New Stimulators and a New Mechanism of

Regulated Secretion in Pituitary Gonadotropes

by

Julia Billiard

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Program Authorized
to Offer Degree Physiology and Biophysics

Date 3/19/97

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Abstract

New stimulators and a new mechanism of regulated secretion in pituitary gonadotropes

by **Julia Billiard**

Chairperson of the Supervisory Committee: Professor Bertil Hille

Department of Physiology and Biophysics

The major regulator of gonadotropin (luteinizing hormone, LH; and follicle-stimulating hormone, FSH) secretion is the peptide gonadotropin-releasing hormone (GnRH). It acts via a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). I used cultured pituitary gonadotropes of male rats to seek other possible agents and mechanisms that might regulate gonadotropin secretion. The GnRH-evoked $[Ca^{2+}]_i$ rise is oscillatory and accompanied by oscillations of outward K^+ current that is activated by Ca^{2+}. Substances that caused current responses similar to those with GnRH were hypothesized to evoke gonadotropin secretion via the Ca^{2+}-dependent mechanism. Endothelin-1, oxytocin, neurotensin, pituitary adenylate cyclase-activating polypeptide, and serotonin raised $[Ca^{2+}]_i$ and evoked LH release as assayed by the reverse hemolytic plaque assay (RHPA). These agents affected only subpopulations of gonadotropes establishing functional heterogeneity of pituitary gonadotropes. On the other hand, a protein kinase C (PKC) activator -- phorbol-12-myristate-13-acetate (PMA) -- caused LH release from pituitary gonadotropes without evoking changes in $[Ca^{2+}]_i$. After 1 hour incubation in RHPA, PMA elicited an increase in LH secretion comparable to that caused by GnRH. A PKC inhibitor did not alter GnRH-induced LH release indicating that PKC activation is not important for GnRH-induced secretion in a 1 hour application. Buffering of intracellular Ca^{2+} by AM-loaded BAPTA reduced GnRH-induced LH secretion almost to control levels without significantly affecting PMA-induced secretion. Single-cell photometry on

gonadotropes previously identified by incubation with GnRH in RHPA, revealed no changes in $[Ca^{2+}]_i$ during a one hour application of PMA. Hence, there appears to be a Ca^{2+}-independent pathway of regulated secretion in gonadotropes. Its nature and physiological ligands remain to be elucidated. Finally, I attempted to develop a technique to study differential regulation of LH and FSH release. This attempt failed due to methodological problems but the method may allow study of secretion of two hormones from cell systems other than pituitary gonadotropes. Thus, gonadotropes possess several mechanisms of regulated secretion and can be regulated by a variety of neuromodulators, suggesting possible ways of differential regulation of LH and FSH secretion.

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Acknowledgments

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Finally, I want to thank my family for being with me every step of the way.

Introduction

Pituitary gonadotropes are one of the key elements of the reproductive axis -- an intermediary between the brain and the gonads. Gonadotropes secrete into the general bloodstream two glycoprotein hormones collectively known as gonadotropins: luteinizing hormone (LH) and follicle-stimulating hormone (FSH). A simplified overview of the factors involved in the regulation of LH and FSH secretion is shown in Fig. 1. The major regulator of gonadotropin secretion is the peptide gonadotropin-releasing hormone (GnRH) that is secreted from the hypothalamus and carried to the pituitary by the portal blood. GnRH secretion by the brain is pulsatile with a period of about 1-2 hr, and it translates into a pulsatile secretion of gonadotropins, with LH following GnRH pulses somewhat more faithfully than does FSH. In the gonads, LH and FSH stimulate gamete maturation and secretion of gonadal hormones. The gonadal hormones, in turn, control production and release of both GnRH and gonadotropins (Fig. 1).

GnRH exerts a biphasic stimulatory effect on LH and FSH secretion: a rapid release of a previously formed hormonal pool and a delayed stimulation of hormone synthesis. The simplified diagram of intracellular events that take place upon GnRH binding to its receptor is shown in Fig. 2. The GnRH receptor is coupled to a G protein (G_q) whose activation leads to stimulation of phospholipase C (PLC), and production of the two intracellular messengers, inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 causes release of Ca^{2+} from the IP_3-sensitive intracellular Ca^{2+} stores (the endoplasmic reticulum). The IP_3-induced rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) occurs in an oscillatory manner (Fig. 2). The $[Ca^{2+}]_i$ rises as Ca^{2+} is released from stores and falls due to a combined action of endoplasmic reticulum pumps, intracellular Ca^{2+} buffers, and other cellular processes like uptake into the mitochondria and extrusion through the plasma membrane. Each $[Ca^{2+}]_i$ elevation causes a burst of exocytosis. At the same time, each $[Ca^{2+}]_i$ rise opens Ca^{2+}-activated K^+ channels in the plasma membrane (see Chapter I). In addition, DAG and Ca^{2+} synergistically activate PKC that is known to promote LH and

Fig. 1. The major factors regulating LH and FSH secretion. + -- stimulation; -- -- inhibition. In females, low concentrations of estrogen inhibit and high concentrations, stimulate GnRH and gonadotropin production. The stimulation (a positive feedback effect) leads to a gonadotropin surge.

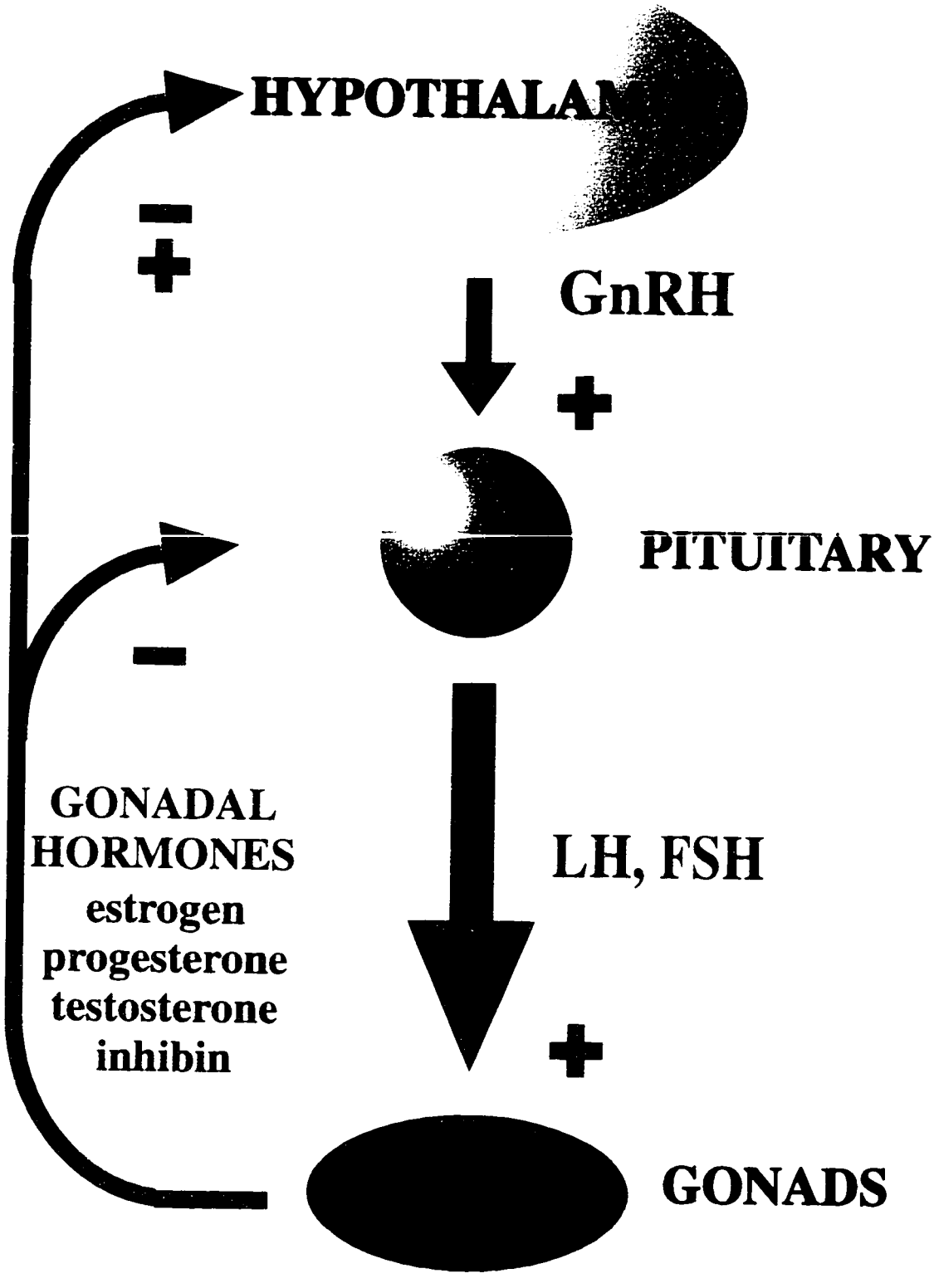
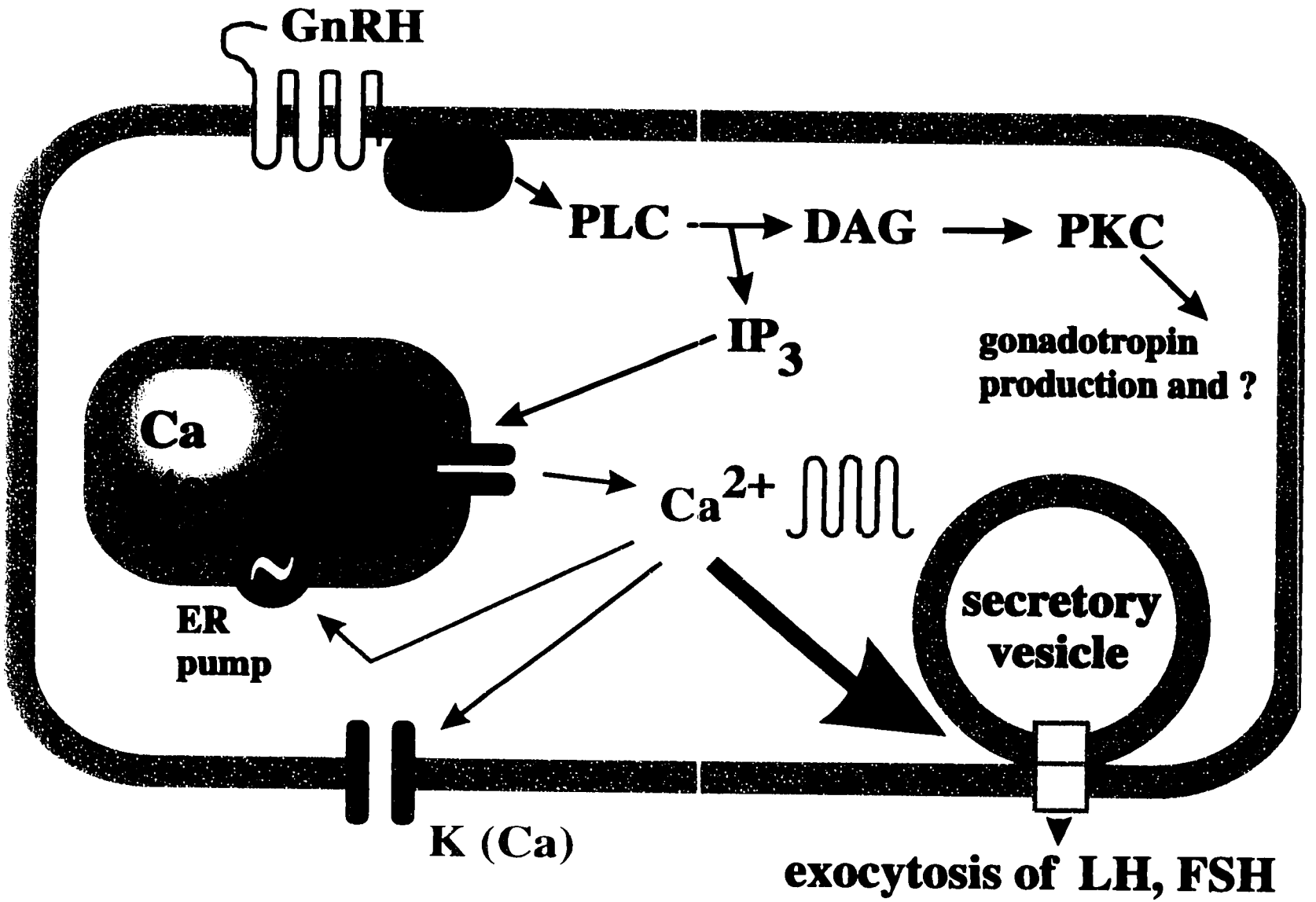

HYPOTHALA
GnRH
PITUITARY
GONADAL
HORMONES
estrogen
progesterone
testosterone
inhibin
LH, FSH
GONADS

Fig. 2. Activation of a pituitary gonadotrope by GnRH. G_q -- GTP-binding protein of G_q type; PLC -- phospholipase C; DAG --diacylglycerol; PKC -- protein kinase C; IP_3 -- inositol 1, 4, 5-trisphosphate; K(Ca) -- Ca^{2+}-activated K^+ channel; ER -- endoplasmic reticulum.


GnRH
PLC
DAG
PKC
IP3
gonadotropin
production and ?
Ca
Ca2+
ER
pump
secretory
vesicle
K (Ca)
exocytosis of LH, FSH

FSH *biosynthesis*, possibly through mitogen-activated protein kinase [1]. The issue of PKC involvement in GnRH-induced LH and FSH *release* is controversial (see Chapter II for discussion).

Our understanding of the regulation of LH and FSH secretion is by no means complete. In this thesis, I attempted to address a few questions related to regulation of gonadotropin release using as a model cultured pituitary gonadotropes of adult male rats. In Chapter I, I used electrophysiology and incubation studies to identify hormones and neuromodulators, other than GnRH, that can rapidly regulate gonadotropin secretion. I found several gonadotropin-releasing factors and also showed that gonadotropes are functionally heterogeneous with only subpopulations responding to various neuromodulators. In Chapter II, I investigated the role of protein kinase C (PKC) in gonadotropin secretion. Incubation studies and single-cell photometry revealed that an activator of PKC rapidly stimulates LH release via a Ca^{2+}-independent mechanism. This is the first conclusive evidence for existence of a Ca^{2+}-independent secretory pathway in rat gonadotropes. Finally, in Chapter III, I attempted to develop a tool to study how LH and FSH secretion can be differentially regulated. This attempt failed due to methodological problems but Chapter III and Appendix contain the description of methods that may be used to study secretion of two hormones in cell systems other than pituitary gonadotropes.

Chapter I

FUNCTIONAL HETEROGENIETY OF PITUITARY GONADOTROPES IN RESPONSE TO A VARIETY OF NEUROMODULATORS

Rationale

GnRH is the major direct physiologic regulator of gonadotropin secretion from pituitary gonadotropes. Other hormones and neuromodulators affect release of gonadotropins as well and may discriminate between subsets of gonadotropes and differentially regulate LH and FSH release. However, for most of these substances it is not known if the action is on gonadotropes or on upstream cells. It was important therefore to assess a list of candidate hormones and neuromodulators for their ability to regulate pituitary gonadotropes directly.

All of the agents studied here were previously implicated in some manner in the control of the hypothalamo-pituitary-gonadal axis. Some affect the concentration of serum gonadotropins when administered to the whole animal and their precise site of action remains unknown. Others regulate hormonal release from pituitary cells in culture. Thus, oxytocin (OT) [2, 3], endothelin 1 (ET-1) [4], pituitary adenylate cyclase-activating polypeptide (PACAP) [5], ATP [6], and substance P [7] are reported to upregulate basal and/or GnRH-induced gonadotropin secretion from pituitary fragments as well as from dissociated pituitary cells. On the other hand, γ-aminobutyric acid (GABA) [8], and a nitric oxide (NO) donor, sodium nitroprusside [9], are reported to inhibit GnRH-evoked gonadotropin release. Effects of neuropeptide Y (NPY) depend upon the steroid milieu [10, 11]. When I began my research, only five substances had been shown to have direct rapid effect on single pituitary gonadotropes. They are GnRH itself; neuropeptide Y (NPY), which suppressed GnRH-induced $[Ca^{2+}]_i$ elevation [12]; and ET-1 [4], PACAP [13, 14], and ATP [15], which increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in a manner similar to that of GnRH.

In my work, I used changes in the whole-cell membrane ionic current as an assay of gonadotrope responsiveness to different agents. In rat pituitary gonadotropes, GnRH induces rhythmic release of Ca^{2+} from inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} stores [16] and each elevation of $[Ca^{2+}]_i$ triggers a burst of exocytosis [17]. Each $[Ca^{2+}]_i$ elevation also opens Ca^{2+}-activated K^+ channels (SK channels) [16, 18] resulting in oscillations of the whole-cell current. Therefore, this current can be used to monitor $[Ca^{2+}]_i$ and to assess the responsiveness of gonadotropes to the test agents. The agents found capable of increasing $[Ca^{2+}]_i$ were then used in the reverse hemolytic plaque assay (RHPA) to test their ability to stimulate LH release from cultured rat pituitary gonadotropes.

Materials and Methods

Cell identification and culture

Experiments were performed on pituitary gonadotropes obtained from adult (5-7 weeks) Sprague-Dawley male rats, intact or castrated at 3 weeks of age. Anterior pituitary cells were enzymatically dispersed as described [19] and gonadotropes were identified by the RHPA as described [20, 21]. Briefly, pituitary cells were mixed with protein A-coupled red blood cells and plated on glass coverslips glued onto a hole drilled in the bottom of a culture dish. Cells were incubated for 2 hr with GnRH (50 nM) and rabbit polyclonal antibody against bovine luteinizing hormone (LH) (1:40 dilution; two antibodies used were kind gifts of Dr. J. D. Neill, University of Alabama, Birmingham, AL, and Dr. D. A. Leong, University of Virginia, Charlottesville, VA. For more information about the antibodies, see Refs. 22 and 23, respectively). Following this incubation, cells were exposed to complement for 30 min for plaque formation. After the plaque assay, cells were maintained in 5% CO_2 at 37 °C in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY) supplemented with 10% horse serum (Sigma, St. Louis, MO), 100 U/ml Penicillin V and 100 mg/ml Streptomycin (Gibco or Sigma) for 1-4 days prior to current recordings.

In some experiments, in order to increase the yield of plaques, rats were castrated 3 weeks prior to sacrifice. For castration, rats were anesthetized with 0.9 ml/kg of a mixture of 100 mg/ml Ketamine (Aveco Co., Inc., Fort Dodge, IA) and 20 mg/ml Xylazine (Phoenix Pharmaceutical, Inc., St. Joseph, MO). All animals were given an injection of Bicillin L-A (Wyeth Laboratories Inc., Philadelphia, PA) postoperatively and were closely monitored during surgery and recovery.

Current recording

Membrane ionic currents were recorded at room temperature (20-25 °C) using the whole-cell configuration of the patch-clamp technique [24]. The electrodes were made from 75 µl hematocrit glass pipettes (VWR Scientific, Seattle, WA) and had resistances around 2-3 MΩ after filling. Currents were measured by an Axopatch-1B amplifier (Axon Instruments, Foster City, CA) from cells held at a constant voltage of -50 mV. Currents were filtered at 10 kHz, digitized with a modified SONY PCM-501 EC digital audio processor and stored on video tapes. The signals from the video tape were later played back through the digital audio processor, refiltered at 10-1,000 Hz and analyzed with BASIC-FASTLAB (Indec Systems, Capitola, CA). Currents were also recorded on a chart recorder. Where appropriate, membrane potentials were corrected for a 9 mV junction potential.

Regular external saline (see below) with or without a test substance was continuously perfused into the bath via the whole-bath perfusion system at a rate of 1.5-2 ml/min.

Solutions

The regular external saline contained (in mM): 150 NaCl, 5 $CaCl_2$, 2.5 KCl, 1 $MgCl_2$, 8 glucose, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.4 with NaOH) and the regular internal saline contained (in mM): 120 K-aspartate, 20 KCl, 0.1 Na_4GTP, 2 $MgCl_2$, 2 Na_2ATP, 20 HEPES (pH 7.4 with KOH). Solutions used for isolation of ATP-dependent current contained: external (in mM): 160.5 NaCl, 1 $CaCl_2$, 1

$MgCl_2$, 8 glucose, 10 HEPES, (pH 7.4 with NaOH) and internal (in mM): 120 aspartic acid, 20 CsCl, 0.1 Na_4GTP, 2 $MgCl_2$, 2 Na_2ATP, 20 HEPES (pH 7.4 with CsOH).

Chemicals

Baclofen hydrochloride, dopamine hydrochloride, serotonin hydrochloride, 2-methylthio-ATP, and β,γ-methylene-L-ATP were obtained from Research Biochemicals International (Natick, MA). GTP and its analogues were from Boehringer Mannheim (Indianapolis, IN). Calcitonin gene-related peptide and isotocin were from Bachem Bioscience Inc. (King of Prussia, PA), and all other polypeptides, from Peninsula Laboratories Inc. (Belmont, CA). Melatonin, prostaglandin E_2, and suramin were obtained from Calbiochem (La Jolla, CA); 3-morpholinosydnonimine, from Molecular Probes (Eugene, OR); and morphine and testosterone, from University of Washington Hospital Pharmacy (Seattle, WA). All other chemicals were purchased from Sigma.

Polypeptides were diluted in water or 0.1 M acetic acid, aliquoted, lyophilized and kept at -20 °C. ATP solution was prepared fresh daily. All other substances were diluted to about 1,000 times their final concentration either in water or in aqueous solutions of ethanol or NaOH (adenosine only), aliquoted and kept at -20 or +4 °C. All substances were diluted to their final concentrations in the external solution on the day of the experiment. The concentration of ethanol in the final solution never exceeded 0.1%. Where necessary, precautions were taken against light exposure.

Measurement of secretory response

The secretory responsiveness of gonadotropes to various agents was assessed by means of the RHPA designed to detect secretion of LH. The assay was performed as described in *cell identification and culture* but GnRH was either omitted (control) or replaced by the test substance. Cells were maintained in culture for 2-4 days after the assay to allow for complete disintegration of any cells that formed plaques as a result of lysis during the assay as opposed to secretion. For measurements, cells were viewed under the light

microscope (250x) and plaques were counted by eye. Once counted, the cell inside the plaque was destroyed by a glass pipette mounted on a micromanipulator to avoid duplicate counting of plaques. To obtain plaque density, the total number of plaques in a dish was normalized by the area covered by the cell mixture. Cell density was assumed to be uniform throughout all dishes in each experiment. Plaques and cells were approximated by circles and the diameters of eight randomly selected plaques and the cells that produced them were measured in each dish. When there were fewer than eight plaques, all plaques were measured. Relative plaque size was determined by normalizing the diameter of each plaque by the diameter of the cell inside it. Each experiment included at least six dishes with a given treatment and at least six control dishes. Every experiment was repeated at least three times (total $n \geq 18$ dishes per treatment).

For each experiment, the mean plaque density was calculated for the control dishes, and the plaque densities in control and treatment dishes within the experiment were normalized to this mean. The normalized plaque densities for treatment dishes from all experiments with the same agent were pooled and mean ± SEM was calculated as an indicator of the agent's effectiveness. Differences between the normalized plaque densities for control dishes and treatment dishes as well as between the normalized plaque densities for treatment dishes obtained with different agents within the same experiment were assessed using a non-parametric method of statistical analysis, the Mann-Whitney U test. $P<0.05$ was considered significant. For relative plaque sizes, values were pooled from all treatment dishes from all experiments with a given agent. Another pool was created for all control dishes from experiments with this agent. Means ± SEM were calculated for each pool and differences were assessed by the Mann-Whitney U test. $P<0.05$ was considered significant. Only preparations from intact male rats were used for measurements of secretory response.

Results

The electrical studies were performed on gonadotropes from intact and castrated male rats. As the number of pituitary gonadotropes and their sensitivity to GnRH increase after castration, rats castrated 3 weeks prior to the plaque assay gave an increased yield of distinct plaques. The use of castrated rats was limited to electrophysiological studies of substances already found effective on at least 5 cells from intact animals. There was no detectable difference in electrical behavior of gonadotropes obtained from castrated rats versus that of cells from intact animals.

Thirty three substances, other than GnRH, were tested for their ability to evoke electrical responses in single pituitary gonadotropes. Of these, six caused rhythmic oscillations of the whole-cell current; one (ATP) increased an inward current; and the rest were without effect in my electrophysiological assay.

Seven substances stimulated oscillations

The percentage of gonadotropes responding to GnRH and six other agents that stimulated oscillations of outward current is listed in Table 1, and Fig. 3 shows examples of the evoked oscillations. As can be seen in Fig. 3, unstimulated gonadotropes voltage-clamped at -50 mV exhibited only a few picoamperes of standing inward or outward currents. Consistent with previous reports from our lab (see, for example, Ref. 17), extracellular application of GnRH induced a large oscillatory outward current, known to be caused by oscillations in $[Ca^{2+}]_i$ (Fig. 3A). Such oscillations were induced in all gonadotropes previously identified by their ability to form plaques in response to GnRH. The oscillations did not desensitize in the continuous presence of GnRH for up to tens of minutes, and stopped completely after a wash-out of GnRH. Fig. 3 also shows examples of oscillations induced by extracellular application of serotonin (5-HT) (Fig. 3B), oxytocin (OT) (Fig. 3C), neurotensin (NT) (Fig. 3D), and arginine-vasopressin (AVP) (Fig. 3E). The amplitudes of the observed oscillations were similar to those obtained with GnRH in the same cell whereas the time courses were usually slower. In contrast to

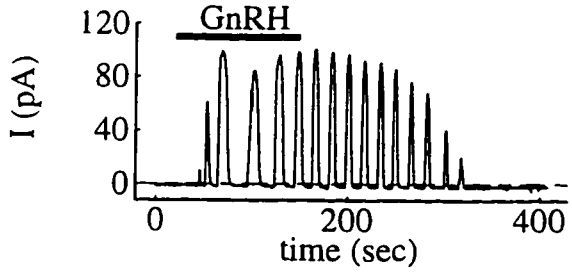
Table 1

Substances that cause $[Ca^{2+}]_i$ oscillations

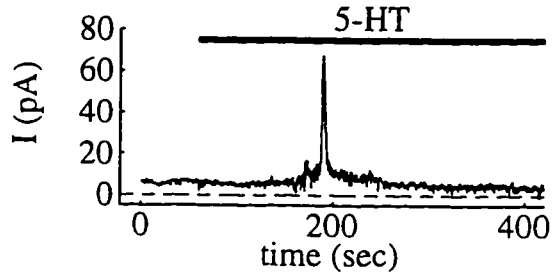
substance	*concentration, nM*	*% responsive cells (n tested)*
Gonadotropin-releasing hormone	0.25	100 (318)
Serotonin	100	44 (9)
Oxytocin	100	36 (22)
Neurotensin	5,000	24 (21)
Arginine-vasopressin	100	11 (9)
Endothelin 1	100	62 (8)
Pituitary adenylate cyclase-activating polypeptide	100	43 (7)

FIG. 3. Membrane current oscillations in gonadotropes induced by GnRH (A) 5-HT (B), OT (C), NT (D), and AVP (E). Gonadotropes were held at -50 mV. GnRH (250 pM), 5-HT (100 nM), OT (100 nM), NT (5 μM), and AVP (100 nM) were applied for the duration of the respective bars. The outward current spikes are a characteristic signature of opening of Ca^{2+}-activated K^+ channels due to Ca^{2+} elevations in these cells. Dashed lines denote zero current. Regular external and internal salines. Different cells.

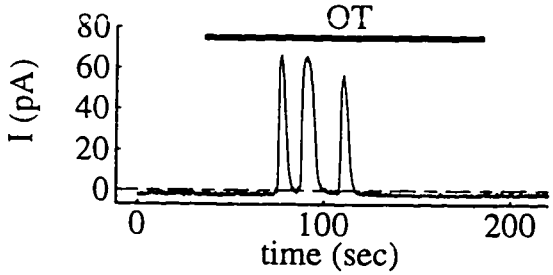
A.


GnRH
120
80
40
0
I (pA)
0
200
400
time (sec)

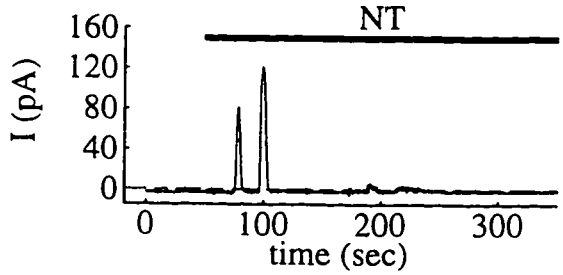
B.


5-HT
80
60
40
20
0
I (pA)
0
200
400
time (sec)

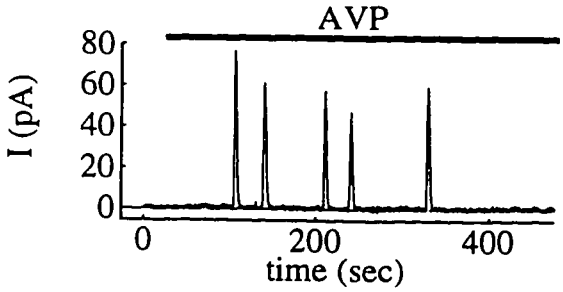
C.


OT
80
60
40
20
0
I (pA)
0
100
200
time (sec)

D.


NT
160
120
80
40
0
I (pA)
0
100
200
300
time (sec)

E.


AVP
80
60
40
20
0
I (pA)
0
200
400
time (sec)

GnRH-evoked behavior, the responses to 5-HT, OT, NT, and AVP desensitized so that only a few current oscillations were seen upon agonist application, and not all gonadotropes identified by their sensitivity to GnRH were responsive. The percentage of responsive cells is listed in Table 1. AVP was effective only on one gonadotrope that was also OT-responsive and on a subpopulation of the cells identified by their sensitivity to OT in the RHPA (these cells were not included in the calculations of the percentage of responsive cells). These results are consistent with the report that AVP acts through low-affinity interactions with the OT receptors [2].

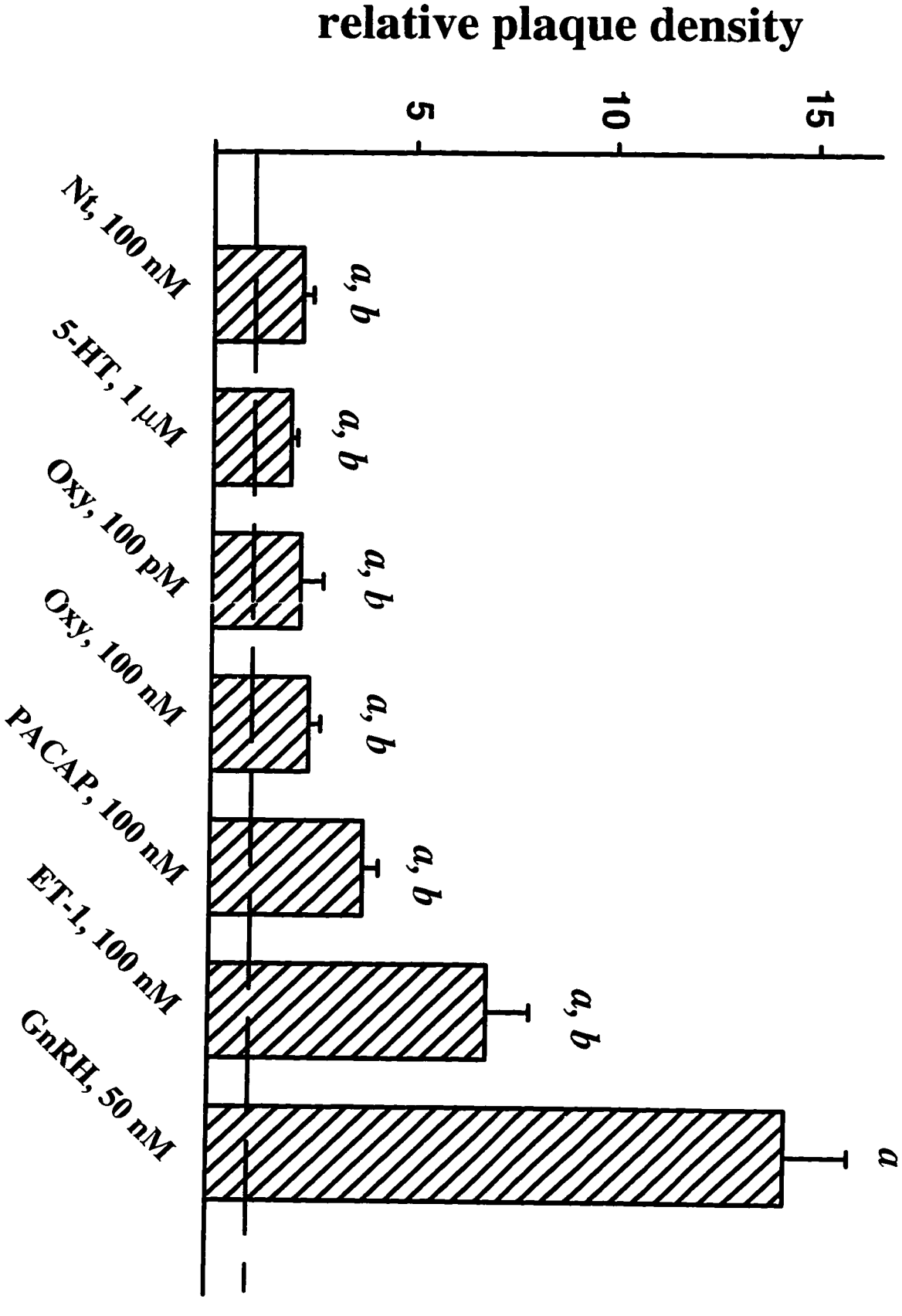
Five neuromodulators stimulated gonadotropin secretion

The ability to evoke an electrical response suggested that the substances listed in Table 1 might induce gonadotropin secretion from pituitary gonadotropes. This hypothesis was tested by assessing the ability of these substances to act as secretagogues in the RHPA. Fig. 4 summarizes the relative number of plaques (LH-secreting cells) obtained with different agents. GnRH increased the number of LH-secreting cells 1400% compared to unstimulated conditions. All test substances also significantly increased the number of LH-secreting cells compared to unstimulated conditions, yet they were significantly less effective than GnRH ($P<0.005$ for both comparisons). GnRH caused a 19% increase in the average plaque sizes (an index of the amount of LH secreted per cell). The average plaque sizes obtained in the presence of all the other test substances were not significantly different from control but were significantly smaller than those obtained in GnRH ($P<0.05$, data not shown). All gonadotropes that made plaques in response to agents other than GnRH in RHPA also proved to be GnRH-sensitive when tested subsequently in electrophysiological experiments ($n = 31$).

ATP increased an inward current

When ATP (10-100 μM) was added to the bath, almost all the gonadotropes tested (41 out of 45) showed a marked increase in *inward* current at the -50 mV holding potential

FIG. 4. LH release induced by NT, 5-HT, OT, PACAP, and ET-1. Dissociated anterior pituitary cells were exposed to the indicated concentrations of each agonist for 2 hr in RHPA. The number of LH-secreating cells (the number of plaques) was normalized to the area occupied by the cell mixture to obtain plaque density. Average plaque density obtained without any agonist was set at 1(dashed line, control). *a* indicates significant difference from basal release; *b* indicates significant difference from GnRH-evoked release. $P<0.005$, means ± SEM.

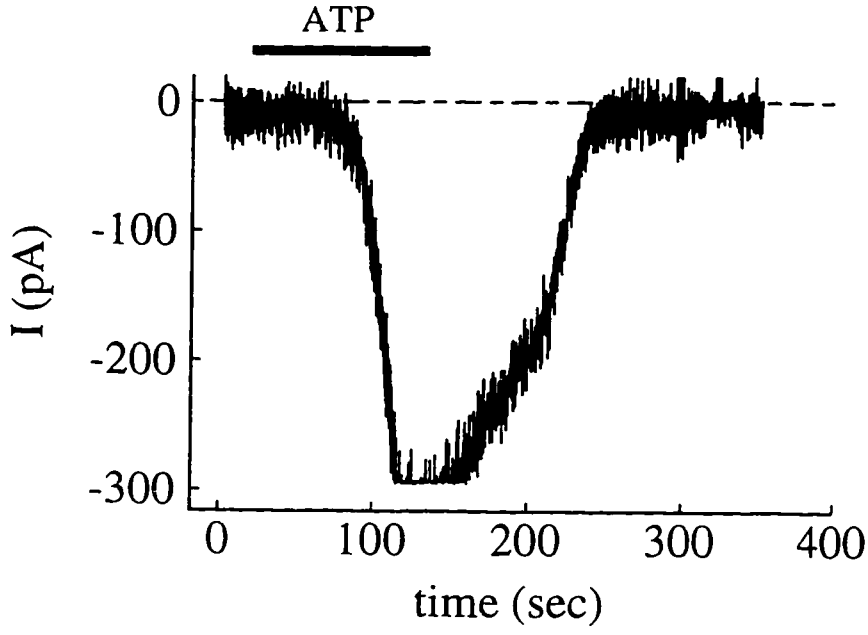

relative plaque density
5
10
15
Nt, 100 nM
5-HT, 1 μM
Oxy, 100 pM
Oxy, 100 nM
PACAP, 100 nM
ET-1, 100 nM
GnRH, 50 nM
—
a
a, b
a, b
a, b
a, b
a, b
a, b

(Fig. 5A). The ATP-evoked current desensitized in tens of seconds in the continuous presence of ATP. The recovery of the response following several minutes of wash-out varied among the cells from complete to only ~10%. Replacement of external Na^+ with an equimolar concentration of N-methyl-D-glucamine (NMDG) consistently reduced the ATP-dependent current to less than 5 pA (n = 9; data not shown), indicating that this inward current was mainly carried by inflow of Na^+ ions. For studying the ATP effect, external and internal salines were replaced by the ones designed to isolate ATP-dependent current (see Methods). With these salines, the outward current did not exceed tens of picoamperes across the voltage range employed and inward current was virtually undetectable in the absence of ATP. A typical current-voltage (I-V) relation for the ATP-evoked current is shown on Fig. 5B. The current exhibited prominent inward rectification (little current in the outward direction) and its reversal potential fell between -6 and +9 mV in different cells. The current was completely and reversibly abolished by 100 μM of the P_2-purinergic receptor antagonist suramin (4 out of 4 cells, data not shown).

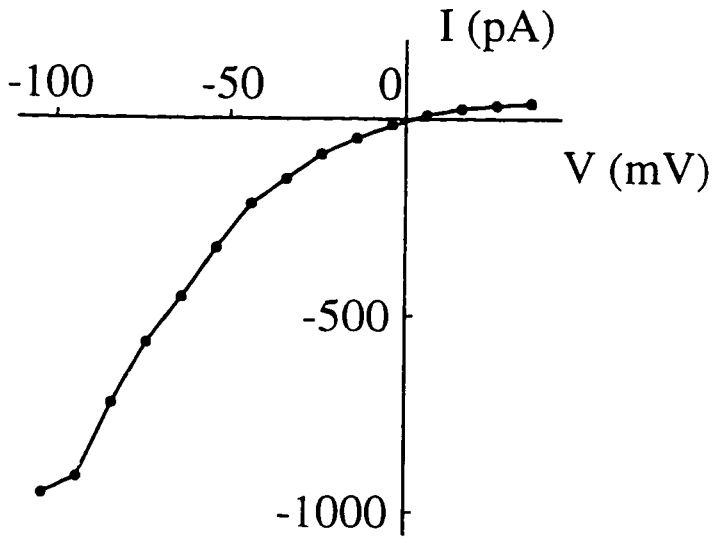
The purinergic receptor was further classified by assessing its responsiveness to different ATP analogues. In these experiments, the currents evoked by ATP analogues were compared with the current evoked in the same cell by subsequent application of 100 μM ATP. Currents evoked by 100 - 500 μM 2-methylthio-ATP were consistently much smaller than those in 100 μM ATP, with some cells completely lacking a response to the analogue. The same was true for the currents evoked by 100 - 500 μM α,β-methylene-ATP. When 2-methylthio-ATP and α,β-methylene-ATP were tested on the same cell, 2-methylthio-ATP evoked a bigger current. Thus, the order of agonist efficacy at the receptor was ATP >> 2-methylthio-ATP > α,β-methylene-ATP. Adenosine, AMP, ADP, diadenosine tetraphosphate (AP_4A), and β,γ-methylene-L-ATP were without effect at 100 μM concentration and uridine triphosphate (UTP) had no effect at concentrations up to 1 mM.

FIG. 5. ATP-evoked inward current in a gonadotrope voltage-clamped at -50 mV. A. Current evoked by ATP. ATP (100 μM) was applied for the duration of the bar. Dashed line denotes zero current. Regular external and internal salines. The current trace appears truncated due to saturation of the amplifier. **B. The current-voltage (I-V) relation of ATP-evoked current.** The I-V relation was obtained by holding the cell at -50 mV and stepping every 250 ms for 50 ms to potentials between -95 and 45 mV in 10 mV increments. The I-V relations were obtained before, during and approximately 4 min after ATP application. The mean was found between the currents before and after application of ATP, and this value was subtracted from the current in the presence of ATP to give the ATP-dependent current. The voltages were corrected for the junction potential. External and internal salines were designed to isolate the ATP-dependent current.

A.


ATP
0
-100
-200
-300
I (pA)
0
100
200
300
400
time (sec)

B.


I (pA)
-100
-50
0
V (mV)
-500
-1000

Many other substances had no rapid electrophysiological effect

Agents that caused no consistent change in the whole-cell membrane current when applied alone or in combination with GnRH ($n \geq 7$ for each agent) are listed in Table 2. The experimental concentrations were chosen based on data available from literature.

Examples of current records obtained with substances concluded to be ineffective are shown in Fig. 6. When neuropeptide Y (NPY, 100 nM), corticotropin-releasing factor (CRF, 100 nM), galanin (GAL, 100 nM), and angiotensin II (A II, 100 nM) were applied alone to quiescent gonadotropes, no change in the background current could be observed (Fig. 6A; $n = 4$ for each substance). Fig. 6B and C show the lack of effect of CRF and galanin on oscillations evoked by GnRH (open bars). To allow detection of small possible inhibitory or potentiating influence of a test agent, the GnRH concentration used in all electrophysiological experiments was 250 pM, the lowest capable of evoking continuous, reproducible oscillations. When 100 nM CRF was added to GnRH-containing bath (filled bar in Fig. 6B), there was no detectable change in either the time course or amplitude of GnRH-induced oscillations ($n = 7$). Similarly, addition of 100 nM GAL did not change the pattern of oscillations ($n = 5$; Fig. 6C).

Overall, 26 substances evoked no current response by themselves and did not change the response to GnRH. They were classified as ineffective in short exposures.

Discussion

Six substances were found to cause current oscillations in pituitary gonadotropes in a manner similar to that of GnRH but only in subpopulations of cells. Of these six agents, AVP and OT are hormones secreted from posterior pituitary, whereas ET-1, NT, 5-HT, and PACAP are neuromodulators secreted from the hypothalamus. Actions of ET-1 and PACAP on rat pituitary gonadotropes have already been well described [4, 25, 13, 14, 26], and the ability of OT to upregulate LH release from dissociated pituitary cells has been reported [2, 3]. PACAP was reported to elevate $[Ca^{2+}]_i$ in about 70% of gonadotropes [13] and ET-1, in all gonadotropes [25]; both values are higher than those

Table 2

Ineffective substances *

substance	*concentration, μM*
Dopamine	0.1-1
Histamine	1-100
Norepinephrine	10
Leucine-enkephalin	0.5
Morphine	1-50
γ-aminobutyric acid	0.1-10
Baclofen	10
Glutamate	20-100
Corticotropin-releasing factor	0.1
Isotocin	0.1
NO donor (3-Morpholinosydnonimine)	100
Pancreatic polypeptide	0.1
Neuropeptide Y	0.1
Substance P	1
Prostaglandin E_2	10
Testosterone	0.1
Angiotensin II	0.1
Bombesin	0.5

(continues)

* for all substances, $n \geq 7$

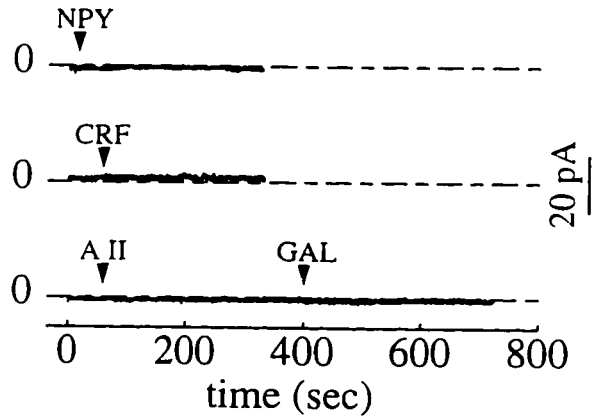
Table 2

(continued)

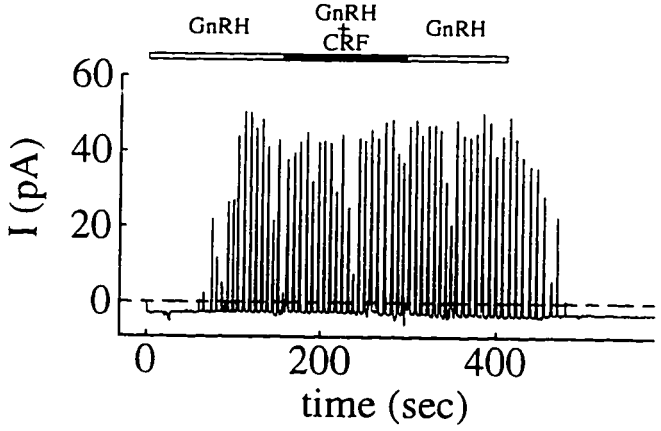
Calcitonin	0.5
Calcitonin gene-related peptide	0.5
Galanin	0.1
Melanocyte-stimulating hormone	0.5
Melatonin	10
3-α-hydroxy-4-pregnen-20-one	10^{-4}
Somatostatin	0.1
Vasoactive intestinal peptide	0.1-1

FIG. 6. Time course of membrane currents in identified gonadotropes upon application of substances classified as ineffective. All cells were held under voltage-clamp at -50 mV. Dashed lines denote zero current. Regular external and internal salines. **A. NPY, CRF, A II, and GAL are applied to quiescent identified gonadotropes.** Application of agents began at times indicated by arrowheads. Arrowhead for GAL also denotes the start of A II wash-out. Three different cells. **B, C. CRF and GAL are applied to identified gonadotropes oscillating in GnRH.** Cells were exposed to 250 pM GnRH for the time marked by the open bars. During the time marked by the closed bar, 100 nM CRF (A) or 100 nM GAL (B) were added to the same concentration of GnRH.

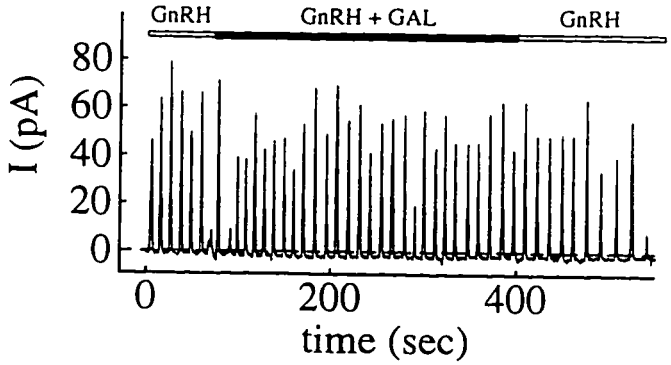
A.


NPY
0
CRF
0
20 pA
A II
GAL
0
0
200
400
600
800
time (sec)

B.


GnRH
GnRH + CRF
GnRH
60
40
20
0
I (pA)
0
200
400
time (sec)

C.


GnRH
GnRH + GAL
GnRH
80
60
40
20
0
I (pA)
0
200
400
time (sec)

found here (see Table 1). ET-1 and PACAP are thought to use the same mechanism of $[Ca^{2+}]_i$ elevation as GnRH does: they bind G-protein-coupled receptors and can induce formation of IP_3 and release of Ca^{2+} from IP_3-sensitive stores. AVP, OT, NT, and 5-HT also have G-protein coupled receptors coupled to IP_3 production, but to assess the pathway these substances activate in gonadotropes will be difficult because only subpopulations of GnRH-sensitive cells responded to 5-HT, OT, NT, and AVP (see Table 1), and the responses desensitized after a few spikes of current. When these agents were used to identify gonadotropes in RHPA (see later), cells became unresponsive to them in subsequent electrophysiological experiments.

Any agent producing $[Ca^{2+}]_i$ elevations is a candidate secretagogue. As was anticipated, OT, NT, 5-HT, ET-1, and PACAP evoked LH secretion from some but not all gonadotropes. It is conceivable that not all the gonadotropes were responsive to these agents because the agents were used at less than optimal concentrations that evoked only the threshold responses. However, OT evoked similar responses at 100 pM and 100 nM (see Fig. 4) indicating that at least for this substance the concentration was not at the threshold for the response. Various concentrations of ET-1 and PACAP have been previously applied to rat pituitary cells. For both ET-1 and PACAP, 100 nM caused maximal LH release [4 and 26, respectively]. The K_D for NT receptor in the pituitary is 1.4 nM [27] and the K_D and the EC_{50} for non-channel 5-HT receptors do not exceed 300 nM [28].

All gonadotropes that responded to OT, NT, 5-HT, ET-1, and PACAP in the RHPA were GnRH-sensitive when tested in subsequent electrophysiological experiments, indicating that these agents stimulated subsets of GnRH-responsive cells. The amount of LH secretion per cell in the presence of these agents was less than that evoked by GnRH. Fractions of GnRH-sensitive cells that responded to these other test substances in the RHPA were lower than the fractions responding electrophysiologically (cf. Fig. 4 with Table 1). This difference most likely results from rapid desensitization of the response in most of the gonadotropes. The LH release produced by OT, ET-1, and PACAP as

compared to basal levels is in agreement with the values obtained in various incubation studies [2, 25, 26].

Thus, a multiplicity of hormones and neuromodulators can stimulate pituitary gonadotropes. We suggest that under some circumstances they may act as physiological "gonadotropin-releasing hormones." Unlike GnRH, the six additional agonists affected only subpopulations of gonadotropes. Thus, gonadotropes are functionally heterogeneous.

Application of ATP opened inwardly rectifying channels in the plasma membrane. The lack of effect of adenosine, AMP, and ADP as well as inhibition of the ATP-evoked current by suramin indicated that the response was mediated via P_2-purinoceptors. So far, P_{2U} purinoceptors have been suggested to exist on pituitary gonadotropes and ATP was found to induce LH secretion presumably via P_{2U} receptors [15]. The lack of effect of up to 1 mM of a specific P_{2U} agonist, UTP, argues against involvement of classical P_{2U} receptors in the response observed here. After this part of my thesis was submitted for publication, Tomic *et. al.* [29] reported that in female rat gonadotropes, ATP activates P_{2X_2} and/or P_{2X_3} purinoceptors that are ion channels. The response observed here had similar pharmacological and electrical properties and hence was probably mediated through the same type(s) of purinoceptors. The current here had a reversal potential that did not correspond to the equilibrium potential of any single ion but could be accounted for if the channel was non-selective for cations. Replacement of Na^+ in the bath by a large cation species (N-methyl-D-glucamine, NMDG) reduced the inward current to almost undetectable levels, indicating that Na^+ was the major physiological current carrier. Tomic *et. al.* [29] reported that P_{2X} receptor channels conduct Na^+ and Ca^{2+} which cause depolarization of the cells and activation of voltage-sensitive Ca^{2+} channels (VSCC). They also observed ATP-induced LH release that they attributed to the Ca^{2+} influx through the P_{2X_2}/P_{2X_3} and the VSCC. In addition, GnRH stimulated ATP secretion from rat gonadotropes [29], which suggested a physiological role for ATP as a positive feedback mechanism on GnRH-induced LH secretion.

The rest of the agents tested did not induce rapid electrical effects on male rat pituitary gonadotropes (see Table 2). The time frame of my measurements was limited to tens of minutes. Such time limits are probably too short to see actions of testosterone or of another steroid 3-α-hydroxy-4-pregnen-20-one. It should also be added that each agent was tested at one concentration or in a fairly narrow concentration range. Whenever the data were available, concentrations were chosen that were reported to be the most effective on pituitary cells. Otherwise, the data obtained in other systems were used.

The substances listed in Table 2 could be concluded to lack rapid effects upon gonadotropin secretion if I could demonstrate that 1) changes in $[Ca^{2+}]_i$ are accurately reflected by the membrane current and 2) a Ca^{2+} rise is required for exocytosis in presence of these agents as it is in the presence of GnRH. As for the first assumption, GnRH-induced gonadotropin release requires around 300 nM intracellular free Ca^{2+} [17], the concentration at which the SK current becomes detectable [30]. It is unlikely that test substances can make SK current less sensitive to Ca^{2+}, since none of the test agents diminished the current oscillations seen in GnRH. Test agents, however, might increase the Ca^{2+} sensitivity of secretion by activating other second messengers, for example, arachidonic acid and its metabolites [31]. The second assumption is probably not true since gonadotropes possess at least one Ca^{2+}-independent mechanism of secretion (see Chapter II) and it is conceivable that some of the test agents can stimulate such a mechanism. Overall, it can only be safely concluded that the test agents listed in Table 2 did not have rapid effects on Ca^{2+} mobilization in male rat pituitary gonadotropes.

Substance P [7] has been reported to stimulate LH secretion from cultured rat pituitary cells when used at the same concentration as was employed here. This makes substance P a potential physiological activator of a Ca^{2+}-independent secretory pathway in rat gonadotropes, possibly of a pathway described in Chapter II. In addition, GABA [9], a NO donor [10], and 3-α-hydroxy-4-pregnen-20-one [32] inhibited GnRH-induced gonadotropin secretion. These substances can conceivably inhibit GnRH-induced or basal gonadotropin release without affecting $[Ca^{2+}]_i$.

In summary, six substances commonly present in the hypothalamus induce both Ca^{2+} mobilization and LH secretion from subpopulations of male rat gonadotropes. Gonadotropes differ in their sensitivity for different agonists. A multiplicity of agents capable of mobilizing various pools of gonadotropes should provide multiple sites for control of gonadotropin secretion and may also allow for differential regulation of LH and FSH release.

Chapter II

A PHORBOL ESTER ACTIVATES A Ca^{2+}-INDEPENDENT SECRETORY PATHWAY IN RAT PITUITARY GONADOTROPES

Rationale

In neurons and chromaffin cells, where exocytosis has been well studied, a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is known to be necessary and sufficient to elicit secretion of neurotransmitters. In pituitary gonadotropes, GnRH induces rhythmic release of Ca^{2+} from inositol 1, 4, 5-trisphosphate (IP_3)-sensitive Ca^{2+} stores [16] and each elevation of $[Ca^{2+}]_i$ causes a burst of exocytosis [17]. Artificial $[Ca^{2+}]_i$ rise created in absence of GnRH by introducing IP_3 into the cell, also causes exocytosis [17]. In addition, phorbol esters which activate protein kinase C (PKC) trigger LH release from pituitary gonadotropes of a variety of species, including rat [33, 34, 35], and the effect is pronounced within minutes of exposure [36]. At first, this observation suggested that PKC may mediate the response to GnRH. Indeed, GnRH stimulates production of diacylglycerol and Ca^{2+} that synergistically activate PKC, and application of GnRH causes a shift in PKC distribution from cytosolic to membrane fraction, linked to PKC activation [37]. Recently, however, it was proposed that PKC is not necessary for GnRH-induced gonadotropin secretion since GnRH action was not impaired in rat pituitary cells upon depletion of PKC [38] or upon inhibition of PKC by a variety of specific inhibitors [39].

The LH secretion induced by phorbol esters could be Ca^{2+}-independent. Indeed, our laboratory found that a potent phorbol ester, phorbol-12-myristate-13-acetate (PMA) when applied to rat gonadotropes for several minutes, had modulatory effects on ionic channels and that these effects were blocked by a PKC inhibitor staurosporin [30]. But there was no suggestion of a $[Ca^{2+}]_i$ rise [30]. Millar and his colleagues [40] found that in permeabilized sheep gonadotropes, PMA stimulates exocytosis in the virtual absence of

Ca^{2+}. This suggests that gonadotropes may have a secretory pathway that does not need $[Ca^{2+}]_i$ rise. Here I examine existence of such PMA-activated Ca^{2+}-independent secretory pathway in rat gonadotropes.

Reverse hemolytic plaque assay (RHPA) introduced by Molinaro and Dray in 1974 [41] and adopted for pituitary cells by Neill and coworkers [42] reports hormone secretion from individual cells in pituitary cell populations. I use RHPA to determine if PKC activation by PMA causes LH secretion from rat gonadotropes during a 1 hr incubation and if PKC inhibition impairs GnRH signaling. I combine RHPA with single-cell photometry to assess Ca^{2+} requirements of PMA-induced LH release.

My results demonstrate that PMA is as effective as GnRH in causing LH release from rat pituitary gonadotropes, but that PKC activation is not required for GnRH signaling. Unlike GnRH, PMA does not require a $[Ca^{2+}]_i$ rise for action, which suggests the existence of a Ca^{2+}-independent secretory pathway in rat pituitary gonadotropes.

Materials and Methods

Animals

In order to increase the yield of gonadotropes in pituitary preparations, male Sprague-Dawley rats were castrated at 3 weeks of age and used in experiments 1-2 weeks later. Before castration, rats were anesthetized with 0.9 ml/kg of a mixture of 100 mg/ml Ketamine (Aveco Co., Inc., Fort Dodge, IA) and 20 mg/ml Xylazine (Phoenix Pharmaceutical, Inc., St. Joseph, MO). They were given an injection of Bicillin L-A (Wyeth Laboratories Inc., Philadelphia, PA) postoperatively and closely monitored during surgery and recovery.

Chemicals and solutions

Phorbol myristate acetate (PMA), 4α-phorbol-12,13-didecanoate (4α PDD) and bisindolylmaleimide I hydrochloride (BIS) were obtained from Calbiochem (La Jolla,

CA), 8-(4-chlorophenylthio)-cAMP (CPTcAMP) was from Boehringer Mannheim (Indianapolis, IN), and emetine dihydrochloride was from Sigma (St. Louis, MO). PMA, 4α PDD, BIS, CPTcAMP, and emetine were dissolved in water to about 1,000 times their final concentration and kept in aliquots at -70 °C (PMA, 4α PDD, and emetine) or -20 °C. GnRH (Peninsula Laboratories Inc., Belmont, CA) was dissolved in water, aliquoted, lyophilized and kept at -20 °C. 1,2-*bis*-(*o*-Aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA AM), indo-1 acetoxymethyl ester (indo-1 AM), and pluronic F-127 were obtained from Molecular Probes (Eugene, OR). Indo-1 AM was kept at -20 °C in the dark; BAPTA AM was dissolved in dimethylsulfoxide (DMSO, Sigma) at 20 mM, aliquoted and kept at -20 °C; pluronic F-127 was dissolved in DMSO at 20% and kept at room temperature. Easy Tag™ protein labeling mix ([^{35}S]methionine) was obtained from DuPont NEN Research Products (Boston, MA) and kept at -20 °C. All other chemicals were purchased from Sigma.

The *external saline* contained (in mM): 150 NaCl, 5 $CaCl_2$, 2.5 KCl, 1 $MgCl_2$, 8 glucose, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.4 with NaOH).

The *incubation medium* consisted of Dulbecco's Modified Eagle Medium with HEPES buffer (DMEM with HEPES, Gibco, Grand Island, NY) supplemented with 0.1% bovine serum albumin (Sigma), 100 U/ml Penicillin V and 100 mg/ml Streptomycin (Gibco or Sigma).

Measurement of secretory response

The secretory responsiveness of gonadotropes to various agents was assessed by means of the reverse hemolytic plaque assay (RHPA).

The assay was performed as described in *Cell identification and culture* in Chapter I. Briefly, pituitary cells were mixed with protein A-coupled red blood cells and injected into a narrow chamber constructed in a culture dish. Cells were incubated for 1 hr in *incubation medium* with or without (control) test substance(s) in the presence of rabbit polyclonal antibody against bovine luteinizing hormone (LH) (1:40 dilution; kind gift of

Dr. J. D. Neill, University of Alabama, Birmingham, AL, see Ref. 22). Following this incubation, cells were exposed to complement for 30 min for plaque formation. After the plaque assay, the top of the chamber was removed and cells were maintained in 5% CO_2 at 37 °C in DMEM without HEPES (Gibco) supplemented with 10% horse serum (Sigma), 100 U/ml Penicillin V and 100 mg/ml Streptomycin for 1-2 days prior to measurements.

Where indicated, the modifying agents were added to cells before addition of PMA and GnRH. Cells were incubated for the indicated time in *incubation medium* containing only the modifying agent followed by 1 hr incubation with the modifying agent and LH antibody and PMA or GnRH. The rest of the RHPA was carried out as usual.

To buffer intracellular free Ca^{2+}, I used BAPTA AM, a membrane-permeant form of the Ca^{2+} chelator BAPTA. BAPTA AM was mixed with equal volume of 20% pluronic F-127 and diluted into *incubation medium* to a final concentration of 20 μM; the concentration of Pluronic F-127 in the final solution was 0.02%. The enzymatically dispersed cells were divided into two groups: one group was resuspended in *incubation medium* and the other, in 10 μM BAPTA AM. Both groups were incubated at 37 °C for 15 min with constant agitation, cells were centrifuged out and washed twice in *incubation medium*. Each group was mixed with protein A-coupled red blood cells, plated on dishes, and subjected to RHPA. The AM ester was cleaved regenerating active BAPTA during the 1 hr incubation used for cell attachment in the RHPA protocol.

For measurements of plaques, cells were viewed under the light microscope (250x) with the built-in photo mask frame in the field of view. The dish was moved until the photo mask frame was in its upper left-hand corner, and the position of the bottom side of the frame was noted in reference to the configuration of blood cells, pituitary cells, and plaques in a dish. After all plaques inside the frame were counted by eye, the dish was moved up so that the top side of the photo mask frame was positioned where the bottom side had been. This procedure was repeated until the bottom of the cell layer was in the frame. Then the dish was moved one frame to the left so that the left side of the frame

was positioned where the right side had been. Now the dish was moved down frame-by-frame until the frame was on top again. The whole dish was counted in this manner. Plaque density and plaque sizes in each dish were calculated as described in *Measurement of secretory response* in Chapter I. Each experiment included at least four dishes with a given treatment and at least four control dishes, and every experiment was repeated at least three times (total $n \geq 12$ dishes per treatment).

Relative plaque densities and relative plaque sizes were assessed as described in *Measurement of secretory response* in Chapter I. Differences were assessed by the Mann-Whitney U test, $P<0.05$ was considered significant. Percent inhibition was calculated from the means.

Measurement of average free $[Ca^{2+}]_i$ (Single-cell photometry)

Anterior pituitary cells were enzymatically dispersed, RHPA with GnRH as an agonist was performed to identify gonadotropes (see *Measurements of secretory response*), and cells were used for Ca^{2+} measurements 1-2 days later. Intracellular free Ca^{2+} was measured with the membrane-permeant form of the fluorescent dye indo-1, indo-1 AM, as previously described [43]. Briefly, indo-1 AM was solubilized into DMSO at 10 mM, mixed with equal volume of 20% pluronic in DMSO and diluted into *external saline* at 1 μM final concentration. Cells were incubated in indo-1 AM-containing *external saline* for 1 hr in 5% CO_2 at 37 °C and then washed with *external saline* and placed on the microscope stage. Indo-1 was excited at 365 nm and the fluorescence at 405 nm (F_{405}) and 500 nm (F_{500}) was measured. Excitation by the 100-watt mercury lamp was restricted with an electronic shutter to periods of 20-25 msec every 0.2-1 s. Average background fluorescence measured without red blood cells or pituitary cells in the field was subtracted. Fluorescence intensity ratios can be converted into $[Ca^{2+}]_i$ [43] with higher F_{405}/F_{500} ratios corresponding to higher $[Ca^{2+}]_i$. However, since no novel changes in $[Ca^{2+}]_i$ were observed here and since $[Ca^{2+}]_i$ ranges for gonadotropes have been reported [see, for

example, 4, 17], no conversion was performed and the results are expressed as the ratio F_{405}/F_{500}.

Measurement of inhibition of protein synthesis by emetine

Rates of protein synthesis were assessed by measuring the rate of incorporation of [^{35}S]methionine in trichloroacetic acid (TCA)-precipitable material. Anterior pituitary cells were enzymatically dispersed and divided into 5 groups. Groups 1 and 2 were resuspended in *incubation medium* as controls, groups 3, 4, and 5, in *incubation medium* containing 1, 10, and 100 μM emetine, respectively. All groups were incubated for 20 min in 5% CO_2 at 37 °C. [^{35}S]methionine was added to the medium in all groups at 221 μCi/ml and cells were incubated under the same conditions for additional 30 min (group 1) or 60 min (group 2, and groups 3, 4, and 5). After the incubation, cells were removed from the medium by centrifugation, and each group was resuspended in 8% TCA and incubated at 90 °C for 3 min. The TCA/cell mixtures were spotted on strips of Whatman 3MM filter paper which were washed in 7.5% TCA and 95% ethanol at 0 °C. After drying, the amount of radioactive label in the papers was counted in toluene-based scintillant by Beckman LS-30 Liquid Scintillation Counter. The validity of the method was confirmed by obtaining higher counts in control group incubated for 60 min than in control incubated for 30 min.

Results

For RHPA, the mixture of pituitary cells and red blood cells (RBCs) is injected into a narrow chamber constructed in a culture dish, and allowed to attach. The final area covered by cell mixture is approximately 70-90 mm^2. The density of pituitary cells in a dish cannot be measured since RBCs cover most of pituitary cells that did not form plaques. The pituitary cell density can not be calculated either because, although we know cell density in a mixture before injection into the chamber, we do not know the probabilities for the big pituitary cells and the small mobile red blood cells to take up the

space in the chamber. However, we do not need to know the pituitary cell density as long as it stays constant throughout each experiment.

Only a few plaques (LH-secreting cells) were observed when RHPA was performed under basal conditions. The basal plaque density in all experiments was $0.084\pm0.008/mm^2$, which corresponds to about 5-8 plaques per dish. Addition of 100 nM phorbol-12-myristate-13-acetate (PMA) to the incubation medium, increased the number of LH-secreting cells more than 800% (Fig. 7A). There was also a statistically significant increase in the plaque size (the amount of LH secreted per cell), but it was small, only 17% (Fig. 7B). As Fig. 7 shows, PMA was as effective as GnRH in potentiating LH release, whereas the inactive PMA analogue, 4α-phorbol-12, 13-didecanoate (4α PDD), had no effect even at 1 μM. The effectiveness of both GnRH and PMA in increasing the number of LH-secreting cells varied between experiments (cf. Fig. 7 with Figs. 8 and 9) but the effectiveness was always similar for both secretagogues. Fig. 7 also shows that the effects of GnRH and PMA were not additive on either the number of LH-secreting cells or on the plaque sizes. Although the plaque sizes were smaller in the presence of both GnRH and PMA than in the presence of either one alone, the difference was not statistically significant.

To investigate if the effect of PMA on LH release was indeed due to PKC activation, I performed experiments in the presence of a selective PKC inhibitor, bisindolylmaleinide (BIS). BIS, applied 10 min before addition of PMA and present throughout the PMA application, inhibited the effect of PMA on recruitment of LH-secreting cells (Fig. 8) and on the relative plaque sizes (18% inhibition, $P<0.05$, data not shown). In the same experiment, the GnRH effect on the number of LH-secreting cells was not changed by BIS (Fig. 8). The GnRH effect on the relative plaque sizes was not significantly changed either (11% inhibition and big data variability, $P>0.5$, data not shown). These findings indicate that PKC activation is not necessary for GnRH to stimulate secretion.

Fig. 7. LH release in response to GnRH and to PMA. Dissociated pituitary cells were exposed for 1 hr in RHPA to 50 nM GnRH; 100 nM PMA; 50 nM GnRH + 100 nM PMA; or 1 μM 4α PDD. **A.** The number of LH secreting cells (the number of plaques) was normalized to the area occupied by the cell mixture to obtain plaque density. Average plaque density obtained without any agonist (around 0.08/mm^2) was set at 1 (dashed line, control). **B.** Relative plaque size (an indicator of LH secretion per cell) was obtained by normalizing plaque diameters to diameters of the forming cells. *a* indicates significant difference from basal release. $P<0.05$, means ± SEM.

A

relative plaque density
5
10
15
GnRH
PMA
GnRH+PMA
4α PDD
a
a
a

B

relative plaque size
1
2
3
4
control
GnRH
PMA
GnRH+PMA
4α PDD
a
a
a

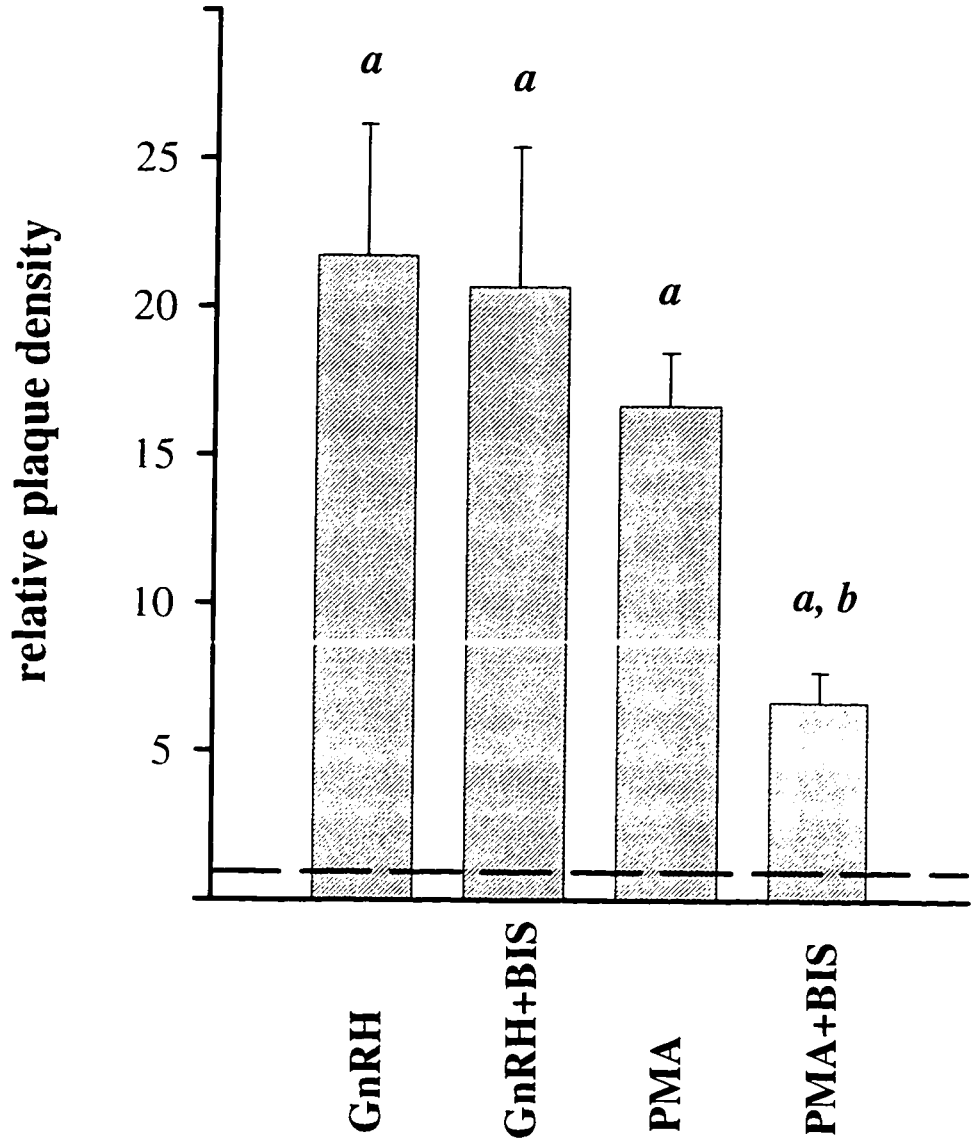


Fig. 8. Effect of a specific PKC inhibitor, bisindolylmalemide, on PMA-evoked and GnRH-evoked LH release. Where indicated, dissociated pituitary cells were incubated for 10 min with 100 nM BIS. Then all cells were exposed for 1 hr in RHPA to 50 nM GnRH; 50 nM GnRH + 100 nM BIS; 100 nM PMA; or 100 nM PMA + 100 nM BIS. Relative plaque density obtained as in Fig. III.1. Average plaque density without any agonist (around 0.08/mm^2) is set at 1 (dashed line, control). *a* indicates significant difference from basal release. *b* indicates significant difference from GnRH- and PMA-evoked release. $P<0.05$, means ± SEM.

Since PKC activation stimulates protein synthesis in a variety of cell systems including pituitary gonadotropes [44], I wanted to check if protein synthesis contributes to the effect of PMA on LH secretion observed here. I used emetine, a blocker of translation, to inhibit protein synthesis in gonadotropes. Emetine inhibited incorporation of [^{35}S]methionine into total cellular protein during 1 hr incubation by 70% at 1 μM, by 90% at 10 μM, and by 80% at 100 μM. Actual counts of radioactivity are given on the inset to Fig. 9. The addition of 1 μM emetine 20 min prior to and during 1 hr incubation with PMA, did not significantly affect the number of LH-secreting cells mobilized by PMA or GnRH (Fig. 9). Emetine also had no effect on the relative plaque sizes (7% inhibition of the GnRH-evoked effect and 2% inhibition of PMA-evoked effect, $P>0.1$ for both, data not shown). I concluded that protein synthesis does not contribute to LH release evoked by PMA during the 1 hr incubation.

Three experimental approaches were used to assess if PMA requires a rise in $[Ca^{2+}]_i$ for signaling: In the first experiment, the flux of Ca^{2+} into the cell from the bathing medium was blocked by blocking Ca^{2+} channels in the plasma membrane. In the second experiment, $[Ca^{2+}]_i$ was buffered by introducing a Ca^{2+} chelator BAPTA into the cell. In the third experiment, the intracellular Ca^{2+}-sensitive dye indo-1 was used to monitor $[Ca^{2+}]_i$ during a 1 hr application of PMA (performed with the help of Dr. D. F. Babcock).

Fig. 10 shows a comparison of the effects of lowering $[Ca^{2+}]_i$ on GnRH-evoked (Fig. 10A) and PMA-evoked (Fig. 10B) LH release. Blocking Ca^{2+} channels by addition of 100 μM Cd^{2+} to the incubation medium, significantly decreased the number of LH-secreting cells recruited by GnRH while having no effect on recruitment by PMA. The GnRH effect on the relative plaque sizes was inhibited by 17% ($P<0.05$) and the PMA effect was not changed significantly (3% inhibition, $P>0.1$). Buffering $[Ca^{2+}]_i$ by loading cells with BAPTA prior to incubation, rendered GnRH incapable of recruiting LH-secreting cells but only decreased the response to PMA by about 50% (Fig. 10). The GnRH effect on the relative plaque sizes was reduced to below control level (16% inhibition, $P<0.001$, not

emetine concentration, μM	Incubation time, min	Radioactivity, counts/min
0	30	47, 932
0	60	375, 254
1	60	121, 376
10	60	44, 326
100	60	77, 230

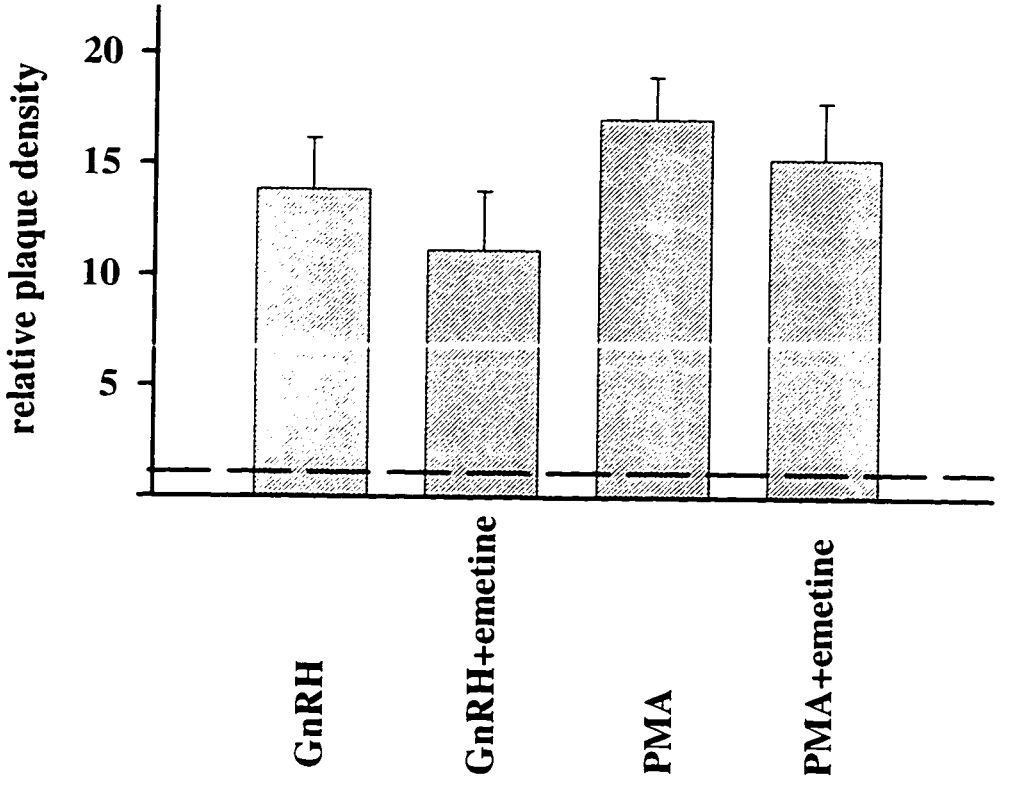
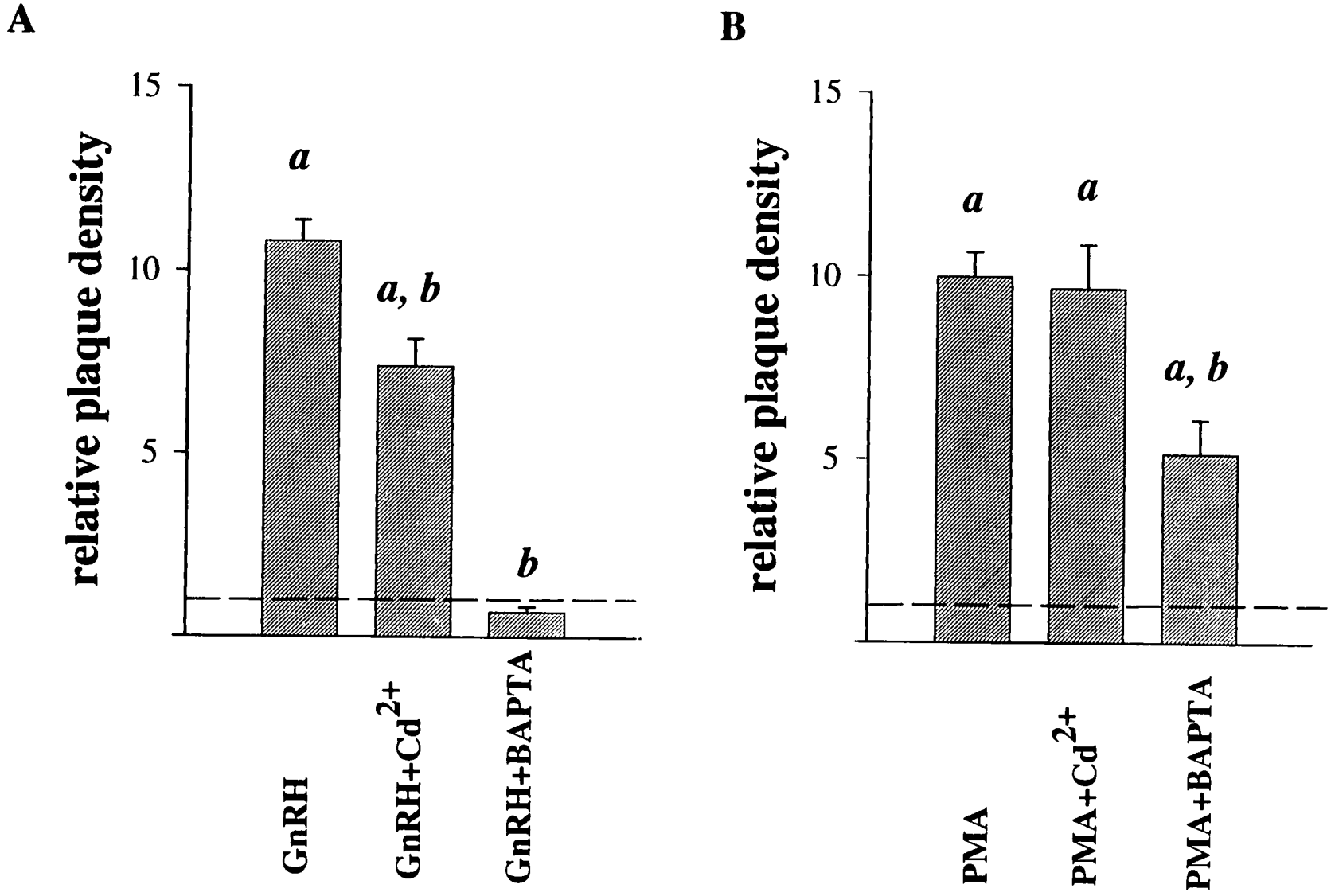


Fig. 9. Effect of a protein synthesis inhibitor, emetine, on PMA-evoked and GnRH-evoked LH release. Where indicated, dissociated pituitary cells were incubated for 10 min with 10 μM emetine. Then all cells were exposed for 1 hr in RHPA to 50 nM GnRH; 50 nM GnRH + 1 μM emetine; 100 nM PMA; or 100 nM PMA + 1 μM emetine. Relative plaque density obtained as in Fig. III.1. Average plaque density without any agonist (around 0.08/mm^2) is set at 1 (dashed line, control). Results for all treatments are not significantly different from each other. $P<0.05$, means ± SEM. **Inset: Inhibition of protein synthesis by emetine.** Pituitary cells were incubated for 20 min with emetine at indicated concentrations, [^{35}S]methionin was added and cells were further incubated for the indicated time. Radioactivity in each sample was counted for 20 s since longer exposures saturated the counter.

Fig. 10. Effect of lowering $[Ca^{2+}]_i$ on PMA-evoked and GnRH-evoked LH release. Dissociated pituitary cells were exposed for 1 hr in RHPA to: **A.** 50 nM GnRH; 50 nM GnRH + 100 μM Cd^{2+}; or 50 nM GnRH. **B.** 100 nM PMA; 100 nM PMA + 100 μM Cd^{2+}; or 100 nM PMA. In addition, where indicated, cells were loaded with BAPTA by incubation for 15 min in 10 μM BAPTA prior to RHPA. Relative plaque density obtained as in Fig. III.1. Average plaque density without any agonist (around 0.08/mm^2) is set at 1 (dashed line, control). *a* indicates significant difference from basal release. *b* indicates significant difference from GnRH- (A) and PMA- (B) evoked release. $P<0.05$, means ± SEM.


A
B
relative plaque density
15
10
5
a
a, b
b
a
a
a, b
GnRH
GnRH+Cd2+
GnRH+BAPTA
PMA
PMA+Cd2+
PMA+BAPTA

shown) whereas the PMA effect on the plaque sizes was unchanged (0% inhibition, not shown).

Fig. 11 shows traces of $[Ca^{2+}]_i$ obtained with indo-1 during 1 hr application of PMA at room temperature. Gonadotropes previously identified by their sensitivity to GnRH in RHPA were capable of producing $[Ca^{2+}]_i$ oscillations in response to GnRH in the beginning as well as in the end of the monitoring experiment (Fig. 11A). However, no change in $[Ca^{2+}]_i$ was observed during a 1 hr PMA application (n=5, Fig. 11A), although in RHPA, 100 nM PMA stimulated plaque formation as effectively at room temperature as at 37 °C (740% increase in plaque number and 19% increase in plaque size, n=8 dishes in two experiments). In a different experimental paradigm, several gonadotropes in one dish were monitored consecutively over about 1.5 hrs in the constant presence of PMA (Fig. 11B). GnRH was added to the bath on top of PMA at the end of monitoring periods and all cells showed $[Ca^{2+}]_i$ oscillations (these recording portions are omitted for clarity). But no $[Ca^{2+}]_i$ rise was detected in any of the cells in the presence of PMA alone.

Based on these results, I conclude that PMA evokes LH secretion without a rise in $[Ca^{2+}]_i$.

In permeabilized sheep gonadotropes, PMA also evokes LH release in a Ca^{2+}-independent manner [40]. In addition, cAMP evokes LH release from permeabilized sheep gonadotropes [45]. To check if cAMP evokes LH release in rat gonadotropes as well, I incubated pituitary cells with a membrane-permeant cAMP analogue, CPTcAMP. In 1 hr incubations, 100 μM CPTcAMP did not change the number of LH-secreting cells or the size of plaques (Fig. 12). Fig. 12 also shows that CPTcAMP was unable to potentiate GnRH-induced LH release.

Discussion

The plaque assay showed that in rat gonadotropes, PMA is as effective as GnRH in evoking LH release. PMA, like GnRH, is much more effective in recruiting new gonadotropin-releasing cells than in increasing secretion per gonadotrope (Fig. 7). The

Fig. 11. Intracellular Ca^{2+} concentration during 1 hr application of PMA. Pituitary cells were dissociated, gonadotropes were identified by RHPA and cells were loaded with indo-1 AM. Changes in the fluorescence intensity ratio (F_{405}/F_{500}) reflect changes in $[Ca^{2+}]_i$. **A.** A gonadotrope identified by RHPA exhibited $[Ca^{2+}]_i$ oscillations in presence of 10 nM GnRH (solid bars) but no changes in $[Ca^{2+}]_i$ during 1 hr application of 100 nM PMA. Representative of 5 similar experiments. **Inset: GnRH-evoked $[Ca^{2+}]_i$ oscillations on a faster time scale. B.** Eight different cells monitored over 1.5 hrs in continuous presence of PMA. Each cell was confirmed to be a gonadotrope by GnRH application (10 nM, not shown). Each cell was monitored for about 2 min with 5-15 min between consecutive cells. Representative of 2 similar experiments.

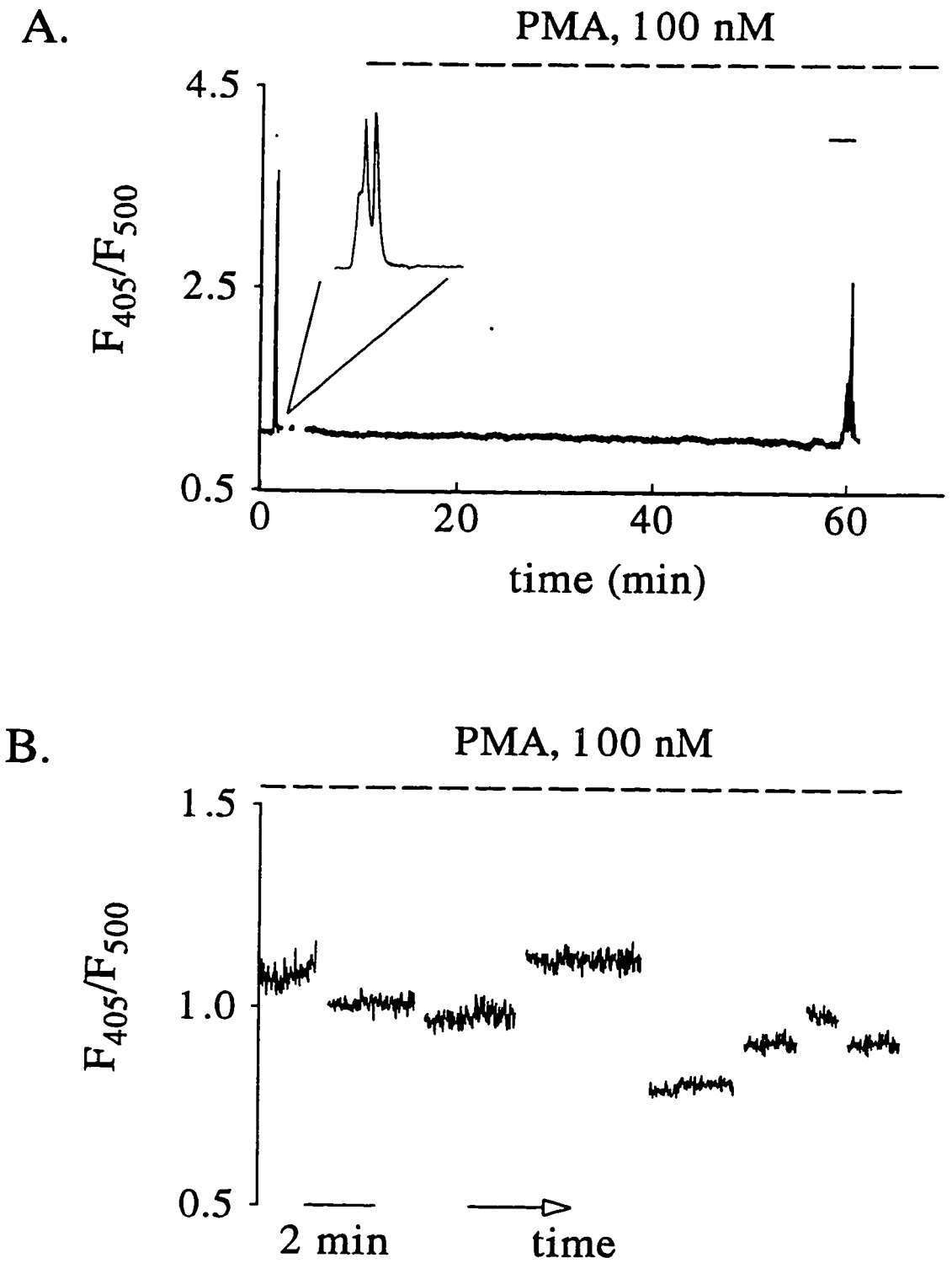
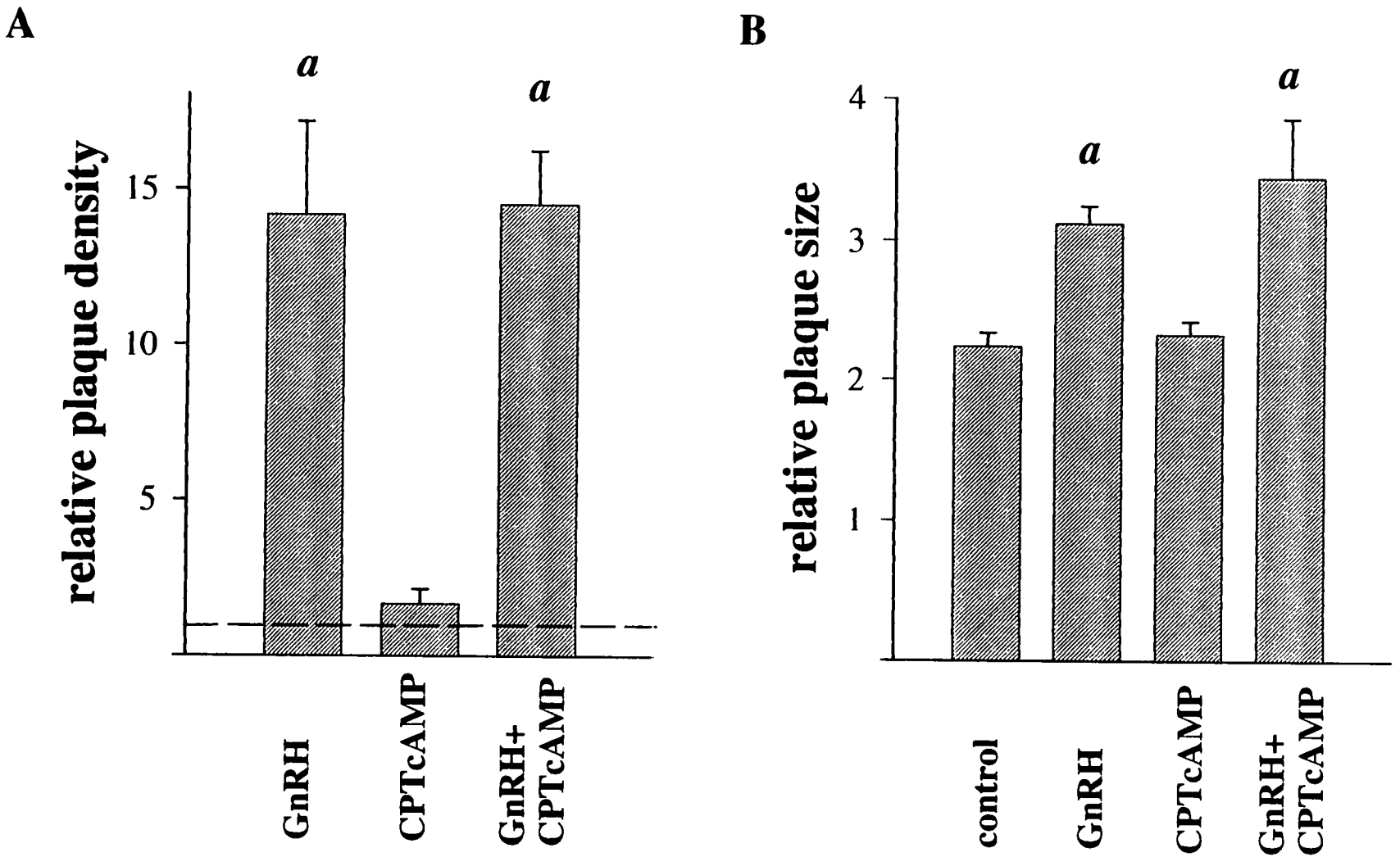

A.
PMA, 100 nM
4.5
2.5
0.5
F_{405}/F_{500}
0
20
40
60
time (min)
B.
PMA, 100 nM
1.5
1.0
0.5
F_{405}/F_{500}
2 min
time

Fig. 12. LH release in response to GnRH and to CPTcAMP. Dissociated pituitary cells were exposed for 1 hr in RHPA to 50 nM GnRH; 100 μM CPTcAMP; or 50 nM GnRH + 100 μM CPTcAMP. **A.** and **B.** obtained as in Fig. III.1. Average plaque density without any agonist (around 0.08/mm^2) is set at 1 (dashed line, control). *a* indicates significant difference from basal release. $P<0.05$, means ± SEM.


A
a
a
relative plaque density
15
10
5
GnRH
CPTcAMP
GnRH+
CPTcAMP
B
a
4
3
2
1
relative plaque size
control
GnRH
CPTcAMP
GnRH+
CPTcAMP

effect of PMA is structurally specific (no action of 4α PDD, Fig. 7) and is inhibited by a specific PKC inhibitor, BIS (Fig. 8).

Prolonged activation of PKC can also stimulate synthesis of LH α and β subunits [34]. In order to study rapid, protein synthesis-independent action of a PKC activator on secretion, I picked the shortest secretagogue exposure time that allowed for plaque formation in RHPA, found to be 1 hr in my system. Indeed, protein synthesis does not considerably contribute to secretion during this time since emetine, a blocker of translation, had no effect on secretion evoked by either GnRH or PMA in 1 hr incubations (Fig. 9). The action of PMA, unlike that of GnRH, is largely irreversible and plaques may keep forming after its removal. Removal of the complement should stop plaque formation, but the complement is not removed until 30 min after removal of PMA. In effect, I may be comparing a 1 hr exposure to GnRH with a 1.5 hr exposure to PMA, but the number and the size of plaques evoked by GnRH did not change when its incubation time was increased to 1.5 hr (data not shown).

Stimulation by PMA proved to have significantly lower requirements for Ca^{2+} than does stimulation by GnRH: **1)** Blocking Ca^{2+} channels reduced the ability of GnRH to cause LH release, presumably due to depletion of internal Ca^{2+} stores, but had no effect on PMA-induced secretion (Fig. 10). **2)** Buffering $[Ca^{2+}]_i$ by intracellular BAPTA completely abolished GnRH-induced LH release but only partially inhibited PMA-induced secretion (Fig. 10). The free BAPTA concentration achieved inside the cells after BAPTA-AM loading is unknown, and the buffered $[Ca^{2+}]_i$ cannot be calculated, but at all times it must have been lower than the 300 nM required for GnRH-induced secretion [17] and it might have been lower than the resting $[Ca^{2+}]_i$ (100 nM, [17]). The observed partial inhibition of PMA-induced secretion could result from intrinsic Ca^{2+} requirements of exocytotic machinery rather than from Ca^{2+} requirements for signaling. **3)** Our single-cell photometry revealed no changes in $[Ca^{2+}]_i$ during a 1 hr application of PMA (Fig. 11) although the same cells showed clear $[Ca^{2+}]_i$ oscillations when tested with GnRH. This result is different from that obtained by Shangold *et. al.* [46] and

Stojilkovic *et. al.* [47] who did see weak increases in $[Ca^{2+}]_i$ in single gonadotropes of female rats seconds upon application of 1 μM PMA. The difference can conceivably result from different sex of animals or the lower PMA concentration used here. Stojilkovic and his coworkers also reported [36] that an acute (5 min) increase in LH release by PMA is reduced 80-90% upon application of the Ca^{2+} channel antagonist, nifedipine. They attributed this acute effect to activation of voltage-sensitive Ca^{2+} channels in the plasma membrane and the resultant Ca^{2+} influx. We saw no changes in $[Ca^{2+}]_i$ at any time upon application of PKC (Fig. 11). Taken together, the lack of Ca^{2+} requirements for PMA-evoked exocytosis and the absence of changes in $[Ca^{2+}]_i$ during exposure to PMA for 1 hr (sufficient time to evoke secretion) indicate that PKC activation stimulates LH release through a Ca^{2+}-independent mechanism.

It is currently not clear if PKC is involved in rapid GnRH signaling in rat gonadotropes. I found that GnRH-induced secretion is insensitive to a specific PKC inhibitor, BIS (Fig. 8). Interestingly, the general protein kinase inhibitor, staurosporin, is reported to inhibit GnRH-induced LH release even at 1 nM [47] whereas a variety of more specific PKC inhibitors have no effect [39, 35]. I found that 100 nM staurosporin inhibits GnRH action by about 30% (data not shown) perhaps due to inhibition of protein kinases other than PKC. My results indicate that even if activation of PKC by GnRH contributes to LH secretion, GnRH can cause secretion of the same amount of hormone in absence of PKC stimulation. Whether there are physiological ligands that can evoke LH release without $[Ca^{2+}]_i$ rise by stimulating PKC, remains to be elucidated. It has to be noted that screening for stimulators of gonadotropin secretion by monitoring $[Ca^{2+}]_i$ (see Chapter I) will miss such ligands.

In permeabilized sheep pituitary cells, application of cAMP also leads to rapid LH release with PKC and cAMP effects being additive [45]. In the rat, however, I found no effect of a cAMP analogue on either basal or GnRH-evoked LH release in a 1 hr incubation (Fig. 12). This observation is consistent with the consensus opinion that in rats, PKA activation may stimulate LH subunits biosynthesis but not LH secretion.

There seems to be another marked difference between signaling in rat and sheep gonadotropes: in the sheep, effects of GnRH and phorbol ester were additive [35] whereas I found no additivity in the rat (Fig. 7). Therefore, in rat gonadotropes, PKC must act upon the same final pool of vesicles as GnRH does.

What might be the mechanism of PKC signaling in rat gonadotropes?

In rat gonadotropes, PKC has been proposed to modulate voltage-sensitive Ca^{2+} channels [36] and Ca^{2+}-activated K^+ channels [30], to slow $[Ca^{2+}]_i$ oscillations [30], to inhibit the GnRH-induced production of inositol phosphates [48], to enhance gonadotropin biosynthesis [44], and to increase GnRH receptor number and affinity [49]. None of these actions can account for the rapid massive LH release observed here. In endocrine cells, as in neurons, secretory vesicles accumulate beneath the plasma membrane, as if a 'fusion clamp' (possibly, synaptotagmin) blocks a late step in exocytosis [50]. So far, Ca^{2+} has been thought to relieve the block and trigger fusion of vesicles with the plasma membrane. I propose that at least in gonadotropes, PKC also provides a means for removal of the block. A simple model would be that PKC phosphorylates synaptotagmin or synaptotagmin-binding proteins and either removes the 'fusion clamp' or increases its affinity for Ca^{2+}. The amino acid sequence of synaptotagmin contains several potential PKC phosphorylation sites and the isolated protein has been shown to be phosphorylated upon addition of PKC [51]. Interestingly, in chromaffin cells, PKC increases the pool of readily-releasable vesicles by acting before the final Ca^{2+}-dependent step -- possibly by speeding up the maturation of granules to a fully fusion-competent state after they dock with the plasma membrane [52]. The difference in sensitivity of vesicle fusion to PKC may arise from differences in isoforms of synaptotagmins expressed in different cell types [53]. Phosphorylation of synaptotagmin is not the only possible mechanism of PKC action. SNAP-25 and α-SNAP, the components of 20S SNAP-SNARE complex, contain consensus sequences for PKC phosphorylation [54, 55], and one can imagine that upon phosphorylation, SNAP-25 and α-SNAP may undergo conformational changes that cause synaptotagmin unbinding. Alternatively, PKC may phosphorylate a GTP-binding

protein related to that implicated in the late steps of exocytosis in mast cells -- G_E [56] -- or a variety of other PKC targets involved in exocytosis (summarized in 52).

In conclusion, I have demonstrated that in rat pituitary gonadotropes, a PKC activator evokes secretion via a Ca^{2+}-independent mechanism. I propose a model of PKC action on gonadotropin secretion that may also apply to other endocrine cells.

Chapter III

TWO-HORMONE CELL IMMUNOBLOT

Rationale

The work of this chapter was undertaken to develop a new tool for investigating if LH and FSH are secreted by the same cells in a gonadotrope population and if there are agents that can preferentially regulate LH or FSH secreting cells. Unfortunately, these goals were not fully realized. Nevertheless, the rationale and approach are discussed here to aid future reseach.

The LH and FSH *content* in pituitary cell populations has been well studied using immunocytochemistry on ultrathin sections and a dual-labeling technique developed by Childs and coworkers [see 57 for review]. In adult male rat, 70% of all gonadotropes express both gonadotropins, 17% express LH alone and 13% express FSH alone [58]. Gonadotropin *secretion* from individual gonadotropes has been studied less, mainly because of the difficulty of developing good FSH antibodies. Lloyd and Childs [59] used reverse hemolytic plaque assay (RHPA, see Chapter I) to study LH and FSH secretion from female rats but they did not look at secretion of both gonadotropins from the same cells.

The secretion of LH and FSH raises a very interesting question of how does one cell type process and release two hormones differentially. LH and FSH secretion can be regulated with considerable independence: the minute to minute regulation of FSH secretion is not as dependent on GnRH as is the regulation of LH [reviewed in 60]; steroid feedback is more successful in lowering serum LH than FSH [61, 62]; follistatin and inhibin suppress and activin increases FSH but not LH production [reviewed in 63]. The percentage of monohormonal gonadotropes is too low to account for divergent regulation of the two gonadotropins, especially since secretion from monohormonal gonadotropes is lower than that from bihormonal cells [57]. It is possible that appropriate physiological conditions

can drive bihormonal gonadotropes to synthesize and package preferentially only one gonadotropin. Indeed, activin and inhibin change the rate of biosynthesis of FSH but not LH mRNA, and FSH secretion rate changes in parallel to its biosynthesis [64]. Alternatively, some cells that express both gonadotropins may secrete only one to provide for divergent regulation. LH and FSH are reported to be stored in separate regions of the same cell [65] and in different vesicles [66]. These might express or bind different isoforms of proteins involved in exocytosis with different Ca^{2+} affinities or different phosphorylation sites that could provide for alternative ways of regulating exocytosis. In support of this hypothesis, Kile and Nett [67] reported that secretion of LH and of FSH from ovine gonadotropes are regulated quite differently. They found that in 2 hr incubations, GnRH, a Ca^{2+} ionophore and a phorbol ester (phorbol-12-myristate-13-acetate, PMA) all effectively stimulated secretion of LH, but only PMA effectively stimulated FSH secretion. Contribution of *de novo* protein synthesis to LH and FSH secretion during 2 hr was not addressed, but it alone probably could not account for the observed effect. In rat gonadotropes, PMA and GnRH cause LH release from the same pool of vesicles (see Chapter II) but there may be other agents that stimulate LH and FSH vesicles differently there as well.

Here, I wanted to address the following questions. How does the distribution of LH and FSH *secreting* cells compare to that of the LH and FSH *containing* cells? How does this distribution change upon incubation with activin or inhibin? Does GnRH increase the secretion of LH to a greater extent than that of FSH? If GnRH challenge is repeated after 24 hr incubation with inhibin, is FSH secretion preferentially inhibited and if so, did the treatment affect FSH-secreting cells only? In Chapter I, I identified agents that stimulate secretion in subpopulations of gonadotropes in a time period that is probably too short for action via protein synthesis. Do any of these agents preferentially stimulate secretion of LH or FSH? If they do, they would be agents that preferentially act on monohormonal gonadotropes or stimulate secretion of only LH or FSH containing vesicles.

To study divergent regulation of LH and FSH secretion, I need to measure or at least detect LH and FSH secreted by the same gonadotrope. To address rapid, protein synthesis-independent secretion, the method will have to be sensitive enough to detect secretion in exposures of up to 1 hr (in Chapter II, I found that protein synthesis does not contribute to GnRH- or PMA-induced gonadotropin release in 1 hr). To study changes in secretory patterns of a gonadotrope upon incubation with inhibin or activin, I will have to compare secretion from the same cell before and after the incubation. RHPA could be modified to study two hormones secreted by the same cells [68] but it requires very good antibodies at high concentrations. Instead, I chose to modify an immunostaining technique called cell immunoblot assay (CIBA). CIBA was introduced by Kendall and Hymer in 1987 [69] and modified by Arita and coworkers in 1991 [70]. Since 1991, the modified CIBA has been used successfully in several laboratories around the world including those of Jun Arita [70, 71, 72] and Nobuyuki Masumoto [73] on pituitary cells, Hiroshi Matsushita on parathyroid cells [74], Gerry Oxford on pancreatic β-cells [75], and Erwin Neher on dorsal root ganglion neurons [76].

In CIBA as modified by Arita, a protein transfer membrane is placed in contact with plated living cells to absorb peptide hormones as they are secreted. When the membrane is removed, it can be stained by immunocytochemical procedures to visualize spots of hormones secreted by single cells. Arita also introduced two modifications of CIBA that allow detection of two hormones. In *sequential CIBA* [70], the same coverslip of cells is used to blot sequentially onto several membranes. This method can be used for *sequential* detection of two hormones, but it would have the disadvantage that the first hormone challenge probably will alter the response to the second. In *sandwich CIBA* [71], a cell suspension is injected into a chamber formed between two immunoblot membranes that are subsequently processed with different antibodies. A disadvantage of sandwich CIBA is that it might blot the entire protein content of the cell instead of just the secreted hormone.

Here, an alternative method was developed for two-hormone detection -- dual-label CIBA. Also, CIBA and sequential CIBA were modified to increase their sensitivity and to obtain easy-to-analyze permanent records of secretion. Sequential CIBA was not intended for two-hormone detection but for studying how various treatments between two blottings might affect the distribution of LH/FSH secreted ratios. Unfortunately, methodological problems prevented me from answering the original questions. The problems I encountered may be specific to anterior pituitary culture and the methods developed here can feasibly be used in other cell systems.

Results

The main result of this chapter is adaptation of CIBA and sequential CIBA for detection of LH and FSH and development of dual-label CIBA. The detailed protocols of these methods with a list of materials and solutions are given in the Appendix. Here, I will describe the general outlines of the methods and the major modifications that I introduced. I will also discuss the results and the reasons why CIBA developed here was concluded to be inadequate for LH and FSH detection.

The CIBA assay

The sequence of events in CIBA is shown in Fig. 13. Dissociated anterior pituitary cells of adult male rats (see Materials and Methods in Chapter I for dissociation protocol) are plated on coverslips and allowed to attach. Coverslips with etched grids are used. Once cells are attached, they are examined under the microscope and positions of the cells in several grid squares are diagrammed with respect to grid lines and numbers. This allows determination of whether the cells remain after blotting. The coverslip is placed cell-side down onto a protein transfer membrane (PVDF membrane) with a drop of medium in between. Control medium contains low concentration of bovine serum albumin (BSA) for protein transfer; challenge medium contains GnRH or other secretagogues in addition to BSA. The coverslip-membrane sandwich is incubated for 1 hr to allow hormones to be

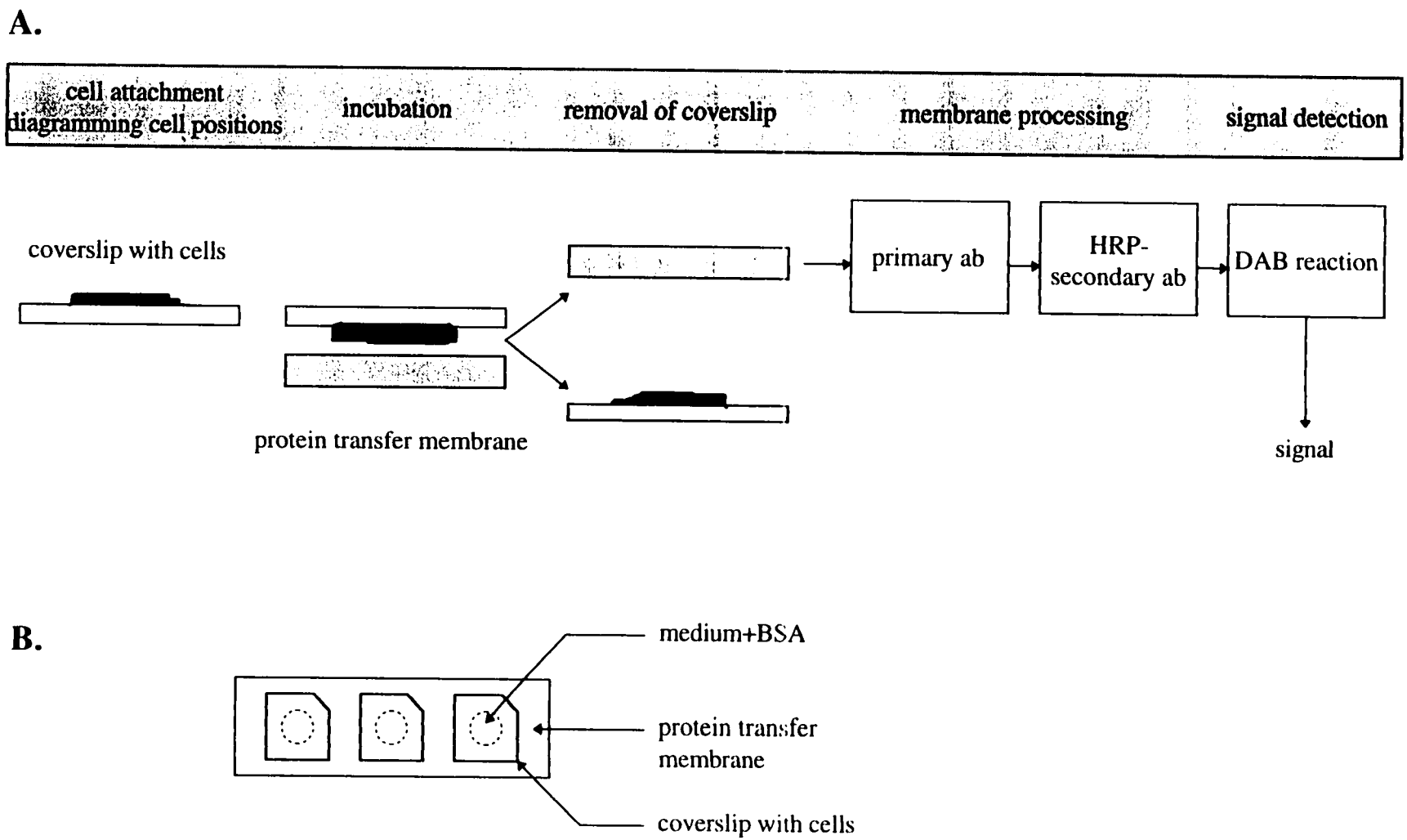


Fig. 13. Diagram of CIBA. A. Steps in CIBA. See text for explanations. primary ab -- primary antibody; HRP-secondary ab -- HRP-coupled secondary antibody. **B. Top view of membrane-coverslip sandwich.**

absorbed by the membrane as they are secreted. Once the coverslip is removed, it is checked under the microscope to make sure that the diagrammed cells are still attached and look healthy. The general cell loss in my experiments did not exceed 5-10% and cells lived for at least 6 days after the blotting. The cell coverslip can be fixed and stained to compare cell positions with positions of secreted hormones (see Discussion). One corner of the coverslip is cut off before the blotting to make orientation of cell positions with respect to blots easier. For sequential CIBA, the coverslip is not fixed but is blotted again onto a different membrane.

The transfer membrane with the absorbed proteins is incubated with BSA at high concentration to block the unoccupied sites and then with antibody against LH or FSH (primary antibody, see Fig. 13). This is followed by incubation with a horseradish peroxidase (HRP) conjugate of immunoglobulin raised against the animal where the primary antibody was raised (HRP-coupled secondary antibody). In the standard CIBA method, a chromagen, diaminobenzidine (DAB), is added at this point and it is converted into a colored precipitate by HRP. This results in the dark brown spots that correspond to hormone secreted by single cells (cell blots). For sequential CIBA, there are two membranes that are developed with antibodies in the same manner.

The major modification of CIBA introduced here was the use of a highly sensitive enhanced chemiluminescence (ECL) technique in place of the traditional DAB reaction. In ECL, HRP catalyzes a chemical reaction that produces light that is captured on photographic film. The darkest spots will correspond to maximal light production, or spots of secretion. We reasoned that ECL is not only a thousand times more sensitive than the DAB reaction but also would give a final record on photographic film leaving the membrane with hormone signal for possible additional future processing. ECL is widely used for protein detection in Western blots where the light is captured on x-ray film. Since x-ray film has too large a grain size for localization and detection of femtograms of protein secreted by single cells, it was replaced here by fine-grain tech pan film. To minimize light scattering, the membrane was positioned as close to the film as possible

with only 0.3 mil plastic in between to avoid wetting the film. In addition, the membrane was pressed onto film with weight.

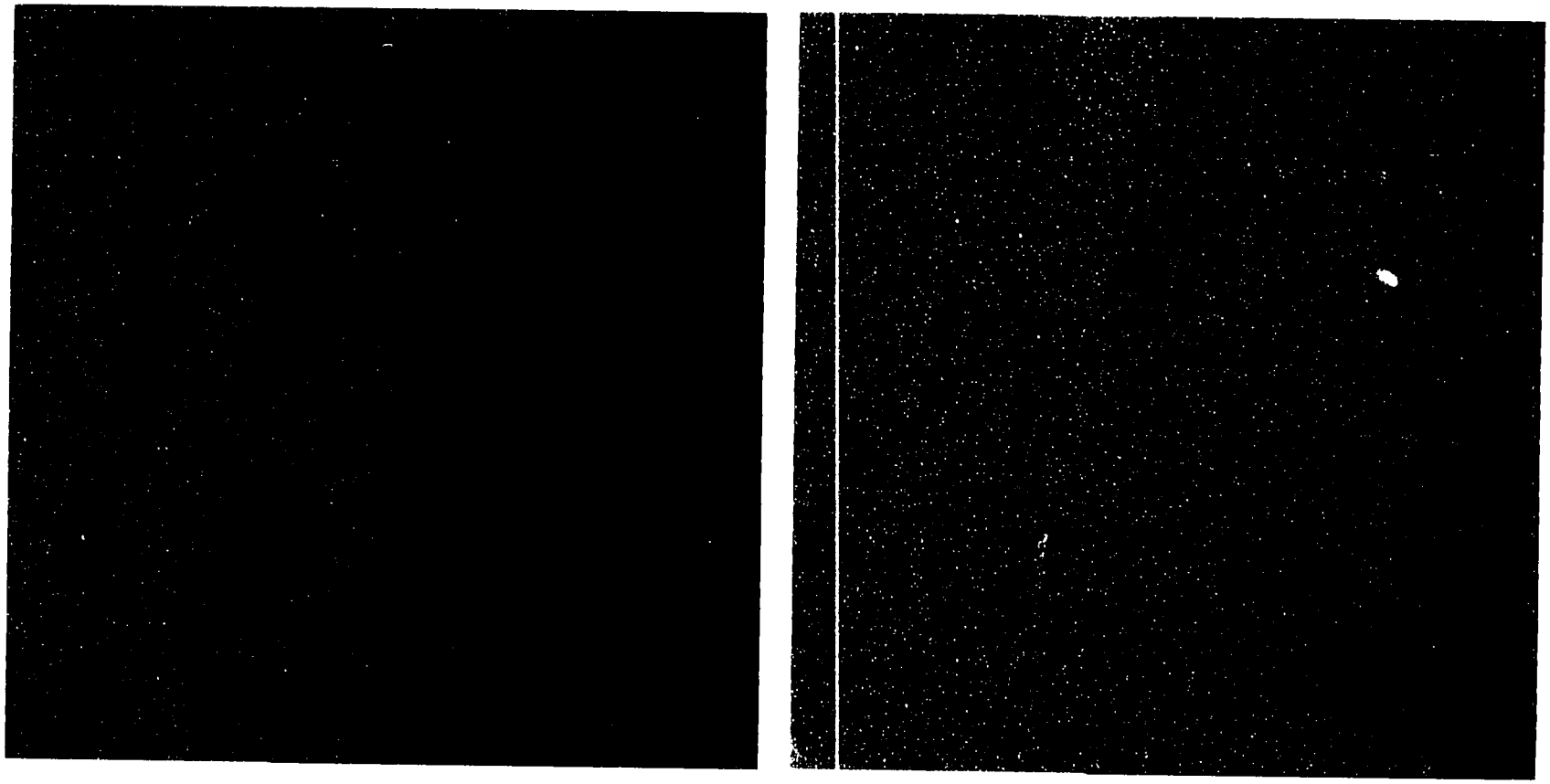
CIBA reports FSH or LH secretion

The developed ECL film is examined under the light microscope equipped with a CCD camera and computerized image-acquisition and analysis program. The image is digitized and stored as an array of gray values between 0 for black and 256 for white, but it usually does not span the entire range. Fig. 14 shows digitized images of two FSH blots obtained with (Fig. 14A) and without (Fig. 14B) 10 μM GnRH in the medium between membrane and coverslip. The dark spots, hundreds of micrometers in diameter, represent spots of FSH release. For comparison, the image of a stained coverslip with cells is shown in Fig. 15, where some cells are marked with red arrows. Notice that cells are much smaller than the spots of secretion. Fig. 14 shows that addition of secretagogue greatly increased the number of secretory spots. Similar pictures were obtained for LH release using LH antibody. Fig. 16 shows digitized images of three LH blots obtained with medium alone (A) and with 1 μM (B) and 10 μM (C) GnRH. In these images, staining intensities are coded by the height and color of bars with higher areas of warmer color corresponding to spots of higher hormone concentration. The concentration of GnRH chosen to obtain Figs. 14A and 16A as well as that used in other experiments was very high (10 μM) because PVDF membrane is designed to absorb proteins and may absorb GnRH. However, as Fig. 16 shows, GnRH was equally potent at 1 μM and 10 μM in evoking LH release (cf. Figs. 16B and C). Several experiments done with 100 nM GnRH also gave similar results, suggesting that absorbtion does not present a problem for a small peptide like GnRH.

Quantification of CIBA

To quantify the amount of secreted hormone, LH and FSH standards of known concentrations are pipetted onto the membrane that is developed in parallel with cell

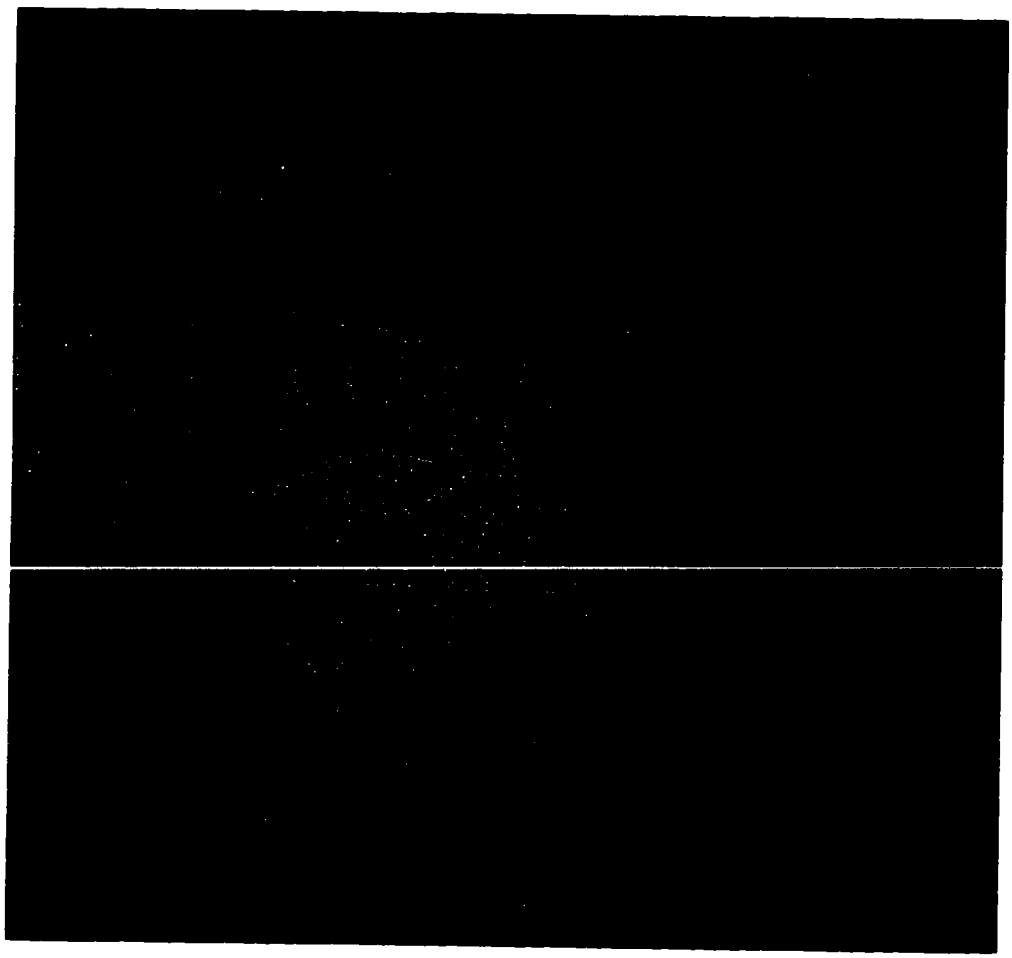
Fig. 14. FSH release reported by CIBA. Pituitary cells cultured on coverslips were incubated with transfer membranes in medium with (**A**) and without (**B**) 10 μM GnRH. Transfer membranes were immunostained using anti-FSH antibody at 1:1,000 final dilution as primary antibody. The staining signal was detected on film by ECL reaction and the image was computerized using a microscope equipped with CCD camera and image acquisition program. The dark spots represent hormone release from individual cells.



A

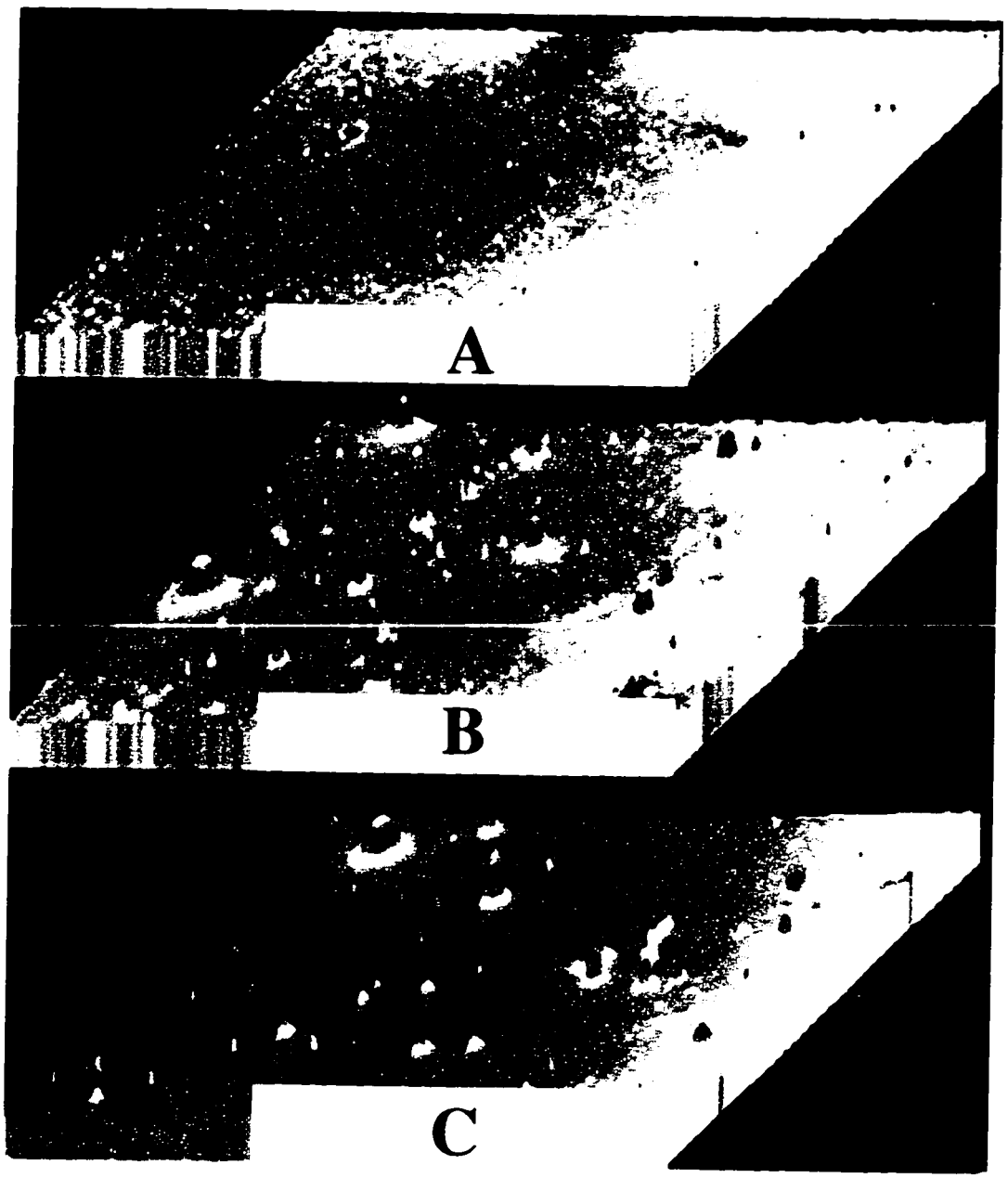
B

Fig. 15. Dissociated pituitary cells on a gridded coverslip. Cells were dissociated, attached to coverslip, blotted onto PVDF membrane and then fixed and stained. Some of the cells are marked with arrows. Fixative:100% MeOH, stain: Giemsa stain at 1:20 dilution. A representative coverslip; not the one used to obtain Fig. 14 or any other blots shown.



250 μm

Fig. 16. False-color three dimensional representation of LH secretion. Pituitary cells cultured on coverslips were incubated with transfer membranes in medium alone (**A**) or in presence of 1 μM GnRH (**B**) or 10 μM GnRH (**C**). The transfer membranes were processed as in Fig. 14 but anti-LH primary antibody was used and the image was further transformed to give a three-dimetional picture where intensity of staining is coded by color and hight of bars. Higher bars of warmer colors represent higher staining intensities and higher hormone concentrations.


A
B
C
1 mm

blots. Average gray values of hormone standards are subtracted from their individual backgrounds and these values are plotted against known concentrations of standards to get a calibration curve. An example of LH calibration curve is shown in Fig. 17. According to this curve, CIBA could be used to quantify LH secretion of between 1 ng/μl and 10 ng/μl. Concentrations of LH below 1 ng/μl did not produce staining intensities darker than background. Similar curves giving the same calibration range were obtained in other experiments for both LH and FSH.

The dual-label CIBA assay

The dual-label CIBA developed for two-hormone detection is diagrammed in Fig. 18. The membrane is incubated with a mixture of two primary antibodies raised in different animals: anti-FSH antibody raised in rabbit and anti-LH raised in guinea pig. This is followed by incubation with HRP-coupled secondary antibody against rabbit IgG, to detect only one hormone -- FSH. The final record of FSH secretion is obtained on the tech pan film using ECL reaction. The signal is then stripped off the membrane by incubation in 0.1 M glycine - HCl, pH 2.2 for 2 hr (acid wash). The acid wash removes the secondary antibody but leaves primary antibodies intact, and the anti-LH antibody can be detected by biotinylated secondary antibody against guinea pig IgG followed by HRP-coupled streptavidin. The two-step reaction (primary antibody followed by HRP-coupled secondary antibody) detects no signal after the membrane stripping. When the anti-LH primary antibody is omitted, no cell blots are detected on the membrane after the acid wash and incubation with biotinylated secondary antibody against guinea pig IgG and HRP-coupled streptavidin.

The dual-label CIBA reports FSH and LH secretion on the same membrane

The dual-label CIBA gives two pictures of the same coverslip that can be aligned to compare positions of FSH and LH secretion spots. Fig. 19 shows secretion pictures of the transfer membrane incubated with a cell coverslip in 10 μM GnRH. Red arrows indicate

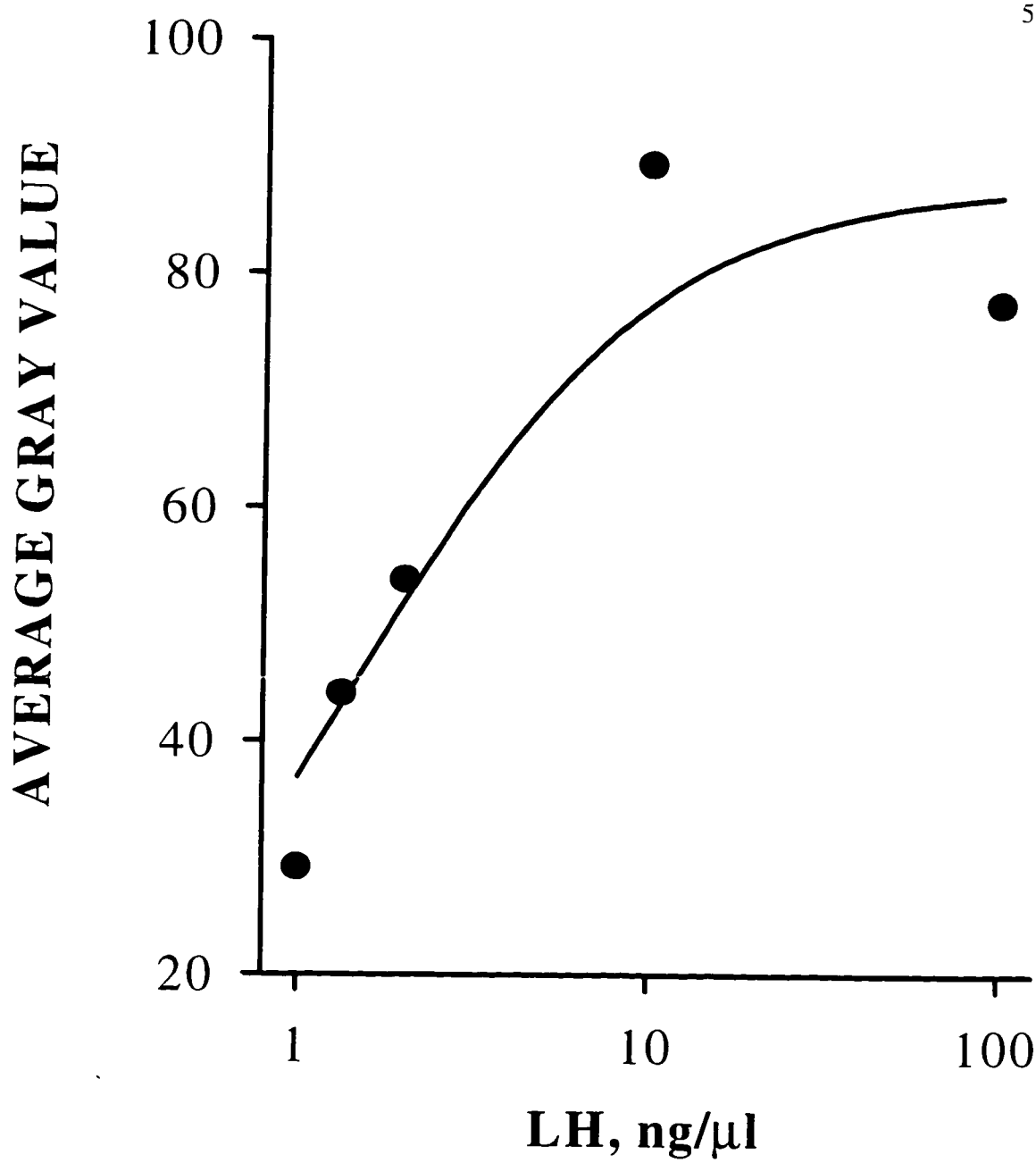


Fig. 17. LH calibration curve. Different concentrations of LH in 1 µl aliquots were applied to transfer membranes and detected with anti-LH antibody. Average gray values on the ordinate were obtained by subtracting average gray values of hormone spots from average gray values of the immediate background. Representative result.

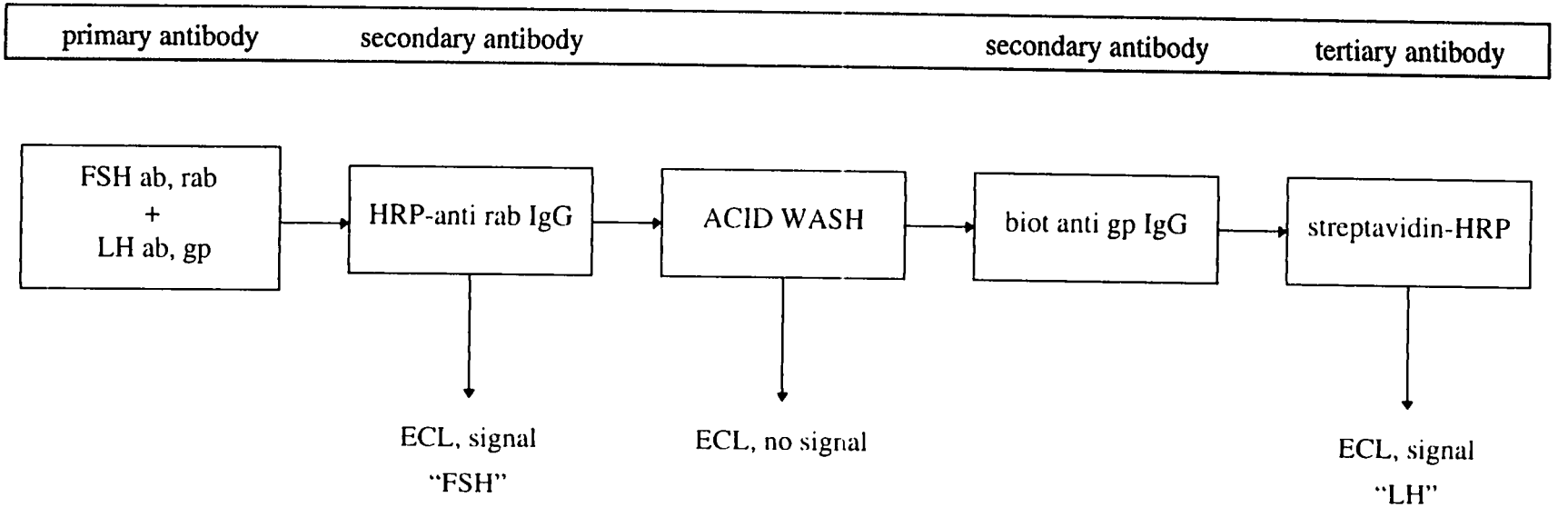


Fig. 18. Diagram of dual-label CIBA. See text for explanations. FSH ab, rab -- anti-FSH antibody raised in rabbit; LH ab, gp -- anti-LH antibody raised in guinea pig; HRP-anti rab IgG -- HRP-coupled anti-rabbit immunoglobulin; biot anti gp IgG -- biotinylated anti-guinea pig immunoglobulin.

cell blots common for both FSH secretion (Fig. 19A) and LH secretion (Fig. 19B). If we assume that each spot comes from one cell (see discussion), then spots with red arrows arose from cells releasing both LH and FSH whereas unmarked spots arose from cells releasing only one hormone above the detection limit. By this criterion, this coverslip had many more LH-releasing than FSH-releasing cells. Fig. 20 shows secretion pictures of another transfer membrane obtained in the same experiment with a different coverslip on which more cells released FSH (Fig. 20A) than LH (Fig. 20B). This coverslip also had a higher percentage of cells secreting both gonadotropins than that in Fig. 19. Fig. 19 has low enough magnification to show that coverslips leave dark imprints on film, with darker circles in the middle where BSA-containing medium first touched the membrane (Fig. 19A). These details of the background are lost after the acid wash (Fig. 19B).

Controls

The CIBA method met several control standards. Cell blots could not be detected after (1) replacement of the primary or secondary antibody or streptavidin-HRP by 3 % BSA; or (2) preabsorption of LH and FSH antibodies with 1 μg/ml LH and FSH for 24 hrs at 4 °C.

Discussion of Problems with the Assay

When regular and dual-label CIBA were used to detect LH and FSH, the final digitized images were consistent with the methods reporting hormone secretion. Indeed, Fig. 14 shows that GnRH greatly increased the number of dark spots on the membrane (cf. Figs. 14A and B) indicating that CIBA detects the released protein and does not blot the entire cell. The protein content of the cell does not depend on GnRH in 1 hr incubation and would be the same in both conditions. There are other indications that CIBA does not blot the entire cell: (1) cell loss did not exceed 5-10 % and (2) no correlation existed between the percent of cells lost and the number of dark spots on the membrane.

Fig. 19. FSH and LH release reported by dual-label CIBA. More LH-secreting cells. Cell coverslip was incubated with transfer membrane in 10 μM GnRH. The membrane was developed to see FSH first (**A**), stripped, and developed again to see LH (**B**). Red arrows indicate cell blots common for both pictures.

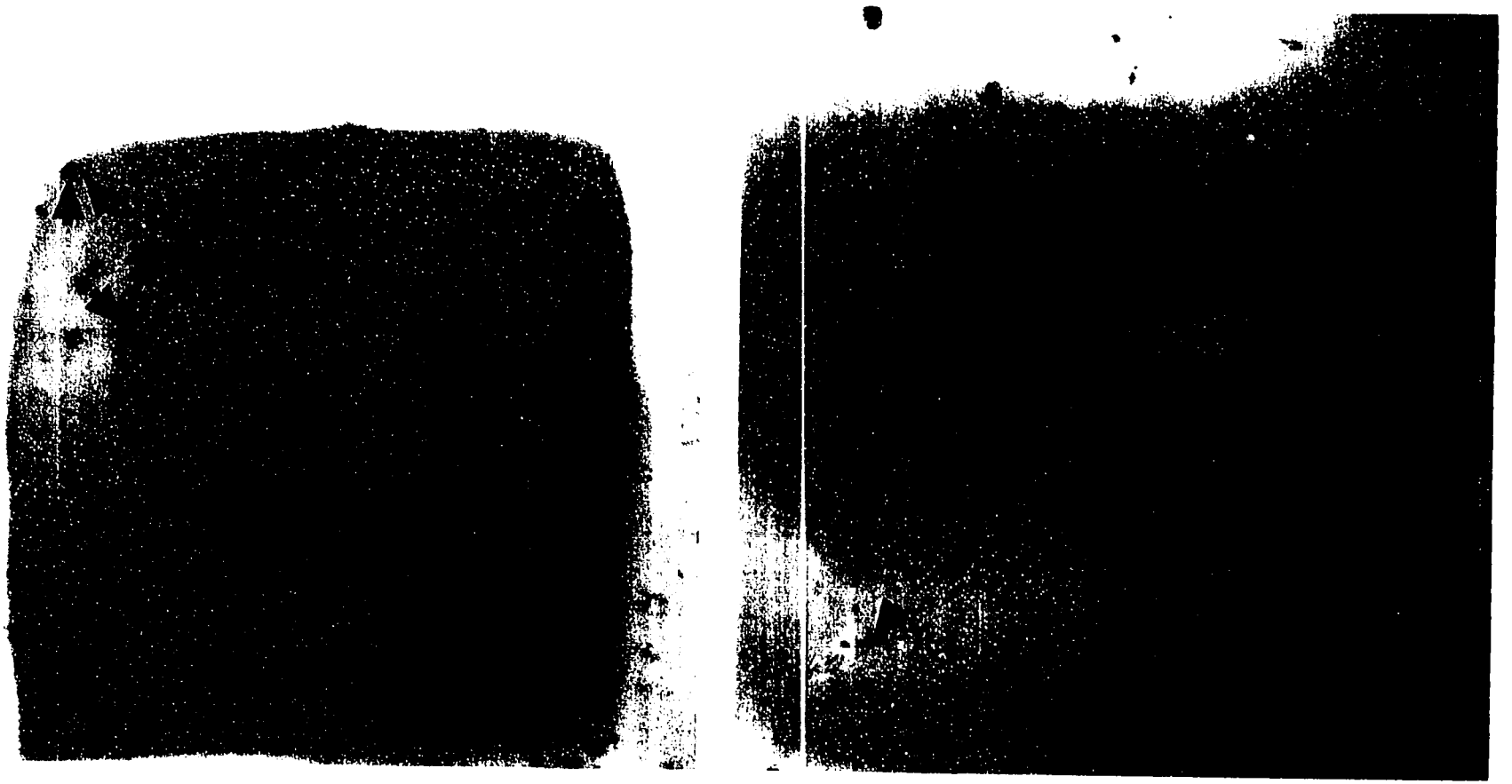
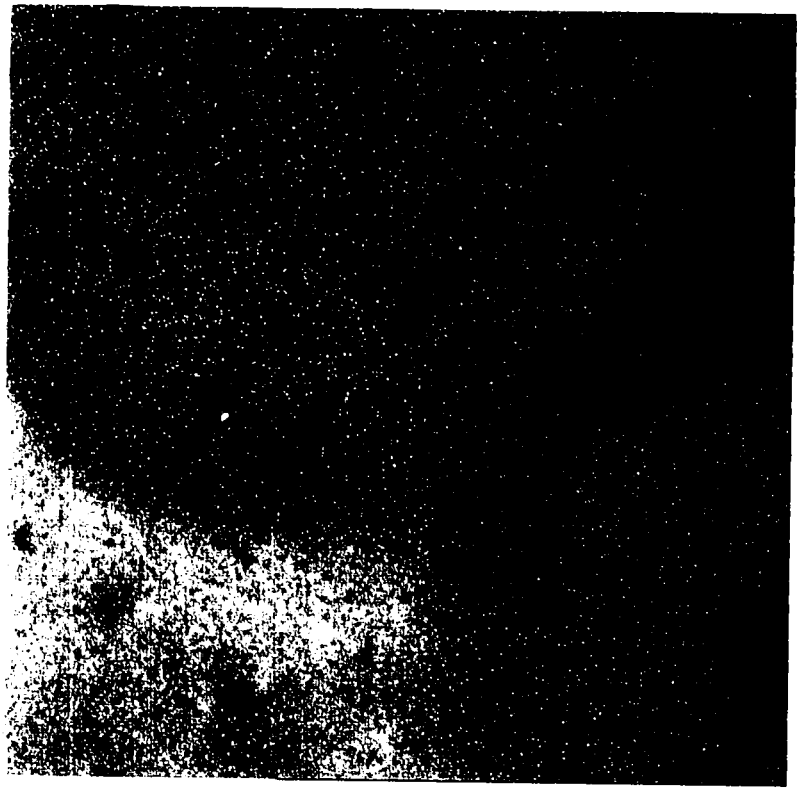
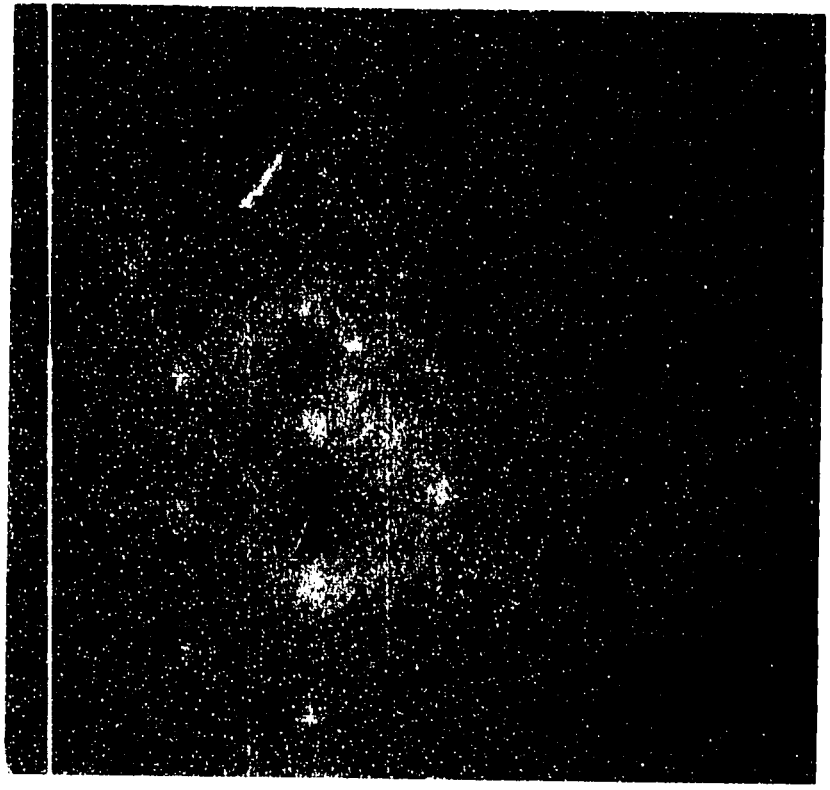

A
1 mm
B

Fig. 20. FSH and LH release reported by dual-label CIBA. More FSH-secreting cells. Obtained as in Fig. 19. **A.** FSH secretion; **B.** LH secretion.



A



B

Antibodies

For dual-label CIBA, I needed anti-LH and anti-FSH antibodies with no cross-reactivity that were raised in different animals. After testing a long succession of antibodies that recognized either both hormones or none, I finally obtained from NIDDK the antibodies with low cross-reactivities (NIDDK-anti-rβLH-IC-2 raised in guinea pig; and NIDDK-anti-rβFSH-IC-1 raised in rabbit). NIDDK was the best source of antibodies that provided me with an opportunity to perform the dual-label CIBA. According to dual-label CIBA, some coverslips had predominantly LH-secreting cells whereas others had mostly FSH secretors (cf Figs. 19 and 20). The percentage of cells secreting both gonadotropins also varied from coverslip to coverslip within the same experiment. Lloyd and Childs [59] found that in enriched gonadotrope populations of female rat gonadotropes, 10% of the cells secreted FSH and 43% secreted LH, but they did not look at the percentage of cells secreting both gonadotropins.

I encountered several problems with using regular and dual-label CIBA on gonadotropes.

Problem of comparison of cell secretion with the distribution of cells on a coverslip

The cell density used in CIBA experiments was low to ensure that each spot comes from one cell. But to confirm that each spot indeed arises from a single gonadotrope, cell blots on the membrane have to be identified with individual cells on the stained coverslip. Such identification must be possible since others report it [71] but I could not do it because cells and cell blots have vastly different sizes and have to be examined at different magnifications (cf. Figs. 15 and Fig. 19).

Problem of quantification

The calibration curve gave usable results in a concentration range of 1-10 ng/μl (Fig. 17). The hormone standards were applied in 1 μl aliquots, so the calibration range corresponds to a mass of 1-10 ng. After blotting, the immunostains of these standards occupied areas

of about $2.6x10^6\mu m^2$, giving the calibration range of approximately 0.4-4 $fg/\mu m^2$. Secretion from individual gonadotropes fell in this range of 0.4-4 $fg/\mu m^2$, with average sizes of secretion spots being about 10^4 μm^2, so according to my assay gonadotropes secrete **4-40 pg/cell**. These values are higher than what other authors report for other peptides. Thus, Arita *et. al.* using CIBA to detect prolactin secretion, report that pituitary lactotropes secrete **0.03-1.92 pg/cell** in 1 hr [70]. Huang and Neher [76] report the average substance P secretion from dorsal root ganglion cells as 0.065-0.08 fmol/cell in 30 min, which corresponds to about **0.1 pg/cell**. Matsushita *et. al.* studying secretion of parathyroid hormone-related protein from parathyroid adenoma and hyperplasia cells [74], report secretion of 0.3-3 fmol/cell in 2 hr (an average of **0.6-6 pg/cell** per hour), and Arita *et. al.* [72] detect that pituitary cells secrete **0.00014-0.14 pg/cell** of substance P in 1 hr.

These comparisons suggest both that my assay is considerably less sensitive than the CIBA assay used by others and that gonadotropes can secrete much more peptide in an hour than other cells do. Such conclusions are quite unexpected as I used ECL that is supposed to be 1,000 times more sensitive than the color reactions used in all the work cited here. Several things may account for the observed discrepancies. Firstly, the low sensitivity of my detection system may reflect a low sensitivity of the antibodies. Indeed, to detect any signal, I had to use both anti-LH and anti-FSH antisera at 1:1,000 dilution which is much higher antibody concentrations than those used for detection of other hormones (for example, 1:60,000 in [70]). Secondly, I applied the standards in 1 µl aliquots and got areas of immunostains of about $2.6x10^6\mu m^2$, whereas Arita *et. al.* had 50 nl drops which gave rise to immunostains of $7x10^5$ μm^2. So, I had 20 times bigger volume (and mass) that resulted in only 4 times bigger blot area making my calibration spots 5 times more concentrated. One difference is that Arita *et. al.* pipetted their standards onto the dry membrane and used wet membrane for cell blotting only, whereas I used wet membranes for both. Finally, there is an issue of the hormone standards themselves. I first attempted to use LH and FSH reference preparations of known concentration (rLH-RP-3 and rFSH-RP-2 obtained from NIDDK). However, I found that not only was the LH standard

recognized by anti-FSH antibody, and the FSH standard, by anti-LH antibody, but that both standards were recognized by secondary anti-IgG antibodies when the primary antibodies were omitted. The standards may contain residual serum that secondary antibodies bind to. Therefore I obtained from NIDDK highly purified preparations of LH and FSH normally used for iodination (rLH-I-9 and rFSH-I-8). These preparations did not give any non-specific binding but they were not designed as standards and their concentration was not given precisely. Also, the purification procedure could have denatured some of the protein making the concentration that is recognizable by the antibody smaller than the total concentration. Arita *et. al.* used similar highly purified standard for prolactin (NIDDK-rPRL-I-5) which might have had a different concentration than LH and FSH samples. Such factors leave uncertainty in the absolute calibrations of these assays.

My calibration curve is also questionable since tech pan film is non-linear and the membrane background luminescence is uneven. Therefore the CIBA assay described here allows to determine the number of secreting cells but not the amount of hormone secreted per cell. This problem might be solved by replacing ECL with the DAB reaction, which will eliminate the use of non-linear photographic film and give more even background due to less sensitivity. Indeed, several laboratories report calibration of hormone secretion using various chromagen reactions [70, 71, 74, 75, 76]. However, the DAB signal cannot be stripped off the membrane and dual-label CIBA would have to be replaced by a double-staining technique. I tried a double-staining technique in which one secondary antibody was coupled to HRP and the other, raised in different species, was coupled to alkaline phosphatase. Then, ECL was used to detect HRP and CSPD or its more sensitive analogue, CDP-Star™, were used to detect alkaline phosphatase. Unfortunately the alkaline phosphatase reaction was so weak that no signal could be detected even with the ultra-sensitive and fast chemiluminiscence substrate CDP-Star™.

Problem of cell detachment

The most serious problem for me became apparent when sequential CIBA was performed. Sequential blots gave two completely different pictures of secretion. Most of the spots were not in the same positions on the two membranes. Moreover, the more secretion spots there were after the first blotting, the fewer there were after the second. It appears that cells that were detected in the first round were unable to secrete again. This suggests that not only sequential CIBA, but also CIBA and dual-label CIBA report hormone release from cells that cannot be considered healthy. Interestingly, Dr. Gwen Childs (personal communication to Dr. Bertil Hille) has seen movements of gonadotropes associated with secretion. It is conceivable that secreting gonadotropes detach from the coverslip as a result of such movements while quiescent gonadotropes and other cells stay on the coverslip and account for the low percentage of cell loss. If this were the case, the results of CIBA and dual-label CIBA would be obtained from healthy cells and could be used, provided also that I am not blotting the entire content of the detached cells. But such "physiological" detachment of secreting cells would be very hard to prove. Masumoto *et. al.* performed CIBA on enriched gonadotropes and did not report any cell loss [73].

Conclusion

We reasoned that the problems with the CIBA methods developed here would prevent reliable study of the secretory behavior of pituitary gonadotropes and analysis of LH and FSH released by the same population of cells. The CIBA project was abandoned once I performed sequential CIBA and found these results.

Nevertheless, the dual-label CIBA developed here as well as the suggested modifications to CIBA can feasibly be used in cell systems other than anterior pituitary cells. The dual-label CIBA allows study of secretion of two different hormones released simultaneously by the same population of cells.

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Appendix

CELL IMMUNOBLOT ASSAY (CIBA) PROTOCOL

The protocol below is that for CIBA but it contains necessary steps to turn it into sequential or dual-label CIBA.

MATERIALS AND SOLUTIONS

Before starting the assay, make sure that you have bought and made the following:

1. Materials

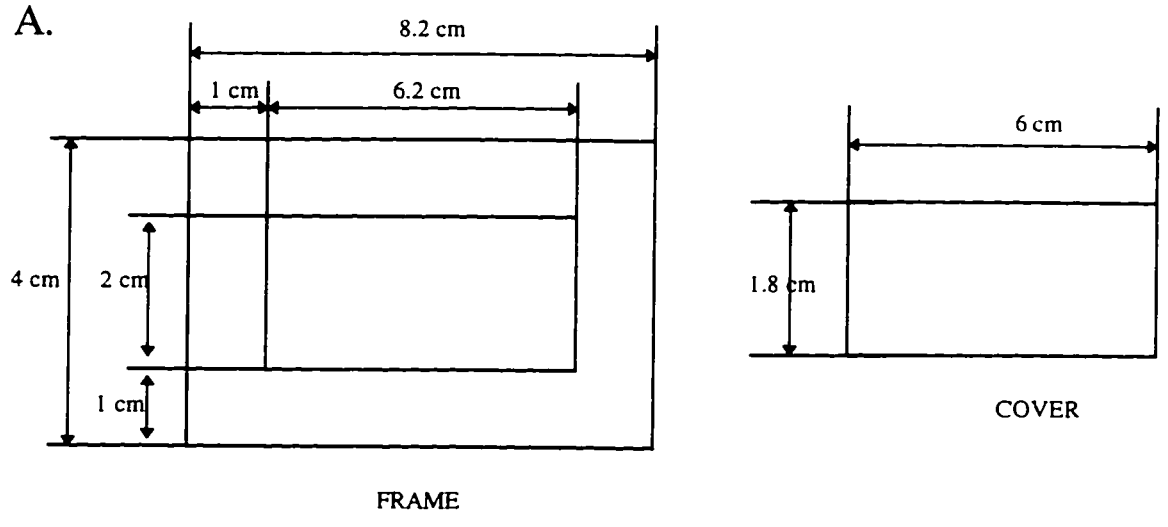
- Everything needed for making cells that you are going to use
- Agonist(s) that stimulates secretion of hormone(s) of interest
- Microscope equipped with CCD camera and computerized image acquisition and analysis program (I used Image-1 and MetaMorph, Universal Imaging Corp, West Chester, PA, for data aquisition and data analysis, respectively)
- Immobilon-P Transfer membrane (PVDF, Millipore Inc., Bedford, MA)
- Two #5 forceps
- Curved or blunt-end forceps (forceps)
- Sterile Petri dishes (35 mm, 100 mm, and 150 mm, VWR Scientific, Seattle, WA)
- Double stick 12 mm scotch tape
- Microslides (25x75 mm, VWR Scientific)
- Slide incubation tray (Newcomer Supply, Middleton, WI)
- Pap Pen hydrophobic slide marker (Newcomer Supply)
- Enhanced Chemiluminescence (ECL) Detection Kit (Amersham, Buckinghamshire, England)
- Technical Pan Film, b/w 4x5 in sheets (Eastman Kodak, Rochester, NY)
- Plastic film, o.3 ml thick (painting dropcloth used for furniture protection, sold in hardware stores)

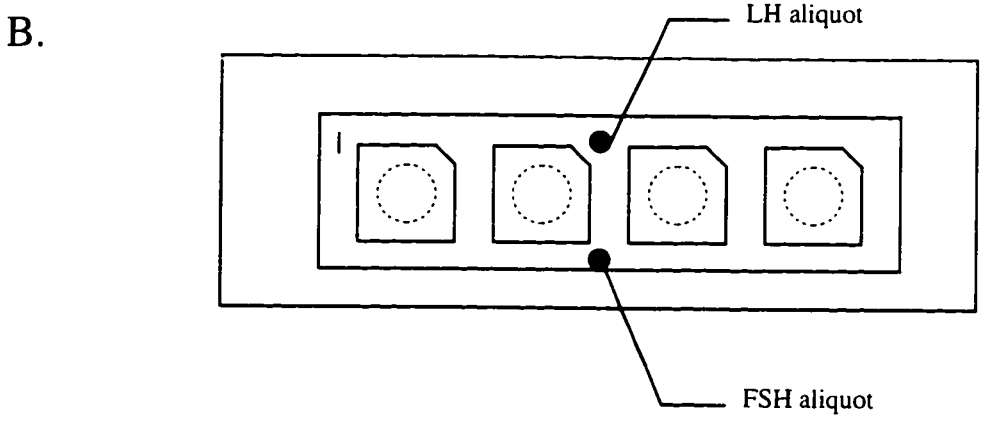
- Developer (HC-110, Eastman Kodak)
- Rapid Fixer: solution A and solution B (Eastman Kodak)
- Ebonite frame for stretching plastic and ebonite cover (better to have two of each since two can fit on 4x5 in film, Fig. 21.A)
- Giemsa Stain Solution (Sigma, St. Louis, MO); keep in fume hood
- ddH_2O, sterile filter
- Methanol, sterile filter
- Poly-L-lysine (M.W. 70, 000 - 150, 000, Sigma); dilute into ddH_2O at 1 mg/ml, store in 1 ml aliquots at -20 °C
- Dulbecco's Modified Eagle Medium (DMEM), low glucose, no HEPES (Gibco, Grand Island, NY)
- Penicillin-V/Streptomycin (Pen V/Strep, Sigma or Gibco); aliquot, keep at -20 °C. One aliquot should be enough for preparation of DMEM-Horse Serum solution (see Assay, Day 1)
- Heat-Inactivated Horse Serum (Gibco), aliquot; keep at -20 °C. One aliquot should be enough for preparation of DMEM-Horse Serum solution (see Assay, Day 1)
- Antibodies and hormone standards:

  Regular and sequential CIBA require one primary and one HRP-coupled secondary antibody. Dual-label CIBA allows to detect two different hormones on the same membrane. It requires primary antibodies against both hormones raised in different animals and secondary antibodies against both of these animals. One secondary antibody should be HRP-coupled; the other, used for second detection, should be biotinylated. Hormone standards for each hormone you want to detect are also needed.

  Below, is the sample list of antibodies and standards for detection of LH and FSH:

  - Anti-LH antibody raised in guinea pig (NIDDK-anti-rβLH-IC-2, AFP-22238790GPOLHB, Dr. A. F. Parlow and NIDDK, Bethesda, MD); reconstitute with ddH_2O, aliquot, keep at -20 °C





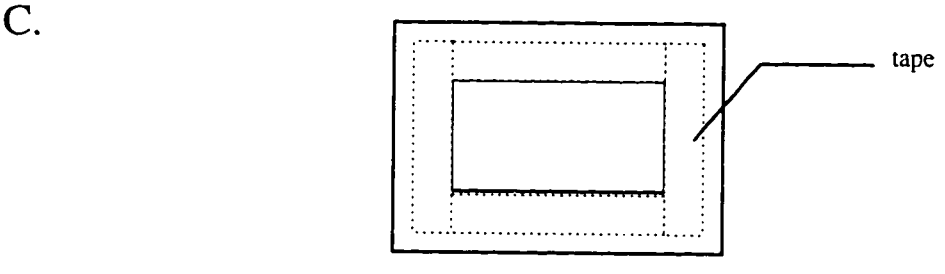


Fig. 21. Apparatus for CIBA protocol. See text for explanations.

Anti-FSH antibody raised in rabbit (NIDDK-anti-rbFSH-IC-1, AFP7798-1289, from Dr. A. F. Parlow and NIDDK); reconstitute with ddH_2O, aliquot, keep at -20 °C

- Biotinylated anti-guinea pig IgG (Vector, Burlingame, CA); keep at +4 °C
- HRP-coupled anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA); reconstitute with 50% ddH_2O: 50% glycerol, keep at -20 °C
- Streptavidin-HRP (Vector); keep at +4 °C
- Rat LH standard (NIDDK-rLH-I-9 AFP-10250C) and FSH standard (NIDDK-rFSH-I-8 AFP-11454B) from Dr. A. F. Parlow and NIDDK; solubilize in PBS at 100 µg/ml, aliquot, keep at -20 °C

2. Solutions

- DMEM-0.04%BSA solution (DMEM/0.04% BSA)
  DMEM, low glucose, with HEPES (Gibco), 50 ml
  0.04% Bovine Serum Albumin fraction V (BSA, Sigma)
  Sterile filter; keep at +4 °C
- Earle's Balanced Salt Solution with HEPES (EBSH)
  20 mM HEPES in 500 ml Earle's Balanced Salt Solution (Sigma)
  Sterile filter; keep at +4 °C
- Phosphate Buffered Saline (PBS)
  Buy powdered PBS in 5 l bottles (Sigma). Use one bottle to make 1 l of 5xPBS.
  Keep at +4 °C. Dilute it out to 1 l 1xPBS as needed, keep at +4 °C
- 10% BSA in PBS (10% BSA/PBS)
  10% BSA in 50 ml PBS
  keep at +4 °C
- 3% BSA in PBS (3% BSA/PBS)
  3% BSA in 50 ml PBS
  keep at +4 °C

2a. Additional solutions for dual-label CIBA

- Glycine - HCl

  0.1 M Glycine in 50 ml ddH_2O

  pH to 2.2 with HCl, keep at +4 °C

- PBS/Tween

  0.1% Polysorbate 20 (Tween 20) in 1 l PBS

  keep at +4 °C

3. Coverslips

Use glass coverslips with photoetched grid (25 mm x 25 mm, Bellco Glass Inc., Vineland, NJ) if you want to monitor cell position throughout experiment. Otherwise, use plastic or glass coverslips (18 mm x 18 mm, VWR Scientific).

Cut out ~10 mm squares; for gridded coverslips, cut out the upper left-hand corner of the grid. Cut off one corner of the square for marking orientation on the membrane. Plastic coverslips can be cut with scissors, and glass, with diamond pencil using reflected light to see the grid.

Soak in MeOH (glass) or EtOH (plastic) for 3-4 days.

Rinse in ddH_2O several times.

Place on filter paper in 150 mm Petri dish and dry in 50 °C oven overnight. Filter paper prevents sticking of coverslips to plastic dish.

Expose each side to UV light for 20-30 minutes. Keep in sterile 150 mm Petri dish.

THE CIBA ASSAY PROTOCOL

Day 1

COATING COVERSLIPS (tissue culture hood)

1. Sterilize forceps in 70% EtOH and then flame.

2. With forceps take out needed number of sterile coverslips and place in 150 mm Petri dish.

3. Place poly-L-lysine solution on coverslips for 5 minutes.

4. Suck up poly-L-lysine. Rinse coverslips in several changes of sterile ddH_2O. Do not remove last change of water to avoid sticking of coverslips to dish.

5. Put coverslips into 35 mm Petri dishes (1 coverslip per dish) and let dry for at least two hours prior to use. Cover the Petri dishes and number them.

MAKING CELLS (mostly in tissue culture hood)

1. Prepare fresh DMEM-Horse Serum solution (DMEM/HS):

10% Horse Serum (1 frozen aliquot)

1% Pen V/Strep (1 frozen aliquot)

in DMEM (low glucose, no HEPES)

Make up enough solution to flood all dishes (~2.5 ml/dish). The size of HS and Pen V/Strep aliquots should be chosen such that one aliquot can be used for preparation. Sterile filter and warm up to 37°C.

2. Euthanize the animal, dissect the organ of interest and digest the cells. (See Ref. 9 for dispersion of pituitary gonadotropes). Resuspend cells at ~100 cells/µl in DMEM/HS. This may take several rounds of dilution.

3. Put 50 µl of cell suspension on each coverslip and leave in 5% CO_2 37 °C incubator for 1 hr for cell attachment. Put cell suspension only in the center of coverslip since cells on the periphery are likely to detach during blotting.

4. Flood the dishes with DMEM/HS and leave in the incubator overnight for further cell attachment.

CUTTING MEMBRANE

1. Handle membrane with forceps. The PVDF membrane has two sides, one of which is water-repellent. Determine the water-repellent side by cutting out a small piece of

membrane and dipping it into water. When taken out, the water-repellent side has only droplets of water whereas the other side is evenly moist.

2. Sheets of membrane are protected on both sides by pieces of blue paper. Take out one sheet with its protective paper. Draw 18x60 mm rectangles on the paper and use a sharp razor blade to cut them out of all three layers. Cut enough strips for all coverslips (at 4 coverslips per strip) plus one strip for calibration. For sequential CIBA, multiply this number by two for two sets of membranes.

3. Lift the corner of blue paper with forceps and use sharp #2 pencil to put numbers in the left-hand corner of water-repellent side of the membrane. Since there will be 4 coverslips per membrane, skip 4 digits between successive membrane numbers: 1, 5, 9 etc. In sequential CIBA, number the second set of membranes by 1., 5., 9., etc. Store with protective paper in 100 mm Petri dish.

Day 2

WETTING MEMBRANES (tissue culture hood)

1. Pour sterile MeOH into 50 ml tube to >6 cm liquid height. Pour sterile ddH_2O and EBSH into 150 mm Petri dishes.

2. Dip one strip of PVDF membrane (no paper!) into MeOH for 20 sec.

3. Put into ddH_2O for 5 minutes. If membrane floats, submerge it with forceps. This is easier if the water-repellent side is up.

4. Put into EBSH for 1 hr to equilibrate. Repeat steps 2-4 for all membrane strips. Try to position strips in EBSH so that they do not cover each other. If they are too crowded, make another dish of EBSH.

-- If you left the membrane in EBSH longer, check the color of the EBSH to make sure that the pH did not change too much. If it turned intensely purple, make up fresh EBSH and reequilibrate the membrane. Usually, 2-3 hours in EBSH are still OK.

-- After the membrane is equilibrated, never let it dry out during the assay. Although the manufacturer says that it can be rewetted by repeating steps 1-4, it never worked in my hands. You can tell the membrane is dry if it turns opaque white.

DIAGRAMMING CELL POSITIONS

-- If you have gridded coverslips, you may draw diagrams of cell positions for some or all of them. This allows you to determine if cells are lost during blotting. If they are, the assay is unreliable since you may be blotting entire cells instead of cell secretion. A good time to diagram cell positions is while the membrane is being equilibrated in EBSH.

Take dishes out of the incubator (no more than 4 at a time). Put a dish under the microscope, pick one grid square and draw a diagram indicating grid lines, grid square number and the position of all the cells inside. Diagram two squares per coverslip: one in the center and one on the periphery. When all four dishes are done, put them back in the incubator and take out the next batch.

PREPARING MOIST CHAMBERS

Put 3 sheets of Kimwipes into 150 mm Petri dish and wet with PBS. Put one 25x75 mm microslide into each chamber; each chamber will hold one strip of membrane.

MAKING COVERSLIP-MEMBRANE SANDWICHES (tissue culture hood)

1. Pour DMEM/0.04% BSA into 100 mm Petri dish. Sterilize #5 forceps in 70% EtOH (do not flame).

2. Take the first four dishes out of the incubator. With forceps take membrane strip #1 out of EBSH and use Kimwipes to suck up the excess of liquid at the edge of the membrane. Put the membrane, water-repellent side up, onto the microslide and put 1 µl drops of undiluted hormone aliquots near the edge of the membrane. Pipette aliquots of all hormones you want to detect -- they are needed as an internal control to make sure that

immunostaining worked. Replace the cover of the moist chamber to prevent membrane from drying out.

3. Put 4 drops of ~100 µl DMEM/0.04% BSA (control) or DMEM/0.04% BSA supplemented with secretagogue (in my case, 1-10 µM GnRH) onto the membrane at equal intervals. Keep the cover of moist chamber on whenever possible.

-- The original protocol calls for 30 µl of medium. I found that more medium helps to keep cells on coverslips intact. Indeed, with only 30 µl of medium, cells around the edges of coverslips are mostly gone, perhaps, because medium dries out there. The problem of more medium is possible coverslip sliding but I did not see any.

-- If using proteins as agonists, keep in mind that they can be absorbed by PVDF membrane, so higher concentrations may be needed to get an effect. However, it does not seem to be a problem for small peptides like GnRH since 1 µM and even 100 nM gave good results.

4. With #5 forceps, take out coverslip #1 and dip it into the dish with DMEM/0.04% BSA for a couple of seconds to rinse off the serum. While keeping the coverslip in solution, lift the cover of the moist chamber with your free hand. Gently position the coverslip onto the membrane with cell side down. Lowering one edge initially and then gradually bringing the rest of the coverslip down works pretty well. Immediately replace the cover of the moist chamber.

5. Repeat steps 3-4 for coverslips 2, 3, and 4, making sure that all the cut-off corners are facing the same direction (Fig. 21.B).

6. Put the moist chamber in the incubator for 1 hr for hormone transfer (blotting). Put empty dishes with medium in the incubator. Finish all moist chambers.

PIPETTING DILUTION SERIES FOR CALIBRATION (tissue culture hood)

1. Prepare serial dilutions of hormone standards.

-- Start with dilutions from 1 to 1:10,000 in 10 times increments. After immunostaining, determine what dilutions result in different staining intensities. Photographic film is non-linear and saturates fast. In my experience, different staining intensities were obtained in the range of 1-10 ng/µl for both LH and FSH. In all subsequent experiments, prepare 5 dilutions within the range of different intensities. You need to have a calibration membrane in every experiment.

2. Put calibration membrane on a slide in a moist chamber.

3. Pipette 1 µl of each dilution onto the membrane. Prepare and pipette dilution series for all hormones you want to detect. Put in the incubator for 1 hr.

PREPARING INCUBATION TRAY FOR MEMBRANE IMMUNOSTAINING

Slide incubation tray is a moist chamber that consists of a saline reservoir and slide supports elevated above the level of liquid. Fill the saline chamber with PBS and position microslides on supports (one microslide per membrane.)

LIFTING COVERSLIPS (tissue culture hood)

1. Take out the first moist chamber and the first four culture dishes. Raise the first coverslip slightly above the membrane by adding DMEM/0.04% BSA around it. Add the minimum amount necessary to raise the coverslip without letting it slide on the membrane because sliding may cause cells to come off the coverslip.

2. Take two #5 forceps and position one tip of each in the liquid between the coverslip and the membrane. Simultaneously lift two opposite ends of the coverslip. Try to avoid moving the coverslip around. Put the coverslip back into the dish with its number. Once all four coverslips are removed, put the dishes in the incubator.

3. Go to 'blocking unoccupied sites on membranes' for membrane #1 before taking out the next moist chamber to avoid membrane drying.

BLOCKING UNOCCUPIED SITES ON MEMBRANES

1. Put the membrane, water-repellent side up, on the microslide in the incubation tray. With pap pen draw two lines on a microslide along the vertical sides of the membrane to restrict the spread of blocking solution. Do not touch membrane with pap pen.

2. Using 5 ml Pipetman, pour 10% BSA/PBS solution onto the membrane until it is completely covered. This may take a while since the membrane is water-repellent.

3. Take the next moist chamber out of the incubator and lift the coverslips. When all membranes including calibration membrane are covered with 10% BSA/PBS, incubate for 4 hrs at room temperature.

-- You can also incubate overnight at +4 °C. If so, washing membranes and incubation with primary antibody will be done on day 3, and all other days will shift accordingly.

WASHING MEMBRANES

Wash membranes in PBS three times for 10 minutes. For best results, pour PBS into 100 mm Petri dishes and put each membrane in an individual dish. Put on rotator for 10 min. Pour out used PBS and replace with fresh PBS. Repeat twice (three washes total).

INCUBATING WITH PRIMARY ANTIBODY

1. Put membranes into the incubation tray, water-repellent side up; draw pap pen lines and apply primary antibody in 3% BSA/PBS solution. For dual-label CIBA, apply a mixture of two primary antibodies in 3% BSA/PBS solution.

-- Concentration of antibody has to be determined experimentally. I used anti-FSH antibody at 1:1,000 final dilution. For dual-label CIBA, I used anti-FSH antibody and anti-LH antibody at 1:1,000 and 1:2,500 final dilution, respectively. Since good antibodies are hard to find/expensive, make up the minimum amount of solution necessary to cover entire membrane (there is no way to tell where the coverslips were). You need ~65 $\mu l/cm^2$.

2. Incubate overnight at RT. If the background signal is too high, you may lower the antibody concentration and incubate at +4 °C.

CHECKING CELL POSITIONS

Look at the same grid squares as before to make sure that blotting did not alter cell positions. Use different color pencil to mark any cells that are gone or new cells (i.e. moved from somewhere else.) Decide if your cell loss is indeed negligible. If it is not, you need to be more careful in handling the coverslips and may try different coating. Coverslips can be fixed and stained or used again on another membrane (see Day 3 continued, sequential CIBA and Day 4, sequential CIBA).

FIXING AND STAINING CELLS (fume hood)

-- Cell coverslips can be kept in the incubator for 1-2 days before fixing and staining. Or it can be done during membrane incubation with 10% BSA/PBS.

1. Using serological pipette with pipette pump, pour filtered MeOH into N wells of a 24-well plate, where N is the number of cell coverslips. Take coverslips out of the incubator. Dip each coverslip into a well with forceps, making sure the number of the coverslip and the well coincide. Incubate for 10 min.

2. Rinse in ddH_2O by positioning in successive order around the perimeter of a water-filled 150 mm Petri dish.

3. Dilute Giemsa 1:20 with ddH_2O to a final volume of ~3 ml*N. Prepare N numbered 35 mm Petri dishes and fill them with Giemsa solution using syringe with 0.22 μm filter. Take each coverslip out of water and put into a dish with its number. Incubate for 10 min. If cells come out too pale, increase incubation time to 15-20 min.

4. Rinse in ddH_2O as after formalin.

5. Dry between two layers of filter paper in covered 150 mm Petri dish for dust protection. Coverslips are now ready for microscopic examination.

Day 3

WASHING MEMBRANES

Wash the membranes in PBS three times for 10 minutes.

INCUBATING WITH SECONDARY ANTIBODY

1. Put in the incubation tray, water-repellent side up; draw pap pen lines and apply ~65 $\mu l/cm^2$ of HRP-coupled secondary antibody at 1:500 in 3% BSA/PBS. I used HRP-coupled anti-rabbit IgG.

2. Incubate for 1 hr at RT.

WASHING MEMBRANES

Wash the membranes in PBS twice for 10 minutes. Keep each membrane in 50 ml Falcon tube completely covered by fresh PBS at +4 °C until ECL detection.

-- If performing sequential CIBA, go to Day 3, sequential CIBA, continued.

ECL DETECTION (photo dark room)

-- ECL detection does not have to be done on the same day membranes are made. Signal can still be detected 2-3 days later. I did not have longer delays and would not recommend it although signal may still be there.

-- ECL detection is based on capturing luminescence on extremely light-sensitive film. Light, including red light, can be on only during preparatory steps. In the dark room, all membranes can be kept at RT in a 150 mm Petri dish filled with PBS. Another such dish should be prepared for washing. The ECL detection kit (Solution 1 and Solution 2) should be kept on ice.

1. Put double-stick scotch tape on all sides of the top of ebonite frames (Fig. 21.C). Make sure there are no gaps or overlaps of tape. Stretch out 0.3 ml plastic sheet on the

taped side of the frames until plastic is absolutely smooth. Plastic will be held in place by tape, which is why tape has to cover frames evenly.

2. Prepare developer and fixer according to manufacturer's instructions (use dilution B for developer).

3. Take one membrane out of PBS, suck up excess fluid with Kimwipes, and put in 100 mm Petri dish, water-repellent side up. Put on 250 µl of Solution 1, add 250 µl of Solution 2 and shake the membrane gently side to side for mixing. Do not premix Solutions 1 and 2, as mixing activates them. 500 µl of mixture is the minimum amount necessary to cover an 18x60 mm membrane. Prepare one more membrane.

-- According to manufacturer's specifications ECL reaction peaks in 5-20 min after mixing solutions, but in my experience it peaks in 2-5 min. Do not exceed 5-6 min between exposure of membrane to solutions and film to membrane. The reaction decays slowly thereafter with a half-life of ~1 hr but for extremely low signal you may try to collect light for 5 hrs or more.

4. Shake off excess solution and put membranes, water-repellent side up, on the ebonite covers. To avoid air bubbles, use forceps to position the membrane with one end touching the cover and slowly pull the membrane onto the cover.

5. Put covers inside frames so that membranes lie on plastic. Use Kimwipes to gently remove any air bubbles but avoid tearing or stretching the plastic.

-- If plastic tears or thins out unevenly, disassemble the frame, return the membrane to PBS to avoid drying, put new plastic on, and repeat steps 3-5.

6. Turn off the light; take out a piece of Technical Pan film and position it on a hard flat surface using the notch for orientation. Position both frames with membranes on film and put a heavy, evenly distributed weight on top (you may use a lead brick). Insufficient weight will result in a blurred picture.

7. Expose film for the necessary time.

-- Exposure time can vary between 10 sec and 5 hrs depending on the amount of the signal. This, in turn, depends on strength and dilution of primary and secondary antibodies as well as on the amount of hormone secreted by cells. You have to determine it each time you change anything in the protocol. Start off with one membrane in a frame, leave it on film for 30 sec, move it lower on the same film, leave on for 90 sec, move lower, leave on for 5 min, then develop the film.

8. Lift off the frames, but do not disassemble them until film is developed and fixed and light is on so you can return membranes to PBS.

9. Put film into fixer for 6 min, agitate constantly. Rinse with running H_2O and place in developer for 2 min, agitate constantly. Put in running H_2O for 5 min. Turn on the light.

10. Lift off ebonite covers and put membranes into fresh PBS. If you are not satisfied with the picture on film, you can repeat detection with the same membrane. The membrane can be exposed to ECL solutions any number of times provided it is well rinsed in between. New plastic has to be put on the frame every time. For dual-label CIBA, the membrane can be stripped of the signal and developed again to detect a different hormone (see Day 4, dual-label CIBA).

11. Hang up the film to dry. It is now ready for microscopic examination.

Day 3 continued, sequential CIBA

Repeat all steps of day 2 using the second set of membranes. Hopefully, you will find that the cells stayed in their places and there is no need to draw new diagrams.

Day 4, sequential CIBA

Repeat all steps of day 3 with the second set of membranes. Keep each membrane in 50 ml Falcon tube completely covered by fresh PBS at +4 °C until ECL detection.

Day 4, dual-label CIBA

WASHING MEMBRANES

Take membranes that have already been immunostained and photographed out of the fridge. Wash in PBS twice for 10 min.

STRIPPING MEMBRANES

1. Prepare the incubation tray as before, place membranes on microglasses and draw pap pen lines.

2. Use 5 ml Pipetman to completely cover with glycine - HCl. Incubate for 2 hrs.

WASHING MEMBRANES

1. Wash in PBS/Tween twice for 10 minutes.

2. Wash once in PBS for 10 min.

INCUBATING WITH SECONDARY ANTIBODY

Incubate in the incubation tray with biotinylated secondary antibody at 1:200 in 3% BSA/PBS for 1.5 hrs at RT. I used biotinylated anti-rabbit IgG.

WASHING MEMBRANES

Wash in PBS three times for 10 minutes.

INCUBATING WITH STREPTAVIDIN-HRP

Incubate in streptavidin-HRP at 1:800 in PBS for 1 hr at RT.

WASHING MEMBRANES

Wash the membranes in PBS two times for 10 minutes. Keep each membrane in 50 ml Falcon tube completely covered by fresh PBS at +4 °C until ECL detection.

Later

ANALYZING DATA

The ECL film is a negative with darker areas corresponding to more light production. Coverslips leave dark imprints on film, with darker circles in the middle where DMEM/ 0.04%BSA first touched the membrane. Film is examined under the light microscope equipped with CCD camera and computerized image-acquisition and analysis program. The image is digitized and stored as an array of gray values.

To convert gray values into hormone concentrations, use calibration membranes immunostained in the same experiment. Determine average gray values of hormone standards, subtract them from individual backgrounds and plot these values against known concentrations of standards to get a calibration curve.

In sequential CIBA, compare secretion pictures on two membranes incubated with the same coverslip.

In dual-label CIBA, align the two pictures of the same coverslip obtained with different antibodies. This might be difficult if all that coincides is the imprint of the coverslip. But if there is some overlap in secretion spots of two hormones, the alignment is quite easy.

Vita

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