THE REGULATION OF LUTEINIZING HORMONE EXOCYTOSIS IN α-TOXIN PERMEABILIZED SHEEP ANTERIOR PITUITARY CELLS

by

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ABSTRACT

Although exocytosis is the major mechanism by which cells secrete products into their environment, little is known about the mechanism of this fundamental process. Previous studies on the regulation of luteinizing hormone (LH) exocytosis have used intact cells exclusively. It is not possible, however, to determine the precise requirements for exocytosis in intact cells since the cytosol is not directly accessible. Permeabilization of the plasma membrane allows experimental manipulation of the intracellular milieu while preserving the exocytic apparatus. The diameter of the α-toxin pores (2-3 nm) allowed the exchange of small molecules such as ATP while larger cytosolic proteins such as lactate dehydrogenase were retained. Because of the slow exchange of small molecules through α-toxin pores a protocol was developed which combines prolonged pre-equilibration of the permeabilized cells at 0°C before stimulation with strong Ca^{2+} buffering. Under these conditions an increase in the $[Ca^{2+}]_{free}$ stimulated a 15-20 fold increase in LH exocytosis (EC_{50} pCa 5.5). After 12-15 minutes the rate of exocytosis declined and the cells became refractory to Ca^{2+}. At resting $[Ca^{2+}]_{free}$ (pCa 7), cAMP stimulated a rapid, 2 - 3 fold, increase in LH exocytosis. cAMP caused a modest enhancement of Ca^{2+}-stimulated LH exocytosis by causing a left shift in the EC_{50} for Ca^{2+} from pCa 5.6 to pCa 5.9. Activation of protein kinase C (PKC) with phorbol 12-myristate 13-acetate (PMA) synergistically enhanced cAMP-stimulated LH exocytosis, an effect which was further augmented by increasing the $[Ca^{2+}]_{free}$. Gonadotrophin-releasing hormone (GnRH) was found to stimulate cAMP production in intact pituitary cells. Since previous studies have shown that GnRH activates PKC and stimulates a rise in cytosolic $[Ca^{2+}]_{free}$, these results suggest that a synergistic interaction of the cAMP, PKC and Ca^{2+} second messenger systems is of importance in the mechanism of GnRH-stimulated LH exocytosis.

When permeabilized cells were equilibrated for prolonged periods in the absence of MgATP, Ca^{2+}-stimulated LH exocytosis declined. The time course of the decline closely followed the leakage of intracellular ^{14}C-ATP. Addition of MgATP rapidly restored full Ca^{2+} -stimulated LH exocytosis. Ca^{2+}-, cAMP-, and PMA-stimulated LH exocytosis were all dependent on millimolar MgATP concentrations (EC_{50} 1.5-3 mM).

It has been postulated that PKC is a mediator of Ca^{2+}-stimulated exocytosis. Several findings in the present study argue against this hypothesis. Firstly, PMA and Ca^{2+} had additive effects on LH exocytosis at all $[Ca^{2+}]_{free}$. Secondly, PMA was able to stimulate further LH release from cells made refractory to high $[Ca^{2+}]_{free}$. Thirdly, the PKC inhibitor staurosporine did not inhibit Ca^{2+}-stimulated LH exocytosis under conditions in which it inhibited PMA-stimulated exocytosis. Fourthly, in cells desensitized to PMA by prolonged exposure to a high PMA concentrations, Ca^{2+}-stimulated LH exocytosis was not inhibited. And finally, Ba^{2+} was able to stimulate LH exocytosis to a maximal extent similar to Ca^{2+} despite the fact that Ba^{2+} is an extremely poor activator of PKC. Since Ba^{2+} is also a poor activator of calmodulin, this latter result implies that calmodulin does not mediate the effect of Ca^{2+}. In agreement with this, the calmodulin inhibitor calmidazolium did not inhibit Ca^{2+}-stimulated LH exocytosis.

Since GTP-binding proteins have been implicated in regulated exocytosis in other cell systems, the effects of guanine nucleotides on LH exocytosis were examined. At resting cytosolic $[Ca^{2+}]_{free}$ (pCa 7), the GTP analogues GTPγS and GMPPNP stimulated LH exocytosis with similar potencies (EC_{50} 20-50 μM). Additional experiments indicated that the effects of these GTP analogues could not be explained by activation of either PKC alone or cAMP-dependent protein kinase alone.

In the presence of both PMA and cAMP, GMPPNP did not stimulate a further increase in the rate of LH exocytosis, suggesting that the stimulatory actions of guanine nucleotides may be mediated by the combined activation of PKC and generation of cAMP, as a result of activation of signal-transducing G proteins. In contrast, pretreatment of cells with GTPγS at low $[Ca^{2+}]_{free}$ markedly inhibited subsequent responses to Ca^{2+}, cAMP, PMA, and cAMP plus PMA. This inhibitory effect required lower GTPγS concentrations than the stimulatory effect (IC_{50} 1-10 μM), and was not observed with GMPPNP. These findings indicate the involvement of a distinct guanine nucleotide-binding protein in exocytosis at a site distal to second messenger generation.

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(2) van der Merwe, P.A., Millar, R.P., Wakefield, I.K., and Davidson, J.S. Mechanisms of luteinizing hormone exocytosis in α-toxin-permeabilized sheep gonadotropes (1989) Biochemical Journal 264:901-908

(3) van der Merwe, P.A., Millar, R.P., and Davidson, J.S. Calcium stimulates luteinizing hormone (LH) exocytosis by a mechanism independent of protein kinase C (1990) Biochemical Journal 268:493-498

(4) van der Merwe, P.A., Millar, R.P., Wakefield, I.K., Macrae, M.B., and Davidson, J.S. Staurosporine enhances gonadotropin-releasing hormone-stimulated luteinizing hormone secretion (1990) FEBS Lett 267:111-113

(5) Macrae, M.B., Davidson, J.S., Millar, R.P., van der Merwe, P.A. cAMP stimulates luteinizing hormone exocytosis in permeabilized sheep anterior pituitary cells : synergism with protein kinase C and Ca^{2+} (1990) Biochemical Journal 271:635-639

(6) van der Merwe, P.A., Millar, R.P., Wakefield, I.K., Davidson, J.S. Inhibition of luteinizing hormone exocytosis by GTPγS reveals involvement of a GTP-binding protein distal to second messenger generation (submitted)

CONTENTS PAGE

LIST OF TABLES

LIST OF ABBREVIATIONS USED

$[Ba^{2+}]_{free}$: free Ba^{2+} concentration

$[Ca^{2+}]_{free}$: free Ca^{2+} concentration

ATPγS : adenosine 5'-O-(3-thiotriphosphate)

BSA : bovine serum albumin

Ca/CAM Kinase II : Ca^{2+} and calmodulin dependent protein kinase II

DAG : diacylglycerol

EC_{50} : concentration effecting half-maximal response

EGTA : [ethylenebis(oxyethylenenitrilo)]tetraacetic acid

ER : endoplasmic reticulum

F-actin: filamentous actin

GDPβS : guanosine 5'-(β-thio)diphosphate

GMPPMP : guanosine 5-(β,γ-imido)triphosphate

GnRH : gonadotrophin-releasing hormone

GTPγS : guanosine 5'-(γ-thio)triphosphate

HEPES : 4-(2-hydoxyethyl)-1-piperazineethanesulfonic acid

IBMX : 3-isobutyl-1-methylxanthine

LH : luteinizing hormone

MEM : Minimal Essential Medium

Me_2SO : dimethylsulfoxide

pBa : $-\log[Ba^{2+}]$

pCa : $-\log[Ca^{2+}]$

PIC : phosphoinositidase C

PIPES : 1,4-piperizinediethanesulfonic acid

PKA : protein kinase A

PKC : protein kinase C

PMA : phorbol 12-myristate 13-acetate

SEM : standard error of the mean

TFP : trifluoperazine

TGN : trans-Golgi network

1 GENERAL INTRODUCTION

Exocytosis is the major mechanism by which cells export material into their environment. Until recently little was known about the mechanism of this fundamental process. However, the development of cell permeabilization techniques over the past decade has coincided with a recent dramatic increase in exocytosis research. The first part of this introduction (Section 1.1) reviews what is known about exocytosis. The second part of the introduction (Section 1.2) provides an overview of the models used for studying exocytosis as well as the various cell permeabilization techniques available. α-Toxin permeabilization is described in some detail. The regulation of exocytosis in gonadotropes is discussed in the final part of the introduction (Section 1.3) but only briefly since it is dealt with in more detail in later sections.

1.1 EXOCYTOSIS

1.1.1 Regulated and constitutive exocytosis.

Proteins destined for export from the cell are synthesized by ribosomes associated with the endoplasmic reticulum (ER), and pass into the ER cisternal space during or soon after synthesis (Alberts et al., 1989; Verner and Schatz, 1988). After preliminary processing in the ER these proteins are transported within membrane bound vesicles through the Golgi apparatus where processing is continued (Palade, 1975; Burgess and Kelly, 1987). On leaving the Golgi apparatus, secretory proteins enter the trans-Golgi network (TGN; Griffiths and Simons, 1986) where they are sorted into one of two pathways, both of which are present in anterior pituitary cells (Kelly, 1985; Burgess and Kelly, 1987). Some proteins (eg. albumin, immunoglobulin G) are transported directly to the plasma membrane in membraneous vesicles

which fuse with the plasma membrane, releasing their contents to the cell exterior in a continuous, unregulated manner. This process is referred to as **constitutive exocytosis**. In contrast, proteins such as hormones are packaged in large amounts into secretory vesicles which are stored in the cell, only fusing with the plasma membrane when an appropriate signal is received. The latter process is called **regulated exocytosis** and is the mechanism by which specialized secretory cells such as neurons, exocrine, and endocrine cells release their secretory products. Biogenic amines such as catecholamines and acetylcholine are also stored in specialized secretory vesicles before release by regulated exocytosis although clearly their synthetic pathway differs to that of proteins (Pollard et al., 1985).

1.1.2. Stimulus-secretion coupling.

Many cell types secrete hormones or other products in response to a stimulus. For example, anterior pituitary gonadotropes secrete the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) when stimulated by the hypothalamic decapeptide gonadotrophin-releasing hormone (GnRH) (for reviews see Hazum and Conn, 1988; Huckle and Conn, 1988). The process by which GnRH stimulation leads to gonadotropin secretion is referred to as stimulus-secretion coupling and may be divided into two stages. The first stage begins with the binding of GnRH to its cell surface receptor and results in the generation of intracellular second messengers such as Ca^{2+}, cAMP, and diacylglycerol (Borgeat et al., 1972, Andrews and Conn, 1986; Limor et al., 1987). The second stage is initiated by increased levels of intracellular second messengers and culminates in the release, by regulated exocytosis, of stored LH and FSH.

Although much is known about how cell stimulation results in generation of intracellular second messengers (Berridge,

1987; Levitzki, 1988), the precise mechanisms by which intracellular second messengers stimulate regulated exocytosis are not well understood in any cell type. Ca^{2+} was long held to be the most important intracellular second messenger responsible for regulating secretory exocytosis in a wide variety of cell types (see Rink and Knight, 1988; Knight et al. ,1989). Recently, however, it has become clear that other second messengers, including cAMP (Takuma and Ichida, 1988; and this study) and diacylglycerol [or its analogue phorbol 12-myristate 13-acetate (PMA)] (Di Virgilio et al., 1984; Pozzan et al., 1984; and this study), can stimulate secretory exocytosis in the absence of any change in cytosolic $[Ca^{2+}]_{free}$. Furthermore, guanine nucleotide analogues can stimulate exocytosis independently of changes in $[Ca^{2+}]_{free}$ (Haslam and Davidson, 1984; Barrowman et al., 1986). Although the mechanism of the guanine nucleotide-stimulation is not known (see Section 5), this finding confirms that an increase in $[Ca^{2+}]_{free}$ is not essential for secretory exocytosis to occur.

1.1.3 Steps in regulated exocytosis.

1.1.3.1 Formation of secretory vesicles

Proteins destined for regulated exocytosis need to be sorted from constitutively-secreted proteins and packaged into specialized secretory vesicles in concentrated form for storage (Burgess and Kelly, 1987). These secretory vesicles are also termed secretory granules since their high content of electron-dense material results in a granular appearance in electron micrographs (Burgess and Kelly, 1987). The sorting process takes place in the trans-Golgi network (Griffiths and Simons, 1986), and appears to involve the active separation of "regulated" proteins from the bulk-flow secretory pathway down which "constitutive" proteins are passively transported (Moore et al., 1983). Sorting may be mediated by a group of M_r 25 000 proteins purified from

pancreas Golgi which are able to distinguish "regulated" from "constitutive" proteins (Chung et al., 1989). Morphological studies suggest that clathrin may be involved in the formation of secretory granules, perhaps by removing excess membrane from the condensing secretory vesicle and/or by recycling resident TGN proteins from the newly formed secretory vesicle back to the TGN (Brodsky, 1988).

1.1.3.2 Transport of secretory vesicles to their plasma membrane fusion sites.

Newly formed secretory vesicles need to move from the Golgi to their sites of fusion at the plasma membrane. There are two distinct components to this movement. Immediately after formation, secretory vesicles need to be transported from the Golgi to a storage site near the plasma membrane. This is often, as in neurons, a considerable distance. There is evidence that microtubules are involved in this process and that it is not regulated by second messengers (Burgess and Kelly, 1987; Rivas and Moore, 1989). In contrast, second messenger systems do regulate the movement of secretory vesicles from these sub-plasmalemmal storage sites into the the immediate vicinity of the plasma membrane fusion sites. The microfilament (actin-containing) cytoskeleton may be involved in this movement but its role seems to be mainly passive in that it acts as a barrier between secretory vesicles and the plasma membrane (see Section 1.1.4.2). In some cell types, including neurons (Heuser et al., 1979) and oocytes (Sasaki and Epel, 1983) as well as the in protozoa Tetrahymena (Satir et al., 1973) and Paramecium (Plattner et al., 1982), a large proportion of secretory vesicles appear to be prepositioned at their plasma membrane fusion sites for rapid release.

1.1.3.3. Fusion of the secretory vesicle with the plasma membrane.

As a result of fusion of the secretory vesicle membrane with the plasma membrane, the vesicle lumen becomes continuous with the extracellular space. Theoretical considerations (Blumenthal, 1987) suggest that the major barriers to membrane fusion are the water associated with membrane surfaces and the electrostatic repulsion of like-charged phospholipid head groups (both of which prevent close approach of the membranes). Any model proposed for membrane fusion will need to suggest mechanisms for overcoming these barriers. Recent morphological (Chandler, 1984) and electrophysiological (Fernandez et al., 1984; Breckenridge and Almers, 1987; Zimmerberg, 1987; Spruce et al., 1989) studies indicate that the first step in membrane fusion is the reversible formation of a small "fusion pore". Measurements of ion conductance indicate that this pore has a diameter similar to that of gap junctions, the only known biological channel structure which traverses two membranes (Loewenstein, 1981). Presumably it is this pore which widens to complete the fusion process. Interestingly, structural studies of synaptophysin, a major integral membrane protein of synaptic vesicles, suggest that it forms hexameric transmembrane channels with the same conductance as a fusion pore (Thomas et al., 1988). Since this conductance is voltage-sensitive (Thomas et al., 1988), being apparent only at depolarizing voltages, synaptophysin may confer the voltage-sensitivity to Ca^{2+}-stimulated exocytosis which has been demonstrated in neurons (Hochner et al., 1989).

1.1.3.4 Expulsion of secretory vesicle contents.

The expulsion of secretory vesicle contents may occur by simple diffusion or by active expulsion. Two mechanisms have been proposed for active expulsion. In one, the contraction of a microfilament network surrounding fused secretory vesicles expels the vesicle contents (Segawa and Yamashina,

1989; for further discussion see Section 1.1.4.2). In the other, the granule content swelling which is associated with exocytosis assists in the dispersal of vesicle contents (Bilinski et al., 1981; Green, 1982). The role of osmotic forces in exocytosis is discussed in Section 1.1.5.

1.1.3.5 Retrieval of secretory vesicle membrane.

The retrieval of secretory vesicle membrane which has been incorporated into the plasma membrane is essential since the plasma membrane surface area can increase as much as four fold following stimulation of exocytosis (Fernandez et al., 1984). In the adrenal chromaffin cell, membrane retrieval increases two- to three-fold immediately following secretory exocytosis (von Grafenstein et al., 1986). Significantly, it is not Ca^{2+}-dependent but is temperature-sensitive and requires ATP (von Grafenstein et al., 1986; Rink and Knight, 1988). It appears that secretory vesicle membrane does not mix with the plasma membrane (Phillips et al., 1983; Patzak and Winkler, 1986; Torri-Tarelli et al., 1990) and is efficiently retrieved (Thilo, 1985; Torri-Tarelli et al., 1990), thus minimizing the turnover of plasma membrane which would be wasteful in an active secretory cell. After retrieval, some secretory vesicle membrane is recycled into newly formed secretory vesicles, probably at the TGN (Burgess and Kelly, 1987).

1.1.4 Proteins implicated in regulated exocytosis.

1.1.4.1 Introduction

Several lines of evidence indicate that one or more protein(s) must be involved in the mechanism of regulated exocytis. Firstly, not only does exocytosis require the overcoming of considerable energy barriers, but it is also generally dependent on ATP (see Section 3). Secondly, in the absence of proteins, phospholipid vesicles do not fuse in the presence of physiological concentrations of Ca^{2+}

(Blumenthal, 1987). Thirdly, second messenger-stimulated exocytosis is abolished by N-ethylmaleimide which modifies protein sulphydryl groups (Knight and Baker, 1982).

Although no protein has been conclusively demonstrated to be essential for regulated exocytosis, many proteins have been tentatively implicated. In most cases it is not known at which stage in regulated exocytosis these proteins are involved. Nor is it known whether these proteins are essential for exocytosis or merely regulatory. For these reasons they are discussed separately from the previous section and in no particular order.

1.1.4.2 Microfilament proteins

Many secretory cells possess a dense, subplasmalemmal cytoskeletal network composed of filamentous actin (F-actin) and various actin binding proteins including fodrin and calpactin (Burgoyne and Cheek, 1987; Aunis and Bader, 1988; Segawa and Yamashina, 1989; Ikebuchi and Waisman, 1990; Nakata et al., 1990). Secretory vesicles are embedded within this cytoskeletal network and may be bound to it through actin-binding proteins such as synapsin I/II, α-actinin, caldesmon, and calpactin, all of which also bind secretory vesicle membranes (Burgoyne et al., 1986; Walker and Agoston, 1987; Drust and Creutz, 1988).

In support of a functional role for microfilament proteins in exocytosis, F-actin modifying agents such as the cytochalasins, phalloidin and botulinum C2 toxin have been shown to modify regulated exocytosis (Lelkes et al., 1986; Lew et al., 1986; Sontag et al., 1988; Nüsse and Lindau, 1988; Matter et al., 1989). Furthermore, antibodies to the α-subunit of fodrin inhibited Ca^{2+}-stimulated exocytosis when introduced into permeabilized adrenal chromaffin cells (Perrin et al., 1987). Fodrin is thought to crosslink F-actin to the plasma membrane and there is evidence that

antibodies to fodrin inhibit fodrin degradation by Ca^{2+}-activated proteases (Siman et al., 1985).

Most of the evidence suggests that the sub-plasmalemmal microfilament network has a passive role, preventing movement of secretory vesicles to the plasma membrane (Stossel, 1981). Nicotine stimulation of intact - and Ca^{2+} stimulation of permeabilized - adrenal chromaffin cells leads to disassembly of the sub-plasmalemmal microfilament network and exocytosis (Cheek and Burgoyne, 1986; Sontag et al., 1988). Furthermore, F-actin destabilizing agents such as cytochalasins and botulinum C2 ADP-ribosyltransferase generally enhance Ca^{2+}-stimulated exocytosis (Lelkes et al., 1986; Lew et al., 1986; Sontag et al., 1988; Matter et al., 1989). There are several Ca^{2+}-sensitive cytoskeletal proteins such as gelsolin (Matsudaira and Janmey, 1988) activation of which could promote F-actin disassembly.

In neurons synapsin I (which is not present in non-neuronal cells) appears to mediate the binding of synaptic vesicles to the subplasmalemmal microfilament network (see Section 1.1.4.4 for references). Ca^{2+} stimulated phosphorylation of synapsin I leads to its dissociation from synaptic vesicles, providing an attractive mechanism for the effects of Ca^{2+}.

Consistent with a barrier role for microfilaments is the finding that in sea urchin eggs, where secretory vesicles are prepositioned at their fusion sites, and are therefore presumably are not restrained by microfilaments, agents which disrupt microfilaments have no effect on Ca^{2+}-stimulated exocytosis (Whitaker and Baker, 1983).

There is no direct evidence for two other roles proposed for microfilaments which are that actin, together with myosin, pull secretory vesicles toward the plasma membrane or that the subplasmalemmal network provides the force to expel the

secretory vesicle contents once fusion with the plasma membrane has occured (Segawa and Yamashina, 1989). However, a recent study demonstrating that calpactin, a member of the annexin protein family which is required for Ca^{2+}-stimulated exocytosis (see Section 1.1.4.3), mediates Ca^{2+}-stimulated formation of F-actin bundles provides indirect evidence for an active role for the microfilament network (Ikebuchi and Waisman, 1990).

Although microtubules are involved in the transport of secretory vesicles from the TGN to their storage sites near the plasma membrane (see Section 1.1.3.2), this process is not regulated by second messengers. Indeed, there is no evidence that microtubules play a role in second messenger-stimulated exocytosis of stored secretory products (Burgess and Kelly, 1987).

1.1.4.3 Annexins

One strategy used to identify proteins involved in exocytosis was the purification and characterization of soluble proteins which bind chromaffin granule membranes in a Ca^{2+}-dependent manner (Geisow and Burgoyne, 1982; Creutz et al., 1983). It should be emphasized that these proteins also bind artificial membranes, indicating that this binding is not specific (Creutz et al., 1983). Creutz et al. (1983, 1987) have identified 23 such proteins and refer to them as "chromobindins". Some of these proteins were subsequently identified as calmodulin, protein kinase C, caldesmon, synexin and calelectrin (Creutz et al. 1987 and references therein). Others have been shown to have sequence similarity to the proteins synexin and calelectrin and are clearly part of a homologous family of proteins which are collectively referred to as "annexins" (Geisow et al., 1987; Burgoyne and Geisow, 1989; Burns et al., 1989). Annexins share a common sequence motif of about 70 amino acids which is repeated at

least four times within the protein (Burgoyne and Geisow, 1989).

Synexin is an extremely hydrophobic M_r 47 000 protein which, in the presence of Ca^{2+} and cis-unsaturated fatty acids such as arachidonic acid, mediates the fusion of chromaffin granules in vitro (Pollard et al., 1988). A "hydrophobic bridge" model has been proposed in which it is postulated that Ca^{2+} stimulates the formation of a synexin polymer, which traverses both the plasma membrane and the secretory vesicle membrane, thus forming a "hydrophobic bridge" along which membrane phospholipids can move and eventually fuse (Pollard et al., 1988). However, this model is somewhat speculative and direct evidence is required.

In contrast, there is direct evidence that another annexin, **calpactin**, may mediate Ca^{2+}-stimulated exocytosis. Like synexin, calpactin has been shown to mediate Ca^{2+}-dependent aggregation of chromaffin granules (Drust and Creutz, 1988). However, whereas synexin required >80 μM $[Ca^{2+}]_{free}$ for detectable aggregation, calpactin induced aggregation at 0.7 μM $[Ca^{2+}]_{free}$, well within the physiological cytosolic $[Ca^{2+}]_{free}$ range (Drust and Creutz, 1988). Calpactin also reconstituted Ca^{2+}-dependent exocytosis in permeabilized adrenal chromaffin cells which had lost their ability to secrete because of leakage of cytosolic proteins (Ali et al., 1989). And finally, a synthetic 20 amino acid annexin consensus peptide inhibited Ca^{2+}-stimulated exocytosis in these cells and this inhibition was reversed by calpactin (Ali et al., 1989). Calpactin is a tetramer composed of two large (M_r 36 000) and two small (M_r 10 000) subunits and has a wide tissue distribution (Burgoyne and Geisow, 1989). In adrenal chromaffin cells, calpactin is localized on the inner face of the plasma membrane (Nakata et al., 1990) and is phosphorylated after nicotine stimulation (Burgoyne and Geisow, 1989). In vitro and in vivo studies indicate that

calpactin is a substrate for retroviral tyrosine kinases, protein kinases A and C, as well as Ca^{2+} /calmodulin dependent protein kinase (Burgoyne and Geisow, 1989). The name calpactin originally derived from its ability to bind actin in the presence of high Ca^{2+} concentrations (Glerk and Weber, 1984). More recently calpactin has been shown to bind F-actin at physiological $[Ca^{2+}]_{free}$ (Km < 1 µM) and mediate Ca^{2+}-stimulated bundling of F-actin fibres (Ikebuchi and Waisman, 1990; see Section 1.1.4.2).

1.1.4.4 Synapsin I

Synapsin I is a heterodimer of closely related polypeptides of M_r 86 000 and 80 000 and is found exclusively in neuronal tissue (for review see Hemmings et al., 1989). It is present in virtually all synaptic termini where it is localized to the cytosolic surface of small synaptic vesicles, constituting 6 % of total vesicle protein. Morphological studies (Hirokawa et al., 1989) indicate that synapsin I binds secretory vesicles to the presynaptic microfilamentous cytoskeletal network. Phosphorylation of synapsin I by Ca^{2+}/calmodulin-dependent protein kinase II (Ca/CAM kinase II) in vitro leads to its dissociation from synaptic vesicles (Schiebler et al., 1986). Depolarization of isolated rat nerve terminals, which is followed by an increase in intraterminal $[Ca^{2+}]_{free}$, leads to phosphorylation of synapsin I and its dissociation from the particulate to the soluble fraction (Sihra et al., 1989). Intracellular introduction of Ca/CAM kinase II enhances Ca^{2+}-stimulated neurotransmitter release whereas both dephospho-synapsin I and a peptide inhibitor of Ca/CAM kinase II are inhibitory (Llinás et al., 1985; Nichols et al., 1990). Based on these findings it has been postulated that in neurons Ca^{2+} stimulates exocytosis at least in part by activating Ca^{2+}/CAM kinase II. Activated Ca/CAM kinase II phosphorylates synapsin I, decreasing its affinity for synaptic vesicles. As a result, synaptic vesicles which are

bound to the cytoskeleton through synapsin I are free to fuse with the plasma membrane (Hemmings et al., 1989).

1.1.4.5 Parafusin

In the ciliated protozoa, Paramecium tetraulia, stimulation of exocytosis requires the Ca^{2+}-dependent dephosphorylation of a 63 000 M_r cytosolic phosphoprotein termed parafusin (Satir et al., 1988) which may be mediated by a Ca^{2+}/calmodulin dependent protein phosphatase similar to calcineurin (Momayesi et al., 1987). In several P. tetraulia temperature-sensitive secretory mutants dephosphorylation of parafusin does not occur at the non-permissive temperature (Gilligan and Satir, 1982; Zeiseniss and Plattner, 1985). Using polyclonal antibodies against parafusin Satir et al. (1989) have identified this protein in yeast, insects, and mammals, suggesting that it may have a universal role in exocytosis.

1.1.4.6 51K Chromaffin Granule Binding Protein (CGBP)

Meyer and Burger (1979) have purified a M_r 51 000 plasma membrane protein from adrenal chromaffin cells which binds selectively, and in a Ca^{2+}-independent manner, to chromaffin granules and not to other membranous organelles. CGBP differs from chromobindins (see Section 1.1.4.3) in that: (i) it is a plasma membrane protein rather than a soluble protein; (ii) its binding is selective for chromaffin granule membrane; and (iii) it binds chromaffin granules in a Ca^{2+}-independent manner. These properties, taken together with the recent finding that intracellular application of antibodies to CGBP abolished depolarization-induced exocytosis from rat pheochromocytoma (PC12) and adrenal chromaffin cells (Schweitzer et al., 1989), implicate CGBP in regulated exocytosis.

1.1.4.7 Miscellaneous Proteins

Studies using **metalloendoprotease** active site analogue inhibitors suggest that this enzyme(s) may be involved in Ca^{2+}-stimulated exocytosis in mast cells (Mundy and Strittmatter, 1985) and in both secretory and endocytic pathways in hepatoma cells (Strous et al., 1988). However, recent studies cast doubt on this intepretation since these inhibitors have non-specific effects on phospholipid bilayers (Aiello, et al., 1986; Epand et al., 1987; Thomas and Meizel, 1989).

Intracellular application of **botulinum A, D, and E neurotoxins and tetanus neurotoxins** inhibit Ca^{2+}-stimulated exocytosis in adrenal chromaffin cells (Knight et al., 1985; Penner et al., 1986; Bittner et al., 1989). Since these neurotoxins may act on proteins, attempts have been made to identify their substrates. Unfortunately, the finding that botulinum D toxin apparently ADP-ribosylates M_r 20-22 000 proteins (Adam-Vizi and Knight, 1988), one of which is a small ras-related GTP-binding protein (Morii et al., 1988; Narumiya et al., 1988), has been shown to be due to contamination with a botulinum ADP-ribosyl transferase which does not inhibit exocytosis (Rösener et al., 1987; Narumiya et al., 1988). Therefore the target(s) for these neurotoxins remain(s) unidentified.

An attractive new approach to identifying proteins involved in regulated exocytosis is based on the finding that permeabilized cells which leak proteins frequently lose their ability to secrete in response to stimuli such as Ca^{2+} (Sarafian et al., 1987; Ali et al., 1989; Koffer and Gomperts, 1989; Martin and Walent, 1989a). Martin and Walent (1989a) found that responsiveness to Ca^{2+} could be reconstituted in permeabilized GH_3 pituitary cells by adding back soluble proteins prepared from various tissues. Using these permeabilized cells as a bioassay, they have purified

a single M_r 135 000 protein, termed **calekkrin**, from rat brain, which is apparently sufficient to restore Ca^{2+}-stimulated exocytosis (Martin and Walent, 1989a; Martin and Walent, 1989b).

Dekker et al. (1989) have recently shown that introduction of antibodies to **B-50** (a neuron-specific, pre-synaptic, protein kinase C substrate) into permeabilized synaptosomes inhibits Ca^{2+}-stimulated exocytosis suggesting a role for this protein in regulated exocytosis.

Other proteins which have been implicated in exocytosis are discussed below. These include **protein kinase C** (Section 4), **calmodulin** (Section 4) , and **GTP-binding proteins** (Section 5).

1.1.5 The Role of Osmotic Forces in Regulated Exocytosis

Despite extensive research a role for osmotic forces in regulated exocytosis has not been estabilished but nor has it been conclusively ruled out (for reviews see Baker and Knight, 1984; Holz, 1986). Two distinct roles for osmotic forces have been proposed.

First, osmotic forces have been postulated to provide the driving force for membrane fusion (Pollard et al., 1978; Zimmerberg et al., 1980). It is proposed that stimulation leads to swelling of secretory vesicles which somehow provides the force necessary to overcome the barriers to membrane fusion. In favour of this hypothesis are the findings that (1) osmotic gradients enhance fusion of lipid vesicles with planar lipid membranes (Zimmerburg et al., 1980); (2) hyperosmolar solutions inhibit exocytosis in intact cells (for review see Holz, 1986); and (3) Ca^{2+} stimulates swelling of secretory vesicles in vitro (Zimmerberg and Whitaker, 1985). Against this hypothesis are

the findings that (1) visible swelling in vivo occurs only after membrane fusion (Breckenridge and Almers, 1987; Zimmerberg et al., 1987) and that (2), in permeabilized as opposed to intact cells, hyperosmolar solutions do not inhibit exocytosis (Holz, 1986).

Second, osmotic forces have also been proposed to assist dispersal of granule contents following membrane fusion by mediating the swelling of granule contents (Bilinski et al., 1981; Green, 1982). This hypothesis is compatible with the finding that granule swelling appears to follow vesicle fusion.

Mechanisms proposed for granule swelling can be divided into those that postulate an influx of osmotically active particles driven, for example, by a granule membrane H^+-ATPase (for review and critique see Baker and Knight, 1984) and those that postulate an increase in the osmotic activity of the granule contents as a result, for example, of dissociation of protein oligomers into monomers (Lorenson and Jacobs, 1984). Both would result in increased osmotic activity within the granule which draws water into the granule, causing swelling.

1.2 PERMEABILIZATION

1.2.1 Models used to Study Regulated Exocytosis

Early work on regulated exocytosis relied on studies in intact cells. Two problems with intact cells are (1) that the intracellular environment is inaccesible to precise experimental manipulation and (2) that they are too complex, making it impossible to elucidate the molecular mechanism of exocytosis and identify the proteins involved.

In an attempt to overcome these problems, cell-free models of exocytosis have been developed. The simplest models use **artificial lipid membranes** (Blumenthal, 1987; Rand and Parsegian, 1986). Studies have examined fusion of lipid vesicles with cells, with other lipid vesicles, or with planar lipid membranes (Blumenthal, 1987). A clear advantage of these models is that the components of the system are defined and they have provided useful information on the forces which may be involved in membrane fusion (Blumenthal, 1987). However, it is doubtful whether fusion observed in these models resembles in vivo exocytosis (Blumenthal, 1987).

The rationale for models in which the **fusion of isolated secretory vesicles with each other** is examined is that this is what occurs in compound exocytosis in which secretory vesicles expel their contents by fusing with secretory vesicles that have already fused to the plasma membrane. Although this system is attractive it is clearly doubtful that vesicle-vesicle fusion is identical to exocytosis. Furthermore, chromaffin granules are the secretory vesicle most frequently used in these studies (Ekerdt et al., 1981) even though compound exocytosis is rare in adrenal chromaffin cells (Burgoyne, 1984).

More promising are models in which the **fusion of isolated secretory vesicles with plasma membranes** are examined. In what is arguably the best of these models, isolated sea urchin egg secretory vesicles can be induced, by micromolar $[Ca^{2+}]_{free}$, to fuse with an egg "plasma membrane lawn" attached to a microscope slide (Crabb and Jackson, 1985). Recently, Nadin et al. (1989) claim to have demonstrated fusion between purified pancreatic secretory vesicles and plasma membrane fragments **in suspension**. In the sea urchin egg model exocytosis occurs without adding any other component except Ca^{2+} (in Crabb and Jackson, 1985). The

pancreas secretory vesicle model remains to be validated since it is not clear that fusion rather than simple vesicle lysis is occuring.

Undoubtedly the most widely used, and arguably the most successful, model for studying exocytosis is the **permeabilized cell** (for Reviews see Gomperts and Fernandez, 1985; Knight and Scrutton, 1986; Ahnert-Hilger et al., 1989). In permeabilized cells the integrity of the plasma membrane is disrupted in a manner which allows manipulation of the intracellular environment but does not disrupt the exocytic process. The most widely used methods for cell permeabilization are described below (see Section 1.2.2). The existence of so many different methods can be ascribed to (1) the particular advantages that individual methods have and (2) the adoption of permeabilization techniques originally developed for use in fields other than exocytosis research.

1.2.2. Permeabilization Techniques

The techniques used for permeabilization can be divided according to (a) whether the permeabilization is reversible and (b) whether they render the plasma membrane permeable to small or large molecules (Table 1). Some of these techniques have been found to be effective in only a limited number of cell types, usually for obscure reasons. For example, ATP^4 permeabilizes rat masts cells (Gomperts, 1983), several mouse cell lines (Rozengurt, et al., 1977; Greenberg et al., 1988), as well as rat anterior pituitary cells (Andrews et al., 1986) but is without effect in many other cell types examined including sheep anterior pituitary cells (van der Merwe et al., 1989b).

TABLE 1 CELL PERMEABILIZATION TECHNIQUES

I. Reversible Permeabilization

(a) Large Molecules (M_r > 2000)

Scrape loading	McNeil et al. (1984)
Liposomes	Friedman et al. (1987)
Free-thawing	Nichols et al. (1989)
Microinjection	Bar-Sagi & Feramisco (1986)
Hyposmotic lysis	Winicov & Gershengorn (1989)

(b) Small Molecules (M_r < 2000)

ATP^{4-}	Gomperts (1983)
Dextran sulphate	Kucera & Paulus (1982)

II. Irreversible permeabilization

(a) Large Molecules (M_r > 2000)

Detergents (e.g. digitonin)	Dunn & Holz (1983), Wilson & Kirshner (1983)
Streptolysin O	Howell & Gomperts (1987)
Proteases	Lemons et al. (1988)
Patch pipette	Neher & Marty (1982)
"Cell cracking"[a]	Martin (1989)

(b) Small molecules (M_r < 2000)

Staphyloccocal α-toxin	Ahnert-Hilger et al. (1989)
High voltage shock	Baker & Knight (1983
Sendai virus	Gomperts et al. (1983)

[a]Cell cracking involves passing cells through a narrow aperture (typically < 4 μM) which produces a stable lesion in the plasma membrane (Martin, 1989)

The advantage of **reversible** permeabilization is that, after manipulation of the intracellular milieu, the cells can be returned to their intact state. This is more physiological and allows prolonged survival of the cells after permeabilization (hours to days). However, reversibile permeabilization does not allow precise control of compounds which are sequestered (e.g. Ca^{2+}), or degraded (e.g. ATP and many proteins) by the cells. Conversely, cells permeabilized **irreversibly** survive for shorter periods (minutes to hours), and their intracellular milieu, being continuously accessible, can be precisely controlled. Since experiments on permeabilized cells seldom need to continue for more than one hour, irreversible permeabilization techniques are satisfactory for most purposes and, indeed, are far more widely used in exocytosis research.

The major advantage of permeabilization to **large molecules** is that it results in the leakage of many soluble intracellular proteins from the cell (Sarafian et al., 1987) and allows the introduction of large proteins, including antibodies, into the cell (Ali et al., 1989; Dekker et al., 1989; Schweizer et al., 1989). After leaking proteins cells frequently lose their ability to secrete in response to stimuli such as Ca^{2+} (Sarafian et al., 1987; Ali et al., 1989; Koffer and Gomperts, 1989; Martin and Walent, 1989a). Responsiveness to Ca^{2+} can sometimes be reconstituted by adding back proteins, providing a convenient assay for purifying proteins essential for Ca^{2+}-stimulated exocytosis. This approach has already led to the purification of at least one protein, termed **calekkrin** (see Section 1.1.4.7), which appears to be essential for Ca^{2+}-stimulated exocytosis

in "cracked" GH_3 pituitary cells (Martin and Walent, 1989a; Martin and Walent, 1989b).

Although the protein content of cells permeabilized to small molecules cannot be manipulated, an advantage of using these cells is that, since they do not lose soluble proteins, they are more both physiological as well as more stable (Koffer and Gomperts, 1989). Therefore, when experiments on regulated exocytosis do not require protein manipulation, permeabilization to small molecules is to be preferred.

A major advantage of using the patch-pipette is that exocytosis can be monitored with excellent time resolution by measuring changes in plasma membrane electrical capacitance. Exocytosis results in the incorporation secretory vesicle membrane into the plasma membrane enlarging the plasma membrane area and therefore increasing the plasma membrane capacitance. This technique has been used successfully in a range of cell types (Neher and Marty, 1982; Fernandez et al., 1984; Nüsse and Lindau, 1988; Lim et al., 1990).

1.2.3 α-Toxin Permeabilization

In considering which permeabilization technique to use to study regulated exocytosis in the pituitary gonadotrope, we favoured selective permeabilization to small molecules since our experiments did not require protein manipulations. In addition, our experiments required continuous precise control of Ca^{2+}, ATP, and cAMP concentrations, which excluded permeabilization techniques which are necessarily transient (I(a) in Table 1). Although ATP^{4-}, and dextran sulphate can be used for prolonged permeabilization [since reversal of their effect is under experimental control (Kucera and Paulus, 1982; Gomperts, 1983)], neither of these agents successfully permeabilized sheep anterior pituitary

cells (van der Merwe et al., 1989b; P.A. van der Merwe, J.S. Davidson, unpublished data). Of the three remaining methods which permeabilize cells to small molecules (II(b) in Table 1), we favoured staphylococcal α-toxin since it can be used in substratum-attached cells [unlike high voltage shock (Baker and Knight, 1983)], and is easier to use than Sendai virus which must grown in the laboratory (Gomperts et al., 1983).

Staphylococcus aureus α-toxin is a water-soluble monomeric protein exotoxin of M_r 34 000 which is secreted by most strains of Staphylococcus aureus (Bhakdi and Tranum-Jensen, 1988). Monomeric α-toxin inserts into phospholipid bilayers (including biological membranes) after which it assembles into a transmembranous, hexameric, ring-shaped protein with a central pore of diameter 2-3 nM (Füssle et al., 1981; Reichwein et al., 1987; Harshman et al., 1989). Membranes treated with α-toxin are rendered permeable to molecules with M_r up to 5 200 (Füssle et al.,1981), and because monomeric α-toxin is too large to pass through such pores, only the plasma membrane is permeabilized. Once formed, α-toxin pores are apparently stable for hours (Bhakdi and Tranum-Jensen, 1988; and this study) and do not close except in the presence of millimolar concentrations of divalent cations (Bashford et al., 1986). These properties make α-toxin a suitable permeabilizing agent to investigate the mechanism of exocytosis as well as other cellular processes (Table 2).

TABLE 2 STUDIES USING CELLS PERMEABILIZED WITH STAPHYLOCOCCUS AUREUS α-TOXIN

Problem	Cells	Reference
Exocytosis	PC12	Ahnert-Hilger et al. (1985)
	Adrenal Chromaffin	Bader et al. (1986)
	T lymphocytes	Schreizenmeier et al. (1988)
	Rat basophilic leukemia	Hohman (1988)
	Pancreatic islets	Metz (1989)
	Anterior Pituitary	van der Merwe et al. (1989a)
Muscle contraction	Smooth muscle	Kitizawa (1989)
Ca^{2+} homeostasis	RINA2 & PC12	Föhr et al. (1989)

1.3 STIMULUS-SECRETION COUPLING IN THE GONADOTROPE

1.3.1 The GnRH Receptor

GnRH is the primary regulator of gonadotropin secretion from anterior pituitary gonadotropes. The first step in gonadotrope stimulus-secretion coupling is the binding of GnRH to its cell surface receptor (for reviews see Conn et al., 1987; Hazum and Conn, 1988). The GnRH structural requirements for receptor binding and activation have been extensively studied and reviewed and will not be discussed further (Millar and King, 1987; Millar et al., 1989). The GnRH receptor has a monomeric M_r of 60 000 (Hazum and Conn, 1988). There is evidence that GnRH-induced crosslinking of two receptors is important for receptor activation (Conn et al., 1987). Following activation, GnRH receptors form large-

scale clusters which are internalized and then either degraded or recycled to the cell surface (Hazum and Schvartz, 1987). Although neither large-scale cluster formation nor internalization are important for receptor activation (Conn et al., 1987), these processes probably mediate the decrease in cell-surface receptor number, called down-regulation, which occurs after prolonged GnRH stimulation. After prolonged exposure to GnRH, gonadotropes become unresponsive to GnRH, a process referred to as desensitization (Badger et al., 1983; King et al., 1986). Interestingly, receptor down-regulation appears to play little role in the developement of desensitization (Conn et al., 1987). Recent studies implicates GnRH-induced impairment of voltage-sensitive Ca^{2+} channel activity in this process (Stojilkovic et al., 1989a).

1.3.2 Generation of Second Messengers

GnRH stimulation of gonadotropes is followed by increases in cytosolic $[Ca^{2+}]_{free}$ (Chang et al., 1986; Limor et al., 1987; Shanghold et al., 1988; Tasaka et al., 1988), activation of protein kinase C (PKC; Hirota et al., 1985; Naor et al., 1985; Strulovici et al., 1987), and the generation of cAMP (Borgeat et al., 1972) and arachidonic acid (Naor and Catt, 1981). There is some evidence that these effects of GnRH may be mediated through a signal-transducing GTP binding protein or G protein (Andrews et al., 1986; Perrin et al., 1989), as is the case for many hormone receptors (Gilman, 1987).

The increase in cytosolic $[Ca^{2+}]_{free}$ results both from the release of intracellular stored Ca^{2+} and the entry of extracellular Ca^{2+} (Shangold et al., 1988; Tasaka et al., 1988). Release of stored Ca^{2+} probably results from the action of inositol 1,4,5-trisphosphate (Guillemette et al., 1987; Naor et al., 1988) which is one of two products

generated by the GnRH-stimulated action of phosphoinositidase C (PIC) on phosphatidylinositol 4,5-bisphosphate (Naor et al., 1986), the other being diacylglycerol (DAG; Andrews and Conn, 1986). Entry of extracellular Ca^{2+} results from activation of dihydropyridine-inhibited, voltage-sensitive Ca^{2+} channels as well as at least one other class of Ca^{2+} channels (Chang et al., 1986; Davidson et al., 1988; Smith et al., 1989). Activation of PKC probably results mainly from DAG generation, although Ca^{2+} may also play a role (Hermon et al., 1986).

GnRH may stimulate cAMP generation either directly or indirectly, through Ca^{2+} or PKC, both of which regulate adenylate cyclase (Brostrom et al., 1982; Summers and Cronin, 1986; Minocherhomjee, et al., 1988). Arachidonic acid generation may result from (1) the action of phospholipase A_2 on phospatidylcholine or phosphatidylethanolamine, or (2) through the sequential actions of PIC, DAG lipase, and monoacylglycerol lipase on phosphatidylinositol 4,5-bisphosphate (Chang et al., 1988). As is the case with cAMP generation, arachidonic acid generation may be secondary to increased $[Ca^{2+}]_{free}$ and/or PKC activation (Burch et al., 1986; Burch and Axelrod, 1987; Feinstein and Halenda, 1988; Churcher et al., 1990). Arachidonic acid may exert its effect on gonadotropin secretion after conversion to leukotrienes by 5-lipoxygenase (Samuelsson et al., 1987; Hulting et al., 1985; Naor et al., 1985a).

Elucidating the precise mechanisms by which second messengers are generated in response to agonists such as GnRH is complicated by the fact that numerous interactions between second messenger pathways exist, some of which have been described above. For example, an increase in cytosolic $[Ca^{2+}]_{free}$ could cause activation of PKC as well as

increased cAMP and arachidonic acid generation, thus accounting for all the observed effects of GnRH on second messengers.

1.3.3 Regulation of gonadotropin exocytosis

As is the case in many other secretory cells (Berridge, 1985; Baker and Knight, 1986; Rink and Knight, 1988), Ca^{2+} appears to be the dominant regulator of gonadotropin exocytosis (for reviews see Conn et al., 1987; Huckle and Conn, 1988; Stojilkovic et al., 1989b). Roles for cAMP, PKC and arachidonic acid and its metabolites have been proposed on the basis of studies in intact cells (Borgeat et al., 1972; Smith and Vale, 1980; Naor et al. 1985a). A pitfall of these studies is that cAMP (Luini et al., 1985), activators of PKC (Albert et al., 1987; Stojilkovic et al., 1988a; Restrepo et al., 1989; Yada et al., 1989), and arachidonic acid (Kolesnick et al., 1984) may all induce increases in cytosolic $[Ca^{2+}]_{free}$, which itself stimulates LH exocytosis. For this and other reasons the role of these second messengers is controversial and will be discussed further in Section 3. The precise mechanism by which Ca^{2+} stimulates LH exocytosis is not known. Clearly, any of the calcium binding proteins wich have been implicated in exocytosis are potential targets for calcium (see Section 1.2). Recently attention has focused on calmodulin and PKC as possible mediators of Ca^{2+}-stimulated LH exocytosis. The role of these proteins in LH exocytosis is discussed further in Section 4.

2 MATERIALS AND METHODS

2.1 MATERIALS

Purified, lyophilized Staphylococcus aureus α-toxin was obtained from Dr. Sucharit Bhakdi (Institute of Medical Microbiology, Justus-Liebig University, Giessen, Germany) and was stored at -20°C. Stock aliquots of α-toxin in solution (0.5 mg/ml) were stored at -20°C for up to 6 months with no loss of activity as determined by monitoring induced 2-deoxy[^{3}H]glucose efflux (see Section 2.5). Myo-[2-^{3}H]inositol (10-20 Ci/mmol), [8-^{14}C]adenosine (50-60 mCi/mmol), [5,6-^{3}H]uridine (35-50 Ci/mmol), 2-deoxy-D[2,6-^{3}H]glucose (30-60 Ci/mmol), and I^{125} (10-15 mCi/μg) were obtained from The Radiochemical Centre (Amersham, Bucks., UK). ATPγS, GDPβS, GTPγS, GMPPNP, and staurosporine were supplied by Boehringer-Mannheim Biochemicals (Mannheim, Germany). Staurosporine was dissolved in Me_2SO at 1 mM (w/v) and stored at 4°C. Ovine LH (NIADDK-oLH-I-3) and ovine LH antiserum (NIADDK-anti-oLH-1) were kindly provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), U.S.A.. Mammalian GnRH was synthesized by Dr R.C. deL. Milton, Department of Chemical Pathology, University of Cape Town Medical School. All other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

2.2 CELL CULTURE

Pituitaries were removed from 6-12 month old castrated male sheep within 30 minutes of slaughter at the Cape Town municipal abattoir and immediately transported to the laboratory in ice-cold Minimal Essential Medium (MEM; Gibco, Paisley, Scotland) containing 20 mM HEPES (pH 7.4) and amphotericin B (2.5 mg/L). Within 1 hour the anterior pituitaries were dissected free from the capsule and posterior pituitary, minced, and incubated in collagenase

solution [collagenase 0.9% (w/v) (155 U/mg, Worthington Biochemical Corp., Freehold, NJ) and 18 mg/L deoxyribonuclease (Miles Laboratories, Elkhardt, IN) in buffer B] for 2-3 h at 37°C. Buffer B comprised (mM): NaCl, 137; KCl, 5; Na_2HPO_4, 0.7; HEPES, 25 (pH 7.2); $CaCl_2$, 0.36; glucose, 10; and 1% (w/v) BSA (fatty acid free, Pentex fraction V, Miles Laboratories). Undigested material was filtered out with nylon gauze and the suspension centrifuged at 400 x g for 10 min at 20°C. The pellet was resuspended in buffer B, centrifuged again, and then resuspended in MEM containing $NaHCO_3$ (1 g/L), 10% (v/v) fetal calf serum (Gibco, Grand Island, NY), penicillin (60 mg/L), and streptomycin (100 mg/L). The suspension was drawn twice through a 19-gauge needle, filtered through nylon gauze, and dispensed at a density of 4 x 10^5 cells/well in 12-well cell culture plate (Nunc, Copenhagen, Denmark). All experiments were performed after 48 h of culture in 5% CO_2/95% air, at which time the pituitary cells were firmly attached to the substratum.

2.3 CELL PERMEABILIZATION AND STIMULATION

An outline of the basic protocol which was adopted for most experiments in this thesis is shown in Table 3.

TABLE 3 BASIC PROTOCOL FOR PERMEABILIZED CELL EXPERIMENTS

Procedure	Buffer	Time	Temperature
1. Washing	I	15 min	37°C
2. Permeabilization	IC	10 min	37°C
3. Cooling	IC	10 min	0°C
4. Equilibration	IC	30 min	0°C
5. Stimulation	IC	10-30 min	37°C

Adherent anterior pituitary cells were **washed** twice (once briefly, once for 10 min) with buffer I and then once (for 5 min) with Ca^{2+}-free buffer I at 37°C on a slowly rotating shaker. Buffer I comprised (mM): NaCl, 140; KCl, 4; $MgCl_2$, 1; $CaCl_2$, 1; glucose 8.3; HEPES, 20 (pH 7.4); phenol red, 6 mg/L; and 0.1% (w/v) BSA. The cells were then **permeabilized** by incubation in buffer IC (0.3 ml/well) containing 3 µg/ml α-toxin, 0.5 mM EGTA, 6.5 mM $MgCl_2$, and 6 mM Na_2ATP for 10 min at 37C on a slowly rotating shaker. Buffer IC comprised (mM): Na propionate, 140; KCl, 4; NaPIPES, 25 (pH 6.6); phenol red, 6 mg/L; and 0.1% (w/v) BSA. A pH of 6.6 was chosen because it allows buffering of free Ca^{2+} in the concentration range 0.1 to 30 µM using EGTA (see Figure 1). Buffers at this pH are widely used in experiments on permeabilized cells (Knight and Baker, 1982; Wilson and Kirshner, 1983; Dunn and Holz, 1983; Knight et al., 1984; Vallar et al., 1987; Luini and De Matteis, 1988). After 10 min the cell culture plates were **cooled** by placing them directly on ice in a 4°C refrigerator for 10 min, after which time the medium temperature was less than 4°C. The permeabilization medium was aspirated and replaced with ice-cold stimulation buffer (0.3-0.5 ml/well). Stimulation buffer comprised IC buffer with $MgCl_2$ (6.5 mM), Na_2ATP (6 mM) and 10 or 30 mM CaEGTA buffer with the indicated $[Ca^{2+}]_{free}$ (prepared as described in Section 2.6). In all experiments additional nucleotides were added with equimolar $MgCl_2$ unless otherwise indicated. After **equilibration** on ice for the indicated time (usually 30 minutes), LH exocytosis was initiated by warming to 37°C in a waterbath or by replacement with identical medium at 37°C and transferring the cell culture plates to a 37°C incubator. After the indicated **stimulation** time (10-30 min), the medium was removed for LH determination. Detached cells were removed from all stimulation media by centrifugation (400 x g, 8

min, 4°C), and the supernatant was stored at -20°C until LH determination (see Section 2.4).

This basic protocol was modified in some experiments as follows:

(a) In experiments examining staurosporine inhibitory effects (Section 4), cells were permeabilized for 20 min. Staurosporine was added at the indicated concentration after 5 min of permeabilization and was present until the end of the experiment. This allowed a 15 min pre-incubation with staurosporine at 37°C.

(b) In all experiments examining the effects of nucleotides, ATP was omitted during the permeabilization step and was included in the equilibration steps only when indicated in the figure legend. When ATP was omitted $MgCl_2$ 2 mM was present instead of 6.5 mM.

(c) In experiments where guanine nucleotide inhibitory effects were examined (Section 5.2.2), an additional pre-incubation step at 37°C was introduced (see Figure 36). Cells were equilibrated for 30 min at 0°C in the presence of guanine nucleotides and then were warmed to 37°C and pre-incubated for a further 30 min in the same medium. After preincubation the medium was removed and the cells were stimulated for 30 min in stimulation buffer (containing 6.5 mM $MgCl_2$ and 6 mM Na_2ATP).

LH released is expressed as a percentage of the total cellular LH present at the beginning of the stimulation period. Total cellular LH was measured after solubilizing the cells in Nonidet NP40 (1%, v/v). The average cellular LH content calculated from a large number of experiments was 680 ± 60 ng/well (mean ± SEM, n = 38) which is the

equivalent of 1.7 ± 0.2 µg per 10^6 cells or 16.4 ± 1.5 µg/pituitary.

2.4 LH DETERMINATION

Luteinizing hormone was measured by radioimmunoassay using antiserum (NIADDK-anti-oLH-1) and ovine LH (NIADDK-oLH-I-3) kindly provided by the NIDDK and used as recommended. Ovine LH antiserum was used at a final titre of 1 : 2 000 000. LH was iodinated by Chloramine-T method and free I^{125} was separated from I^{125}-LH on a Sephadex G-100 (Pharmacia, Uppsala, Sweden) column. Radioactivity determinations were performed using a Crystal gamma counter (Packard, United Technologies). The antiserum-bound LH was separated from unbound LH by second antibody attached to cellulose (Sac-Cel RD70, Wellcome Reagents Limited, Beckenham, England). After iodination, the proportion of radioactivity in the I^{125}-LH preparation which bound the LH antiserum (B_0/T x 100; B_0 = radiolabel which bound the LH antiserum in the absence of unlabelled LH; T = total radiolabel) was typically 40-50 % and a particular preparation of I^{125}-LH was used until this value fell below 25 percent. Non-specific binding (N/T x 100; N = radioactivity which remained in the tube in the absence of LH antiserum) was routinely < 5 percent. A standard curve was generated using unlabelled ovine LH (NIH-LH-S18) and fitted by the logistic method. The useful range of the assay (B/B_0 x 100 between 80% and 20%; B = antiserum-bound radioactivity in the presence of unlabelled LH) was typically 0.4 to 8 ng/mL ovine LH. Intraassay and interassay coefficients of variation were less than 5% and 8% respectively.

2.5 MEASUREMENT OF PLASMA MEMBRANE PERMEABILITY

Adherent cells were incubated for 1 h at 37°C with 2-deoxy[^{3}H]glucose (0.4 µCi/ml) in glucose-free buffer I (0.75

ml/well). Cells were then washed 7 times (3 times briefly and 4 times for 5 min on a slowly rotating shaker) in buffer I (1 ml/well). The remaining radioactivity represents intracellularly trapped, membrane-impermeant, phosphorylated metabolites of 2-deoxy[^{3}H]glucose. The buffer was then replaced with buffer IC (1 ml/well) containing EGTA (0.5 mM), $MgCl_2$ (1 mM) and the indicated additions, and the radioactivity appearing in the medium was determined on a Beckman LS 3801 liquid scintillation counter after dissolving the medium in scintillation fluid (Instagel, Packard). Total cellular radioactivity was determined after solubilizing cells with 0.5% (w/v) sodium dodecyl sulfate.

Lactate dehydrogenase efflux was measured under the same conditions. Lactate dehydrogenase activity was assayed by spectrophotometric monitoring of NADH disappearance in the presence of excess pyruvate. LDH assays were performed on a Centrifchem System 800 centrifugal analyser (Union, Cambridge).

Efflux of [^{14}C]adenosine- and [^{3}H]uridine- labelled pools was measured in the same way as 2-deoxy[^{3}H]glucose except that the effluxed label is expressed as a percentage of total trichloroacetic acid(TCA)-soluble cellular radioactivity. Total TCA-soluble radioactivity was determined by washing cells twice in 1 ml of 5% TCA at 4°C on a rotating shaker for 5 min. The wash medium was pooled and insoluble material was removed by centrifugation at 1000 x g (0°C, 10 min). The radioactivity in the supernatant was determined by liquid scintillation counting after adjusting the pH to 7 with 0.5 M NaOH.

2.6 CaEGTA AND BaEGTA BUFFERS

2.6.1 Caculation of CaEGTA and BaEGTA ratios

The indicated $[Ca^{2+}]_{free}$ and $[Ba^{2+}]_{free}$ were obtained using EGTA buffers with varying Ca^{2+} to EGTA and Ba^{2+} to EGTA ratios (Figure 1 and Table 4). The required ratios were calculated using a computer program written by us which used equations given in Fabiato and Fabiato (1979) (see Appendix).

The apparent metal-ligand stability constants for ATP and EGTA which were used in the program (Table 5) were calculated from absolute stabilitity constants compiled by Fabiato (1981) which were adjusted for use at 37°C using enthalpy changes as described by Martell and Smith (1976) (see Appendix). In medium containing nucleotide triphosphates, sufficient $MgCl_2$ was always added to maintain free Mg^{2+} at 1-2 mM, calculated using the computer program. The stability constants for Ca^{2+} and Mg^{2+} binding to non-ATP nucleotides were assumed to be similar to those for ATP. Neither nucleotides nor $MgCl_2$ (at the concentrations used experimentally) could have altered the calculated pCa by more than 0.04. Since the pH critically affects the $[Ca^{2+}]_{free}$ and $[Ba^{2+}]_{free}$ at a particular CaEGTA or BaEGTA ratio (Figure 1), all nucleotide triphosphate stock solutions were prepared at pH 6.6 and the effect of all test substances on the pH of the buffer was checked. In experiments performed at pH 7.1 (insets to Figures 9, 14, and 33) the pH of the final stimulation media was adjusted to 7.1 with NaOH.

TABLE 4 CALCULATED CaEGTA AND BaEGTA RATIOS USED TO OBTAIN SPECIFIED $[Ca^{2+}]_{free}$ AND $[Ba^{2+}]_{free}$

	pH 6.6	pH 7.1
(a) pCa	Ca/EGTA Ratios*	
9	-	0.005
8	0.005	0.047
7	0.051	0.332
6.5	0.145	0.611
6	0.349	0.832
5.5	0.629	0.940
5	0.845	0.981
4.5	0.947	-
(b) pBa	Ba/EGTA Ratios*	
4.5	0.043	-
4	0.125	-
3.5	0.316	-
3	0.620	-
2.5	0.942	-

*These ratios were calculated assuming the following final total concentrations (mM): EGTA, 30; Na+, 165, K+, 4, MgCl2, 6.5, Na2ATP 6. However, these ratios vary less than 1 % for total EGTA concentrations ranging from 5 to 30 mM.

TABLE 5 CACULATED APPARENT STABILITY CONSTANTS FOR TEMPERATURE 37°C AND IONIC STRENGTH 0.16 M

Ligand	Cation	Apparent Stability Constant (M^{-1})	
		pH 6.6	pH 7.1
EGTA	Ca2+	546320	5370716
EGTA	Ba2+	1401	13138
EGTA	Mg2+	20	82
ATP	Ca2+	2095	4340
ATP	Ba2+	358	731
ATP	Mg2+	7737	15678
ATP	Na+	5	6
ATP	K+	2	4

2.6.2 Preparation of CaEGTA and BaEGTA buffers

Because of the uncertain purity of EGTA (Miller & Smith, 1984) accurate Ca:EGTA ratios could not be determined by mixed weighed quantities of $CaCl_2$ and EGTA. Instead, CaEGTA buffers of varying Ca:EGTA ratios were prepared by mixing a stock solution of Ca^{2+}-free EGTA with a stock solution of CaEGTA which had a Ca^{2+} to EGTA ratio of precisely 1:1 [CaEGTA(1:1)]. Since both stocks were prepared from the same batch of EGTA they had nearly identical EGTA concentrations (160 mM assuming 97% EGTA purity). Precise CaEGTA ratios were then easily obtained by accurate mixing of these stocks in different ratios. An additional advantage of this method is that if the pH each stock is adjusted to 6.6, the pH of the mixed stocks is invariably also 6.6 irrespective of final Ca:EGTA ratio.

CaEGTA(1:1) was prepared by end-point titration with Ca.oxalate precipitation as the end-point (modified from Tatham and Gomperts, 1987; Miller and Smith, 1984). Briefly, CaEGTA at a ratio of approximately 0.9:1 was prepared by mixing weighed quantitities of $CaCO_3$ (180 mM) and EGTA (200 mM) in water and stirring at 80°C until dissolved. Potassium oxalate (final concentration 50 mM) was added to precisely 25 ml of this CaEGTA(0.9:1) solution. After bringing the pH to > 11 with NaOH, $CaCl_2$ was added from a 0.5 M stock solution in 0.02 ml aliquots using a precision burette while stirring continuously. CaEGTA was assumed to be 1:1 when, after adding 0.02 ml of $CaCl_2$, the solution remained cloudy for > 1 min, indicating Ca.oxalate precipitation. With repeat titrations the volume of $CaCl_2$ required for Ca.oxalate precipitation varied less than 2%. The mean of three determinations was used for further calculations. The appropriate amount of the same 0.5 M $CaCl_2$ stock was then added from the same burette to precisely 200 ml of CaEGTA (0.9:1) to give CaEGTA(1:1). This was brought to pH 6.6 with NaOH and the volume increased to 250 mM to give 160 mM CaEGTA(1:1).

BaEGTA buffers were prepared in the same way except that BaEGTA(1:1) was prepared by mixing weighed quantities of $BaCl_2$ and EGTA. $[Ba^{2+}]_{free}$ concentrations are therefore less accurate than the $[Ca^{2+}]_{free}$ concentrations.

2.6.3 Measurement of $[Ca^{2+}]_{free}$ by Ca^{2+} electrode

The $[Ca^{2+}]_{free}$ of CaEGTA buffers (at the final concentration used experimentally) were checked with a Ca^{2+} selective electrode (Radiometer Selectrode F2112Ca). All determinations were performed at 37°C in Buffer E [comprising (mM): NaCl, 140; KCl, 4; NaPIPES, 25 (pH 6.6)] which resembles Buffer IC but differs in that (1) NaCl is used instead of Na propionate, since propionate binds Ca^{2+}

weakly and (2) phenol red and BSA are not included since they shorten the life of the electrode membrane. Although weak, the binding of propionate to Ca^{2+} interferes with the Ca^{2+} standards since these are unbuffered and the Ca^{2+} concentrations are high. Ca^{2+} standards consisted of $CaCl_2$ in Buffer E, and were calibrated by atomic absorbtion spectrophotometry using a Varian Spectra AA10. Semi-logarithmic plots of the electrode potential produced by the Ca^{2+} standards (pCa 2, 2.5, 3, 3.5, 4) in Buffer E were consistently linear with a slope of 30 ± 1 mV/pCa unit (n = 8). The $[Ca^{2+}]_{free}$ produced by the CaEGTA buffers (pCa 4 to pCa 6.5) diluted in buffer E were checked against the extrapolated standard line. The measured $[Ca^{2+}]_{free}$ produced by the prepared CaEGTA buffers varied less than 0.1 pCa unit from the expected pCa. Below pCa 6.5 the electrode response was non-linear and so the $[Ca^{2+}]_{free}$ could not be directly checked at these low concentrations.

2.7 ASSESSMENT OF NUCLEOTIDE PURITY

Nucleotides were kindly analysed for purity by Dr. Peter Cole on a Spectra Physics 3500 HPLC system with a Hi Chrom APSHYP2537 anion exchange column, using a linear gradient of potassium phosphate from 50 mM, pH 2.5 to 500 mM, pH 3.8.

2.8 STIMULATION OF INTACT CELLS AND CELLULAR CAMP DETERMINATION

Attached cells were washed four times (twice briefly and then twice for 10 min) with Buffer I followed by stimulation in buffer I for 60 minutes at 37°C. The medium was collected and processed for LH determination as described in sections 2.3 and 2.4. For cAMP extraction cells were dissolved in 0.4 ml 0.1 M HCl which was neutralised with 0.1 ml 100 mM Na-Tris (pH 13) before cAMP determination by competitive binding assay (Amersham kit no. TKR 342)

according to the kit instructions. The assay principle is the competition by cold cAMP with [8-^{3}H]cAMP for binding to a specific cAMP-binding protein. Free [8-^{3}H]cAMP is removed by coated charcoal. The unknown [cAMP] is calculated with reference to a standard curve generated with cAMP standards. The intraassay coefficient of variation was less than 5 percent.

2.9 DATA PRESENTATION

Data points and error bars represent the mean and the SEM of triplicate or the mean and range of duplicate determinations. The absence of error bars indicates that the error was smaller than the dimensions of the symbol. ANOVA and the modified Student's t-test were used to test statistical significance where indicated.

3 PERMEABILIZATION OF CELLS AND CHARACTERIZATION OF CALCIUM-, PHORBOL ESTER-, AND cAMP-REGULATED LH EXOCYTOSIS

3.1 INTRODUCTION

Studies in intact pituitary gonadotropes have implicated a rise in cytosolic $[Ca^{2+}]_{free}$ as the major intracellular mechanism mediating GnRH-stimulated LH exocytosis (see Section 1.3). The ability of raised cytosolic $[Ca^{2+}]_{free}$ to stimulate LH exocytosis has been studied in intact cells using Ca^{2+} ionophores (Hopkins and Walker, 1978; Conn et al., 1979b). It is not possible, however, to achieve sustained and accurately defined $[Ca^{2+}]_{free}$ using this method. To determine the precise Ca^{2+} requirements for LH exocytosis, direct access to the cytosol is required.

Although GnRH clearly activates PKC (Hirota et al., 1985; Naor et al., 1985b; Strulovici et al., 1987) and activators of PKC stimulate LH exocytosis in intact cells (Smith and Vale, 1980), the role of PKC in mediating GnRH-stimulated LH exocytosis remains controversial (McArdle et al., 1987; Stojilkovic et al., 1988a,b; Conn, 1989). One problem with studies in intact cells is that activators of PKC can stimulate changes in cytosolic $[Ca^{2+}]_{free}$ (Albert et al., 1987; Stojilkovic et al., 1988a; Restrepo et al., 1989; Yada et al., 1989), presumably through effects of PKC on Ca^{2+}-regulating proteins (Nishizuka, 1986; Yamaguchi et al., 1987; Smallwood et al., 1988; Yada et al., 1989; Yoshida and Nachmias, 1989). As a result, experiments in intact cells leave open the possibility that effects of PKC activation on LH exocytosis are secondary to increases in cytosolic $[Ca^{2+}]_{free}$.

As is the case with PKC, the role of cAMP in mediating GnRH-stimulated LH exocytosis is controversial. Early reports claiming a second messenger role for cAMP (Borgeat et al.,

1972; Kaneko et al., 1973; Makino, 1973; Bonney and Cunningham, 1977; Kercret et al., 1977) were not confirmed in subsequent work (Naor et al., 1975; Conn et al., 1979a; Sen and Menon, 1979; Benoist et al., 1981). Since all these studies were performed in intact cells, the effects of cAMP on LH exocytosis were examined using high concentrations of membrane-permeable cAMP analogues or by activating adenylate cyclase with forskolin. These approaches have pitfalls since cAMP analogues are used at concentrations at which they interact with the GnRH receptor (Smith et al., 1982; Capponi et al., 1984) while forskolin is not entirely specific (Hoshi et al., 1988). More importantly, cAMP can activate voltage sensitive plasma membrane Ca^{2+} channels in gonadotropes (Mason and Sikdar, 1989) and other cell types (Osterrieder et al., 1982; Curtiss and Catterall, 1985; Armstrong and Eckert, 1987) and thereby stimulate an increase in cytosolic $[Ca^{2+}]_{free}$ (Luini et al., 1985; Leong, 1988; Lau and Bourdau, 1989). This could explain the finding that forskolin-stimulated LH secretion is dependent on extracellular Ca^{2+} and inhibited by voltage-sensitive Ca^{2+} channel blockers (Cronin et al., 1984).

By utilizing permeabilized cells, several of the pitfalls of previous studies can be bypassed because the cytosolic $[Ca^{2+}]_{free}$ can be clamped at the desired level using Ca^{2+} buffers and furthermore, membrane-impermeable agents such as cAMP can be introduced directly into the cell at known concentrations. An additional advantage of permeabilized cells is that the requirements of exocytosis for endogenous small molecules (such as ATP) may be determined.

In this section the permeabilization procedure is described, the regulation of LH exocytosis by Ca^{2+}, PKC, and cAMP is characterized, and the role of ATP in regulated exocytosis is investigated. Although exocytosis has been investigated in permeabilized cells of the pituitary-derived GH_3 and

AtT20 tumor cell lines (Ronning and Martin, 1986a; Luini and De Matteis, 1988), this is the first study on the regulation of exocytosis in permeabilized primary anterior pituitary cells.

3.2 RESULTS

3.2.1 α-Toxin permeabilization

Permeabilization of primary sheep anterior pituitary cells was monitored using 2-deoxy[^{3}H]glucose. Plasma membrane-impermeant phosphorylated metabolites of 2-deoxy[^{3}H]glucose (M_r 250) effluxed very slowly from intact cells, whereas after addition of 3-30 µg/ml staphylococcal α-toxin, 85% of trapped 2-deoxy[^{3}H]glucose metabolites was released within 18 minutes (Figure 2). Increasing the α-toxin above 3 µg/ml did not significantly increase the final extent or the maximal rate of 2-deoxy[^{3}H]glucose efflux but did shorten the lag time before onset of efflux (Figure 2). When the efflux of phosphorylated metabolites of [^{14}C]adenosine and [^{3}H]uridine (which comprise mostly [^{14}C]ATP and [^{3}H]UTP, respectively) were used to monitor permeabilization, α-toxin (3 µg/ml) evoked efflux of 85% (Figure 19) and 80% (Figure 3), respectively, of total trichloroacetic acid-soluble cellular label within 20 minutes. Under the same conditions α-toxin caused efflux of less than 2% of cellular lactate dehydrogenase (Mr 134 000, Figure 3), indicating that the cells are rendered selectively permeable to small molecules including nucleotide triphosphates. In cells exposed to α-toxin (3 µg/ml) for the initial 5 minutes only, the rate and extent of efflux of 2-deoxy[^{3}H]glucose label was as great as during continuous exposure, indicating that permeabilization was complete within 5 minutes (Figure 4). The efflux rate of 2-deoxy[^{3}H]glucose label (half-time 5-10 minutes) is therefore probably limited by diffusional constraints (limited size and number of pores) rather than the rate of

pore formation. Permeabilization does not occur at 0°C (Table 6). Decreasing the temperature from 37°C to 0°C after five minutes caused only a small (15%) decrease in the efflux of label (Table 6). This lack of temperature dependence suggests that efflux occurs by simple diffusion once permeabilization has occured and confirms that permeabilization is complete within 5 min. None of the agents used in subsequent experiments on permeabilized cells affected α-toxin permeabilization as monitored by efflux of 2-deoxy[^{3}H]glucose label (Table 6).

3.2.2 Ca^{2+} dependence for LH Exocytosis

A five-step protocol was employed for most experiments on LH exocytosis (Table 3). After (1) **washing**, cells were (2) **permeabilized** at 37°C in low Ca^{2+} medium. The cells were then (3) **cooled**, after which they were (4) **equilibrated** with stimulation medium containing defined buffered $[Ca^{2+}]_{free}$ at 0°C for at least 30 minutes. This was designed to allow adequate time for equilibration of small extracellular molecules such as CaEGTA (Figure 7B) and ATP (Figures 19 and 20) with the cytoplasm. Permeabilization and equilibration were performed separately because α-toxin permeabilization is strongly retarded at low temperatures (Table 6). After equilibration, exocytosis was (5) **stimulated** by increasing the stimulation medium temperature to 37°C. Under these conditions minimal LH release occurred during the permeabilization (1 ± 0.2 %, n = 2) and equilibration periods (Table 7).

TABLE 6 THE EFFECTS OF VARIOUS AGENTS ON α-TOXIN PERMEABILIZATION

Permeabilization was monitored by measuring efflux of intracellular 2-deoxy[^{3}H]glucose metabolites over 20 min. Experiments were conducted in buffer IC with EGTA 0.5 mM and $MgCl_2$ 1 mM except when Ca^{2+} and Ba^{2+} were tested (where EGTA was 30 mM) or when ATP was tested (where $MgCl_2$ was 6.5 mM).

Addition	2-deoxy[^{3}H]glucose efflux (% of control)[a]
control	100
no α-toxin	14 ± 1
37°C (5 min) then 0°C (15 min)[b]	85 ± 1
0°C[c]	4 ± 1
0°C (no α-toxin)[c]	3 ± 1
pCa 8	99 ± 1
pCa 6	99 ± 1
pCa 4	100 ± 2
pBa 2.5	104 ± 4
cAMP (300 μM)	105 ± 4
IBMX (0.5 mM)	101 ± 6
ATP (6 mM)	97 ± 1
GTPγS (100 μM)	98 ± 3
trifluoperazine (30 μM)	102 ± 1
staurosporine (1 μM)	99 ± 1

[a]The control value represents permeabilization by 3 μg/ml α-toxin at 37°C without additions and is normalized to 100 %. The mean and range duplicate determinations are shown.
[b]After permeabilization for 5 min at 37°C, the medium was replaced with identical medium at 0°C and incubated for a further 15 min on ice. Total efflux over 20 min is shown.
[c]After washing, permeabilization medium was added at 0°C.

TABLE 7 LH RELEASE DURING THE EQUILIBRATION PERIOD

Permeabilized cells were equilibrated at 0°C in stimulation buffer with CaEGTA 30 mM and the indicated $[Ca^{2+}]_{free}$ for 30 min after which time the medium was removed for LH determination.

	LH released (%)[a]
pCa 7	0.7 ± 0.2
pCa 5	1.1 ± 0.3[b]

[a]Mean and SEM of three independent experiments.
[b]Not significantly different from pCa 7 ($p > 0.05$).

When permeabilized cells which had been equilibrated with CaEGTA (pCa 5) and MgATP-containing medium were warmed to 37°C, rapid exocytosis of LH was initiated (Figure 5). Exocytosis was maximal during the first 3 minutes and declined to basal levels after 12 minutes (Figure 5). This decline could result either from the development of refractoriness to maintained high $[Ca^{2+}]_{free}$ or, alternatively, from a lowering of cytosolic $[Ca^{2+}]_{free}$ by cellular Ca^{2+} sequestration mechanisms activated by warming to 37°C. The latter possibility is unlikely since exocytosis of LH at sub-maximal free Ca^{2+} concentrations (pCa 5.5) continued after 12 minutes (Figure 5). Maximal LH exocytosis occurred at pCa 5.25-4.75 (Figure 6) with half-maximal exocytosis at pCa 5.7-5.4 (Figure 6, Table 8). Although the Ca^{2+} EC_{50} for LH exocytosis was highly reproducible, the extent of LH release was quite variable between experiments (Table 8). For this reason, instead of combining results from different experiments, results of representative experiments are shown.

TABLE 8 Ca^{2+}-STIMULATED LH EXOCYTOSIS

All experiments were performed as described in the legend to Figure 6. The EC_{50}s for Ca^{2+} were deduced by visual inspection of the Ca^{2+} dose-response curve.

Experiment	Ca^{2+} EC_{50} (pCa)	Maximal LH Exocytosis (%)
1.	5.6	27
2.	5.7	12
3.	5.4	21
4.	5.4	20
5.	5.5	26
6.	5.7	46
7.	5.5	18
8.	5.5	20
9.	5.4	19
10.	5.4	32
11.	5.7	12
12.	5.5	29
13.	5.5	13
14.	5.6	22
Mean (± SEM)	5.5 (± 0.1)	23 (± 3)

A consistent finding in experiments was an inhibition of exocytosis at $[Ca^{2+}]_{free}$ above pCa 4.5 (Figure 6). Permeabilization was unaffected by varying the Ca^{2+} concentration over the range pCa 8 to 4 (Table 6), indicating that the decrease in exocytosis at high $[Ca^{2+}]_{free}$ is not due to closure of the pores. Basal LH exocytosis from intact cells was unaffected by varying the $[Ca^{2+}]_{free}$ from pCa 7 to 4 (Figure 6).

High total EGTA concentrations were necessary to achieve adequate Ca^{2+} buffering. When the EGTA concentration was varied while keeping the $[Ca^{2+}]_{free}$ constant (verified by Ca^{2+} electrode), exocytosis was dependent on EGTA concentration up to 20 mM (Figure 7A). In addition, at low [EGTA] the apparent Ca^{2+}-dependence of exocytosis was markedly shifted towards higher Ca^{2+} concentrations (Figure 8). This indicates that Ca^{2+} buffering is inadequate when EGTA concentrations are below 20 mM and that, as a result, the cytosolic $[Ca^{2+}]_{free}$ is lowered by cellular Ca^{2+} sequestration mechanisms. Under conditions of inadequate Ca^{2+} buffering the true Ca^{2+} dependence of exocytosis cannot be determined. Furthermore, with weak buffering any manipulation which altered the rate of Ca^{2+} sequestration would be likely to alter the cytosolic $[Ca^{2+}]_{free}$. Consequently, CaEGTA buffers were used at 30 mM (EGTA concentration) in all experiments in which the $[Ca^{2+}]_{free}$ was different to resting values (pCa 7). Increasing the equilibration period from 10 to 30 minutes at a suboptimal EGTA concentration (10 mM) did not increase LH exocytosis (Figure 7B), indicating that the 30 minute equilibration period used allows ample time for equilibration of CaEGTA.

3.2.3 PKC activation and LH exocytosis

The PKC-activating phorbol ester PMA stimulated LH exocytosis to a similar extent at all $[Ca^{2+}]_{free}$ without significantly modifying the Ca^{2+} sensitivity of LH exocytosis (Figures 9, 26 and 27). PMA was able to stimulate LH exocytosis at Ca^{2+} concentrations as low as pCa 8, suggesting that Ca^{2+}-independent exocytosis is possible (Figure 9). The stability constant for Ca-EGTA binding is such that it is not possible to accurately decrease the $[Ca^{2+}]_{free}$ below pCa 8 using EGTA at pH 6.6 (Figure 1, Table 4). The experiment was therefore performed at pH 7.1 at which the tighter binding of Ca^{2+} to EGTA permits lowering

of the $[Ca^{2+}]_{free}$ to pCa 9 (Figure 9, inset). PMA was able to stimulate exocytosis even at this very low $[Ca^{2+}]_{free}$, confirming that PKC-stimulated Ca^{2+}-independent exocytosis can occur. In a time course, maximal PMA-stimulated LH exocytosis occurred in the first 3 minutes followed by a period of slow release which continued for at least 15 minutes (Figure 10). PMA-stimulated LH exocytosis was maximal at 100-300 nM and half-maximal at 20-50 nM (Figures 16 and 34).

TABLE 9 EFFECT OF cAMP ON LH EXOCYTOSIS IN INTACT CELLS

The standard protocol was used (Table 3) except that α-toxin was omitted from the permeabilization medium for experiments on intact cells. Cells were then equilibrated in stimulation buffer for 30 min at 0°C with CaEGTA 10 mM (pCa 7) and the indicated additions. Exocytosis was initiated by replacing with identical medium at 37°C and LH released after 10 min was determined.

	LH released (%)[a]
(a) Permeabilized Cells	
control	3.2 ± 0.7
cAMP 300 μM	8.3 ± 0.1
(b) Intact Cells	
control	3.7 ± 0.5
cAMP 300 μM	2.3 ± 0.4

[a]Mean ± range (n = 2)

3.2.4. cAMP-stimulated LH exocytosis

At resting $[Ca^{2+}]_{free}$ (pCa 7) cAMP stimulated LH exocytosis with half-maximal LH release at 30 μM cAMP (Figure 11). LH exocytosis from intact cells was unaffected by cAMP over a similar concentration range (Table 9). cAMP stimulated LH exocytosis within 5 min and stimulation was sustained for at least 30 min (Figure 12). This differs from Ca^{2+}-stimulated LH exocytosis which is transient with no further exocytosis evident after 12-15 min (Figure 5).

The presence of the cyclic nucleotide phosphodiesterase inhibitor IBMX (0.25 mM) decreased the EC_{50} of cAMP from 30 μM to 10 μM (Figure 11). When used alone, or in combination with IBMX, cGMP (300 μM) did not stimulate LH exocytosis (Table 10). This result suggests that cGMP does not have a role in mediating acute GnRH-stimulated LH exocytosis, a finding which differs from an earlier study in intact cells (Snyder et al., 1978). It also indicates that IBMX enhances cAMP-stimulated LH exocytosis by inhibiting cAMP hydrolysis rather than cGMP hydrolysis. All subsequent experiments with cAMP were conducted in the presence of 0.25 mM IBMX, a concentration which caused optimal enhancement of cAMP-stimulated LH exocytosis without increasing basal LH release (Figure 13). Neither cAMP nor IBMX affected α-toxin permeabilization as measured by leakage of phosphorylated 2-deoxy[^{3}H]glucose metabolites (Table 6).

TABLE 10 THE EFFECTS OF cGMP ON LH EXOCYTOSIS

Permeabilized cells were equilibrated at 0°C for 30 minutes with stimulation buffer containing 10 mM CaEGTA (pCa 7) and the indicated additions. Exocytosis was initiated by replacing with identical buffer at 37°C and LH release after 10 minutes was determined.

Addition	% LH release[a]
control	2.50 ± 0.11
cGMP 300µM	2.90 ± 0.10*
IBMX 0.25mM	2.78 ± 0.32*
cGMP 300µM + IBMX 0.25mM	2.96 ± 0.33*
cAMP 300µM + IBMX 0.25mM	7.04 ± 0.22**

[a]Mean and SEM of triplicate determinations.
*not significantly different from control
**significantly different from control ($p < 0,001$)

cAMP caused a modest increase in the the sensitivity of LH exocytosis to Ca^{2+}, shifting the EC_{50} for Ca^{2+} from pCa 5.6 to pCa 5.9, but had little effect at high $[Ca^{2+}]_{free}$ (Figure 14). To investigate the effect of cAMP at very low $[Ca^{2+}]_{free}$, pH 7.1 (rather than 6.6) was used, for the same reasons explained in Section 3.2.3. Although cAMP-stimulated LH exocytosis was much reduced at low $[Ca^{2+}]_{free}$, some stimulation was still evident at $[Ca^{2+}]_{free}$ as low as pCa 9 (Figure 14, inset; Table 11).

TABLE 11 cAMP-STIMULATED LH EXOCYTOSIS AT LOW $[Ca^{2+}]_{free}$

The combined results of several independent experiments similar to Figure 14 are shown.

	LH released (%)[a]
(a) pCa 9 (n = 3)	
Control	3.0 ± 0.4
cAMP 100 μM + IBMX 0.25 mM	4.2 ± 0.4[b]
(b) pCa 8 (n = 5)	
Control	2.3 ± 0.3
cAMP 100 μM + IBMX 0.25 mM	3.9 ± 0.4[b]

[a]Mean ± SEM.

[b]Significantly different from the control ($p < 0.05$)

PMA (100 nM) synergistically enhanced cAMP-stimulated LH exocytosis by both decreasing the EC_{50} for cAMP from 10 μM to 3 μM (Figures 11 and 15) and increasing the maximum LH response 5-7 fold (Figures 15 and 16). This synergistic interaction was present at low concentrations of PMA (1-3 nM) which did not stimulate LH exocytosis in the absence of cAMP (Figure 16). Synergism between cAMP and PMA was decreased at low $[Ca^{2+}]_{free}$ (pCa 8) but was fully expressed at resting $[Ca^{2+}]_{free}$ (pCa 7) and was not further enhanced at higher $[Ca^{2+}]_{free}$ (Figure 17).

To further examine whether cAMP may mediate the effects of GnRH on LH exocytosis, the ability of GnRH to stimulate cAMP generation in intact cells was examined. In the presence of IBMX, GnRH stimulated LH release from intact cells with $EC_{50} = 10^{-9}$ M (Figure 18). Under the same conditions, cellular

cAMP content was increased by GnRH with EC_{50} = 3 x 10^{-8} M (Figure 18).

3.2.5 ATP Requirements for LH Exocytosis

All the preceding experiments were conducted in the presence of 5 mM MgATP. Prolonged equilibration of permeabilized cells at 0°C in the absence of MgATP resulted in a gradual decrease in LH exocytosis when cells were subsequently stimulated with Ca^{2+} (pCa 5) at 37°C (Figure 19). The half-time of this decrease (10-15 min) was similar to the half-time of efflux of [^{14}C]adenosine label (8-10 min; Figure 19). Inclusion of 5 mM MgATP entirely prevented this decrease (Figure 19). When MgATP (final concentration 5mM) was added to permeabilized cells equilibrated for 70-90 minutes in the absence of MgATP, Ca^{2+}-stimulated LH exocytosis was restored (Figure 20). Full restoration of Ca^{2+}-stimulated LH exocytosis required addition of MgATP 10 minutes before exocytosis was initiated (Figure 20), the time lag presumably reflecting equilibration of extracellular MgATP with the cytosol. In addition to estabilishing the MgATP dependence of Ca^{2+}-stimulated LH exocytosis, these results demonstrate that α-toxin pores remain patent for at least 90 minutes at 0°C despite the absence of α-toxin in the medium. The continued ability of α-toxin-permeabilized cells to undergo undiminished Ca^{2+}-stimulated exocytosis after more than 1 hour of equilibration at 0°C demonstrates the stability of the exocytotic mechanism in α-toxin permeabilized cells and argues against a requirement for any small molecule other than ATP for stimulated LH exocytosis.

To further characterize the role of MgATP the concentration dependence of MgATP-supported Ca^{2+}-stimulated exocytosis was examined. Half-maximal support of LH exocytosis occurred at 1.5 mM MgATP (Figure 21A). Of several nucleotide

triphosphates tested only UTP and ATPγS caused significant enhancement of Ca^{2+}-stimulated exocytosis in MgATP-depleted cells but both were less effective than ATP at the same concentration (Figure 22). High performance liquid chromatography analysis of these nucleotides performed by Dr. Peter Cole (Dept. Chemical Pathology, University of Cape Town) indicated that these compounds were not detectably contaminated with ATP (less than 2% contamination). It is notable that ATPγS stimulated LH exocytosis at pCa 7 (Figure 22), an effect which may result from the formation of GTPγS from ATPγS, catalyzed by the action of nucleoside diphosphate kinases acting on endogenous GDP (see Section 5).

Like Ca^{2+}-stimulated LH exocytosis, both PMA- and cAMP-stimulated exocytosis were also dependent on millimolar MgATP concentrations (Figures 21B and 23). Half-maximal support of cAMP- and PMA-stimulated LH exocytosis occurred at about 3 mM and 2.5 mM MgATP respectively (Figures 21B and 23).

3.3 DISCUSSION

3.3.1 α-Toxin permeabilized cells as a model for regulated exocytosis

Several lines of evidence suggest that stimulated LH release from α-toxin permeabilized pituitary cells occurs by exocytosis. (1) LH release is stimulated by micromolar $[Ca^{2+}]_{free}$ and is dependent on millimolar MgATP concentrations. These are properties typical of regulated exocytosis in other permeabilized cell systems (Knight and Baker, 1982; Knight et al., 1984; Ronning and Martin, 1986a). (2) Ca^{2+}-stimulated LH release from permeabilised cells does not occur at 0°C, consistent with exocytosis rather than leakage of LH from damaged cells. Efflux of 2-

deoxy[^{3}H]glucose metabolites was only slightly decreased at 0°C, indicating that inhibition of LH release at 0°C was not due to closure of α-toxin pores. (3) Whereas α-toxin causes efflux from cells of small molecules such as 2-deoxy[^{3}H]glucose-, [^{3}H]uridine- and [^{12}C]adenosine-labelled metabolites, large molecules such as LDH (M_r 134 000) do not leak out. It is unlikely, therefore, that LH-containing secretory vesicles, which are much larger than LDH, could leak out. (4) And finally, since α-toxin pores are are too small to transmit the α-toxin monomer (Mr 34 000), a direct effect of α-toxin on secretory vesicles is unlikely to occur.

Although staphylococcal α-toxin has advantages over more widely used permeabilization tools such as digitonin or high voltage electric fields (see Section 1.2.3), the small size of the pores has the disadvantage that exchange of permeant molecules is slow ($t_{1/2}$ 8-10 min). To compensate for this an equilibration step at 0°C was introduced, during which exchange of small molecules through the pores continues while exocytosis is prevented. Slow exchange through the pores probably also explains the requirement for strong Ca^{2+} buffering (see below).

3.3.2 Ca^{2+} dependence of LH exocytosis

The Ca^{2+} dependence of LH exocytosis from α-toxin permeabilized sheep gonadotropes (half-maximal stimulation at 2-3 μM $[Ca^{2+}]_{free}$) is similar to that found for exocytosis in cells permeabilized using other techniques (Knight and Scrutton, 1986; Peppers and Holz, 1986; Howell and Gomperts, 1987; Luini and De Matteis, 1988). In contrast, the Ca^{2+} requirements of exocytosis in α-toxin-permeabilized adrenal chromaffin (Bader et al.,1986) and PC12 (Ahnert-Hilger et al., 1987) cells were reported to be much higher, with half-maximal secretion requiring $[Ca^{2+}]_{free}$ greater than 10 μM. An explanation for these

discrepant results is suggested by the finding that high CaEGTA buffer concentrations are required to overcome cellular Ca^{2+} sequestration in α-toxin permeabilized gonadotropes. Indeed, at low CaEGTA buffer concentrations (5 mM), half-maximal LH exocytosis occurred at apparent $[Ca^{2+}]_{free}$ greater than 10 μM. Grant et al. (1987) have compared the Ca^{2+} sensitivity of exocytosis in digitonin-permeabilized- and α-toxin-permeabilized adrenal chromaffin cells using identical Ca^{2+} buffering systems. They found that half-maximal exocytosis occurred at 3 and 30 μM $[Ca^{2+}]_{free}$ in digitonin-permeabilized- and α-toxin-permeabilized adrenal chromaffin cells, respectively. Taken together with results in this study, these findings suggest that more effective Ca^{2+} buffering is required to clamp cytosolic $[Ca^{2+}]_{free}$ in α-toxin permeabilized cells than in cells permeabilized using detergents or electric fields. This may be a consequence of a small pore size, resulting in a relatively slower equilibration of CaEGTA in α-toxin permeabilized cells, and allowing more time for cellular Ca^{2+} sequestration.

Shangold et al. (1988) have demonstrated, using fura-2 in single rat gonadotropes, that GnRH can stimulate oscillatory increases in cytosolic $[Ca^{2+}]_{free}$ which occasionally reach 1 μM. The findings of the present study suggests that even this level is too low to stimulate significant LH exocytosis. However, since this value represents the average cytosolic $[Ca^{2+}]_{free}$ over a whole cell, it underestimates the large subcellular variations in $[Ca^{2+}]_{free}$ which are known to occur in many cells, including gonadotropes, upon stimulation (Leong, 1989; Tank et al., 1988; Cheek et al., 1989; Foskett et al., 1989; Kim and Westhead, 1989; Silver et al., 1990). This variation probably results from the localized mobilization of intracellular (Cheek et al., 1989) and extracellular (Silver et al., 1990) Ca^{2+} combined with efficient buffering of increases in cytosolic $[Ca^{2+}]_{free}$

(Rose and Loewenstein, 1975). It is therefore possible that, when intact cells are stimulated, $[Ca^{2+}]_{free}$ of 1 to 10 μM are reached in a localized region at the exocytotic site as a result of activation of plasma membrane Ca^{2+} channels and mobilization of intracellular Ca^{2+} stores close to the plasma membrane. The observation that, in the GH_3B_6 pituitary cell line, thyrotropin releasing hormone stimulates oscillatory increases in cytosolic free Ca^{2+} which reach 10 μM, indicates that very high $[Ca^{2+}]_{free}$ are attainable in response to physiological stimuli (Winiger and Schlegel, 1988).

The time course of Ca^{2+}-stimulated LH exocytosis in permeabilized cells is similar to GnRH-stimulated LH exocytosis in intact cells with an initial spike phase of rapid exocytosis followed by a plateau phase of slow exocytosis (Davidson at al., 1988; Tasaka et al., 1988). This finding suggests that the biphasic pattern of GnRH-stimulated LH exocytosis in intact cells is not merely a consequence of the biphasic changes in cytosolic free Ca^{2+} concentration (Limor et al., 1987; Tasaka et al., 1988) but also reflects a change in the response of the exocytotic mechanism to a raised constant $[Ca^{2+}]_{free}$. The finding of the present study that exocytosis terminates after 12 minutes exposure to maximally effective $[Ca^{2+}]_{free}$ (pCa 5), whereas it continues for longer at submaximal $[Ca^{2+}]_{free}$ (pCa 5.5), suggests that LH exocytosis becomes refractory to high $[Ca^{2+}]_{free}$. This could result either from desensitization to Ca^{2+} at some step in the exocytotic mechanism, or from a depletion of Ca^{2+}-sensitive LH stores.

3.3.3 PKC activation and LH exocytosis

GnRH activates PKC and increases the cytosolic $[Ca^{2+}]_{free}$ (see Section 1.3.2), suggesting that these two simultaneous events may both be involved in GnRH-stimulated LH

exocytosis. This possibility is supported by the finding in the present study that the PKC activator, PMA, stimulated LH exocytosis over a range of Ca^{2+} concentrations. PMA-stimulated exocytosis required millimolar MgATP concentrations, which rules out the possibility that PMA-stimulated LH exocytosis at low $[Ca^{2+}]_{free}$ results from the action of PMA on a residual population of intact cells since, in that case, PMA-stimulated exocytosis would not be MgATP dependent. The observation that, like Ca^{2+}, PMA stimulates biphasic LH exocytosis with an early spike phase followed by a late plateau phase suggests that PKC-activation may be involved in both the spike and plateau phases of GnRH-stimulated LH exocytosis in intact cells. The ability of PMA to stimulate LH exocytosis at very low $[Ca^{2+}]_{free}$ suggests that exocytosis is regulated by an isoenzyme of PKC which has a very low Ca^{2+} requirement. In this context it is of interest that the predominant PKC subtype found in the pituitary is the type II (or type ß) isozyme (Yoshida et al., 1988) which retains considerable activity in vitro at very low $[Ca^{2+}]_{free}$ (Nishizuka, 1988).

3.3.4 cAMP-stimulated LH exocytosis

Since cAMP is known to increase the cytosolic $[Ca^{2+}]_{free}$ in a variety of cell types (see Section 3.1), the ability of cAMP analogues or forskolin to stimulate LH exocytosis in intact cells may be a consequence of increased $[Ca^{2+}]_{free}$. In the present experiments, cytosolic $[Ca^{2+}]_{free}$ is strongly buffered with high concentrations of EGTA. The results establish conclusively that cAMP can stimulate LH exocytosis directly without any change in the $[Ca^{2+}]_{free}$. The rapid effect of cAMP (evident at 5 min) indicates that cAMP could play a role in acute GnRH-stimulated LH exocytosis during which stores of previously-synthesized LH are released. Previous studies using intact rat pituitary cells have demonstrated stimulatory effects of cAMP analogues or

forskolin on LH secretion but stimulation was generally observed only after 1 to 4 hours (Makino, 1973; Naor et al., 1975; Cronin et al., 1984; Bourne and Baldwin, 1987). The effect of cAMP analogues may be delayed because of their slow entry into the cell or because, in the rat, the effects of cAMP are mediated by an increase in LH synthesis (Bourne and Baldwin, 1987).

The ability of raised $[Ca^{2+}]_{free}$ to stimulate an increase in cytosolic cAMP concentrations by activating Ca^{2+}/calmodulin dependent adenylate cyclases (Brostrom et at., 1982; Minocherhomjee et al., 1988) raises the possibility that Ca^{2+} stimulates LH exocytosis indirectly through effects on cAMP. This is clearly not the case since high $[Ca^{2+}]_{free}$ stimulated much more extensive LH exocytosis than maximally-effective cAMP concentrations and Ca^{2+} and cAMP, when used together, showed a synergistic interaction in stimulating LH exocytosis.

When used alone, high concentrations of cAMP were necessary to stimulate exocytosis (EC_{50} 30 μM in the absence of IBMX) and the maximal effect was small when compared to stimulation with Ca^{2+} or phorbol ester. These findings might cast doubt on the physiological relevance of cAMP as a mediator of exocytosis. However, in the presence of the phorbol ester PMA, cAMP stimulated extensive LH exocytosis at low micromolar concentrations, well within the range of cAMP concentrations expected in vivo. Since GnRH stimulation results in an increase in cytosolic $[Ca^{2+}]_{free}$ and activates PKC (see Section 1.3.2), even a small increase in the cytosolic cAMP concentration would result in significant enhancement of LH exocytosis. Because PMA has previously been shown to be cabable of stimulating adenylate cyclase activity (Brostrom et al., 1982; Summers and Cronin, 1986) it could be argued that the effects of phorbol ester result from an increase in cAMP production. However, our finding

that very low concentrations of cAMP dramatically enhance PMA-stimulated LH exocytosis suggest that cAMP does not mediate the effect of PMA.

The stimulation of cAMP production by GnRH in intact gonadotropes supports a role for cAMP in GnRH-stimulated LH exocytosis. The different dose-dependence of GnRH-stimulated LH exocytosis and GnRH-stimulated cAMP production (EC_{50} 10^{-9} M and $3x10^{-8}$ M respectively) is not unexpected since theoretical modelling of second messenger cascades predicts a shift in agonist dose-dependence to lower agonist concentrations when responses further down the cascade are examined (Strickland and Loeb, 1981).

In conclusion, these results demonstrate that cAMP is able to directly stimulate LH exocytosis independently of its ability to cause changes in the $[Ca^{2+}]_{free}$ and that cAMP synergistically enhances PMA- and Ca^{2+}-stimulated LH exocytosis. These findings suggest that cAMP plays a major role in GnRH-stimulated LH exocytosis, through its synergistic interactions with PKC and Ca^{2+}.

3.3.5 ATP requirements for LH exocytosis

The results presented in Section 3.2.5 show that Ca^{2+}-, PMA-, and cAMP-stimulated LH exocytosis are absolutely dependent on the presence of millimolar concentrations of ATP. In studies on several other cell types, including the GH_3 and AtT20 pituitary tumour cell lines, stimulated exocytosis was only partially decreased in the absence of ATP (Ronning and Martin, 1986a; Peppers and Holz, 1986; Luini and Matteis, 1988). Our finding that prolonged equilibration in the absence of ATP is required before complete loss of Ca^{2+}-stimulated LH exocytosis occurs suggests that ATP depletion may have been incomplete in these other studies. A recent study by Holz et al. (1989)

suggests that, in adrenal chromaffin cells, a component of Ca^{2+}-stimulated exocytosis can occur in the absence of ATP, provided that the cells are exposed to ATP before Ca^{2+} stimulation. Since stimulated exocytosis was lost rapidly (within 8 min) in the absence of ATP, these results do not contradict the present findings, but suggest instead that ATP may be required for a reversible priming reaction which occurs independently of Ca^{2+}. The ATP dependence that we observed may result from a reversal of this priming step.

The results of the present study allow limited speculation concerning the precise nature of ATP-requiring step(s) involved in stimulated LH exocytosis. The finding that millimolar ATP concentrations are required for Ca^{2+}-, PMA-, and cAMP-stimulated LH exocytosis, while most protein kinases (Edelman et al., 1987), including PKC (Kishimoto et al., 1977; Wise et al., 1982) and PKA (Beebe and Corbin, 1986), require only micromolar ATP concentrations (Km typically < 20 μM), suggests that there is an ATP-dependent step other than protein kinase activation involved in second-messenger-stimulated LH exocytosis. An alternative explanation is that the cytosolic ATP concentrations obtained using our experimental protocol are considerably (at least 100 fold) lower than the extracellular ATP concentrations. Failure of the added ATP to reach the cytosol seems unlikely in view of the permeability of the cells to radiolabelled nucleotides. Rapid breakdown of ATP is also unlikely because it would require the consumption of more than 99% of the available cytosolic ATP within minutes of the initiation of LH exocytosis by warming to 37°C. Furthermore, if exocytosis required only micromolar concentrations of ATP then the time dependence of the loss of Ca^{2+}-stimulated LH exocytosis (Figure 19) would not be similar to the time-dependence of the efflux of [^{14}C]adenosine-labelled metabolites. The lower relative effectiveness of the slowly-hydrolysing ATP analogue, ATPγS,

in supporting Ca^{2+}-stimulated LH exocytosis suggests that ATP hydrolysis is involved.

In conclusion, a model of regulated exocytosis using Staphylococcus aureus α-toxin as a permeabilizing agent in primary anterior pituitary cells has been developed and characterized. Using this model it was shown that Ca^{2+}, PMA, and cAMP are able to stimulate LH exocytosis and that regulated exocytosis is absolutely dependent on millimolar ATP concentrations.

4 DEFINING THE CALCIUM TARGET : CALCIUM STIMULATES LH EXOCYTOSIS BY A MECHANISM INDEPENDENT OF PKC OR CALMODULIN

4.1 INTRODUCTION

Although it has long been known that Ca^{2+} is the dominant stimulus of regulated exocytosis in a variety of cells (Section 1.1), including gonadotropes (Section 1.3), the exact mechanism by which Ca^{2+} stimulates exocytosis is not known (see Section 1.1). One possibility is via activation of the Ca^{2+}-sensitive protein kinase, PKC (Baker and Knight, 1986; Nishizuka, 1986). In view of the absence of specific inhibitors of PKC (Rando, 1988; Huang, 1989), studies on a putative role for PKC in Ca^{2+}-stimulated exocytosis have relied on the down-regulation of PKC by prolonged incubation with phorbol esters (Phillips and Jaken, 1983; Rodriguez-Pena and Rozengurt, 1984). Ca^{2+}-stimulated exocytosis was decreased following PKC down-regulation in adrenal chromaffin cells (Burgoyne et al., 1988) but not in PC12 cells (Matthies et al., 1988) nor in pancreatic islets (Hii et al., 1987), suggesting heterogeneity in the mechanism of Ca^{2+}-stimulated exocytosis. However, experiments utilising PKC down-regulated cells have several drawbacks (see Section 4.3) which makes their interpretation difficult. This is illustrated by the conflicting results obtained in two

recent studies which used PKC down-regulated anterior pituitary cells to investigate the requirement for PKC in GnRH-stimulated LH exocytosis (McArdle et al., 1987; Stojilkovic et al., 1988b).

A second possible target for Ca^{2+} is the ubiquitous Ca^{2+}-binding protein calmodulin (Conn et al., 1987). Calmodulin is present in the anterior pituitary and redistributes from the cytosol to the plasma membrane following GnRH stimulation (Conn et al., 1981a). Further evidence for a role for calmodulin is the finding that antipsychotic calmodulin inhibitors inhibit Ca^{2+} ionophore-stimulated LH exocytosis in intact cells (Conn et al., 1981b; Davidson et al., 1987a). However, these hydrophobic calmodulin inhibitors lack specificity in that they have effects on surface membrane potential (McLaughlin and Whitaker, 1988), inhibit PKC (Sanchez et al., 1983), and bind to several non-calmodulin proteins in a Ca^{2+}-dependent way (Moore and Dedman, 1982).

In the present study several different approaches, in addition to PKC down-regulation, were used to provide evidence that Ca^{2+} stimulates LH exocytosis by a mechanism independent of PKC activation. Furthermore, evidence is presented which argues against a role for calmodulin.

4.2 RESULTS

When permeabilized gonadotropes which have been equilibrated at 0 °C with high $[Ca^{2+}]_{free}$ (pCa 5) are warmed to 37 °C there is burst of LH exocytosis (Figures 5, 24, and 28). LH release is maximal during the first 3-5 minutes and declines to near basal levels after 12-20 minutes despite the continued presence of high $[Ca^{2+}]_{free}$. This refractoriness to Ca^{2+} could be due either to a depletion of Ca^{2+}-sensitive LH stores, or to desensitization of the exocytotic apparatus

to Ca^{2+}. Addition of the PKC-activating phorbol ester PMA to cells which had been made refractory to Ca^{2+} resulted in further release of LH (Figure 24). The quantity of LH released from Ca^{2+}-refractory cells in response to PMA was comparable to that released from cells kept at low $[Ca^{2+}]_{free}$ (pCa 8) (Figure 24) which suggests that the PMA-sensitive LH stores are not markedly depleted by prolonged exposure to high $[Ca^{2+}]_{free}$. This finding demonstrates that Ca^{2+} and PMA stimulate LH exocytosis, at least in part, by different mechanisms.

Staurosporine, a potent, though non-specific, inhibitor of PKC (Tamaoki et al., 1986; Rüegg and Burgess, 1989), blocked PMA-stimulated LH exocytosis (IC_{50} 10-20 nM) but had little effect on Ca^{2+}-stimulated LH exocytosis (Figure 25 and Table 12). Staurosporine also blocked the enhancement by PMA of Ca^{2+}-stimulated exocytosis over a range of Ca^{2+} concentrations, while having little effect on Ca^{2+}-stimulated exocytosis itself (Figure 26). The effects of other PKC inhibitors were also examined (Table 13). Sphingosine (Hannun and Bell, 1989) was a poor inhibitor of PMA-stimulated LH exocytosis except at high concentrations at which basal LH release was elevated (Table 13), probably as a result of cell damage (Hannun and Bell, 1989). Unlike staurosporine, acridine orange (Hannun and Bell, 1988) inhibited both PMA- and Ca^{2+}-stimulated LH exocytosis. However inhibition was only observed at high concentrations, casting doubt on the specificity of this effect (Table 13).

TABLE 12 INHIBITION OF PHORBOL ESTER-STIMULATED EXOCYTOSIS BY STAUROSPORINE

The ability of staurosporine to inhibit LH exocytosis stimulated by 100 nM PMA (pCa 8) was examined using the same protocol as in Figure 25.

Experiment	IC_{50} (nM)	Maximal inhibition (%)
1.	*	86
2.	20	84
3.	20	95
4.	*	95
5.	*	81
6.	10	72

*Insufficient data for determination of IC_{50}.

TABLE 13 EFFECTS OF ACRIDINE ORANGE AND SPHINGOSINE ON LH EXOCYTOSIS

Permeabilized cells were equilibrated at 0°C for 30 min with stimulation buffer containing 30 mM CaEGTA (pCa 8 or 5) with the indicated additions. Exocytosis was initiated by replacing with identical medium at 37°C and LH exocytosis after 10 min was determined.

	LH released (%) with..[a]		
Additions	pCa 8	pCa 8 + PMA 100 nM	pCa 5
(a) Sphingosine (μM)			
0	2.1 ± 0.1	10.8 ± 0.9	n.d.[b]
10	1.7 ± 0.1	9.3 ± 0.7	n.d.
30	1.9 ± 0.3	7.5 ± 0.2	n.d.
100	5.7 ± 0.9	6.1 ± 0.1	n.d.
(b) Acridine Orange (μM)			
0	1.8 ± 0.2	6.2 ± 0.1	21.0 ± 0.2
30	n.d.	6.8 ± 0.1	22.4 ± 0.5
100	n.d.	7.7 ± 0.1	19.9 ± 0.1
300	n.d.	6.6 ± 0.5	16.3 ± 0.8
1000	< 1	< 1	< 1

[a]Mean ± range of duplicate determinations. Similar results were obtained in two independent experiments.
[b]Not done

Cells incubated for prolonged periods with high concentrations of PMA become desensitized to PMA as a result of proteolytic degradation of cellular PKC (Phillips and

Jaken, 1983; Rodriguez-Pena and Rozengurt, 1984; Melloni et al., 1986; Young et al., 1987). Sheep anterior pituitary cells exposed to a high concentration of PMA for 24 hours did not release LH in response to subsequent stimulation with PMA, indicating desensitization (Figure 27). Ca^{2+} was still able to stimulate LH exocytosis in these PMA-desensitized cells although the absolute amount of LH released was decreased (Figure 27A). This decrease may reflect, in part, a depletion of releasable LH since cells desensitized to PMA contained less cellular LH than untreated cells (Table 14). When LH release was normalized to a percentage of cellular LH present immediately before stimulation, Ca^{2+}-stimulated exocytosis was not inhibited in PMA-desensitized cells (Figure 27B).

TABLE 14 EFFECT OF PMA-PRETREATMENT ON CELLULAR LH CONTENT

The LH content of cells used in the experiment shown in Figure 27 is shown.

	Cellular LH (ng/well)[a]
Control cells (n = 16)	760 ± 10
PMA pretreated cells (n = 4)	270 ± 20

[a]Mean ± SEM of n determinations (where n = number of wells)

Since Ba^{2+} is a poor activator of either PKC (Takai et al., 1979; Sekiguchi et al., 1988) or calmodulin (Chao et al., 1984; Kuret and Schulman, 1984) in vitro and yet is a powerful LH secretagogue in intact pituitary cells (Davidson et al., 1987b, Smith et al., 1989) we examined Ba^{2+}-stimulated LH exocytosis in permeabilized cells to determine

whether Ba^{2+} activates the same exocytotic mechanism as Ca^{2+}. High $[Ba^{2+}]_{free}$ stimulated LH exocytosis in permeabilized gonadotropes with a time course very similar to Ca^{2+}-stimulated LH exocytosis (Figure 28A). As was the case with Ca^{2+}, cells eventually became refractory to high $[Ba^{2+}]_{free}$ but could still release LH in response to PMA (Figure 28A). Ba^{2+} was not able to stimulate LH exocytosis in cells made refractory to Ca^{2+} whereas Ba^{2+} could stimulate LH exocytosis when added to cells which had been maintained at low $[Ca^{2+}]_{free}$ (pCa 7) (Figure 28B). Ba^{2+}-stimulated LH exocytosis was half-maximal at pBa 3.6, a concentration 100 fold higher than for Ca^{2+} (Figure 29). Concentrations of Ba^{2+} higher than pBa 3 inhibited LH exocytosis (Figure 29) in a manner similar to the inhibition of LH exocytosis by very high Ca^{2+} concentrations (Figure 6). Maximally effective concentrations of $[Ba^{2+}]_{free}$ (pBa 3) and $[Ca^{2+}]_{free}$ (pCa 5) stimulated LH exocytosis to a similar extent (Table 15). Ba^{2+}-stimulated LH exocytosis, like Ca^{2+}-stimulated LH exocytosis, was not inhibited by staurosporine (Figure 25). Taken together, these findings suggest that Ba^{2+} and Ca^{2+} stimulate exocytosis by the same mechanism. Since Ba^{2+} does not activate PKC or calmodulin these findings provide further evidence against a major role for PKC in Ca^{2+}-stimulated LH exocytosis, and also suggest that calmodulin does not mediate the the effects of Ca^{2+}.

TABLE 15 COMPARISON OF MAXIMAL Ca^{2+}- AND Ba^{2+}-STIMULATED LH EXOCYTOSIS

Results are shown of several experiments similar to Figure 28 in which Ca^{2+} and Ba^{2+} where used at maximally effective concentrations (pCa 5 and pBa 3, respectively) within the same experiment. LH exocytosis was measured over 10 min.

Experiment	LH exocytosis (%)	
	pBa 3	pCa 5
1.	14.9	15.6
2.	16.4	16.8
3.	15.8	14.9
4.	13.4	10.4
5.	11.4	9.0
6.	10.2	15.7
Mean ± SEM	13.7 ± 1	13.7 ± 1.3

The role of calmodulin was examined further using calmodulin inhibitors. The antipsychotic calmodulin inhibitor trifluoperazine inhibited PMA-, as well as Ca^{2+}- and Ba^{2+}-stimulated LH exocytosis at similar doses (Figure 30), confirming doubts about its specificity (see Section 4.1). In contrast, the more potent and specific calmodulin inhibitor calmidazolium (Gietzen et al., 1981) inhibited neither PMA- nor Ca^{2+}-stimulated LH exocytosis at concentrations up to 30 µM (Figure 31). Higher concentrations of the drug caused an increase in basal LH release, probably as a result of cell damage (Figure 31).

4.3 DISCUSSION

McArdle et al. (1987) examined the role of PKC in Ca^{2+}-stimulated LH exocytosis in intact cells and found that PKC down-regulation did not inhibit calcium ionophore (A23187)-stimulated LH exocytosis. However, PKC can modulate the activity of several important Ca^{2+}-regulating proteins including plasma membrane Ca^{2+} channels (Yamaguchi et al., 1987; Yada et al., 1989), plasma membrane Ca^{2+}-ATPases (Nishizuka, 1986; Smallwood et al., 1988), and Ca^{2+}-ATPases associated with intracellular Ca^{2+} stores (Yoshida and Nachmias, 1989; Rogers et al., 1990) and this may affect the extent of ionophore-induced increases in cytosolic $[Ca^{2+}]_{free}$. Furthermore, experiments involving PKC down-regulation are difficult to interpret for reasons outlined below. Therefore several different approaches, in addition to PKC down-regulation, were used in the present study to investigate the possible role of PKC and these experiments were conducted in permeabilized cells, which allow direct control of the cytosolic $[Ca^{2+}]_{free}$.

From the results presented above, several lines of evidence suggest that Ca^{2+}-stimulated LH exocytosis is not mediated by PKC.

(1) The PKC activator PMA enhances LH exocytosis at all Ca^{2+} concentrations (Figures 9, 26, and 27). If Ca^{2+} were acting via PKC, LH exocytosis at maximal $[Ca^{2+}]_{free}$ should not be further enhanced by PMA since PMA does not enhance maximal Ca^{2+}-stimulated PKC activity in vitro (Sekiguchi et al., 1988). The effects of PMA and Ca^{2+} on LH exocytosis are additive which suggests that PMA-stimulated LH exocytosis is largely Ca^{2+}-independent. In this context it of interest that the major rat pituitary PKC isoenzyme (type II or ß) (Yoshida et al., 1988) shows only slight Ca^{2+}-dependence

when compared with the type I(γ) and III(α) isoenzymes (Sekiguchi et al. 1988).

(2) PMA was able to stimulate LH exocytosis in cells made refractory to high $[Ca^{2+}]_{free}$. Two possible explanations for this finding are: (a) that there exists a pool of releasable LH which is sensitive to PMA but not Ca^{2+}; or (b) that PMA and Ca^{2+} stimulate LH release from the same store but that the Ca^{2+} mechanism desensitizes before depletion of releasable LH occurs. Although our results cannot distinguish between these two possibilities, both these explanations imply that Ca^{2+} is not acting through PKC.

(3) The PKC inhibitor, staurosporine, had little effect on Ca^{2+}-stimulated LH exocytosis though it blocked PMA-stimulated LH exocytosis. The fact that staurosporine is a non-specific PKC inhibitor (Rüegg and Burgess, 1989) in that it also inhibits protein kinase A (Tamaoki et al., 1986) and myosin light chain kinase (Watson et al., 1988) does not alter this conclusion. The absence of an inhibitory effect of stuarosporine is strong evidence that Ca^{2+}-stimulated LH exocytosis is not mediated by PKC.

(4) Ca^{2+} stimulates LH exocytosis in cells completely desensitized to PMA. Although not demonstrated directly here, it is likely that this desensitization results from depletion of PKC (Phillips and Jaken, 1983; Rodriguez-Pena and Rozengurt, 1984; McArdle et al., 1987; Stojilkovic et al., 1988b). Experiments of this type in which PKC is depleted by prolonged phorbol ester stimulation are difficult to interpret for the following reasons: (a) Prolonged activation of PKC by phorbol ester is likely to result in pleiotropic cellular changes due to phosphorylation of multiple proteins (Nishizuka, 1986). Thus observed changes may be due to mechanisms other than down-regulation of PKC. (b) Prolonged exposure to phorbol ester

results in depletion of LH stores (McArdle et al., 1987; Stojilkovic et al., 1988b; and this study) and this is likely to reduce the amount of releasable LH. In the present study in which acute LH exocytosis (over 10 min) was studied LH depletion was corrected for by expressing LH exocytosis as a percentage of the available cellular LH at the beginning of the experiment. However, when LH exocytosis is measured over several hours, as in previous studies (McArdle et al., 1987; Stojilkovic et al., 1988b), substantial LH synthesis takes place during the course of the experiment (Stojilkovic et al., 1988b) and it is therefore not clear how one should calculate the percentage of LH released. Finally, (c) the rate of PKC down-regulation varies between cell types (Adams and Gullick, 1989) and between different isoenzymes of PKC (Huang et al., 1989; Kishimoto et al., 1989; Isakof et al., 1990). Together these considerations suggest that experiments conducted on PKC down-regulated cells should be interpreted with caution and should not be used as the only evidence for or against a role for PKC in cellular processes.

(5) Ba^{2+} was able to stimulate LH exocytosis even though Ba^{2+} is a poor activator of PKC in vitro and in fact inhibits the type II or ß isoenzyme (Takai et al., 1979; Sekiguchi et al., 1988) which is the major isoenzyme present in the rat pituitary (Yoshida et al., 1988). This is in agreement with studies on catecholamine exocytosis using PC-12 cells (Mattheis et al., 1988). Our finding that PMA is able to stimulate LH exocytosis from cells refractory to Ba^{2+} also indicates that Ba^{2+} does not stimulate exocytosis by activating PKC. The following findings suggest that Ba^{2+} and Ca^{2+} stimulate LH exocytosis by the same mechanism: (a) Ba^{2+} and Ca^{2+} stimulated LH exocytosis with the same time course; (b) maximal Ba^{2+}- and Ca^{2+}-stimulated LH exocytosis were the same; (c) Ba^{2+} was unable to stimulate LH exocytosis from cells made refractory to Ca^{2+}; (d) high

concentrations of both Ba^{2+} and Ca^{2+} inhibited LH exocytosis; and (e) neither Ba^{2+}- nor Ca^{2+}-stimulated LH exocytosis was inhibited by staurosporine.

While the evidence presented above argues strongly against a role for PKC as the mediator of Ca^{2+}-stimulated LH exocytosis, this does not necessarily imply that Ca^{2+} and PMA stimulate exocytosis by completely separate and parallel pathways. The data is compatible with Ca^{2+} and PMA activating a final common exocytotic pathway distal to PKC and the Ca^{2+} target.

In contrast to the present study, studies bovine adrenal chromaffin cells permeabilized by digitonin or high-voltage electric field have been interpreted to implicate PKC as a target for Ca^{2+} in regulated exocytosis (Burgoyne et al., 1988; Knight et al. 1988). Burgoyne et al. (1988) demonstrated partial inhibition of Ca^{2+}-stimulated exocytosis in PKC down-regulated cells and concluded that PKC has a major role in Ca^{2+}-stimulated exocytosis. However, the inhibitory effect observed may have resulted from phorbol ester effects other than PKC depletion. Alternatively, PKC may play a modulatory role in catecholamine exocytosis without being the major Ca^{2+} target. Knight et al. (1988) tested a range of PKC inhibitors (but not staurosporine) and fo nd none that did not also inhibit Ca^{2+}-stimulated exocytosis. However, their results may simply reflect a lack of specificity of the agents used, as is the case many protein kinase C inhibitors (Rando, 1988; Huang, 1989) such as TFP and acridine orange as demonstrated in the present study (Figure 30 and Table 13). Knight et al. (1988) also found that exocytosis and PKC (purified from bovine adrenal medulla) had similar cation and nucleotide specificities. While these data are consistent with a role for PKC in exocytosis they do not constitute proof of one.

The finding of the present study that Ba^{2+} can stimulate LH exocytosis, and that it acts by the same mechanism as Ca^{2+}, argues strongly against a role for calmodulin in Ca^{2+}-stimulated LH exocytosis, since Ba^{2+} does not bind to calmodulin or activate calmodulin dependent enzymes even at concentrations as high as 1 mM (Chao et al., 1984; Kuret and Schulman, 1984). Further evidence against a role for calmodulin is the finding that calmidazolium, a much more potent and specific calmodulin inhibitor than trifluoperazine (Gietzen et al., 1981), does not inhibit Ca^{2+}-stimulated LH exocytosis.

The results of the present study indicate that PKC is not a mediator of Ca^{2+}-stimulated LH exocytosis, but they do not exclude a role for PKC in GnRH-induced LH exocytosis. Other studies from this laboratory (not shown in this thesis) demonstrated that, in intact cells, the PKC-inhibitor staurosporine enhanced GnRH-stimulated LH exocytosis under conditions where it inhibited PMA-stimulated exocytosis (van der Merwe et al., 1990). This argues against a role for PKC in GnRH stimulus-secretion coupling. Although the precise role of PKC is not known, there is some evidence that it mediates (a) GnRH stimulation of LHß-subunit gene transcription (Andrews at al., 1988) and (b) feedback effects of GnRH-stimulation on the GnRH receptor (Huckle et al., 1988; Huckle et al., 1989).

In conclusion, these results indicate that Ca^{2+} stimulates LH exocytosis by a mechanism which does not involve PKC or calmodulin. Although the data do not suggest what the Ca^{2+} target is, there are a large number of Ca^{2+}-sensitive proteins which have already been identified which may mediate Ca^{2+}-stimulated exocytosis (see Section 1.1).

5 DUAL STIMULATORY AND INHIBITORY EFFECTS OF GUANINE NUCLEOTIDES ON LH EXOCYTOSIS

5.1 INTRODUCTION

GTP-binding proteins play diverse roles in several cellular processes including protein synthesis, microtubule assembly, intracellular vesicle trafficking, targeting of secretory proteins to the endoplasmic reticulum, and stimulus-secretion coupling (Gilman, 1987; Allende, 1988; Balch, 1989; Rothman, 1989). A family of heterotrimeric GTP-binding proteins (G-proteins) couple certain cell surface receptors to plasma membrane ion channels (Houslay, 1987; Rosenthal et al., 1988) and intracellular effectors such as adenylate cyclase, phospholipase A_2, phospholipase D, phosphatidylinositol-specific phospholipase C (phosphoinositidase C, PIC), and phosphatidylcholine-specific phospholipase C (Gilman, 1987; Fain et al., 1988; Martin and Michaelis, 1989; Pelech and Vance, 1989). In addition, there is a family of small (M_r 20 000 - 27 000) proteins which includes the ras proto-oncogenes which can be detected by their ability to bind GTP after separation on SDS-PAGE (Burgoyne, 1989). The functions of this group of GTP-binding proteins are not clear.

Recently, two lines of evidence have accumulated supporting the involvement of GTP-binding proteins in exocytosis. Firstly, studies on a temperature-dependent yeast mutant with a defect in constitutive exocytosis have identified the gene product SEC4 as a 23.5 kDa ras-like GTP-binding protein which associates with yeast secretory vesicles (Salminen and Novick, 1987; Goud et al., 1988). Secondly, intracellular application of GTP analogues has been reported to modulate exocytosis in several cell types. GTP analogues stimulate exocytosis in mast cells (Gomperts, 1983; Howell et al., 1987), platelets (Haslam and Davidson, 1984), adrenal

chromaffin cells (Knight and Baker, 1985; Bittner et al., 1986), neutrophils (Barrowman et al., 1986), parathyroid cells (Oetting et al., 1986), RINm5F cells (Vallar et al., 1987), gonadotropes (Andrews et al., 1986) and lactotropes (Sikdar et al., 1989). In some of these studies the GDP analogue GDPßS inhibited second-messenger-stimulated exocytosis (Barrowman et at., 1986; Bittner et al., 1986; Vallar et al., 1987). In contrast, GTP analogues have also been reported to inhibit exocytosis in bovine adrenal chromaffin cells (Knight and Baker, 1985), PC12 cells (Anhert-Hilger et al., 1987), and AtT20 cells (Luini and De Matteis, 1988 & 1990).

In the present study guanine nucleotides were used to examine whether GTP-binding proteins have a role in regulating LH exocytosis.

5.2 RESULTS

5.2.1 Stimulatory effects of GTPγS and GMPPNP on LH exocytosis

In permeabilized cells buffered at resting cytosolic $[Ca^{2+}]_{free}$ (pCa 7), GTPγS and GMPPNP stimulated rapid LH exocytosis. LH release was maximal between 5 and 10 min and declined to basal levels after 20 min (Figure 32A). Although GTPγS and GMPPNP stimulated LH exocytosis with similar potency (EC_{50} 20-50 µM, n = 5 independent experiments) the maximal effect of GMPPNP was consistently greater than that of GTPγS (Figure 32B) and also more sustained (Figure 32A). GTP had little stimulatory effect at concentrations up to 3 mM (Figure 32B). GDPßS did not stimulate LH exocytosis and inhibited the stimulatory effect of GTPγS (Table 16). GTPγS-stimulated LH exocytosis was ATP dependent (Figure 32B), as was the case for Ca^{2+}-, phorbol ester- and cAMP-stimulated LH exocytosis (see Section 3). The Ca^{2+}-dependence of GMPPNP-stimulated LH exocytosis was examined (Figure 33).

GMPPNP stimulating LH exocytosis most effectively at pCa 6, but was effective at both very low (pCa 9-8) and high $[Ca^{2+}]_{free}$ (pCa 5-4.5) (Figure 33).

TABLE 16 EFFECT OF GDPβS ON GTPγS-STIMULATED LH EXOCYTOSIS

Permeabilized cells were equilibrated at 0°C for 30 min in stimulation buffer containing 30 mM CaEGTA (pCa 6) and the indicated nucleotides. LH exocytosis was initiated by replacing with identical stimulation buffer at 37°C and LH released after 15 min was determined.

Addition	% LH Released[a]
Control	6.5 ± 0.1
GTPγS 300μM	28.6 ± 0.9
GDPβS 2 mM	4.4 ± 0.8
GTPγS 300μM + GDPβS 2 mM	5.8 ± 0.5

[a]Mean ± range of duplicate determinations. Similar results were obtained in three independent experiments.

Since both PKC activation and cAMP can stimulate LH exocytosis, a probable mechanism for the stimulatory effect of guanine nucleotides is through the activation of signal-transducing G-proteins which would result in the generation of diacylglycerol (if a phospholipase C or phospholipase D were activated), or cAMP (if adenylate cyclase was activated).

GMPPNP was able to stimulate a further increase in LH exocytosis in the presence of maximally effective concentrations of the PKC-activating phorbol ester PMA (Figure 34). GMPPNP was approximately additive with PKC at

all doses. To examine further whether activation of PKC mediates the effects of GMPPNP, cells were desensitized to PMA by prolonged treatment with high concentrations of phorbol ester (Table 17). Cells incubated overnight with PMA were unresponsive to subsequent acute stimulation with PMA, indicating PKC desensitization (Table 17). However, GMPPNP was still able to stimulate LH exocytosis in PKC depleted cells (Table 17). Taken together, these findings indicate that GMPPNP does not act solely by activation of PKC and suggest that other mechanisms are involved.

GMPPNP was able to stimulate a further increase in LH exocytosis in the presence of maximally-effective concentrations of cAMP, although the effects of GMPPNP and cAMP were slightly less than additive (Figure 34). Therefore, the stimulatory effects of guanine nucleotides are not fully explained either by stimulation of cAMP generation alone or by the isolated activation of PKC. However, it is possible that simultaneous activation of both of these second messenger pathways could account for the guanine nucleotide effects. In support of this GMPPNP was unable to further stimulate LH exocytosis from cells stimulated with maximal concentrations of cAMP plus PMA (cAMP/PMA) (Table 17).

TABLE 17 EFFECTS OF cAMP, PHORBOL ESTER, AND GMPPNP ON LH EXOCYTOSIS IN PHORBOL ESTER-DESENSITIZED CELLS

Cells were pre-treated with vehicle [Me_2SO 0.13%] or PMA (250 nM) for 18 h. They were then washed and permeabilized as usual and equilibrated at 0°C for 30 min in stimulation buffer with CaEGTA 30 mM (pCa 7) and the indicated additions. LH exocytosis was initiated by replacing with identical stimulation buffer at 37°C and LH released after 20 min was determined. LH release is expressed as a percentage of the cellular LH content immediately before stimulation which was: Me_2SO pre-treated cells, 920 ± 40 ng/well; PMA pre-treated cells, 340 ± 10 ng/well (mean ± SEM, n = 4 wells). Similar results were obtained in three independent experiments.

	% LH Released[a]
(a) no phorbol ester pre-treatment	
control	3.7 ± 0.1
GMPPNP (500 µM)	11.0 ± 0.3
PMA (250nM)	19.0 ± 0.1
PMA + GMPPNP	23.9 ± 1.2
PMA + cAMP (30µM) + IBMX (250µM)	29.3 ± 0.7
PMA + cAMP + IBMX + GMPPNP	29.2 ± 2.4
(b) phorbol ester pre-treated	
control	7.5 ± 0.6
GMPPNP (500 µM)	15.2 ± 0.6
PMA (250nM)	8.1 ± 0.4
PMA + GMPPNP	13.3 ± 1.2
PMA + cAMP (30µM) + IBMX (250µM)	19.1 ± 1.6
PMA + cAMP + IBMX + GMPPNP	17.8 ± 0.9

[a]Mean ± range of duplicate determinations.

5.2.2 Inhibitory effects of GTPγS on LH exocytosis

In contrast to the acute stimulatory effects of guanine nucleotides described above, GTPγS was found to inhibit LH exocytosis under certain conditions. In cells stimulated with cAMP/PMA, low concentrations of GTPγS (which were not stimulatory) inhibited LH exocytosis after a lag of 5 min (Figure 35). In subsequent experiments cells were pre-incubated with guanine nucleotides for 30 min at 37°C in the absence of ATP (see Figure 36 for an outline of the protocol). The absence of ATP prevented guanine nucleotide stimulation of LH exocytosis during the pre-incubation (Figure 32B). After pre-incubation cells were stimulated in the presence of ATP but in the absence of guanine nucleotides. Since the inhibitory effect of GTPγS pretreatment did not require its continued presence during the stimulation period (Table 18), guanine nucleotides were omitted during the stimulation period. This was desirable in order to minimize their stimulatory effects. Using this protocol GTPγS inhibited Ca^{2+}-stimulated LH exocytosis (IC_{50} 10 μM), an effect not observed with GMPPNP or GTP at concentrations up to 100 μM (Figure 36). This inhibition by GTPγS does not result from depletion of releasable LH during the preincubation since GTPγS stimulated minimal LH exocytosis in the absence of ATP (Figure 32B). GTPγS inhibited cAMP-,PMA-, and cAMP/PMA-stimulated LH exocytosis even more potently (IC_{50}s 1 μM) (Figure 37). The inhibitory effect of 30 μM GTPγS was prevented by including GMPPNP (0.1 mM) and partly prevented by GDP (1 mM) and GTP (1 mM) (Figure 38) whereas ATP (6 mM) was without effect (Table 19).

TABLE 18 INHIBITORY EFFECT OF GTPγS PREINCUBATION ON LH EXOCYTOSIS

Permeabilized cells were equilibrated for 30 min at 0°C in buffer IC containing 2 mM $MgCl_2$ and 10 mM CaEGTA (pCa 7) with or without GTPγS (30 µM). Cells were then warmed to 37°C and pre-incubated for 30 min in the same medium. This medium was removed and replaced with stimulation buffer containing 10 mM CaEGTA (pCa 7) with or without cAMP (30 µM) **plus** IBMX (0.25 mM) **plus** PMA (100 nM) (cAMP/PMA) and with or without GTPγS (30 µ). LH released after 30 min was determined. Results are expressed as the percent of cAMP/PMA-stimulated LH release in the absence of GTPγS. Values for basal LH release (in the absence of cAMP/PMA) were subtracted.

Pre-inc.	Stim.	cAMP/PMA-stimulated LH release (%)[a]
Control	Control	100
Control	GTPγS	40 ± 3
GTPγS	Control	30 ± 3
GTPγS	GTPγS	34 ± 1

[a]Mean ± range of duplicate determinations. Similar results were obtained in two independent experiments.

TABLE 19 THE EFFECT OF ATP ON GTPγS-INHIBITION OF LH EXOCYTOSIS

Permeabilized cells were equilibrated for 30 min at 0°C in buffer IC with 2 mM $MgCl_2$ and 10 mM CaEGTA (pCa 7) with indicated additions. Cells were then warmed to 37°C and pre-incubated for 30 min in the same medium. This medium was removed and replaced with stimulation buffer containing 30 mM CaEGTA (pCa 5) and MgATP 5 mM ($MgCl_2$ 6.5 mM plus Na_2ATP 6 mM) and LH released after 30 min was determined.

Preincubation	LH release (%)[a]
$MgCl_2$ 2 mM	23.0 ± 1
MgATP 5 mM	31.2 ± 4
GTPγS 100 µM plus $MgCl_2$	8.6 ± 0.2
GTPγS plus MgATP	7.0 ± 0.1

[a]Mean and range of duplicate determinations. Similar results were obtained in two independent experiments.

High concentrations of ATPγS also inhibited both Ca^{2+}-stimulated (IC_{50} 200 µM, Figure 39) and cAMP/PMA-stimulated LH exocytosis (IC_{50} 20 µM, Figure 40). However, this inhibitory effect of ATPγS was antagonized by UDP (Figures 39 and 40), a competitive inhibitor of nucleotide diphosphate kinase (Seifert et al., 1988), suggesting that ATPγS-inhibition results from nucleotide diphosphate kinase-mediated synthesis of GTPγS.

5.3 DISCUSSION

These results demonstrate that intracellularly applied guanine nucleotides have both stimulatory and inhibitory

effects on exocytosis in sheep gonadotropes. Several findings suggest that the stimulatory effects result from activation of one or more signal-transducing G-proteins with consequent generation of stimulatory second messengers. Firstly, in time course experiments, the stimulatory effects of GTPγS and GMPPNP were maximal between 5 and 10 min whereas the stimulatory effects of Ca^{2+}, PKC and cAMP are maximal in the first 5 min (see Section 3). The delay is compatible with guanine nucleotides having less direct mechanisms of action. Secondly, the combined stimulatory effects of GMPPNP and saturating cAMP concentrations were less than additive. And finally, in cells stimulated with saturating concentrations of PMA plus cAMP, GMPPNP was unable to stimulate further LH exocytosis. Although this latter finding suggests that the stimulatory effects of guanine nucleotides may mediated solely by PKC activation together with cAMP generation, it is possible that, when the cells were stimulated with cAMP/PMA, LH exocytosis had reached an inherent maximal rate, thus obscuring any additional GMPPNP effect. Our results do not, therefore, entirely exclude additional mechanisms for the stimulatory effect of guanine nucleotides.

Several differences between the stimulatory effects and the inhibitory effects of guanine nucleotides indicate that they are exerted at different sites (Figure 41). Unlike the stimulatory effect, inhibition was observed only with GTPγS (not GMPPNP), it had a slower onset, and was produced by a 10 fold lower concentration of GTPγS. The finding that GTPγS inhibits LH exocytosis stimulated by a variety of second messenger pathways (Ca^{2+}, PMA, and cAMP) indicates t at GTPγS inhibits exocytosis distal to the generation of second messengers. The delayed inhibitory effect of GTPγS, not found with GMPPNP, probably explains the lower maximal stimulation observed with GTPγS and shorter duration of GTPγS-stimulation when compared with GMPPNP (Figure 32A).

The finding that the inhibitory effect was observed with ATPγS as well as GTPγS but not GMPPNP raises the question whether the inhibition results from protein thiophosphorylation (Eckstein, 1985; Li et al., 1988). In support of this, GTP (and GTPγS) is preferred to ATP as the phosphate donor in some phosphorylation reactions (Amir-Zaltsman et al., 1980; Amir-Zaltsman and Salomon, 1989) Since thiophosphorylated proteins are resistant to dephosphorylation (Li et al., 1988), processes requiring dephosphorylation, of which exocytosis may be one (Momayezi et al., 1987), will be inhibited. Our findings that (a) much higher concentrations of ATPγS than GTPγS are required for inhibition and (b) that the ATPγS effect is inhibited by a nucleotide-diphosphate kinase inhibitor (UDP) suggests that ATPγS-inhibition results from its intracellular conversion to GTPγS (Seifert et al., 1988).

Intracellular application of guanine nucleotides can stimulate exocytosis in diverse cell types (see Section 5.1). In several previous studies these results have been interpreted in terms of a stimulatory GTP-binding protein (G_E) directly involved in exocytosis (Cockcroft et al., 1987; Regazzi et at., 1989). In neutrophils (Barrowman et al., 1986), mast cells (Cockcroft et al., 1987), and RINm5F cells (Vallar et al., 1987; Regazzi et al., 1989) there is evidence that the stimulatory effect of guanine nucleotides on exocytosis is not entirely due to the activation of PIC and/or adenylate cyclase. However, it remains possible that guanine nucleotides stimulate exocytosis through effects on other G-protein coupled effector systems such as phosphatidylcholine-specific phospholipase C, phospholipase A_2, or phospholipase D (Burgoyne et al., 1987; Irving and Exton, 1987; Martin and Michaelis, 1989).

In a few studies inhibitory effects of guanine nucleotides on second messenger-stimulated exocytosis have been demonstrated, and these provide more convincing evidence for the involvement of GTP-binding proteins other than signal-transducing G-proteins in exocytosis. GTPγS has been reported to inhibit Ca^{2+}-stimulated exocytosis in bovine adrenal chromaffin cells (Knight and Baker, 1985) and PC12 cells (Anhert-Hilger et al., 1987) over a concentration range similar to that found in the present study. Interestingly, GMPPNP was not inhibitory in bovine adrenal chromaffin cells (Knight and Baker, 1985), as we have found in gonadotropes. These similarities suggest that the mechanism of GTPγS inhibition may be common to many secretory cell types.

It is attractive to hypothesize that GTPγS inhibition is mediated by a GTP-binding protein directly involved in exocytosis. Recent studies have identified such a protein (SEC4) which is essential for constitutive exocytosis in yeast (Salminen and Novick, 1987; Goud et al., 1988). SEC4 is a member of a large family of small (20-25 kDa), ras-like GTP-binding proteins some of which have been implicated in intracellular vesicle tafficking pathways in higher eukaryotes (Bourne, 1988; Balch, 1989; Burgoyne, 1989). In support of this, GTPγS inhibits vesicle trafficking in several mammalian cell free systems (Melançon et al.,1987; Balch, 1989). Interestingly, and in agreement with our findings, GMPPNP is sometimes a considerably less potent inhibitor of vesicle trafficking than GTPγS (Melançon et al., 1987). Furthermore, small GTP binding proteins have been found tightly bound to the cytosolic surface of bovine adrenal chromaffin (Burgoyne and Morgan, 1989) and sheep anterior pituitary* secretory granules. Based on these findings Bourne (1988) has proposed a model in which GTP-

*Unpublished work of J.S. Davidson and I.K. Wakefield in our laboratory.

binding proteins may direct membrane traffic in the secretory pathway. He postulates, by analogy with the GTP-binding proteins which participate in protein synthesis, that hydrolysis of GTP is an essential prerequisite for vesicle fusion and that it establishes unidirectional movement along the secretory pathway. Since this model predicts that non-hydrolysable GTP analogues will inhibit such a pathway, our results are compatible with such a mechanism.

An alternative mechanism has been proposed in which guanine nucleotides may inhibit exocytosis by activating GTP-binding proteins coupled to inhibitory receptors such as the somatostatin and α_2-adrenergic receptors (Ullrich and Wollheim, 1988; Luini and De Matteis, 1990). However, this mechanism seems unlikely to apply to our results since no cell surface receptors on the gonadotrope are known to inhibit exocytosis.

In conclusion, the findings of the present study indicate that guanine nucleotide-binding proteins are involved in LH exocytosis at least two distinct sites. At proximal site(s) G-proteins regulate the generation of second messengers which stimulate LH exocytosis. In addition, GTP-binding-protein(s) are involved in exocytosis at a site distal to second messenger generation.

6 APPENDIX

CALCULATING METAL-LIGAND EQUILIBRIA

When solutions contain more than one metal and/or ligand multiple equilibria exist. Although each equilibrium is simple to describe, multiple equilibria are complex and their solution is greatly aided by the use of a computer. The following equations were employed in a computer program which was written in Turbo PASCAL version 4 (Borland International, Scotts Valley, California) and run on an IBM XT computer.

(a) Absolute stability constants (K) were adjusted for use at 37°C using the following equation (Martell and Smith, 1976):

$$\log K = \log K_O + 0.00246 \times H \times (T-T_O)$$

where

T = desired temperature (usually 37°C)
T_O = temperature for which stability constant is given.
K = absolute stability constant at T
K_O = absolute stability constant at T_O (usually 20-25 0°C; obtained from Fabiato (1981)
H = enthalpy change; obtained from Martell and Smith (1976)

Although routinely done, the adjustments for temperature made little difference to the calculations since in most cases $-5 < H < 5$, and $T-T_O < 17$.

(b) Apparent stability constants (K_{APP}) take into account the existence of protonated forms of the ligands (such as EGTA, ATP) which may also bind Ca^{2+} and Mg^{2+}, although with smaller stability constants (i.e. lower affinity). Since the

proportion of the ligand which is protonated varies with pH, its affinity for Ca^{2+} and Mg^{2+} is pH dependent. K_{App} is calculated from the absolute stability constants using the following equation (program 3 in Fabiato and Fabiato, 1979):

$$K_{APP} = K_{m1}/SUM + K_{m2}K_1[H^+]/SUM$$

where

K_{m1}, K_{m2} = absolute stability constant for binding of metal to unprotonated and monoprotonated ligand, respectively. Binding to diprotonated ligands was insignificant.

$$SUM = \frac{[\text{Total ligand}]}{[\text{unprotonated ligand}]}$$

or

$$SUM = 1 + K_1[H^+] + K_1K_2[H^+]^2 + K_1K_2K_3[H^+]^3 + K_1K_2K_3K_4[H^+]^4$$

where

K_1, K_2, K_3, K_4 = absolute stability constants for binding of ligand to 1st, 2nd, 3rd, and 4th protons.

(c) The $[metal]_{total}$ required to give specified $[metal]_{free}$ in the presence of specified [ligand] can be calculated from the following equations (modified from program 2 in Fabiato and Fabiato, 1979).

$$[metal]_{total} = [metal]_{free} + [metal.ligand(1)] + [metal.ligand(2)]..etc...$$

where

[metal.ligand] = concentration of metal bound to ligand

$$= K_{App} \times [metal]_{free} \times [ligand]_{free}$$

$[ligand]_{free}$ can be calculated since the total ligand concentration is specified:

$$[ligand]_{free} = \frac{[ligand]_{total}}{(1 + [metal(x)]_{free}K_{APP}(x) + ..etc...)}$$

where x refers to all metals in the solution which bind the ligand.

(d) Calculation of $[metal]_{free}$ from specified $[metal]_{total}$ and $[ligand]_{total}$ requires reiterative solution of the several equations, one for each ligand and metal in the solution (program 4 in Fabiato and Fabiato, 1979):

$$[ligand]_{free} = \frac{[ligand]_{total}}{(1 + [metal(x)]_{free}K_{APP}(x) + ..etc....)}$$

where x refers to all metals in the solution which bind the ligand.

$$[metal]_{free} = \frac{[metal]_{total}}{(1 + [ligand(y)]_{free}K_{APP}(y) + ..etc.....)}$$

where y refers to all ligands in the solution which bind the metal.

As new results are calculated for $[ligand]_{free}$ and $[metal]_{free}$ these are used in subsequent calculations. Iteration is terminated when new results differ from old results by < 0.005 percent.

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8 FIGURES

Figure 1. Calculated relationship between $[Ca^{2+}]_{free}$ and Ca/EGTA ratio at pH 6.6 and pH 7.1

The Ca/EGTA ratios were calculated using the apparent stability constants given in Table 5 using a computer program described in the appendix.

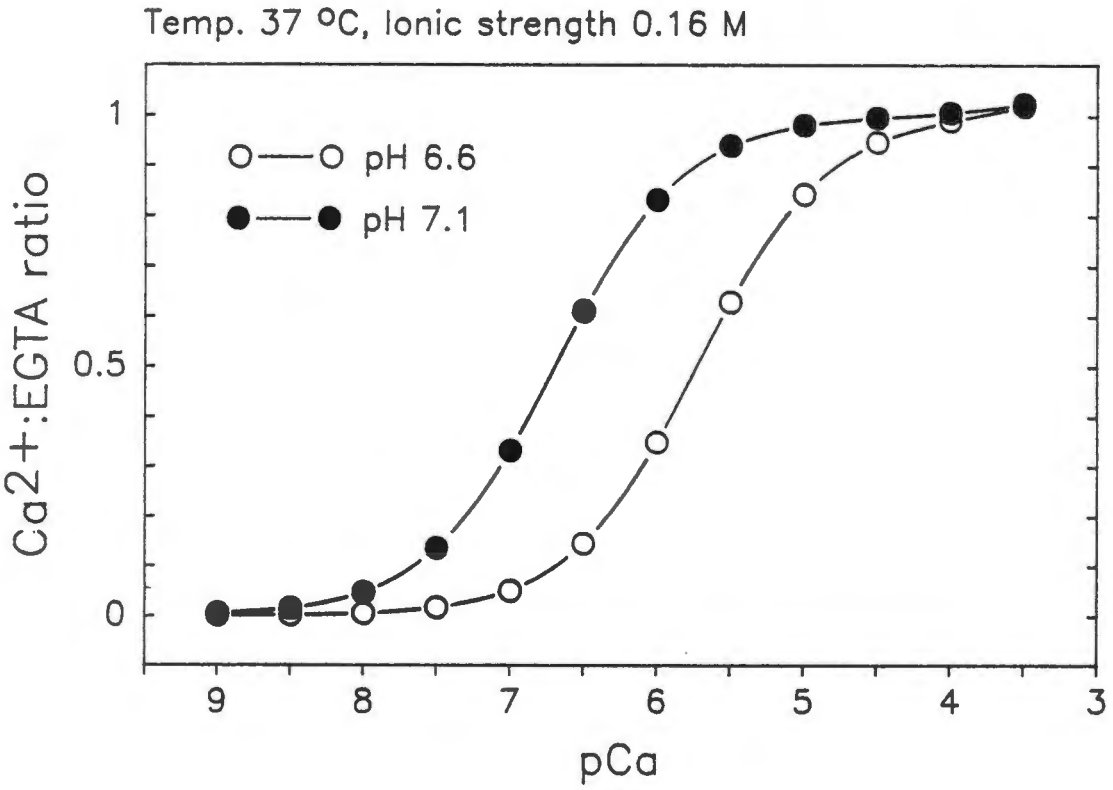

Figure 2. Time course and concentration-dependence of α-toxin-induced efflux of 2-deoxy[3H]glucose

After labelling with 2-deoxy[3H]glucose cumulative efflux of radioactivity in the presence of the indicated α-toxin concentration (added at t = 0) was measured. Results are expressed as a percentage of the initial total cellular radioactivity. Similar results were obtained in six independent experiments.

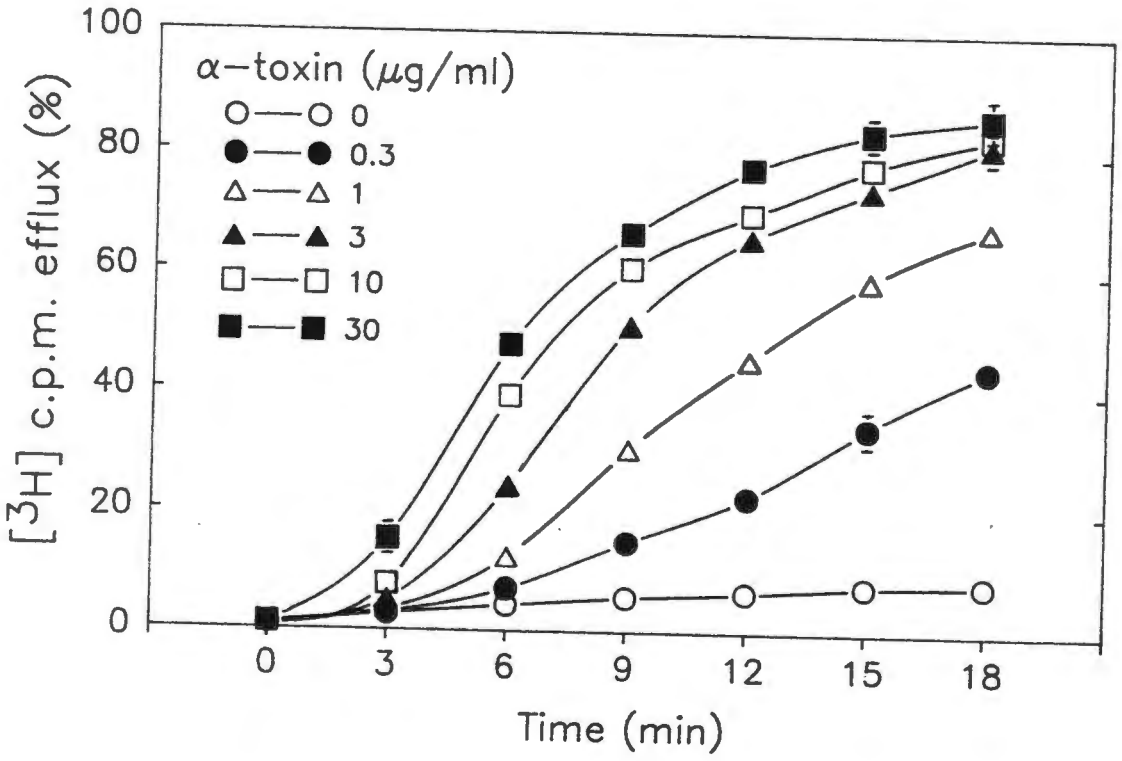

Figure 3. Comparison of LDH and [^{3}H]uridine efflux in the presence of α-toxin

After labelling with [^{3}H]uridine the efflux of radioactivity (O) and LDH (□) was measured over 20 min and is presented as a percentage of the initial total cellular radioactivity and LDH content, respectively. Similar results were obtained in three independent experiments.

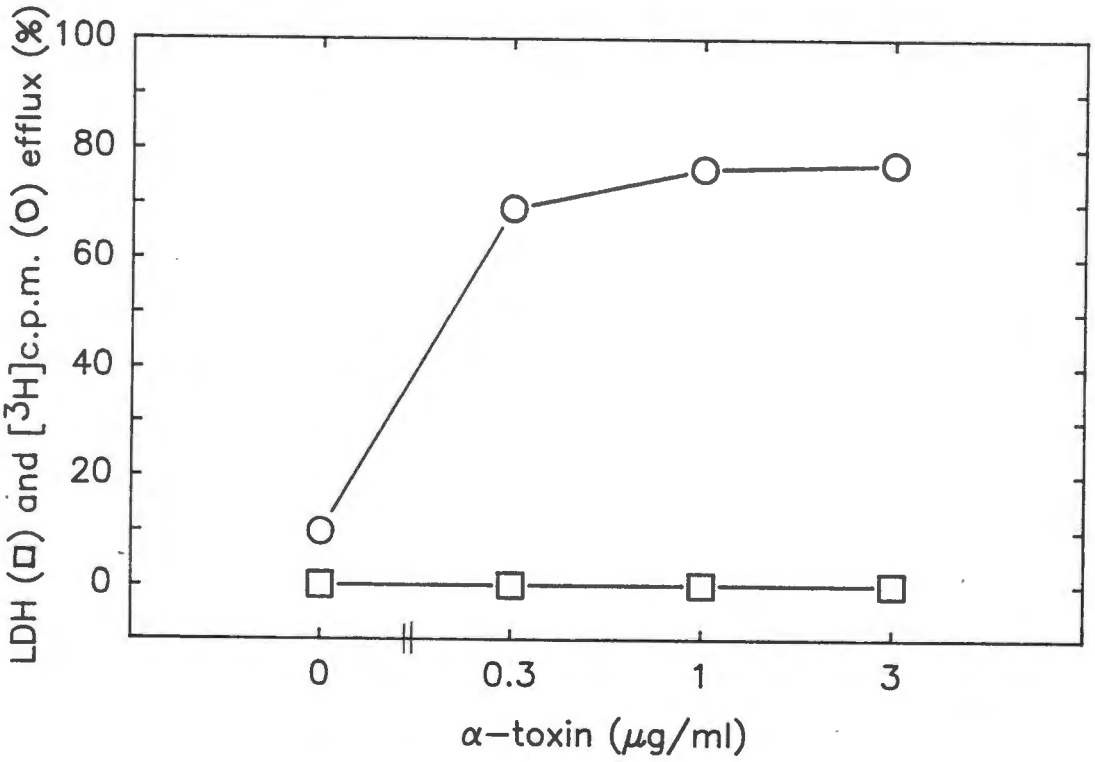

Figure 4. α-Toxin permeabilization : Time of onset

After labelling with 2-deoxy[3H]glucose cumulative efflux of radioactivity was measured and is presented as a percentage of the initial total cellular radioactivity. α-Toxin was either omitted (●) or present from either 0-5 min (O) or 0-20 min (Δ). Similar results were obtained in three independent experiments.

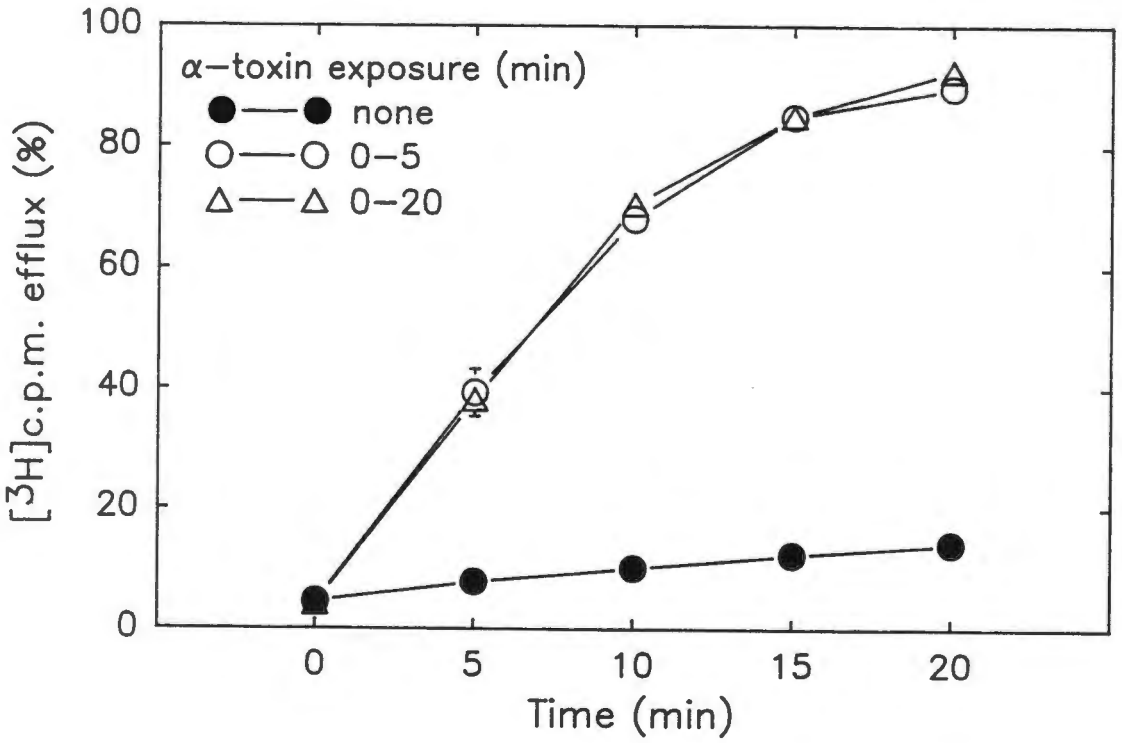

Figure 5. Time course of Ca^{2+}-stimulated LH exocytosis

Permeabilized cells in were equilibrated at 0°C for 30 min in stimulation buffer containing 30 mM CaEGTA with pCa 7 (○), 5.5 (●), and 5 (▲). LH exocytosis was initiated by replacing with identical buffer at 37°C which was exchanged at 3 minute intervals. The values at each time point represent LH released during the preceding 3 minute interval. The t = 0 points represent the rate of LH release (per 3 minutes) during the cold equilibration period. Similar results were obtained in six independent experiments.

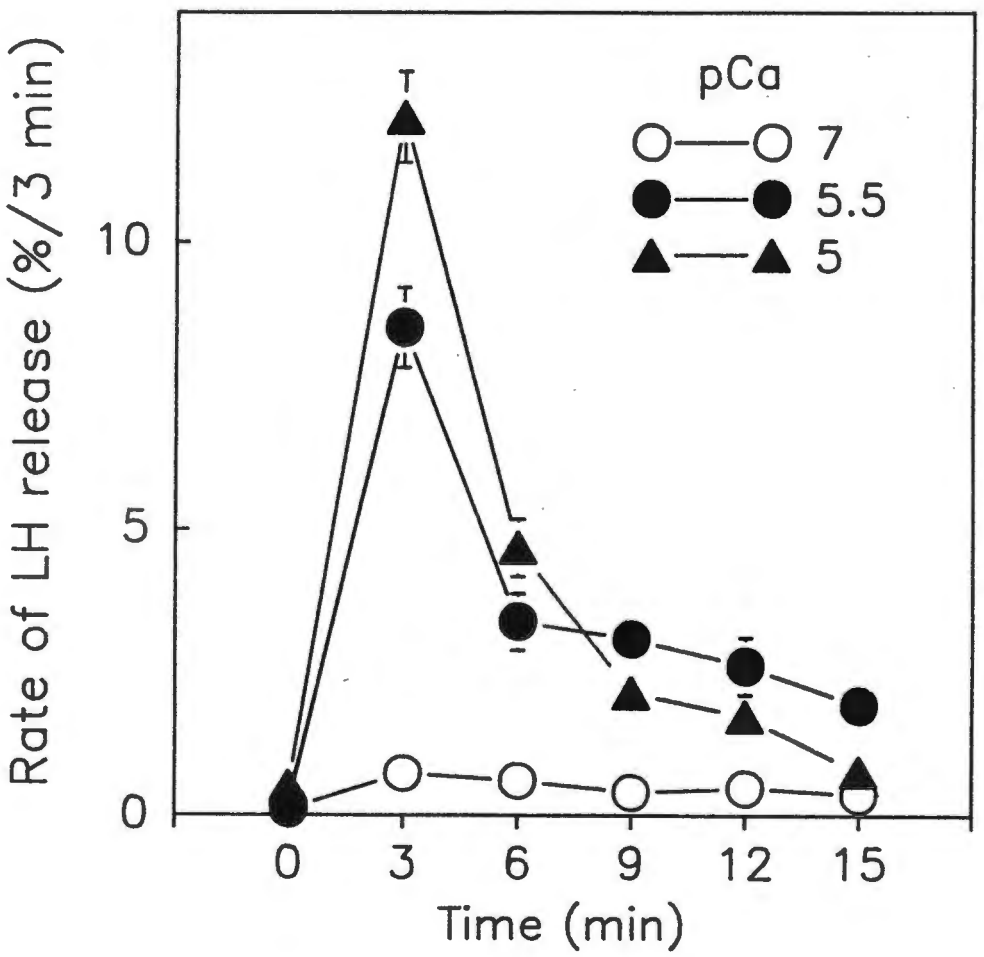

Figure 6. Ca^{2+} dependence of LH exocytosis

Permeabilized (●) and intact (O) cells were equilibrated at 0°C for 30 minutes in stimulation buffer containing 30 mM CaEGTA with the indicated $[Ca^{2+}]_{free}$. LH exocytosis was initiated by replacing with identical buffer at 37°C and the LH released after 10 minutes was determined. Similar results were obtained in fourteen independent experiments.

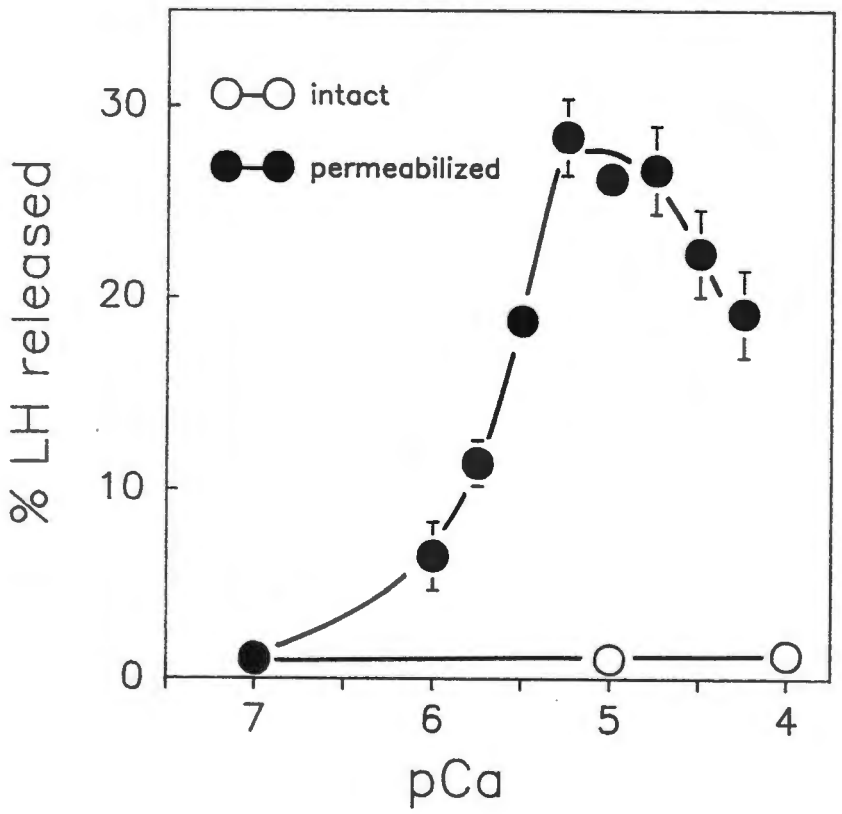

Figure 7. Effect of CaEGTA (A) concentration and (B) equilibration time on LH exocytosis

In (A) permeabilized cells were equilibrated for 30 minutes at 0°C in stimulation buffer containing the indicated concentrations of CaEGTA with pCa 7 (O), 5.5 (●), and 5 (▲). In (B) permeabilized cells were equilibrated for 10 (O) and 30 (●) minutes at 0°C in stimulation medium containing 10 mM CaEGTA with the indicated $[Ca^{2+}]_{free}$. Exocytosis was initiated by replacing with identical buffer at 37°C and LH released after 10 minutes was determined. Similar results were obtained in three (A) and two (B) independent experiments.

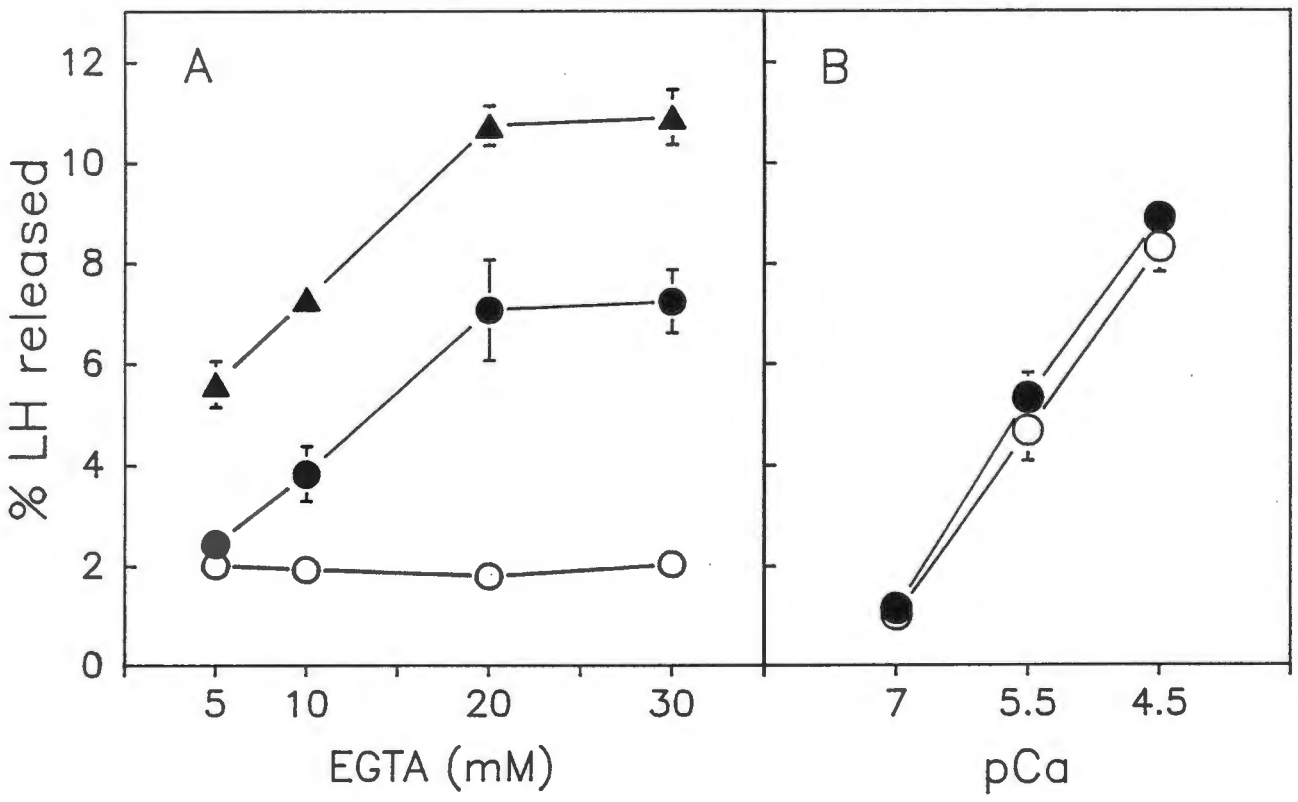

Figure 8. Ca^{2+}-dependence of LH exocytosis at different EGTA concentrations

This figure is drawn from the same data presented in Figure 7A.

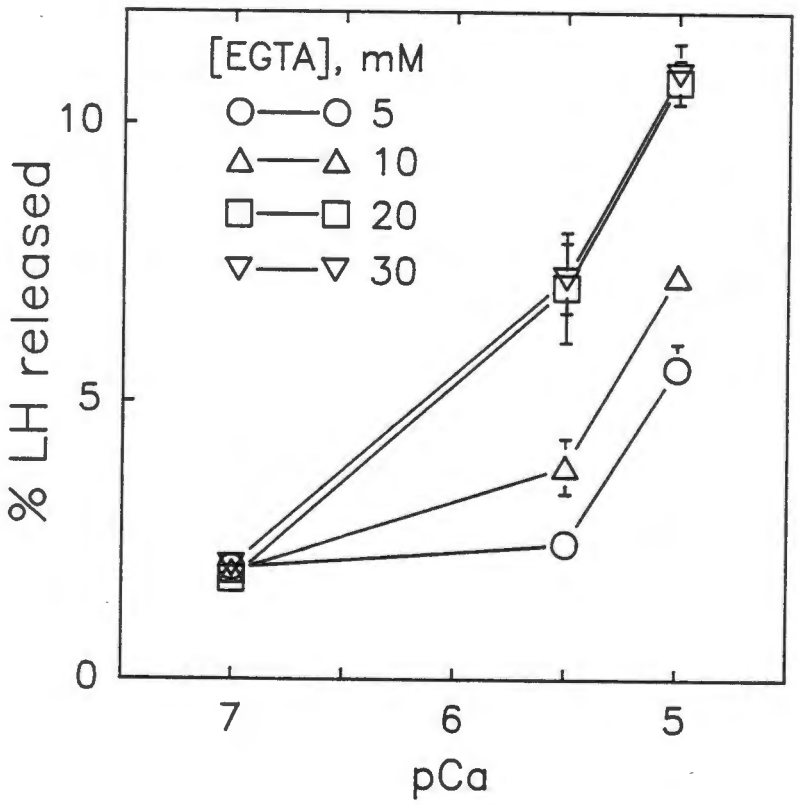

Figure 9. Effect of PMA on Ca^{2+}-stimulated LH exocytosis
Permeabilized cells were equilibrated for 30 minutes at 0°C in stimulation buffer containing 30 mM CaEGTA with the indicated $[Ca^{2+}]_{free}$ and 50 nM PMA (●) or vehicle alone [0.025% (v/v) Me_2SO] (○). LH exocytosis was initiated by replacing with identical buffer at 37°C and the incubation was continued for 10 minutes. In the inset the same protocol was used as above except that the stimulation buffer pH was 7.1. Similar results were obtained in six independent experiments.

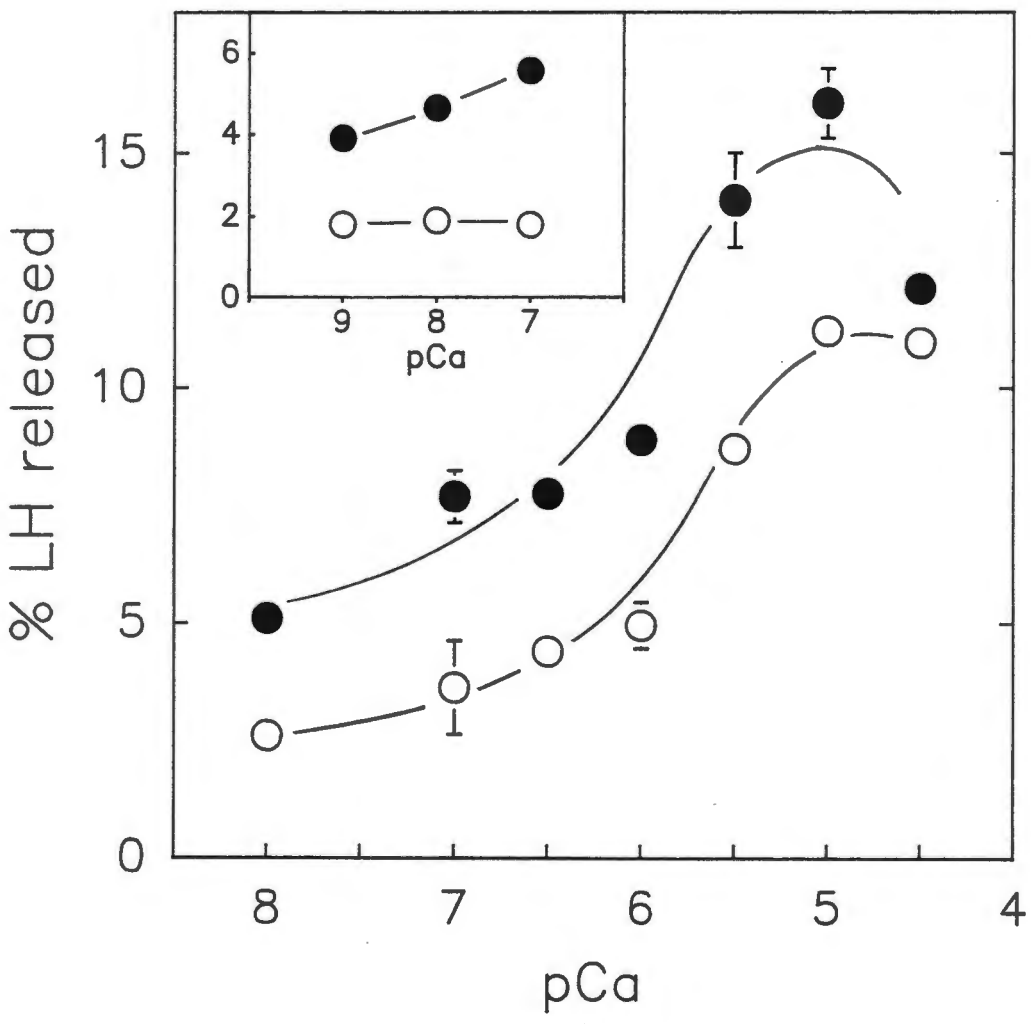

Figure 10. Time course of phorbol ester-stimulated LH exocytosis

Permeabilized cells in were equilibrated at 0°C for 30 minutes in stimulation buffer containing 30 mM CaEGTA (pCa 7) and PMA 50 nM (●) or vehicle alone (○) [0.025% Me_2SO (v/v)]. LH exocytosis was initiated by replacing with identical buffer at 37°C which was exchanged at 3 minute intervals. The values at each time point represent LH released during the preceding 3 minute interval. The t = 0 points represent the rate of LH release (per 3 minutes) during the cold equilibration period. Similar results were obtained in two independent experiments.

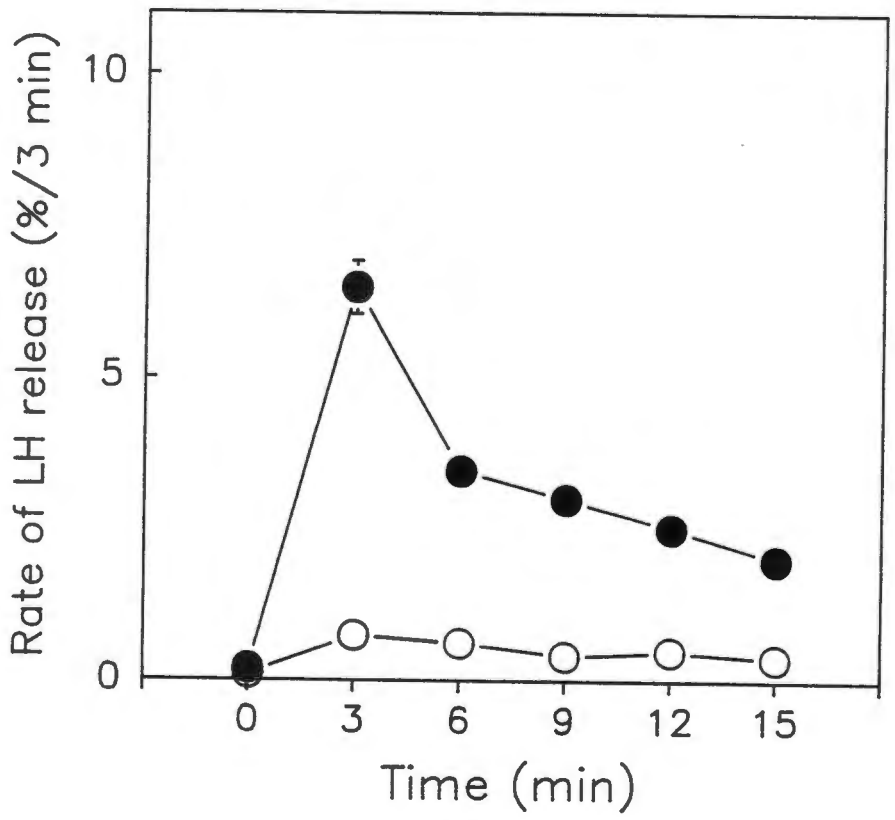

Figure 11 The effect of cAMP, with or without IBMX, on LH exocytosis

Permeabilized cells were equilibrated at 0°C for 30 minutes in stimulation buffer containing 10 mM CaEGTA (pCa 7) and cAMP at the indicated concentration alone (O) or in the presence of 0.25 mM IBMX (●). Exocytosis was initiated by replacing with identical buffer at 37°C and the LH released after 10 minutes was determined. Similar results were obtained in three independent experiments.

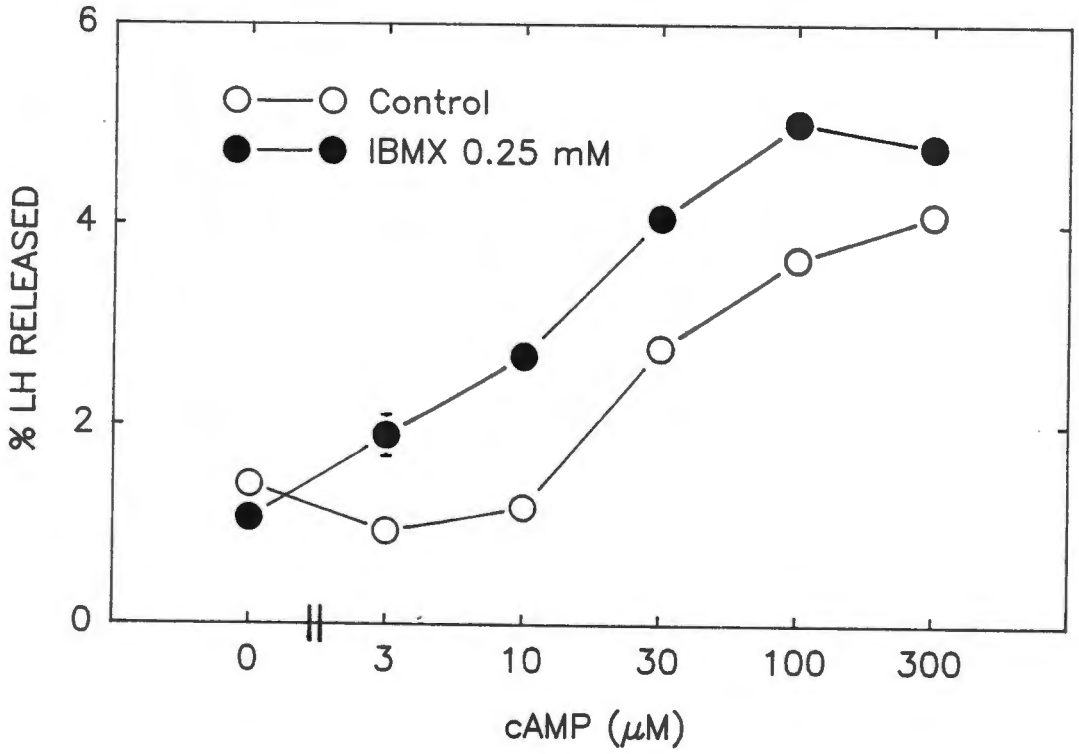

Figure 12. Time course of cAMP-stimulated LH exocytosis
Permeabilised cells were equilibrated at 0°C for 30 minutes in stimulation buffer containing 10 mM CaEGTA (pCa 7) alone (O) or with 100 µM cAMP (●). Exocytosis was initiated by replacing with identical buffer at 37°C which was exchanged at 5 min intervals. The values at each time point represent the rate of LH release during the preceeding 5 min period. The zero time points represent the rate of LH release (per 5 min) during the cold equilibration period. Similar results were obtained in three independent experiments.

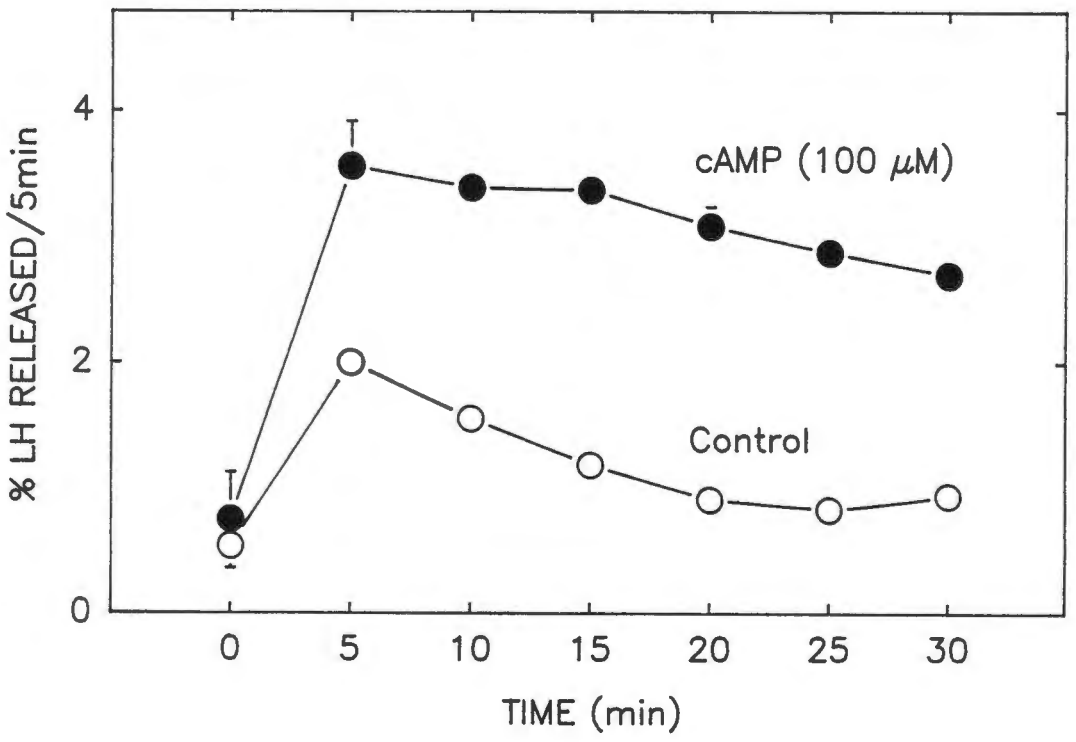

Figure 13 The effect of IBMX concentration on cAMP-stimulated LH exocytosis

Permeabilized cells were equilibrated at 0°C for 30 minutes in stimulation buffer containing 10 mM CaEGTA (pCa 7) and IBMX at the indicated concentration either alone (O) or in the presence of 100 µM cAMP (●). Exocytosis was initiated by replacing with identical buffer at 37°C and the LH released after 10 minutes was determined. Similar results were obtained in two independent experiments.

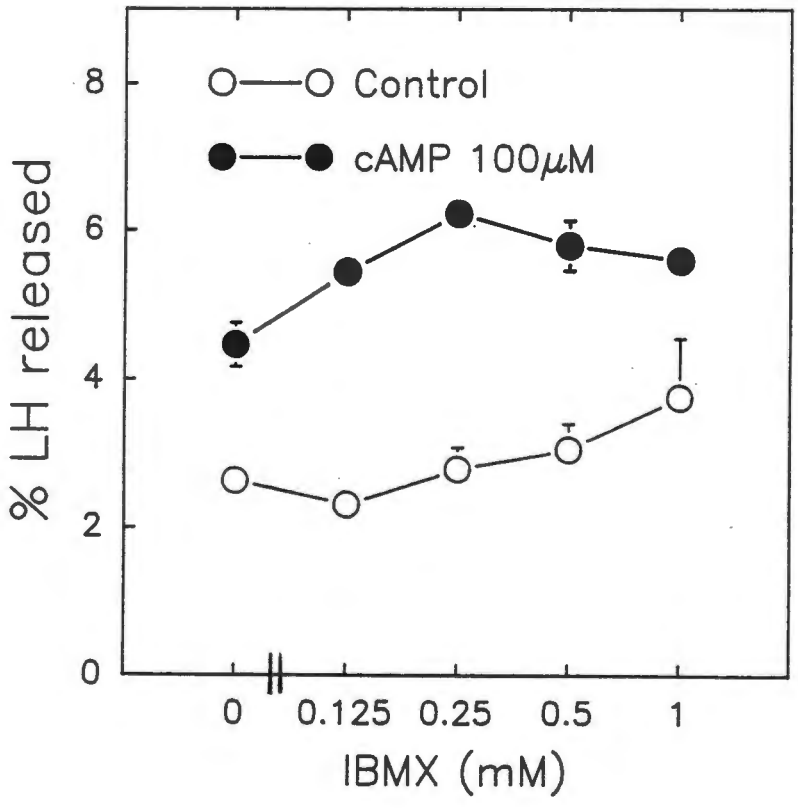

Figure 14. The effect of cAMP on Ca^{2+}-stimulated LH exocytosis

Permeabilized cells were equilibrated at 0°C for 30 minutes in stimulation buffer with 30 mM CaEGTA at the indicated $[Ca^{2+}]_{free}$ alone (*O*) or in the presence of cAMP (100 μM) plus IBMX (0.25 mM) (●). LH exocytosis was initiated by replacing with identical buffer at 37°C and the LH released after 10 minutes was determined. In the inset the same protocol was used except the stimulation buffer pH was 7.1. Similar results were obtained in three independent experiments.

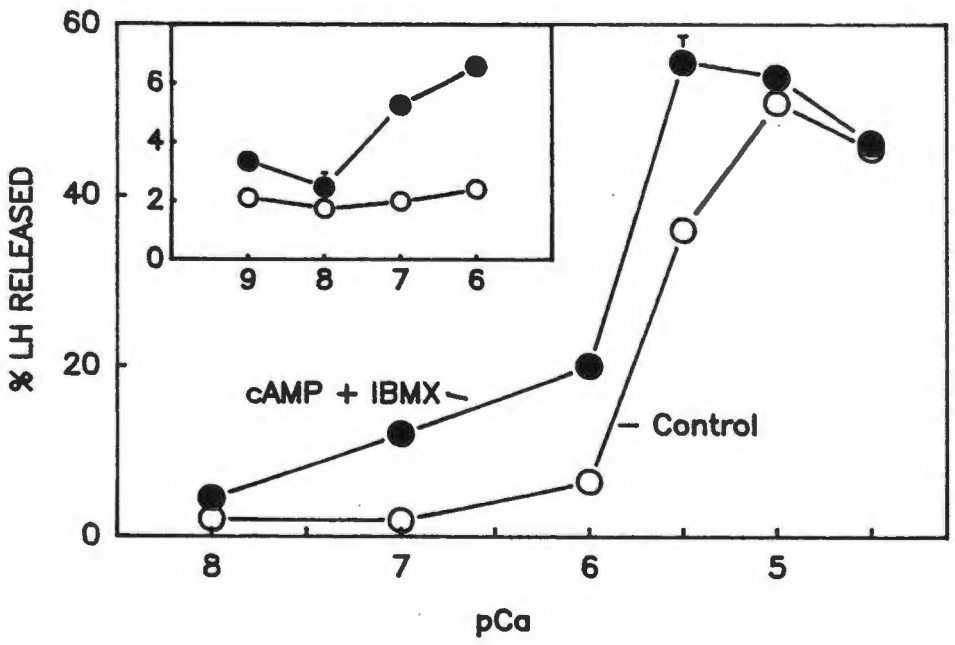

Figure 15. The effect of PMA on cAMP-stimulated LH exocytosis

Permeabilized cells were equilibrated at 0°C for 30 minutes in stimulation buffer containing 10 mM CaEGTA (pCa 7), IBMX (0.25 mM), and the indicated cAMP concentrations alone (O) or in the presence of 100 nM PMA (●). LH exocytosis was initiated by replacing with identical buffer at 37°C and the LH released after 10 minutes was determined. Similar results were obtained in five independent experiments.

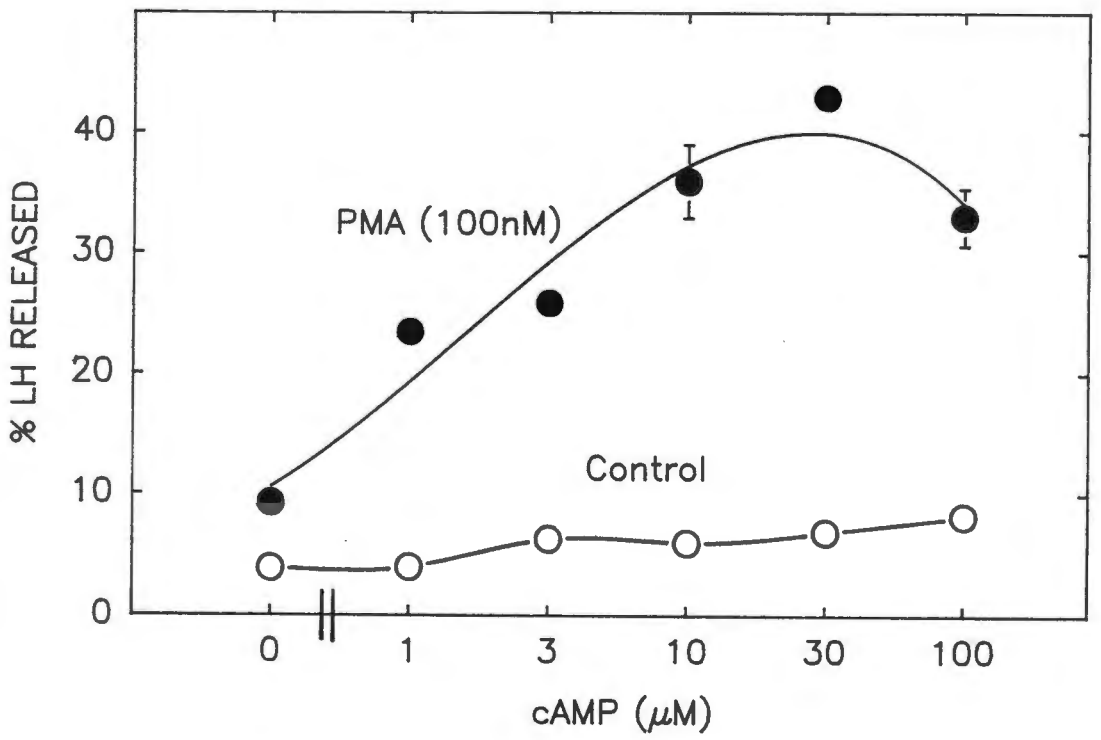

Figure 16. The effect of cAMP on PMA-stimulated LH exocytosis

Permeabilized cells were equilibrated at 0°C for 30 minutes in stimulation buffer containing 10 mM CaEGTA (pCa 7), IBMX (0.25 mM), and the indicated PMA concentrations alone (O) or in the presence of 30 µM cAMP (●). LH exocytosis was initiated by replacing with identical buffer at 37°C and the LH released after 10 minutes was determined. Similar results were obtained in three independent experiments.

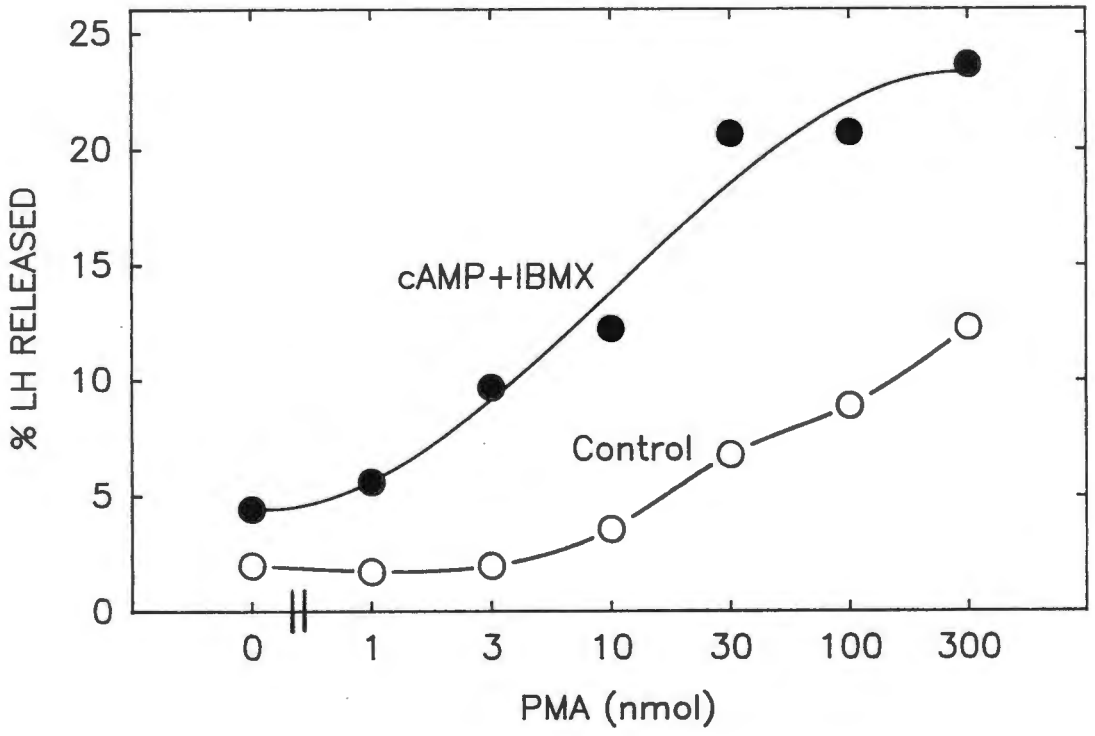

Figure 17. Ca^{2+}-dependence of cAMP plus PMA-stimulated LH exocytosis

Permeabilized cells were equilibrated at 0°C for 30 min in stimulation buffer containing 30 mM CaEGTA with the indicated pCa and with the following additions: None (*O*); cAMP (30 µM) plus IBMX (0.25 mM) (●); PMA (100 nM) (□); or cAMP (30 µM) plus IBMX (0.25 mM) plus PMA (100 nM) (■). LH exocytosis was initiated by replacing with identical buffer at 37°C and the LH released after 10 minutes determined. Similar results were obtained in two independent experiments.

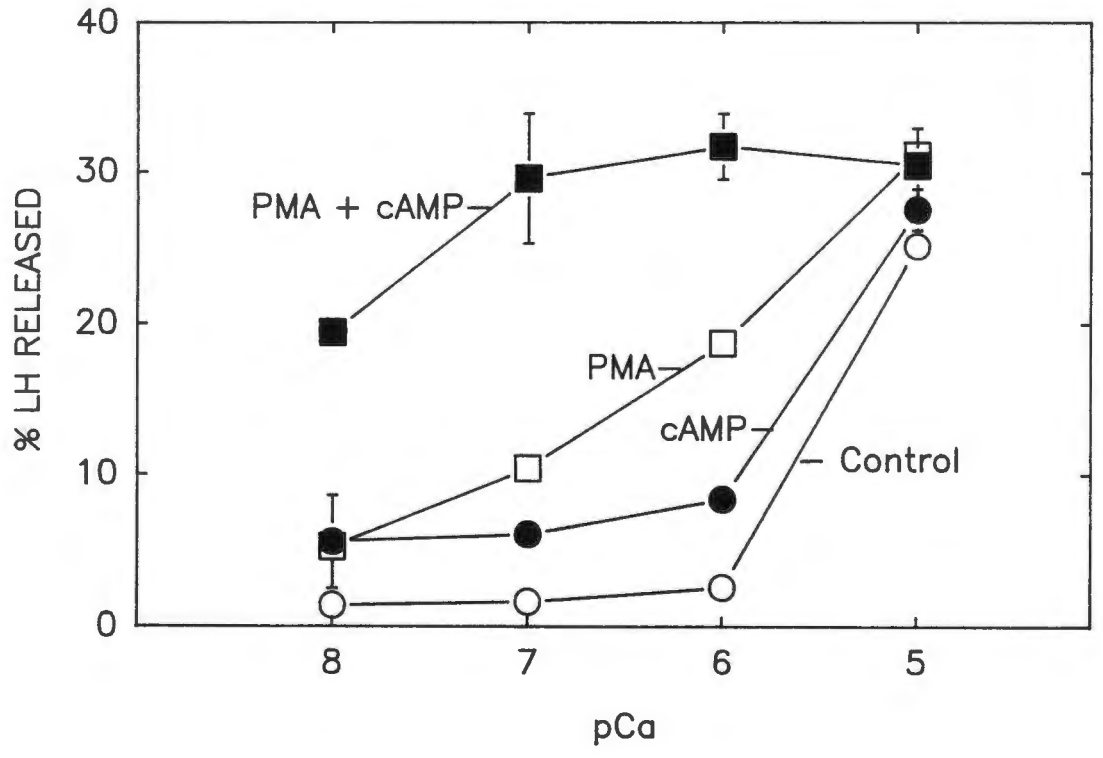

Figure 18. The effects of GnRH on cAMP production and LH exocytosis in intact cells

Cells at a density of 3 pituitaries per 6-well plate were stimulated for 1 h at 37°C in Buffer I with IBMX (0.25 mM) and the indicated GnRH concentrations, after which the LH released and the cellular cAMP were determined as described in Section 2. Similar results were obtained in two independent experiments.

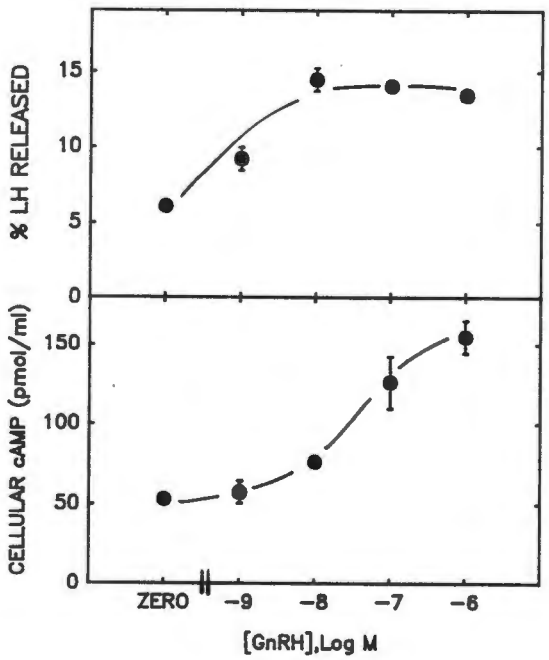

Figure 19. The effect of MgATP depletion on LH exocytosis
Permeabilized cells were equilibrated for the indicated times at 0°C with Buffer IC containing 30 mM CaEGTA with the following composition: (●) pCa 7, 5 mM MgATP; (□) pCa 5, no MgATP; (■) pCa 5, 5 mM MgATP. Exocytosis was initiated by replacing with identical buffer at 37°C and LH released after 10 minutes was determined. The time course of α-toxin-induced loss of radioactivity from the trichloroacetic acid-soluble [^{14}C]adenosine labelled cellular pool (▲) is shown for comparison. Similar results were obtained in three independent experiments.

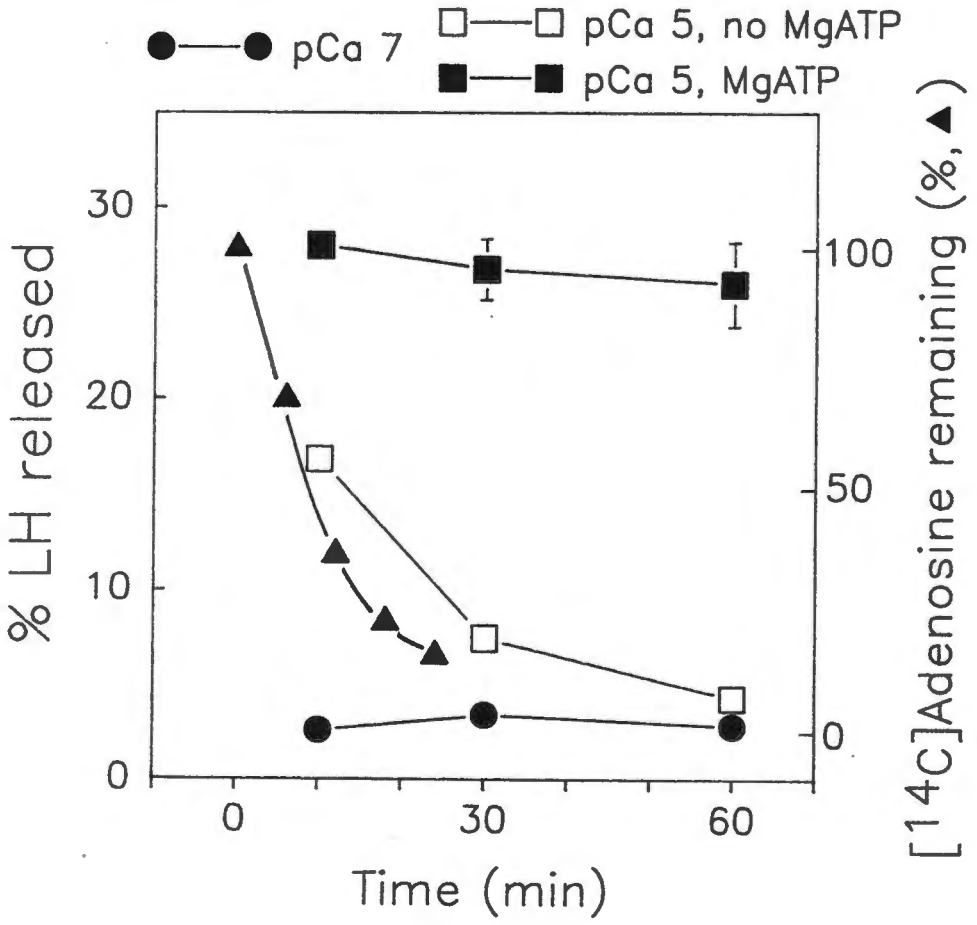

Figure 20. Restoration of Ca^{2+}-stimulated LH exocytosis by MgATP

Permeabilized cells were equilibrated for 90 minutes at 0°C in Buffer IC containing 30 mM CaEGTA with pCa 7 (open) or 5 (closed). MgATP (final concentration 5 mM) was added (circles) at the indicated times before stimulation. Exocytosis was initiated by replacing with identical buffer at 37°C with (circles) or without (bars) 5 mM MgATP and LH released after 5 minutes was determined. Similar results were obtained in three independent experiments.

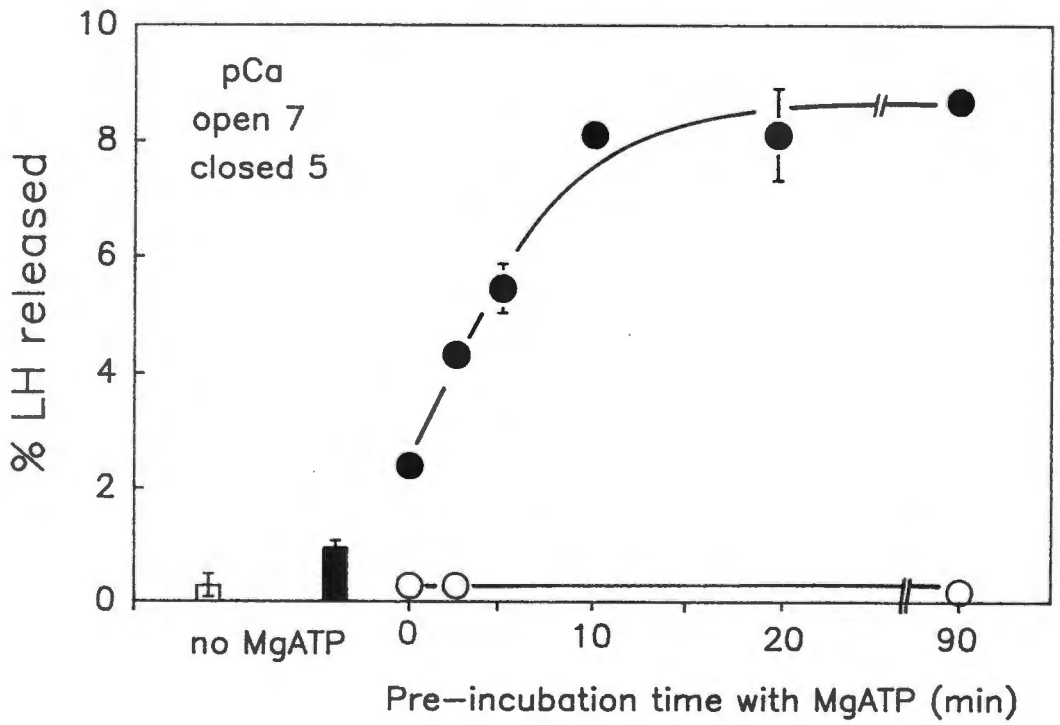

Figure 21. MgATP dependence of (A) Ca^{2+}-stimulated and (B) phorbol ester-stimulated LH exocytosis

Permeabilized cells were equilibrated for 60 minutes at 0°C in Buffer IC containing the indicated MgATP concentrations and 30 mM CaEGTA with: (A) pCa 7 (O) or 5 (●); (B) pCa 8 and 100 nM PMA (●) or vehicle alone [0.05% (v/v) Me_2SO] (O). Exocytosis was initiated by replacing with identical buffer at 37°C and LH released after 10 minutes was determined. Similar results were obtained in four (A) and three (B) independent experiments.

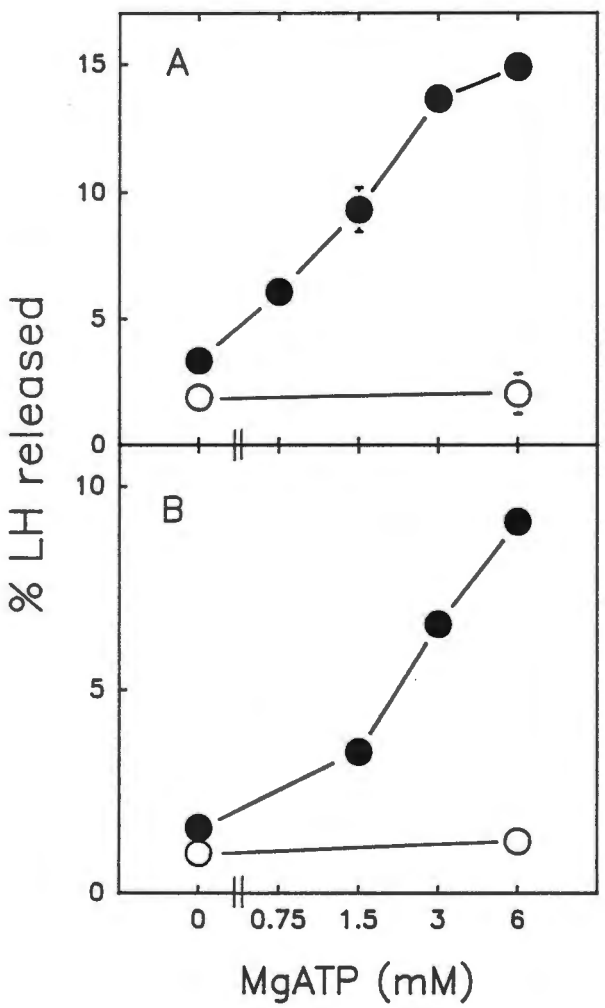

Figure 22. Nucleotide-dependence of Ca^{2+}-stimulated LH exocytosis

Permeabilized cells were equilibrated for one hour at 0°C in Buffer IC containing 30 mM CaEGTA with pCa 7 (open) or pCa 5 (closed) and the indicated nucleotide. Exocytosis was initiated by replacing with identical buffer at 37 °C and LH released after 10 minutes was determined. $MgCl_2$ was include to maintain a $[Mg^{2+}]_{free}$ of about 1 mM. Similar results were obtained in two independent experiments.

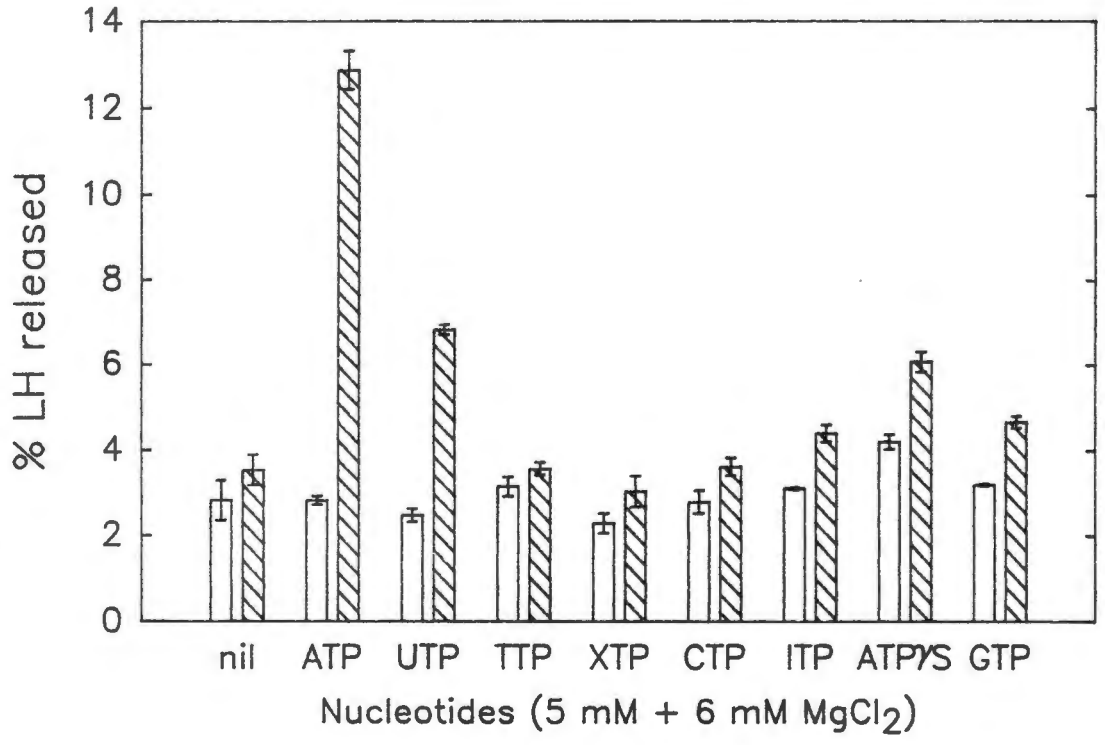

Figure 23. MgATP dependence of cAMP-stimulated LH exocytosis
Permeabilized cells were equilibrated at 0°C for 90 minutes in Buffer IC containing 10 mM CaEGTA (pCa 7) and the indicated MgATP concentration alone (O) or with cAMP (100 μM) plus IBMX (0.25 mM) (●). Exocytosis was initiated by replacing with identical buffer at 37°C and the LH released after 10 min was measured. Similar results were obtained in three independent experiments.

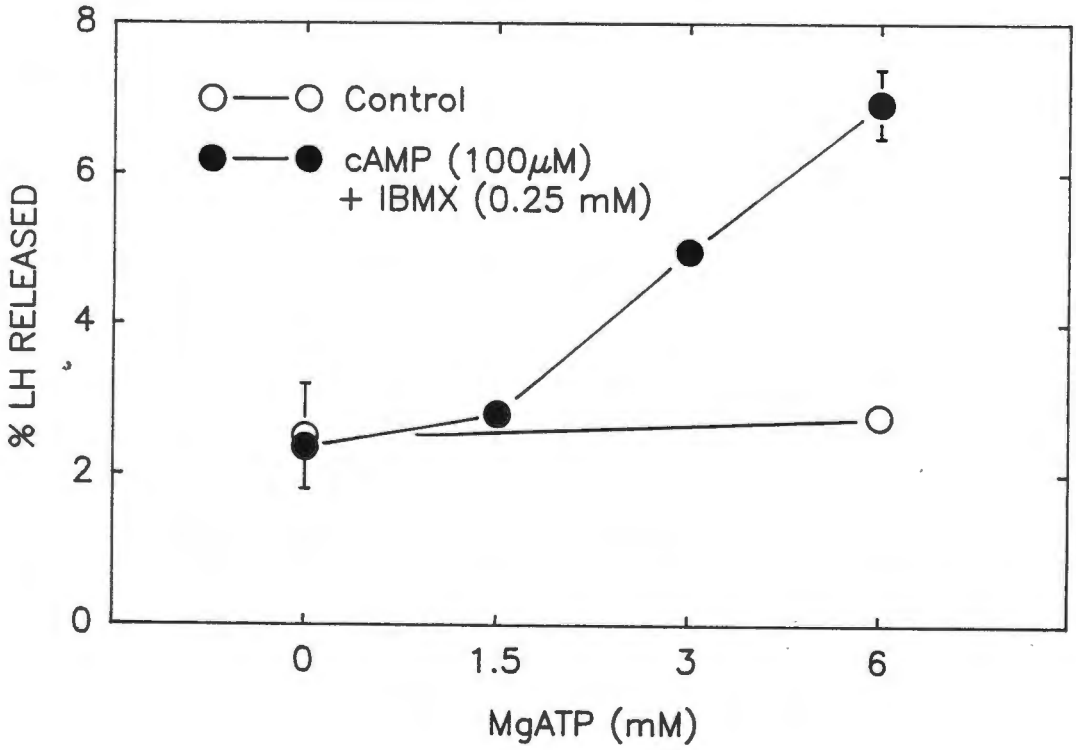

Figure 24. PMA-stimulated LH exocytosis in cells refractory to Ca^{2+}

Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer containing 30 mM CaEGTA with pCa 8 (○,●) or 5 (△,▲). LH exocytosis was initiated by replacing with identical buffer at 37 °C which was exchanged at 5 min intervals. Buffer added from t = 20 min onwards included PMA (100 nM)(●,▲) or vehicle [0.05% Me_2SO (v,v)] (○,△). The values at each time point represent the LH released during the preceding 5 min interval. The t = 0 point represents the rate of LH release (per 5 minutes) during the cold equilibration period. Similar results were obtained in three independent experiments.

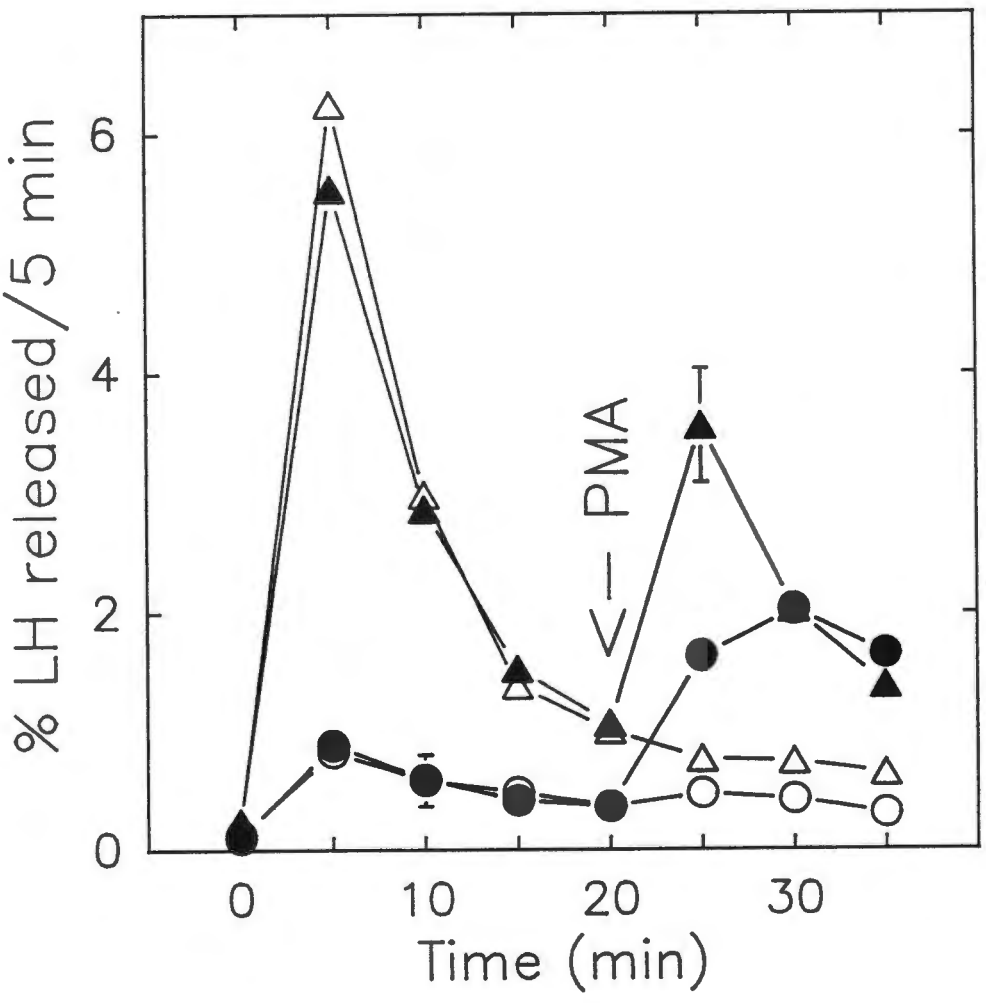

Figure 25. The effect of staurosporine on PMA-, Ca^{2+}-, and Ba^{2+}-stimulated LH exocytosis

Staurosporine was added at the indicated concentration 5 min after the start of the permeabilization step and was present until the end of the experiment. Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer containing 30 mM EGTA and with: pCa 8 plus 100 nM PMA (○), pCa 5 (●), or pBa 3 (△). LH exocytosis was initiated by replacing with identical buffer at 37 °C and LH released over the next 10 min was measured. LH release is expressed as a percentage of the release in the absence of staurosporine. Control values were (% of cellular LH ± range): pCa 5, 15.7 ± 0.3; 100 nM PMA (pCa 8), 4.3 ± 0.3; pBa 3, 10.2 ± 0.2. Similar results were obtained in three independent experiments.

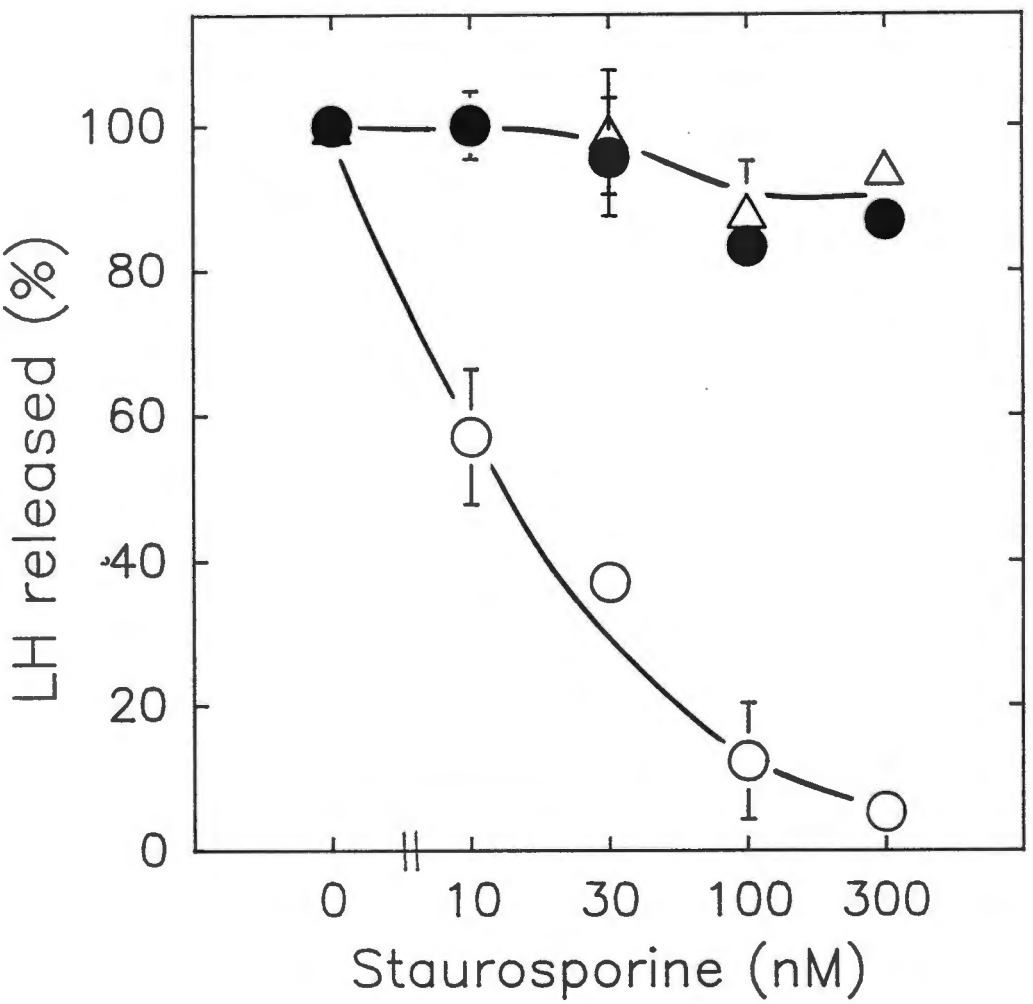

Figure 26. The effect of staurosporine on combined Ca^{2+}-and PMA-stimulated LH exocytosis

Staurosporine (1 μM)(●,▲) or vehicle [Me_2SO, 0.1% (v,v)] (O,Δ) were added 5 min after the start of the permeabilization step and were present until the end of the experiment. Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer containing 30 mM CaEGTA with the indicated $[Ca^{2+}]_{free}$ and 100 nM PMA (Δ,▲) or vehicle [Me_2SO 0.05% (v/v)] (O,●). LH exocytosis was initiated by replacing with identical buffer at 37 °C and LH released over the next 10 min was measured. Similar results were obtained in three independent experiments.

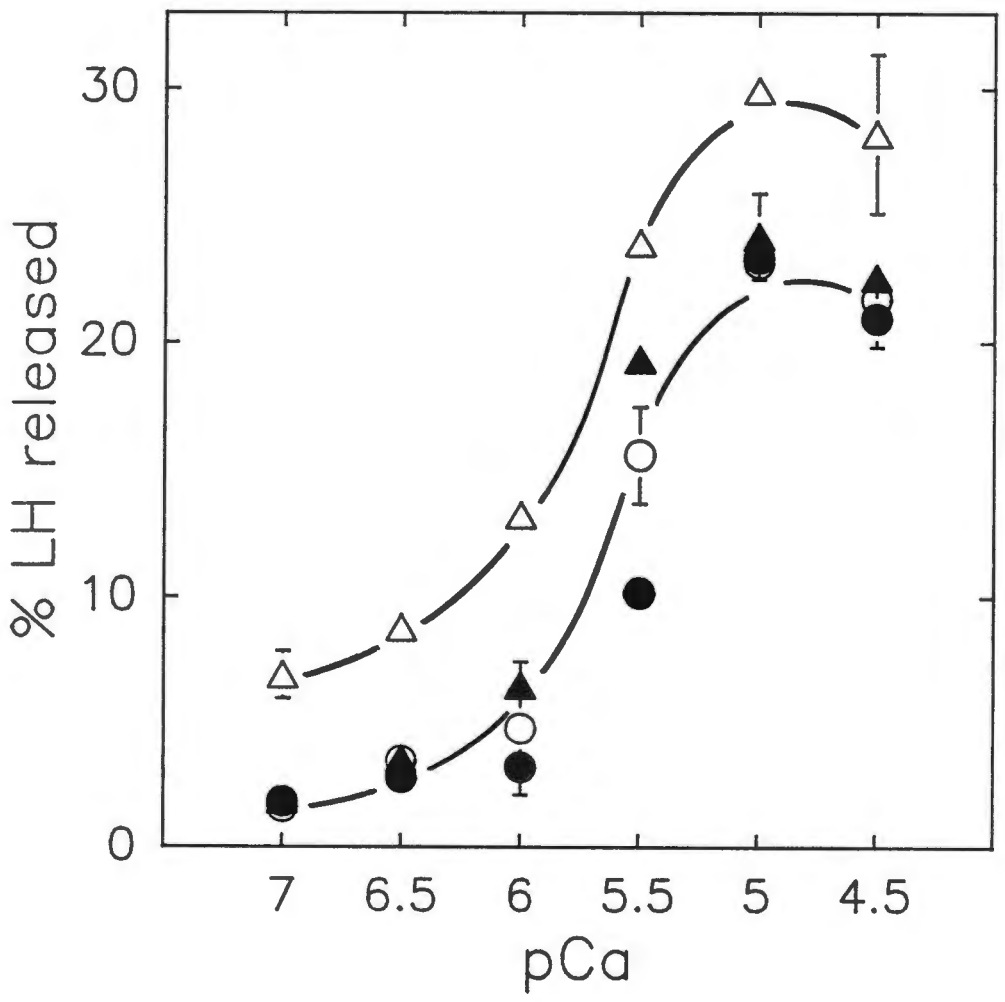

Figure 27. The effect of PMA-desensitization on Ca^{2+}-stimulated LH exocytosis

Pituitary cells were exposed to PMA (500 nM) (Δ,▲) or vehicle [Me_2SO 0.25% (v/v)] (O,●) for 24 h after which the cells were washed and permeabilized. Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer containing 30 mM CaEGTA with the indicated $[Ca^{2+}]_{free}$ and 100 nM PMA (●,▲) or vehicle [Me_2SO 0.05% (v/v)] (O,Δ). LH exocytosis was initiated by replacing with identical buffer at 37 °C and LH released over the next 10 min was measured. In (a) absolute amounts of LH released are shown whereas in (b) LH release is expressed as a percentage of cellular LH present immediately before stimulation. Similar results were obtained in three independent experiments.

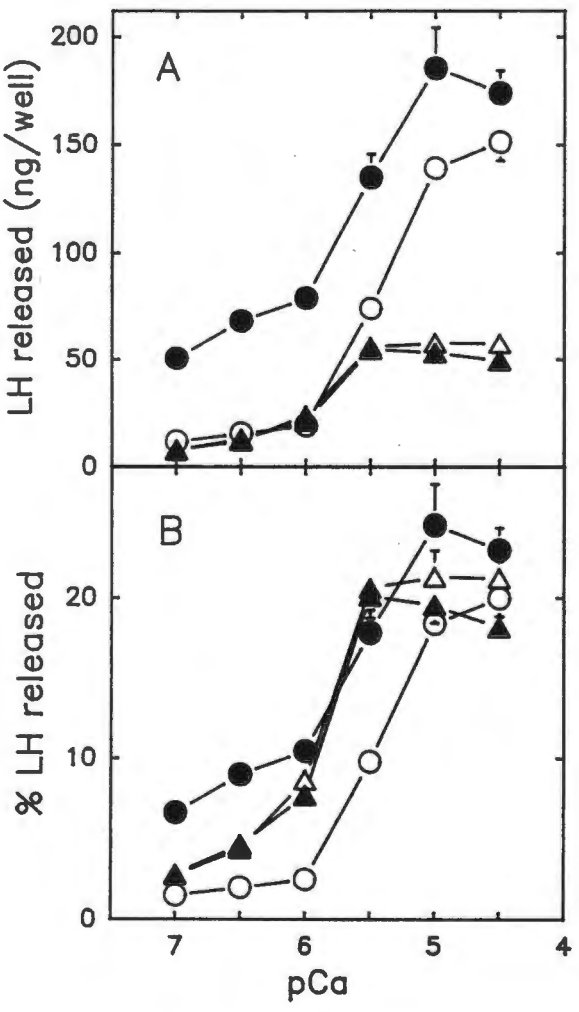

Figure 28. (A) Time-course of Ba^{2+}- and Ca^{2+}-stimulated LH exocytosis and the effect of phorbol ester on cells refractory to Ba^{2+}. (B) The effect of Ba^{2+} on cells refractory to Ca^{2+}.

In (A) permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer containing 30 mM EGTA with pCa 8 (○,●), pCa 5 (△), or pBa 3 (□,■). LH exocytosis was initiated by replacing with identical buffer at 37 °C which was exchanged at 5 min intervals. PMA 100 nM was added from t = 20 onwards (arrow) (●,■). In (B) permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer containing 30 mM EGTA with pCa 7 (○,●) or 5 (△,▲). LH exocytosis was initiated by replacing with identical buffer at 37 °C which was exchanged at 5 min intervals. BaEGTA (30 mM, pBa 3) was added from t = 20 onwards (arrow) (●,▲). In (A) and (B) the values at each time point represents the LH released during the preceding 5 min interval and the t = 0 point represents the rate of LH release (per 5 minutes) during the cold equilibration period. Similar results were obtained in three independent experiments.

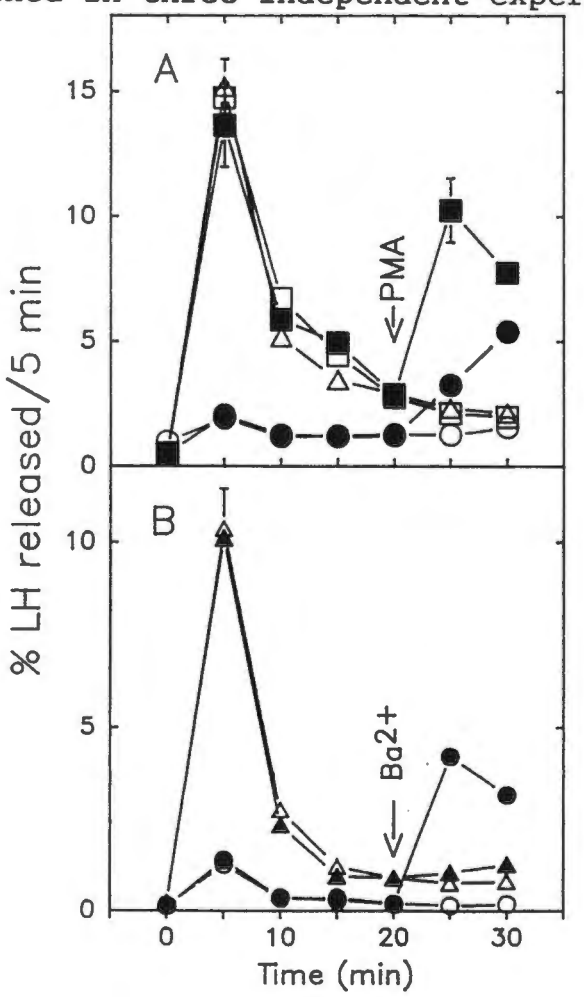

Figure 29. Concentration dependence of Ba^{2+}-stimulated LH exocytosis

Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer containing BaEGTA 30 mM with the indicated $[Ba^{2+}]_{free}$ (*O*). LH exocytosis was initiated by replacing with identical medium at 37 °C and LH released over the next 10 minutes was measured. Similar results were obtained in three independent experiments. Also shown is the concentration dependence of Ca^{2+}-stimulated LH exocytosis (– – –) drawn from Figure 6.

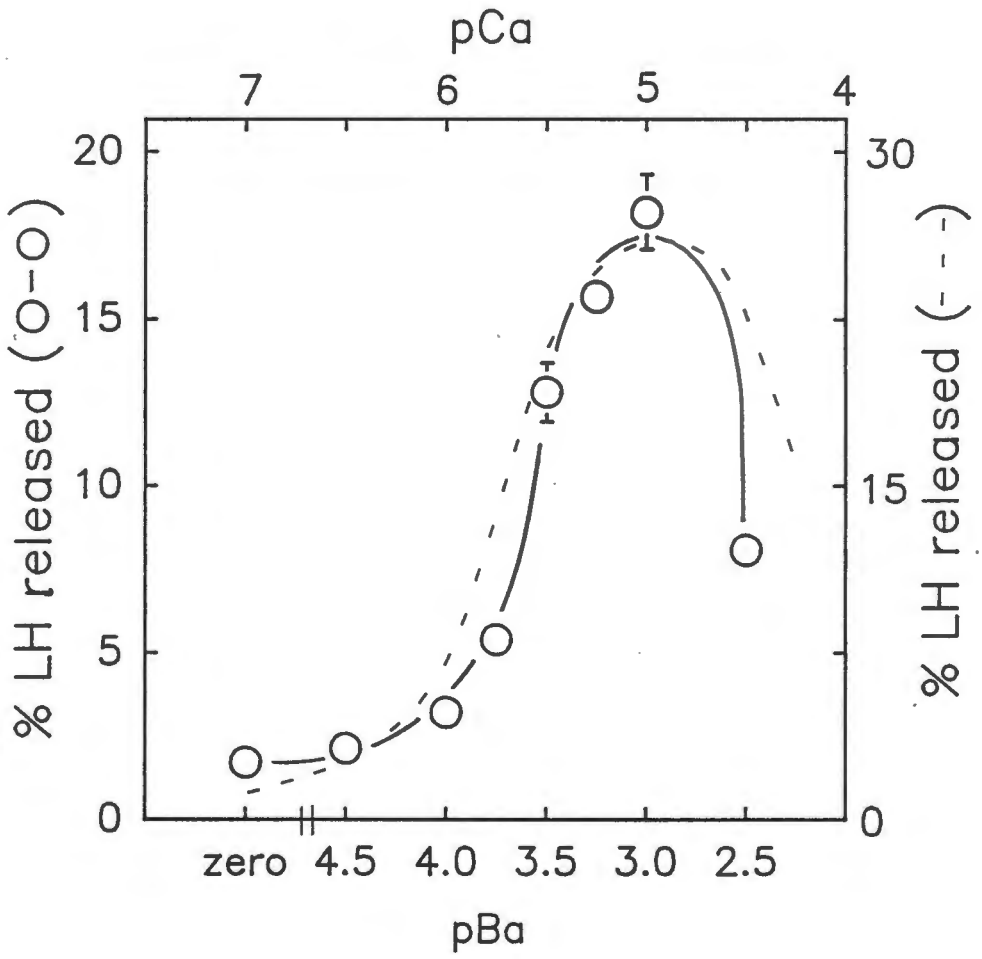

Figure 30. Effect of trifluoperazine on Ca^{2+}-, Ba^{2+} -, and phorbol ester-stimulated LH exocytosis

Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer (EGTA 30 mM) with the indicated concentration of trifluoperazine and: pCa 7 (●), pCa 7 plus 100 nM PMA (O), pCa 5 (■), or pBa 3 (Δ). LH exocytosis was initiated by replacing with identical buffer at 37 °C and LH released over the next 10 min was measured. Similar results were obtained in two independent experiments.

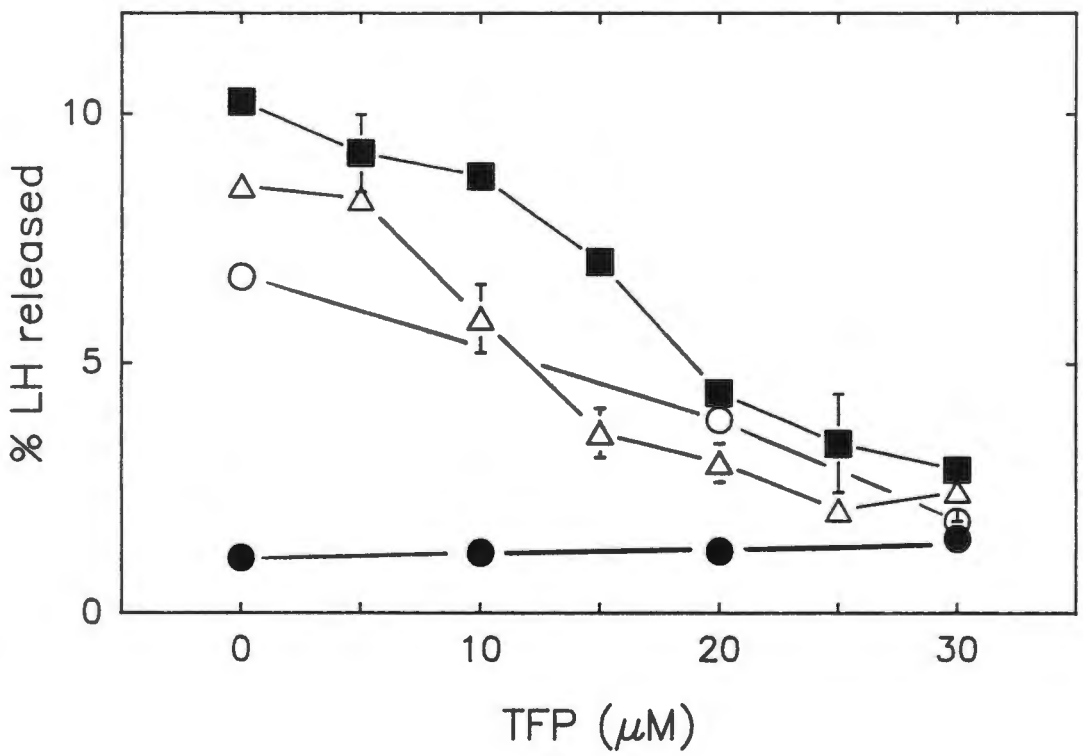

Figure 31. Effect of calmidazolium on Ca^{2+}- and phorbol ester-stimulated LH exocytosis

Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer (CaEGTA 30 mM) with the indicated concentration of calmidazolium and: pCa 7 (○), pCa 7 plus 100 nM PMA (▲), or pCa 5 (●). LH exocytosis was initiated by replacing with identical buffer at 37 °C and LH released over the next 10 min was measured.

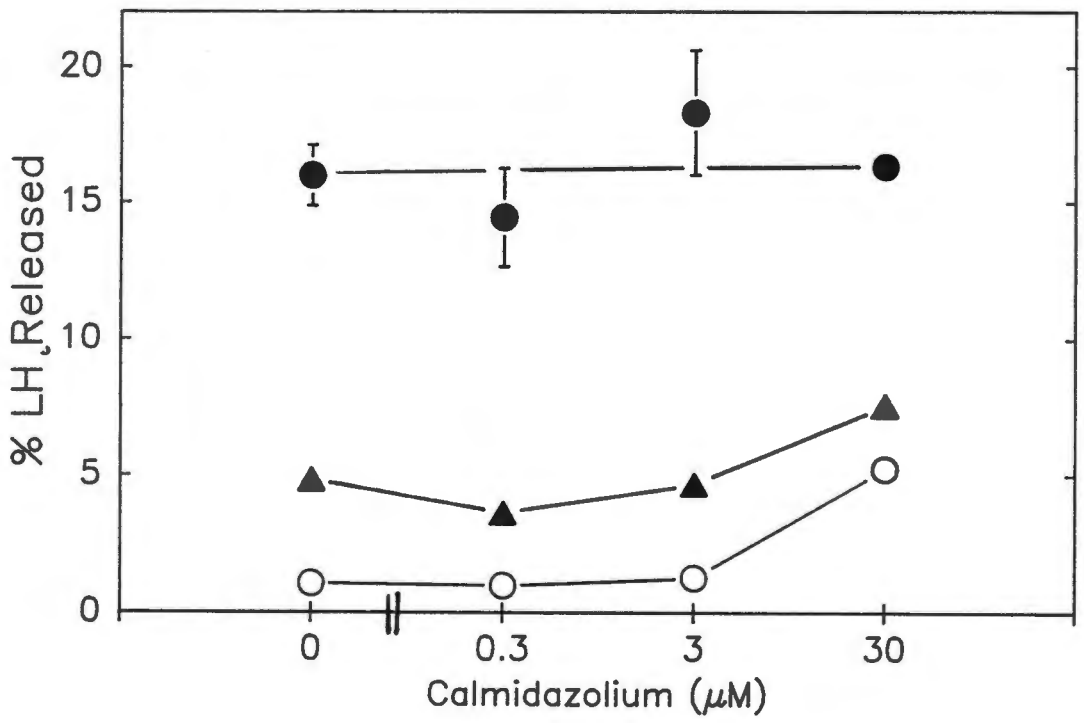

Figure 32. Stimulatory effects of guanine nucleotides on lH exocytosis

(A) Time course. Permeabilized cells were equilibrated at 0°C for 30 min in stimulation buffer containing 10 mM CaEGTA (pCa 7) with no guanine nucleotide (■), 300 μM GMPPNP (▲), or 300 μM GTPγS (●). LH exocytosis was initiated by replacing with identical stimulation buffer at 37°C which was replaced at 5 min intervals. LH release at each time point represents the rate of LH released in the preceding 5 min. The t = 0 point represents the rate of LH released per 5 min during the 30 min equilibration period. Similar results were obtained in two independent experiments.

(B) Concentration-dependence. Permeabilized cells were equilibrated at 0°C for 30 min in buffer IC containing 10 mM CaEGTA (pCa 7) with 2 mM $MgCl_2$ (open) or 6.5 mM MgCl plus 6 mM Na_2ATP (closed) and the indicated concentration of GTPγS (○,●), GMPPNP (▲), or GTP (◆). LH exocytosis was initiated by replacing with identical buffer at 37°C and LH released after 20 min was determined. Similar results were obtained in five independent experiments.

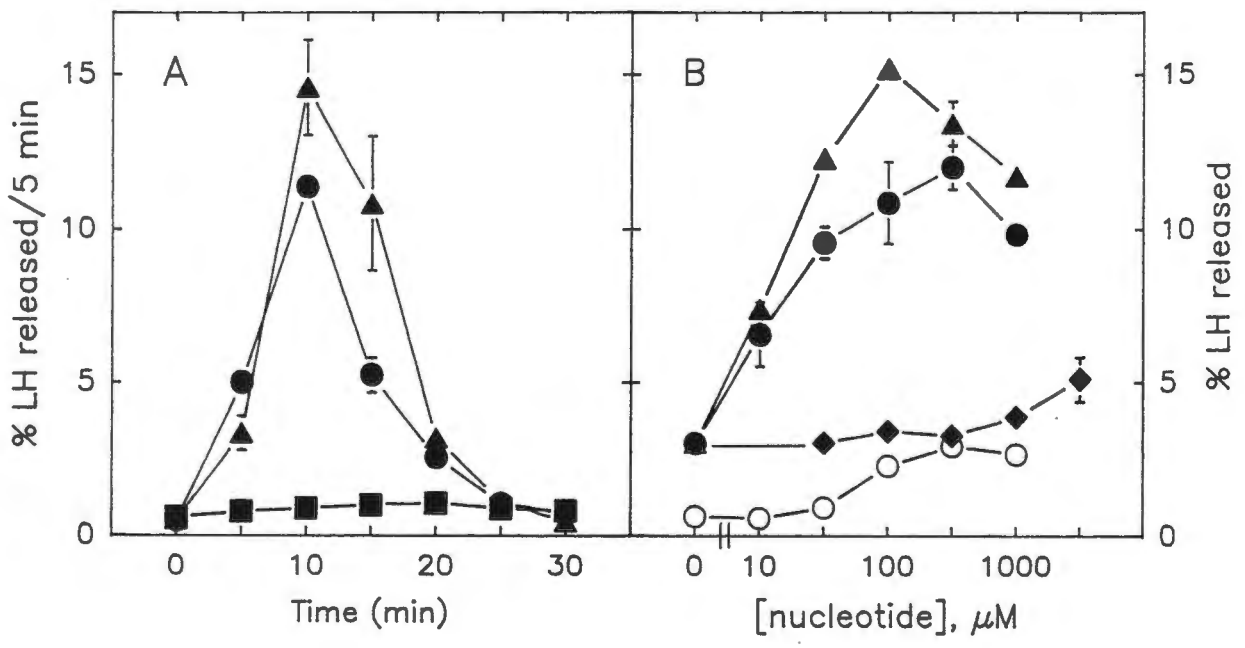

Figure 33. Effect of $[Ca^{2+}]_{free}$ on GMPPNP-stimulated LH exocytosis

Permeabilized cells were equilibrated at 0°C for 30 min in stimulation buffer containing 30 mM CaEGTA at the indicated $[Ca^{2+}]_{free}$ with (●) or without (*O*) 300 µM GMPPNP. LH exocytosis was initiated by replacing with identical buffer at 37°C and LH released after 20 min was determined. In the inset the experiment was conducted at pH 7.1 to allow buffering of the $[Ca^{2+}]_{free}$ down to pCa 9. Similar results were obtained in three independent experiments.

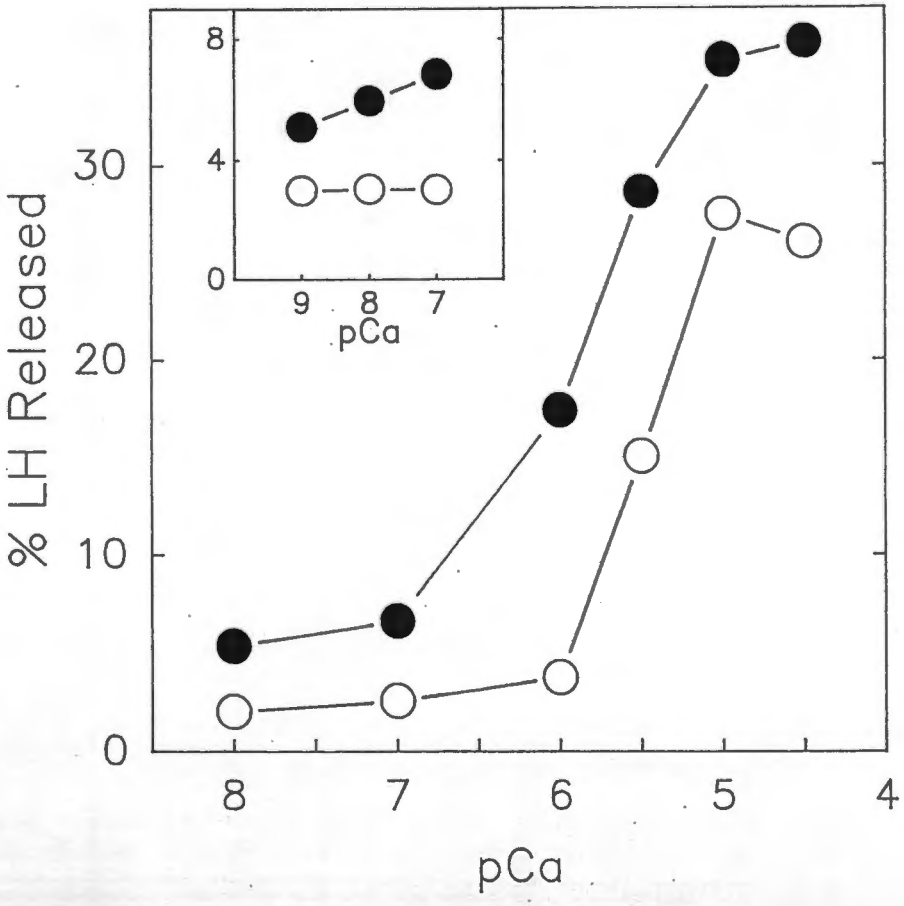

Figure 34. Effect of GMPPNP on LH exocytosis in the presence of phorbol ester or cAMP

Permeabilized cells were equilibrated at 0°C for 30 min in stimulation buffer containing 30 mM CaEGTA (pCa 7) with (●) or without (○) GMPPNP (300 μM) and the indicated concentrations of PMA or cAMP. LH exocytosis was initiated by replacing with identical buffer at 37°C and LH released after 20 min was determined. Similar results were obtained in three independent experiments.

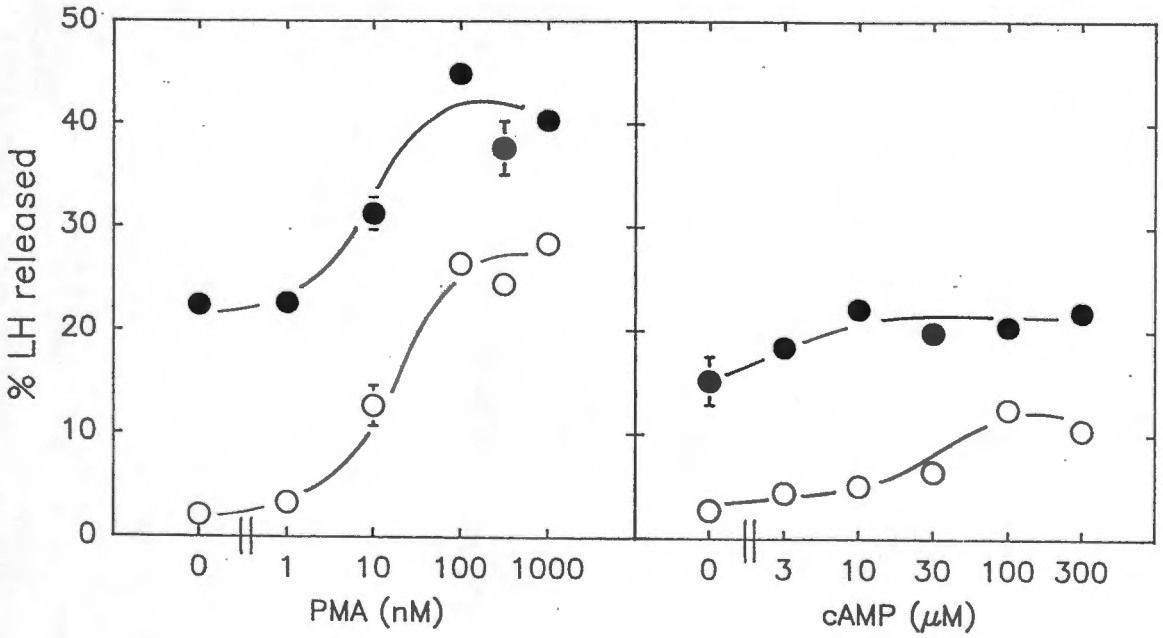

Figure 35. Time-course of inhibition of cAMP/PMA-stimulated LH exocytosis by GTPγS

Permeabilized cells were equilibrated at 0°C for 30 min in stimulation buffer containing 10 mM CaEGTA (pCa 7) with (closed) or without (open) 3 μM cAMP plus 10 nM PMA together with the following guanine nucleotides: none (○,●), 3 μM GMPPNP (□,■), or 3 μM GTPγS (△,▲). LH exocytosis was initiated by replacing with identical stimulation buffer at 37°C which was replaced at 5 min intervals. LH release at each time point represents the rate of LH released in the preceding 5 min. The t = 0 point represents the rate of LH released per 5 min during the 30 min equilibration period. Similar results were obtained in three independent experiments.

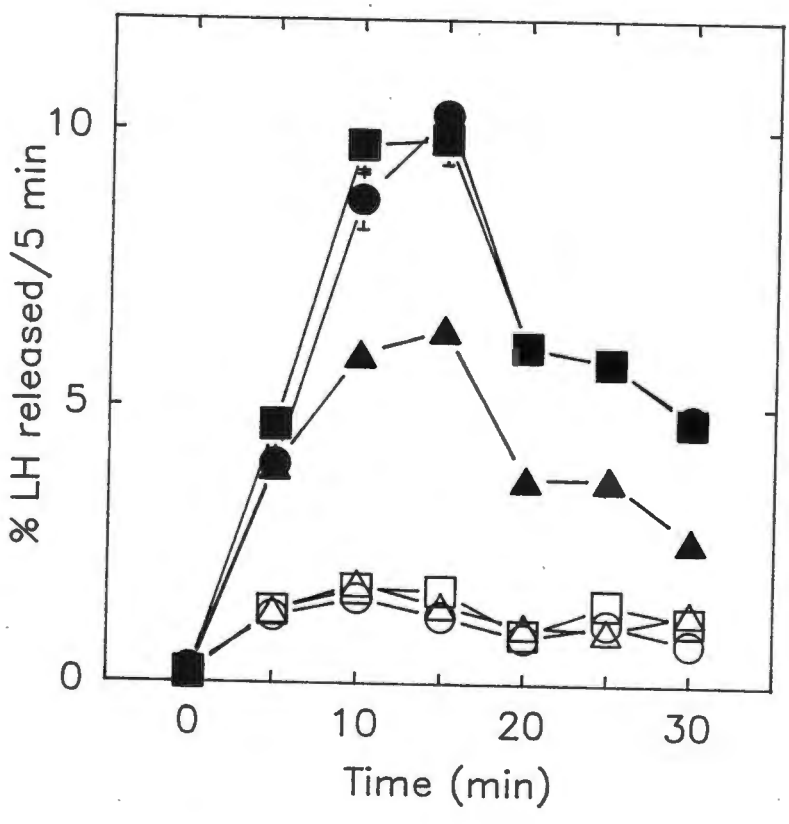

Figure 36. Inhibitory effects of GTPγS on Ca^{2+}-stimulated LH exocytosis

An outline of the protocol used in all experiments examining GTPγS-inhibitory effects is shown. Permeabilized cells were equilibrated for 30 min at 0°C in buffer IC with 2 mM $MgCl_2$, 10 mM CaEGTA (pCa 7) and the indicated concentration of GTP (♦), GTPγS (*O*,●), or GMPPNP (▲). Cells were then warmed to 37°C and pre-incubated for 30 min in the same medium. This medium was removed and replaced with stimulation buffer [which contains MgATP (see Section 2.3)] with 30 mM CaEGTA at pCa 5 (closed) or pCa 7 (open) and LH released after 30 min was determined. To allow the comparison of results from two experiments LH release is presented as percent of LH release evoked by pCa 5 in the absence of guanine nucleotides. Similar results were obtained in three independent experiments.

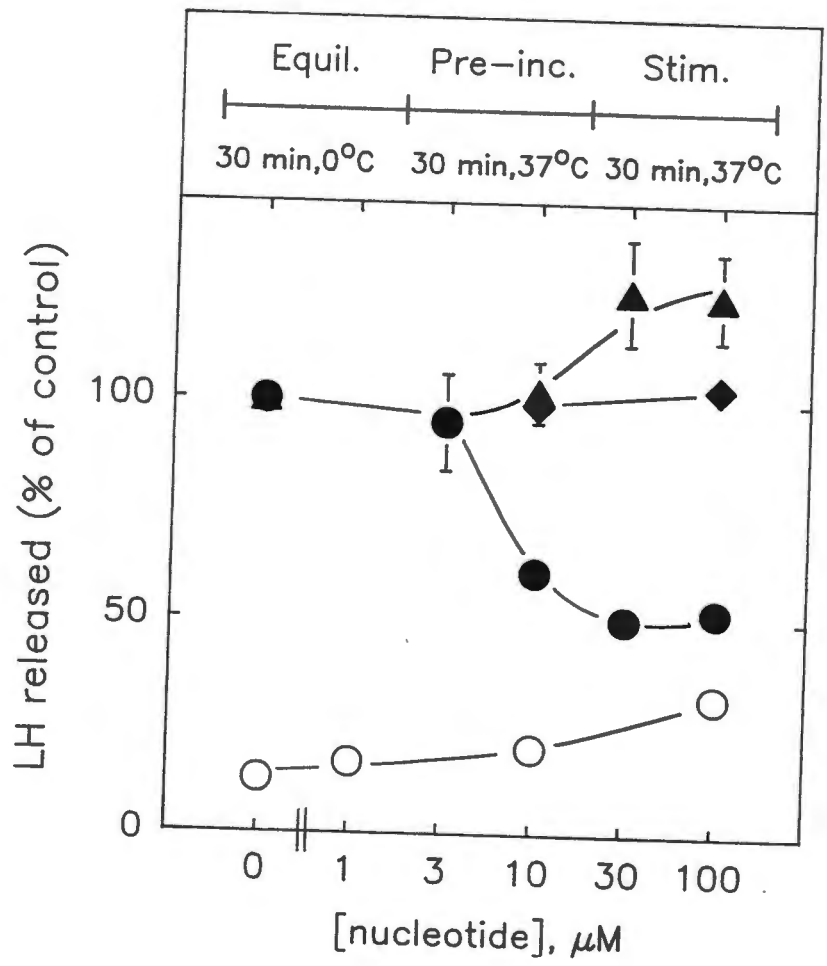

Figure 37. Inhibitory effects of GTPγS on cAMP- and PMA-stimulated LH exocytosis

Permeabilized cells were equilibrated for 30 min at 0°C in buffer IC with 2 mM $MgCl_2$, 10 mM CaEGTA (pCa 7) and the indicated concentration of GTPγS. Cells were then warmed to 37°C and preincubated for 30 min in the same medium. This medium was removed and replaced with stimulation buffer containing 10 mM CaEGTA (pCa 7) with no addition (●) or with cAMP (300 µM) (▲), PMA (100 nM) (◆), or cAMP(300 µM) plus PMA (100 nM) (▼) and LH released after 30 min was determined. Similar results were obtained in three independent experiments.

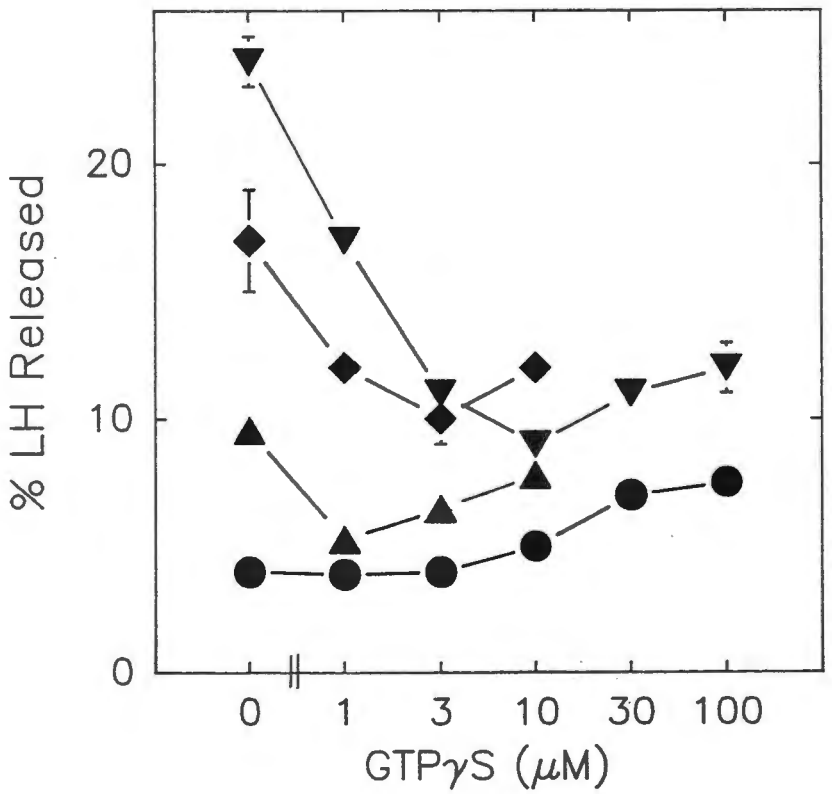

Figure 38. Antagonism of GTPγS-inhibition by GTP, GMPPNP, and GDP

Permeabilized cells were equilibrated for 30 min at 0°C in buffer IC containing 2 mM $MgCl_2$ and 10 mM CaEGTA (pCa 7) without (open and closed bars) or with (hatched bars) 30 µM GTPγS, together with the indicated guanine nucleotides. Cells were then warmed to 37°C and pre-incubated for 30 min in the same medium. This medium was removed and replaced with stimulation buffer containing 30 mM CaEGTA at pCa 7 (open bars) or pCa 5 (closed and hatched bars) and LH released after 30 min was determined. Similar results were obtained in two independent experiments.

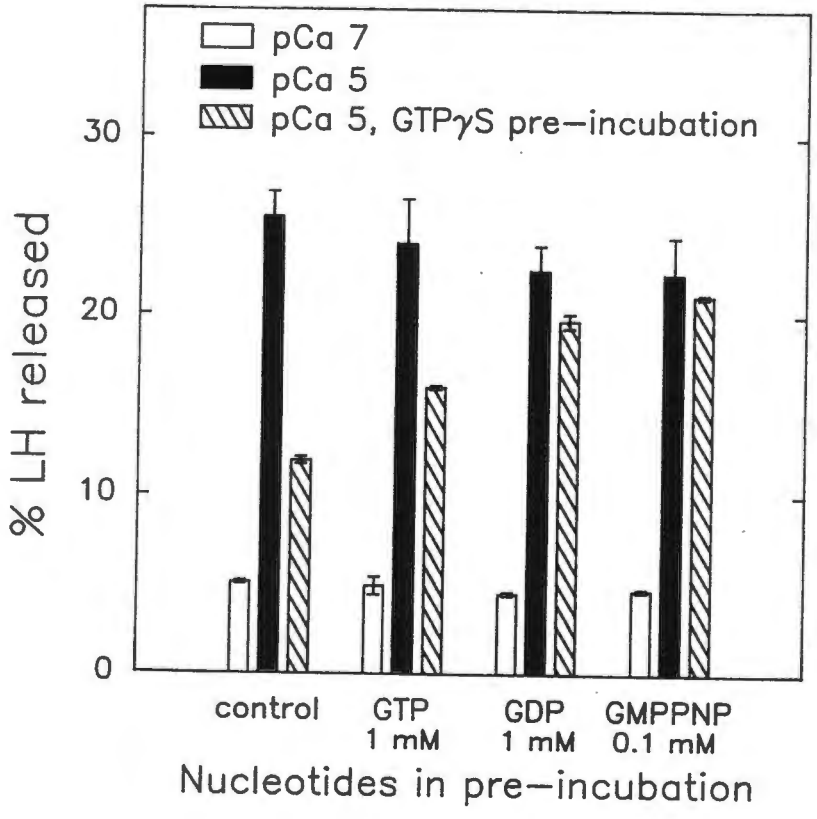

Figure 39. Inhibitory effects of GTPγS and ATPγS on Ca^{2+}-stimulated LH exocytosis

Permeabilized cells were equilibrated for 30 min at 0°C in buffer IC with 2 mM $MgCl_2$, 10 mM CaEGTA (pCa 7) and the indicated concentration of GTPγS (○,●) or ATPγS (△,▲) with (●,▲) or without (○,△) 2 mM MgUDP. Cells were then warmed to 37°C and preincubated for 30 min in the same medium. This medium was removed and replaced with stimulation buffer containing 30 mM CaEGTA (pCa 5) and the LH released after 30 min was determined. Similar results were obtained in three independent experiments.

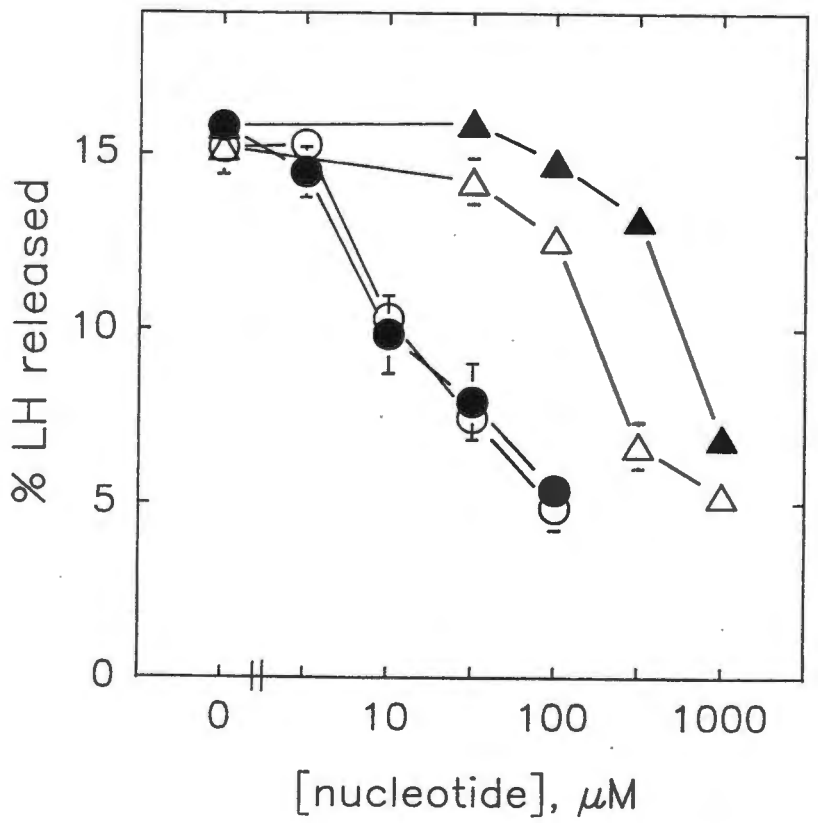

Figure 40. Inhibitory effects of GTPγS and ATPγS on cAMP/PMA-stimulated LH exocytosis

Permeabilized cells were equilibrated for 30 min at 0°C in buffer IC with 2 mM $MgCl_2$, 10 mM CaEGTA (pCa 7) and the indicated concentration of GTPγS (○,●) or ATPγS (△,▲) with (●,▲) or without (○,△) 2 mM MgUDP. Cells were then warmed to 37°C and preincubated for 30 min in the same medium. This medium was removed and replaced with stimulation buffer containing 10 mM CaEGTA (pCa 7), cAMP (30 μM), and PMA (100 nM) and the LH released after 30 min was determined. Similar results were obtained in two independent experiments.

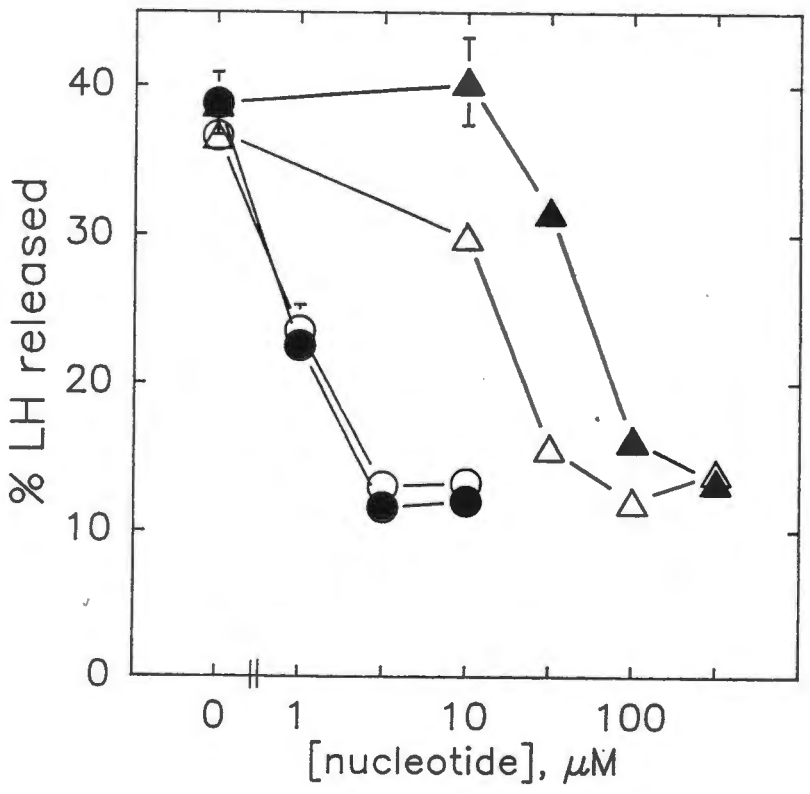

Figure 41. A hypothetical model depicting the dual stimulatory and inhibitory effects of GTPγS (GTPS).

The stimulatory effects result from activation of one or more heterotrimeric signal transducing G protein(s) (G_s, G_p, $G_?$) coupled to cell-surface receptors (R), leading to generation of cAMP, DAG, and possibly other second messengers. The inhibitory effects result from binding to one or more unidentified GTP-binding protein(s) involved in exocytosis at a site distal to the generation of second messengers.