recticulum (ER) (Berridge and Irving, 1984). However, no conclusive proof of the IP_3-sensitive storage properties of the ER has been given. Recent data have suggested that the "calciosomes", which are discrete Ca^{2+}-binding cytoplasmic organelles, might be the intracellular target of IP_3 (Volpe et al., 1988).

In addition to investigating Ca^{2+} entry mechanisms involved in GnRH-stimulated LH release, the role of cAMP as a second messenger in GnRH-stimulated LH release was examined. Forskolin, which activates the

catalytic subunit of adenylate cyclase,and increases cellular cAMP levels, stimulated LH release from chicken gonadotropes. In addition, IBMX, a cAMP phosphodiesterase inhibitor, which inhibits the degradation of cAMP thereby resulting in an accumulation of cAMP within cells, also stimulated LH release. The cAMP analogue dBu-cAMP also stimulated the release of LH. Its effect was small in comparison to forskolin-stimulated LH release. This may be due to an inability of dBu-cAMP to efficiently cross the plasma membrane of the gonadotrope. It has been reported that dBu-cAMP interacts directly with the GnRH receptor thereby stimulating LH release (Smith et al., 1982). This, together with a possible permeability problem, would have made interpretation of data in which dBu-cAMP was used difficult. Further studies with dBu-cAMP were therefore discontinued and forskolin was used as the primary tool to examine the role of cAMP in LH release from the chicken gonadotrope. Forskolin both increases the cellular cAMP levels and stimulates LH release (Fig. 25). Since a mixed pituitary cell culture preparation was used in these studies, an increase in the cellular cAMP levels is representative of an increase in the cellular cAMP of all the cell types present. However, an increase in LH release is specific for gonadotropes.

In the chicken, the stimulatory effect of forskolin on LH release was rapid in onset but prolonged. Release of LH occurred within minutes after the cells had been exposed to forskolin. In previous studies, in which the temporal aspects of forskolin-stimulated LH release in rat gonadotropes were investigated (Turgeon and Waring ,1986), there was only a slight difference in the secretion rate between control and forskolin-stimulated cells and a significant increase in secretion

rate occurred only 70 minutes after exposure to forskolin. In the original studies by Cronin et al. (1983 and 1984), a similar delayed effect of forskolin on LH secretion from rat gonadotropes was reported. However, in a recent study by the latter group, LH release was reported to be evident within 5 minutes after exposure to forskolin (Evans et al., 1985). These data are similar to the data obtained in the chicken where the effect of forskolin is maximal in the first 20 minutes after which the rate of LH release diminishes.

Forskolin stimulates LH release on its own and potentiates the action of GnRH in both chicken (Fig.30) and rat (Cronin et al., 1983 and 1984; Evans et al., 1985; Turgeon and Waring, 1986) gonadotropes. An unusual phenomenon reported by Turgeon and Waring (1986), was that the enhancement of GnRH-stimulated LH release was less at 10 μM forskolin than at a dose of 1 μM.

It has been reported that forskolin has more than one mode of action depending on the dose at which it is used. Nelson and Seamon (1986), using human platelet membranes, found that forskolin bound to adenylate cyclase at two binding sites for which it had different affinities. It binds to a low affinity site (in the μM range) which is responsible for the direct activation of adenylate cyclase and a high affinity site (Kd = 20 nM) which is responsible for forskolin's synergistic interaction with hormones. Similarly, Khanum and Dufau (1986), using rat testicular Leydig cells, demonstrated that forskolin at concentrations of 1-10 μM, directly activates adenylate cyclase, stimulating cAMP production and testosterone release. However, at lower doses, where forskolin did not affect cAMP production and

stimulate testosterone release, it caused on increase in sensitivity to hormonal stimulation of testosterone production. This ability of forskolin to bind to adenylate cyclase at more than one site does not appear to play a major role in regulating forskolin's ability to stimulate LH release or augment GnRH-stimulated LH release. Over a range of low doses at which forskolin was tested (Fig.29), there was only one dose, 10^{-9}M, at which forskolin enhanced GnRH-stimulated LH release and did not stimulate LH release alone. At doses lower than this, forskolin had no effect on basal or GnRH-stimulated LH release, whereas, at high doses, it stimulated LH release on its own and enhanced GnRH-stimulated LH release.

How does forskolin interact with GnRH to enhance GnRH-stimulated LH release? Possible sites at which forskolin interacts with GnRH could be at the level of: (1) the GnRH receptor; (2) inositol phosphate metabolism; (3) intracellular Ca^{2+} mobilization; (4) activation of protein kinase C; and (5) activation of the Ca^{2+} second messenger system.

In the presence of forskolin, the EC_{50} of GnRH-stimulated LH release was unchanged. This suggested that forskolin only enhanced the ability of GnRH to stimulate LH release without apparently increasing the affinity of GnRH for its receptor. It would be expedient to provide data conerning the ability of GnRH to bind to the GnRH receptor in the chicken gonadotrope in the absence and presence of forskolin. However, attempts to establish a reproducible receptor binding assay using cultured chicken pituitary cells have been unsucessful, and this was thus not possible. Therefore, although it is not possible to totally exclude the possibility that forskolin may

interact with the GnRH receptor, it does not seem likely.

One of the immediate post-receptor events that occurs once GnRH has bound to its receptor, is the activation of phospholipase C which hydrolizes PIP_2 into DAG and IP_3 (Davis et al., 1986). This is one of the early events resulting from the action of GnRH since an increase in inositol phosphates can be seen within seconds after stimulation of gonadotropes with GnRH (Naor et al., 1986 and Davidson et al.,1987). Forskolin did not enhance the ability of GnRH to stimulate an increase in total inositol phosphates (Fig.32). An increase in total inositol phosphates is assumed to be representative of an increase in IP_1, IP_2 and IP_3. However, IP_1 is the main species present, since the experiments were done in the presence of Li^+, which inhibits inositol-1- phosphatase, resulting in the accumulation of IP_1. The absence of any effect of forskolin on GnRH-stimulated inositol phosphate production suggests that the synergism between GnRH and forskolin, seen at the level of LH release, is not due to an interaction occurring at this level of GnRH action.

IP_3, one of the products of the phospholipase C hydrolysis of PIP_2, is thought to stimulate the mobilization of Ca^{2+} from stored intracellular Ca^{2+} pools. This mobilization of intracellular Ca^{2+} is another event that appears to occur immediately after GnRH has bound to its receptor, since an increase in cytosolic Ca^{2+} concentration, due to the release of Ca^{2+} from intracellular stores, is evident within seconds after GnRH addition (Limor et al.,1987). In this study the mobilization of stored intracellular Ca^{2+} was monitored by investigating the ability of GnRH to stimulate $^{45}Ca^{2+}$ efflux from a

slowly turning over Ca^{2+} pool. Forskolin, in the absence of GnRH, raised basal levels of $^{45}Ca^{2+}$ efflux. This increase in $^{45}Ca^{2+}$ efflux could be due to an effect of forskolin on another pituitary cell type (eg. lactotropes) and does not necessarily reflect an increase in $^{45}Ca^{2+}$ efflux from gonadotropes. Forskolin did not enhance the ability of GnRH to mobilize stored intracellular Ca^{2+}. This, together with the evidence that forskolin does not enhance the ability of GnRH to stimulate an increase in inositol phosphate metabolism, suggests that forskolin does not interact with GnRH at these early sites of GnRH action.

This thesis also determined whether or not forskolin was able to interact with other second messengers to stimulate LH release. GnRH is known to stimulate the accumulation of DAG (Davis et al., 1986), another product of the hydrolysis of PIP_2 by phospholipase C, which leads to the redistribution of protein kinase C from the cytosol to the plasma membrane and to its activation (Naor et al., 1985; McArdle et al., 1986). The phorbol ester, TPA, was used to directly activate protein kinase C. Forskolin potentiated TPA-stimulated LH release. The interaction between forskolin and secretagogues that elevate the cytosolic Ca^{2+} concetration was also investigated because Ca^{2+} is an important second messenger involved in GnRH stimulation of LH release. Agents that allow extracellular Ca^{2+} entry, such as high concentrations of K^+, veratridine and the Ca^{2+} ionophore, stimulate LH release. In this study forskolin potentiated the stimulation of LH release by these agents that raise cytosolic Ca^{2+} levels through the influx of extracellular Ca^{2+}. LH release in the presence of Ba^{2+}, which can be substituted for Ca^{2+} to stimulate LH release in chicken

pituitary cells (Davidson et al., 1987), was also enhanced by forskolin. The protein kinase C-dependent pathway and the Ca^{2+} second messenger system, when activated simultaneously, interact synergistically to stimulate LH release from gonadotropes (Harris et al., 1985; Naor and Eli, 1985; Davidson et al., 1987). Forskolin enhanced the synergistic interaction between the Ca^{2+} second messenger pathway and the protein kinase C-dependent pathway when these two pathways are activated by veratridine and TPA, respectively (Fig.34). Therefore, the cAMP second messenger pathway, when activated by forskolin, is able to interact synergistically with both the protein kinase C- dependent pathway and the Ca^{2+} second messenger pathway, when these pathways are activated individually or simultaneously.

In conclusion, cAMP may play a direct or at least a modulatory role in mediating the action of GnRH. Due to the fact that the anterior pituitary consists of a number of different cell types, the slight increase in cellular cAMP levels seen due to stimulation of primary chicken pituitary cells with GnRH, could be representative of a larger increase in cellular cAMP levels within the gonadotropes. Even though the change in cellular cAMP levels is small, it could be crucial, since only a small change in cellular cAMP levels may be required to affect LH release. The site of interaction between forskolin i.e. cAMP, and GnRH which results in a synergistic LH response appears to be distal to the activation of protein kinase C and the Ca^{2+} second messenger pathways. The ability of forskolin to interact with both the protein kinase C-dependent pathway and the Ca^{2+} second messenger pathway, in addition to the ability of GnRH to cause a slight increase in cellular cAMP levels, suggests that cAMP may play a role in the

ability of GnRH to stimulate LH release.

The role of cAMP and the Ca^{2+} requirements of GnRH-stimulated LH release have been investigated. It can be concluded that the GnRH-stimulated spike phase of LH secretion is independent of Ca^{2+} entry via D600/nifedipine-sensitive VSCCs. It is, however, largely dependent on extracellular Ca^{2+} that enters via a route that can be blocked by RR. The residual portion (about 10%) of the spike phase is due to the mobilization of stored intracellular Ca^{2+} as shown by kinetic experiments in which the intracellular Ca^{2+} stores were depleted. The plateau phase of release is partially dependent (about 50%) on Ca^{2+} entry via D600- and nifedipine-sensitive VSCCs and on Ca^{2+} that enters via the major mechanism operative during the spike phase which is blocked by RR (about 50%). Thus the Ca^{2+} entry route that can be blocked by RR is important in both phases of release and may be due to a ROCC or capacitative Ca^{2+} entry.

The data indicate that Ca^{2+} is the major second messenger pathway activated by GnRH. However, cAMP may also be involved in the release mechanism but to a lesser extent. Although the generation of DAG and its activation of protein kinase C was not examined in this thesis a role for DAG as a second messenger involved in GnRH-stimulated LH release cannot be excluded. Further investigations should attempt to clearly define the role of the protein kinase C second messenger pathway in GnRH-stimlated LH release. The advent of cAMP antagonists would provide useful tools to understand the role of cAMP as a second messenger in this system.

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