APPROVED:

[redacted]

.....
(Professor in Charge of Thesis)

[redacted]

.....
(Chairperson, Graduate Council)

THE REGULATION OF VOLTAGE-DEPENDENT CALCIUM CURRENTS AND THEIR ROLE IN MODULATION OF STIMULUS-SECRETION COUPLING IN PITUITARY

by

JULIANNE J. STACK

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ABBREVIATIONS

g	gram
h	hour
Hz	hertz
kd	kilodalton
kg	kilogram
kHz	kilohertz
L	liter
M	molar
mCi	millicurrie
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
mmol	millimole
ms	millisecond
mV	millivolt
μM	micrometer
μM	micromolar
μl	microliter
ng	nanogram
nm	nanometer
nM	nanomolar
pA	picoamp
pg	picogram
pS	picosiemens
RMS	root mean square
rpm	revolutions per minute
s	second
vol	volume
wt	weight

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ABSTRACT

Stimulated hormone secretion is a calcium-dependent exocytotic process. Secretagogues induce an elevation of the intracellular ionized calcium concentration to support an increase in the release of hormones above the basal secretory rate. In cell types that are dependent on extracellular calcium to support stimulated secretion, activation of voltage-dependent calcium channels has been implicated as the mechanism by which intracellular calcium may be elevated. Conversely, agents that inhibit stimulated secretion have been postulated to reduce intracellular calcium levels by inhibition of voltage-dependent calcium channels. Mechanisms involved in the modulation of the stimulus-secretion coupling response in proopiomelanocortin (POMC) producing pituitary cells were studied using both electrophysiological and secretion techniques. Cells studied were the rat melanotroph, the major cell type of the intermediate pituitary and the AtT-20 cell, a cell line derived from a mouse anterior lobe corticotroph tumor. The main aim of this project was to determine whether a direct relationship exists between the modulation of electrical activity and the regulation of secretory activity by investigating both voltage-dependent calcium currents and the secretion response. Calcium currents were recorded using the whole-cell variant of the patch-clamp technique and β-endorphin secretion was measured by radioimmunoassay. The concentrations of agents that modulated the calcium currents and the β-endorphin secretion were compared. The effects of secretagogues (BAY K 8644, isoproterenol and forskolin) and inhibitory agents (nifedipine, cadmium, nickel, dopamine and somatostatin) were examined. A direct agreement of the drug concentrations that inhibited secretion and reduced voltage-dependent calcium currents was generally not found. These results indicate that mechanisms other than direct inhibition of the voltage-dependent calcium channels may be responsible for the interruption in stimulus-secretion coupling by inhibitory agonists in endocrine cells.

In AtT-20 cells, biochemical analysis of the secreted POMC products collected during basal, stimulated and inhibited conditions was also performed. The majority of peptides released during stimulated release was the fully processed, bioactive, POMC peptide β-endorphin(1-31). β-endorphin(1-31) release was specifically reduced when basal secretion was inhibited by removal of extracellular calcium or application of somatostatin, whereas the amount of the larger forms of endorphin-containing proteins was not changed. These results also provide evidence that basal secretion is a heterogenous process that involves a calcium-dependent and a calcium-independent pathway and that inhibition of basal secretion by somatostatin specifically reduces the release of the calcium-dependent releasable pool of β-endorphin(1-31).

CHAPTER ONE: STIMULUS-SECRETION COUPLING

1.1. WHAT IS STIMULUS-SECRETION COUPLING

The phrase "stimulus-secretion coupling" was coined in the 1960's by Douglas and Rubin (1963) to describe the cascade of intracellular events that are initiated by the activation of a plasma membrane receptor and culminate with the exocytosis of hormones and neurotransmitters. Their early work demonstrated that activation of the muscarinic receptor by acetylcholine stimulated catecholamine secretion from chromaffin cells in a calcium-dependent manner (Douglas & Rubin, 1961, 1963) and led to the proposal of the calcium hypothesis for stimulus-secretion coupling (Douglas, 1968).

Since then, many investigators have studied the mechanism of stimulus-secretion coupling in a variety of cell systems including neuronal, endocrine and exocrine cells. In endocrine systems, with the exception of the parathyroid gland (Habenener, Rosenblatt & Potts, 1984), calcium plays a supportive role in the stimulus-secretion process. Substances that stimulate secretion also raise intracellular calcium levels while substances that suppress increases in intracellular calcium inhibit secretion. In electrically excitable cell types that require extracellular calcium to support stimulated secretion, voltage-dependent calcium channels have been found and have been implicated as the mechanism by which intracellular calcium may be elevated (Hagiwara & Ohmori, 1982). It has been postulated that the magnitude of increase in the intracellular calcium regulates the amount of hormone or neurotransmitter secreted (Douglas, 1968).

However, the calcium hypothesis has not provided a satisfactory explanation in many investigations and has led to the proposal of the calcium-voltage hypothesis in which the depolarization of the presynaptic terminals or hormone-secreting cells has two functions (Parnas, Parnas & Dudel, 1986). First, the depolarization leads to an increase in calcium conductance, allowing for calcium entry. Second, a conformational change occurs in a membrane molecule that renders it sensitive to calcium and the binding of calcium to this active form triggers release of the neurotransmitter or hormone. When the membrane is repolarized, the molecule is inactivated and release is terminated, regardless of the local calcium concentration (Parnas, Parnas & Dudel, 1986; Hochner, Parnas & Parnas, 1989)

Neither of these two hypotheses really describes completely the stimulus-secretion response. The increase in the intracellular calcium concentration is just the first step in the intracellular cascade of events that facilitate the stimulus-secretion response. With the activation of the plasma receptor, many intracellular systems may be turned on including the activation of G-proteins [guanosine triphosphate (GTP) binding proteins] that are coupled to the production of second messengers, regulation of phosphodiesterases, kinases, phosphorylases, and the modulation of ion channels (Casey & Gilman, 1988; Dunlap, Holz & Rane, 1987). These systems may have many sites of action including the phosphorylation or dephosphorylation of proteins involved in the structural secretory apparatus (De Lisle & Williams, 1986). The final event of this cascade is the fusion of the secretory vesicle with the plasma membrane and release of the hormone or neurotransmitter. Although triggering of these intracellular systems can be induced by the

activation of a plasma membrane receptor, their roles in the coordination of the acute secretory process are still not understood.

In order for vesicle fusion to occur, two of the three forces (van der Waals, coulombic and hydration), which are involved in the interaction of membranes, must be overcome. The van der Waals force is an attractive force but the coulombic (electrostatic) force, which is usually repulsive, and the hydration force, which prevents spontaneous fusion of membranes (Stegmann, Doms & Helenius, 1989), prevent vesicle fusion. The hydration force is due to a layer of water molecules bound to the polar lipid head groups of the bilayer and causes repulsion at small membrane separations, being effective at a distance of 2 nm or less. For fusion to proceed, the repulsive hydration force must be overcome and molecular contact between the membranes must be established.

Calcium has been shown to play an important role in this process. In a cell-free system calcium facilitates fusion of membranes by forming salts with the negatively charged phosphatidyl serine molecules. This leads to the removal of water between bilayers and direct molecular contacts between lipid molecules of the two membranes causing lipid packing deformations and restructuring (Stegmann, Doms & Helenius, 1989). For the fusion event to occur in a cell, however, the reaction may be more complex than just removing the water between bilayers. It may depend on multiple factors including membrane components, cytosolic proteins and low-molecular weight effector molecules such as adenosine triphosphate (ATP) and calcium, and availability of vesicles (De Lisle & Williams, 1986). This suggests that other processes may augment calcium in the sequence of events involved in vesicle fusion.

1.2 MECHANISMS INVOLVED IN REGULATION OF CALCIUM INFLUX

Calcium may move across the plasma membrane through channels or via transporter or exchange mechanisms. With the discovery that many types of endocrine cells exhibit spontaneous electrical activity, it has been hypothesized that this activity has an important role in stimulus-secretion coupling (Douglas, 1968). The spontaneous electrical activity is due to the coordination and interaction of several ion conductances including sodium, potassium, chloride and calcium (Hille, 1984). Determining how activation of plasma membrane receptors may regulate this activity should provide clues in the understanding of the regulation of secretion.

The development of patch-clamp recording technique (Hamill et al. 1981), which allows for high resolution of single channel activity within a circumscribed piece of membrane, has led to the intense study of calcium channels in the past 5 years. In dorsal root ganglion (DRG) neurons, calcium channels have been classified into three groups, "T", "N", and "L", and have been distinguished by their kinetics, voltage dependence, conductance and pharmacological properties (summarized in Table 1.1; Fox, Nowycky & Tsien, 1987, Tsien et al. 1987).

The physiological significance and function of multiple calcium channels is not known. Studies that determine the distribution of calcium channels and their specific regulation may provide insight into their functional significance. For example, in cultured sympathetic neurons it has been shown that "N" calcium channels are responsible for the influx of calcium that supported release of norepinephrine (Hirning et al. 1988). Blockade of "L" calcium channels only inhibited secretion 20 % whereas blockade of the "N" channels inhibited secretion 80 %.

Table 1.1 Properties of Voltage-Dependent Calcium Channels

Characteristic	L	N	T
Activation Threshold	-10 mV	-30 mV	-70 mV
Inactivation Rate (tau, 0 mV)	> 1 s	50-80 ms	20-50 ms
Single Channel Conductance	25 pS	13 pS	8 pS
Ba/Ca Selectivity	Ba>Ca	Ba>Ca	Ba=Ca
Cd Block (50 μM)	90%	90%	50%
Ni Block (100 μM)	No	No	Yes
Dihydropyridine Sensitivity Nifedipine Block (10 μM)	Yes 60 %	No	No

(after Fox et al . 1987)

However, enhancement of "L" channel activity with the agonist Bay K 8644 stimulated secretion. One explanation for these perplexing results may involve the spatial distribution of the channel types rather than attributing a specific function to each type of channel as suggested. For instance, the "L" channels may be located primarily at the soma, and activation of these channels may facilitate the propagation of an action potential to the terminal where "N" channels are located. Blockade of the "N" channels would inhibit secretion even in the presence of an action potential since influx of calcium at the terminal is necessary for secretion, whereas blockade of the "L" channels would not inhibit secretion as long as an action potential could still be generated and propagated to the nerve terminal.

The mechanisms by which calcium currents can be modulated may be divided into two categories, direct or indirect. Direct modulation of calcium channels may involve G-protein subunits, second messengers, and/or chemical modifications such as phosphorylation of calcium channel proteins. Indirect modulation of calcium channels may be achieved by altering the membrane potential. This mechanism involves the regulation of other ion conductances such as potassium.

Whole-cell patch-clamp recording allows for reliable voltage-clamp in small cells and measurement of whole-cell currents (Hamill et al. 1981). This configuration offers unique advantages for the investigation of currents that are not possible by intracellular and extracellular recording. The major advantage to this method is that diffusional exchange of the intracellular solution with the pipette solution allows control over, and quantitative manipulation of, the intracellular environment. This control over intracellular ionic concentrations

permits specific currents, such as calcium, potassium and sodium, to be recorded in isolation from one another. This method can be used to study the regulation of ionic currents by agents that stimulate or inhibit secretion and should provide useful information concerning the mechanisms involved in the regulation of intracellular calcium concentrations. Using these techniques, components of the intracellular mechanisms involved in the modulation of ionic currents also may be studied.

1.3 RATIONALE FOR EXPERIMENTS

It was the aim of this study to determine whether direct modulation of calcium currents could account for the modulation of secretion. Previous investigators of stimulus-secretion coupling have isolated a mechanism of interest and studied in isolation the regulation of that mechanism without regard to the regulation of secretion. Only a few studies have attempted to determine whether a direct concentration-dependent relationship exists between the modulation of the mechanism with the modulation of the secretory response (Munemura, Eskay & Kebabian, 1980; Heisler, 1986). In melanotrophs, secretion of hormone and accumulation of adenosine-3',5'-monophosphate (cAMP) were measured in response to isoproterenol and it was shown that the EC_{50} for the secretory response was 75-fold less than for the cAMP response (Munemura, Eskay & Kebabian, 1980). These results suggest that submaximal stimulation of the formation of cAMP elicits a maximal physiological response.

A two pronged approach was utilized in this study to understand the relationship between the bioelectric activity and the secretory activity of the cell. Parallel experiments were performed that measured the

concentration-dependence of agents that modulated secretion and modulated voltage-dependent calcium currents; results from such studies allow conclusions to be drawn regarding the regulation of voltage-dependent calcium currents and their role in the stimulus-secretion coupling mechanism.

The studies presented here utilized the whole-cell patch-clamp recording configuration on two types of pituitary cells. The cell preparations studied were AtT-20 cells, a mouse anterior pituitary corticotroph tumor cell line, and melanotrophs, the primary cell type of the intermediate lobe of the rat pituitary gland. These cells are similar in that they both synthesize and secrete peptides derived from the prohormone, proopiomelanocortin (POMC). This was especially useful for the secretion experiments where an immunoassay was designed and employed to measure β-endorphin levels in both cell types. Secondly, they are both homogeneous populations of cells which was a requisite for the electrophysiological studies. Conversely, these cells do differ in several ways including their plasma membrane receptors, post-translational processing of POMC, secreted peptides and growth rate in culture. Therefore, these studies also provides a comparison of two endocrine cell types and of a tumor cell to a normal cell.

The following chapter describes in more detail what is known to date about the secretory and electrical activities of the AtT-20 cells. Section 3.1 considers these physiological activities of the melanotroph.

CHAPTER TWO: MODULATION OF THE VOLTAGE-DEPENDENT CALCIUM CURRENT AND STIMULUS-SECRETION COUPLING IN ATT-20 CELLS

2.1 LITERATURE SURVEY

2.1.1 INTRODUCTION

AtT-20 cells were derived from a radiation-induced murine anterior pituitary tumor (Yasumura, 1968). Biochemical studies performed on these cells in the late 1970's by Mains and Eipper provided evidence against the popular hypothesis of the time, "one gene, one protein". It was in these cells that it was discovered that a common precursor hormone contained the amino acid sequences for several hormones. This prohormone is referred to as proopiomelanocortin (POMC) and is post-translationally processed into at least two biologically active peptide hormones, adrenocorticotropin (ACTH) and β-endorphin (Mains, Eipper & Ling, 1977). The post-translational processing and regulation of secretion of POMC-derived peptides from these cells is similar to that which occurs in corticotrophs of the anterior pituitary lobe (Mains & Eipper, 1976). This indicates that although this is a tumor cell line, it may be a valid model to study post-translational protein processing and stimulus-secretion coupling mechanisms in endocrine cells.

Intracellular protein sorting and processing has been studied using immunocytochemical techniques coupled with electron microscopy; this has shown that POMC is synthesized at the endoplasmic reticulum and is shuttled through the Golgi as an intact protein. The first site at which processed POMC products are detected is the trans-most cisterna on the exit side of Golgi (Schnabel, Mains & Farquhar, 1989). These products

are then packaged into secretion granules (Tooze & Tooze, 1986) which fuse with the membrane in a regulated manner (Gumbiner & Kelly, 1982; Burgess & Kelly, 1987).

The AtT-20 cells have a very high basal rate of secretion, approximately 15 % of the cellular hormone content is secreted per hour (Mains & Eipper, 1981a). By comparison, basal secretion from primary rat anterior pituitary cells is approximately 0.4 % of cellular hormone content per hour (Eipper & Mains, 1980b). Even with such a high rate of basal secretion, the secretory rate of these cells may be stimulated or inhibited by a variety of agents.

The literature to date abounds with reports regarding how numerous agents affect hormone secretion, second messenger systems, calcium flux, and intracellular calcium levels in AtT-20 cells. On the other hand, there are only a few reports on the modulation of the electrophysiological properties of these cells by secretagogues. The coordination of various biochemical and electrophysiological changes induced by the secretagogues and how they are related to the physiological response of secretion is not understood.

2.1.2 WHAT IS KNOWN ABOUT SECRETION

ACTH and β-endorphin secretion from AtT-20 cells may be stimulated by activation of plasma membrane receptors. Corticotropin releasing factor (CRF) (Hook et al. 1982), isoproterenol (acting on β-adrenoceptors), and vasoactive intestinal peptide (VIP) (Westendorf, Phillips & Schonbrunn, 1983) have been shown to stimulate secretion as well as activate adenylate cyclase that produces cAMP (Reisine et al. 1982).

Forskolin, which directly activates cAMP, (Reisine et al. 1982), cAMP analogs (Reisine, 1985), and phorbol ester analogs that stimulate protein kinase C (PKC) (Phillips & Tashjian, 1982; Heisler, 1984), also stimulated hormone release from AtT-20 cells. However, a plasma receptor has yet to be reported to be coupled to the PKC pathway in AtT-20 cells. The loss of this type of a functional receptor (whose analogous counterpart receptor would be the vasopressin receptor expressed on anterior pituitary corticotrophs) may be due to a mutation during the formation of this cell line.

These second messenger systems which eventually converge at some intracellular site to stimulate secretion have been shown to be coupled to the phosphorylation of two different sets of proteins in the cytosolic, particulate and nuclear fractions (Rougon, Barbet & Reisine, 1989). However, three proteins were phosphorylated in common by the cAMP and the PKC pathway, a 40 kd cytosolic protein and two membrane proteins that were 60 kd and 32 kd (Rougon, Barbet & Reisine, 1989). The common phosphorylated proteins activated by forskolin and phorbol ester treatment may be sites of convergence for the action of cAMP and PKC; however, further identification and functional characterization of these proteins is required to determine whether the phosphorylation of these proteins is an integral part of the cascade of events involved in the stimulus-secretion coupling response.

Enhancement of calcium channel activity that allows an increase in the inward flux of calcium also stimulated secretion. Depolarization of the membrane with elevated concentrations of potassium stimulated secretion in a concentration dependent manner (Guild et al. 1986). BAY K 8644, the dihydropyridine calcium channel agonist, stimulated secretion

without altering cAMP accumulation in the cells (Heisler, 1985). Concentrations above 1 μM, however, inhibited stimulated secretion. BAY K 8644 is a racemic mixture of molecules, where the S-enantiomer is the agonist form. At high concentrations the R-enantiomer may act as an antagonist (Triggle & Rampe, 1989). The calcium ionophore, A-23187, also augmented ACTH release in a cAMP-independent manner (Reisine et al. 1982).

Verapamil, an organic calcium channel antagonist, inhibited the stimulation of secretion by activation of plasma receptors coupled to the cAMP system (CRF, isoproterenol, and forskolin). However the facilitation of cAMP synthesis by these agonists was not reduced in the presence of nifedipine (Reisine et al. 1982) or the removal of extracellular calcium (Reisine, 1985) suggesting that calcium entry through voltage-dependent calcium channels is mandatory for enhancing the secretory rate, but is not necessary for cyclic nucleotide production. In experiments where the Walsh inhibitor of cAMP-dependent protein kinase (PKI) was introduced into intact AtT-20 cells, agonists that stimulated secretion in a cAMP-dependent manner such as CRF, isoproterenol and forskolin, were rendered ineffective. Whereas, high external potassium and phorbol esters, which stimulate secretion without altering cAMP levels, remained effective stimulators in the presence of PKI (Reisine et al. 1985; Reisine, Rougon & Barbet, 1986). These results indicate that elevated cAMP levels are not sufficient for enhancing secretory activity, whereas extracellular calcium is necessary for secretion. However, the relationship between increases in intracellular cAMP and calcium entry are still not understood.

The hypothesis that the mechanisms involved in cAMP-dependent secretion are different than the mechanisms involved in cAMP-independent secretion is further supported by studies of the time course of the secretory response. The time course of secretion stimulated by a high potassium solution (cAMP-independent secretion) differed from the time course of forskolin (cAMP-dependent) stimulated secretion (Guild et al. 1986). High potassium produced a rapid, large burst of secretion in the first 2 min and then secretion fell to basal levels, whereas secretion stimulated by forskolin peaked at 4-5 min and remained elevated above basal secretion for the entire 10 min test period. When the two agents were applied together, the amount of secretion was additive indicating there may be more than one mechanism involved in stimulating secretion. Due to fact that forskolin did enhance potassium stimulated secretion, it is unlikely that the rapid return of the secretion rate to the basal level in the presence of high potassium alone was due to a depletion of releasable stores of hormone. This also suggests that the change in the secretory rate induced by high potassium must be regulated by another mechanism and may be explained by the time course of the rise in intracellular calcium in response to high potassium (see section 2.1.3). However, the time course of the effect of forskolin or forskolin plus high potassium on an intracellular calcium response has not been reported.

2.1.2(i) INHIBITION OF SECRETION BY SOMATOSTATIN

Secretion may be inhibited by agonists, such as somatostatin [Somatostatin (1-14); SRIF], acting at membrane receptors. Studies have characterized the SRIF binding site as a single class of receptors on

AtT-20 cells (Richardson & Schonbrunn, 1981). SRIF (100 nM) was reported to produce a maximum inhibition cAMP-dependent (Reisine, 1985), cAMP-independent stimulated secretion (Richardson & Schonbrunn, 1981; Heisler, 1985; Reisine, 1985) and basal secretion (Maruyama & Ishikawa, 1977; Pennefather, Heisler & MacDonald, 1988). In all of these cases however, secretion was never completely abolished. SRIF (100 nM) also completely blocked the increase in cAMP induced by forskolin (Reisine, 1985) or CRF (Reisine, 1984; Litvin et al. 1986); however, SRIF did not alter basal levels of cAMP (Srikant & Heisler, 1985; Litvin et al. 1986).

Pretreatment of cells with SRIF desensitized the AtT-20 cells to subsequent SRIF inhibition of stimulated hormone release, cAMP formation and calcium influx (Reisine, 1984, 1985; Reisine et al. 1988) in a concentration- and time-dependent manner. This desensitization was reversible.

When somatostatin is added at the same time as CRF or high potassium, the secretion of ACTH is reduced. The concentrations of SRIF that are effective in reducing secretion are identical for the inhibition of cAMP-dependent and cAMP-independent stimulated secretion. However, increased levels of extracellular calcium can overcome the SRIF inhibitory effects when secretion is stimulated by high potassium but not by forskolin or 8-bromo-cAMP (Reisine, 1985). This evidence suggests that SRIF must have act at multiple sites to inhibit secretion, one site may be involved in the inhibition of calcium influx and another site may involve the reduction of cAMP production.

In recent years, the integral role of G-proteins in intracellular transduction of receptor signals has been recognized (Johnson & Dhanasekaran, 1989). G-protein subunits from AtT-20 cells have been

identified using immunoblot analysis of two-dimensional gels; these experiments showed that G_s, G_i and G_o are present in these cells (Mahy et al. 1988b). Northern blot analysis showed that mRNA is present for many subtypes of G-protein subunits including α_s, $\alpha_{i-1,2,3}$ subtypes, and $\beta_{1,2}$ subtypes. The most abundant mRNA forms are α_s, α_{i-2}, β_1 and β_2 (Thiele & Eipper, 1990). Reisine presented preliminary evidence that the α_{i-2} subunit is specifically coupled to the SRIF receptor (personal communication).

Pertussis toxin is known to ADP-ribosylate the α_i and α_o subunits and renders them inactive. When AtT-20 cells were pretreated with pertussis toxin, SRIF inhibition of hormone secretion was blocked (Luini & De Matteis, 1990) indicating that inhibition of hormone secretion by SRIF involves a G-protein. Mahy et al. (1988a) found that while pertussis toxin pretreatment abolished SRIF inhibition of forskolin-stimulated adenylate cyclase activity, introduction of purified G_i (when activated reduces adenylate cyclase activity) stored in deoxycholate (a detergent) into the cells lowered the level of forskolin-stimulated adenylate cyclase activity but did not reconstitute the inhibitory action of SRIF. The ability of G_o, the other major type of pertussis-sensitive G-protein, was not examined in this study; however, studies in other tissues have shown that a combination of G_i and G_o will reconstitute the function abolished by pertussis toxin treatment such as the inhibition of voltage-dependent calcium currents by the activation of opiate receptors (Hescheler et al. 1987).

Recently, data have been reported that show secretion can be stimulated in a calcium-dependent (10 μM calcium) and a calcium-independent manner in permeabilized cells (Luini & De Matteis, 1990).

In this cell preparation, artificial pores are formed in the plasma membrane either by detergent treatment or electrolytic lesions. The intracellular solution can be altered simply by changing the extracellular solution; hormone secretion may thus be regulated either by agonists binding to plasma receptors from the outside the cell or by small molecules that diffuse into the cell to act from within. In these studies (Luini & De Matteis, 1990) calcium (10 μM) in the extracellular solution stimulated ACTH release, however, this concentration is higher than that observed by indirect measurements when a secretagogue is used to increase intracellular calcium (see Adler et al. 1989). Guanosine-5'-O-(3-thiotriphosphate) (GTPγS), a non-hydrolyzable form of GTP, stimulated ACTH release in the absence of calcium. SRIF (10 nM) did not inhibit GTPγS or calcium-stimulated release, but when calcium and GTPγS were applied together SRIF was able to reduce ACTH secretion. These results imply that perhaps several G-proteins exist in the stimulus-secretion response, and that activation of a G-protein distal to calcium entry may be involved in the regulation of the exocytotic events of secretion.

Several other agents have been reported to reduce secretion; these include glucocorticoids (Phillips & Tashjian, 1982; Westendorf, Phillips & Schonbrunn, 1983), muscarinic receptor agonists (Heisler, Larose & Morisset, 1983), the peripheral-type benzodiazepine receptor ligand, Ro 5-4864 (Bisserbe, Patel & Eskay, 1986) and disulfonic stilbene derivatives, which have been shown to be inhibitors of a calcium-activated chloride conductance (Heisler & Jeandel, 1989).

2.1.3 WHAT IS KNOWN ABOUT INTRACELLULAR CALCIUM

The regulation of the intracellular calcium concentration is very complex. For instance, an increase in intracellular calcium can be due to an increased calcium flux from the extracellular space or from intracellular stores, or a decreased calcium efflux due to a decreased buffering capacity of the intracellular milieu or decreased pumping activity out of the cell or into the intracellular stores. Calcium influx experiments using $^{45}Ca^{2+}$ can determine whether extracellular calcium is the source of calcium entry, and the kinetics of this movement, but do not quantitate the intracellular levels of calcium. Intracellular calcium concentrations can be measured using calcium-sensitive fluorescent indicators such as Fura-2 or Quin-2. However, these are measurements of the average concentration of calcium in the cell and do not represent the local concentration of calcium at the release site. Isolation of mechanisms such as calcium currents must be studied directly, to understand how the regulation of the mechanism contributes to the regulation of intracellular calcium.

$^{45}Ca^{2+}$ uptake experiments have shown that depolarization with 50 mM K^+ will enhance $^{45}Ca^{2+}$ uptake and that this is blocked by nimodipine (10 nM); this indicates the involvement of voltage-dependent calcium currents. CRF (5 nM) did not alter $^{45}Ca^{2+}$ uptake but did stimulate release to 262 % of control; this stimulation of release could be antagonized by EGTA, cobalt, verapamil, and nimodipine, all of which are thought to inhibit calcium influx (Richardson, 1986). However, Quin-2 experiments, a fluorescent calcium buffer technique used to measure calcium concentrations, showed that CRF (100 nM) did increase intracellular calcium levels 50-60 % (Reisine, Wang & Guild, 1988). Experiments using

lower concentrations of CRF were not reported. Therefore, not enough data are available to determine whether the same concentrations of CRF both stimulated hormone release and increased intracellular calcium concentrations.

The concentration of free cytosolic calcium has been determined to be 115 nM in the basal state (Guild et al. 1986). Forskolin (10 μM) elevated intracellular calcium 30 % and enhanced secretion 3-fold, whereas 100 mM K^+ increased intracellular calcium 120 % and stimulated secretion 6-fold (Guild et al. 1986). Further, forskolin augmented potassium stimulated release but, surprisingly, reduced intracellular calcium levels (Guild et al. 1986). This study suggests that a greater increase in intracellular calcium is required to support stimulated secretion by potassium than is secretion stimulated through a cAMP-dependent mechanism, although it does not report complete concentration curves for both forskolin and high potassium on the secretion and the intracellular calcium response.

The time course of the elevation of intracellular calcium varied with each type of secretagogue. CRF (100 nM, a concentration that maximally stimulated secretion) increased intracellular calcium in 30 s and reached a maximum increase of 50-60 % in 2 min, which remained constant thereafter at the elevated level (Reisine, Wang & Guild, 1988). This concentration of CRF stimulated secretion by 368 % (Reisine et al. 1982).

SRIF can block cAMP-dependent and cAMP-independent stimulated calcium influx (Reisine, Wang & Guild, 1988). SRIF (100 nM) inhibited the increase in intracellular calcium evoked by CRF; however, this action of SRIF was lost if it was present 4 min prior to adding the CRF, a finding consistent with the desensitization often associated with SRIF action.

The mechanism that underlies this desensitization is not known. Potassium (10 mM) elevated intracellular calcium within 8 s and a maximal level was achieved by 20 s; however, the intracellular calcium concentration returned to basal level within 2-3 min despite the continued presence of the high potassium solution, similar to the time course of the secretion response to high potassium (Guild et al. 1986). Pertussis toxin blocked the SRIF (100 nM) inhibition of an elevation in intracellular calcium invoked by high potassium (Reisine, Wang & Guild, 1988).

BAY K 8644 also stimulated intracellular calcium levels in a concentration-dependent manner; 100 nM BAY K 8644 elevated intracellular calcium within 8 s and was maximal (67 % increase) by 40 s. SRIF reduced the BAY K 8644 response within 10 s in a concentration-dependent manner; this action was blocked by pertussis toxin (Reisine, 1990).

Phorbol ester treatment transiently increased intracellular calcium. This was blocked by pretreatment with 50 mM tetraethylammonium (TEA) or 40 mM cesium, which block potassium channels (Ruby, 1988), suggesting that phorbol ester is facilitating the calcium influx by turning off a potassium conductance and depolarizing the membrane potential into a range that activates calcium channels (Reisine, 1989).

In an early study, which attempted to correlate the SRIF inhibition of the calcium flux and the inhibition of secretion, it was shown that SRIF inhibited both the spontaneous release of ACTH and the influx of $^{45}Ca^{2+}$ in a concentration-dependent manner. However, comparison of the concentration-response curves for SRIF on these two effects shows poor agreement. For example, 200 nM SRIF reduced ACTH secretion 20 % and reduced $^{45}Ca^{2+}$ influx 75 %, 800 nM SRIF reduced ACTH secretion 75 % and

reduced $^{45}Ca^{2+}$ influx 90 %, and 1.6 μM SRIF reduced ACTH secretion 90 % and $^{45}Ca^{2+}$ influx 99 % (Maruyama & Ishikawa, 1977).

In studies that measured the intracellular concentration of calcium and secretion in response to SRIF, SRIF (1 μM) antagonized both the elevation of secretion and the elevation of intracellular calcium evoked by 15 mM potassium. However, when higher concentrations of potassium were used, stimulated secretion was still reduced by SRIF (1 μM) but SRIF did not reduce the elevation of intracellular calcium (Adler et al. 1989). These results suggests that SRIF may also have a site of action distal to calcium entry in the regulation of the stimulus-secretion coupling mechanism.

2.1.4 WHAT IS KNOWN ABOUT THE ELECTROPHYSIOLOGY

AtT-20 cells are electrically excitable cells, have an average resting membrane potential of -50 mV and fire spontaneous action potentials (Surprenant, 1982; Adler et al. 1983). Action potentials are depolarizing, resulting from inward sodium and calcium currents, and are followed by an after-hyperpolarization. The spontaneous activity is voltage-dependent in that depolarization of the membrane increased the firing rate and hyperpolarization of the membrane decreased the firing rate (Adler et al. 1983). Increased extracellular calcium (from 2 mM to 5 mM) increased the frequency and the amplitude of spontaneous voltage spikes whereas reduced extracellular calcium (0.2 mM) abolished this activity; isoproterenol (5 μM) also increased the frequency of the spontaneous action potentials (Surprenant, 1982).

Single channel studies performed in the cell-attached patch configuration found that the most frequently observed voltage-dependent

calcium channel resembled the "L" channel described in chick dorsal root ganglion neurons (Nowycky, 1987). These channels had a conductance of 23 pS (measured in 110 mM barium). They inactivated little during a depolarizing step pulse, BAY K 8644 increased their mean open time and isoproterenol (1 μM) increased the probability of channel opening (Nowycky, 1987). Voltage-dependent calcium currents measured by whole-cell patch-clamp recording were reported to be enhanced 35 % with 8-bromo-cAMP (10 μM) (Luini et al. 1985). To date, this is the only report of a secretagogue shown to increase the amplitude of the voltage-dependent calcium current in AtT-20 cells. These voltage-dependent calcium currents were reduced by [D-Trp^8]somatostatin (1 μM) by 40 % of control in a cAMP-independent manner (Luini et al. 1986) and the mechanism of this inhibition was coupled to the channel in a pertussis toxin sensitive manner (Lewis, Weight & Luini, 1986). However, these studies do not provide any information regarding the concentration range of SRIF that are effective in reducing the calcium current, and yet, this is an important point in determining whether regulation of the calcium current is the primary mechanism by which stimulus-secretion coupling is being regulated. The concentration of [D-Trp^8] SRIF used (1 μM) is 1000-fold greater than that required to inhibit stimulated secretion completely (Reisine, 1985). Indeed, at this concentration marked desensitization of the SRIF inhibition of the elevation of intracellular calcium response by CRF is also present (Reisine, Wang & Guild, 1988).

SRIF also hyperpolarized the membrane (Pennefather, Heisler & MacDonald, 1988; Adler et al. 1989) by increasing a potassium conductance; this conductance increase was blocked by 5 mM cesium (Pennefather, Heisler & MacDonald, 1988). The same authors reported that

5 mM cesium reduced the effect of SRIF on inhibiting basal secretion and BAY K 8644-stimulated secretion.

Other currents have been identified in these cells; these include a calcium-dependent chloride current (Korn & Weight, 1987) and a calcium-dependent potassium current (Wong, Lecar & Adler, 1982). This calcium-dependent potassium channel had a conductance of 124 pS and was reversibly blocked by 20 mM TEA applied to the internal side of an inside-out patch but only reduced 30 % by TEA applied to the external side (Wong & Adler, 1986). This differential sensitivity to TEA is opposite to other preparations studied (Ruby, 1988). The role of these calcium-dependent currents in the stimulus-secretion process has yet to be determined; however, they may have an important role in regulating calcium influx during the spontaneous action potentials by hyperpolarizing the membrane once a sufficient amount of calcium has entered the cell.

2.1.5 SUMMARY

The reported experiments on AtT-20 cells that have been summarized have shown that extracellular calcium is necessary and sufficient to support stimulated secretion; however, even in the presence of high levels of intracellular calcium, SRIF can still reduce the secretion. Experiments on permeabilized cells have shown that G-proteins are able to facilitate secretion distal to calcium entry. It may be postulated that the inhibition of secretion may be due to regulation of several sites, which would include a direct inhibition of calcium influx, a hyperpolarization that moves the membrane potential out of the activation range of the calcium conductance, and sites distal to calcium entry that

have not been identified.

2.2 METHODS

2.2.1 TISSUE CULTURE

All cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, formula # 430-1600, with 44 mM $NaHCO_3$ and 22 mM D-glucose final concentration) supplemented with 10 % heat inactivated horse serum (Gibco) without antibiotics, and maintained in a humidified environment at 37°C under 5 % CO_2 / 95 % O_2.

2.2.1(i) CELL PREPARATION

AtT-20 cells were obtained from a stock cell line (clone #6) at the Vollum Institute tissue culture facility at Oregon Health Sciences University. Cells were grown to confluence, dispersed into single cells with trypsin buffer [section 3.2.1(i)], and collected by low speed centrifugation (1000 rpm). The cells were resuspended in DMEM and the cells were plated for stock line, secretion experiments, or electrophysiology experiments.

For secretion experiments, cells were plated onto Nunc 24 multiwell plates and grown for three days; the medium was changed the day prior to the experiment. For electrophysiological experiments, a drop of the cell suspension was plated onto glass coverslips [see section 2.2.1(ii)] and after a couple of minutes, the entire plate was flood with DMEM. Recordings were performed on cells that had been growing 2-4 days; the medium was changed the day before recording.

2.2.1(ii) GLASS COVERSLIP PREPARATION

Glass coverslips with a 12 mm diameter (Belco) were boiled in 10 % HCl for 30 min, rinsed 10 times with distilled H_2O, autoclaved and stored in H_2O. Coverslips were placed in 35 mm Falcon dishes (4 coverslips/dish), air dried, scored into quarters with a diamond-tipped instrument and placed under ultraviolet light for 20 min.

2.2.2 IMMUNOCYTOCHEMISTRY

AtT-20 cells were stained for β-endorphin immunoactivity. Cells were dispersed and grown as described for electrophysiological recordings on coverslips for 24 h and 48 h. The cells were fixed by washing the cells two times with Tris-saline (0.05 M Tris, 0.9% NaCl, pH 7.5) and incubating for 30 min in 4 % paraformaldehyde (in phosphate buffered saline) followed by 3 rinses with Tris-saline. Coverslips were stored in Falcon multiwell plates covered with Tris-saline at 4°C until the immunocytochemistry was performed.

Coverslips were washed and incubated with 500 μl volumes of solutions in the Falcon multiwell plates. At room temperature coverslips were: 1) washed twice with Tris-saline (5 min each time), 2) incubated for 30 min in 1 % hydrogen peroxide (1 ml of 30 % H_2O_2 into 29 ml Tris-saline), 3) washed for 5 min with Tris-saline, 4) washed in 0.02 % bovine serum albumin (BSA) in Tris-saline (BSA-Tris-saline). The coverslips were incubated at 4°C overnight in a humidified box with the primary β-endorphin specific antibody (identified as Nora) at a 1:1000 dilution made in BSA-Tris-saline. This was followed by a Tris-saline and a BSA-Tris-saline wash, 5 min each. The coverslips were incubated at room temperature for 45 min in BSA-Tris-saline containing biotinylated-Protein

A (Vector Lab, Inc., B-2001) at 1 mg/ml. This was followed by a Tris-saline and a BSA-Tris-saline wash, 5 min each. The coverslips were incubated at room temperature for 1 h with the avidin/biotinylated peroxidase enzyme solution 1:1000 dilution in BSA-Tris-saline (Vectastain ABC Kit, Vector Lab, Inc.). This was followed by two 5 min washes in Tris-saline. The coverslips were treated with freshly prepared diaminobenzidene (DAB) solution. The coverslips were rinsed every 15 min with Tris-saline and checked for color development. DAB solution was prepared by adding 300 μl of glucose oxidase (3 mg/ml Tris-saline), 800 μl of D-(+)-glucose (250 mg/ml Tris saline), and 200 μl of NH_4Cl (200 mg/ml Tris-saline) to 100 ml of freshly filtered DAB solution (15 mg/ml Tris saline). After development, coverslips were washed twice more in Tris-saline, 5 min each. Coverslips were dipped in increasing concentrations of EtOH (70 %, 95 %, 100 %) for 30 s each, followed by clearant (Anatech L.T.P Anatomical Tech.) and mounted onto slides using mounting medium. Photographs were taken using Ektachrome 50 slide film. Incubation with synthetic β-endorphin blocked the staining for β-endorphin in this procedure (Richard Allen, personal communication).

2.2.3 SECRETION EXPERIMENTS

The protocol for the culture period and secretion experiments is shown in Figure 2.1. The medium was changed the day before the experiment. On the day of the experiment, cells were washed with DMEM (300 μl) 5 times in a 90 min period preceding the medium collection; the subsequent 2 h was divided into 4 equal periods. During the first, second, and fourth periods the cells were exposed to normal DMEM, and during the third period they were exposed to the experimental treatment.

2.1 Secretion protocol

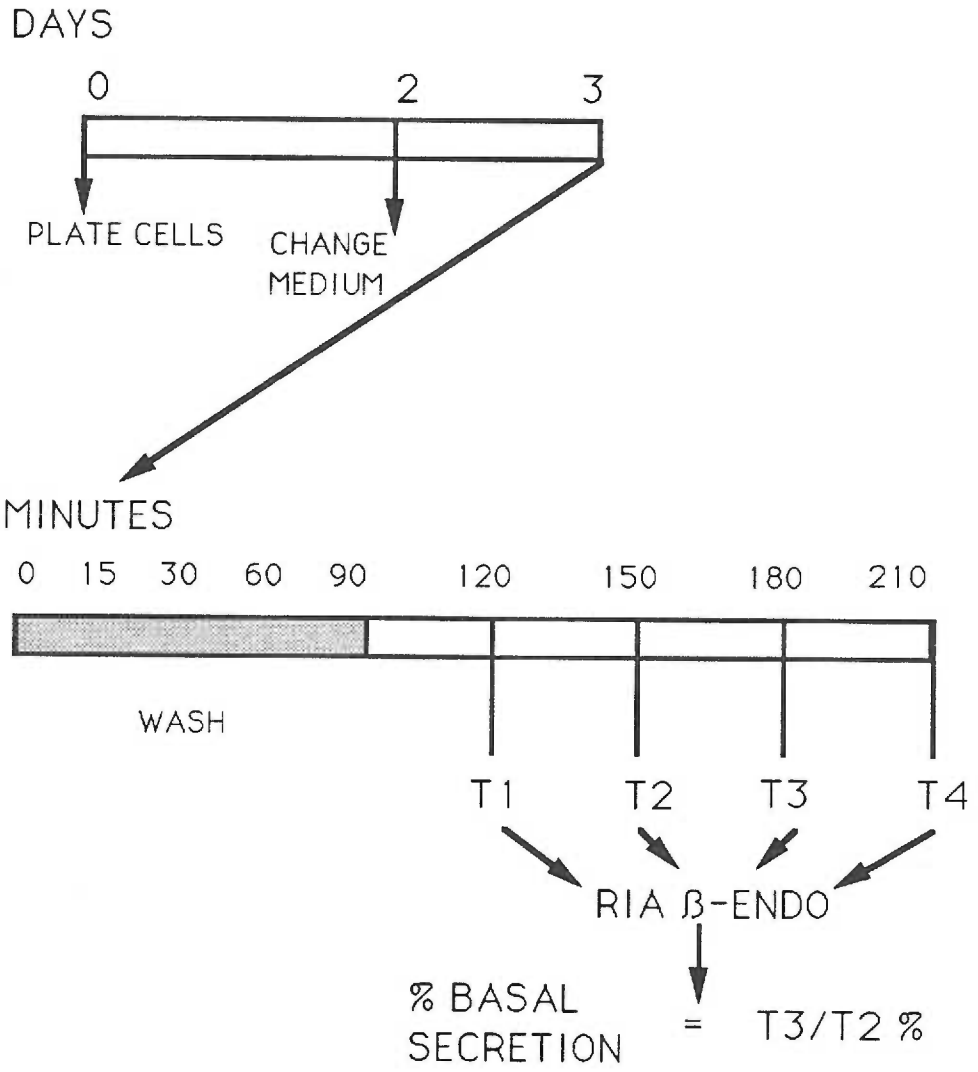


Figure 2.1 Secretion Protocol. AtT-20 cells were plated onto multiwell plates (day 0) and grown in 0.5 ml DMEM. The DMEM was changed on day 2 and the secretion experiment was performed on day 3. Prior to the experimental collection period, the cells were washed with DMEM (300 μl) 5 times in 90 min (at 1, 2, 15, 30, 60, and 90 min). The subsequent 2 h was divided into four 30 min periods; at each period all DMEM (300 μl) was removed and processed and replaced with fresh DMEM (300 μl). Control DMEM was applied during the first (T1), second (T2), and fourth (T4) periods and test DMEM was applied during the third (T3) period. Samples were assayed for β-endorphin immunoactivity by radioimmunoassay (RIA) and the data are expressed as per cent of basal secretion as described in the text.

At the end of each period, the DMEM was removed and replaced with DMEM (or DMEM and appropriate drugs in the case of the third period). The collected samples were centrifuged to remove debris. The supernatant was either frozen or immediately assayed for β-endorphin immunoactivity (Section 2.2.4). Aliquots of medium assayed had volumes of 5-20 μl (dependent upon the number of cells/well).

Since each culture well had its own control secretory rate it could be compared to the change of secretory rate in the test solution. Therefore, the secretion data are expressed as per cent of basal secretion which is defined as:

$$\frac{\text{pg immunoactive } \beta\text{-endorphin secreted during third period}}{\text{pg immunoactive } \beta\text{-endorphin secreted during second period}} \times 100.$$

Parametric statistical testing was performed on this ratio data (Seigel, 1956). Statistical significance between control and drug treatment groups was determined using the unpaired, two-tailed *t*-test, where $p < 0.05$ was significant. In experiments with more than one treatment, analysis of variance where $p < 0.05$ was significant. Multiple *t*-tests were used as the ad hoc test to determine significance between treatments and control and $p < 0.01$ was significant.

2.2.4 β-ENDORPHIN IMMUNOASSAY

β-endorphin immunoactive material was measured with a radioimmunoassay (RIA) according to methods that had been adapted and modified from an ACTH assay (Rees *et al*. 1971).

2.2.4(i) THE ASSAY

The total assay volume was 400 μl/tube which was composed of 100 μl antibody solution, 100 μl iodinated peptide solution (10,000 counts/min), and 200 μl peptide standard or unknown sample. Assay buffer was used as the diluent for the antiserum, the iodinated peptide, and the samples. To make assay buffer, 0.5 ml of β-mercaptoethanol was added to phosphate buffer. Phosphate buffer was composed of a 0.05 M sodium phosphate solution (pH 7.6, made by titrating dibasic sodium phosphate with monobasic sodium phosphate), 2.5 g/L BSA (crystallized and lyophilized, Sigma), and 0.02 % sodium azide. The standard curve was generated from a 12-fold serial dilution of β-endorphin that ranged from 5 ng to 2.5 pg.

The assay was incubated overnight at 4°C and maintained at this temperature for the rest of the assay procedure. Antibody-bound iodinated peptide was separated from iodinated peptide by dextran-coated charcoal, which binds the unbound iodinated peptide. This was made by incubating 2 g of Norit "A" activated charcoal (washed once in H_2O) with 0.5 g of dextran in 40 ml of H_2O for 1 h at room temperature. This solution was diluted to 500 ml with 100 ml of 0.25 M sodium phosphate (pH 7.6) and water for a final concentration of 0.05 M sodium phosphate. The dextran-coated charcoal solution (500 μl) was added to each assay tube, incubated for 15 min, and centrifuged at 3000 rpm for 15 min. A 500 μl sample of the supernatant was transferred to a new tube and radioactivity counted in a gamma counter. The mass values of the unknown samples were read only off the linear portion of the standard curve; this assay was sensitive to 20-40 pg/tube.

2.2.4(ii) IODINATION OF β-ENDORPHIN

β-endorphin was iodinated using the following protocol. Solutions used in this procedure were kept on ice until used. β-endorphin (1 μg, 2 μl from a 0.5 mg/ml stock) was combined with 10 μl of a sodium borate solution (0.1 M, pH 10) and 0.5 mCi or 20 μl of ^{125}I (2 mCi ^{125}I shipped in a 20 μl aliquot from New England Nuclear and diluted to 80 μl with H_2O). Then, 5 μl of $HClO^-$ solution (15 μl of Clorox into 10 ml of the Na borate solution) was added and pipetted up and down for 15 s. This was followed by 20 μl of sodium meta-bisulfite solution (10 mg sodium meta-bisulfite/ml of the Na borate solution) added for 2 min and then 20 μl of NaI solution (100 mg/ml in 60 % acetic acid) added for 2 min. Finally 50 μl of assay buffer was added and the sample was applied to a Sephadex G-10 column and eluted with 10 % acetic acid. [The column was made prior to the iodination reaction where a siliconized pasteur pipette was stuffed with siliconized glass wool at the tapering end and was filled with Sephadex G-10 that had been swelled in 10 % glacial acetic acid. The column was washed with 1 ml of assay buffer followed by 2 ml of 10 % glacial acetic acid.] The drops from the column were monitored with a Geiger Counter; after the first radioactive drop was detected, 7-8 drops were collected per tube. The peptide usually came out in the void volume usually in 3 tubes. Each of these tubes was diluted to 1 ml with H_2O. Trasylol (50 μl; Sigma) was added to each tube to inhibit proteolysis.

2.2.4(iii) GENERATION OF β-ENDORPHIN ANTISERUM

Antiserum was generated in a rabbit that was immunized with a preparation of β-endorphin that was covalently coupled to BSA. Five mg

of β-endorphin(1-27, camel) (Peninsula, Belmont, CA), 1.5 mg BSA (Sigma) and carbodiimide HCl (0.015 mg) (Sigma) were dissolved in 2 ml of distilled water, incubated at room temperature for 30 min and dialyzed overnight at 4°C against distilled water. The dialyzed sample was allocated into volumes that contained approximately 50 μg of β-endorphin and frozen until used for immunization. The rabbit was initially immunized subcutaneously at multiple sites on the back with the 50 μg endorphin-BSA solution emulsified in 2 ml of Freund's Complete Adjuvant (Difco). Thereafter, every two weeks the rabbit was immunized as described above except that Freund's Incomplete Adjuvant (Difco) was used; on these occasions the animal was bled from an ear vein (2-10 ml) and the serum sample was tested for an antibody titer using iodinated β-endorphin. This antiserum (identified as Sugar) recognized the mid-portion region of the β-endorphin molecule and cross-reacted with all forms of β-endorphin as well as β-lipotropin (β-LPH) and POMC (Hatfield et al. 1988). The antiserum was used at a concentration where maximum binding of the iodinated peptide was 50 % (usually 1:3000 but this depended upon the bleed).

2.2.5 ELECTROPHYSIOLOGY

2.2.5(i) THEORY AND ADVANTAGES OF PATCH-CLAMP RECORDING

In 1981 Hamill et al. reported major improvements of the patch-clamp recording techniques that had previously been used for extracellular recording. This improved extracellular patch-clamp technique has dramatically altered electrophysiological research in the past decade. The recording configuration obtained by the formation of a seal between

the pipette electrode and the plasma membrane of intact cells with a resistance of 10-100 GΩ is called the cell-attached patch. The cell-attached recording configuration allows for measurement of current flowing through a single ion channel in the patch of membrane that is electrically isolated at the tip of the pipette. This is possible due to the high resistance seal between the cell membrane and the glass pipette, which ensures that the currents moving across the membrane patch flow into the pipette and from there into the current-measurement circuitry. It also reduces the level of background noise in the recordings to improve single channel current resolution.

The high resistance seal also referred to as a "giga-seal" was found to be mechanically stable. This property allowed for the development of several more recording configurations which included the cell-free and the whole-cell recording configurations. The cell-free configurations involve a membrane patch that spans the opening of the pipette tip and may be either an outside-out or and inside-out patch depending upon the orientation of the membrane to the bath recording solution. In the whole-cell configuration, the membrane that spans the pipette opening in the cell-attached configuration is disrupted leaving the pipette-membrane seal intact. This provides a low resistance pathway to the cell interior for potential recording, voltage clamping and measurement of whole-cell currents. This recording configuration was used in these studies (Figure 2.2).

Whole-cell patch-clamp recording offers advantages for the investigation of stimulus-secretion coupling events that are not possible by intracellular and extracellular recording methods. Whole-cell patch-clamp recording allows for reliable voltage-clamp in small cells and

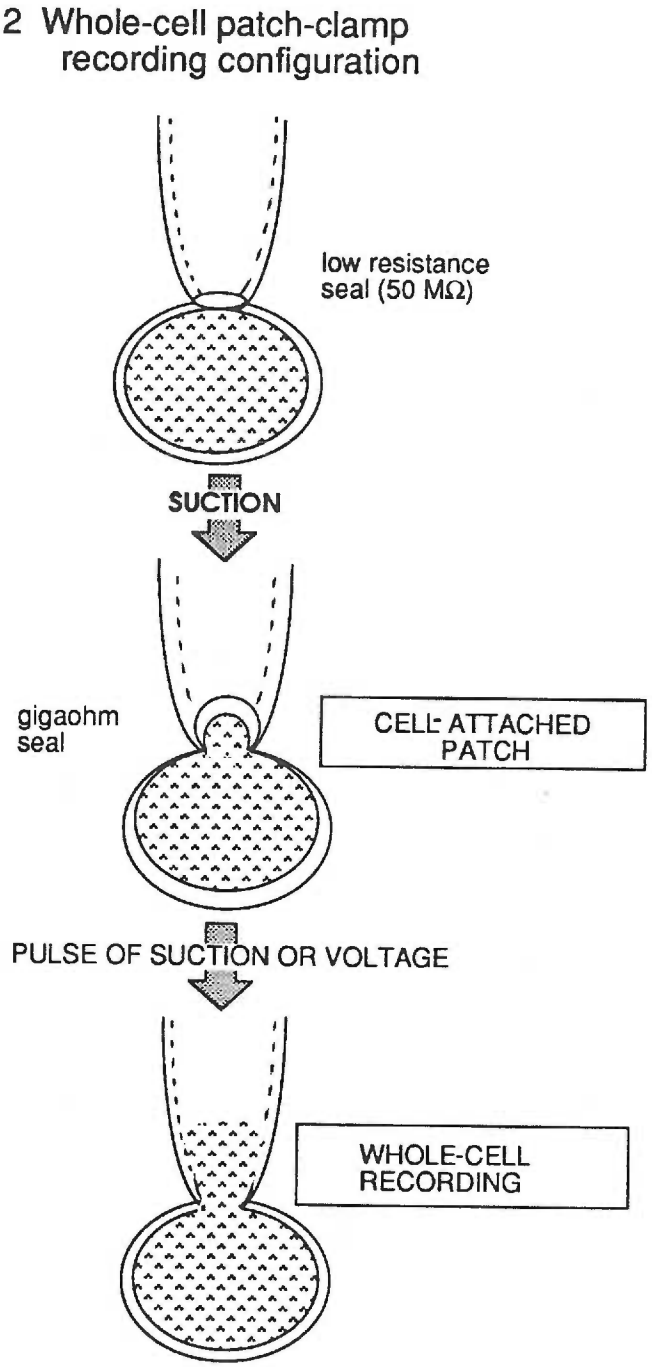


Figure 2.2 Whole-Cell Patch-Clamp Recording Configuration. This recording configuration was described by Hamill et al. (1981). To obtain this configuration, a high resistance seal (gigaohm range) is formed between the glass pipette and the plasma membrane, thus called a "gigaohm seal". This is mechanically stable such that with a sharp pulse of negative pressure or voltage, the membrane that spans the pore of the pipette is ruptured but, the glass-membrane seal remains intact. This produces a low resistance pathway that allows equilibration of the pipette solution with the intracellular solution, good voltage-clamp of small round cells, and measurement of current conducted through the membrane of the entire cell.

measurement of whole cell currents. The diffusional exchange of the intracellular solution with the pipette solution allows a means of perfusing the interior of the cells with ions and macromolecules that may alter intracellular transduction mechanisms and membrane currents. These would include calcium buffering agents such as ethyleneglycol-bis-(β-aminoethyl ether) N,N,N', N'-tetraacetic acid (EGTA), agents that block ion channels such as cesium which blocks potassium channels, and non-hydrolyzable GTP analogs such as GTPγS. Using these strategies, components of the intracellular mechanism involved in the modulation of ionic currents may be studied. However, there are also disadvantages that exist with this technique. Diffusion may also remove intracellular components that are necessary for the maintenance of channel activity or transduction of signals from the receptor to the channels.

In this recording configuration, membrane capacitance may also be measured and used as an indicator of exocytosis (Fernandez, Neher & Gomperts, 1984). Although this method has not been utilized in this study, the technique has been to apply investigate questions of stimulus-secretion coupling. Thus, by utilizing the patch-clamp technique to explore the regulation of ionic currents by stimulatory and inhibitory secretagogues, new and useful information concerning the regulation of intracellular calcium concentrations may be obtained. The ability to measure exocytotic activity in the presence of secretagogues will provide more insight into the process of stimulus-secretion coupling in endocrine cells.

2.2.5(ii) WHOLE-CELL PATCH RECORDING

Experiments were conducted on a Micro-g vibration isolation table (Technical Manufacturing Corporation, Peabody, MA) that was contained within a Faraday cage to reduce exogenous electrical noise. The recording chamber was located on the stage of a Leitz Diavert microscope which was equipped with 16X eye pieces and three objectives, 16X, 20X and 32X. The recording plate was designed for perfusion by the extracellular solutions through the recording chamber. An input reservoir was drained by three short tracks that fed the square recording chamber (12 mm x 12 mm surface area). This was emptied by a long output track (containing a 90° angle turn) to a output reservoir where the solution was removed by a suction tube (Figure 2.3A). The grooves along which solutions entered and left the chamber were positioned so as to ensure even flow across the chamber. The recording plate was milled out of plexiglass and a glass coverslip was epoxied to the bottom of the recording chamber and over the tops of the input and output tracks.

The patch pipette was placed onto the electrode holder (HL-1-17, Axon Instruments) which used a Ag/AgCl pellet assembly and "O" rings with a 1.7 mm hole. To minimize the added noise, these holders used a small (1 mm) pin for the electrical connection that mates firmly with the special teflon connection on a CV-3 headstage (Axon Instruments), a current-to-voltage converter with a gain of 1/100. The headstage was mounted onto a piece of plexiglass that was then mounted onto a Huxley micromanipulator (Sutter Instruments, San Rafael, CA). The holder also had a port on its side; tubing was attached here so that positive or negative pressure could be applied to the patch pipette. A mouthpiece

2.3 Recording chamber and solutions

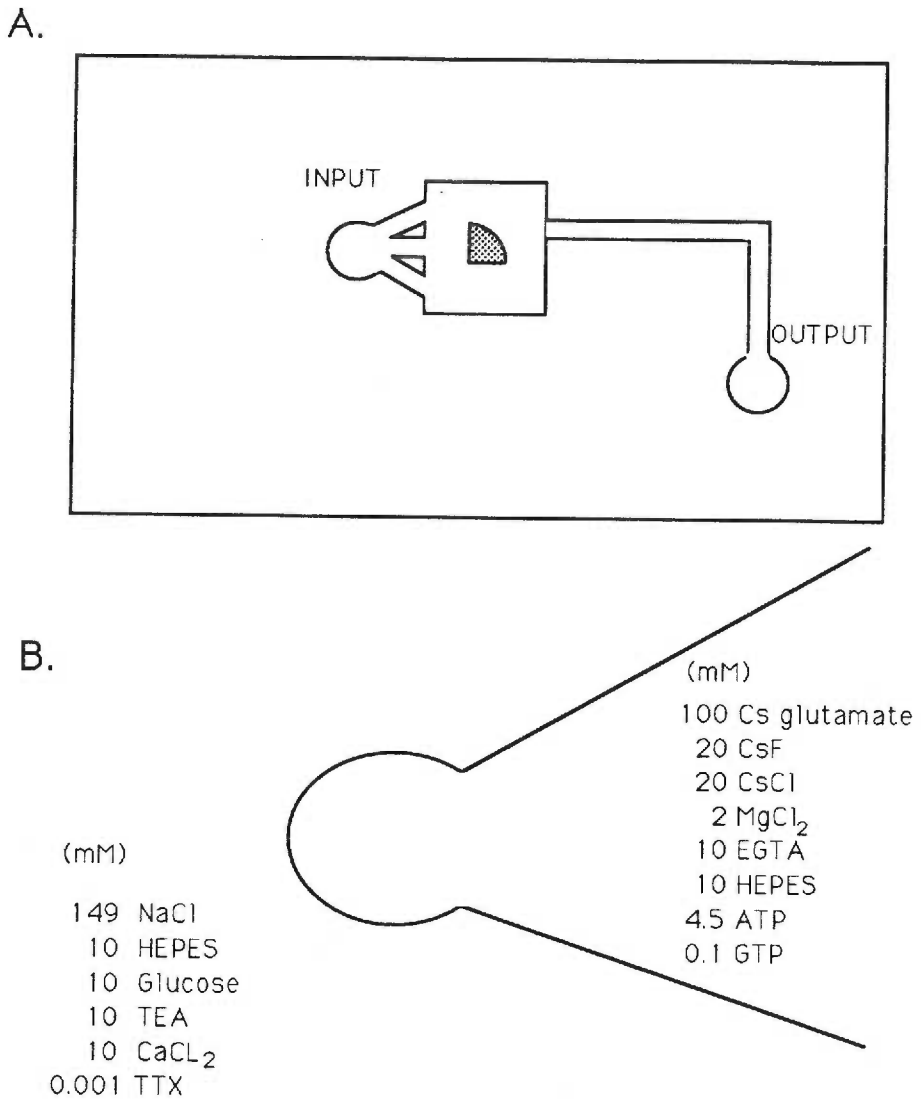


Figure 2.3 Recording Chamber and Solutions. A, schematic diagram of the recording chamber, which was perfused with extracellular recording solution described in B. This solution entered the input through a tube attached to a reservoir of solution. The flow was gravity driven and the solution was heated such the solution in the recording chamber was maintained at 35-37°C. The solution in the output reservoir was removed by aspiration. Coverslips, which had cells attached, were quartered and placed in the recording chamber. B, schematic diagram of a whole-cell recording configuration with the composition of the pipette and the extracellular solutions used to record calcium currents.

was attached to the tubing so the pressure could be regulated by the investigator.

Electrophysiological recordings were performed using the whole-cell patch-clamp configuration (Hamill et al. 1981; Figure 2.2). The formation of high resistance (GΩ range) seals between the plasma membrane and the patch electrode was monitored electrically. The patch pipette was immersed in the recording solution while a constant positive pressure was applied to the pipette to avoid attachment of any debris onto its surface. An oscillating test pulse of 0.2 mV was applied and the current was monitored on a dual beam storage oscilloscope (Model 5113, Tektronics). The patch pipette was positioned close to the cell surface and then the positive pressure was released. When the patch pipette touched the cell surface the current amplitude decreased indicating the initiation of seal formation. The oscillating test pulse was then changed to 20 mV and continued negative pressure was applied gently to the pipette to assist the seal formation. Only cells that formed seals of $\geq$ 2 GΩ were used for recording. To form the whole cell recording configuration, a sharp pulse of negative pressure usually was applied but if this method was not successful, a sharp pulse of voltage was used. The transition from cell-attached to whole-cell configuration was monitored by the capacitative current. A quality whole-cell configuration was achieved when the capacitative current increased in amplitude and had a duration of less 1 ms.

2.2.5(iii) PATCH PIPETTE ELECTRODES

Micropipettes (100 μl, Drummond Scientific Company), which were cut to 80 mm in length, were pulled into two patch pipettes of 4-5 MΩ

resistance using a Narishige two stage puller. The pipettes were fire polished using a Narishige microforge and stored in a pipette holder with desiccant. To fill the pipette, the tip was first back-filled with solution using negative pressure from a syringe and then the rest of the solution was filtered through a syringe filter and injected into the pipette to fill about 30 % of the pipette volume.

2.2.5(iv) MEASUREMENT OF CALCIUM CURRENTS

Voltage-clamp studies were performed using an Axopatch 1B amplifier, Axolab, which is an analog-digital, digital-analog converter, a Grass S88 stimulator, and an IBM AT computer. Data were collected using the Axolab Axessp software version 1.01 and were analyzed with the Axolab Axessa software version 1.00 (Axon Instruments). Voltage steps were applied and the current recorded with a sampling interval of 0.15 ms/point; currents were filtered at 1 kHz.

Whole-cell patch-clamp recordings were made using a recording chamber through which flowed the extracellular solution, which was heated to 35-37°C (the tubing carrying the solution passed through a water jacket prior to entering the chamber). Drugs from mM stock solutions were added in appropriate aliquots to the buffer reservoir to obtain the final concentration. The flow rate was 0.5-1 ml/min and it took 2-3 min for the bath solution to exchange with new drug solution.

The recording solutions were designed to isolate the calcium currents from the other membrane currents. For example, tetrodotoxin (TTX) was added in an attempt to block the inward sodium current and internal cesium and external TEA were added to block the outward potassium currents. To attempt to suppress the calcium activated currents, EGTA

(10 mM) was added to the pipette solution or external barium replaced the extracellular calcium as the charge carrier. The pipette solution was composed of (mM) 100 Cs glutamate, 20 CsF, 20 CsCl, 2 $MgCl_2$, 10 EGTA, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 4.5 ATP disodium salt and 100 μM GTP. The pH was adjusted 7.38 with CsOH and the osmolality was adjusted to 325-330 mmol/kg with Cs glutamate (1 M). The extracellular solution was composed of (mM) 149 NaCl, 10 HEPES, 10 D-glucose, 10 TEA, 10 $CaCl_2$ or $BaCl_2$ and 1 μM TTX. The pH was adjusted to pH 7.38 with NaOH and the osmolality was adjusted to 330 mmol/kg (Figure 2.3B).

The currents shown in this chapter have not been subtracted for leak or capacitative current. Therefore, the amplitude of the currents may be an underestimate since the leak current was not subtracted, and this may account for the large variability seen in the concentration response curves. However, only cells with a leak current of less than 1 pA/mV (measured by evoking the current with a depolarizing pulse from -70 mV to -60 mV) were analyzed. A junction potential existed between the intracellular and extracellular solutions. The voltages reported here are adjusted for the junction potential.

Two measurements of the current amplitude were made. The transient component of the current refers to the largest amplitude of the current measured during the first 10 ms of the voltage pulse. The sustained component of the current refers to the average amplitude of the last 10 ms of the voltage step.

2.3 RESULTS

2.3.1 IMMUNOCHEMISTRY

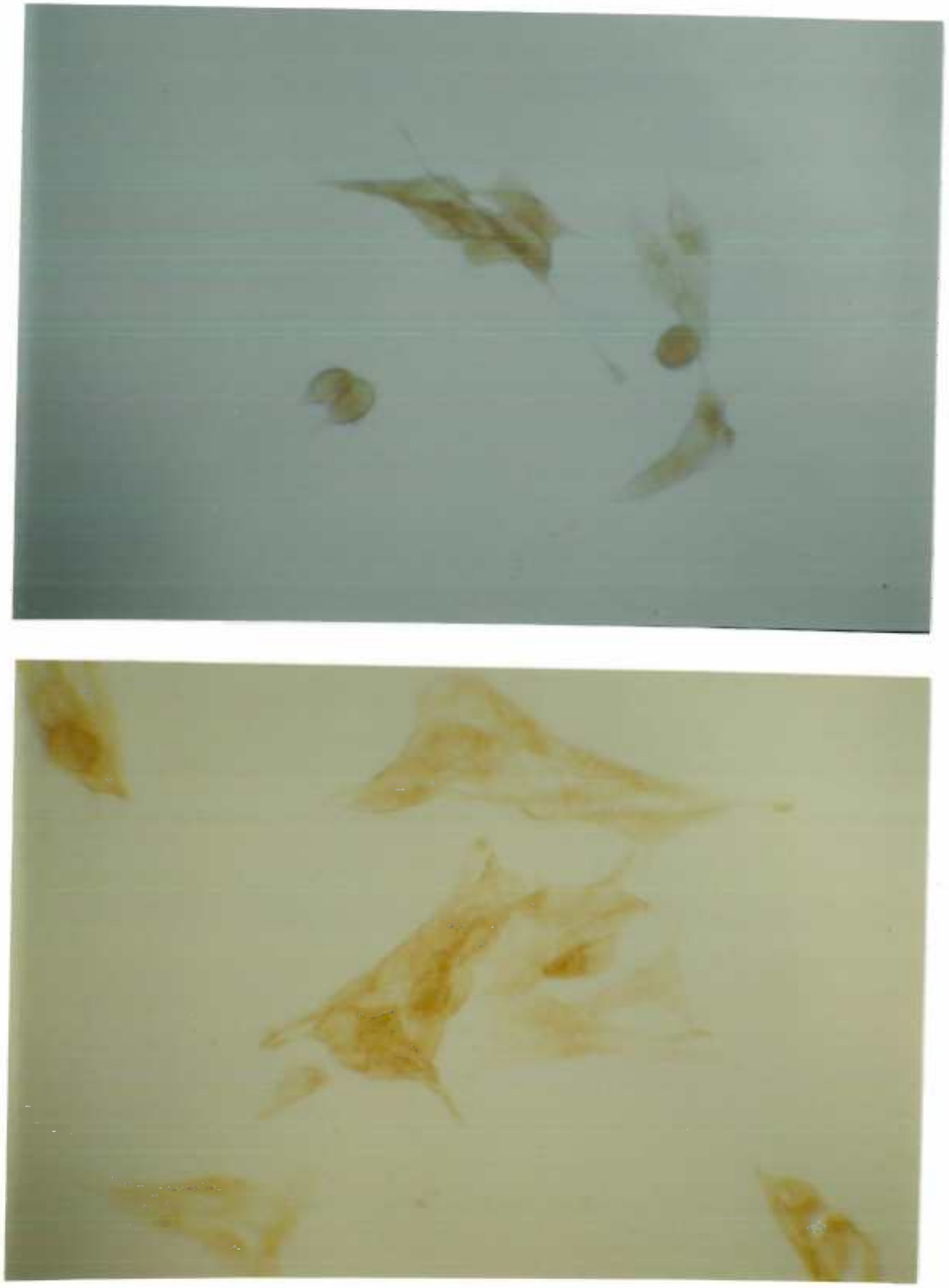
AtT-20 cell cultures stained with a β-endorphin antibody showed that all cells were positive for β-endorphin (Figure 2.4)

2.3.2 SECRETION EXPERIMENTS

2.3.2(i) BASAL SECRETION

All data are expressed as per cent of basal secretion as described in section 2.2.3. When control solution was applied during all four periods, the secretory rate during period three was 95.2 $\pm$ 3.7 % (n = 30) of that during period two. The absolute level of basal secretion in these experiments was 7.78 $\pm$ 0.90 ng immunoactive β-endorphin/well.

Several experimental solutions were tested to determine whether basal secretion could be reduced. The results of these experiments are shown in Figure 2.5A. When calcium was removed from the medium, secretion was reduced to 50.8 $\pm$ 2.2 % of basal secretion (n = 12; $p < 0.0001$). Magnesium (2.4 mM) reduced secretion to 43.8 $\pm$ 6 % of basal secretion (n = 6; $p < 0.0001$). Nifedipine (10 μM) reduced secretion to 52.5 $\pm$ 3.6 % of basal secretion (n = 6; < 0.0001). SRIF reduced basal secretion in a concentration-dependent manner. In the presence of 1 nM SRIF tested, secretion was 74 $\pm$ 6.3 % of basal secretion but this was not a significant reduction (n = 6; $p = 0.0207$). 100 nM SRIF reduced secretion to 71.1 $\pm$ 3.6 % of basal secretion (n = 6; $p = 0.0088$). 300 nM SRIF reduced the to 65.6 $\pm$ 2.8 % of basal secretion (n = 12; $p < 0.0001$).



2.4 Immunocytochemistry of AtT-20 Cells. All cells were stained with β-endorphin specific antibody. Cells were grown on glass coverslips. Top, 24 h. Bottom, 48 h.

2.5 Basal secretion

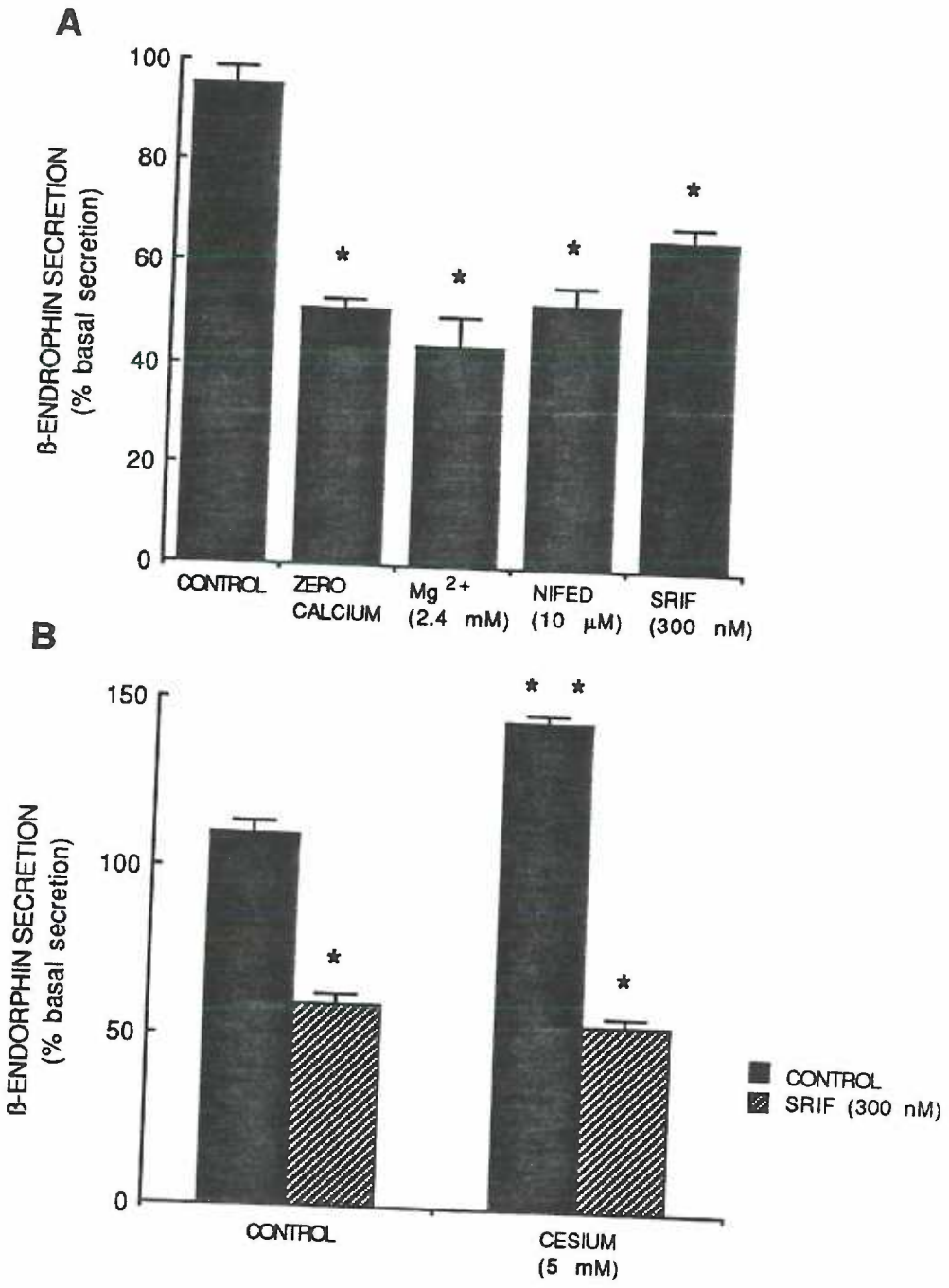


Figure 2.5 Basal Secretion. A, removal of extracellular calcium (n = 12), or addition of magnesium (2.4 mM; n = 6), nifedipine (10 μM; n = 6), and SRIF (300 nM; n = 12) reduced basal secretion (n = 30 for control). The * in this figure and all subsequent figures indicates significantly different from control. B, cesium (5 mM) did not affect the inhibition of basal secretion by SRIF (300 μM) but did enhanced basal secretion (** indicates significantly different from the control without cesium; n = 6 for all points).

A previous study by Pennefather, Heisler and MacDonald (1988) showed that SRIF produced a hyperpolarization due to the activation of a potassium conductance and this conductance increase induced by SRIF was blocked by cesium (5 mM). These authors also reported that the inhibition of basal secretion by SRIF could be reduced by 5 mM cesium. In the present experiments, cesium (5 mM) enhanced secretion to 144.8 ± 4.6 % of basal secretion (n = 6) compared to control which was 110 ± 4.6 % of basal secretion (n = 6; p = 0.0192). SRIF (300 nM) reduced secretion in the presence and absence of cesium [to 54.7 ± 2.4 % of basal secretion (n = 6) and 59.7 ± 3.2 % of basal secretion (n = 6), respectively]. These were not significantly different from one another (p = 0.2355) (Figure 2.5B).

Basal secretion was not significantly reduced by the inorganic calcium channel blockers (Figure 2.6A). Secretion in the presence of cadmium (500 μM) was 80.7 ± 2.5 % of basal (n = 6; p = 0.0922) and in the presence of nickel (500 μM) was 81 ± 10 % of basal (n = 6; p = 0.1374). TTX (1 μM) did not reduce basal secretion; secretion in the absence and presence of TTX was 90 ± 2 % of basal (n = 6) and 91.2 ± 5.4 % of basal (n = 6; p = 0.8573) as shown in Figure 2.6B.

2.3.2(ii) STIMULATED SECRETION

β-endorphin secretion was consistently stimulated by the calcium channel agonist, BAY K 8644 (1 μM), which enhanced secretion to 176.4 ± 7.9 % of basal (n = 6; p < 0.0001). The β-adrenergic receptor agonist, isoproterenol (1 μM), and forskolin (10 μM), an activator of adenylate cyclase, had similar effects and increased secretion to 196.7 ± 6.2 %

2.6 Basal secretion not reduced by cadmium, nickel or tetrodotoxin

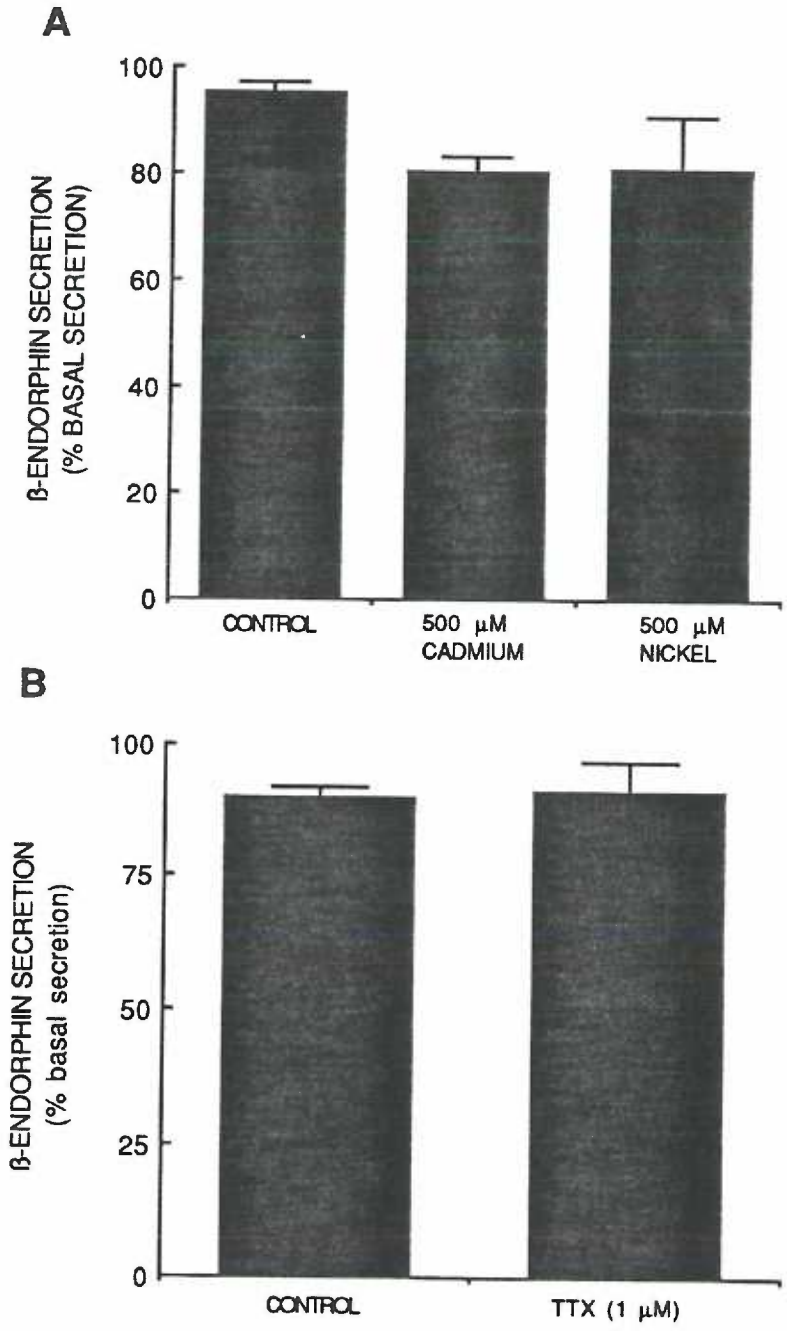


Figure 2.6 Basal Secretion not Reduced by Cadmium, Nickel or Tetrodotoxin. A, cadmium (500 μM; n = 6) and nickel (500 μM; n = 6; n = 30 for control). B, TTX (1 μM; n = 6 for both points).

($n = 68$, $p < 0.0001$) and 145.2 $\pm$ 6.8 % ($n = 12$, $p < 0.0001$) of basal, respectively.

In the absence of extracellular calcium, basal secretion was reduced to 50 $\pm$ 2.2 % (Figure 2.5A) and the above mentioned secretagogues did not stimulate β-endorphin secretion. In the absence of extracellular calcium, secretion in the presence of BAY K 8644 was reduced to 80.5 $\pm$ 2.9 % of basal secretion ($n = 6$, $p < 0.001$), isoproterenol was reduced to 71.9 $\pm$ 2.1 % of basal secretion ($n = 12$, $p < 0.001$), and forskolin was reduced to 76.5 $\pm$ 2.2 % of basal secretion ($n = 6$, $p < 0.001$) (Figure 2.7A). However, secretion in zero calcium in the presence of BAY K 8644, isoproterenol, and forskolin were significantly greater than secretion measured in the zero calcium alone, which was 50.8 $\pm$ 2.2 % of basal ($p < 0.0001$ in all cases). This suggests that although secretion is reduced below the basal levels in the absence of extracellular calcium, these agonists slightly enhanced secretion in the absence of extracellular calcium.

TTX (1 μM) did not alter stimulated secretion. The BAY K 8644-stimulated secretion in the absence and presence of TTX was 156.5 $\pm$ 3.8 % of basal ($n = 6$) and 151.8 $\pm$ 3.8 % of basal ($n = 6$), respectively. Isoproterenol-stimulated secretion in the absence and presence of TTX was 172.3 $\pm$ 4.0 % of basal ($n = 6$) and 167.5 $\pm$ 3.4 % of basal ($n = 6$), respectively. These data are shown in Figure 2.7B.

2.7 Stimulated secretion

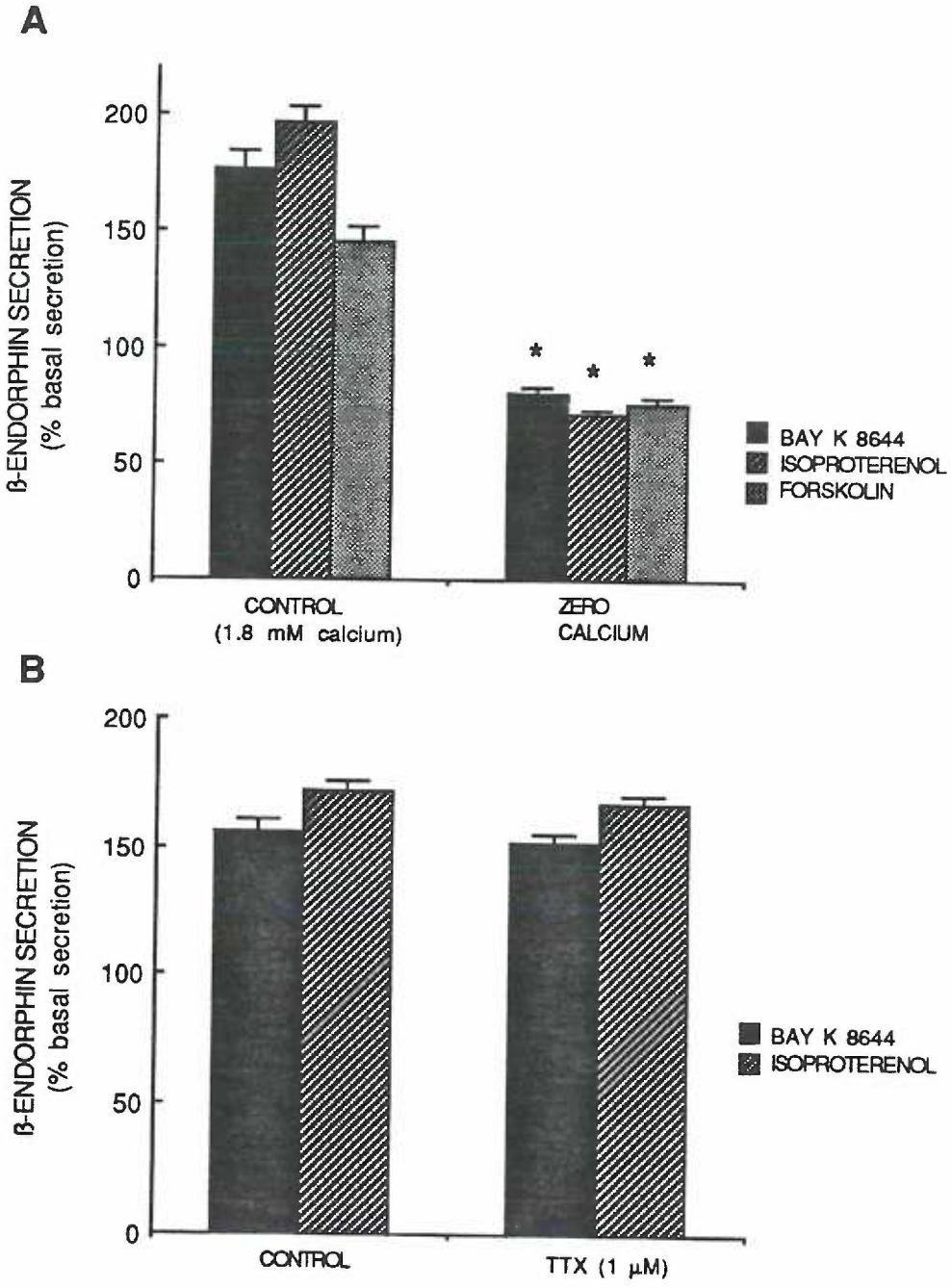


Figure 2.7 Stimulated Secretion. A, secretion was stimulated by BAY K 8644 (1 μM; n = 42), isoproterenol (1 μM; n = 68) and forskolin (10 μM; n = 12). These concentrations of secretagogues were used throughout this study and will not be specifically stated in subsequent figures. Stimulation of β-endorphin release by these secretagogues was abolished when calcium was removed from the external medium (n = 6 for BAY K 8644 and forskolin; n = 12 for isoproterenol). B, TTX (1 μM) did not alter secretion evoked by BAY K 8644 or isoproterenol (n = 6 for all points).

2.3.2(iii) INHIBITION OF STIMULATED SECRETION

MODULATION OF SECRETION BY CALCIUM CHANNEL BLOCKERS

Nifedipine

Secretion of β-endorphin stimulated by isoproterenol [196.7 $\pm$ 6.1 % of basal (n = 68)] was reduced by nifedipine in a concentration-dependent manner (Figure 2.8); 0.1 μM-nifedipine did not significantly reduce secretion and was 175 $\pm$ 7 % of basal (n = 6; p = 0.01822). 0.3 μM-nifedipine reduced secretion to 163 $\pm$ 5 % of basal (n = 6; $p < 0.001$). 1 μM-nifedipine reduced secretion to 89.8 $\pm$ 5.4 % of basal (n = 6; $p < 0.0001$). 10 μM-nifedipine reduced secretin to 94.8 $\pm$ 3.5 % of basal (n = 6; $p < 0.0001$).

Cadmium

BAY K 8644-stimulated secretion [202.8 $\pm$ 13.5 % of basal (n = 6)] was not reduced by concentrations of cadmium below 500 μM. In the presence of 30 μM cadmium secretion was 181.0 $\pm$ 9.8 % of basal (n = 6; p = 0.2197) and in the presence of 100 μM cadmium was 180.5 $\pm$ 9.9 % of basal (n = 6; p = 0.2109). 500 μM-cadmium reduced BAY K 8644-stimulated secretion to 66.7 $\pm$ 5.7 % of basal secretion (n = 6; $p < 0.0001$).

Isoproterenol-stimulated secretion [222.8 $\pm$ 15.4 % of basal secretion (n = 6)] was not reduced by 30 μM cadmium, which was 206.7 $\pm$ 5.9 % of basal secretion (n = 6; p = 0.3512) but was reduced by 100 μM cadmium to 118.2 $\pm$ 4.1 % of basal secretion (n = 6; $p < 0.0001$) and by 500 μM cadmium to 78.5 $\pm$ 5.6 % of basal secretion (n = 6; $p < 0.0001$). These data are shown in Figure 2.9.

2.8 Nifedipine inhibition of stimulated secretion

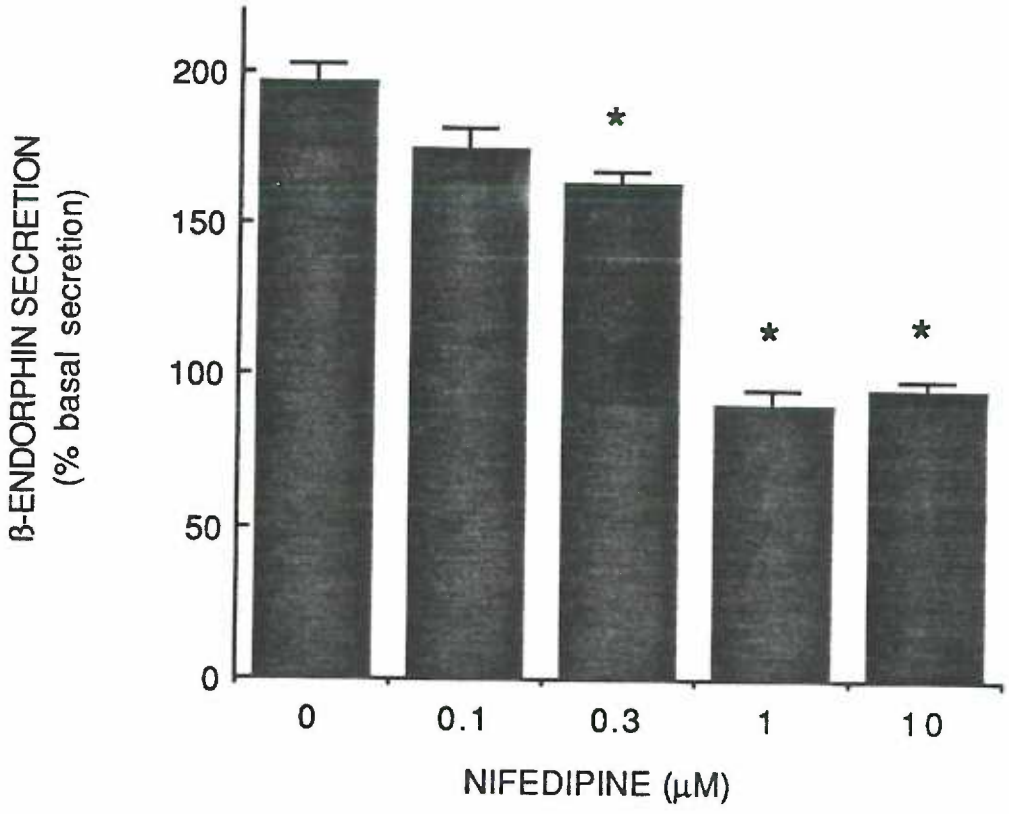


Figure 2.8 Nifedipine Inhibition of Stimulated Secretion. Isoproterenol-stimulated secretion was reduced by nifedipine in a concentration-dependent manner with an IC_{50} of < 0.3 μM. 1 μM-nifedipine produced a maximum inhibition of stimulated secretion reducing secretion to basal levels (n = 68 for control; n = 6 for all nifedipine concentrations).

2.9 Cadmium inhibition of stimulated secretion

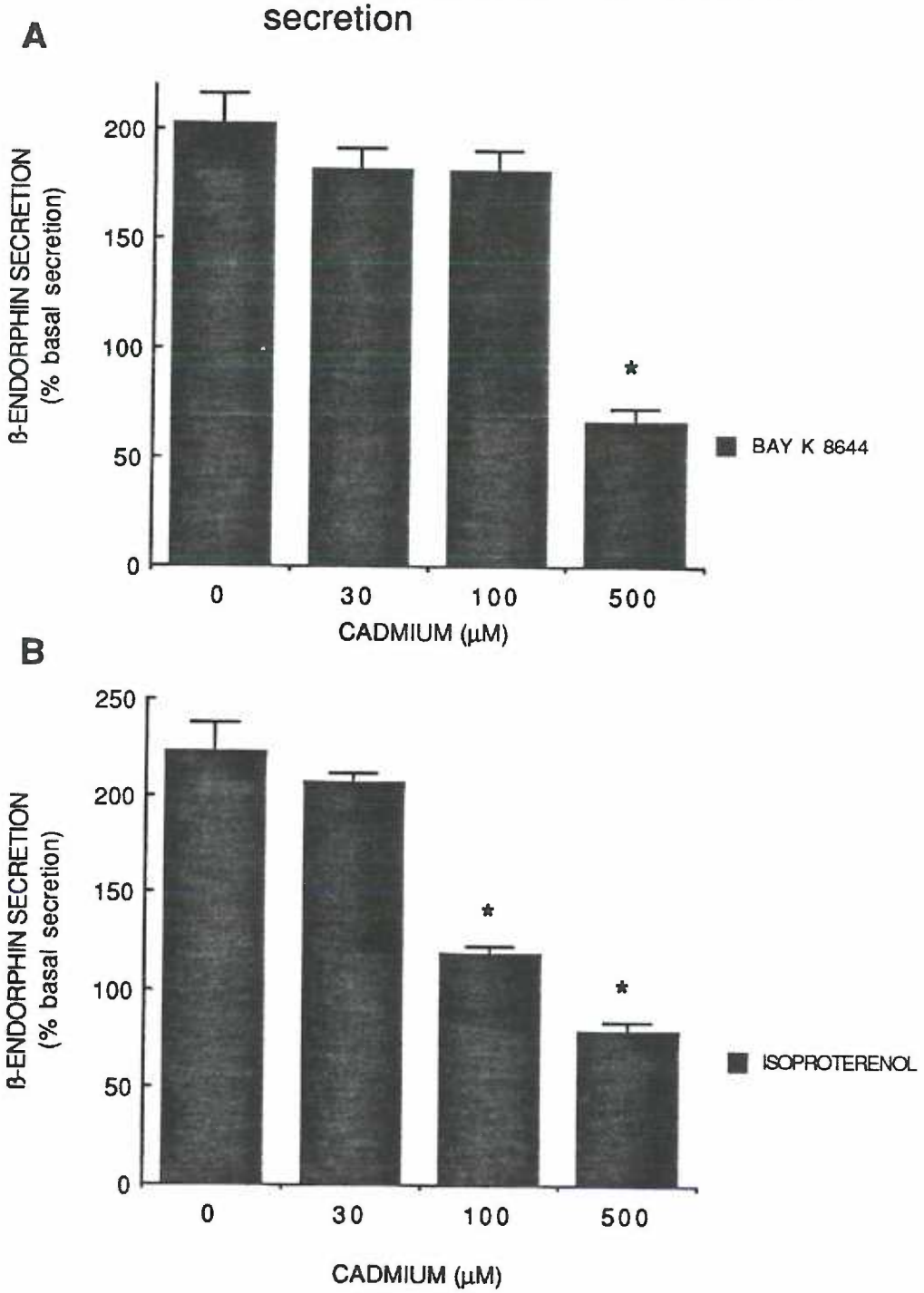


Figure 2.9 Cadmium Inhibition of Stimulated Secretion. A, BAY K 8644-stimulated secretion was inhibited by cadmium (500 μM) reducing secretion below basal levels (n = 6 for all points). B, isoproterenol-stimulated secretion was reduced by cadmium with an IC_{50} between 30 and 100 μM. 500 μM-cadmium reduced secretion to below basal levels (n = 6 for all points).

Nickel

Nickel did not reduce stimulated secretion. BAY K 8644-stimulated secretion was 257.8 ± 18.2 % of basal secretion (n = 6). In the presence of BAY K 8644 and nickel, 100 μM nickel was 228.0 ± 15.7 % of basal secretion (n = 6; p = 0.2481) and 1 mM nickel was 194.6 ± 22.1 % of basal secretion (n = 5; p = 0.0525).

Isoproterenol-stimulated secretion was 289.8 ± 16 % of basal secretion (n = 6) and in 100 μM nickel was 239.5 ± 11.9 % of basal secretion (n = 6; p = 0.0315). 1 mM nickel enhanced secretion to 423.0 ± 13.8 % of basal secretion (n = 6; p < 0.0001). These data are shown in Figure 2.10.

MODULATION OF SECRETION BY SOMATOSTATIN

SRIF reduced secretion in a concentration dependent manner. BAY K 8644-stimulated secretion [164.2 ± 10.9 % of basal secretion (n = 12)] was inhibited by SRIF in a concentration-dependent manner (Figure 2.11A). In the presence of SRIF (1 μM), secretion was 127.0 ± 0.7 % of basal (n = 5; p = 0.0514). 10 nM-SRIF reduced the secretion to 113.8 ± 2.3 % (n = 6) of basal (p = 0.0005). 100 nM-SRIF reduced secretion to 101.6 ± 1.2 % of basal (n = 6; p = 0.0005). 300 nM-SRIF reduced secretion to 103.1 ± 5.0 % of basal (n = 12; p < 0.0001).

Isoproterenol-stimulated secretion [191.6 ± 8.2 % of basal secretion (n = 25)] in the presence of 1 nM SRIF was 148.8 ± 10.7 % of basal secretion (n = 6; p < 0.0221). 100 nM-SRIF reduced secretion to 71.3 ± 4.8 % of basal (n = 6; p < 0.0001). 300 nM-SRIF reduced secretion to 78.8 ± 1.8 % of basal (n = 18, p < 0.0012). These data are shown in Figure 2.11B.

2.10 Nickel does not inhibit stimulated secretion

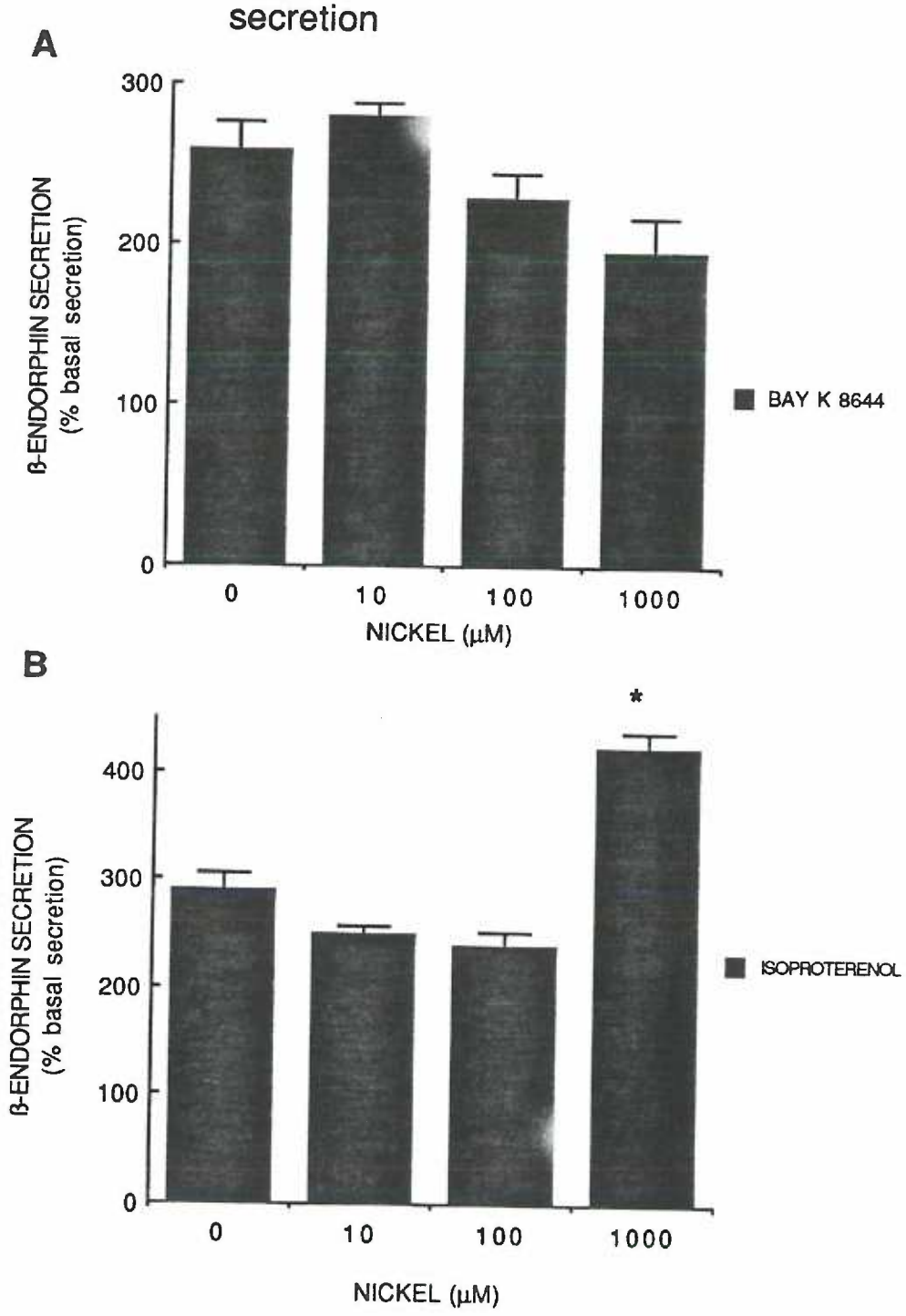


Figure 2.10 Nickel does not Inhibit Stimulated Secretion. A, BAY K 8644-stimulated secretion was not reduced by nickel (n = 6 for all points). B, isoproterenol-stimulated secretion was not reduced by nickel. 1 mM-nickel enhanced the isoproterenol-stimulated secretion (n = 6 for all points).

2.11 Somatostatin inhibition of stimulated secretion

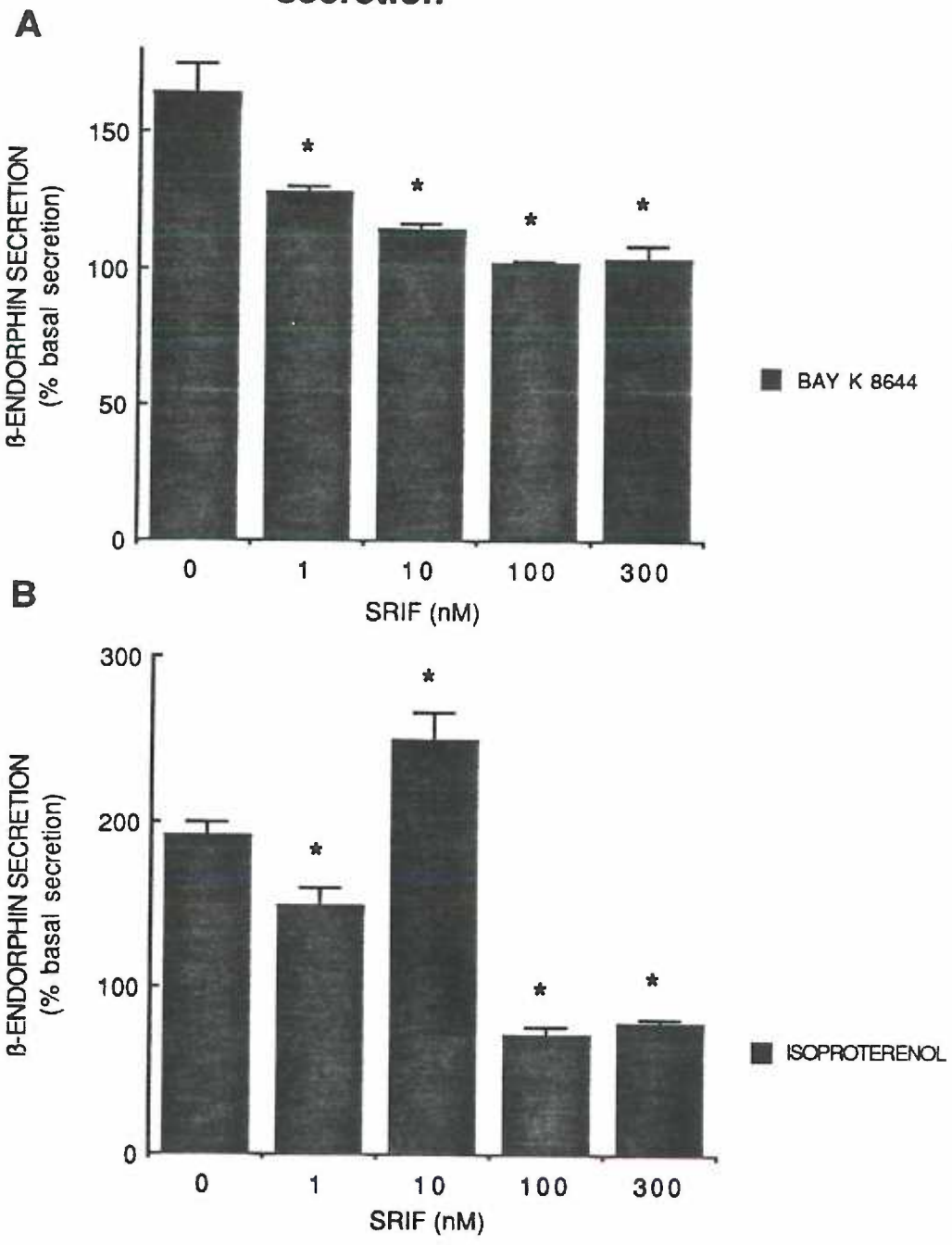


Figure 2.11 Somatostatin Inhibition of Stimulated Secretion. A, BAY K 8644-stimulated secretion was reduced in a concentration-dependent manner with an IC_{50} of 1 nM and 100 nM SRIF produced a maximum inhibition reducing secretion to basal levels (n = 12 for control and 300 nM; n = 5 for 1 nM; n = 6 for 10 nM and 100 nM). B, isoproterenol-stimulated secretion was maximally inhibited by SRIF (100 nM) reducing secretion to below basal levels (n = 25 for control; n = 6 for 1 nM, 10 nM and 100 nM; n = 10 for 300 nM).

Isoproterenol-stimulated secretion was similar in the absence and presence of 5 mM cesium; secretion was 223 ± 15 % of basal (n = 6) in the absence of cesium and 195 ± 3 % of basal (n = 6) in the presence of cesium. These were not significantly different from each other (p = 0.1018). SRIF (300 nM) reduced isoproterenol-stimulated secretion in the absence and presence of cesium; β-endorphin secretion was 78.5 ± 5.3 % of basal (n = 6) in the absence of cesium and 77.3 ± 2.6 % of basal (n = 6; p = 0.8617). These data are shown in Figure 2.12.

Pretreatment of the cell with pertussis toxin (50-100 ng/ml for 16 h) had no effect on BAY K 8644 or isoproterenol-stimulated secretion but blocked the inhibition of stimulated secretion by SRIF. BAY K 8644-stimulated secretion [196.0 ± 10 % of basal secretion (n = 6)] was reduced by 300 nM SRIF to 118.7 ± 3.5 % of basal secretion (n = 6; p < 0.0001). In cells pretreated with pertussis toxin (100 ng/ml), the values of BAY K 8644-stimulated secretion in the absence and presence of SRIF were 179 ± 3.5 % of basal (n = 6) and 162.3 ± 11.1 % of basal (n = 6), respectively, and these values were not significantly different from each other (p = 0.1835). These data are shown in Figure 2.13A.

Isoproterenol-stimulated secretion [144.3 ± 7 % of basal secretion (n = 6)] was reduced by 300 nM SRIF to 79.2 ± 1.3 % of basal secretion (n = 6; p < 0.0001). In cells pretreated with pertussis toxin (50 ng/ml), the values of isoproterenol-stimulated secretion in the absence and presence of SRIF were 148.2 ± 3.7 % of basal (n = 6) and 141.0 ± 2.5 % of basal (n = 6), respectively, and these values were not significantly different from each other (p = 0.1380). In cells pretreated with pertussis toxin (100 ng/ml), the values of isoproterenol-stimulated secretion in the absence and presence of SRIF were 149.0 ±

2.12 Cesium does not affect the inhibition of ß-endorphin secretion by somatostatin

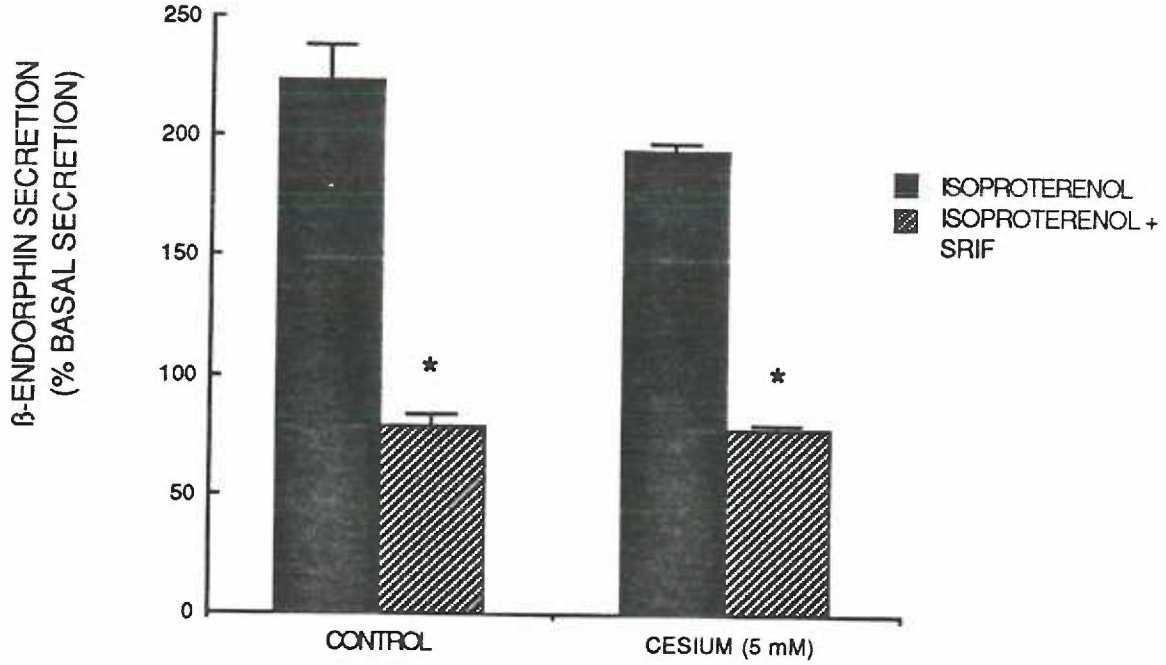
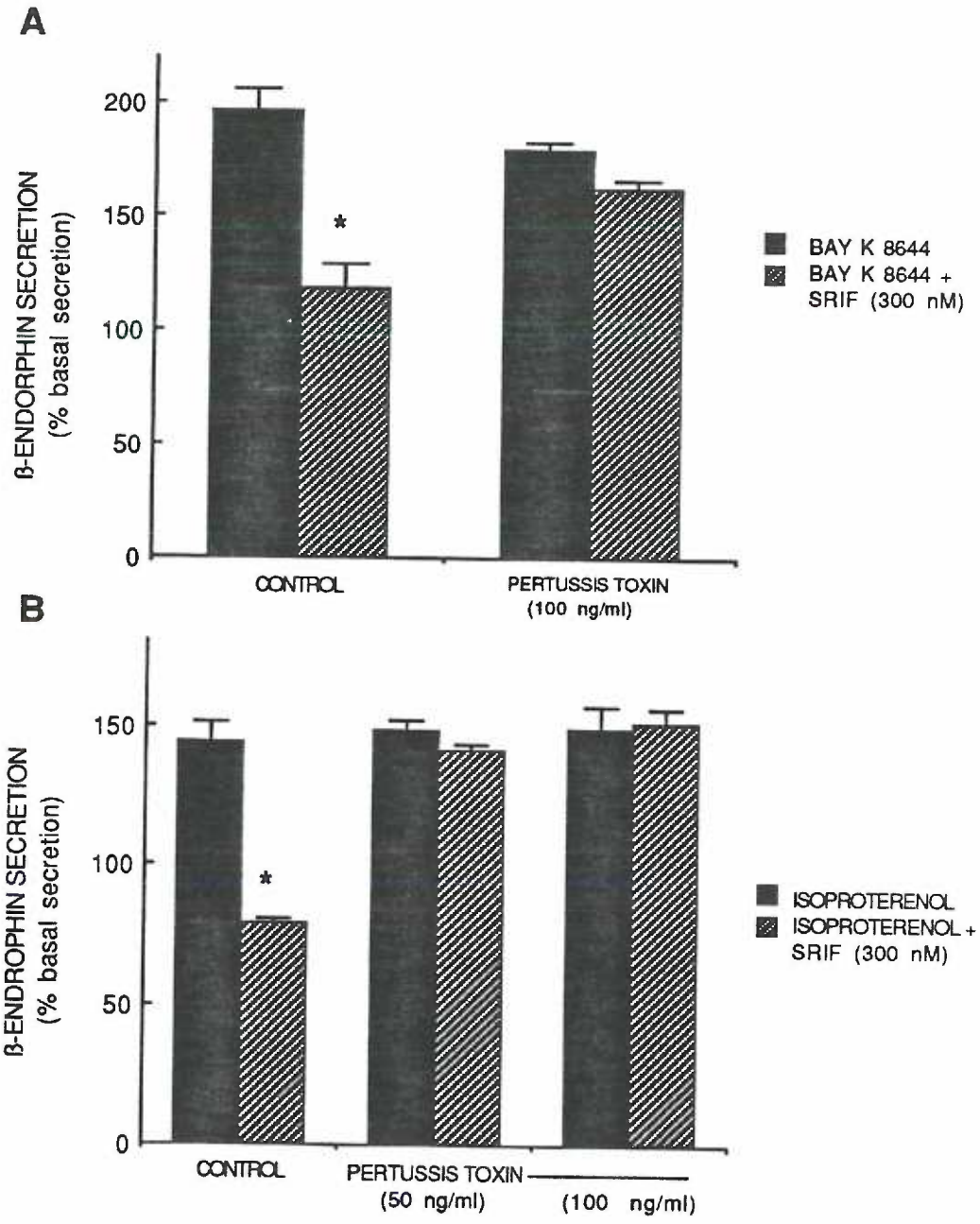


Figure 2.12 Cesium Does Not Affect the Inhibition of β-Endorphin Secretion by Somatostatin. Isoproterenol-stimulated secretion was inhibited with SRIF (300 nM) in the absence and presence of cesium (5 mM). Cesium did not alter isoproterenol-stimulated secretion (n = 6 for all points).

2.13 Pertussis toxin blocks the inhibition by somatostatin of ß-endorphin secretion



2.13 Pertussis Toxin Blocks the Inhibition by Somatostatin of β-Endorphin Secretion. A, BAY K 8644-stimulated secretion was inhibited by SRIF (300 nM) to basal levels. Pertussis toxin pretreatment (100 ng/ml for 16 h) blocked the inhibition of secretion by SRIF and did not alter BAY K 8644-stimulated secretion (n = 6 for all points). B, isoproterenol-stimulated secretion was inhibited by SRIF (300 nM) to below basal levels. Pertussis toxin pretreatment (50 or 100 ng/ml for 16 h) blocked the inhibition of secretion by SRIF and did not alter isoproterenol-stimulated secretion (n = 6 for all points).

7.8 % of basal (n = 6) and 151.3 ± 4.7 % of basal (n = 6), respectively; these values were not significantly different from each other (p = 0.8137). These data are shown in Figure 2.13B.

2.3.3 ELECTROPHYSIOLOGICAL EXPERIMENTS

2.3.3(i) CHARACTERIZATION OF CALCIUM CURRENTS

These voltage-dependent calcium currents were temperature sensitive. When the temperature was increased from 24°C to 32°C, the current amplitude increased at least 3-fold (Figure 2.14). The subsequent recordings were performed at 35-37°C so as to minimize the differences in conditions between the calcium current recordings and secretion experiments.

The I-V relationship was determined in 18 cells when calcium was used as the charge carrier. The transient component activated between -30 mV and -20 mV. The maximum amplitude of the transient component ranged from 60 pA to 1250 pA with an average of 325 ± 63 pA. The maximum amplitude was measured at 0 mV in 3 cells, 10 mV in 9 cells, 20 mV in 6 cells. The sustained component activated between -30 mV and -10 mV. The maximum amplitude of the sustained component ranged from 30 pA to 1010 pA with an average of 248 ± 52 pA. The maximum amplitude occurred at 0 mV in 4 cells, 10 mV in 10 cells, 20 mV in 4 cells. The amplitude of the transient and sustained component were significantly different ($p < 0.0001$) indicating the current was subject to some inactivation during the 150 ms voltage step. The ratio of the amplitudes of the transient to sustained components of the current was 1.45 ± 0.14. A representative current trace is shown in Figure 2.15.

2.14 Calcium current at 24°C and 32°C

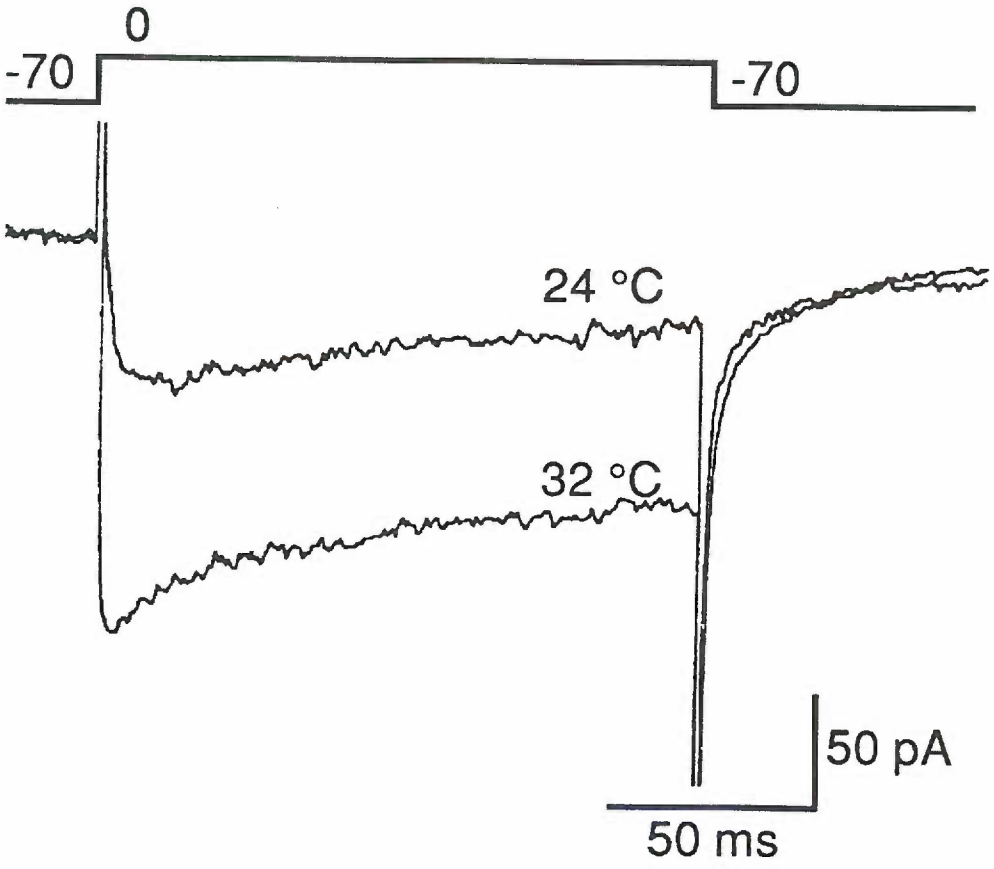


Figure 2.14 Calcium Current at 24°C and 32°C. The calcium currents were evoked with a 150 ms voltage pulse from -70 mV to 0 mV, which were applied every 30 s. The temperature of the recording bath was initially 24°C. At 2 min, the water pump that feeds heated water to the water jacket for the input solution was turned on. Within 1 min the current amplitude increased. The current shown here was measured at 4 min and the temperature was 32°C. (AtT-20 101399c2).

2.15 Calcium current: current-voltage relation

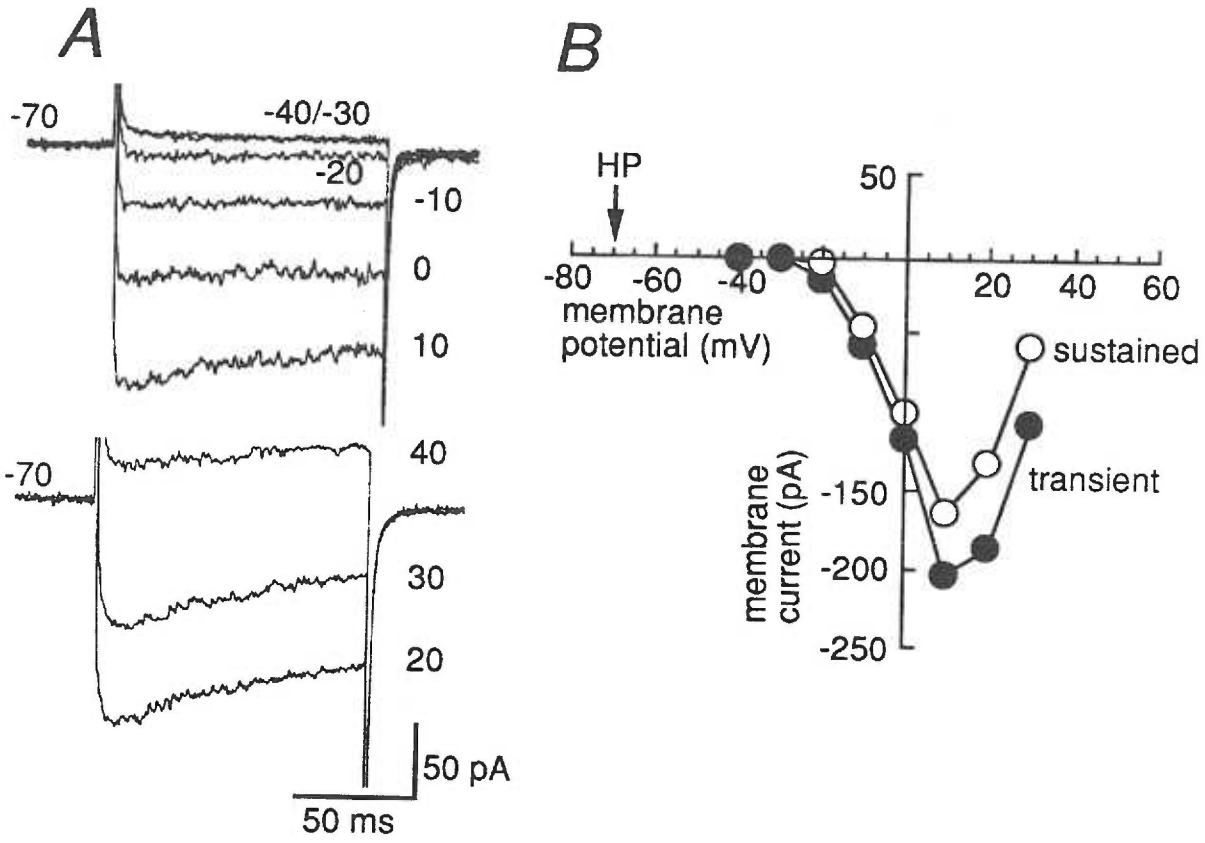


Figure 2.15 Calcium Current: Current-Voltage Relation. A, calcium currents were recorded from one AtT-20 cell in response to 120 ms depolarizing steps of 10 mV increments from a holding potential of -70 mV. B, currents from the experiment illustrated in A are shown; the maximum current amplitude in the first 10 ms (transient, filled circles) and the average current amplitude in the last 10 ms (sustained, open circles) are plotted as a function of potential. (AtT-20 6688c1).

The I-V relationship was examined in 24 cells where barium was used as the charge carrier. The transient component activated between -50 to -30 mV. The maximum amplitude of the transient component ranged from 80 pA to 1430 pA with an average of 322 ± 56 pA. The maximum amplitude occurred at -10 mV for 3 cells, 0 mV for 20 cells, and 10 mV for 1 cell. The sustained component also activated between -50 mV and -30 mV. The maximum amplitude of the sustained component ranged from 65 pA to 1070 pA with an average of 255 ± 43 pA. The maximum amplitude was measured at -10 mV in 4 cells, 0 mV in 18 cells, and 10 mV in 2 cells. The amplitudes of the transient and sustained component were significantly different ($p < 0.0001$), indicating that the barium current was subject to inactivation. The ratio amplitudes of the transient to sustained components of the current was 1.35 ± 0.06. A representative barium current trace is shown in Figure 2.16. The transient-to-sustained ratios measured in calcium and barium were not significantly different from one another ($p = 0.4657$).

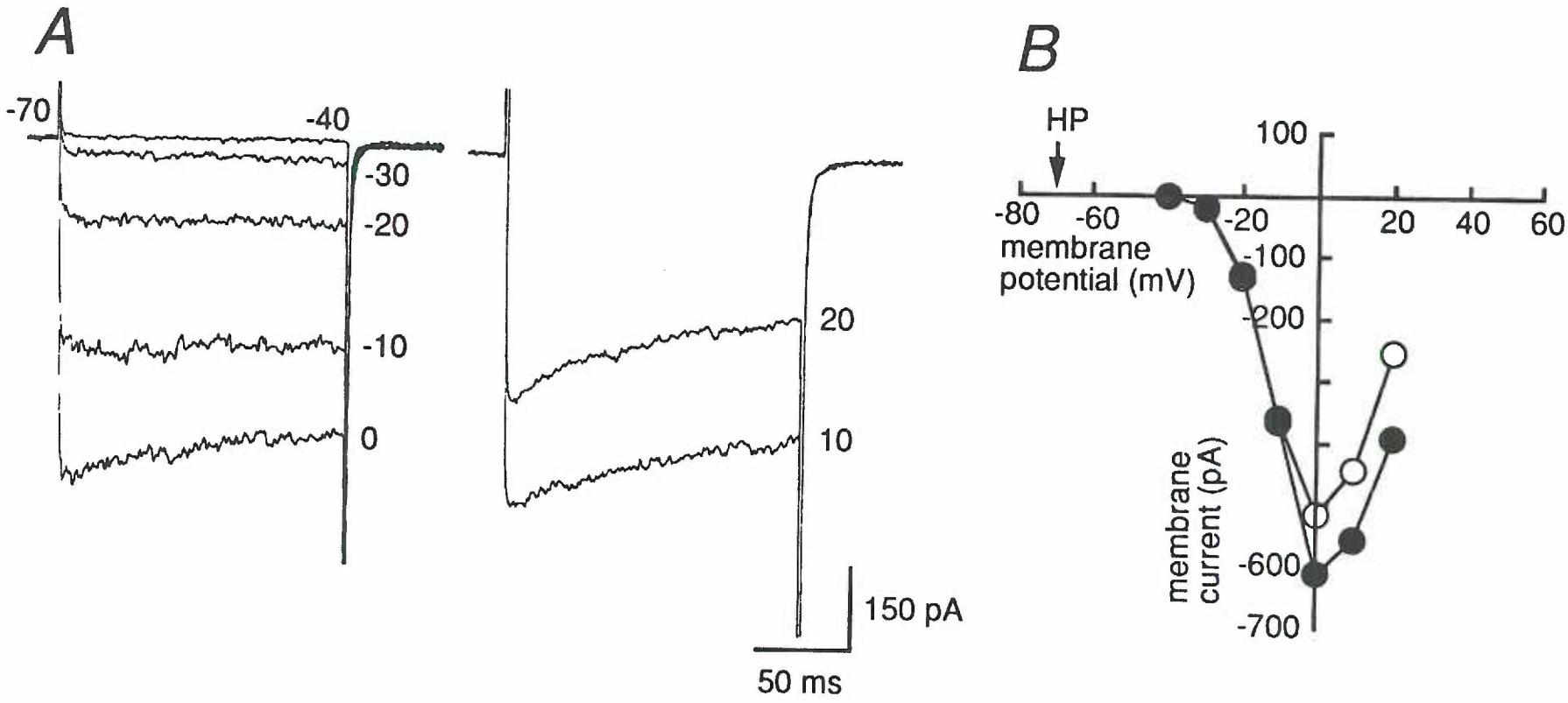
No currents were detected when the cell was held at -100 mV and voltage stepped to -50 mV, suggesting no low threshold currents are present in these cells ($n > 5$).

2.3.3(ii) STIMULATION OF CALCIUM CURRENTS

BAY K 8644 consistently enhanced the calcium currents. When calcium was used as the charge carrier, BAY K 8644 (1 μM) enhanced the transient component to 130 ± 49 % and the sustained component to 117 ± 6 % of control ($n = 2$). When barium was used as the charge carrier, BAY K 8644 enhanced the transient component to 150 ± 11 % and the sustained component to 122 ± 16 % of control ($n = 6$) (Figure 2.17).

Figure 2.16 Barium Current: Current-Voltage Relation. A, barium currents were recorded from on AtT-20 cell in response to 150 ms depolarizing steps of 10 mV increments from a holding potential of -70 mV. B, currents from the experiment illustrated in A are shown; the transient (filled circles) and the sustained (open circles) components of each trace are plotted as a function of potential. (AtT-20 7788c3).

2.16 Barium current: current voltage relation



2.17 BAY K 8644 STIMULATION OF CALCIUM AND BARIUM CURRENTS

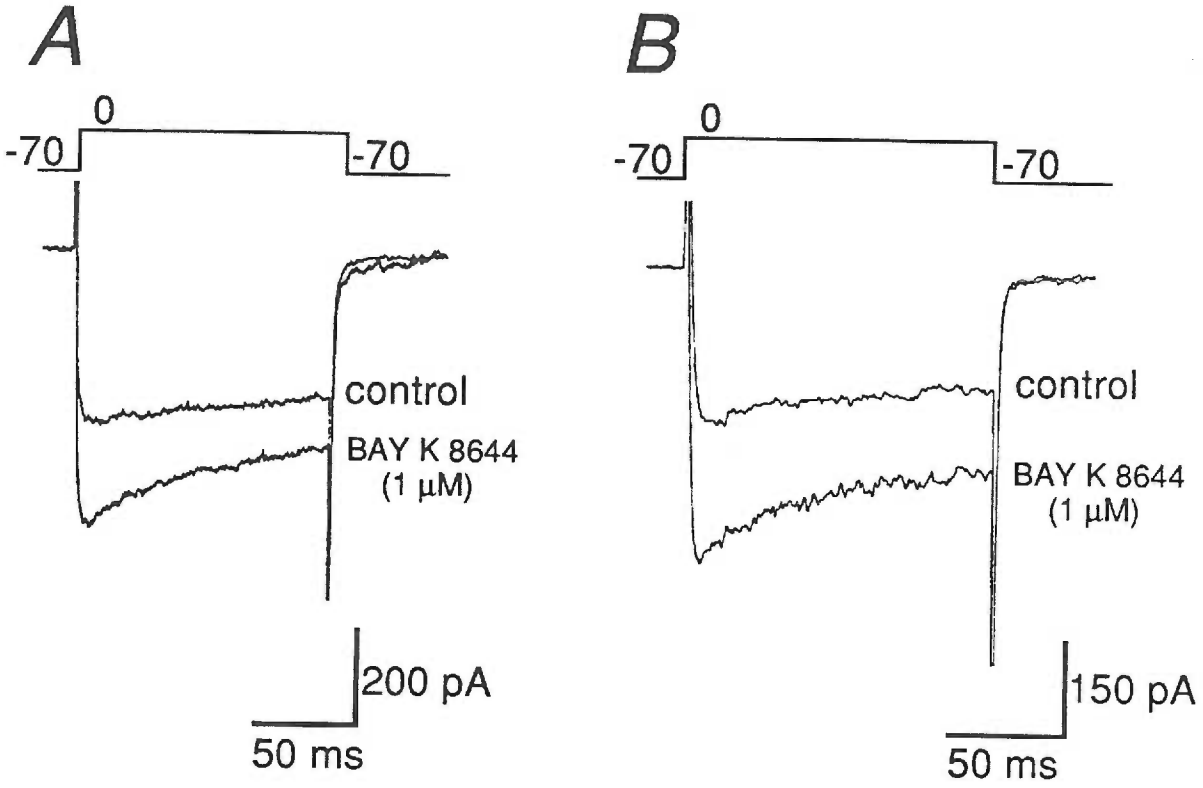


Figure 2.17 BAY K 8644 Stimulation of Calcium and Barium Currents. Currents were evoked from a holding potential of -70 mV with a 120 ms voltage step to 0 mV in the absence and presence of BAY K 8644 (1 μM). A, calcium current using calcium (5 mM) in the recording solution. (AtT-20 4789c2). B, barium current using barium (10 mM) in the recording solution. (AtT-20 7688c4).

Neither isoproterenol nor forskolin consistently enhanced the calcium or barium currents. Isoproterenol was applied to 20 cells and an increase of the current amplitude was observed in 2 cells (Figure 2.18); however, as a qualitative test to confirm that the calcium current could stimulated, BAY K 8644 was subsequently applied to these cells and the current was increased in each cell. This test indicated that the calcium currents in these cells were functional but not responsive to isoproterenol. Forskolin enhanced the current in only 1 cell out of more than 10 cells tested.

2.3.3(iii) INHIBITION OF CALCIUM CURRENTS

Nifedipine

Calcium currents were reduced by nifedipine a concentration-dependent manner as shown in a representative current trace in Figure 2.19. 10 nM-nifedipine reduced the transient component by 29 $\pm$ 19 % and the sustained component by 49 $\pm$ 17 % (n = 4). 100 nM-nifedipine reduced the transient component by 42 $\pm$ 32 % and the sustained component by 55 $\pm$ 30 % (n = 4). 1 μM-nifedipine reduced the transient component by 55.6 $\pm$ 8.6 % and the sustained component by 67.7 $\pm$ 9.5 % (n = 7). 10 μM-nifedipine reduced the transient component by 62.6 $\pm$ 9.2 % and the sustained component by 77.8 $\pm$ 6.8 % (n = 8).

Cadmium

Barium currents were inhibited by cadmium in a concentration-dependent manner (Figure 2.20); currents were completely blocked by 30 μM cadmium. 100 nM-cadmium reduced the transient component by 22.6 $\pm$ 9.7 % and the sustained component by 25.8 $\pm$ 11.6 % (n = 5). 1 μM-

2.18 Isoproterenol stimulation of calcium current

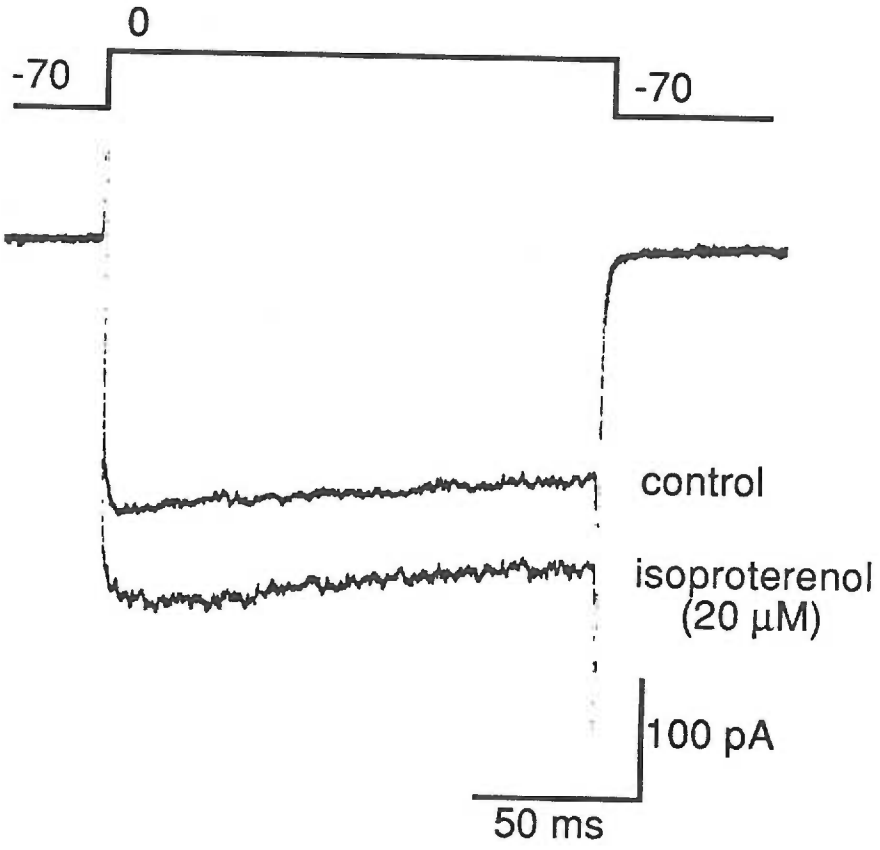


Figure 2.18 Isoproterenol Stimulation of Calcium Current. Currents were evoked from a holding potential of -70 mV with a 150 ms voltage step to 0 mV in the absence and presence of isoproterenol (20 μM). The increase in current amplitude occurred 2.5 min after application to drug to the buffer reservoir. Calcium (10 mM) was used in the recording solution. (AtT-20 7988c2).

2.19 Nifedipine inhibition of barium current

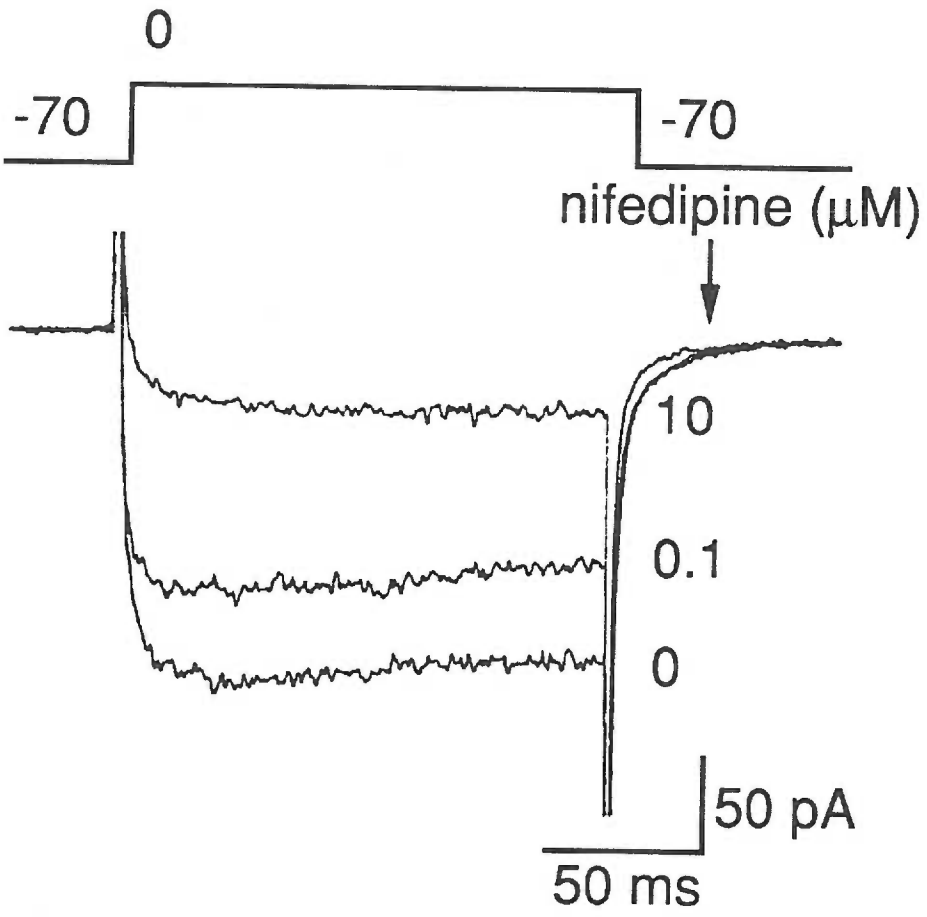


Figure 2.19 Nifedipine Inhibition of Barium Current. Currents were evoked by stepping from -70 mV to 0 mV in control solution and in increasing concentrations of nifedipine (0.1 and 10 μM). Barium (10 mM) was used in the recording solution. (AtT-20 9888c2).

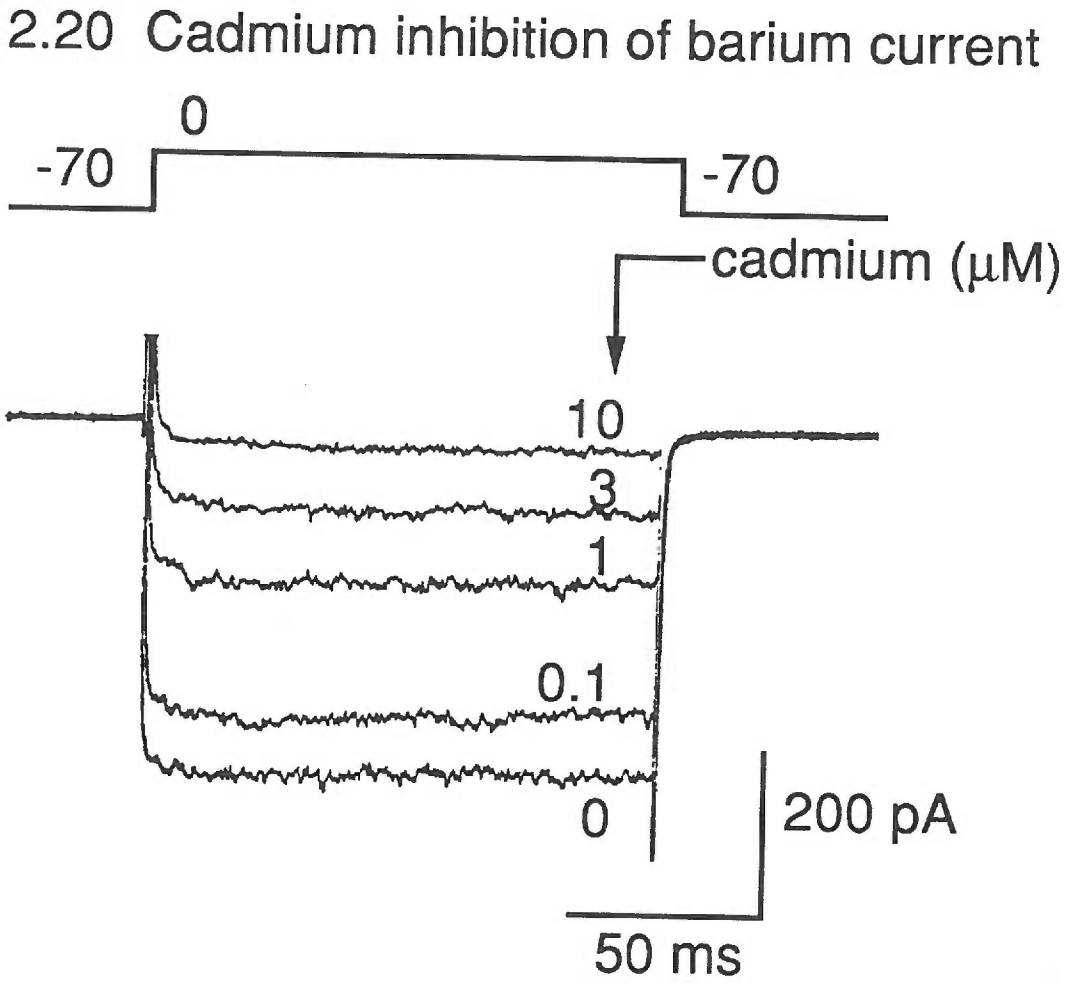


Figure 2.20 Cadmium Inhibition of Barium Current. Currents were evoked by stepping from -70 mV to 0 mV in control solution and in increasing concentrations of cadmium (0.1, 1, 3, and 10 μM). Barium (10 mM) was used in the recording solution. (AtT-20 7888c8).

cadmium reduced the transient component by 45.0 ± 9.8 % and the sustained component by 46.8 ± 10.6 % (n = 4). 3 μM-cadmium reduced the by transient 62 ± 16 % and sustained component by 62 ± 18 % (n = 2). 10 μM-cadmium reduced the transient and sustained component by 93.0 ± 5.0 % (n = 4). Calcium currents were also completely blocked by cadmium (30 μM, n = 5).

Nickel

Calcium currents were reduced in a concentration-dependent manner by nickel and were completely blocked by 100 μM nickel (n = 3; Figure 2.21).

Somatostatin

SRIF reduced both the transient and the sustained component of the calcium current in a concentration-dependent manner, but never completely inhibited the current (Figure 2.22). SRIF (100 nM) produced a maximum inhibition of the calcium current and reduced the transient component by 43 ± 8 % and the sustained component by 39 ± 9 % (n = 14).

2.4 DISCUSSION

The purpose of these studies was to examine the relationship between electrical activity and secretory activity in AtT-20 cells. Specifically, the focus was to determine whether a relationship existed between the inhibition of voltage-dependent calcium currents and the inhibition of secretion of β-endorphin. Using whole-cell patch-clamp recording, voltage-dependent calcium currents were measured in the presence and absence of agents known to reduce calcium currents and/or secretion. Secretion experiments of a short duration were used to

2.21 Nickel inhibition of barium current

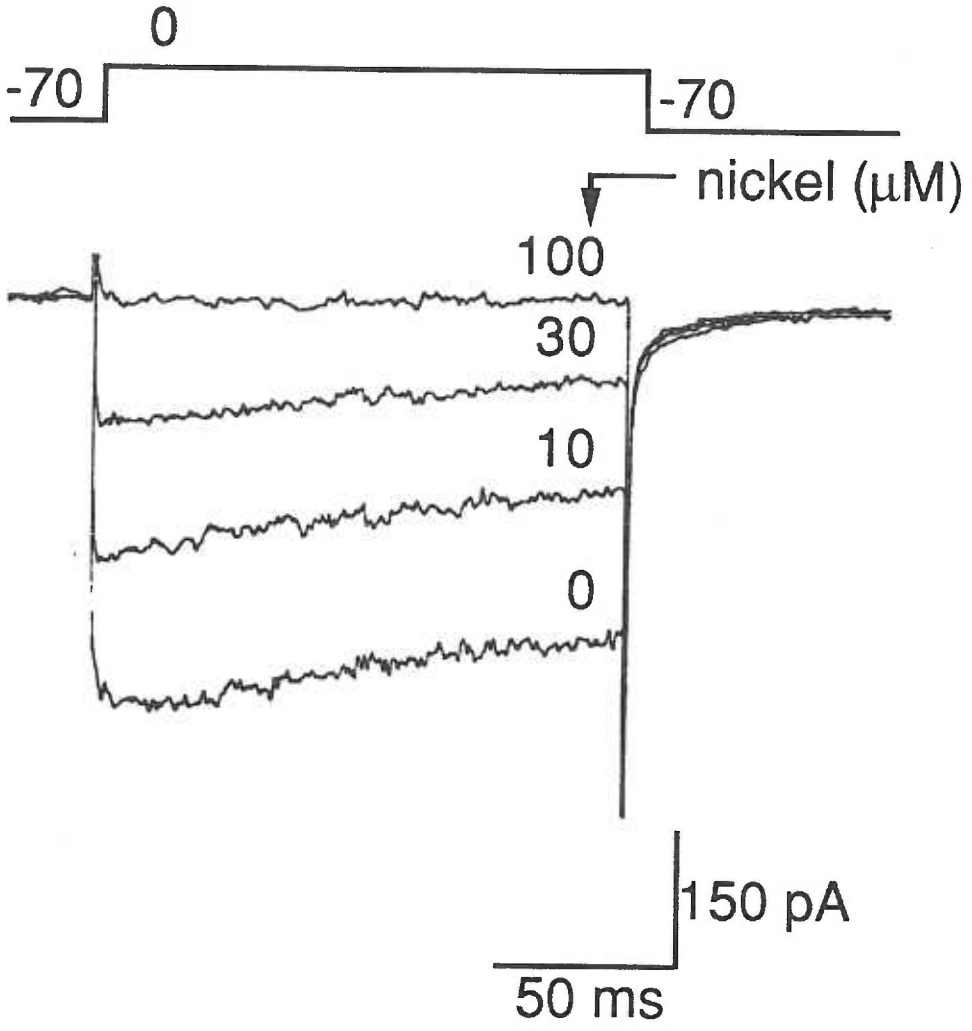


Figure 2.21 Nickel Inhibition of Barium Current. Currents were evoked by stepping from -70 mV to 0 mV in control solution and increasing concentrations of nickel (10, 30 and 100 μM). Barium (2.5 mM) was used in the recording solution. (AtT-20 121288c3).

2.22 SRIF inhibition of calcium current

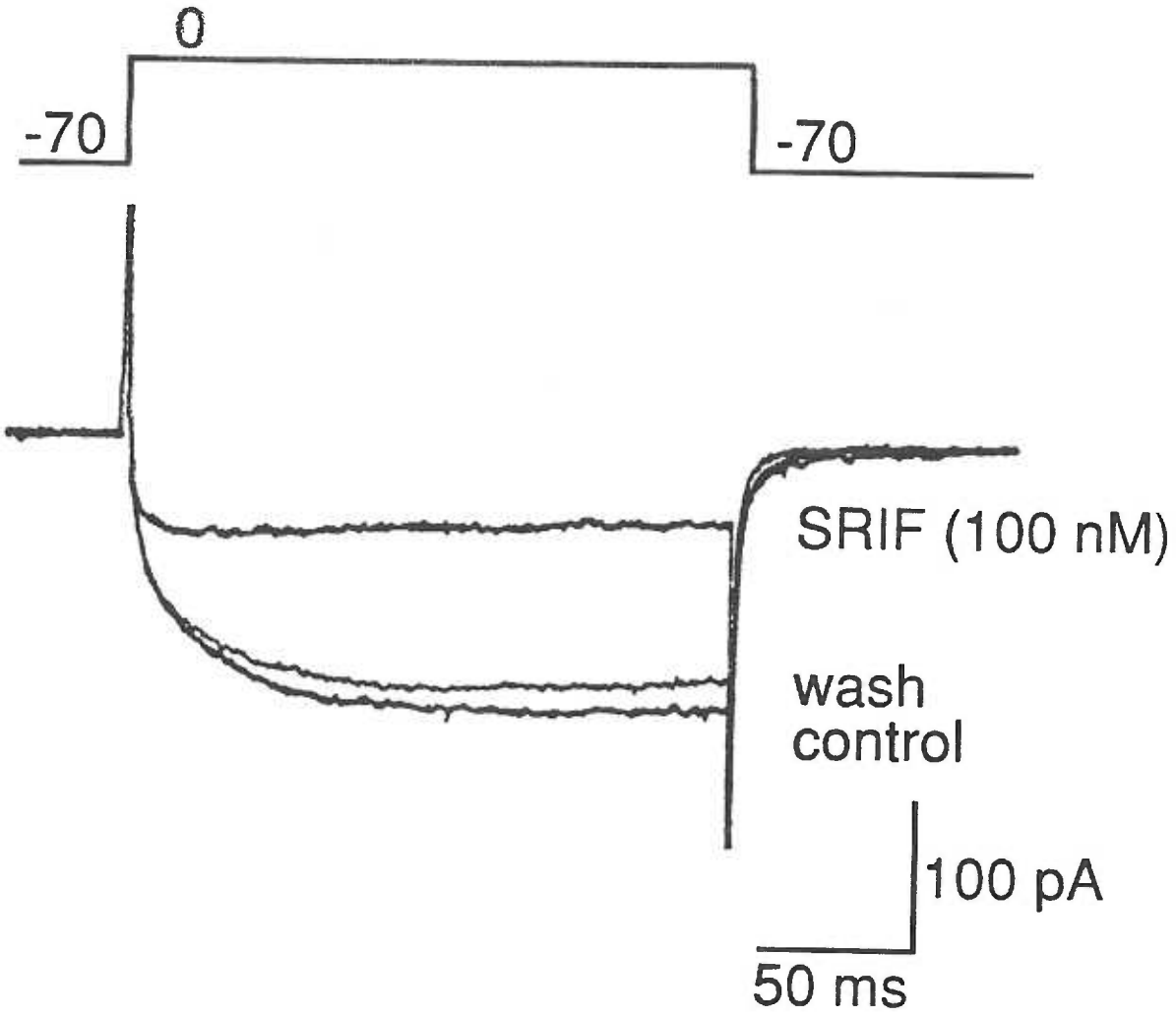


Figure 2.22 Somatostatin Inhibition of Calcium Current. Currents were evoked by stepping from -70 mV to 0 mV in the absence and presence of SRIF (100 nM). Calcium (5 mM) was used in the recording solution.

determine the specific effects of the test agents on release of hormone and avoid complicating factors such as depletion of vesicular stores of hormone, and modulation of gene regulation, protein synthesis or post-translational processing of POMC.

The effects of cadmium, nifedipine and somatostatin on the voltage-dependent calcium currents and β-endorphin secretion were measured and the concentration-response curves are be compared in this section. To determine whether the concentration curves showed any correlation, three parameters of the each curve are compared: the concentration that produced a 50 % inhibition of the response (IC_{50}), the minimum concentration that produced the maximum inhibition of the response (IC_{max}), and the maximum inhibition of the response. In summary, no pharmacological agent tested demonstrated a good correlation for all three of these parameters suggesting that a direct inhibition of voltage-dependent calcium currents may not be the primary mechanism by which inhibition of β-endorphin secretion is facilitated.

2.4.1 THE VOLTAGE-DEPENDENT CALCIUM CURRENT IN ATT-20 CELLS

Three distinct voltage-dependent calcium channels have been described in dorsal root ganglion cells (Nowycky, Fox & Tsien, 1985) and have been classified into three categories ("L", "N", and "T") based on their voltage dependence, inactivation rate, single channel conductance, and pharmacological properties (Fox, Nowycky & Tsien, 1987). Voltage-dependent calcium currents were measured in AtT-20 cells and were evaluated by similar criteria used to distinguish calcium currents in DRG cells (Table 1.1). The activation range of the current was -30 mV to

-10 mV. No current was measured when the cell was held at -100 mV and stepped to -50 mV indicating that no low threshold current ("T" current) was present in these cells. The current slowly inactivated during a 150 ms pulse; inactivation, measured as a ratio of the transient to sustained component of the current, were similar when measured in calcium (1.45) and barium (1.35). The average current amplitudes measured in cells in the presence of calcium and in cells in the presence of barium were similar; however, each experimental group had a large range and therefore it is impossible to determine in these experiments if a selectivity for either ion exists. The calcium currents were reduced by cadmium in a concentration-dependent manner and 30 μM was sufficient to completely block the current. Nickel (100 μM) also blocked this current. These currents were sensitive to dihydropyridine compounds. Nifedipine (10 μM), a calcium channel antagonist blocked 63 % of the calcium current and Bay K 8644, the calcium channel agonist, enhanced the calcium current.

The AtT-20 voltage-dependent calcium current can be described as a high threshold, slowly inactivating, cadmium and dihydropyridine-sensitive current and fits best in the "L" category for calcium currents described for DRG cells. However, the calcium current in AtT-20 cells differed from the "L" current in DRG cells in that it was more sensitive to nickel than is the "L" current in DRG cells.

Classification of this current as an "L"-like calcium current is in agreement with the single channel studies, which showed that AtT-20 cells possessed a single type of voltage-dependent calcium channel that was characterized by a high activation threshold, a large conductance, and was sensitive to dihydropyridines (Nowycky, 1987).

Nowycky (1987) also reported isoproterenol increased the channel activity by increasing the probability of channel opening. This was measured in the cell-attached configuration with isoproterenol applied to the bath (as opposed to the pipette solution) which suggests that a diffusible intracellular message system is regulating the channel activity. The isoproterenol response was observed in 14 out of 21 cells tested, which is much more consistent than the isoproterenol response measured in this studies of whole-cell currents, where only 2 out of 20 cells responded to isoproterenol by an increase in the amplitude of the current. The action of isoproterenol in heart cells is thought to act through the cAMP/protein kinase A pathway and phosphorylate the calcium channel (Belles et al. 1988) and it is postulated that only in the phosphorylated state may the channel open. Perhaps the reason that isoproterenol did not enhance the calcium current consistently in the present study is due to the whole-cell configuration itself. It is possible that critical components of the endogenous intracellular milieu needed to generate or conduct a signal from the β-adrenergic receptor to the calcium channel have been diluted or removed in the whole-cell configuration. Conversely, the phosphorylation system may be maximized with the addition of 4.5 mM ATP to the pipette solution and calcium channels are maximally phosphorylated, therefore no additional enhancement of the current can be detected with the application of isoproterenol. However, the mechanism coupling between the SRIF receptor and the calcium channel was intact and may suggest a coupling mechanism that is closely associated with the membrane rather than a diffusible messenger system. SRIF (1 μM) reduced the calcium currents by a maximum of 40 %. These results are similar to those of Lewis et al. (1986) who

found that [D-Trp$_8$]SRIF [100 nM; a supra-maximal concentration of the SRIF analog to inhibit secretion (Reisine, 1985)] inhibited the AtT-20 cell calcium current 30 %.

2.4.2 MODULATION OF β-ENDORPHIN SECRETION

The modulation of basal and stimulated secretion was examined. Basal secretion was reduced in the absence of extracellular calcium, and in the presence of magnesium (2.4 mM), nifedipine (10 μM) and SRIF (300 nM); however, it was insensitive to cadmium (500 μM), nickel (500 μM), or TTX (1 μM). β-endorphin secretion was stimulated by BAY K 8644, isoproterenol, and forskolin. However, in calcium-free conditions where extracellular calcium was omitted from the medium, these secretagogues were no longer effective stimulators of secretion. Although in these conditions the level of secretion was significantly elevated from basal secretion in zero extracellular calcium, the amount of secretion was reduced from control basal levels. Removal of extracellular calcium reverses the electrochemical gradient for calcium across the cell membrane and also affects calcium transport mechanisms. This alters the intracellular calcium concentrations in a manner different than the application of calcium channel blockers in the presence of extracellular calcium which would not reverse the electrochemical gradient for calcium. Secretion stimulated by BAY K 8644 was not reduced by TTX or nickel but was reduced by cadmium (500 μM) and SRIF. Secretion stimulated by isoproterenol also was not reduced by TTX or nickel but was reduced by nifedipine, cadmium (100 μM) and SRIF. Secretion stimulated by BAY K 8644 and isoproterenol was inhibited by SRIF in a similar concentration-dependent manner, however, isoproterenol-stimulated secretion was more

sensitive to inhibition by cadmium than was BAY K 8644-stimulated secretion. This suggests that SRIF blocked hormone release at a common site in the secretion pathway although these secretagogues had stimulated secretion initially from two different sites on the cell and that BAY K 8644 stimulates secretion by a pathway that is less sensitive to cadmium blockade compared to isoproterenol. BAY K 8644 is a lipophilic compound and may gain intracellular access and stimulate release of calcium from intracellular stores. The inhibition of secretion by SRIF was blocked by pretreatment of the cells with pertussis toxin which is known to ADP-ribosylate certain G-proteins and inactivate them. Such results indicate that activation of the SRIF receptor is coupled to the inhibition of β-endorphin secretion through a G-protein dependent process.

2.4.3 COMPARISON OF THE INHIBITION OF THE CALCIUM CURRENT AND THE INHIBITION OF β-ENDORPHIN RELEASE IN ATT-20 CELLS

To determine whether a correlation exists between the inhibition of calcium currents and the inhibition of stimulated secretion, the concentration-response curves for cadmium, nifedipine, and SRIF are compared in Figures 2.23, 2.24, and 2.25. Three parameters, the IC_{50}, the IC_{max}, and the maximum inhibition of the response, were measured from each concentration response curve and these parameters are compared in Table 2.2.

Cadmium

In Figure 2.23 the concentration-response curves of cadmium inhibition of BAY K 8644-stimulated secretion and the voltage-dependent calcium current are compared and show that there was no agreement between

2.23 Comparison of the concentrations of cadmium that inhibit ß-endorphin secretion and that reduce the calcium current

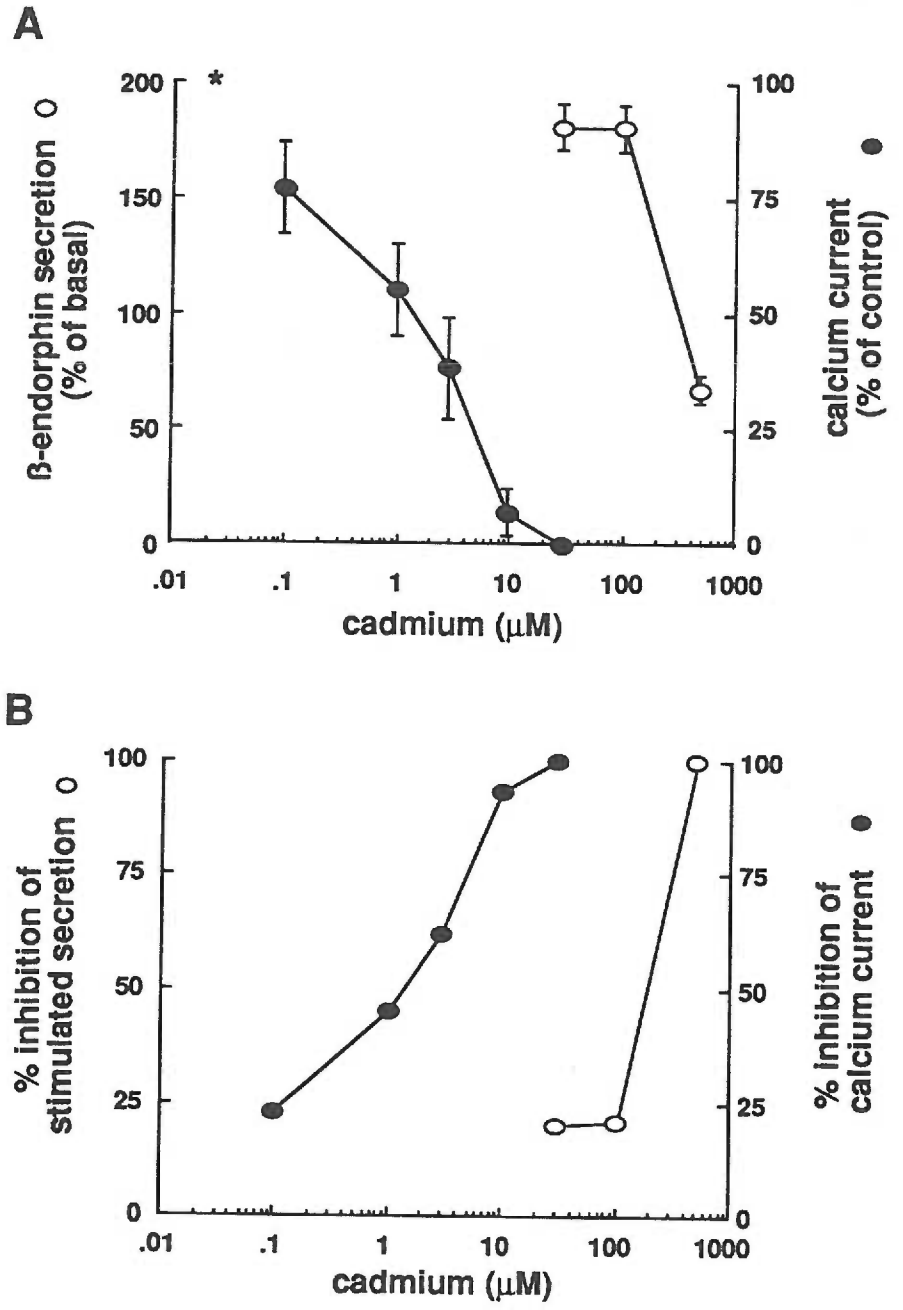


Figure 2.23 Comparison of the Concentrations of Cadmium that Inhibit β-Endorphin Secretion and that Reduce the Calcium Current. A, β-endorphin secretion stimulated by BAY K 8644 (open circles) and the calcium current (filled circles) are plotted as a function of the cadmium concentration. The * indicates the control response of secretion in the absence of cadmium. B, the data shown in A have been transformed such that the inhibition of secretion (open circles) and the inhibition of the calcium current (filled circles) are plotted as a function of the cadmium concentration.

the inhibition of the voltage-dependent calcium current and the inhibition of stimulated secretion. Concentrations of cadmium that completely blocked the voltage-dependent calcium current (30 μM) only reduced stimulated secretion 20 %. 500 μM-cadmium, more than a 10-fold increase in the concentration needed to block the calcium current, was needed to abolish stimulated secretion. The cadmium IC_{50} for inhibition of the current was 2 μM whereas the cadmium IC_{50} inhibition of secretion was 200 μM. Although cadmium completely blocked the calcium current and stimulated secretion, the concentrations needed to abolish these two activities were not equal. These comparisons demonstrate complete dissociation between the inhibition of voltage-dependent calcium current and the inhibition of secretion and suggest that the voltage-dependent calcium current measured in these experiments may not be involved in the regulation of the stimulus-secretion coupling process.

The results from similar experiments using nickel also showed a complete dissociation between the inhibition of the calcium current and secretion. 100 μM-nickel completely blocked the calcium current (Figure 2.21) but did not reduce secretion (2.10).

Nifedipine

In Figure 2.24 the concentration-response curves of nifedipine inhibition of the voltage-dependent calcium current and the inhibition of isoproterenol-stimulated secretion are compared. These concentration-response curves show little agreement. The IC_{50} for the inhibition of the current is 30 nM whereas the IC_{50} for the inhibition of secretion is 400 nM. The IC_{max} for the inhibition of secretion was 1 μM and for the inhibition of the current was 30 μM. At these concentrations nifedipine

2.24 Comparison of the concentrations of nifedipine that inhibit ß-endorphin secretion and that reduce the calcium current

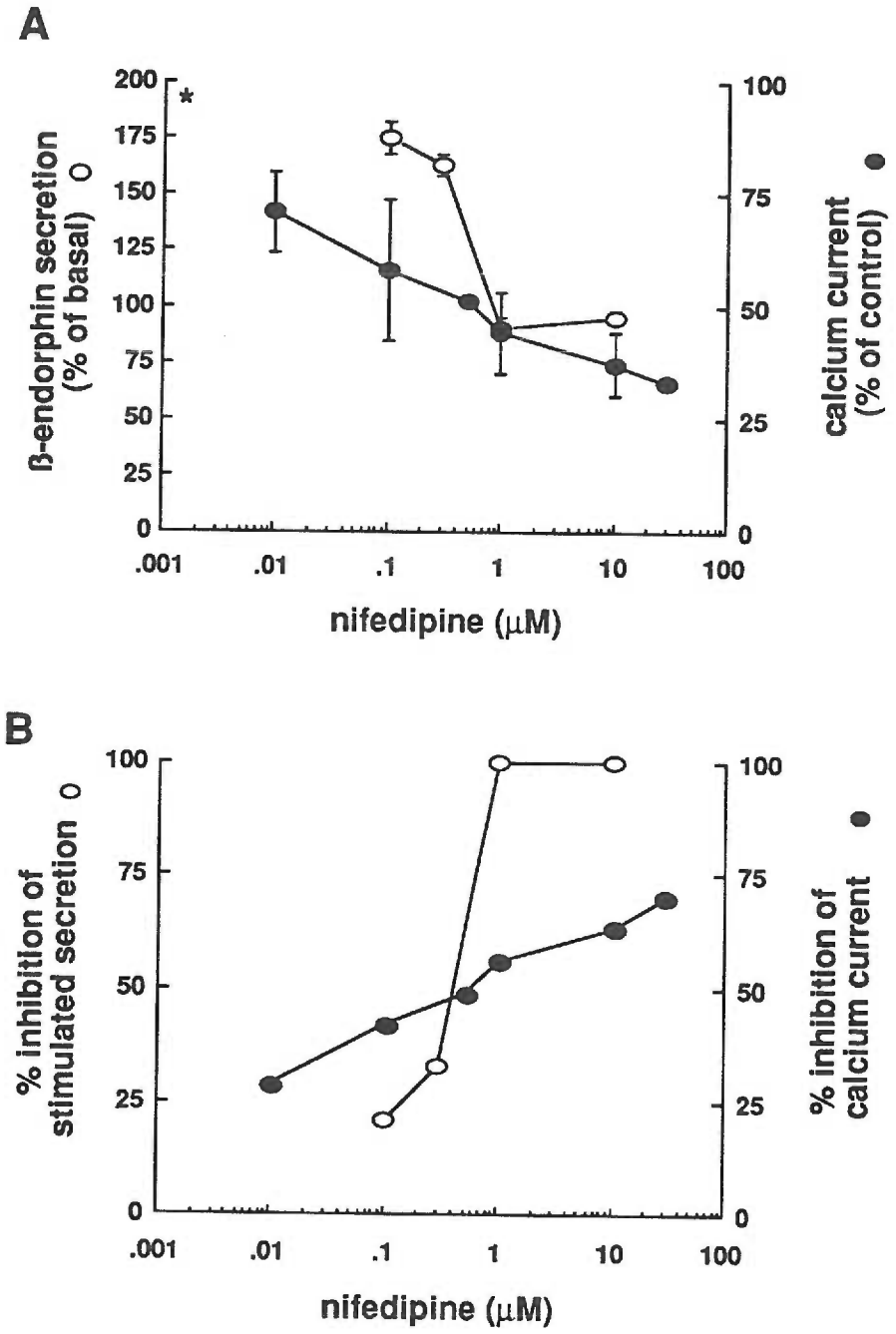


Figure 2.24 Comparison of the Concentrations of Nifedipine that Inhibit β-Endorphin Secretion and that Reduce the Calcium Current. A, β-endorphin secretion stimulated by isoproterenol (open circles) and the calcium current (filled circles) are plotted as a function of the nifedipine concentration. The * indicates the control response of secretion in the absence of nifedipine. B, the data shown in A have been transformed such that the inhibition of secretion (open circles) and the inhibition of the calcium current (filled circles) are plotted as a function of the nifedipine concentration.

completely blocked stimulated secretion however the calcium current was never abolished, maximum inhibition was 67 %. These data also show little with another and suggest that no direct relationship exists between the inhibition of secretion and the reduction of the voltage-dependent calcium current.

Somatostatin

In Figure 2.25 the concentration response curves of SRIF inhibition of the voltage-dependent calcium current and the inhibition of BAY K 8644 stimulated secretion are compared. The IC_{50} for the inhibition of stimulated secretion is about 1 nM whereas the IC_{50} for the inhibition of the calcium current is about 5 nM. The IC_{max} for each response was 100 nM. However, although SRIF reduced stimulated secretion to 90 %, it only reduced the calcium current 40 %. These data show the best agreement between the inhibition of the voltage-dependent calcium current and the inhibition of stimulated secretion of the three pharmacological agents tested. The IC_{50} and the IC_{max} are similar between responses. It may be concluded that somatostatin action in regulating the stimulus-secretion coupling process may involve a direct inhibition of the voltage-dependent calcium current; however, a complete inhibition of the current is not necessary to completely inhibit secretion. However, in view of the comparisons of concentration effects of cadmium and nifedipine on secretion and the calcium current, this may not be the only mechanism involved in the inhibition of secretion at these concentrations of SRIF. The kinetics of the rising phase of the calcium current in the presence of SRIF as shown in Figure 2.22 were slowed in some but not all cells. This could be an alternative mechanism of reducing calcium entry

2.25 Comparison of the concentrations of somatostatin that inhibit ß-endorphin secretion and that reduce the calcium current

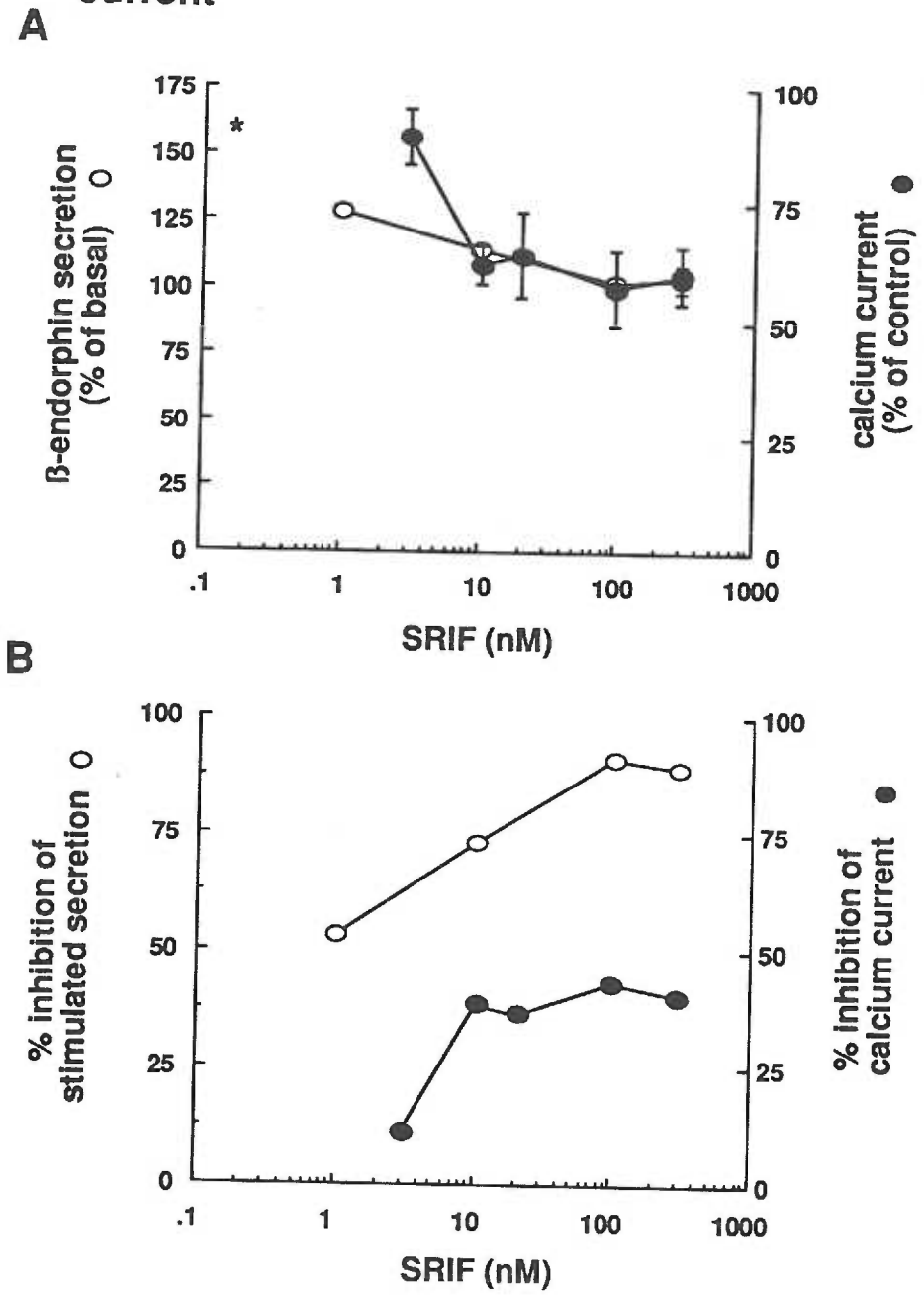


Figure 2.25 Comparison of the Concentrations of Somatostatin that Inhibit β-Endorphin Secretion and that Reduce the Calcium Current. A, β-endorphin secretion stimulated by BAY K 8644 (open circles) and the calcium current (filled circles) are plotted as a function of the somatostatin concentration. The * indicates the control response of secretion in the absence of somatostatin. B, the data shown in A have been transformed such that the inhibition of secretion (open circles) and the inhibition of the calcium current (filled circles) are plotted as a function of the somatostatin concentration.

during spontaneous activity in these cells.

2.4.4 CONCLUDING REMARKS

The results are shown in the previous three figures (2.23, 2.24, 2.25) and are summarized in Table 2.1. SRIF demonstrated the best agreement in this analysis. There was poor concordance between the concentration of cadmium that inhibited calcium currents and that inhibited secretion. Cadmium effects showed no correlation in modulating the calcium current and secretion. In fact, there is a complete dissociation of the two responses. This was an unexpected result since the known action of cadmium is strictly to block calcium currents, and in these experiments produced a concentration-dependent and complete inhibition of the calcium current whereas nifedipine and SRIF did not completely abolish the voltage-dependent calcium current. However, these agonists were more effective at blocking secretion even though they did not completely abolish the calcium current. More detailed analysis and interpretation of these results will be address in section 5.2 in conjunction with the results of the melanotroph studies.

The experiments on the AtT-20 cells provided a relatively easy cell preparation to establish workable conditions for the secretion and the electrophysiology experiments. However, the major problem in these recording studies was obtaining adequate concentration-response data. Since stable recording time for these cells was only 10-15 min and the drugs were applied by a bath perfusion technique, which had an exchange time of at least 3 min, reliable data from usually not more than two drug concentrations could be obtained from a single cell and a wash out recording after drug application was not always obtained. This problem

Table 2.1 Comparison of effectiveness of cadmium, nifedipine and somatostatin to inhibit calcium currents and ß-endorphin secretion in AtT-20 cells.

	Calcium Current Inhibition	Secretion Inhibiton
Cadmium*		
IC_{50}	2 µM	200 µM
IC_{max}	30 µM	500 µM
max inhibition	100%	100%
Nifedipine**		
IC_{50}	0.03 µM	0.4 µM
IC_{max}	30 µM	1 µM
max inhibition	67%	100%
SRIF*		
IC_{50}	0.005 µM	0.005 µM
IC_{max}	0.1 µM	0.1 µM
max inhibition	40%	90%

*Bay K 8644-stimulated secretion

**Isoproterenol-stimulated secretion

also prevented the routine application of a high concentration of a calcium channel blocker (such as cadmium) for subtraction of leak and capacitative currents. These technical difficulties were overcome by developing a micro-perfusion delivery system that allowed rapid application of known concentrations of drugs at 35°C [described in section 3.2.4(i)] and was used in the experiments carried out on melanotrophs. These experiments are the subject of Chapter 3.

The combination of these studies allow for comparisons that yielded conclusions which do not agree with conclusions from studies on neurons where blockage of the voltage-dependent calcium currents completely blocks the release of neurotransmitters (Augustine, Charlton & Smith, 1985). In AtT-20 cells, complete block of voltage-dependent calcium currents does not inhibit secretion. Similar studies on primary cultures of melanotrophs were carried out since these results may be to this tumor cell line rather than a general property of pituitary cells.

CHAPTER THREE: MODULATION OF THE VOLTAGE-DEPENDENT CALCIUM CURRENT AND STIMULUS-SECRETION COUPLING IN THE MELANOTROPH

3.1 LITERATURE SURVEY

3.1.1 INTRODUCTION

The pars intermedia or intermediate lobe (IL) of the rodent pituitary gland is located between the pars distalis or anterior lobe and pars nervosa or posterior lobe. It is composed of a thin layer of relatively homogeneous, polygonal secretory cells that interdigitate with one another (Howe, 1968; Saland, 1980). These cells, referred to as melanotrophs, synthesize POMC and post-translationally process the prohormone into peptides for export. The cells contain cytoplasmic vesicles of 20 nm diameter that are clustered near the plasma membrane and undergo exocytosis to release hormone into the extracellular space of the IL (Saland, 1980). The characteristic hormones secreted by melanotrophs are α-melanocyte stimulating hormone (α-MSH) and acetylated-β-endorphin (Ac-β-EP) (Mains & Eipper, 1979; Zakarian & Smyth, 1979). In rodents, the circulating plasma concentrations of these hormone are elevated in response to immobilization stress challenges (Kvetnansk et al. 1987) and reduced with water deprivation or 2 % NaCl replacement for drinking water (Dave, Rubinstein & Eskay, 1985). However, the biological functions of these hormones are still not understood.

The major regulation of the IL is probably through innervation since this gland is poorly vascularized (Howe, 1968). The IL is directly innervated by the tuberohypophysial neurons that project from the hypothalamus via the infundibular stalk and synapse on melanotrophs and

into extracellular spaces (Baumgarter et al. 1972; Saland, 1980). These neurons contain the neurotransmitters dopamine and γ-aminobutyric acid (GABA); they inhibit the spontaneous electrical activity and produce inhibitory post-synaptic potentials (IPSPs) in melanotrophs (Davis, Haas & Lichtensteiger, 1985; MacVicar & Pittman, 1986; Williams, MacVicar & Pittman, 1989). Excitatory input from these neurons has also been observed; however, the transmitter has not been determined (Davis, Haas & Lichtensteiger, 1985). The removal of dopaminergic input by hypothalamic lesions or administration of dopaminergic antagonists resulted in a transient reduction in the concentration of α-MSH in the IL suggesting that relief from the tonic inhibition by dopamine caused the gland secrete more hormone (Tilders & Smelick, 1978).

3.1.2 WHAT IS KNOWN ABOUT SECRETION

Secretion studies on IL with intact innervation have shown that α-MSH secretion is tonically inhibited by neurotransmitter released from nerve terminals synapsing in IL (Davis, 1986). Stimulation of the infundibular stalk reduced the secretion of α-MSH and this inhibition was abolished by transection of the stalk or antagonized by sulpiride, a D_2 antagonist, indicating that dopamine is a neurotransmitter released by stalk stimulation. In the absence of tonic inhibition, melanotrophs exhibited a high basal rate of secretion (Davis, 1986).

Secretion studies on cultured melanotrophs have identified plasma membrane receptors that are coupled to a stimulation of secretion. Isoproterenol stimulated secretion (Bower, Hadley & Hruby, 1974; Munemura, Eskay & Kebabian, 1980, Vale et al. 1983) through the activation of the β-adrenergic receptor subtype which is coupled to the

cAMP pathway (Cote et al. 1980; Munemura, Eskay & Kebabian, 1980). Corticotrophin-releasing factor (CRF) also stimulated secretion (Proulx-Ferland et al. 1982; Vale et al. 1983; Kraicer, Gajewski & Moor, 1985) in a cAMP-dependent manner (Meunier et al. 1982). GABA has a biphasic effect on secretion in which the initial response to GABA is stimulatory and can be attributed to the activation of a $GABA_A$ receptor (Tomiko, Taraskevich & Douglas, 1983; Demeneix et al. 1984) followed by an inhibitory phase attributed to the activation of $GABA_B$ receptors (Demeneix et al. 1984).

Agents that bypass the plasma membrane may also regulate the secretory rate of these cells. Forskolin-stimulated secretion in a cAMP-dependent manner (Miyazaki, Goldman & Kebabian, 1984). Phorbol ester treatment for 8 h stimulated release; however, long term treatment (24 h) inhibited release. This biphasic response correlates well with the kinetics of the movement of PKC activity from the cytosol to the membrane (Loeffler et al. 1989). Application of the calmodulin (CAM) antagonists, W7 and W13, reduced basal secretion 50 % (Loeffler et al. 1989). At least two pathways involving two different calcium-binding proteins, CAM and PKC, are involved in the conduction of the stimulus-secretion mechanism. However the site of convergence of these two pathways is not known.

Application of high potassium (Tomiko, Taraskevich & Douglas, 1981; Keith et al. 1983), barium (Douglas, Taraskevich & Tomiko, 1983), the calcium channel agonist, BAY K 8644 (Taraskevich & Douglas, 1986), veratridine (Tomiko, Taraskevich & Douglas, 1984; Zein, Lutz-Bucher & Koch, 1986) and the ionophore A23187 (Zein, Lutz-Bucher & Koch, 1986) all stimulated secretion of α-MSH and β-endorphin. Secretion could be

reduced by removing extracellular potassium (Keith et al. 1983), suggesting that a hyperpolarization may reduce secretion. Extracellular calcium is also important for secretion since removal of extracellular calcium resulted in a decrease of basal secretion by 35-50 % in ten min (Tomiko, Taraskevich & Douglas, 1981; Taraskevich & Douglas, 1986). Extracellular calcium was also necessary when veratridine (Tomiko, Taraskevich & Douglas, 1984; Zein, Lutz-Bucher & Koch, 1986), high potassium (Tomiko, Taraskevich & Douglas, 1981), BAY K 8644 (Taraskevich & Douglas, 1986), and the calcium ionophore A23187 (Zein, Lutz-Bucher & Koch, 1986) used to stimulate secretion. Further, nimodipine, a dihydropyridine calcium channel blocker, reduced 100 mM K^+-stimulated release (Taraskevich & Douglas, 1986). TTX, a sodium channel blocker, did not affect basal secretion (Tomiko, Taraskevich & Douglas, 1984) or BAY K 8644-stimulated secretion (Taraskevich & Douglas, 1986) but did inhibit veratridine-stimulated secretion (Tomiko, Taraskevich & Douglas, 1984, Zein, Lutz-Bucher & Koch, 1986).

Both basal and stimulated secretion of α-MSH can be inhibited by dopamine (Munemura, Eskay & Kebabian, 1980; Miyazaki, Goldman & Kebabian, 1984, Beaulieu, Felder & Kebabian, 1986) through the activation of the D_2 receptor subtype (Frey et al. 1982; Beaulieu, Felder & Kebabian, 1986). Dopamine will inhibit the increased levels of cAMP stimulated by isoproterenol (Munemura, Eskay & Kebabian, 1980, Cote et al. 1985) or forskolin (Miyazaki, Goldman & Kebabian, 1984). However, although dopamine inhibits basal secretion, it has no effect on basal levels of cAMP (Munemura, Eskay & Kebabian, 1984). This suggests that dopamine may exert its inhibitory control on stimulus-secretion coupling by more than one mechanism; a cAMP-dependent and a cAMP-independent mechanism.

These reports seem to come to the consensus that extracellular calcium is necessary for secretion, both basal and stimulated. The mechanisms involved in calcium influx may involve voltage-dependent calcium currents since agents which are known to depolarize the membrane also enhance secretion. Secretion in the presence of calcium may be facilitated by second messengers such as cAMP. However a direct correlation does not exist when comparing the physiological response of secretion with the biochemical changes. For instance, isoproterenol, which activates the β-adrenergic receptor, stimulated secretion and the formation of cAMP. However, a 75-fold difference exists between isoproterenol stimulation of cAMP accumulation (EC_{50} = 30 nM) and enhancement of α-MSH release (EC_{50} = 0.4 nM) (Munemura, Eskay & Kebabian, 1980). Secretion can be inhibited by agents known to block calcium channels or produce a membrane hyperpolarization suggesting that reduction of calcium influx will inhibit hormone secretion. Dopamine inhibits cAMP-dependent secretion and reduces elevated levels of cAMP; however, concentration-response curves of dopamine inhibition of secretion and inhibition of cAMP formation are not published. Therefore, it can not be determined whether inhibition of the cAMP formation can account for an inhibition of secretion by dopamine.

Experiments performed on permeabilized cells have shown that secretion is dependent on calcium, Mg-ATP and temperature (Yamamoto et al. 1987a). Secretion was stimulated by calcium in a concentration-dependent manner and 1 mM calcium had a maximum effect. This rate of secretion could be further enhanced with the addition of 1 mM Mg-ATP and when experiments were done at 37°C compared to room temperature. Importantly however, the calcium concentrations needed to stimulate

secretion in the permeabilized cell preparation is more than 1000-fold greater than the measured levels in intact cells (section 3.1.3). This difference may be due either to the disruption of the intracellular structure due to the permealbilization procedure such that the secretory apparatus may not be as sensitive to calcium or, conversely, the measurements of intracellular calcium under estimate the local concentrations of calcium needed to support secretion. However, this system does allow the investigation of the mechanisms involved in secretion distal to the influx of calcium since secretion may be stimulated by other molecules. GTPγS and cAMP enhance secretion in a calcium and magnesium-dependent manner (Yamamoto et al. 1987b) indicating that G-proteins and cAMP-dependent processes are involved in the stimulus-secretion response distal to calcium entry.

3.1.3 WHAT IS KNOWN ABOUT INTRACELLULAR CALCIUM

Measurements of intracellular calcium with Fura-2 have shown that the resting concentrations of calcium are approximately 200 nM and this may be increased by application of veratridine (10 μM and 100 μM; increased intracellular calcium by 77 % and 131 %, respectively), 60 mM K^+ (increased intracellular calcium 390 %), and BAY K 8644 (1 μM; increasing intracellular calcium 60 %) (Nemeth, Taraskevich & Douglas, 1990). Elevated levels of calcium induced by any of the above mentioned stimulators, were blocked by EGTA present in the extracellular solution indicating the rise in intracellular calcium may be due to influx of extracellular calcium. Nimodipine also blocked the rise in intracellular calcium. TTX had a small, transient inhibitory effect on basal calcium levels and only antagonized the veratridine effect and did not reduce

the stimulated levels of calcium induced by high potassium or BAY K 8644 treatment (Nemeth, Taraskevich & Douglas, 1990).

These results agree in a general fashion with secretion experiments i.e. agents that stimulate intracellular calcium levels stimulate secretion and agents that reduce calcium levels inhibit secretion. Presumably if each of these agents stimulates secretion strictly by elevating intracellular calcium it would provide a good model to determine quantitatively whether a concentration-dependent relationship exists between the levels of intracellular calcium and secretion. Unfortunately it is impossible to construct concentration dependent comparisons between rises in intracellular calcium and stimulation of secretion from the published data because the data does not exist. For instance, Bay K 8644 (1 μM) elevated calcium 60 % and stimulated secretion 300 % (Taraskevich & Douglas, 1986), whereas 10 μM veratridine elevated calcium 77 % (similar to 1 μM BAY K 8644) and yet 20 μM veratridine was reported to stimulate a "small but evident" amount of hormone release (Tomiko, Taraskevich, Douglas, 1984). Similarly, no concentration response data is available for potassium concentrations and the concomitant effect on either intracellular calcium or secretion activity.

3.1.4 WHAT IS KNOWN ABOUT THE ELECTROPHYSIOLOGY

Melanotrophs have been shown to be electrically excitable and have been reported to have an average resting membrane potential of -65 mV (Douglas & Taraskevich, 1978; Taraskevich & Douglas, 1985). Extracellular electrical recordings from melanotrophs demonstrated spontaneous action potentials with a duration of 5-10 ms and a frequency

of 1.6 ± 0.2 Hz (Douglas & Taraskevich, 1978; Davis, Haas & Lichtensteiger, 1985). Dopamine (1 μM) (Douglas & Taraskevich, 1978; Douglas & Taraskevich, 1982) and baclofen (100 μM) (Taraskevich & Douglas, 1985) reduced the spontaneous activity of these cells. The frequency of the spontaneous activity was enhanced by application of high potassium (15 mM) or replacement of calcium with barium (2 mM) (Douglas & Taraskevich, 1982). Spontaneous voltage spike amplitude but not spike frequency was considerably decreased by TTX (Douglas & Taraskevich, 1978). Extracellular calcium was also found to be important for spontaneous activity. For instance, blockade of currents with other divalent cations (cobalt, nickel, manganese; 1 mM) or a reduction of the extracellular calcium concentration immediately reduced the spontaneous activity frequency (Douglas & Taraskevich, 1982) and abolished the small TTX insensitive voltage spikes (Douglas & Taraskevich, 1980; Stack, Surprenant & Allen, 1987). Application of the calcium channel blockers, verapamil, D600 and nifedipine, reduced the amplitude of the spontaneous voltage spikes (Douglas & Taraskevich, 1982).

Intracellular recordings of evoked spikes showed a TTX-sensitive and a component which is blocked by cobalt (10 mM) or nickel (10 mM) (Douglas & Taraskevich, 1980), dopamine (100 μM) or nifedipine (100 μM) (Douglas & Taraskevich, 1982), presumably a calcium-dependent component. BAY K 8644 prolonged the duration of the evoked spike, even in the presence of TTX; this appeared to be due to an enhancement of the calcium component because it could be blocked by cobalt (5 mM) or nimodipine (1 μM) (Taraskevich & Douglas, 1989).

Although single channel recording of voltage-dependent calcium channels has yet to be reported in rat melanotrophs. However, tail

current analysis in which the time constants (τ) for the closing kinetics of the calcium current has been reported and two types of calcium currents have been identified in cultured cells (Cota, 1986; Stanley & Russell, 1988). A slow deactivating calcium current (SD, τ = 1.8 ms) that was activated at -40 mV and exhibited inactivation is similar to the high threshold "N" current described in DRG neurons (Fox, Nowycky & Tsien, 1987). A fast deactivating calcium current (FD, τ = 0.11 ms) that was activated at -5 mV and exhibited relatively little inactivation is similar to the high-threshold "L" current described in DRG cells. Bay K 8644 enhanced the calcium current and shifted its activation threshold toward the resting potential (Taraskevich & Douglas, 1989).

William et al. (1990) separated two calcium currents by voltage-dependence in an acute preparation of the IL. These currents are classified as a low-threshold, transient current and a high-threshold, slowly inactivating current. The amplitude of the low threshold calcium current was reduced by the dopamine D_2 agonist, quinpirole (5 μM), or following electrical stimulation of the infundibular stalk. These reductions in the calcium current were blocked by pertussis toxin pretreatment (Williams, MacVicar & Pittman, 1990).

Dopamine produces other effects on the electrical activity of melanotrophs. It increases a potassium conductance (Stack, Surprenant & Allen, 1987; Williams, Pittman & MacVicar, 1987). The activation of the potassium conductance is blocked by pertussis toxin pretreatment (Williams, Pittman & MacVicar, 1987) indicating that the mechanism involves a G-protein. Chronic treatment (2-4 days) of cultured melanotrophs with bromocriptine, a D_2 agonist, reduced the proportion of the FD current with no effect on the SD current (Cota & Armstrong, 1987).

These results suggest that dopamine can selectively regulate calcium channel activity at the level of gene expression.

In the acute intact preparation of the infundibular stalk and the IL, GABAergic neurons have also been shown to synapse in the IL and produce synaptic potentials resulting from an increase in chloride conductance, which can be blocked by the $GABA_A$ antagonist, bicuculline (Williams, MacVicar & Pittman, 1989). GABA effects have also been seen in cultured melanotrophs where GABA evoked macroscopic currents that were identified as chloride currents; these were also blocked by bicuculline, indicating the presence of a functional $GABA_A$ receptor (Taraskevich & Douglas, 1985; Kehl, Hughes & McBurney, 1987). On group reported that baclofen, a $GABA_B$ agonist, reduced the amplitude of voltage-dependent calcium currents (Taleb et al. 1986).

3.1.5 STIMULUS-SECRETION HYPOTHESIS

The spontaneous electrical activity of the melanotroph has been postulated to be involved in the support of hormone secretion; however, suppression of the high amplitude voltage spikes with TTX does not reduce basal secretion. Except in the instance of stimulation of hormone release by veratridine, TTX is ineffective at reducing stimulated secretion. Thus the high amplitude voltage spikes of the spontaneous activity seems to have no significant role in regulating secretion. The evidence from secretion studies indicates that extracellular calcium is mandatory to maintain basal secretion and support stimulated secretion. The low amplitude spikes of the spontaneous activity which are calcium-dependent may have a significant role in supporting secretory activity. The experiments with BAY K 8644 have shown that this drug can increase

calcium influx by two mechanisms. First, BAY K 8644 increases the duration of the evoked spike and, second, it shifts the activation curve of the voltage-dependent calcium current towards the resting potential (Taraskevich & Douglas, 1989). This would allow more calcium entry at the resting potential which could enhance the secretory activity of the cells.

High concentrations of extracellular potassium depolarized the melanotroph cell membrane (Douglas & Taraskevich, 1978) in a calcium-dependent manner, stimulated spontaneous activity (Douglas & Taraskevich, 1982) and secretion (Tomiko, Taraskevich & Douglas, 1981). This stimulation of secretion by high potassium was transient and returned to basal levels within 5 min.

Barium also depolarized the cell membrane (Douglas & Taraskevich, 1980) in a calcium-independent manner, stimulated spontaneous action potential activity (Douglas & Taraskevich, 1982) and secretion (Douglas, Taraskevich & Tomiko, 1983). The time course of the secretory response to barium are very different from that to high potassium in that the barium response stimulated secretion, which does not return to basal level even after a 10 min exposure. The addition of calcium in the presence of barium decreased barium-stimulated release. Barium is conducted through calcium channels and facilitates secretion (Douglas, Taraskevich, Tomiko 1983), however, it blocks potassium currents (Ruby, 1988). Therefore, during exposure to barium (in the absence of calcium), cells may be maintained depolarized due to the blockade of potassium current and thus voltage-dependent calcium channels may remain open until they inactivate themselves, rather than being deactivated by a repolarization of the membrane. In the presence of barium and calcium,

the current conducted through the voltage-dependent calcium currents may be reduced due to the activation of a calcium-dependent potassium current which could repolarize the cell. Thus, it can be postulated that a calcium-dependent potassium current may be involved in the regulation of membrane potential and calcium entry in melanotrophs.

The channels involved in the low amplitude spontaneous activity have not been identified. The activation potential of the SD, low threshold current is -40 mV is closer to the resting membrane potential (-45 mV to -60 mV) and could be responsible for maintaining calcium entry for basal secretion. However, no conclusive evidence to date has been reported to support this idea.

Inhibition of secretion by activating the D_2 receptor may be accomplished by a variety of mechanisms given that dopamine and D_2 agonists have been shown to have a variety of electrophysiological effects. Dopamine has been shown to directly inhibit voltage-dependent calcium currents in dorsal root ganglia (Marchetti, Carbone & Lux, 1986), lactotrophs (Lledo et al. 1990a) and melanotrophs (Williams, MacVicar & Pittman, 1990). Dopamine has also been shown to hyperpolarize the membrane potential by the activation of a potassium conductance in neurons from the substantia nigra (Lacey, Mercuri & North, 1987), in lactotrophs (Lledo et al. 1990b), and in melanotrophs (Stack, Surprenant & Allen, 1987). This hyperpolarization could indirectly reduce calcium entry by moving the membrane potential out of the range of activation of the calcium currents. However, it has not been shown whether one or both of these two actions of dopamine are responsible for regulation of secretion. Concentration-response curves of the changes in the electrical activity and secretion activity have not been reported.

Therefore, a comparative study of concentration-response curves of each of these activities may elucidate which action of the drug is the primary physiological mechanism in the regulation of secretion in melanotrophs. The experiments described herein were designed to investigate this question.

3.2 METHODS

3.2.1 TISSUE CULTURE

All cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, formula # 430-1600, with 44 mM $NaHCO_3$ and 22 mM D-glucose final concentration) supplemented with 10% heat inactivated horse serum (Gibco) without antibiotics, and maintained in a humidified environment at 37°C under 5 % CO_2 / 95 % O_2.

3.2.1(i) MELANOTROPH DISPERSAL

Neurointermediate lobes (NIL) of the pituitary were dissected from adult male Sprague Dawley rats (150-220 g) supplied by Banton and Kingman and collected in a HEPES buffer, (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2PO_4, 10 mM D-glucose, 25 mM HEPES, titrated to pH 7.4 with NaOH). The NILs were diced into quarters and the tissue fragments were transferred to a 15 ml plastic conical test tube for centrifugation at 1000 rpm for 5 min. The tissue pellet was resuspended in a collagenase buffer [HEPES buffer with 2 mg/ml collagenase type V (Sigma), 4 mg/ml BSA (Sigma), and 20 μg/ml DNAse I (Sigma)] at 0.5 ml/NIL and incubated for 30 min in 37°C water bath. The tube was manually agitated 3-5 times during the incubation. Using a pasteur pipet with a small bore obtained by fire

polishing its tip, the tissue fragments were triturated 5-10 times without forming bubbles; this resulted in a partial dispersal. Trypsin buffer [HEPES buffer with 0.5 mg/ml trypsin (Sigma) and 4 mg/ml BSA; 0.5 ml/NIL] was added to the tissue fragment solution, incubated for 15 min in the 37°C water bath and agitated a few times during the incubation. Final dispersal of the cells was obtained by trituration, about 25 times. The cells were collected by centrifugation at 1000 rpm for 5 min. The cell pellet was washed once with zero Ca^{2+} DMEM (Gibco, formula # 81-5210 supplemented with 44 mM $NaHCO_3$, 22 mM D-glucose, 4 mM KCl, and 0.5 mg/ml BSA) and resuspended to the appropriate volume with zero Ca^{2+} DMEM. For secretion experiments, 12 dispersed NILs were resuspended in 900 μl of zero Ca^{2+} DMEM and plated into 36 poly-L-lysine coated microwell Nunc plates [see section 3.2.1(ii)]. 25 μl of the cell suspension (or approximately 1/3 NIL) was plated in each well. After one hour, 75 μl of normal DMEM was added to each well. The medium was changed every three days. Secretion experiments were performed on day seven. For electrophysiological experiments, the cell pellet was resuspended in 100 μl zero Ca^{2+} DMEM per NIL and 25 μl aliquots of the cell suspension were plated on a poly-L-lysine coated coverslips [see section 3.1.1 (ii)]. One hour later, 45 μl of normal DMEM was added to each coverslip avoiding spillage of DMEM over the coverslip edge. Finally, 1 hour later, the plates were flooded with normal DMEM and the medium was changed every three days, or the day before the experiments. Experiments were carried out on cells cultured for 5-8 days.

3.2.1(ii) COVERSLIP AND CULTURE DISH PREPARATION

To facilitate the adherence of the melanotrophs to the glass coverslips and the culture dishes, these surfaces were pretreated with poly-L-lysine (hydrobromide, 260 kd; Sigma). Glass coverslips prepared as described in section 2.2.1(ii) and the culture dishes used in secretion experiments were pretreated with a 0.05 % poly-L-lysine solution (wt/vol; g/100 ml) for 30 min at room temperature, rinsed with sterile H_2O, and exposed to ultraviolet light for 15-20 min.

3.2.2 IMMUNOCYTOCHEMISTRY

The same protocol that was used for the immunocytochemical staining of β-endorphin in AtT-20 cells (section 2.2.2) was used on primary cultures of cells from the anterior lobe and intermediate-posterior lobe of the pituitary cultured as described for electrophysiological studies in section 3.2.1(i).

3.2.3. SECRETION EXPERIMENTS

The protocol for the culture period and secretion experiments is shown in Figure 3.1. The medium was changed the day before the experiment. On the day of the experiment, cells were washed with DMEM (50 μl) 5 times in a 90 min period; the subsequent 2 h was divided into 4 equal periods. During the first, second, and fourth periods the cells were exposed to normal DMEM, and during the third period they were exposed to the experimental treatment. At the end of each period, the DMEM was removed and replaced with DMEM (or DMEM and appropriate drugs in the case of the third period). The collected samples were centrifuged to remove debris and a 30 μl aliquot was immediately assayed for β-

3.1 Secretion protocol

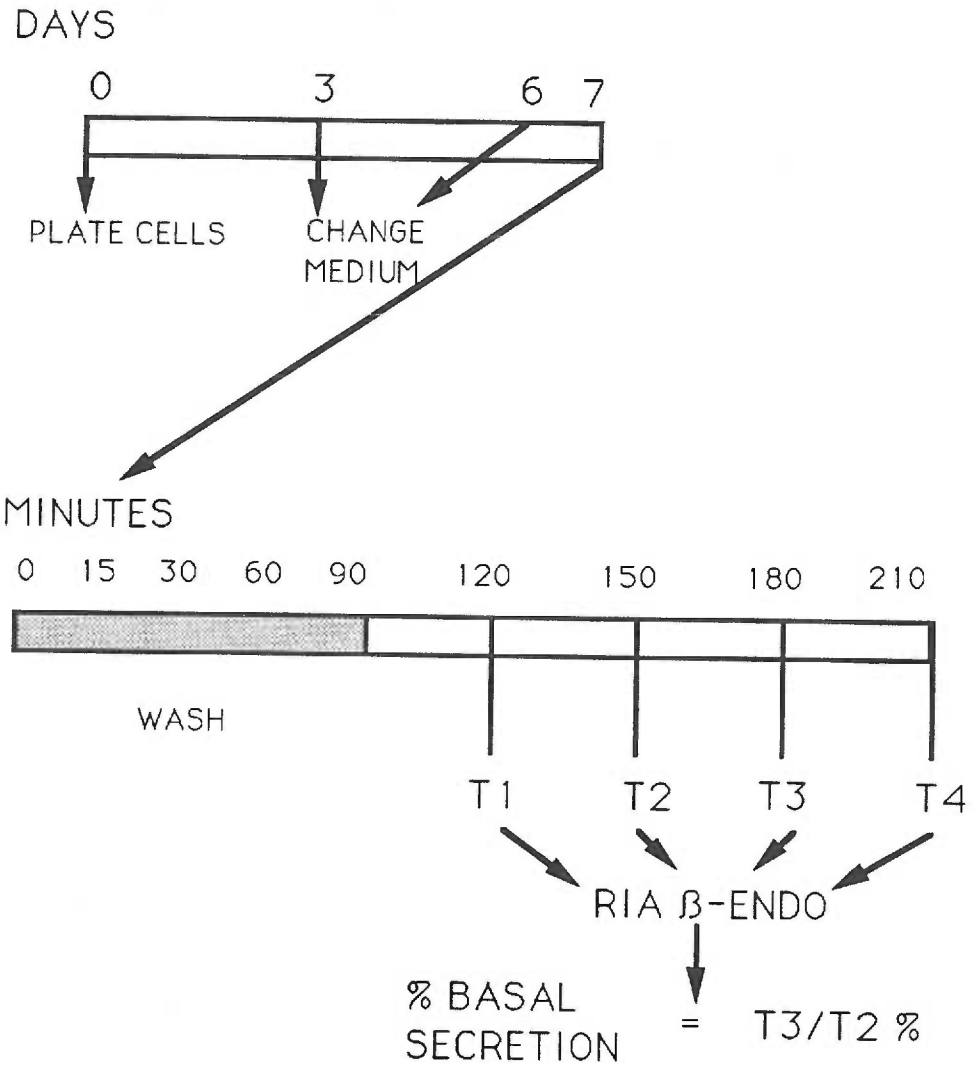


Figure 3.1 Secretion Protocol. Melanotrophs were plated onto poly-L-lysine coated microwell plates (day 0) and grown in 100 μl of DMEM. The DMEM was changed on day 3 and day 6 and the secretion experiment was performed on day 7. Prior to the experimental collection period, the cells were washed with DMEM (50 μl) 5 times in 90 min (at 1, 2, 15, 30, 60, and 90 min). The subsequent 2 h was divided into fout 30 min periods; at each period all DMEM (50 μl) was removed and processed and replaced with fresh DMEM (50 μl). Control DMEM was applied during the first (T1), second (T2) and fourth (T4) periods and test DMEM was applied during the third (T3) period. Samples were assayed for β-endorphin immunoactivity by RIA and the data are expressed as per cent of basal secretion as described in the text.

endorphin immunoactive material. The assay for β-endorphin immunoactive material is described in section 2.2.4.

3.2.4 ELECTROPHYSIOLOGY

Whole-cell patch-clamp recording was performed on melanotrophs as described in section 2.2.5(iii) and 2.2.5(iv). The pipettes were filled with a cesium solution containing (mM): 140 Cs gluconate, 10 NaCl, 10 EGTA, 5 HEPES, 4.5 MgATP, 100 μM GTP and adjusted to pH 7.4 with CsOH and 315 mmol/kg with Cs gluconate (1 M) prior to being used for recording. The extracellular solution was composed of (mM): 166 NaCl, 10 D-glucose, 3 CsCl, 5 HEPES, and 2.5 $CaCl_2$, 0.025-1 μM TTX and adjusted to pH 7.4 with NaOH and 320 mmol/kg (Figure 3.2A).

Melanotrophs that were grown on coverslips were placed in the 35-37°C recording chamber (Figure 2.3A) which was perfused at a rate of 0.5-1 ml/min by a gravity fed system. Before the solution entered the recording chamber, it traversed a heated water jacket. Drugs were applied to the cells via a temperature controlled micro-perfusion drug delivery system [see section 3.2.4(i)].

3.2.4(i) RECORDING AT 37°C

To reduce variables between secretion and electrophysiological experimental conditions, perfusion of drugs 35-37°C during whole-cell recording was used. A micro-perfusion drug delivery system was developed that not only allow for a fast application of known concentrations of drugs and the washout of drugs, but was adapted to work at 35-37°C (Figure 3.2B).

3.2 Recording solutions and micro-perfusion system

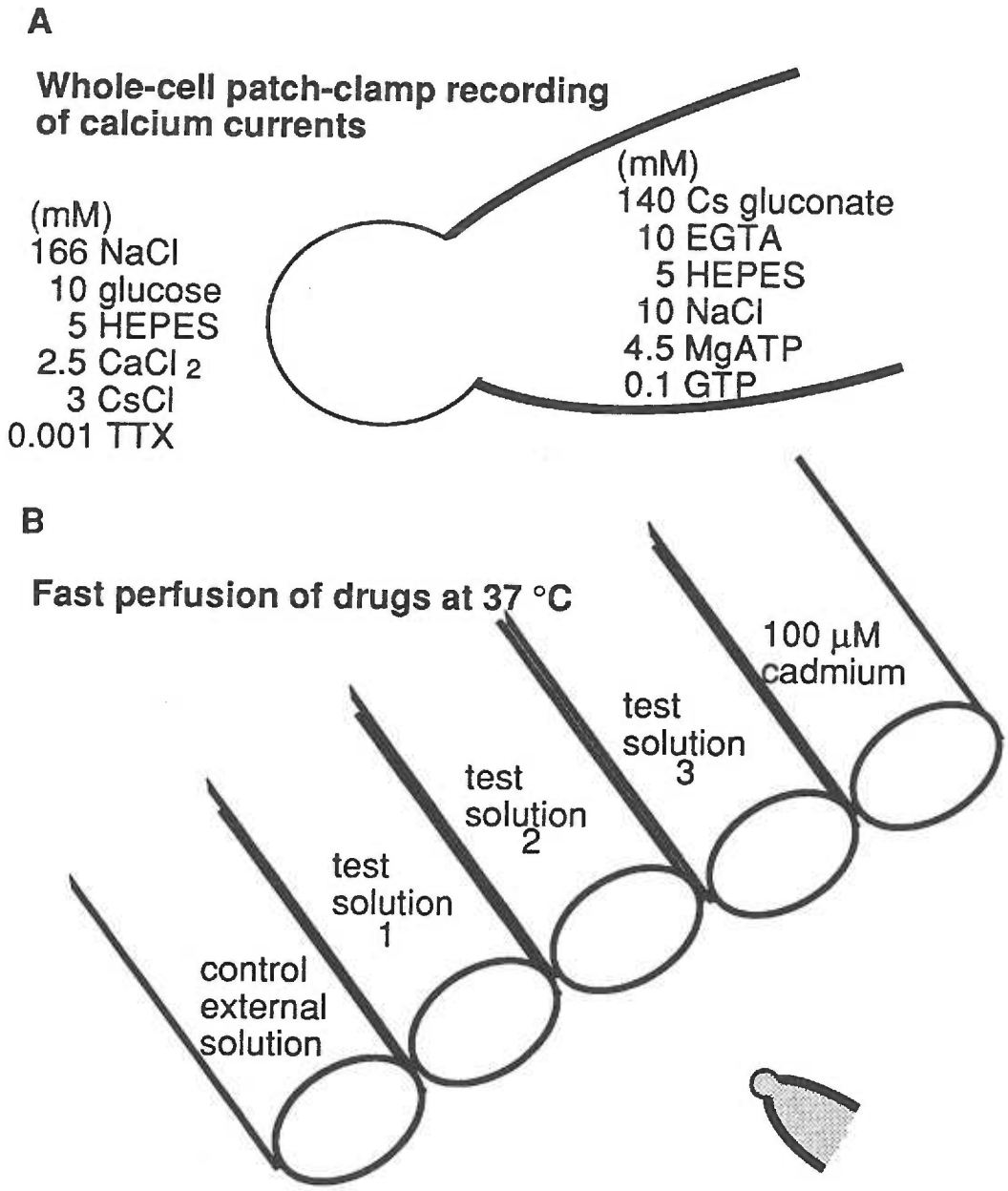


Figure 3.2 Recording Solutions and Micro-Perfusion System. A, a schematic of a cell in the whole-cell recording configuation with the composition of the extracellular and pipette recording solutions used to isolate calcium currents. B, a schematic of the micro-perfusion drug delivery system relative to a cell in the whole-cell recording configuration (not exactly drawn to scale). This system allowed for fast application and washout of drugs, and application of cadmium (100 μM) for cadmium-subtraction of current traces.

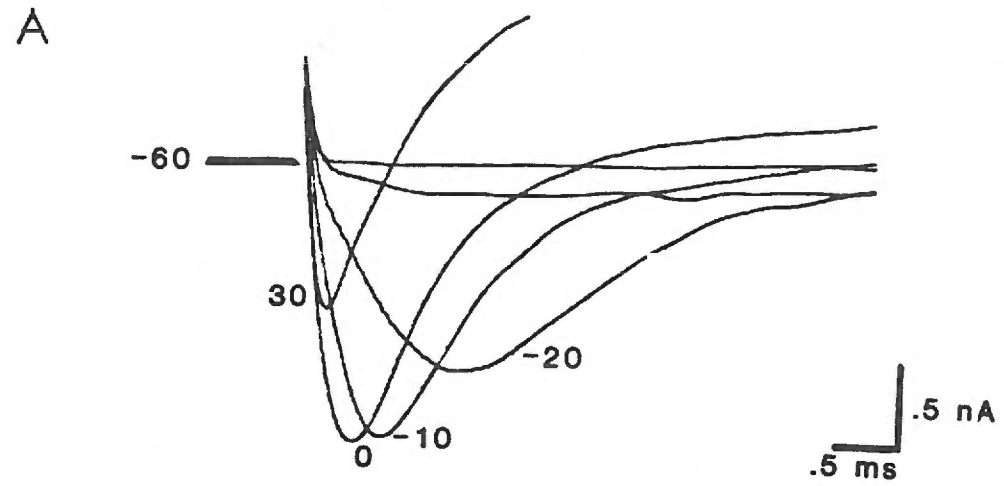
The micro-perfusion system was constructed using glass micropipettes (Drummond, "microcaps", 2 μl) attached to polyethylene tubing 0.58 mm I.D, 0.965 mm O.D. (Intramedic, Clay Adams of Becton Dickinson, Cat. No. 7410) and connected to a 10 ml reservoir of buffer. Four to six glass micropipettes (barrels) were arranged in parallel and their attached tubing traversed a water jacket, which was constructed from a 10 ml syringe that was attached to a recirculating water bath. Solutions were gravity fed through the system continuously to maintain constant temperature of the outflow. The flow had to be equal and constant in each barrel in order to avoid artifacts due to variations in temperature between barrels. The flow rate was monitored by the graticules of the 10 ml reservoir. The system was attached to a Narishige micro-manipulator which allowed for the positioning of a barrel 10 μm from the cell. Each barrel delivered a different solution however, always in each experiment, one barrel contained control solution and one barrel contained 100 μM cadmium. Stock drug solutions were diluted 1:1000 with the external recording solution. These included $CdCl_2$, $NiCl_2$, nifedipine (Sigma), Bay K 8644 (Sigma), quinpirole (Lilly, Ly171555, Lot #162-848-200A) and isoproterenol (Sigma).

3.2.4(ii) MEASUREMENT OF CALCIUM CURRENTS

Recording solutions were designed to isolate calcium currents from the inward sodium current and the outward potassium current by using TTX and cesium to block these currents. These sodium and potassium currents are shown in Figure 3.3 and Figure 3.4, respectively. Comparison of these currents with the calcium currents in Figure 3.5 shows that the sodium and potassium currents were adequately blocked.

Figure 3.3 Sodium Current: Sensitivity to Tetrodotoxin. The inward sodium current and the outward potassium current (Figure 3.4) were measured with a pipette solution composed of (values are mM) 100 Kgluconate, 1 MgCl, 20 KCl, 5 NaCl, 1 $CaCl_2$, 11 EGTA, 10 HEPES, 1.5 ATP, and 0.1 GTP. A, the sodium current was recorded when the potential was stepped to depolarized potentials. The holding potential was -60 mV. B, The I-V relation of the sodium current shown in A in the absence and presence of TTX (1 μM). The maximum amplitude of the current was plotted as a function of voltage.

3.3 Sodium current: sensitivity to tetrodotoxin



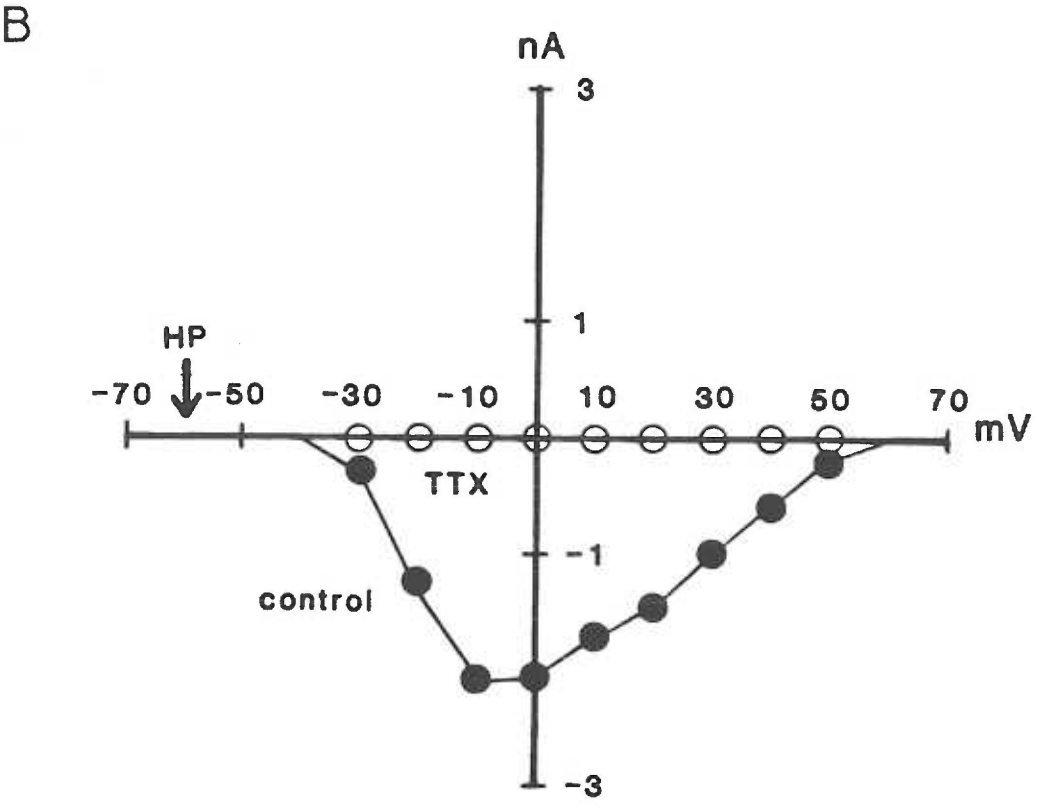


Figure 3.4 Potassium Current: Sensitivity to Tetraethylammonium. A, the transient inward current and delayed outward current were evoked by stepping to a series of depolarizing potentials (10 mV increments) from a holding potential of -60 mV in the absence and presence of TEA (10 mM). B, The I-V relation of the outward current shown in A in the absence and presence of TEA (10 mM). The amplitude of the current at 10 ms was plotted as a function of voltage.

3.4 Potassium current: sensitivity to tetraethylammonium

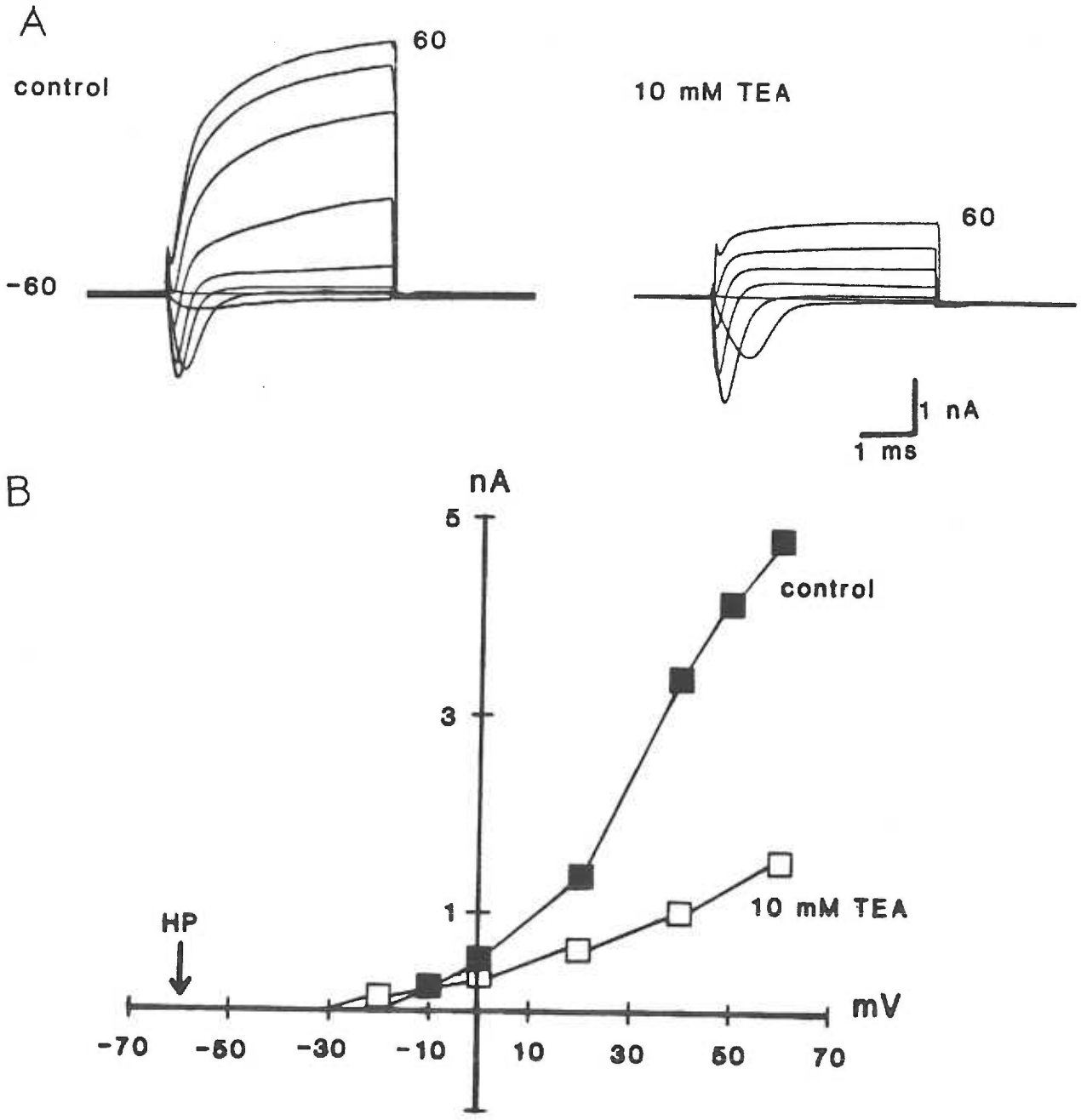
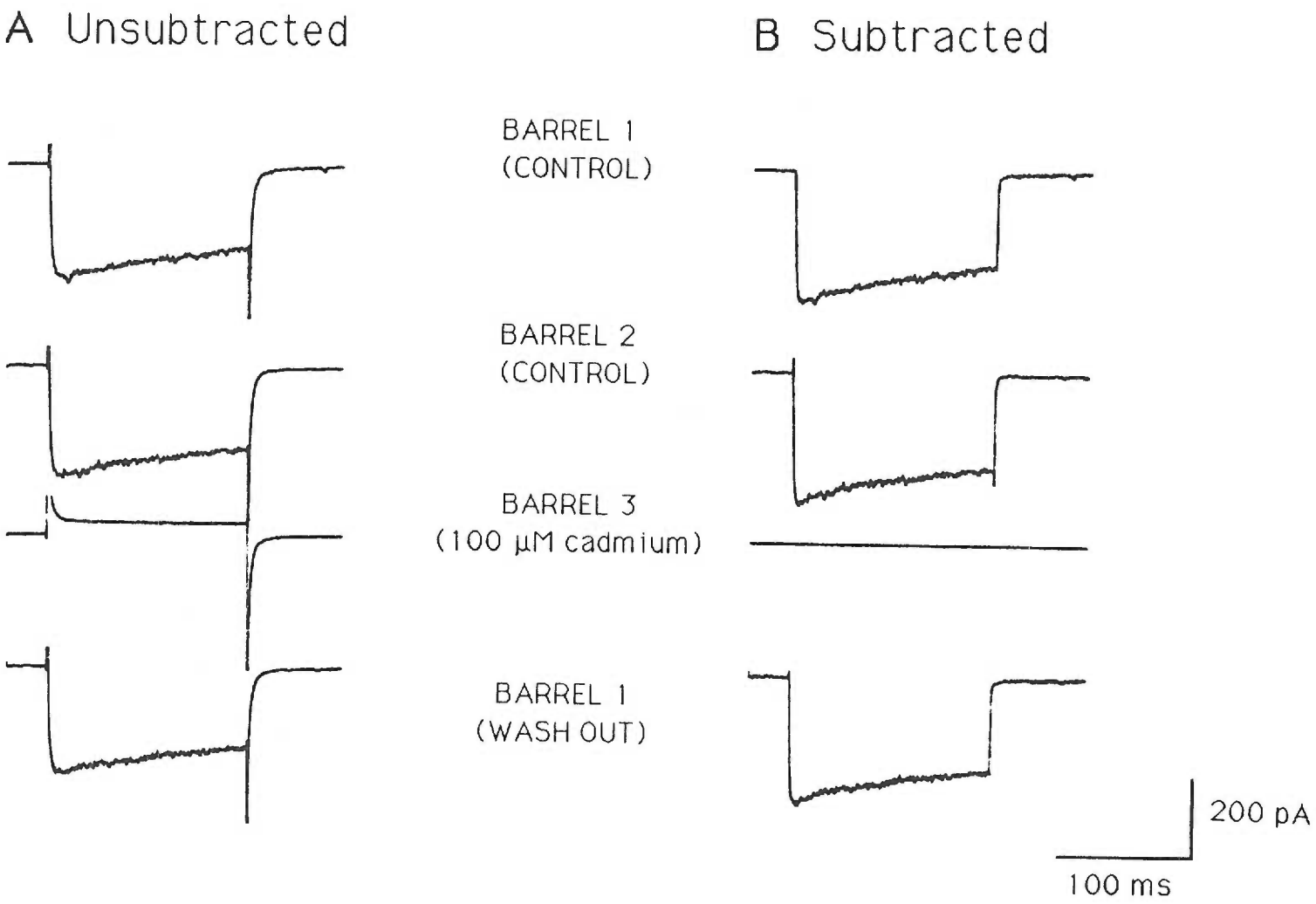


Figure 3.5 Cadmium Subtraction of the Current Trace. Unaltered calcium currents are compared with cadmium subtracted currents. Control and cadmium (100 μM, a concentration sufficient to completely block the calcium current) recording solutions were applied by the micro-perfusion drug delivery system. Currents evoked by stepping the cell from -70 mV to 0 mV while the cell was 20 μm from the opening of barrel 1 (control), barrel 2 (control), or barrel 3 (100 μM). A, unaltered calcium currents. Note that the current amplitude in the two control traces are not different. B, cadmium subtracted currents. The current trace generated in cadmium is subtracted from the other traces. Note the removal of the capacitative transient current and the small increase in amplitude of the current due to the subtraction of the leak current. (AtT 112289c1).

3.5 Cadmium subtraction of current trace



Calcium currents were measured in each of these solutions: control, test(s), and 100 μM cadmium. The reported calcium currents are cadmium-subtracted current traces. These are the currents remaining after the subtraction of a current trace evoked by the identical voltage protocol but recorded in 100 μM cadmium, a dose that completely blocked the inward calcium current. This procedure is shown in Figure 3.5. Cadmium subtraction was opted over a strictly leak subtracted current because these cells exhibit a voltage-dependent outward current that activates at -10 mV even in the presence of cesium (Figure 3.6). This current appears to be similar to an outward current reported in bullfrog sympathetic neurons (Jones & Marks, 1987); it has not been further studied in these experiments. These manipulations of the current trace isolate the cadmium-sensitive (or calcium component) of the measured current and removes the capacitative and leak currents.

Transient and steady-state currents evoked from 150 ms depolarizing pulses were measured in the following way. The largest amplitude of the cadmium-subtracted calcium currents was measured in the first 10 ms of the pulse and this is referred to as the transient component. The amplitudes of the last 10 ms of the cadmium-subtracted current were averaged and this is referred to as the sustained component. Statistical significance was determined by the unpaired, two-tailed $\underline{t}$-test where $p < 0.05$ was considered significant.

3.6 Outward currents in 100 μM cadmium

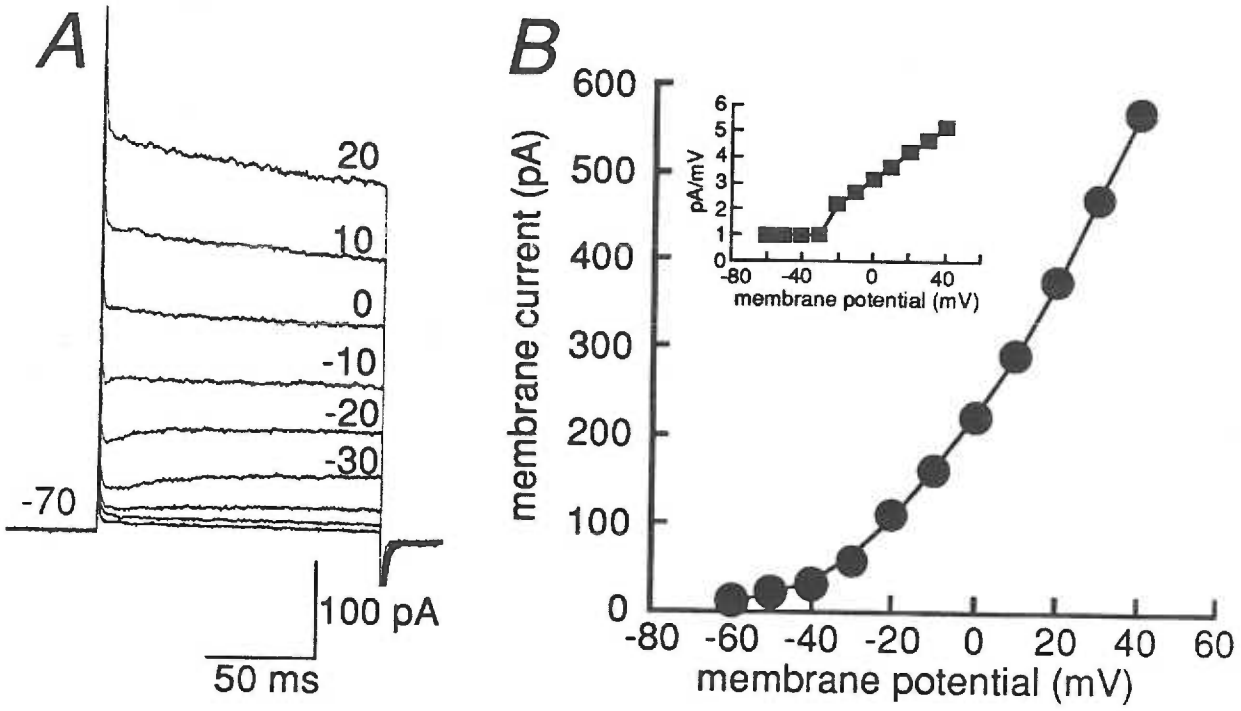


Figure 3.6 Outward Currents in 100 μM Cadmium. A, the currents were recorded in response to 150 ms depolarizing steps of 10 mV increments from a holding potential of -70 mV. B, currents from the experiment illustrated in A are shown; the average amplitude of the current of the last 10 ms of the pulse is plotted as a function of potential. The inset plots the current/voltage as a function of potential and shows that the current was linear between -70 mV and -30 mV (leak current) and at -20 mV an outward current is activated. (Mel 101889c1).

3.3 RESULTS

3.3.1 IMMUNOCYTOCHEMISTRY

Dispersed rat pituitary cells from the neurointermediate lobe and anterior lobe were stained with a β-endorphin antibody. The anterior lobe preparations showed positive staining in only a small fraction of the cells (Figure 3.7), correlating with reports that anterior corticotrophs compose only about 5-10 % of the cell population in the anterior lobe (Hatfield et al. 1989). In contrast, virtually every cell in the preparation derived from the neurointermediate lobe showed positive staining for β-endorphin. These results show that the cell preparation used in this study may be considered a homogeneous population of POMC-producing cells (Figure 3.8).

3.3.2 SECRETION EXPERIMENTS

Secretion is expressed as per cent of basal secretion as described in section 2.2.3.

3.3.2(i) BASAL SECRETION

When control solution was applied during all four time points of the secretion experiment, there was a decrease in the secretory rate between the second period and the third period. The average reduction of basal secretion was 18 %, to 81.7 $\pm$ 4.2 % (n = 23) of basal secretion, measured during the second period. Secretion levels significantly greater than this indicate stimulated release and secretion values significantly less than this indicate an inhibition of basal secretion. The absolute levels of basal secretion were 577.8 $\pm$ 89.9 pg immunoactive β-endorphin/well

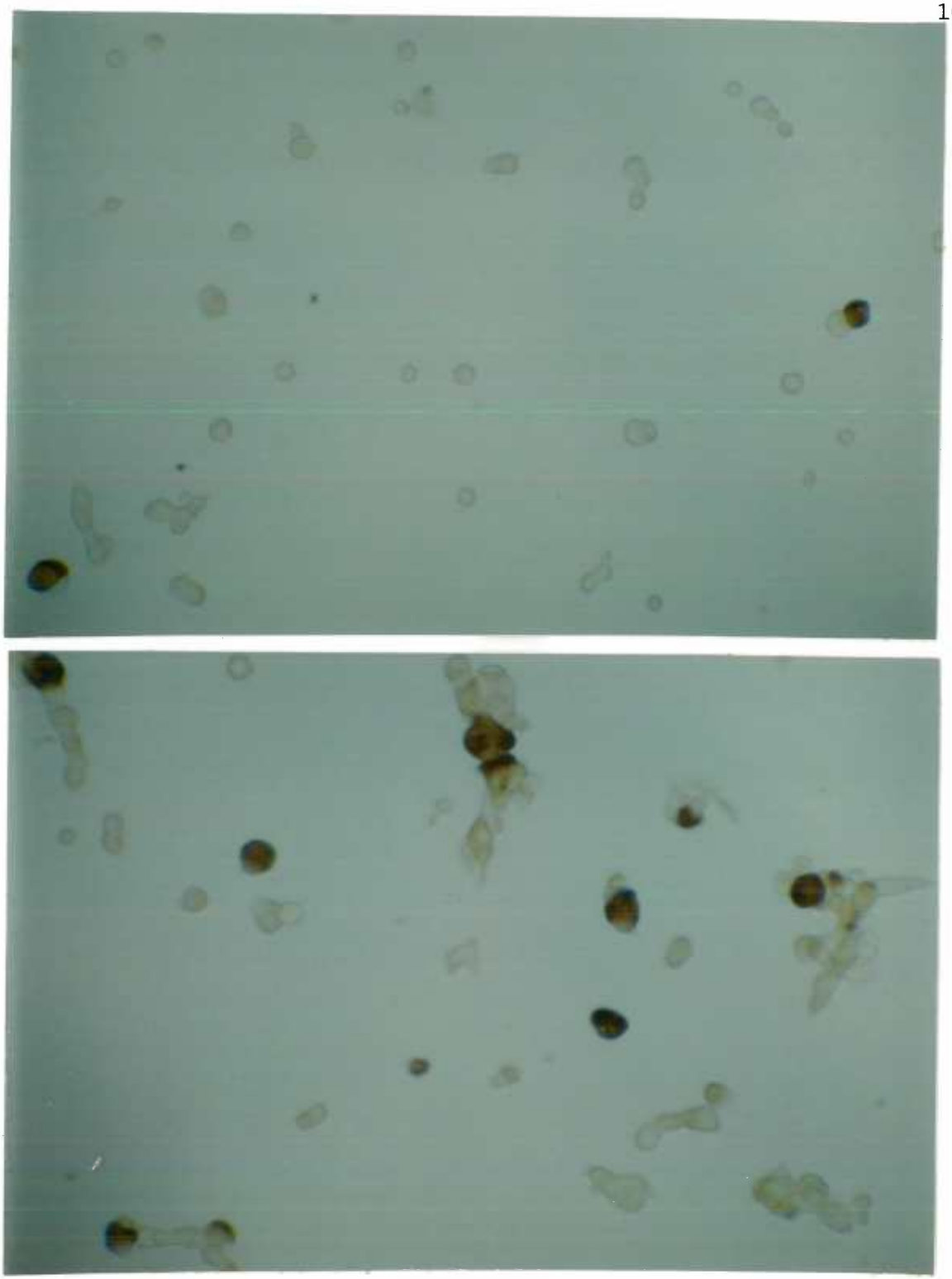


Figure 3.7 Immunocytochemistry of Cells From The Rat Anterior Pituitary. Cell preparations were stained with β-endorphin specific antibody and only a small proportion of the cells stained. This was expected, since only about 5 % of the cells in the anterior lobe of the pituitary produce POMC. This preparation serves as a control for the β-endorphin staining of the cells prepared from the intermediate-posterior lobe of the pituitary (Figure 3.8). Cells were grown on glass coverslips. Top, 24 h. Bottom, 7 days.

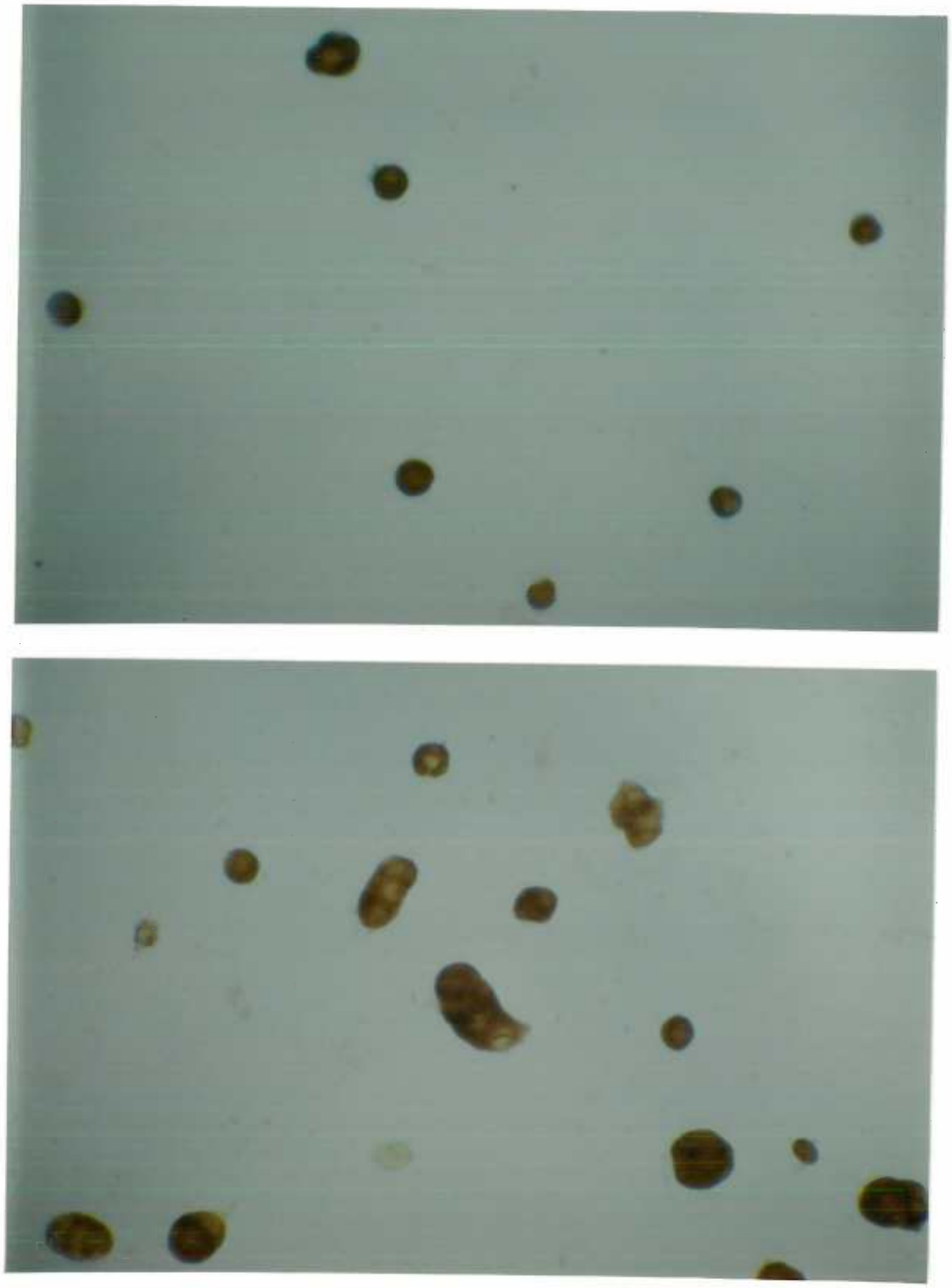


Figure 3.8 Immunocytochemistry of Cells From The Rat Intermediate Pituitary. Cell preparation were stained with β-endorphin specific antibody and virtually every cell was stained. Cells were grown on glass coverslips. Top, 24 h. Bottom, 7 days.

(n = 23).

Several agents were tested to determine whether basal secretion could be inhibited. The calcium channel blocker nifedipine did not significantly reduce basal levels of β-endorphin secretion. Secretory activity in the presence of 100 nM and 1 μM nifedipine was 79 ± 13 % of basal (n = 6) and 94 ± 14 % of basal (n = 6), respectively. However, 10 μM nifedipine significantly stimulated β-endorphin release to 265 ± 36 % of basal (n = 8; p = 0.0001; Figure 3.9A). This augmentation of secretion by high levels of nifedipine has not been studied further.

Quinpirole reduced basal secretion in a concentration-dependent manner and 100 nM quinpirole produced a maximum inhibition, reducing basal secretion to 41 ± 4 % (n = 6; p < 0.0001; Figure 3.9B).

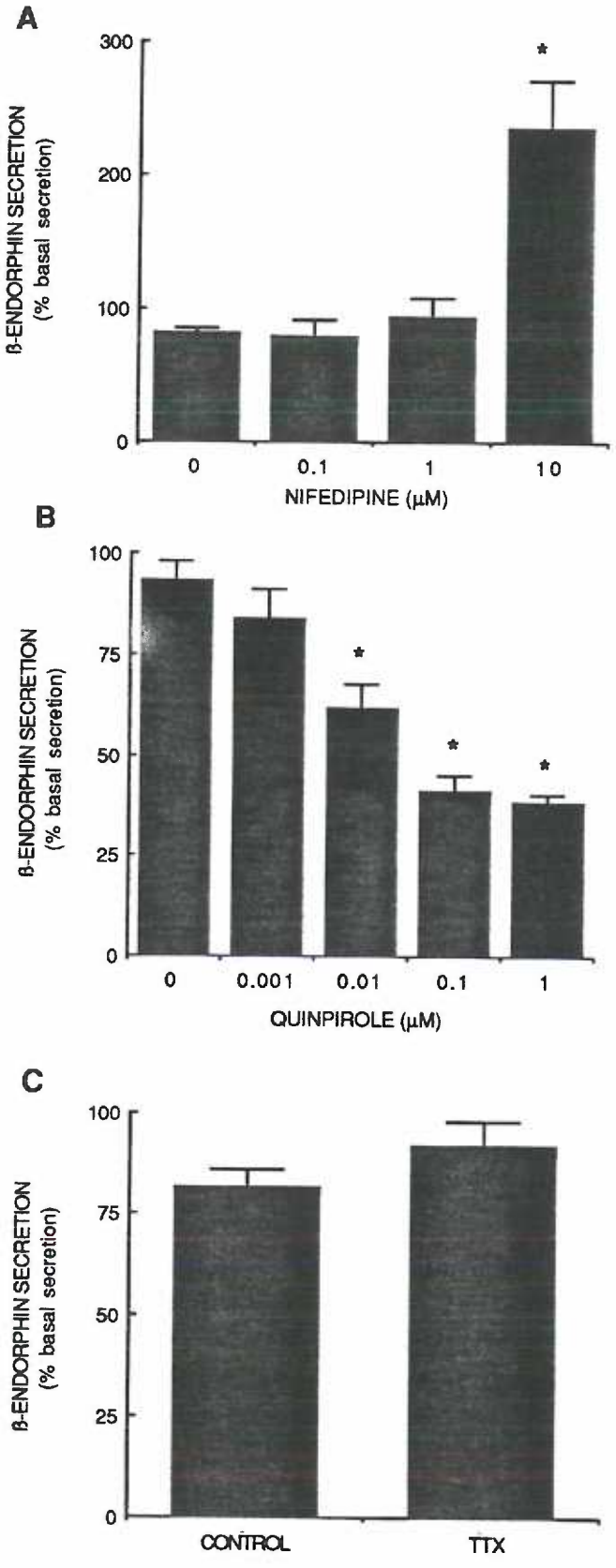
TTX (1 μM) abolished the sodium dependent component of the spontaneous voltage spikes during extracellular recordings (Douglas & Taraskevich, 1980); this concentration of TTX also abolishes the sodium current when measured with whole-cell patch-clamp recording (Figure 3.3) but did not reduce basal secretion. In the presence of TTX (1 μM) basal secretion was 92 ± 6.1 % of basal secretion (n = 6; p = 0.0422; Figure 3.9C).

3.3.2(ii) STIMULATED SECRETION

β-endorphin secretion was consistently stimulated by the calcium channel agonist, Bay-K-8644 (1 μM), which stimulated secretion to 147 ± 9 % of basal (n = 34; p < 0.0001) and by the adrenergic receptor agonist, isoproterenol (1 μM), which stimulated secretion to 165 ± 7 % of basal (n = 39; p < 0.0001; Figure 3.10A). TTX had no effect on β-endorphin secretion. In the absence and presence of TTX (1 μM), secretion

Figure 3.9 Basal Secretion. A, nifedipine (0.1 μM and 1 μM, n = 6) did not reduce basal secretion. 10 μM nifedipine (n = 8) enhanced basal secretion (n = 23 for control). B, quinpirole reduced basal secretion in a concentration-dependent manner with an IC_{50} of 5 nM and 100 nM produced a maximal inhibition of secretion (n = 5 for control, 0.01 μM and 1 μM; n = 6 for 0.001 μM and 0.1 μM). C, TTX (1 μM, n = 6) did not reduce secretion compared to control (n = 23). The * indicates in this figure and all subsequent figures indicates a significant difference from control.

3.9 Basal secretion



3.10 Stimulated secretion

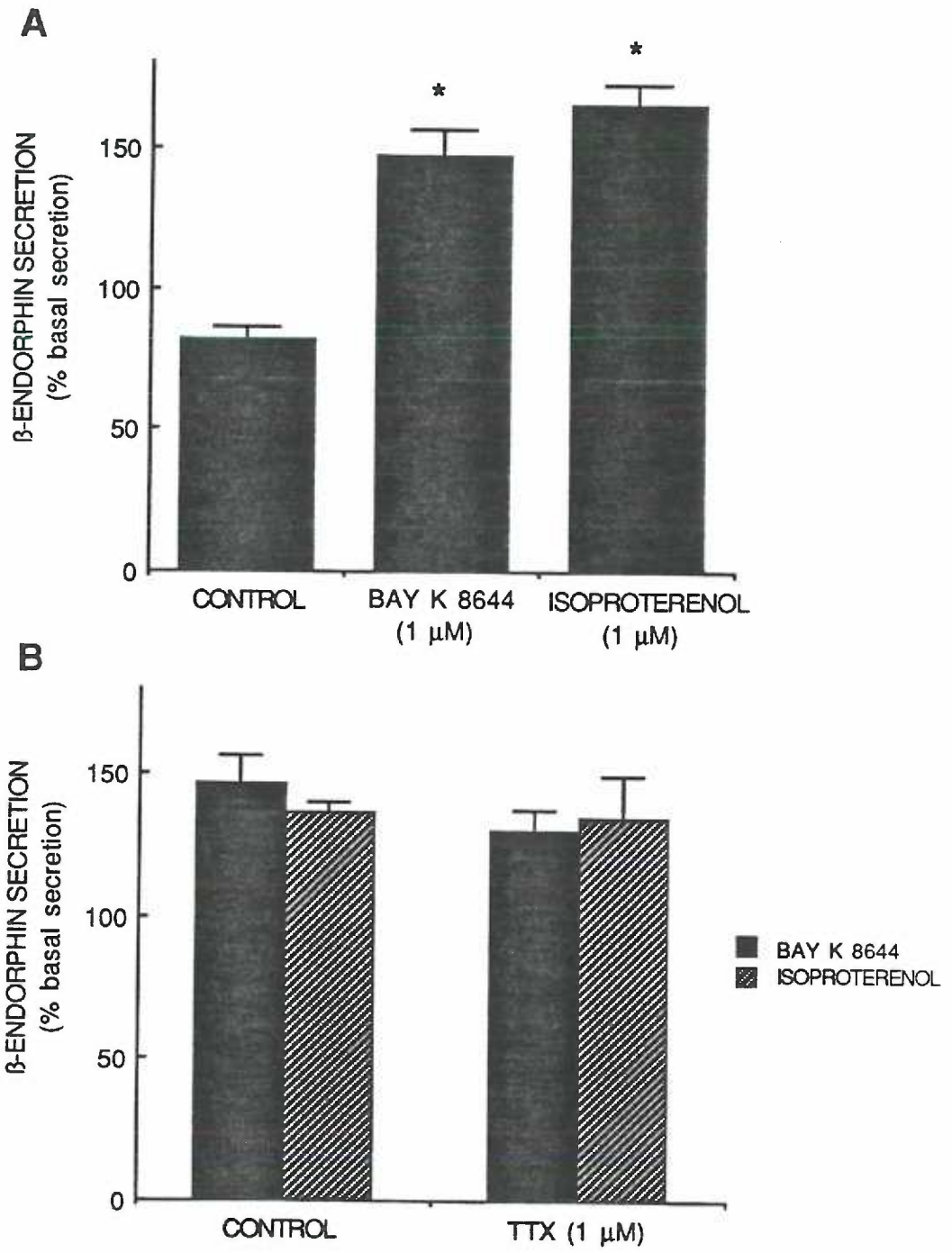


Figure 3.10 Stimulated Secretion. A, secretion was stimulated by BAY K 8644 (1 μM, n = 34) and by isoproterenol (1 μM, n = 39). Only these concentrations of secretagogues were used throughout the secretion study and will not be indicated hereafter. B, TTX (1 μM, n = 5) did not reduce BAY K 8644 (n = 4) or isoproterenol (n = 6) stimulated secretion.

stimulated by BAY K 8644 was 146 ± 10 % of basal (n = 4) and 130 ± 7 % of basal (n = 5), respectively and secretion by isoproterenol was 136 ± 4 % of basal (n = 6) and 134 ± 15 % of basal (n = 5), respectively (Figure 3.10B).

3.3.2(iii) INHIBITION OF STIMULATED SECRETION

MODULATION BY CALCIUM CHANNEL BLOCKERS

Nifedipine

Isoproterenol-stimulated secretion [142 ± 16 % of basal (n = 5)] was not reduced by nifedipine as shown in Figure 3.11. In the presence of 0.01 μM, 0.1 μM, 0.3 μM and 1 μM nifedipine secretion was 140 ± 7 % of basal (n = 6; p = 0.8860), 122 ± 7 % of basal (n = 6; p = 0.2387), 92 ± 11 % of basal (n = 6; p = 0.0260) and 88 ± 14 % of basal (n = 6; p = 0.0280), respectively. However, 10 μM nifedipine enhanced β-endorphin secretion above the isoproterenol stimulated level to 355 ± 58 % of basal secretion (n = 6; p = 0.0098).

Cadmium

BAY K 8644-stimulated secretion [134 ± 8 % of basal (n = 6)] was not reduced by concentrations of cadmium below 500 μM as shown in Figure 3.12A. In the presence of 10 μM, 30 μM and 100 μM cadmium secretion was 123 ± 3 % of basal (n = 6; p = 0.2321), 121 ± 3 % of basal (n = 6; p = 0.1448) and 118 ± 5 % of basal (n = 6; p = 0.1297), respectively. However, 500 μM cadmium significantly reduced BAY K 8644 stimulation to 93 ± 5 of basal (n = 6; p = 0.0012) and 1 mM cadmium further reduced secretion to 69 ± 4 % of basal (n = 6; $p < 0.0001$).

3.11 Nifedipine effects on stimulated secretion

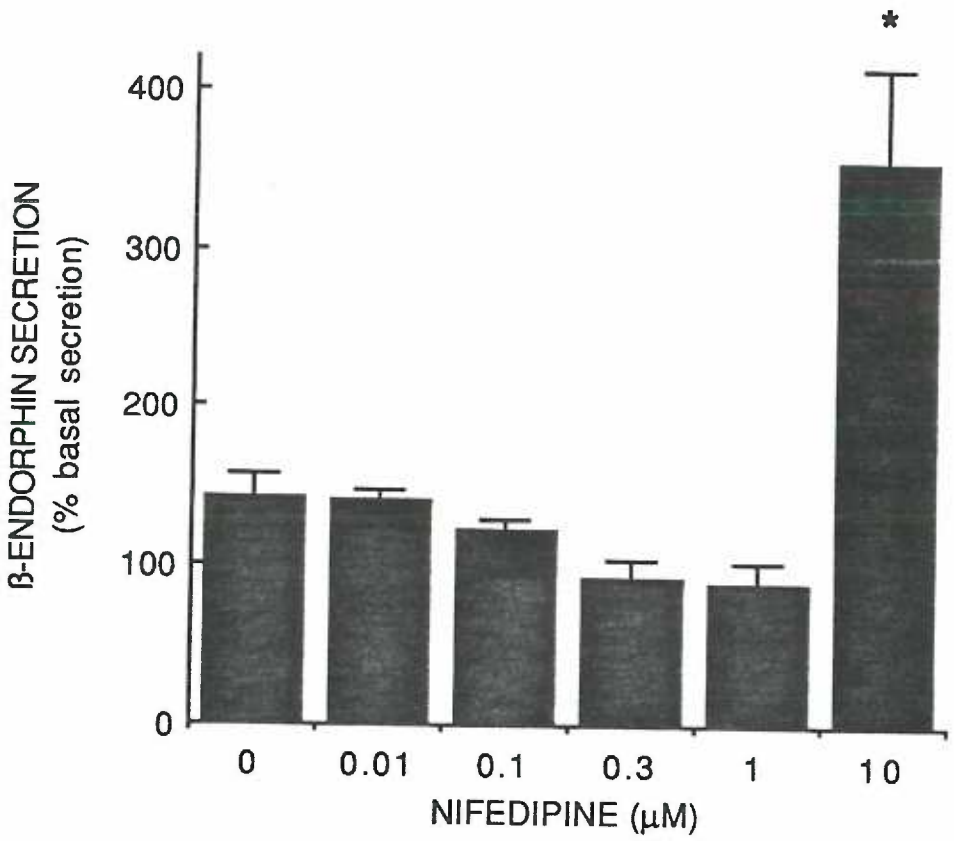
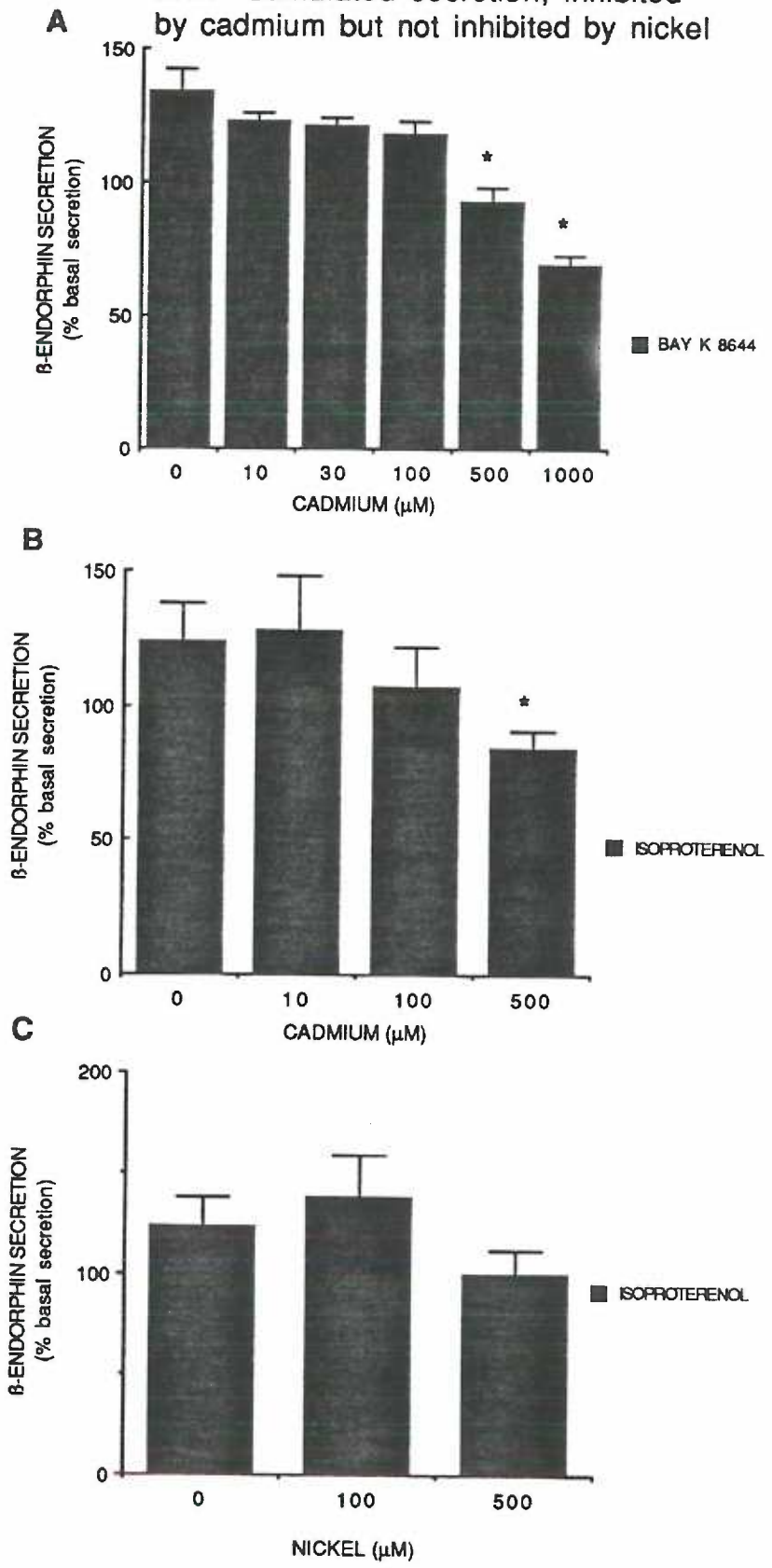


Figure 3.11 Nifedipine Effects on Stimulated Secretion. Isoproterenol-stimulated secretion was not significantly reduced by nifedipine at 0.3 μM and 1 μM nifedipine. 10 μM nifedipine enhanced stimulated secretion. This is similar to the enhancement of basal secretion shown in Figure 3.8A (n = 5 for control; n = 6 for all nifedipine concentrations).

Figure 3.12 Stimulated Secretion; Inhibited by Cadmium but not Inhibited by Nickel. A, Bay K 8644-stimulated secretion was inhibited to basal levels with 500 μM cadmium (n = 6 for all groups). B, isoproterenol-stimulated secretion was inhibited to basal levels with 500 μM cadmium (n = 6 for control and 10 μM and 100 μM cadmium; n = 5 for 500 μM cadmium). C, isoproterenol-stimulated secretion was not significantly reduced nickel (n = 6 for all groups).

3.12 Stimulated secretion; inhibited by cadmium but not inhibited by nickel



Cadmium did not significantly reduce isoproterenol-stimulated secretion as shown in Figure 3.12B. Isoproterenol-stimulated release was 124 ± 14 % of basal (n = 6). In the presence of 10 μM, 100 μM and 500 μM cadmium secretion was 128 ± 20 % of basal (n = 6; p = 0.9066), 107 ± 15 % of basal (n = 6; p = 0.4074) and 84 ± 7 % of basal (n = 6; p = 0.0429), respectively.

Nickel

Nickel did not reduce isoproterenol-stimulated secretion as shown in Figure 3.12C. Secretion stimulated by isoproterenol was 124 ± 14 % of basal (n = 6). In the presence of 100 μM and 500 μM nickel secretion was 138 ± 21 % of basal (n = 6; p = 0.6074) and 100 ± 12 % of basal (n = 6; p = 0.2227), respectively.

MODULATION BY QUINPIROLE

The dopamine D_2 receptor agonist, quinpirole, was used in all experiments in which dopamine receptor actions were examined. This agonist was used in preference to dopamine or other dopaminergic agonists because it is stable in solution and is selective for the D_2 dopamine receptor (Lacey, Mercuri & North, 1987).

BAY K 8644-stimulated secretion [188 ± 28 % of basal (n = 6)] was reduced by quinpirole in a concentration-dependent manner as shown in Figure 3.13A. In the presence of 3 nM quinpirole secretion was 138 ± 14 % of basal (n = 6; p = 0.1471). Secretion was reduced in the presence of 10 nM, 30 nM, 100 nM and 1 μM where secretion was 88 ± 8 % of basal (n = 6; p = 0.0068), 69 ± 4 % of basal (n = 6; p = 0.0020), 44 ± 3 % of basal (n = 6; p = 0.0005) and 43 ± 4 % of basal (n = 6; p = 0.0005),

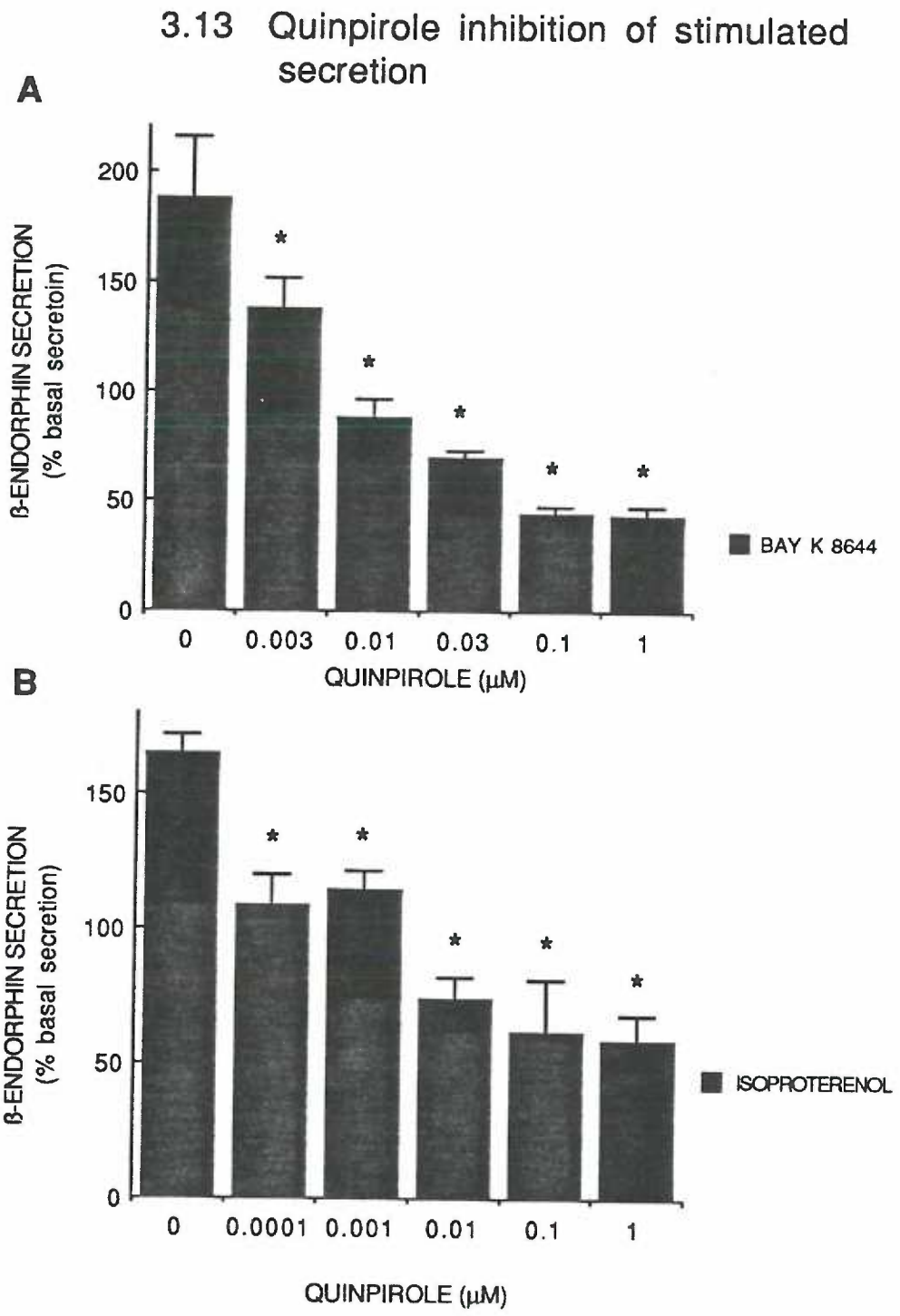


Figure 3.13 Quinpirole Inhibition of Stimulated Secretion. A, BAY K 8644-stimulated secretion was inhibited in a concentration-dependent manner with an IC_{50} of 5 nM and 100 nM quinpirole produced a maximum inhibition of secretion (n = 6 for all groups). B, isoproterenol-stimulated secretion was inhibited by quinpirole and 100 nM quinpirole produced an maximum inhibition of secretion (n = 39 for control; n = 6 for 0.1 nM, 1 nM and 100 nM; n = 10 for 10 nM and 1 μM).

respectively.

Isoproterenol-stimulated secretion [165 ± 7 % of basal secretion (n = 39)] was also reduced by quinpirole in a concentration-dependent manner as shown in Figure 3.13B. Secretion was reduced in the presence of 0.1 nM, 1nM, 10 nM, 100 nM and 1 μM quinpirole; secretion was 108 ± 12 % of basal (n = 6; p = 0.0061), 114 ± 7 % of basal (n = 6; p = 0.0117), 74 ± 8 % of basal (n = 10; p < 0.0001), 62 ± 19 % of basal (n = 6; p < 0.0001) and 59 ± 9 % of basal (n = 6; p < 0.0001), respectively. The maximum inhibition of BAY K 8644 and isoproterenol-stimulated secretion by quinpirole (100 nM) was not significantly different (p = 0.3572).

Activation of D_2 receptors can increase a potassium conductance as well as reduce voltage-dependent calcium currents (see section 3.1.4). Therefore, it was of interest to examine whether potassium channel blockers might antagonize the inhibition of stimulated secretion by quinpirole. In particular, cesium, which is known to block potassium conductances (Ruby, 1988), has been reported to block the action of SRIF in inhibiting ACTH secretion in AtT-20 cells (Pennefather, Heisler & MacDonald, 1988). In this study, the action of cesium in the presence of isoproterenol was examined. When both cesium (5 mM) and isoproterenol (1 μM) were present, β-endorphin secretory activity was measured as 340 ± 72 % of basal (n = 5); this is a significant increase over the degree of stimulation produced by isoproterenol alone which was 180 ± 24 % of basal (n = 6; p = 0.0489). Quinpirole (1 μM) inhibited secretion stimulated by isoproterenol plus cesium to 62 ± 6 % of basal (n = 6); this inhibition was not significantly different from inhibition by quinpirole in the presence of isoproterenol alone which was 59 ± 9 % of

basal secretion (n = 10; Figure 3.14).

INVOLVEMENT OF G-PROTEINS

Pertussis toxin pretreatment (100 ng/ml for 16 h) had no effect on the stimulation of β-endorphin secretion by BAY K 8644. The BAY K 8644-stimulated secretion measured in the absence and presence of pertussis toxin pretreatment was 143.8 $\pm$ 5.5 % (n = 6) and 138.8 $\pm$ 4.8 % (n = 6) of basal secretion, respectively (p = 0.5101). However, pertussis toxin pretreatment abolished the inhibition of secretion by quinpirole (1 μM) of secretion. The effect of quinpirole on the BAY K 8644-stimulated secretion measured in the absence and presence of pertussis toxin pretreatment was 30.5 $\pm$ 2.7 % (n = 6) and 129.83 $\pm$ 5.0 % (n = 6) of basal secretion, respectively ($p < 0.0001$). The secretory level in cells pretreated with pertussis toxin in the absence and presence of quinpirole was not significantly different (p = 0.2222). The results of these experiments are shown in Figure 3.15A.

Pertussis toxin pretreatment for 16 h did not significantly alter isoproterenol-stimulated secretion. The isoproterenol-stimulated secretion with no treatment was 165 $\pm$ 7 % of basal (n = 39), with pertussis toxin pretreatment at 50 ng/ml was 205 $\pm$ 13 % of basal (n = 6) and with pertussis toxin pretreatment at 100 ng/ml was 186 $\pm$ 7 % of basal (n = 6). Quinpirole (1 μM) inhibited stimulated secretion in untreated cells to 59 $\pm$ 9 % of basal secretion. Pertussis toxin pretreatment (50 ng/ml) partially blocked the quinpirole (1 μM) inhibition of stimulated secretion; secretion was 126 $\pm$ 10 % of basal (n = 6), which was significantly greater than secretion in the presence of quinpirole in untreated cells (p = 0.0003) and significantly less than the secretion

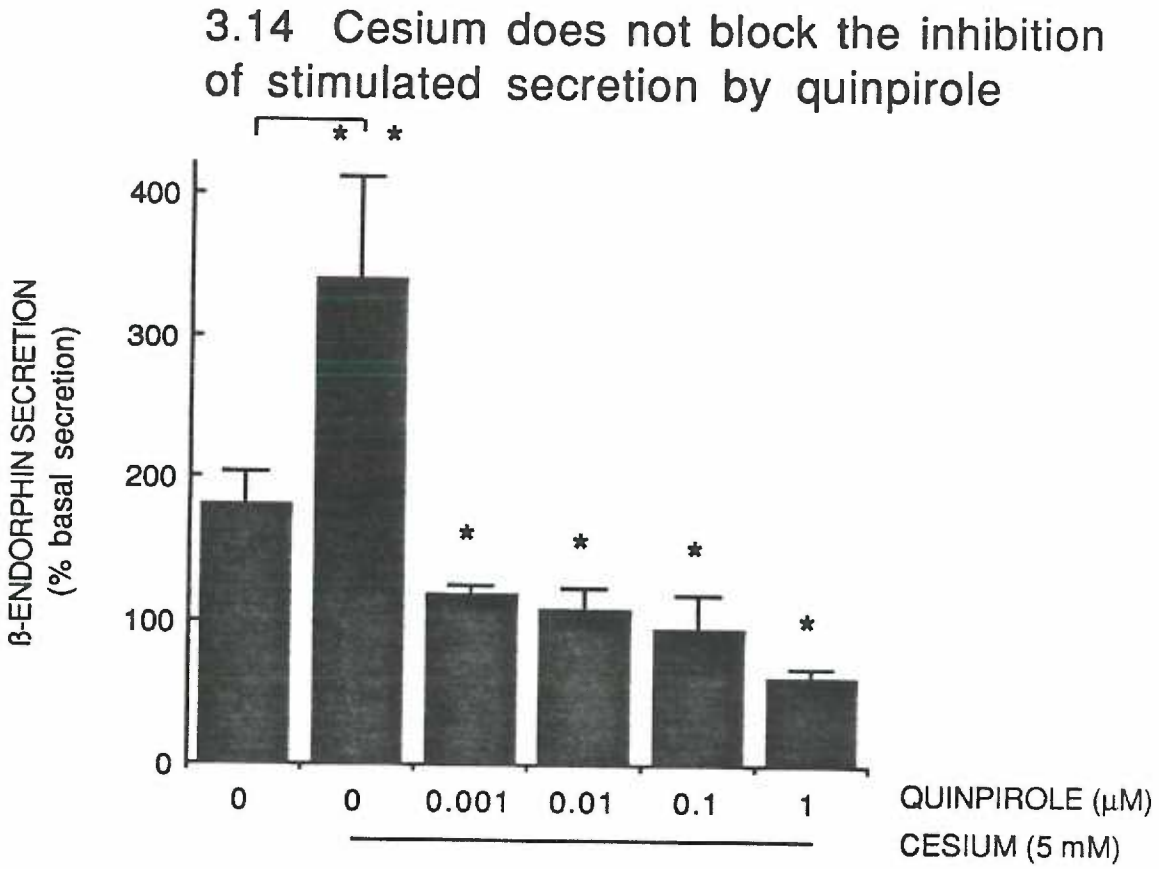


Figure 3.14 Cesium does not Block the Inhibition of Stimulated Secretion by Quinpirole. Isoproterenol-stimulated secretion was enhanced by cesium (5 mM); however, in the presence of isoproterenol and cesium, quinpirole still inhibited secretion and quinpirole (1 μM) maximally inhibited secretion (n = 6 for all except 0.001 μM and 0.1 μM where n = 5).

3.15 Pertussis toxin blocks the inhibition by quinpirole of ß-endorphin secretion

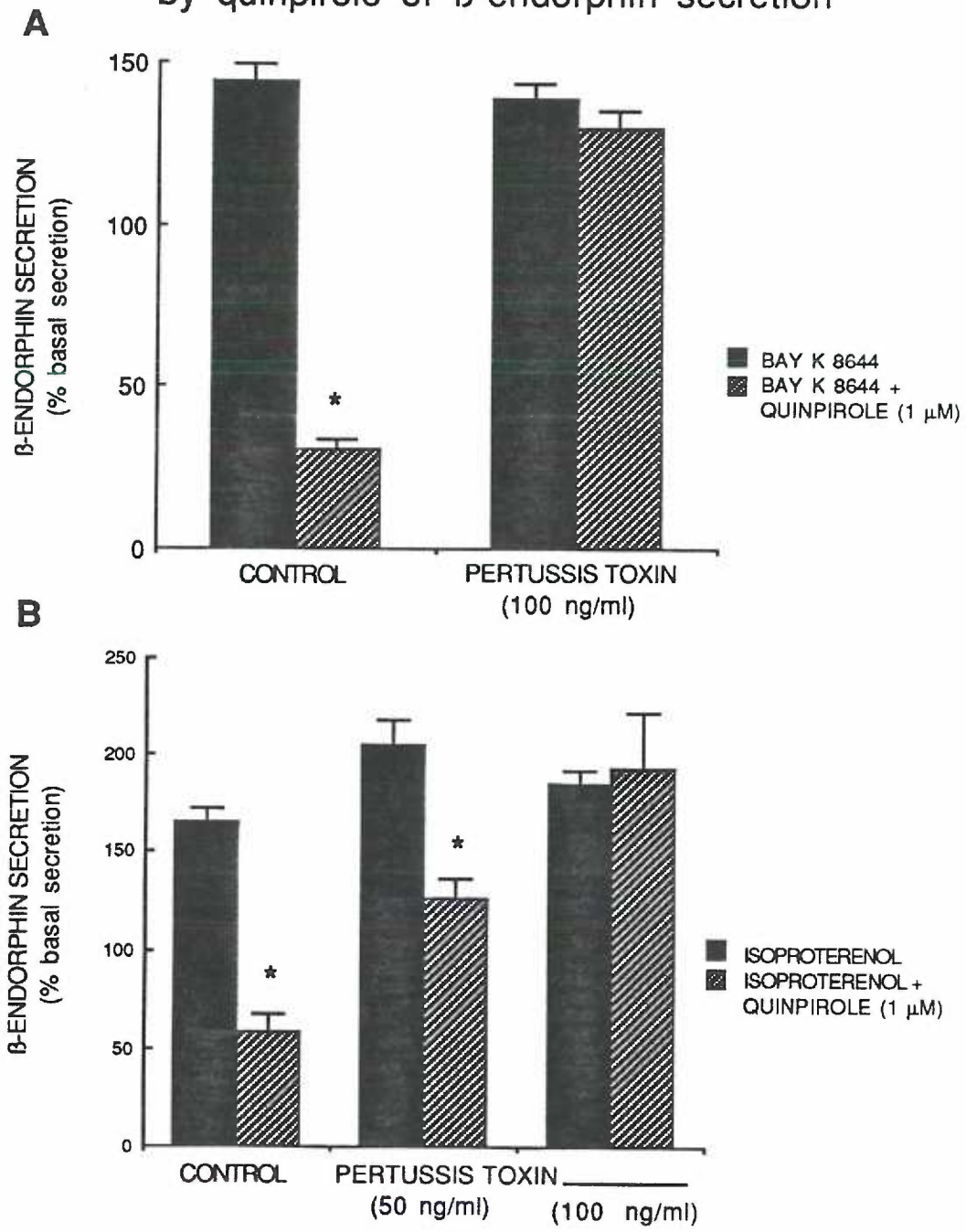


Figure 3.15 Pertussis Toxin Blocks the Inhibition by Quinpirole of β-Endorphin Secretion. A, BAY K 8644-stimulated secretion was inhibited by quinpirole (1 μM) to below basal levels. Pertussis toxin pretreatment (100 ng/ml for 16 h) blocked the inhibition of secretion by quinpirole and did not alter the BAY K 8644-stimulated secretion (n = 6 for all groups). B, isoproterenol-stimulated secretion (n = 39) was inhibited by quinpirole (1 μM, n = 10) to below basal levels. Pertussis toxin preteatment (50 ng/ml for 16 h; n = 6) reduced the inhibition of secretion by quinpirole. Pertussis toxin preteatment (100 ng/ml for 16 h; n = 6) abolished the inhibition of secretion by quinpirole.

in the absence of quinpirole in pertussis toxin treated cells ($p = 0.0008$). Pertussis toxin pretreatment (100 ng/ml) abolished the inhibition of secretion by quinpirole, which was 193 $\pm$ 29 % of basal ($n = 6$) and was not significantly different from the pertussis toxin treated control stated above ($p = 0.8157$). The results of these experiments are shown in Figure 3.15B.

3.3.3 ELECTROPHYSIOLOGICAL RESULTS

3.3.3(i) CHARACTERIZATION OF CALCIUM CURRENTS

Calcium currents were recorded as described in section 3.2.4. The calcium currents were temperature dependent. The peak amplitude of calcium currents measured at room temperature was never greater than 100 pA ($n = 5$); whereas currents at 35°C were typically 300-400 pA (see below). Calcium currents were recorded in 16 cells at 35-37°C; the current-voltage relationship was measured with a holding potential of -70 mV and 10 mV increments of depolarizing step pulses with a 150 ms duration. Two components of the current were measured, the transient component and the sustained component [see section 3.2.4(ii)]. The transient component activated between -50 mV and -30 mV. The maximum amplitude of the transient component of the calcium currents ranged from 140 pA to 610 pA with an average of 367 $\pm$ 30 pA. In 11 of the 16 cells, the maximum transient component was recorded at 0 mV in 3 cells, at 10 mV in two cells, and at -10 mV and -20 mV in one cell each. The sustained current also activated between -50 mV and -30 mV and the maximum amplitude ranged from 80 pA to 305 pA with an average of 195 $\pm$ 17 pA. The maximum amplitude of the sustained component was measured at

0 mV in 11 of the 16 cells. The amplitudes of the transient and sustained components were significantly different from each other ($p < 0.0001$). The ratio of the transient to sustained component of the current was 1.92 ± 0.08. Representative current-voltage traces recorded from one cell are shown in Figure 3.16.

The amplitude of the transient component evoked when the potential was stepped to 0 mV was more sensitive to the holding potential compared to the sustained component. This was measured in experiments where the cells were exposed to two, consecutive, 150 ms voltage steps from a holding potential of -70 mV. The amplitude of the first step was progressively increased in increments of 20 mV; the amplitude of the second step was fixed at 0 mV. Increased depolarization of the first voltage step reduced the transient component of the current measured during the second voltage step (Figure 3.17). A maximum reduction of the transient component of the current measured during the second voltage step was observed when the first voltage step was -10 mV; the transient component evoked at 0 mV was reduced 50 % while the sustained component was only reduced 10 % ($n = 4$) (Figure 3.18). Larger depolarizations to 50 mV were not as effective in reducing the transient component of the second pulse.

No currents were recorded when the membrane was held at -100 mV and the potential stepped to -50 mV ($n = 5$), which indicated that a low-threshold current was not present in these cells.

3.3.3(ii) STIMULATION OF CALCIUM CURRENTS

Results described herein were obtained only from cells in which the response (the calcium current amplitude) returned to within 10 % of the

3.16 Calcium current: current-voltage relation

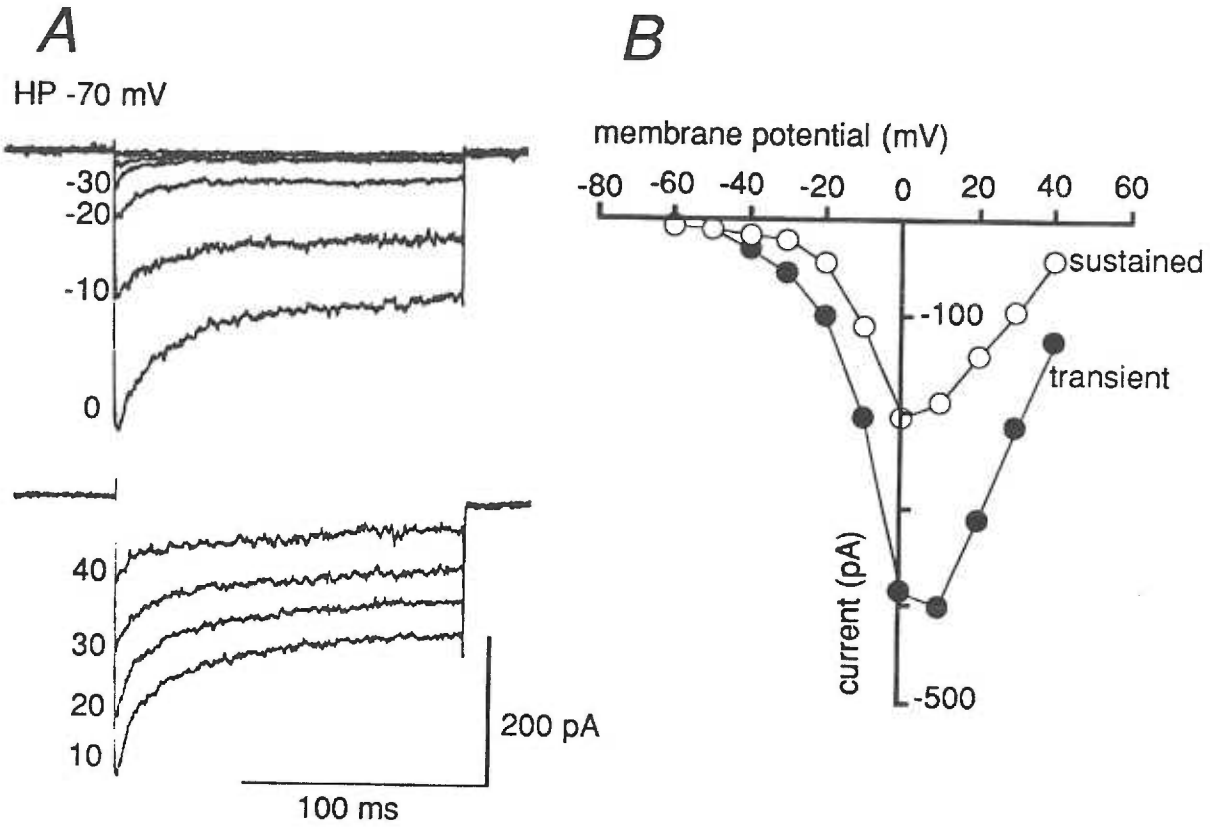
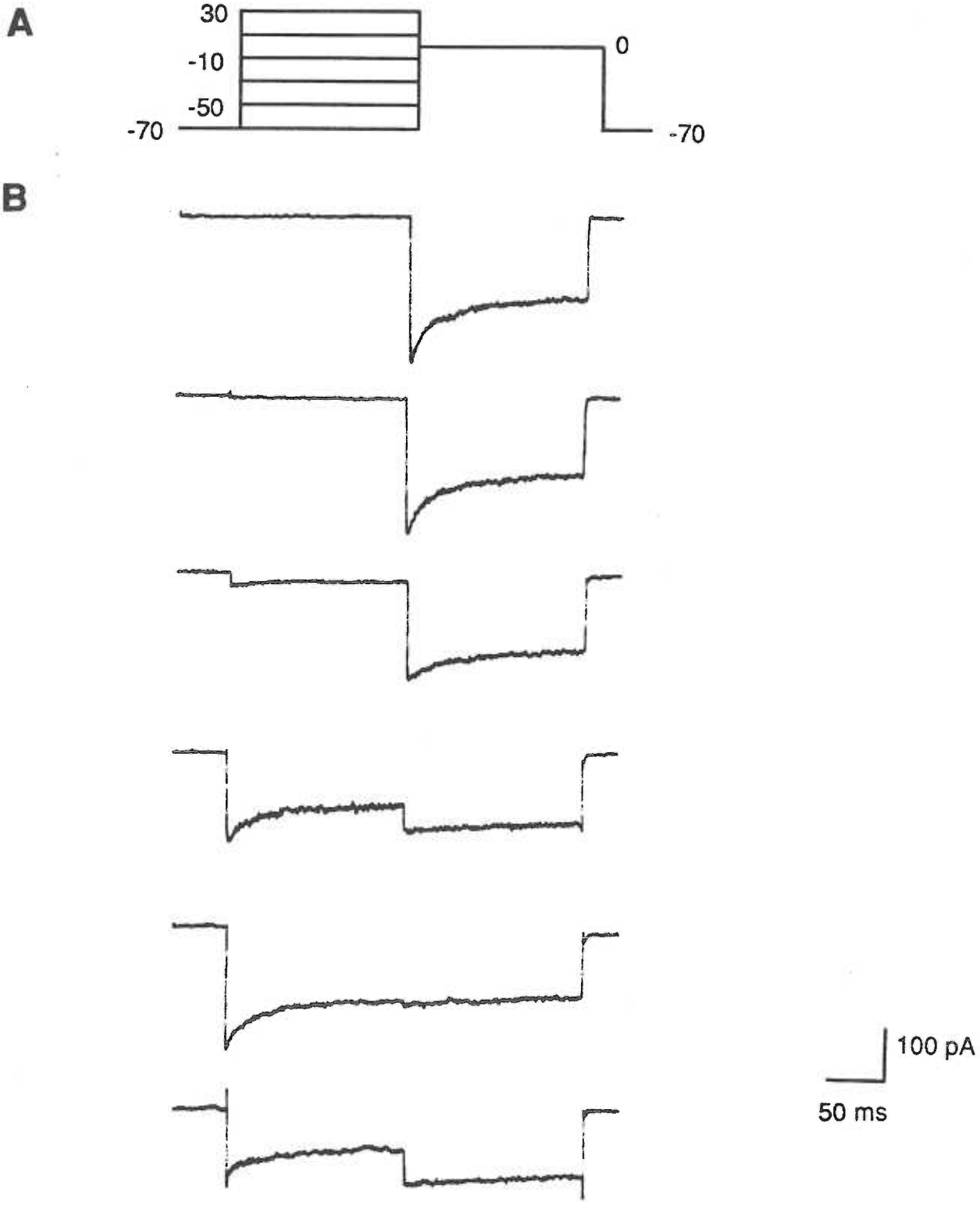


Figure 3.16 Calcium Current: Current-Voltage Relation. A, calcium currents were recorded in response to 150 ms depolarizing steps of 10 mV increments. Holding potential was -70 mV. B, current-voltage relation from the experiment illustrated in A are shown; the maximum current amplitude in the first 10 ms (transient, filled circles) and the average current amplitude in the last 10 ms (sustained, open circles) are plotted as a function of potential. (Mel 111589c5).

Figure 3.17 Steady-State Inactivation of Calcium Current. A, voltage step protocol. The holding potential of -70 mV was depolarized in 20 mV increments for 150 ms followed by a voltage step to 0 mV. B, currents recorded from the voltage protocol described in A. (Mel 11789c4).

3.17 Steady-state inactivation of calcium current



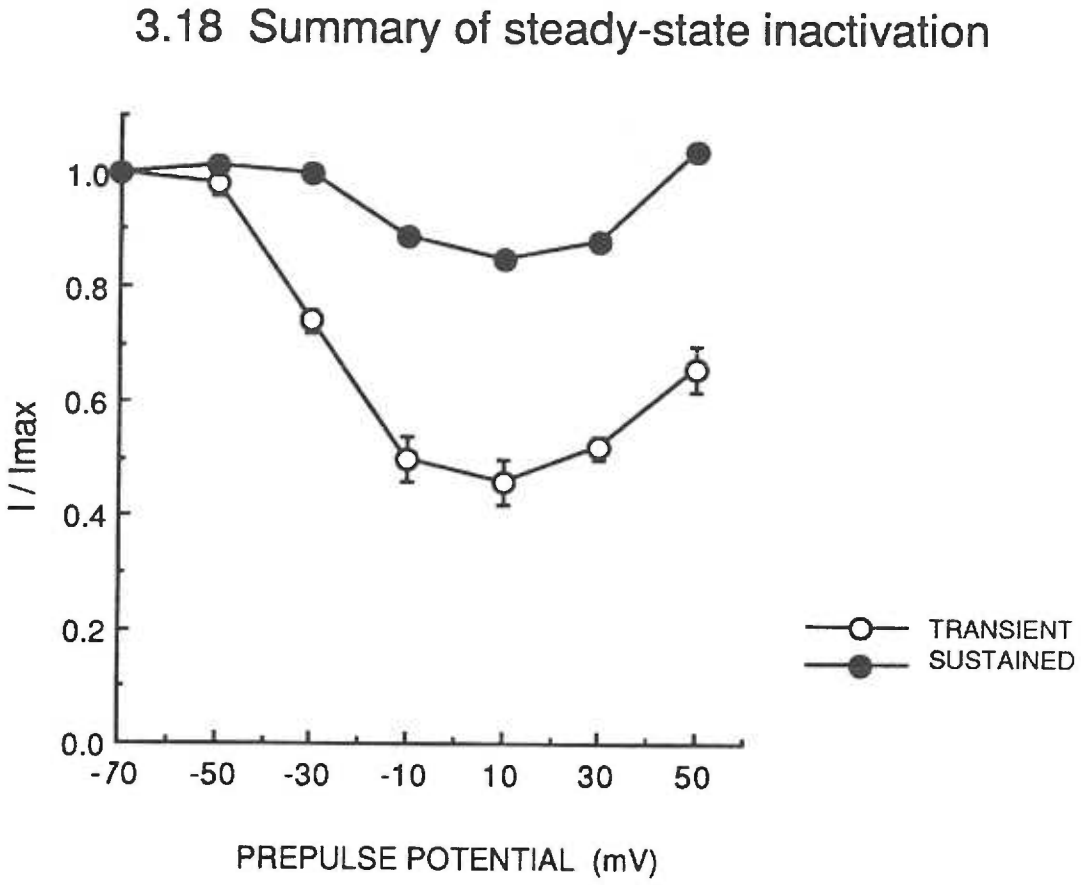


Figure 3.18 Summary of Steady-State Inactivation. Data from 3 cells exposed to the voltage protocol shown in Figure 3.17. Data are reported as current evoked by the test pulse (I) relative to its value when no prepulse was applied (Imax). Filled circles represent the transient component of the current and open circles represent the sustained component of the current.

control response after washout of the drug.

The actions of BAY K 8644 (1 μM) on calcium currents and their I-V relations were examined in 3 cells. Both the transient and the sustained component of the calcium current were enhanced at all potentials between -40 mV and 40 mV within 10 s after application of BAY K 8644 (Figure 3.19). The currents measured at the potentials that evoked the maximum current in the control solution were compared. The maximum transient component was increased an average of 139 $\pm$ 1 % (measured at -20 mV in 1 cell and 0 mV in 2 cells) and the maximum sustained component was increased 126 $\pm$ 9 % (measured at -10 mV in 1 cell and 0 mV in 2 cells). The difference in the enhancement of the transient and sustained component was not significantly different ($p = 0.0718$). The voltage at which the maximum transient component was measured in the presence of BAY K 8644 was shifted 10 mV in the hyperpolarizing direction in 2 of the 3 cells but voltage dependence of the maximum sustained component measured in the presence of BAY K 8644 remained the same .

Isoproterenol (1-10 μM) had no effect on the calcium current even after 5 minutes of application ($n = 6$).

3.3.3(iii) INHIBITION OF CALCIUM CURRENTS

MODULATION BY CALCIUM CHANNEL BLOCKERS

Nifedipine

The actions of nifedipine on the calcium current evoked by stepping the membrane from -70 mV to 0 mV were examined in a total of 14 cells. Nifedipine reduced the calcium current in a concentration-dependent manner but did not abolish the calcium current (Figure 3.20). The

3.19 BAY K 8644 stimulation of calcium current

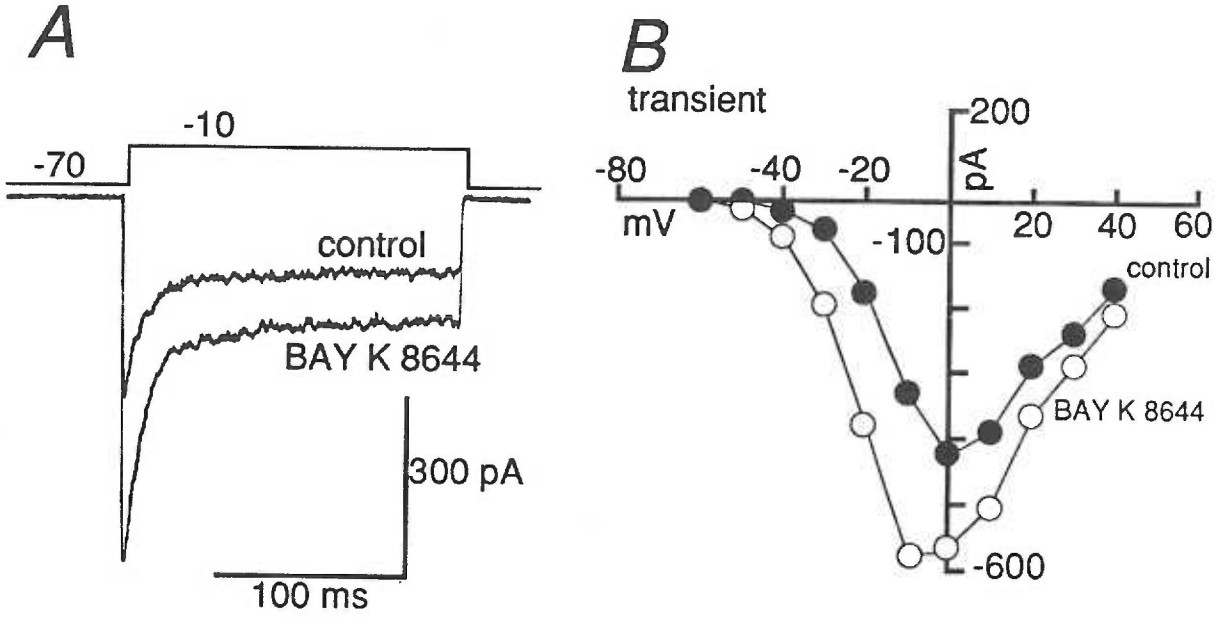


Figure 3.19 BAY K 8644 Stimulation of Calcium Current. A, calcium currents were evoked by depolarizing pulses from -70 mV to -10 mV in the presence and absence of BAY K 8644 (1 μM). (Mel 101889c3). B, the transient component of the calcium currents evoked from a series of depolarizing pulses in the presence and absence of BAY K 8644 (1 μM) is plotted as a function of potential. Current traces are from the same experiment shown in A.

3.20 Nifedipine inhibition of calcium current

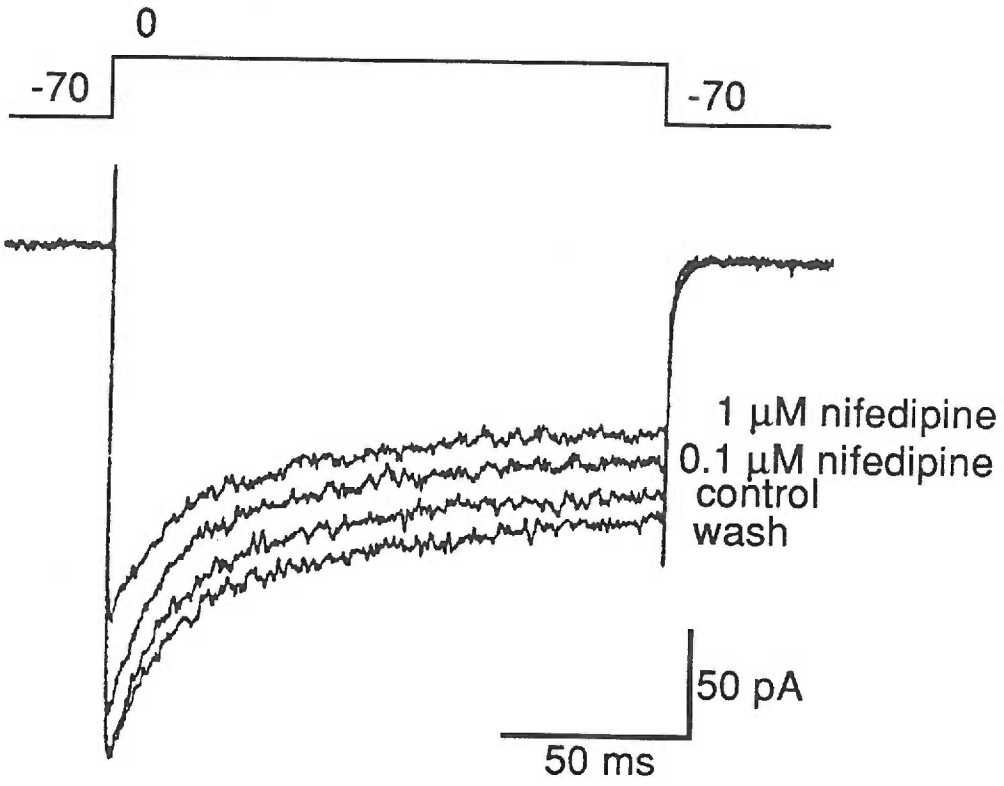


Figure 3.20 Nifedipine Inhibition of Calcium Current. Currents were evoked by stepping from -70 mV to 0 mV in control solution and increasing concentrations of nifedipine (0.1 and 1 μM). (Mel 102889c4).

threshold concentration required to inhibit the calcium current was about 100 nM; this concentration inhibited the transient and sustained components 10 ± 2 % and 21.25 ± 9.7, respectively, (n = 4). 10 μM-nifedipine maximally inhibited the current; this concentration reduced the transient component by 31 ± 4 % and reduced the sustained component by 24 ± 8 % (n = 11). There was no significant difference between the reduction of the transient and sustained components of the calcium current at any concentration of this antagonist.

Cadmium

Cadmium (100 μM) completely blocked the calcium current since cadmium (500 μM) had no additional blockade of the calcium current (n = 3). However, cadmium (500 μM) increased the RMS noise which indicated that the seal was becoming "leaky" when exposed to this high concentration of cadmium. This change in RMS noise was not seen when the cell was exposed to 100 μM cadmium therefore, 100 μM cadmium was routinely used for the cadmium-subtraction of calcium currents.

The following concentration-response experiments with cadmium were analyzed from cadmium-subtracted currents. Cadmium reduced the transient and sustained components of the current in a similar concentration-dependent manner (Figure 3.21). The threshold concentration required to inhibit the current was approximately 1 μM; this concentration reduced the transient and sustained component 12 ± 5 % and 20 ± 1.15 %, respectively, (n = 3). Calcium currents were inhibited greater than 90 % by 30 μM cadmium (n = 3).

3.21 Cadmium inhibition of calcium current

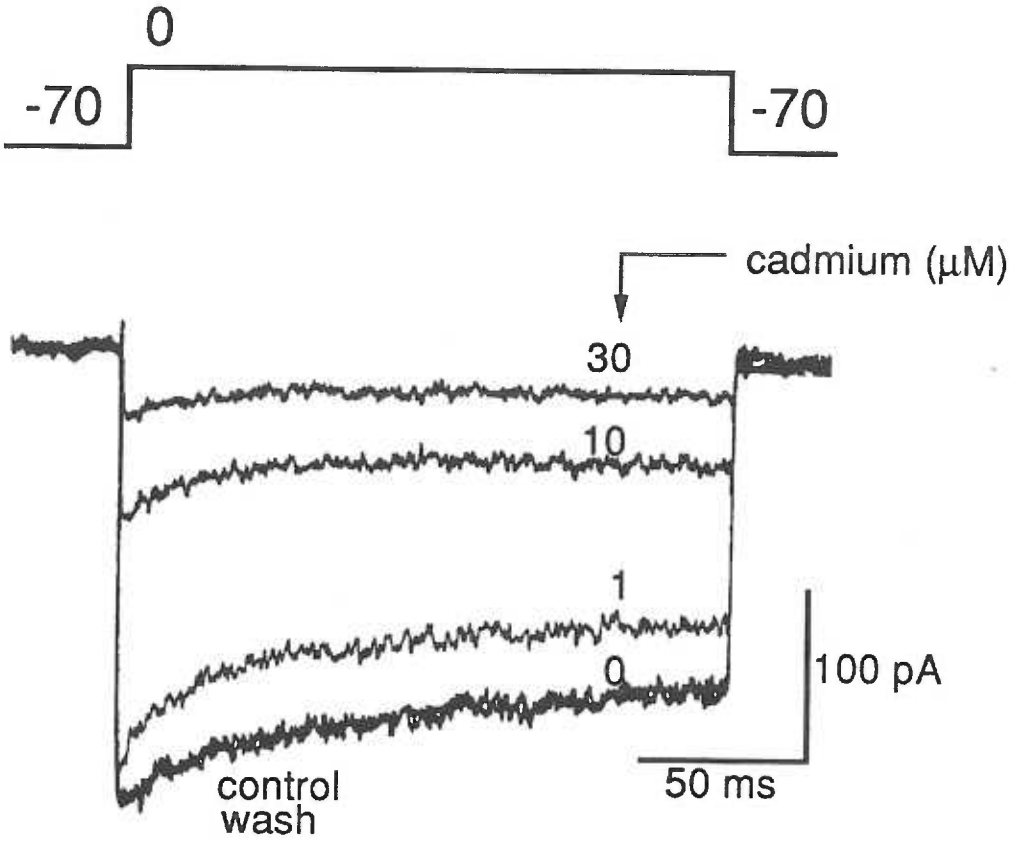


Figure 3.21 Cadmium Inhibition of Calcium Current. Currents were evoked by stepping from -70 mV to 0 mV in control solution and increasing concentrations of cadmium (1, 10, 30, and 100 μM). (Mel 101789c1).

Nickel

The calcium current was reduced by nickel but not completely blocked. 100 μM-nickel reduced the transient component 7.8 $\pm$ 7.4 % and the sustained component 20.0 $\pm$ 9.8 % (n = 5). 500 μM nickel reduced the transient current 68.0 $\pm$ 4.3 % and the sustained component 48.2 $\pm$ 5.4 % (n = 5; Figure 3.22).

MODULATION BY QUINPIROLE

Quinpirole reduced both the transient and sustained component of the calcium current although the maximum inhibition was never greater than 50% (n = 24; Figure 3.23). Quinpirole inhibited the transient component significantly more that the sustained component at all concentrations tested ($p < 0.0122$); inhibition of the transient component by 0.1, 1 and 3 μM quinpirole was 24.0 $\pm$ 2.5 % (n = 7), 36 $\pm$ 2 % (n = 22) and 40.0 $\pm$ 2.5 % (n = 4), respectively, while inhibition of the sustained component in the same cells was 10 $\pm$ 4 %, 24 $\pm$ 3 %, and 18 $\pm$ 3 % at these concentrations of quinpirole, respectively.

The I-V relation of the calcium current suggested that quinpirole reduced the calcium current at each potential and shifted the activation of the transient component from -40 mV to -30 mV and the sustained component from -30 mV to -20 mV; however, I-V relation of the maximum currents was not altered (Figure 3.24).

Both nifedipine and quinpirole only partially inhibited the calcium current (Figures 3.20, 3.23). The "L current" is defined as that component calcium current, which is sensitive to dihydropyridines (Tsien et al. 1987). Nifedipine partially inhibited the calcium current (Figure 3.20) suggesting that all of the channel activity underlying the calcium

3.22 Nickel inhibition of calcium current

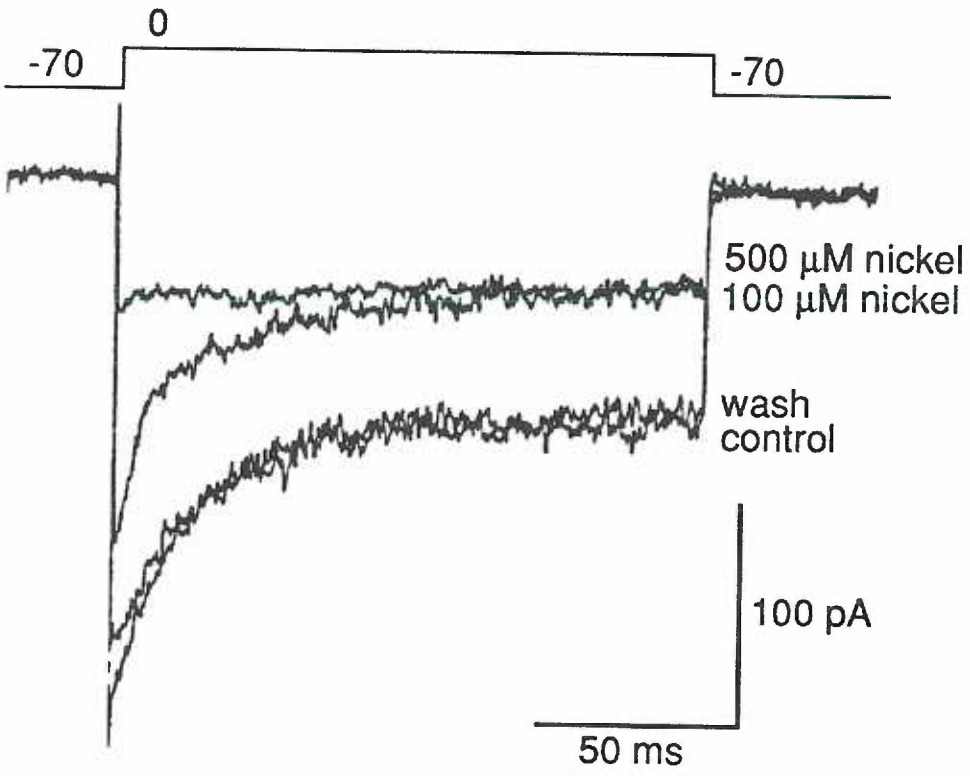


Figure 3.22 Nickel Inhibition of Calcium Current. Currents were evoked by stepping from -70 mV to 0 mV in control solution and increasing concentrations of nickel (100 and 500 μM). (Mel 102489c3).

3.23 Quinpirole inhibition of calcium current

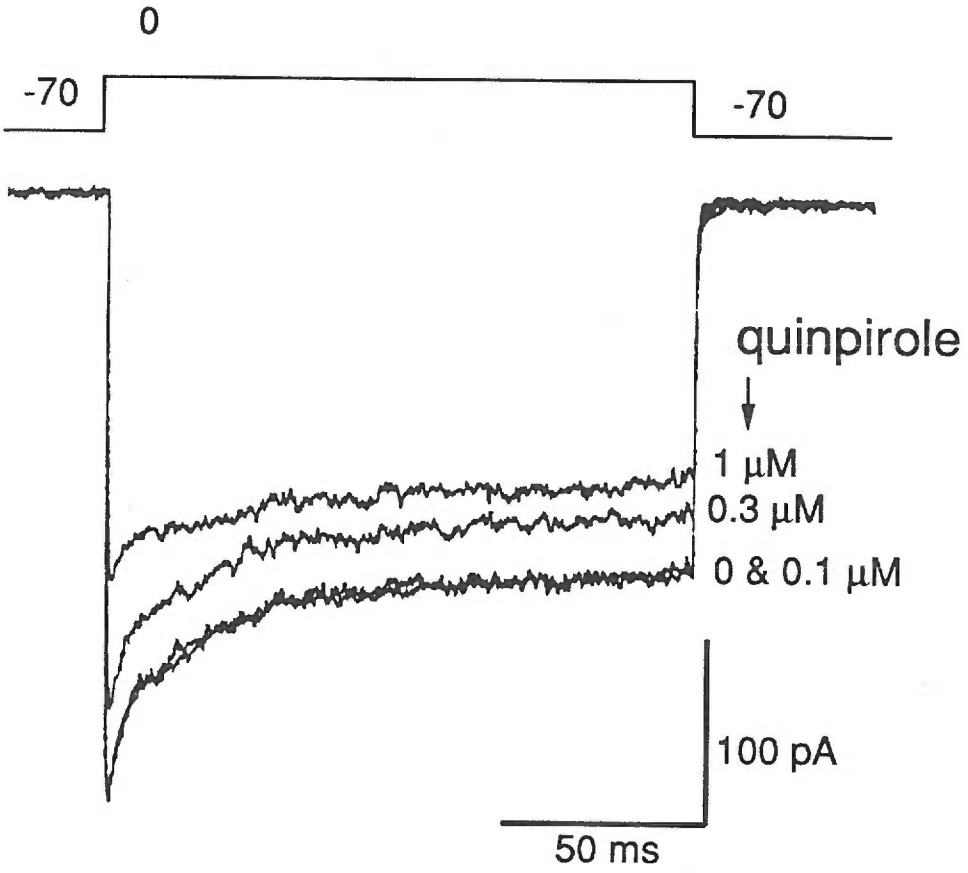
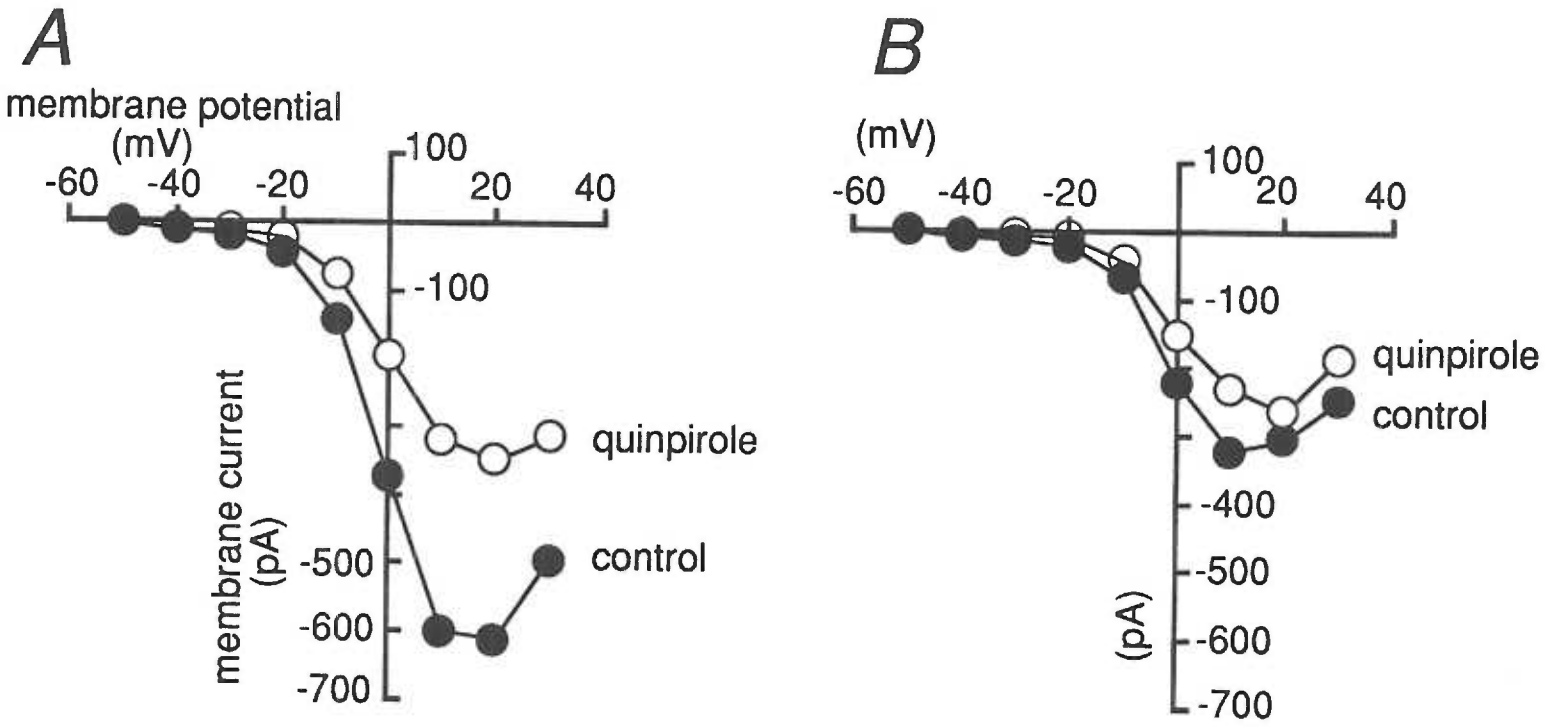


Figure 3.23 Quinpirole Inhibition of Calcium Current. Currents were evoked by stepping from -70 mV to 0 mV in control solution and increasing concentrations of quinpirole (0.1 and 1 μM). (Mel 11689c4).

Figure 3.24 Quinpirole Inhibition of Calcium Current: Current-Voltage Relation. Currents were recorded in response to 150 ms depolarizing steps of 10 mV increments from a holding potential of -70 mV. A, transient component of the calcium current in the absence (filled circles) and presence (open circles) of quinpirole (1 μM). B, sustained component of the calcium current in the absence (filled circles) and presence (open circles of quinpirole. (Mel 12689c2).

3.24 Quinpirole inhibition of calcium current: current-voltage relation



current is not sensitive to dihydropyridines. Quinpirole also only partially inhibited the calcium current (Figure 3.23). To determine whether quinpirole and nifedipine were reducing similar or different components of the calcium current, two sets of experiments were carried out. In the first set of experiments, quinpirole and nifedipine were presented separately and then simultaneously to determine whether the inhibition by each drug was additive. In 3 cells examined, quinpirole (1 μM) reduced the transient component by 37.5 $\pm$ 5.7 % and reduced the sustained component by 20.5 $\pm$ 8.4 %. Addition of nifedipine (1 μM) further reduced the transient component by 47.5 $\pm$ 6.2 % but this reduction was not significantly different from the reduction of the transient component by quinpirole alone ($p = 0.1408$). Addition of nifedipine had no further effect on the sustained component, which was reduced by 21.0 $\pm$ 6.1 %. However, in 3 cells examined where nifedipine (10 μM) was applied first, the transient component was reduced by 30.3 $\pm$ 3.3 % and the sustained component was reduced by 23.7 $\pm$ 2.9 %. The addition of quinpirole (1 μM) further reduced the transient component 52 $\pm$ 8 % and reduced the sustained component 33.3 $\pm$ 2.3 %. Both of these values were significantly different from nifedipine alone ($p = 0.0345$ and $p = 0.0302$, respectively). These results showed that the reduction of the calcium current by nifedipine and quinpirole was not completely additive. Application of quinpirole in the presence of nifedipine further reduced the calcium current indicating that quinpirole inhibited the calcium current at a dihydropyridine-insensitive site. In the converse experiment application of nifedipine after the application of quinpirole did not further reduce the current indicating that the dihydropyridine-sensitive current was already reduced. These data

suggest that the inhibition of the calcium current by quinpirole may involve the inhibition of a dihydropyridine-sensitive and a dihydropyridine-insensitive component of the calcium current. These results are shown in Figure 3.25.

The second experiment involved the application of quinpirole during the two step voltage paradigm for steady-state inactivation described in section 3.3.3(i) and Figure 3.17. Quinpirole (1 μM) reduced the transient component by 31.2 $\pm$ 5.6 % (n = 3) when the current was recorded in response to a voltage step from -70 mV to 0 mV; however the agonist inhibition of the transient component was reduced or abolished when the prepulse potential was -10 mV, 10 mV, and 30 mV (Figure 3.26). At these potentials, the transient component was reduced about 50 % (Figure 3.18). This experiment indicate that the portion of the transient component that is voltage sensitive is also blocked by quinpirole. The inhibition of the sustained component by quinpirole was 11.5 $\pm$ 3.2 % (n = 3). Inhibition of the sustained component by quinpirole was not significantly reduced by depolarizing prepulses. These data suggest that quinpirole inhibits the inactivating portion of the calcium current and also partially inhibits the non-inactivating portion of the current.

INVOLVEMENT OF G-PROTEINS

Pretreatment of the cells with pertussis toxin completely blocked quinpirole (1 μM) inhibition of the transient and sustained component of the current (n = 3; Figure 3.27). Calcium currents, measured in cells from the same culture preparation but not pretreated with pertussis toxin, were reduced by quinpirole (n = 2).

3.25 Quinpirole plus nifedipine inhibition of calcium currents

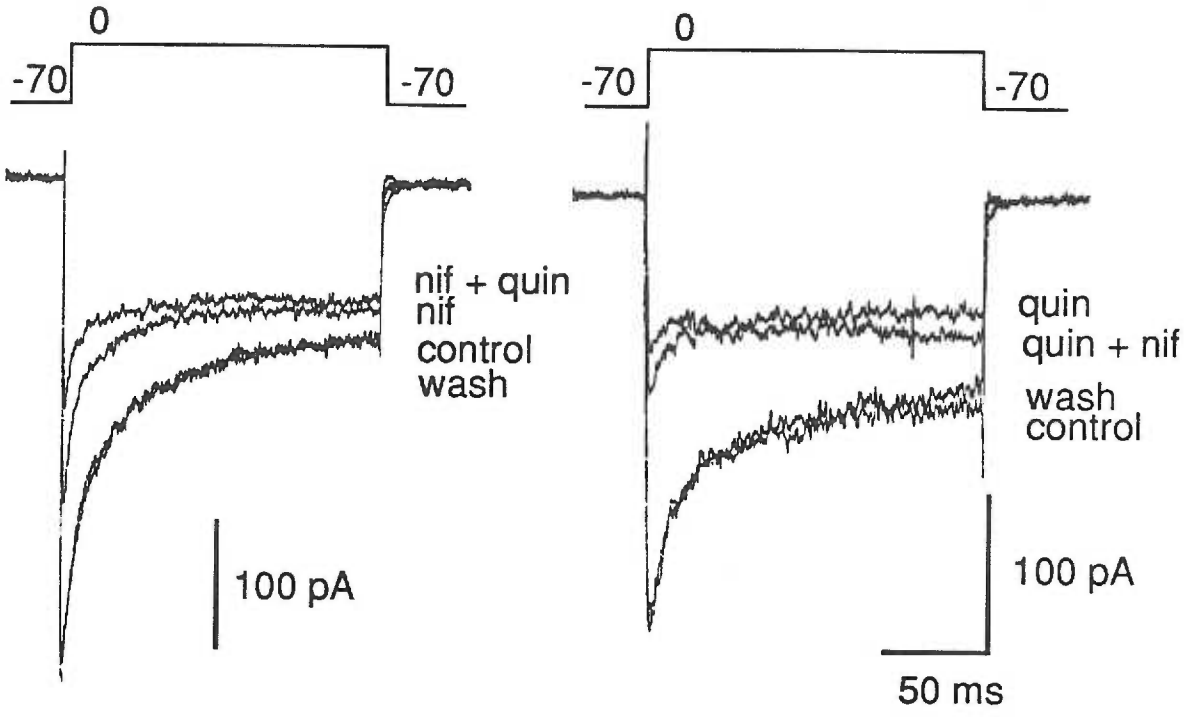


Figure 3.25 Quinpirole Plus Nifedipine Inhibition of Calcium Currents. A, currents were evoked by stepping from -70 mV to 0 mV in control solution, nifedipine (10 μM), and nifedipine plus quinpirole (1 μM). (Mel 121689c3). B, currents were evoked by stepping from -70 mV to 0 mV in control solution, quinpirole (1 μM), and quinpirole plus nifedipine (10 μM). (Mel 121689c5).

3.26 Voltage sensitivity of quinpirole inhibition

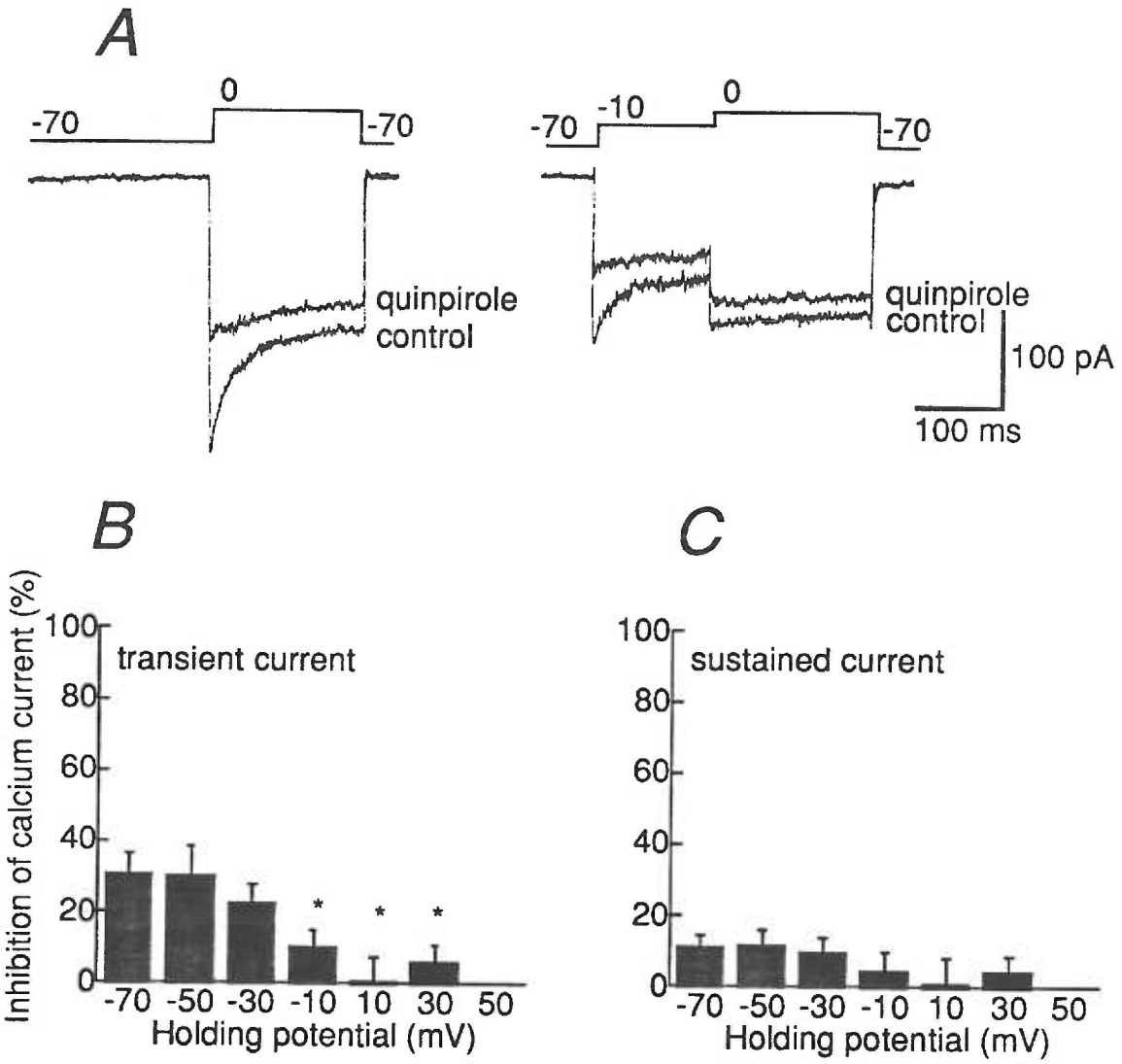


Figure 3.26 Voltage Sensitivity of Quinpirole Inhibition. Currents were recorded in response to 20 mV depolarizing prepulses of 150 ms followed by a 150 ms voltage step to 0 mV (protocol described in Figure 3.17A) from a holding potential of -70 mV. A, currents recorded without a prepulse and with a -10 mV prepulse in the presence and absence of quinpirole (1 μM). (Mel 11789c4). B, the inhibition of the transient component by quinpirole is plotted as per cent inhibition as a function of voltage (n = 3). C, the inhibition of the sustained component by quinpirole is plotted the same as B.

3.27 Pertussis toxin blocks the inhibition of the calcium current by quinpirole

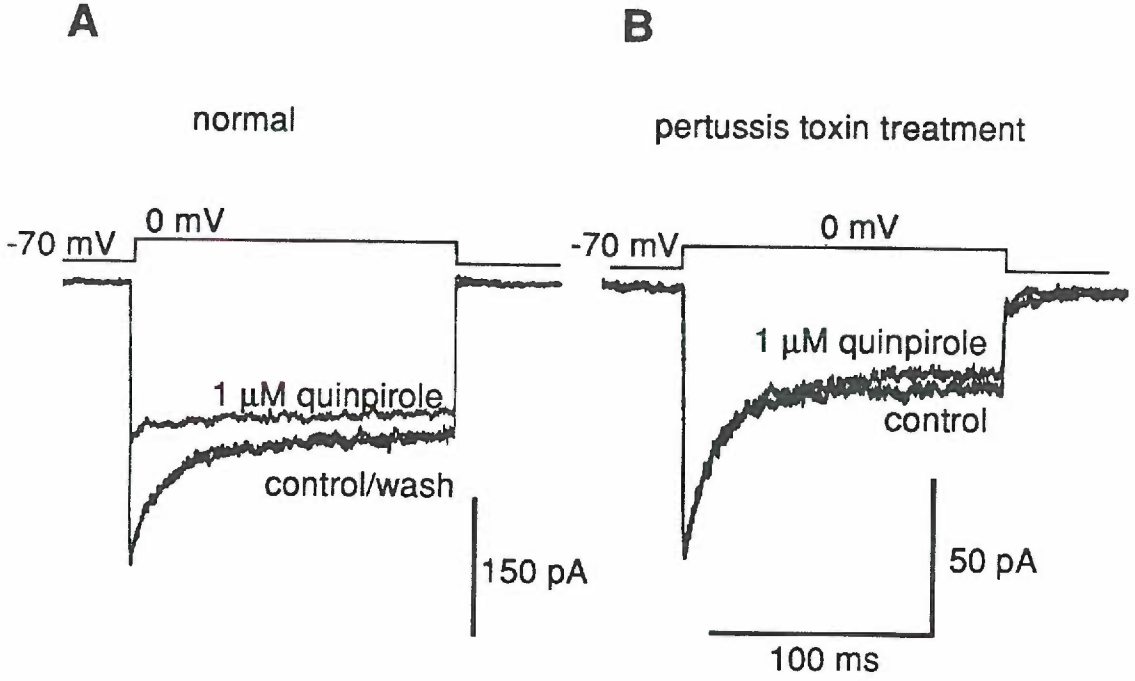


Figure 3.27 Pertussis Toxin Blocks the Inhibition of the Calcium Current by Quinpirole. Currents were evoked by stepping from -70 mV to 0 mV in control solution and quinpirole (1 μM). A, control. (Mel 11689c1). B, pretreated with pertussis toxin (100 ng/ml for more than 16 hours). (Mel 11989c4).

GTPγS (100 μM) was included in the recording pipette solution and calcium currents were recorded in six cells in the absence and presence of quinpirole (1 μM). In two cells, quinpirole produced an irreversible reduction in the current (Figure 3.28). In 2 cells, quinpirole reduced the calcium current in a reversible manner to the first application and in one of these cells application of the drug 1.5 minutes later had no effect on the calcium current. In two cells, quinpirole had no effect on the calcium current. In the absence of GTPγS or pertussis toxin pretreatment, quinpirole (1 μM) always decreased the calcium current (n = 22).

3.4 DISCUSSION

The purpose of these studies was to determine the relationship between the direct inhibition of voltage-dependent calcium currents and inhibition of secretion of β-endorphin in melanotrophs. The concentration responses of calcium channel blockers and quinpirole, a dopamine D_2 specific agonist, were measured on voltage-dependent calcium currents and on secretion. Concentration-response curves of each agonist are compared. In summary, little agreement was found between concentrations of calcium channel blockers that inhibited secretion and reduced calcium currents. The concentration of quinpirole that reduced calcium currents exceeded the concentration of agonist that inhibited secretion. These results suggest that direct (or voltage-independent) inhibition the calcium current is not the primary mechanism by which activation of the D_2 receptor inhibits secretion of β-endorphin. Evidence of a voltage-dependent mechanism to reduce calcium influx is suggested as the primary mechanism by which the stimulus-secretion process is

3.28 Irreversible inhibition of calcium current by quinpirole with GTP-γ-S in pipette solution

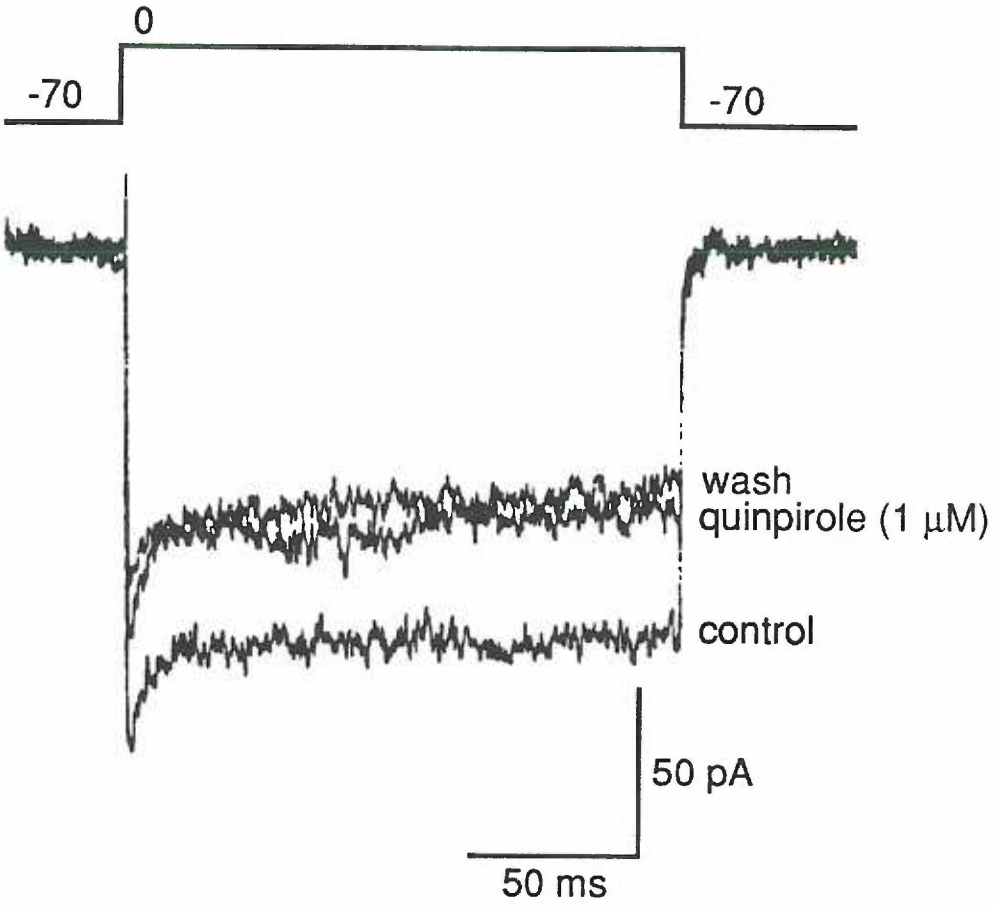


Figure 3.28 Irreversible Inhibition of Calcium Current by Quinpirole with GTPγS (100 μM) in the Pipette Solution. Currents were recorded in response to a voltage step from -70 mV to 0 mV in the absence and presence of quinpirole (1 μM). Inhibition of the current by quinpirole was irreversible (compare with Figure 3.27A) in two of the four cells that were sensitive to quinpirole. (Mel 123089c1).

interrupted upon activation of the D_2 receptor.

3.4.1 THE VOLTAGE-DEPENDENT CALCIUM CURRENT IN THE MELANOTROPH

Voltage-dependent calcium currents were measured in melanotrophs. The calcium currents exhibited partial inactivation during the 150 ms voltage pulse. Therefore, two phases of the current were measured; the transient component, which was measured in the first 10 ms of the pulse, and the sustained component which was measured in the final 10 ms of the pulse. A ratio of 1.92 was obtained when the current amplitudes of the transient to sustained components were compared (using the largest current of 16 cells) which indicates that approximately 50 % of the current inactivated by 150 ms.

In light of the fact that calcium channel types have been described by different inactivation rates (e.g. "N" and "L"), it was of interest to determine whether more than one type of calcium channel was activated to produce the transient component and sustained component of the current. Although single channel recording is the only definitive proof of calcium channel subtypes, the voltage-dependence and inhibition of the current by various calcium channel blockers can provide clues to the type(s) of channels present. Calcium currents were analyzed to characterize the current and determine whether any experimental manipulations would dissociate the transient from the sustained component of the current.

The voltage dependence of the two current components was similar when evoked from a holding potential of -70 mV. They were activated in the same voltage range, -50 mV to -30 mV, and the maximum current was generally evoked at 0 mV. No calcium currents were activated when the

cell was held at -100 mV and stepped to -50 mV indicating that a low threshold current was not present.

Cadmium, a non-selective blocker of calcium channel subtypes, blocked the transient and sustained components of the current; 100 μM cadmium produced a complete block. Nickel, a specific calcium channel blocker for the "T" channel in DRG cells (Seabrook and Adams, 1989), reduced both components of the current at 100 μM. However, 500 μM nickel further reduced only the transient component without producing any further depression of the sustained component.

The current was partially sensitive to dihydropyridine compounds which are specific for the "L" type channel in DRG cells (Fox et al. 1987). Nifedipine (10 μM) maximally reduced the transient and sustained component in a similar manner; the current was inhibited by 40 %. BAY K 8644 enhanced the transient component and sustained component of the calcium current. Augmentation of the calcium current was like that described in cardiac cells (Hamilton et al. 1987) where BAY K 8644 enhanced the transient component of the current and shifted the I-V relation 10 mV in the hyperpolarizing direction.

Isoproterenol, when applied acutely during the whole-cell recording did not enhance the calcium current. Enhancement of the calcium current in melanotrophs by stimulation of the β-adrenergic receptor or by the cAMP analog, 8-bromo-cAMP, has only been measured in cells that have been treated with the agonist for 20 min prior to the formation of the whole-cell configuration (Cota & Hiriart, 1989). It is possible that the exchange of the pipette recording solution with the intracellular milieu in the whole-cell configuration may dilute or remove the critical components needed to couple the activation of the β-adrenergic receptor

to the ion channel and explain why acute application of isoproterenol during recording had no effect. Reconstitution experiments may help elucidate this pathway.

This data suggests that there may be more than one type of calcium channel activity which generates the calcium current. The transient component can be characterized by inactivation and inhibition by nickel (500 μM). However, the transient component is most likely composed of more than one type of calcium channel activity, since nothing exclusively abolished the transient component. The inactivating portion of the transient component shares similarities with both the two inactivating currents described in DRG cells. It is similar to the "T" current based on its sensitivity to nickel (however, the concentration needed is 500 μM compared to 50 μM in DRG cells) and similar to the "N" current based on voltage dependence. The sustained component inactivates slowly and is sensitive to dihydropyridines; it may be composed of an "L" channel but may not exclusively represent a specific calcium channel type since it was only partially inhibited by nifedipine.

In two other studies on calcium currents in melanotrophs, two calcium channels types have also been inferred (described in secretion 3.1.4; Cota, 1986; Williams, MacVicar & Pittman, 1990). However, the description of two currents in each of these two studies along with the present study do not totally agree. The differences may be accounted for by the differences in the methodology used by each of these studies which include culture conditions (acute intact organ preparation verse long term, dispersed cell cultures), recording techniques (intracellular recording verse whole-cell recording), recording conditions (room temperature verse physiological temperature), and current analysis

(activation of the current verse deactivation of the current).

Cota and Hiriart (1989) reported calcium currents in cultured melanotroph using whole-cell recording on cultured melanotrophs at room temperature. By tail currents analysis, they described the fast deactivating channel (FD; also called the high-threshold, non-inactivating channel) and the slow deactivating (SD; also called the low-threshold, inactivating channel). The FD current was increased by isoproterenol and 8-bromo-cAMP, slightly increased by BAY K 8644 and decreased by nifedipine and dopamine. The SD current was increased 120 % by BAY K 8644, decreased by nifedipine and not changed by isoproterenol or dopamine. Tail currents were not examined in the present study because the higher temperatures (36°C in this study compared to room temperature in the study by Cota) markedly increases tail current decay rate, making it unfeasible to measure tail current time course with any accuracy. Williams et al. (1990) using intracellular recording on an acute intact IL preparation at physiological temperature also observed two types of calcium currents in these cells and differentiated the current into a "T" and an "L" current based on their voltage dependence. Williams et al. (1990) found a current that activated at -50 mV from a holding potential of -90 mV and inactivated in 50 ms ("T", low threshold, inactivating) and a non-inactivating current that was activated at step potentials greater than -20 mV ("L", high threshold, non-inactivating). Contrary to the report by Cota, in the Williams' study the low threshold, inactivating current that was reduced by dopamine.

Modulation by quinpirole

The calcium current was inhibited by quinpirole in a concentration-dependent manner where, at each concentration tested, the reduction in the transient component was significantly greater than the reduction in the sustained component. However, the maximum inhibition of the transient component by quinpirole was only 40 % of the control response. The transient component was not only preferentially reduced by quinpirole but also by the steady-state inactivation compared to the sustained component (see Figure 3.17 and 3.27). And, in the presence of steady-state inactivation, the reduction of the transient component by quinpirole was attenuated (Figure 3.26), which indicates that the major portion of the transient component of the current that is reduced is the inactivating portion of the current which is in accord with Williams et al. (1990).

If quinpirole and nifedipine were selectively blocking two different channels, one would expect the inhibitions to be additive but they were not. In the presence of nifedipine (which reduced the current 31 %), quinpirole only increased the inhibition of the current to 55 % (see Figure 3.25). Thus, although quinpirole was more effective in inhibiting the transient than the sustained component of the current, quinpirole clearly does not block the transient component to the exclusion the sustained component.

The mechanism that couples the D_2 receptor to the calcium channel involves a G-protein since pretreatment with pertussis toxin abolished the inhibitory effect of quinpirole on the calcium current amplitude. Experiments with GTPγS were carried out but the results of these experiments were not consistent. In two cells, quinpirole produced an

irreversible inhibition of the calcium current. This was the expected result if indeed the coupling mechanism between the receptor and the channel involved a G-protein. In two other cells, there was no response to quinpirole. This type of result might be expected if there was GTP interaction G-proteins that did not require activation of the receptor. With the introduction of a non-hydrolyzable form of GTP, in time, the G-protein pool would be activated. In this activated state, application of the agonist would have no effect if the response required the induction of a G-protein activation. This time dependence of the loss of a receptor mediated response in the presence of GTPγS has been reported in AtT-20 cells. An initial exposure to SRIF applied 10 min after the formation of the whole-cell configuration did not inhibit the calcium current whereas application of SRIF before 10 min reduced the calcium current in an irreversible manner (Lewis, Weight & Luini, 1986). In the present study, a lack of response to quinpirole was never observed in 22 control cells treated with quinpirole (1 μM). And finally in two cells, quinpirole produced a reversible response. These different results may be due to the rate of diffusion of GTPγS into the cell relative to the time the agonist was applied or to an excess of endogenous GTP that remained effective during the course of the recording.

These results are in agreement with the evidence reported by others (Williams, MacVicar & Pittman, 1990; Lledo et al. 1990a) that the mechanism coupling the D2 receptor to the calcium channel involves a pertussis toxin sensitive G-protein.

3.4.2 MODULATION OF β-ENDORPHIN SECRETION

The modulation of basal and stimulated secretion was examined. Basal secretion was reduced by quinpirole in a concentration-dependent manner with an IC_{50} of 10 nM; however, basal secretion was not reduced by nifedipine or TTX. Secretion was stimulated approximately 2-fold by BAY K 8644 and isoproterenol, and stimulated secretion was not blocked by TTX, indicating that the high amplitude voltage spikes of the spontaneous action potentials in these cells are not involved in the regulation of stimulated secretion. Nifedipine did not significantly reduce secretion. Cadmium was ineffective in reducing stimulated secretion at concentrations less than 500 μM and nickel did not block stimulated secretion at 500 μM. However, quinpirole was very effective in reducing secretion stimulated by BAY K 8644 and isoproterenol with an IC_{50} of about 10 nM for each. The inhibition of stimulated secretion by quinpirole was blocked when cells were pretreated with pertussis toxin, indicating the coupling mechanism between the receptor and the inhibition of secretion involves a G-protein. These results are supported by another study examining the pertussis toxin sensitivity of inhibition of BAY K 8644-stimulated levels of prolactin secretion by dopamine in anterior pituitary cells (Enjalbert et al. 1988). Further since dopamine and quinpirole inhibition of secretion stimulated by a cAMP-independent mechanism and a cAMP-dependent mechanism at similar concentrations supports the hypothesis that activation of the D_2 receptor interrupts the secretion process by a cAMP-independent mechanism.

3.4.3 COMPARISON OF THE INHIBITION OF THE CALCIUM CURRENT AND THE INHIBITION OF β-ENDORPHIN RELEASE IN MELANOTROPHS

To determine whether a direct relationship exists between the inhibition of the calcium current and the inhibition of secretion, the concentration-response curves for cadmium, nifedipine and quinpirole are compared in Figures 3.29, 3.30 and 3.31. Three parameters, the IC_{50}, IC_{max}, and the maximum inhibition of the response, were measured from each concentration-response curve and these parameters are summarized in Table 3.1.

Cadmium

In Figure 3.29 the concentration response curves of cadmium inhibition of BAY K 8644-stimulated secretion and the voltage-dependent calcium current are shown. As seen in the AtT-20 cell, there was no agreement between the concentrations of cadmium that inhibited the calcium current and that inhibited stimulated secretion. Concentrations of cadmium that completely blocked the voltage-dependent calcium current (100 μM) only reduced stimulated secretion 30 %. However, 1 mM cadmium was needed to abolish stimulated secretion. The IC_{50} for the inhibition of the calcium current was 6 μM and for the inhibition of secretion was 200 μM. These results suggest that the voltage-dependent calcium current measured in these experiments may not be involved in the regulation of the stimulus-secretion coupling process.

Nifedipine

In Figure 3.30 the concentration-response curves of nifedipine inhibition of the voltage-dependent calcium current and the effects on

3.29 Comparison of the concentrations of cadmium that inhibit ß-endorphin secretion and that reduce the calcium current

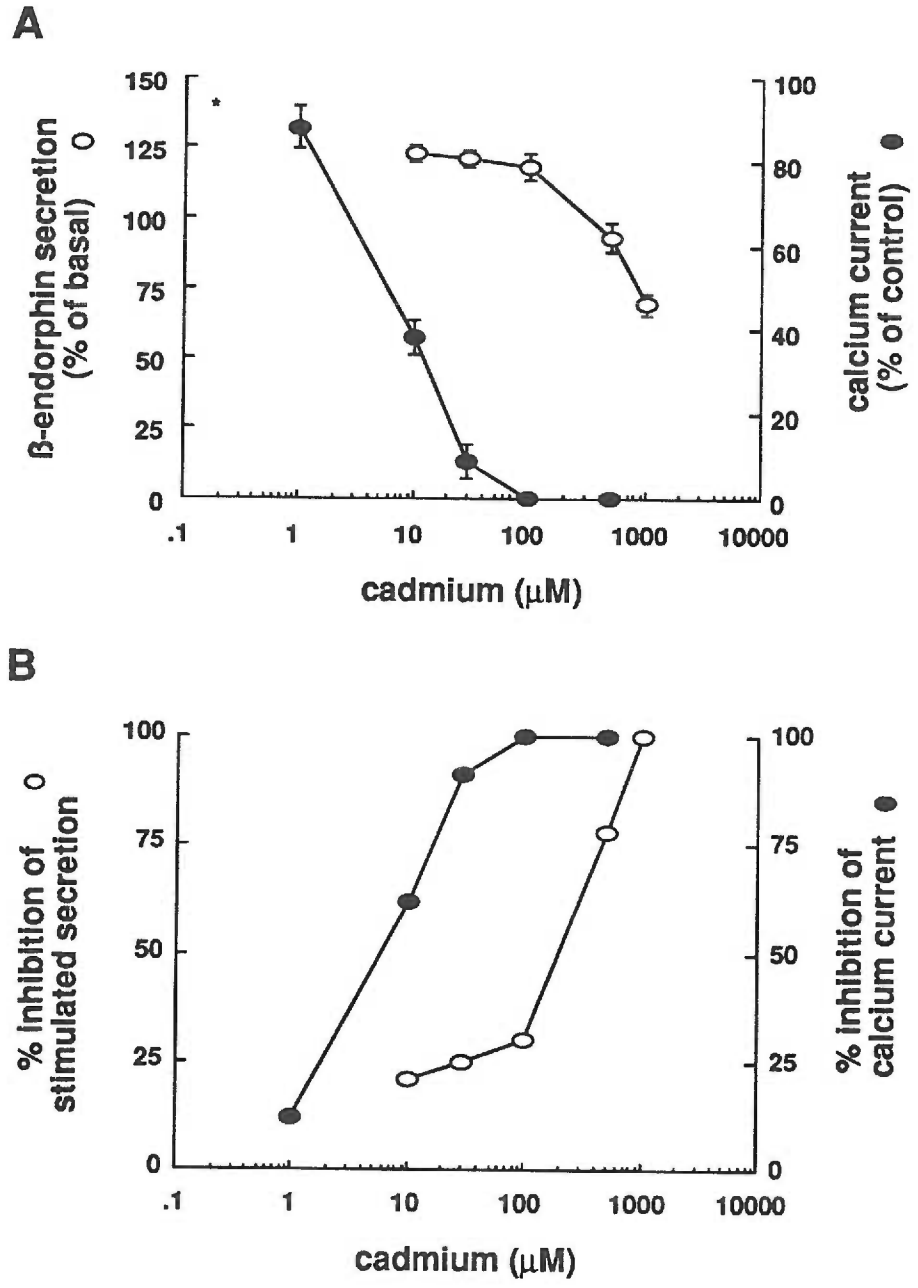


Figure 3.29 Comparison of the Concentrations of Cadmium that Inhibit β-Endorphin Secretion and that Reduce the Calcium Current. A, β-endorphin secretion stimulated by BAY K 8644 (open circles) and the calcium current (filled circles) are plotted as a function of the cadmium concentration. The * indicates the control response of secretion in the absence of cadmium. B, the data shown in A have been transformed such that the inhibition of secretion (open circles) and the inhibition of the calcium current (filled circles) are plotted as a function of the cadmium concentration.

3.30 Comparison of concentrations of nifedipine that inhibit ß-endorphin secretion and that reduce the calcium current

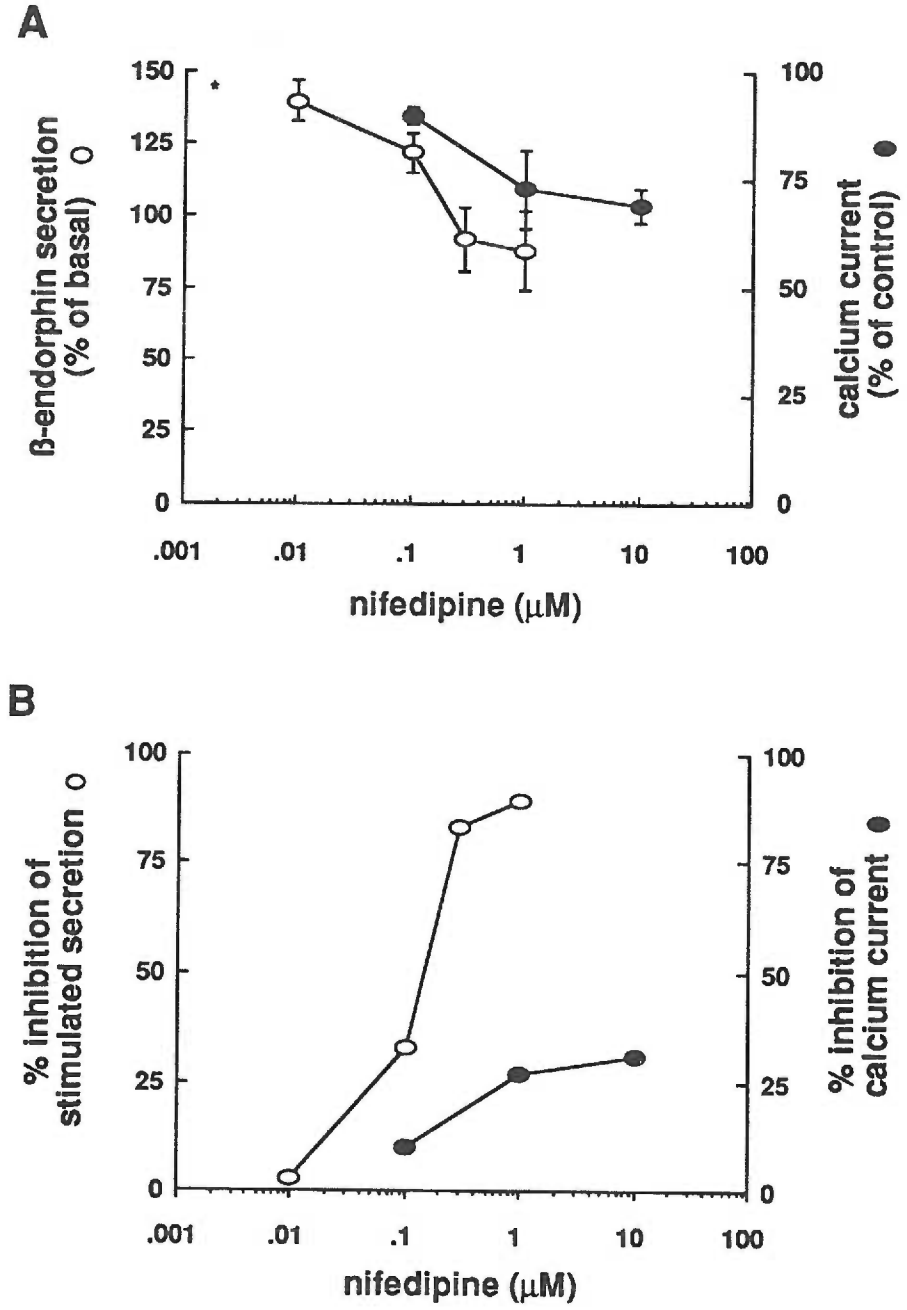


Figure 3.30 Comparison of the Concentrations of Nifedipine that Inhibit β-Endorphin Secretion and that Reduce the Calcium Current. A, β-endorphin secretion stimulated by isoproterenol (open circles) and the calcium current (filled circles) are plotted as a function of the nifedipine concentration. The * indicates the control response of secretion in the absence of nifedipine. B, the data shown in A have been transformed such that the inhibition of secretion (open circles) and the inhibition of the calcium current (filled circles) are plotted as a function of the nifedipine concentration.

inhibition of isoproterenol-stimulated secretion are shown. (Note that although there is a trend of inhibition of secretion with increasing concentrations of nifedipine, this did not prove to be significant as shown in Figure 3.11.) The curves demonstrate some agreement since the IC_{50}s and the IC_{max}s are similar. The IC_{50} for the inhibition of the calcium current is 200 nM and for inhibition of secretion is 100 nM. The IC_{max} for the inhibition of the calcium current is 10 μM and the inhibition of secretion is 1 μM. However, the calcium current was maximally reduced by 31 % whereas the stimulated secretion was reduced 89 %.

Quinpirole

In Figure 3.31 the concentration response curves of quinpirole inhibition of the voltage-dependent calcium current and the inhibition of BAY K 8644 stimulated secretion are shown. The modulation of these two responses by quinpirole show no correlation. At concentrations that completely blocked stimulated secretion (10 nM), the calcium current is not reduced. The IC_{50} for the inhibition of the calcium current is 80 nM whereas for the inhibition of secretion is 3 nM. The IC_{max} for the inhibition of the calcium current is 1 μM whereas for the inhibition of secretion is 0.1 μM. Finally, quinpirole inhibited secretion 100 % but only reduced the calcium current 40 %. These data suggest that although quinpirole can reduce the voltage-dependent calcium current, direct inhibition of the calcium current by activation of the D_2 receptor probably is not the primary mechanism involved in the inhibition of stimulated secretion.

3.31 Comparison of concentrations of quinpirole that inhibit ß-endorphin secretion and that reduce the calcium current

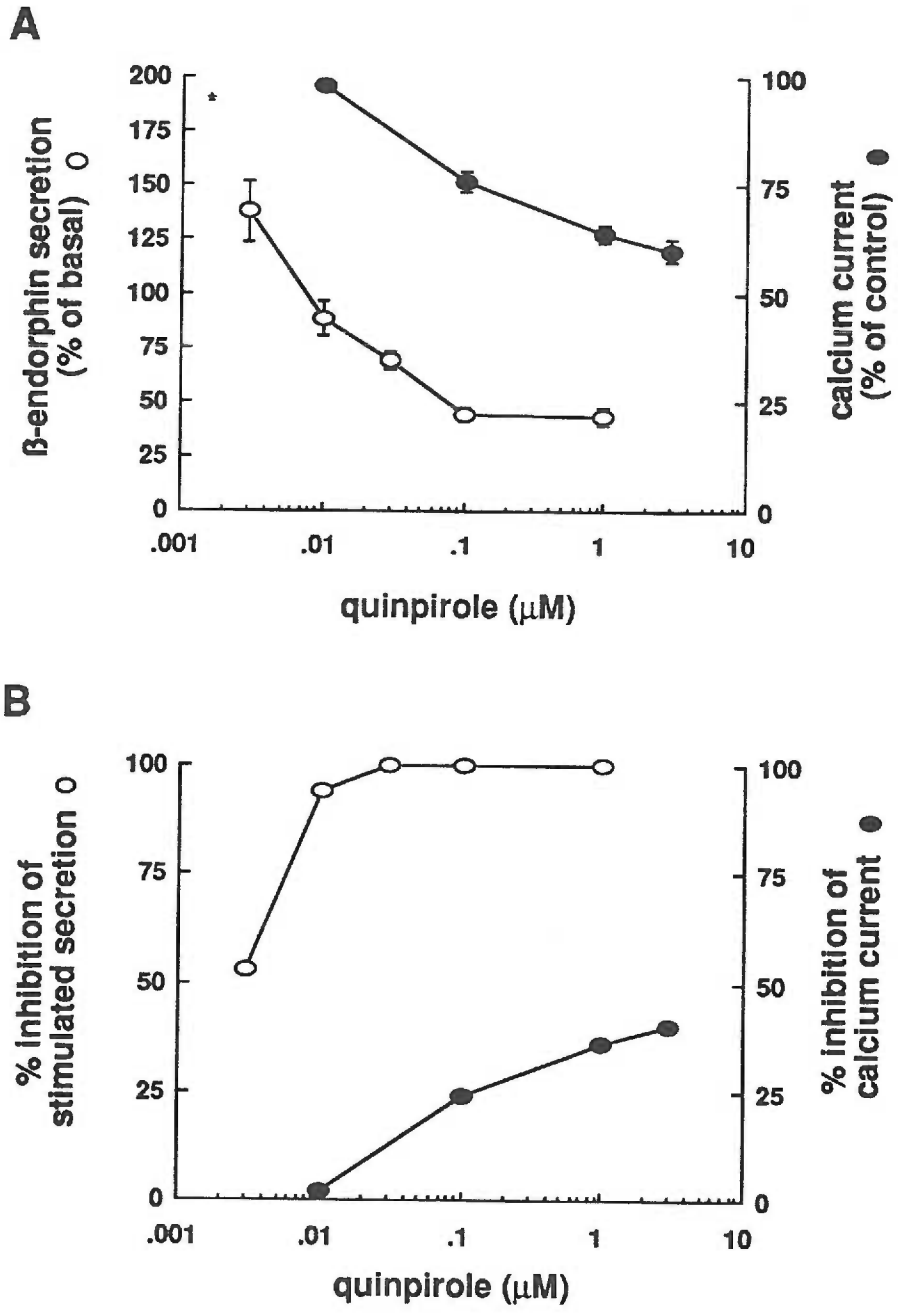


Figure 3.31 Comparison of the Concentrations of Quinpirole that Inhibit β-Endorphin Secretion and that Reduce the Calcium Current. A, β-endorphin secretion stimulated by BAY K 8644 (open circles) and the calcium current (filled circles) are plotted as a function of the quinpirole concentration. The * indicates the control response of secretion in the absence of quinpirole. B, the data shown in A have been transformed such that the inhibition of secretion (open circles) and the inhibition of the calcium current (filled circles) are plotted as a function of the quinpirole concentration.

3.4.4 COMPARISON OF MEMBRANE HYPERPOLARIZATION AND INHIBITION OF β-ENDORPHIN RELEASE IN MELANOTROPHS

Activation of the D_2 receptor has been shown to be linked not only to voltage-dependent calcium currents but also to an increase in a potassium conductance. This has been seen in neurons (Lacey, Mercuri & North, 1987), lactotrophs (Lledo, 1990b), and melanotrophs (Stack, Surprenant & Allen, 1987; Williams, Pittman & MacVicar, 1987). Dr. A. Surprenant has conducted studies on melanotrophs measuring the membrane hyperpolarization in response to quinpirole by the whole-cell current-clamp recording technique (recording solutions are described in Figure 3.3). Quinpirole produced a concentration-dependent membrane hyperpolarization. The maximum hyperpolarization was associated with complete blockade of spontaneous depolarizations (Figure 3.32). Figure 3.33 shows the concentration response curves for the quinpirole activation of the membrane hyperpolarization as well as the inhibition of BAY K 8644-stimulated secretion. When concentrations of quinpirole are compared, these two responses show a direct relationship. The IC_{50} for the membrane hyperpolarization is 4 nM whereas the IC_{50} for the inhibition of stimulated secretion is 3 nM. Quinpirole (100 nM) produced a maximum membrane hyperpolarization (16 mV) and a maximum inhibition of secretion. These results suggest that the primary mechanism by which activation of the D_2 receptor is coupled to the inhibition of secretion may involve hyperpolarization of the membrane, which would inhibit of the calcium influx in a voltage-dependent manner. This mechanism will indirectly reduce the voltage-dependent calcium current since hyperpolarization of the membrane potential moves the membrane potential away from the activation threshold of the voltage-dependent calcium current, reducing

3.2 Quinpirole produces a hyperpolarization

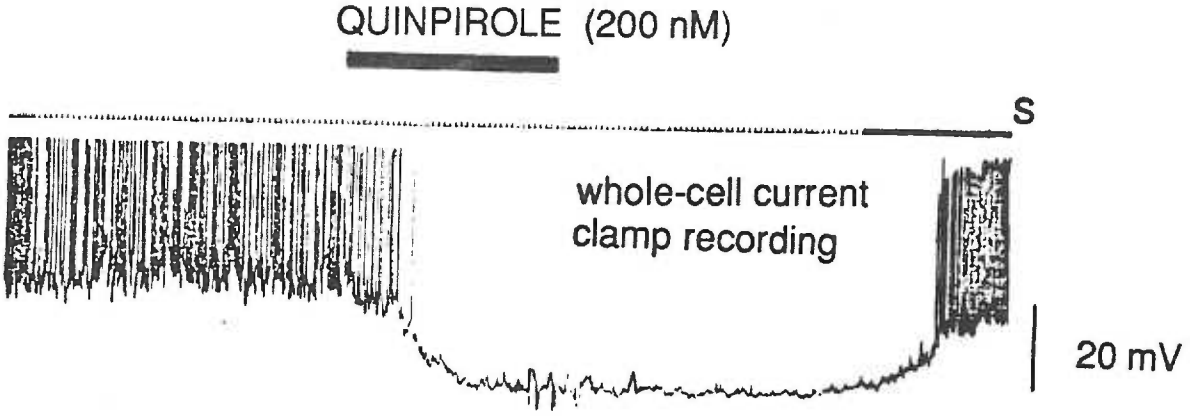


Figure 3.32 Quinpirole Produces a Hyperpolarization. Whole-cell current-clamp recording of a spontaneousely active melanotroph. Quinpirole (200 nM) abolished the spontaneous action potentials and produced a hyperpolarization of the membrane in a reversible manner.

3.33 Comparison of the concentrations of quinpirole that inhibt ß-endorphin secretion and that produce a hyperpolarization

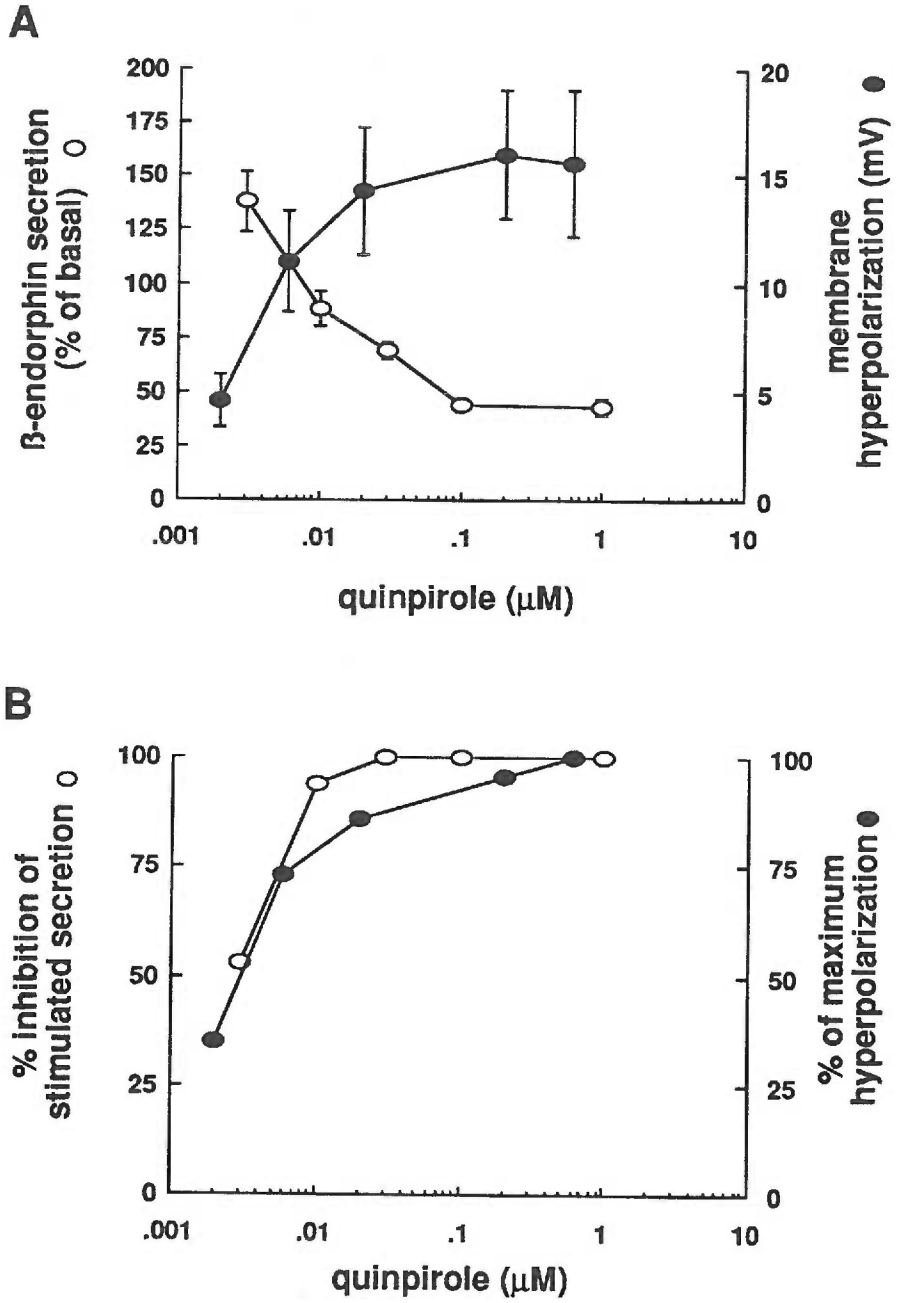


Figure 3.33 Comparison of the Concentrations of Quinpirole that Inhibit β-Endorphin Secretion and that Produce a Hyperpolarization. A, β-endorphin secretion stimulated by BAY K 8644 (open circles) and the membrane hyperpolarization (filled circles) are plotted as a function of the quinpirole concentration. The * indicates the control response of secretion in the absence of quinpirole. B, the data shown in A have been transformed such that the inhibition of secretion (open circles) and the per cent of maximum hyperpolarization (filled circles) are plotted as a function of the quinpirole concentration.

the probability of the calcium channels opening.

3.3.4 CONCLUDING REMARKS

The concentration-response curves of cadmium, nifedipine and quinpirole on the inhibition of voltage-dependent calcium current and the inhibition of stimulated secretion did not show a direct relationship between these two responses. The results are shown in Figures 3.29, 3.30 and 3.31 and are summarized in Table 3.1. It is clear that dissociation of inhibition of calcium currents from inhibition of secretion is the rule rather than the exception. This is discussed in detail in section 5.2.

Table 3.1 Comparison of effectiveness of cadmium, nifedipine and quinpirole to inhibit calcium currents and ß-endorphin secretion in melanotrophs.

	Calcium Current Inhibition	Secretion Inhibiton
Cadmium*		
IC_{50}	6 µM	200 µM
IC_{max}	100 µM	1000 µM
max inhibition	100 %	100%
Nifedipine**		
IC_{50}	0.2 µM	n.s.
IC_{max}	10 µM	
max inhibition	31%	
Quinpirole*		
IC_{50}	0.08 µM	0.008 µM
IC_{max}	1 µM	0.1 µM
max inhibition	40%	100%

*Bay K 8644 stimulated secretion

**Isoproterenol stimulated secretion

CHAPTER FOUR: BIOCHEMICAL ANALYSIS OF PROOPIOMELANOCORTIN-DERIVED PEPTIDES SECRETED BY ATT-20 CELLS

4.1 INTRODUCTION

The physiological response to stress involves the activation of the hypothalamic-pituitary-adrenal axis. Acute stress, such as pain, shock, restraint, or ether administration, cause an immediate and dramatic increase in plasma levels of adrenocorticotropin hormone [ACTH(1-39) or ACTH] (Ganong, Dallman & Roberts, 1987; Jones & Gillham, 1988). The source of ACTH is corticotrophs, which comprise 5-10 % of the cell population of the anterior lobe of the pituitary (Hatfield et al. 1989). The major hypothalamic activator of ACTH secretion is corticotropin releasing hormone (CRH) which is localized in the parvocellular neurons of the paraventricular nucleus of the hypothalamus (Swanson et al. 1983). CRH immunoactive fibers have been found in the external zone of the median eminence (Swanson et al. 1983) where it is thought that upon adequate stimulation, CRH is released into the hypophysial portal blood and delivered to the anterior pituitary to stimulate the release of ACTH from corticotrophs. The major target organ for ACTH is the adrenal gland where it stimulates the fasiculata cells to synthesize and release glucocorticoids. These steroids in turn have actions on peripheral tissue metabolism as well as acting as a negative feedback regulator of the stress response at the level of the hypothalamus and the pituitary (Keller-Wood & Dallman, 1984). It has also been shown that β-endorphin, a peptide with opioid activity, is released concomitantly, and equimolar with ACTH(1-39) (Mains & Eipper, 1981a). In fact, the amino acid sequences of the two molecules are contained in the same precursor

hormone, proopiomelanocortin (POMC) (Mains, Eipper & Ling, 1977). The role of β-endorphin in the stress response is not known; however, since the peptide has opioid bioactivity (Cox et al. 1975), it may be involved in the modulation of pain perception.

In the rodent, POMC is a 260 amino acid protein that is synthesized in anterior and intermediate lobes of the pituitary gland but, the POMC-derived peptides that are produced in these two tissue types are very different (Figure 4.1) (Mains and Eipper, 1981b). The two different sets of POMC-derived peptides are the result of differential post-translational processing and may be due to a combination of differential expression of the post-translational processing enzymes, differential compartmentalization of either one or more proteases, and/or modulation of cleavage site accessibility by differential modification of the precursor (Thomas et al. 1988). One example of such differential processing is the fate of ACTH(1-39). In the anterior lobe, ACTH is cleaved from POMC and is post-translationally processed into glycosylated and phosphorylated forms of ACTH(1-39). However, in the intermediate lobe ACTH(1-39) undergoes further post-translational proteolytic cleavage, N-acetylation and α-amidation to produce α-melanocyte-stimulating hormone (α-MSH) which is α-N-acetyl-ACTH(1-13)NH_2. The bioactivities of these two peptides are completely different: ACTH(1-39) is a potent stimulator of glucocorticoid release from the adrenal gland, whereas α-MSH has virtually no steroidogenic activity.

The β-endorphin portion of the POMC molecule also undergoes differential post-translational processing in the anterior and intermediate lobe of the pituitary. In the anterior lobe, the major forms of β-endorphin peptides are β-endorphin(1-31) and the processing

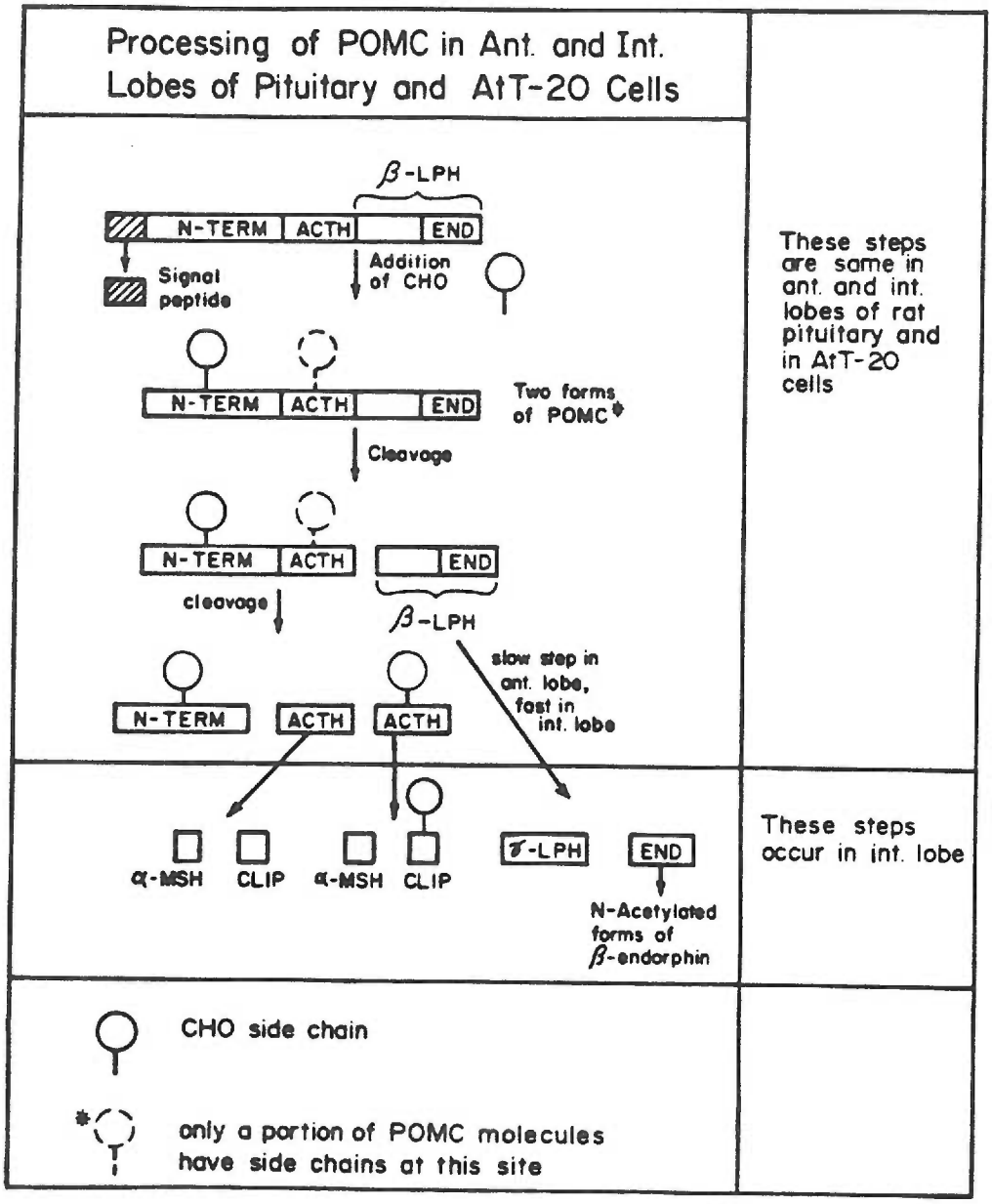


Figure 4.1 Post-translational Processing of POMC. Cell specific processing of POMC in rodent anterior and intermediate pituitary after Herbert et al. (1981).

intermediate, β-lipotropin (β-LPH). In the intermediate lobe, β-endorphin undergoes further post-translational N-acetylation and C-terminal cleavages, producing α-N-acetyl-β-endorphin(1-31), α-N-acetyl-β-endorphin(1-27), and α-N-acetyl-β-endorphin(1-26).

The N-terminal sequence of β-endorphin is the met-enkephalin (tyr-gly-gly-phe-met) which confers opioid activity (Hughes et at. 1975). However, if the N-terminal of β-endorphin remains in peptide linkage as in β-LPH, or is chemically modified by α-N-acetylation of the tyrosine residue, the molecule is devoid of opioid activity.

Since the bioactivities of the β-endorphin containing peptides (β-endorphin compared to β-LPH and POMC) are so different, it was of interest to determine what forms of β-endorphin containing moieties are being released during different secretion states. In this study, AtT-20 cells were used to examine the secreted forms of β-endorphin immunoactivity during basal secretion and in conditions where hormone release was stimulated or inhibited. In AtT-20 cells, the POMC post-translational pattern (Mains & Eipper, 1976) is virtually indistinguishable from the normal anterior lobe processing patterns and therefore is considered to be a valid model for these studies. The β-endorphin containing moieties which have been examined here are POMC, β-LPH and β-endorphin(1-31). AtT-20 cells do not N-acetylate β-endorphin (Mains & Eipper, 1981b).

4.2 METHODS

4.2.1 FRACTIONATION OF POMC PEPTIDES BY RP-HPLC

Cell extracts or culture medium containing β-endorphin immunoactive material were fractionated by reverse phase-high performance liquid chromatography (RP-HPLC) using a Vydac Protein C4 column with a linear gradient of acetonitrile (AcN) in 0.1 % trifluroacetic acid (TFA). Collected fractions were assayed for β-endorphin immunoactivity as described in section 2.2.4. This fractionation technique separates proteins and peptides based on charge and hydrophobicity and POMC-derived peptides previously have been separated and detected by these techniques (Allen, Hatfield & Stack, 1988; Thomas et al. 1988).

With a flow rate of 1 ml/min, two solutions were used to produce the linear gradient: solution 'A' was 0.1 % TFA and solution 'B' was 80 % AcN in 0.1% TFA. The column was equilibrated at 17 % 'B' solution and the 1.5 ml sample was injected onto the column and washed with 17 % 'B' solution before the gradient was initiated. The gradient is shown in Figure 4.2. One minute fractions were collected for 80 min once the gradient was initiated. Synthetic peptides (Peninsula, Belmont, CA; BaChem, Torrance, CA) or purified POMC (a generous gift from Barbara Thorne, OHSU) were used to confirm the elution times which were 45-47 min for β-endorphin(1-31), 55-57 min for β-LPH, and 71-75 min for POMC.

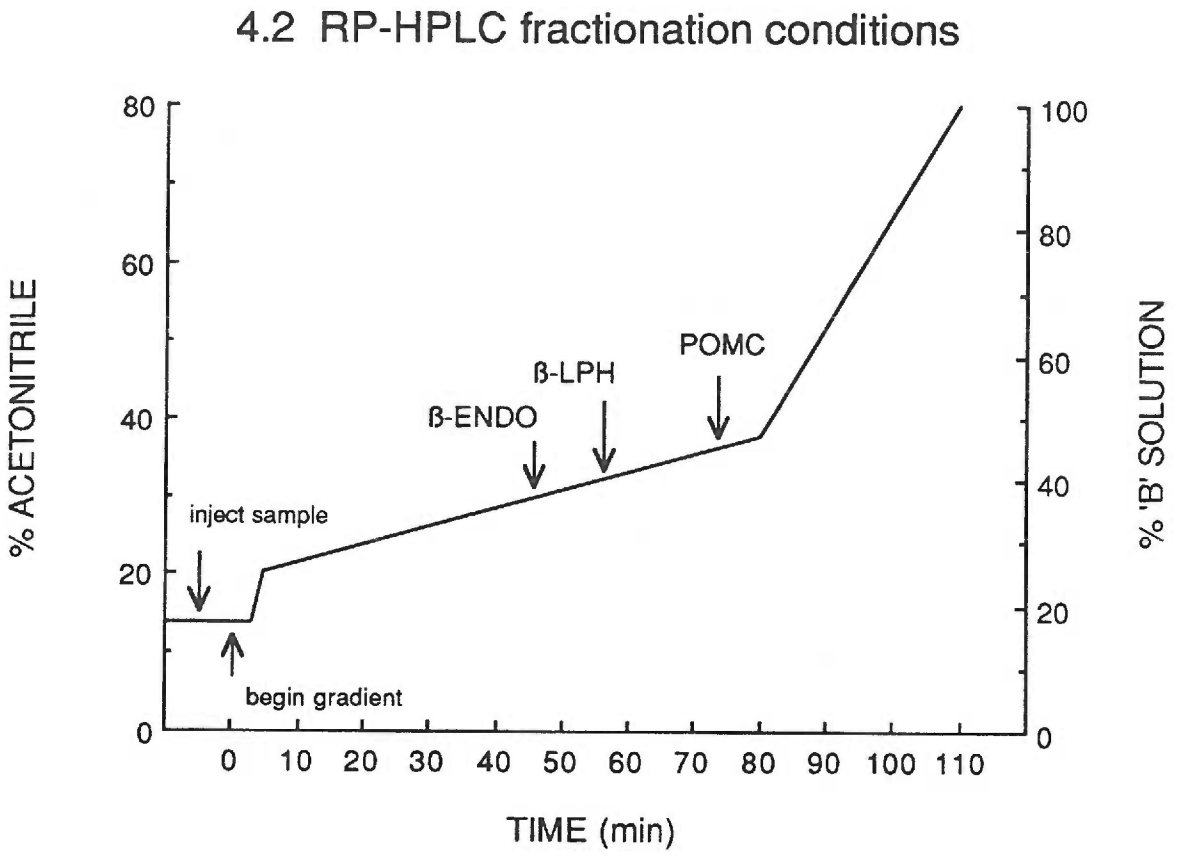


Figure 4.2 RP-HPLC Fractionation Conditions. A linear gradient of acetonitrile was used to elute β-endorphin immunoactive material off the C4 reverse-phase column (Vydac). When the column was equilibrated with 17 % 'B' solution (13.6 % AcN), the sample was injected onto the column and washed with 17 % 'B' solution for 4 min prior to the initiation of the gradient. The gradient protocol was as follows: 17 % 'B' solution for 3 min, 25 % 'B' solution (20 % AcN) at 5 min, 47 % 'B' solution (37.6 % AcN) at 80 min, and 100 % 'B' solution (80 % AcN) at 110 min. The flow rate was 1 ml/min and 1 min fractions were collected once the gradient was initiated. All POMC and POMC-derived peptides elute from the column before 80 min. Using synthetic peptides and purified POMC the elution times of the peptides were as follows: 45-47 min for β-endorphin(1-31), 55-57 min for β-LPH, and 71-75 min for POMC.

4.2.2 PREPARATION AND ANALYSIS OF SAMPLES FOR RP-HPLC

4.2.2(i) CELL EXTRACTS

Extracts of AtT-20 cells were prepared as follows; 1 ml of extraction buffer [30 % glacial acetic acid plus 0.3 mg/ml phenylmethylsufonylfluoride (PMSF)] was added to a confluent 35 mm dish of AtT-20 cells. The cells were scraped from the dish, collected, homogenized, frozen and thawed three times, diluted with 3 ml water, centrifuged (5000 rpm) and supernatant was divided into three equal aliquots and frozen, lyophilized, and stored at -20°C. For fractionation, the samples were resuspended in 17 % 'B' solution.

4.2.2(ii) CULTURE MEDIUM

The medium samples from AtT-20 cell secretion experiments (described in section 2.2.3) were prepared as follows. The secretion experiments were performed in sextuplicate in serum-free medium. The samples from each experiment were pooled for a total volume of approximately 1.8 ml and frozen. Five μl of each sample was assayed for total β-endorphin immunoactivity. For fractionation, 1.5 ml of the sample was acidified with TFA to a final concentration of 0.1 %. The lowest detectable amounts of β-endorphin immunoactivity in these assays is 200-400 pg/ml.

To determine whether protein degradation was occurring during this procedure purified POMC was treated like the medium samples. POMC was incubated in serum free DMEM, frozen, acidified with TFA to 0.1 % final concentration, and fractionated. No evidence of POMC degradation was detected; β-endorphin immunoactivity was only detected at 73-75 min.

4.3 RESULTS

4.3.1 β-ENDORPHIN IMMUNOACTIVITY IN CELL EXTRACT

Analysis of the AtT-20 cell extracts detected three peaks of β-endorphin immunoactivity. These were identified as POMC, β-LPH and β-endorphin(1-31) (Figure 4.3). Approximately 50 % of the β-endorphin immunoactivity was detected in the β-endorphin(1-31) peak. Thirty four per cent of the β-endorphin immunoactivity was in the POMC peak and 13 % was in the β-LPH peak. These represent the intracellular steady-state levels of the molecules.

4.3.2 SECRETED β-ENDORPHIN IMMUNOACTIVITY

Analysis of the medium from secretion experiments detected two major peaks which co-migrated with β-endorphin and β-LPH; very little β-endorphin immunoactive material was detected at the position POMC elutes (n = 8; Figures 4.4-4.7). Therefore, although POMC is contained in the cells in significant amounts, it is not secreted in significant amounts.

4.3.2(i) BASAL SECRETION

Analysis of medium collected during control conditions showed that during basal secretion, β-endorphin and β-LPH were secreted in almost an equimolar ratio. β-endorphin composed 52.5 ± 5.0 % of the β-endorphin immunoactivity secreted and β-LPH composed 41.8 ± 4.8 % of the β-endorphin immunoactivity secreted (n = 4; Figures 4.4A-4.7A). This difference was not statistically significant.

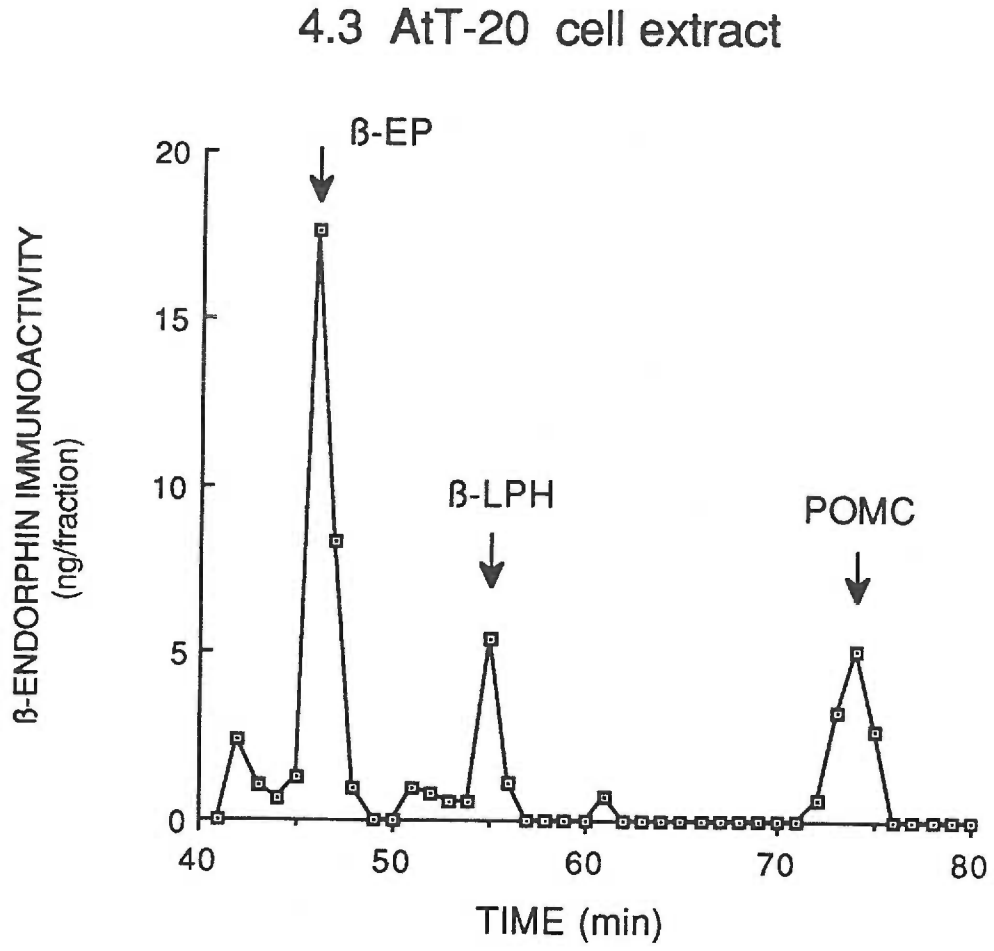


Figure 4.3 AtT-20 Cell Extract: Fractionation by RP-HPLC and Identification of β-Endorphin Containing Moieties. Cell extract samples were resuspended in 1.5 ml of 17 % 'B' solution and fractionated on the C4 reverse-phase column. Fractions were assayed for β-endorphin immunoactivity. The major peaks were detected at 46-47 min which coelutes with β-endorphin(1-31), 55 min which coelutes with β-LPH, and 72-74 min which coelutes with POMC. No β-endorphin immunoactivity was detected in the first 40 min.

4.3.2(ii) STIMULATION OF SECRETION

Analysis of medium collected during test conditions that stimulated secretion showed that the majority of the increased β-endorphin immunoactivity was due to a increase in the release of β-endorphin(1-31) rather than β-LPH. Stimulation of secretion by BAY K 8644 showed a 256 % increase in the release of β-endorphin(1-31) and a 162 % increase in the release of β-LPH (Figure 4.4). Isoproterenol caused a 459 % increase in the β-endorphin(1-31) secreted and only a 115 % increase in the β-LPH secreted (Figure 4.5). These results are similar to those previously reported by Mains and Eipper (1981a) who found a specific increase in the release of β-endorphin(1-31) during stimulation by isoproterenol.

4.3.2(iii) INHIBITION OF SECRETION

Analysis of medium collected during test conditions that inhibited secretion showed that the reduction in secreted β-endorphin immunoactivity was due to a specific reduction in the release of β-endorphin(1-31) rather that β-LPH. Inhibition of secretion by removal of extracellular calcium reduced the secreted β-endorphin(1-31) 70 % and the secreted β-LPH 10 % (Figure 4.6). Inhibition of secretion by 300 nM SRIF reduced the secreted β-endorphin(1-31) 76 % and β-LPH 24 % (Figure 4.7).

4.4 DISCUSSION

As a general process, proteins and peptides that are destined for export must be synthesized, post-translationally modified and packaged as they pass through the endoplasmic reticulum, the Golgi apparatus, and

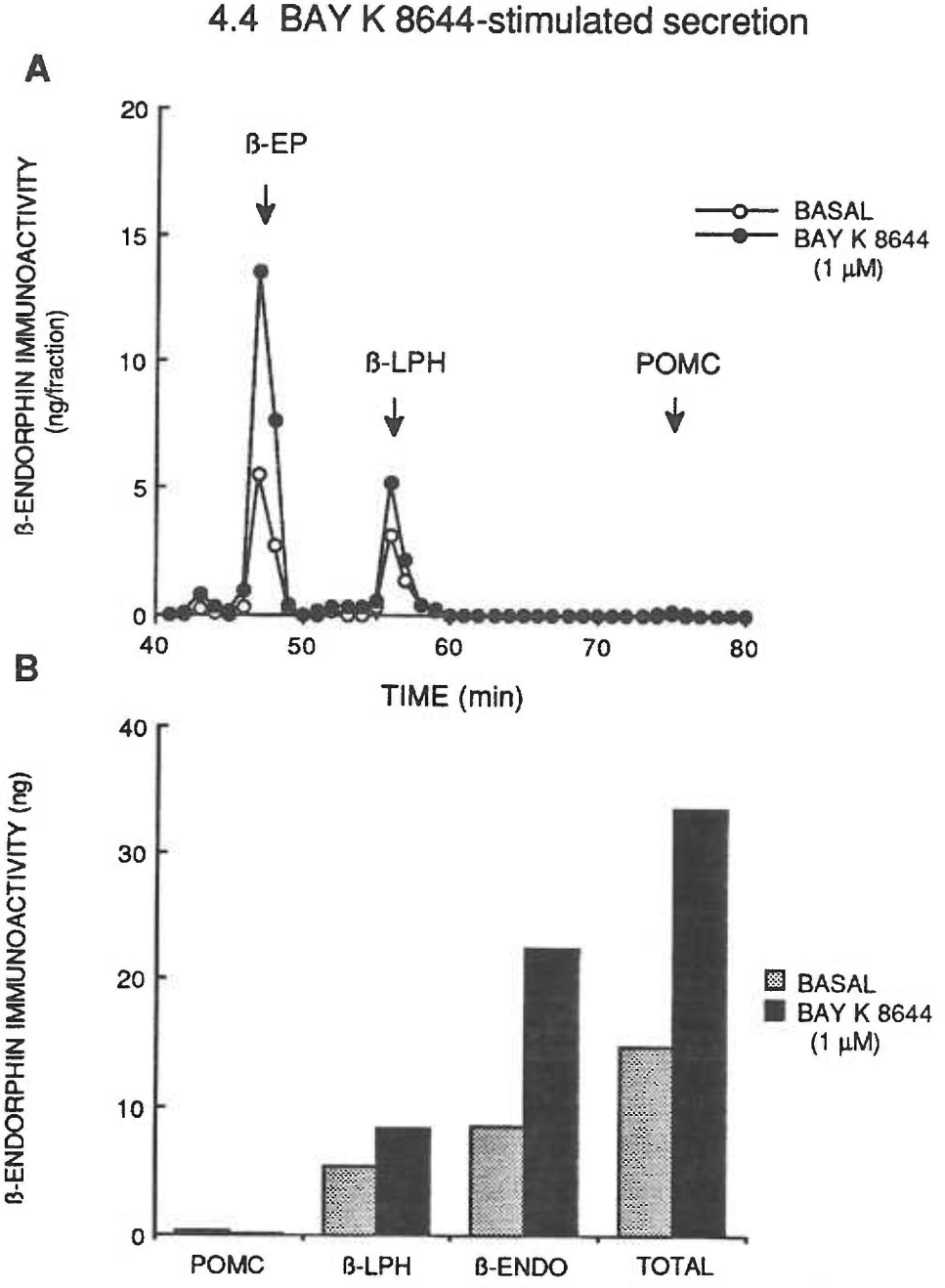


Figure 4.4 BAY K 8644-Stimulated Secretion: Fractionation of Medium by RP-HPLC and Identification of β-Endorphin Containing Moieties. A, DMEM (1.5 ml) collected from secretion experiments in the absence and presence of BAY K 8644 (1 μM) was acidified with TFA (0.1 % final concentration) and fractionated on the C4 reverse-phase column. Fractions were assayed for β-endorphin immunoactivity. Plotted are two RP-HPLC profiles where the open circles represent peptides secreted in basal conditions and the filled circles represent peptides secreted in the presence of BAY K 8644. This protocol was also used in the following figures. B, quantification of β-endorphin immunoactivity. The amounts of β-endorphin immunoactivity of the entire run (total), the POMC peak, the β-LPH peak, and the β-endorphin(1-31) peak were summed during basal and test conditions, and are compared. This analysis is used in the following figures.

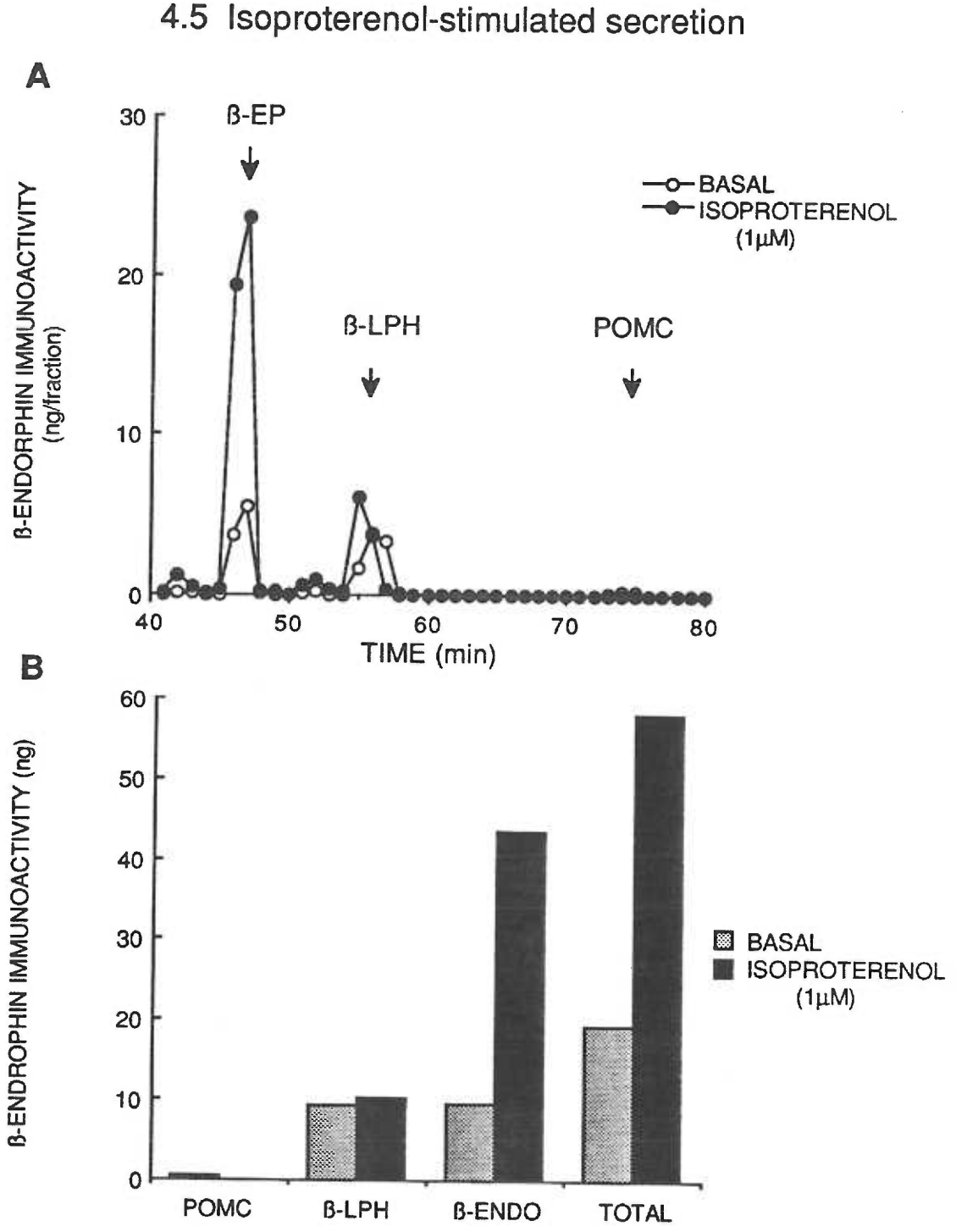


Figure 4.5 Isoproterenol-Stimulated Secretion: Fractionation of Medium by RP-HPLC and Identification of β-Endorphin Containing Moieties. A, fractionation of β-endorphin immunoactivity in DMEM (1.5 ml) collected from secretion experiments in the absence (open circles) and presence of isoproterenol (1 μM; filled circles). B, quantification of β-endorphin immunoactivity.

4.6 Zero calcium inhibition of secretion

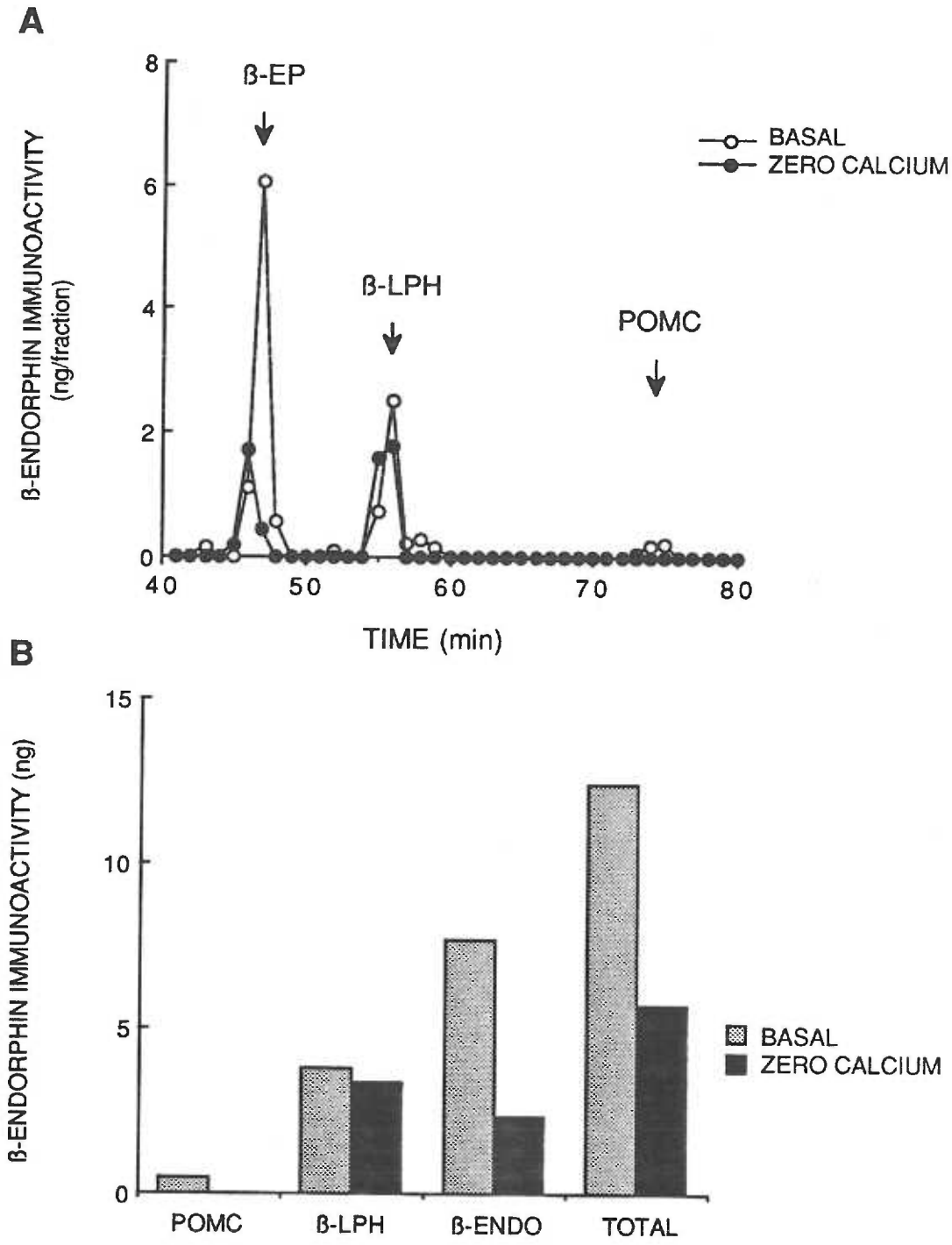


Figure 4.6 Zero Calcium Inhibition of Secretion: Fractionation of Medium by RP-HPLC and Identification of β-Endorphin Containing Moieties. A, fractionation of β-endorphin immunoactivity in DMEM (1.5 ml) collected from secretion experiments in presence of calcium (1.8 mM; open circles) and the absence of calcium (filled circles). B, quantification of β-endorphin immunoactivity.

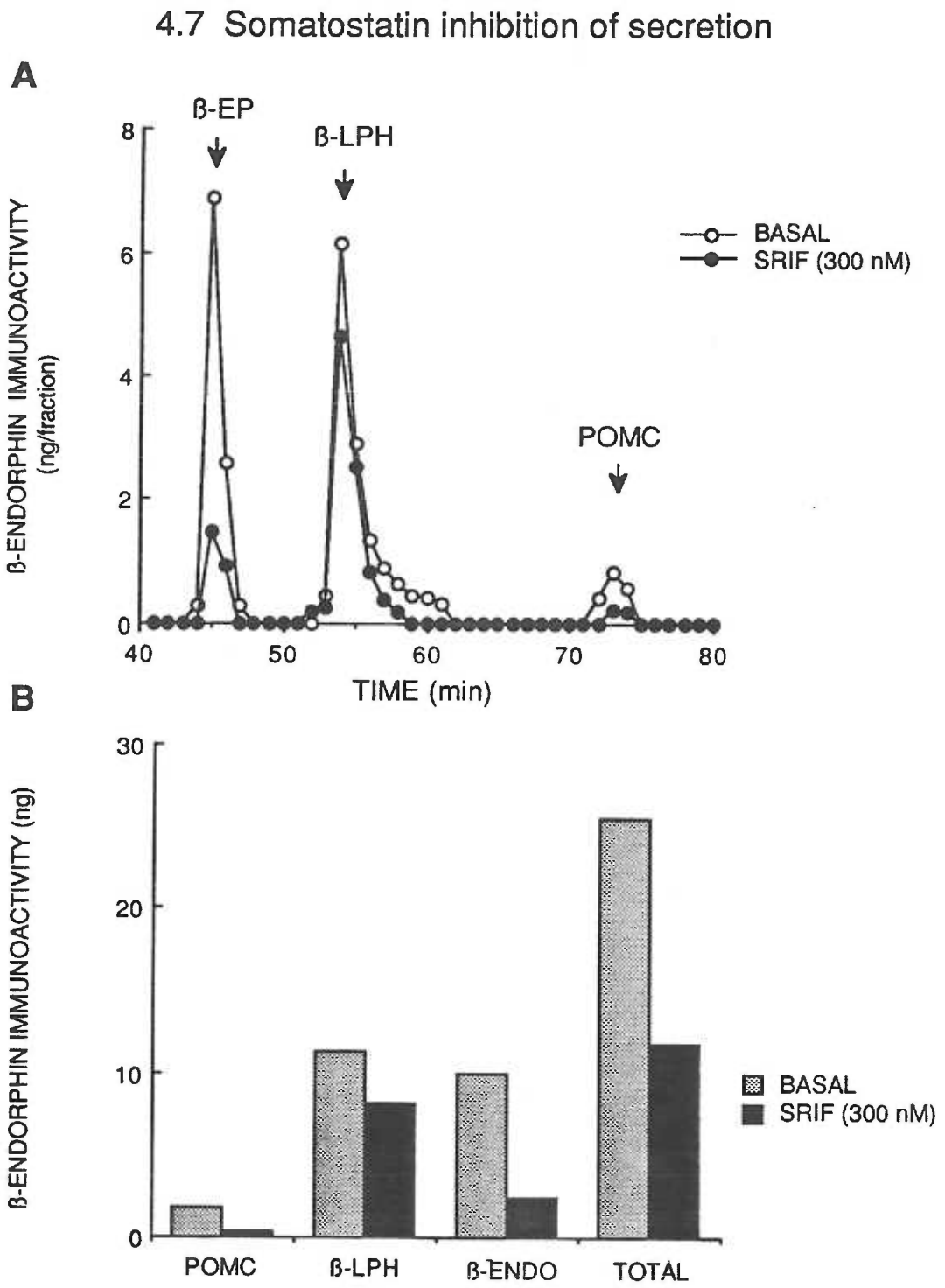


Figure 4.7 Somatostatin Inhibition of Secretion: Fractionation of Medium by RP-HPLC and Identification of β-Endorphin Containing Moieties. A, fractionation of β-endorphin immunoactivity in DMEM (1.5 ml) collected from secretion experiments in the absence (open circles) and the presence of SRIF (300 nM; filled circles). B, quantification of β-endorphin immunoactivity.

secretory vesicles. Two distinct secretory pathways have been described in the AtT-20 cells (Gumbiner & Kelly, 1982; Burgess & Kelly, 1987). "Constitutive" release is a calcium-independent process; the secretory vesicles have a half-life of minutes. "Regulated" release is a calcium-dependent process; the secretory vesicles have a half-life on the order of hours to days. In AtT-20 cells it has been shown that the first site of post-translational processing of POMC occurs in the trans Golgi and processing continues in dense core vesicles (Schnabel, Mains & Farquar, 1989). In order to determine if the release of selected forms of POMC-derived peptides were regulated by secretagogues, or if all forms were regulated, secreted forms of β-endorphin containing peptides were characterized during basal conditions and then compared to forms released under conditions that stimulated or inhibited hormone release.

The β-endorphin containing moieties released during basal secretion conditions were β-LPH and β-endorphin(1-31) and these peptides were released in an equimolar ratio. Very little POMC was detected. If basal secretion were merely a random release of the intracellular contents, one would expect the cell extract profile to resemble the secreted profile. However, the pattern of β-endorphin peptides released during basal secretion is very different from the pattern of peptides detected in the cell extract (compare Figure 4.3 with Figure 4.4A, open circles), the most striking difference being the lack of intact POMC in the medium.

Since regulated release is a calcium-dependent process, it was of interest to determine whether removal of calcium would affect the release of β-LPH and β-endorphin selectively. Figure 4.6 shows indeed that removal of extracellular calcium reduced the total β-endorphin immunoactivity 50 % and selectively reduced the release of β-endorphin(1-

31) compared to β-LPH. Further, these conditions had little effect on the absolute amount of β-LPH released suggesting that calcium-independent secretion involves fusion of secretory vesicles that contain β-endorphin moieties that are not fully processed. Since the release of β-endorphin(1-31) was specifically modulated in the zero calcium conditions it can be concluded that the calcium-dependent releasable pool of peptide is the fully processed form. These data also suggests that there exist at least two types of secretory vesicles based upon the vesicular content and differential sensitivity to extracellular calcium. They could be classified as vesicles that contain mostly β-endorphin(1-31), secretion of which is dependent on extracellular calcium, and vesicles that contain both β-LPH and β-endorphin(1-31) whose liberation is independent of extracellular calcium.

This pattern of inhibition was also seen when secretion was reduced with somatostatin (Figure 4.7) and suggests inhibition of secretion by this receptor activated process modulates the calcium-dependent releasable pool of vesicles.

Stimulation of secretion by either isoproterenol or BAY K 8644 specifically enhanced the release of β-endorphin(1-31) rather than β-LPH or POMC and this stimulation is dependent on extracellular calcium (Figure 2.7). The initial sites of action of these two secretagogues are very different; isoproterenol activates the β-adrenergic receptor which is coupled to the stimulation of adenylate cyclase and BAY K 8644 is a calcium channel agonist. Although these two agonists initially act at two different sites, their actions eventually converge on a common site and preferentially modulate the release β-endorphin(1-31). Again, the calcium-dependent release seems to selectively involve the recruitment

of vesicles that contain the fully processed form of the hormone.

The mechansim by which the fully processed β-endorphin(1-31) peptide is specifically released appears to involve selective recruitment of mature secretory vesicles rather than the coupling of secretion with the processing of the POMC peptides. Processing is not involved because treatment with SRIF inhibited selectively the release of β-endorphin(1-31) and not β-LPH. Since β-endorphin(1-31) is present in the cell extract and already processed product does not go backwards in the processing pathway, it can be ruled out that inhibition of β-endorphin(1-31) secretion is not due to an inhibition of processing. However, the possibility that a component of stimulated release is coupled to processing still remains.

Pulse labeling studies have shown that the processing of POMC begins within 30 min of the synthesis of a POMC molecule and is completed within 4 h (Mains and Eipper, 1978). Studies conducted on anterior corticotroph cultures have shown that CRH preferentially stimulated the release of small forms of ACTH and β-endorphin but there was no marked preference for the secretion of old (days) or new stores (hours) of hormone (Allen, Hinman & Herbert, 1982). Therefore, the birth date of the peptide does not determine when it will be released.

Since the releasable pool of hormone tapped by secretagogues contains the fully processed forms of the hormone and this pathway can also be equated with the calcium-dependent secretory pathway, it may be speculated that this vesicle population may be different from the calcium-independent pathway in their ability to process POMC. The vesicle of the constitutive pathway either do not possess the necessary enzymes and/or conditions for processing to occur. Alternatively the

vesicle half-life is the critical factor; that is they might contain the correct processing machinery but the vesicle does not stay in the cell long enough for processing to be completed. Although the mechansim remains elusive, it appears that the regulated pathway of β-endorphin secretion in these cells involves the selective release of the bioactive and opiate active form of β-endorphin.

CHAPTER FIVE: SUMMARY AND CONCLUSIONS: ROLE OF THE VOLTAGE-DEPENDENT CALCIUM CURRENT IN THE MODULATION OF STIMULUS-SECRETION COUPLING IN PITUITARY

Stimulus-secretion coupling is the process of release of an intracellular product destined for export from the cell upon demand by an extracellular stimulus. In lymphocytes, stimulation of antibody secretion is very slow, taking days, since the antibodies are synthesized de novo upon stimulation of the cell. However, in neurons, endocrine cells and mast cells stimulation of the release of their products is initiated within seconds to minutes after the stimulus has been received by the cell. The difference in the time course of secretion in these cells is that intracellular stores of hormone or neurotransmitter are available for release when the appropriate intracellular conditions are met. Conditions necessary to support secretion in each of these cell types have been studied by measuring the change in membrane capacitance (an indicator of vesicle fusion and secretion at the single cell level) and concomitantly monitoring the intracellular concentration of calcium (Penner & Neher, 1989). In the mast cell, a non-excitable cell, an increase in the membrane capacitance was measured in the absence of a rise in intracellular calcium. In contrast, in electrically excitable cells like the chromaffin cell, an increase in capacitance was dependent upon a preceded and maintained rise in the intracellular calcium.

In neurons it as been demonstrated that extracellular calcium ions must be present at the time of presynaptic depolarization if transmitter release is to occur (Katz & Miledi, 1967). A direct relationship between the magnitudes of the presynaptic calcium current and the postsynaptic

response have been shown in squid giant presynaptic terminal (Augustine, Charlton & Smith, 1985). Application of TTX, which blocks the action potential, inhibits the release of neurotransmitters. In neurons, the depolarization of the terminal by the action potential opens voltage-dependent calcium channels which are responsible for the influx of calcium for secretion of neurotransmitters.

Several types of calcium currents have been described and the most concise method of categorizing has been described for the calcium channels in chick DRG cells named "T", "N" and "L" (Table 1.1; Nowycky, Fox & Tsien, 1985). However, this classification certainly is not complete. The calcium channel in the squid presynaptic terminal has been characterized as a low threshold, non-inactivating channel (Augustine et al. 1989) and synaptic transmission in the rat submandibular parasympathetic ganglia could not be blocked in a consistent manner by calcium channel blockers for the same type of channel described in other preparations (Seabrook and Adams, 1989). The functional significance of the diversity of calcium channels remains to be determined however, the characterization of calcium channels is the first step in understanding this phenomenon.

Similarly, pituitary cells in this study have been shown to be dependent on extracellular calcium for support of secretion of hormones. These are also electrically excitable cells firing spontaneous action potentials that can be blocked by TTX. However, unlike the neuron, TTX does not block secretion in pituitary cells (with the exception of secretion stimulated by veratridine). It has been postulated that calcium component of the spontaneous activity in AtT-20 cells (Surprenant, 1982) and melanotrophs (Douglas & Taraskevich, 1980) is

involved in this process and a likely candidate for this mechanism may involve voltage-dependent calcium currents. The focus of this study was to determine whether inhibition of the voltage-dependent calcium current was the mechanism that would account for the inhibition of secretion.

A direct relationship between the inhibition of the calcium currents and the inhibition of secretion was not observed in either cell preparation. Concentrations of cadmium that completely blocked the calcium current did not block secretion. These results are in contrast to studies in neurons such as squid stellate ganglion (Augustine, Charlton & Smith, 1985) or the guinea pig submucous neurones (Surprenant et al. 1990; Shen & Surprenant, 1990) which demonstrated a direct relationship between the inhibition of the voltage-dependent calcium current and an inhibition of neurotransmitter secretion. These studies suggest that regulation of calcium influx that supports secretion in pituitary cell is different than the regulation of secretion from neurons.

5.1 COMPARISON OF ATT-20 CELLS AND MELANOTROPHS

Table 5.1 compares calcium currents measured in the two cell preparations. The calcium current in the AtT-20 cell was characterized a high-threshold, non-inactivating current that was sensitive to cadmium (100 % block), nickel (100 % block) and nifedipine (63 % block at 10 μM). The calcium current in the melanotroph was classified as a high-threshold current composed of a transient and sustained component. Both components were sensitive to cadmium (100 % block) and dihydropyridine compounds, however the transient component reduced 50 % by steady-state inactivation or by nickel (500 μM). Although these results are not conclusive, they

Table 5.1 Comparison of the regulation of calcium currents in pituitary cells.

Calcium Current	AtT-20	melanotroph
Voltage-Dependence (activation : peak)		
transient component	-30 mV : 10 mV	-40 mV : 0 mV
sustained component	-30 mV : 10 mV	-40 mV : 0 mV
transient/sustained	1.3	1.9
Stimulators		
Bay K 6484	yes	yes
isoproterenol	yes (2 out of 20 cells)	no (0 out of 6 cells)
Inhibititors		
cadmium		
IC 50	2 µM	6 µM
max inhibition	100 %	100 %
nifedipine		
IC 50	0.03 µM	0.2 µM
max inhibition	67 %	31 %
SRIF		
IC 50	0.005 µM	
max inhibition	40 %	
quinpirole		
IC 50		0.08 µM
max inhibition		40 %

suggest that a single calcium channel type is present in the AtT-20 cells whereas calcium currents generated by the melanotroph may represent calcium channel activity by more than one channel.

The currents in both cell preparations were consistently enhanced by BAY K 8644 further supporting the presence of at least an "L" current in both cell preparations. Enhancement of the current amplitude in response to isoproterenol was seldom measured in AtT-20 cells and never seen in melanotrophs in these studies. However, it has been reported in both AtT-20 cells (Nowycky, 1987) and melanotrophs (Cota & Hiriat, 1989). Isoproterenol activates the cAMP pathway, a diffusible second messenger system, while BAY K 8644, nifedipine, cadmium presumably do not, the most likely explanation for the general failure to observe actions of isoproterenol on the current is that internal dialysis of the cell in the whole-cell configuration had washed out (or, alternatively, maximally activated) an essential component of the cascade (see discussion in section 2.4.1).

The currents were inhibited in a concentration-dependent manner by cadmium in both cell types. Nifedipine inhibited the majority of the calcium current in the AtT-20 cells. This would be consistent with the premise that the AtT-20 calcium current is primarily an "L" current. The calcium current in the melanotroph was only partially reduced by nifedipine which indicates that most certainly a "L" current is present in the total current, however this result in combination with the inactivation properties of the current strongly suggest that at least one other current may be present in these cells. However the characteristics of the transient component of the current (high threshold

and inhibited by nickel) do not strictly match the criteria for either inactivating current ("T" or "N") described in DRG cells.

In the AtT-20 cells and the melanotrophs, the calcium currents were reduced by SRIF and quinpirole, respectively. SRIF and quinpirole did not reduce the current more than 40 % of the control current in their respective cell types.

Table 5.2 compares the regulation of β-endorphin secretion in the AtT-20 cells and the melanotrophs. In both cell preparations secretion was stimulated by isoproterenol (cAMP-dependent mechanism) and BAY K 8644 (a cAMP-independent) and isoproterenol was the more effective secretogogue. Stimulated secretion was reduced by cadmium, nifedipine, and either SRIF or quinpirole in a similar manner. The cadmium and nifedipine IC_{50}s are similar when compared between secretogogues within the same cell preparation. They are also similar when compared between cell types. This suggests, that these agents have interrupted secretion at the same site of the secretory pathway in each cell type.

Finally, it is of considerable interest to compare the effects of SRIF and quinpirole. Activation of the SRIF receptor and the D_2 receptor have been reported to be negatively coupled to adenylate cyclase. In both cases, the application of the agonist will reduce stimulated levels of cAMP but will not alter basal levels of cAMP (Srikant & Heisler, 1985; Munemura, Eskay & Kebabian, 1984). However, it has been postulated that the mechanism by which these agonists are acting to inhibit secretion is through a cAMP-dependent mechanism. The data presented here do not support this hypothesis. First, SRIF and quinpirole inhibit basal secretion. Second, SRIF and quinpirole inhibit BAY K 8644-stimulated secretion which is a cAMP-independent response. Third, the effective

Table 5.2 Comparison of the regulation of ß-endorphin secretion from pituitary cells.

		AtT-20	melanotroph
Stimulation of Secretion (% of basal in 30 min)			
isoproterenol		197±6	165±7
Bay K 8644		176±8	147±9
Inhibition of Secretion (IC 50)			
Inhibitor	Stimulator		
cadmium	isoproterenol	<100 µM	100 µM
	Bay K 8644	200 µM	200 µM
nifedipine	isoproterenol	0.4 µM	n.s.
SRIF	isoproterenol	0.001 µM	
	Bay K 8644	0.005 µM	
quinpirole	isoproterenol		0.001 µM
	Bay K 8644		0.008 µM

concentrations of SRIF and quinpirole that inhibit BAY K 8644-stimulated secretion are similar to the concentrations that inhibit isoproterenol-stimulated secretion, a cAMP-dependent response.

5.2 DISCUSSION

Concentration-response curves of the inhibition of the calcium and the inhibition of β-endorphin secretion were compared based on the three parameters: the IC_{50}, the IC_{max}, and the maximum inhibition of the response. None of the pharmacological agents tested (cadmium, nifedipine, quinpirole, or SRIF) demonstrated agreement for these three parameters when the inhibition of the calcium current and the inhibition of secretion were compared.

Cadmium is a non-selective calcium channel blocker. Cadmium has been shown to reduce the fast Na current (Seabrook & Adams, 1989) however in these studies it was shown that blockade of the fast sodium current by TTX does not alter secretion (Figures 2.6, 2.7, 3.9, 3.10). Cadmium reduced the calcium current in a concentration-dependent manner and completely blocked the calcium current at 100 μM. Therefore, cadmium was used in secretion studies as a specific blocker of voltage-dependent calcium currents with the purpose to determine the relationship between the blockade of the calcium current and the inhibition of secretion. As has already been discussed, no direct relationship existed between the inhibition of the calcium current and the inhibition of secretion by cadmium in either the AtT-20 cells or the melanotrophs. These data suggest that reduction of the voltage-dependent calcium current is not the mechanism regulating the inhibition of secretion. Similar results have recently been reported in lactotrophs where is was shown that

cadmium and nickel abolished voltage-dependent calcium currents at concentrations that do this had little effect on secretion (Lledo et al. 1990). Surprisingly, this discrepancy between inhibition of secretion and inhibition of calcium currents by cadmium and nickel was not commented on by these authors.

When the concentration-response curves of quinpirole on the calcium current are compared to that of cadmium, the most striking difference is that quinpirole only partially blocks the current (by a maximum of 40 %). However, as shown in Figure 3.29, when the calcium current is reduce 40 % by cadmium, there is less than a 20 % reduction in stimulated secretion. Yet when the calcium current in reduced only 40 % by quinpirole, secretion is completely inhibited. This is further evidence to suggest that direct inhibition of the voltage-dependent calcium current is not the primary mechanism involved in the inhibition of secretion. Similar conclusions can be drawn from the AtT-20 cell experiments.

The discrepancy in these results might be explained if the antagonists differentially altered the time course of the onset of the current in the first 5 ms of the evoked current. The duration of the spontaneous action potential in these cells is on the order of 5 ms and if the time course of activation of the calcium current was reduced, less calcium would enter the cell to support secretion. However, the current traces shown here do not support this hypothesis since it is the amplitude rather than the kinetics of the current that are reduced with cadmium, nifedipine and quinpirole in the first 5 ms (see Figures 3.20, 3.21 and 3.23).

If direct modulation of the voltage-dependent calcium is not the mechanism by which nifedipine and quinpirole are modulating secretion, where is their site of action?

With respect to nifedipine, dihydropyridine inhibition of calcium currents has been shown to have voltage-dependent characteristics such the high threshold calcium current is insensitive to nifedipine when the current was evoked from holding potential near the resting potential (Dunlap et al. 1989). In contrast, BAY K 8644 can potentiate the calcium current evoked from the resting potential (Dunlap et al. 1989). This voltage-dependent effect of nifedipine could explain why this compound was ineffective in inhibiting secretion in melanotrophs even though BAY K 8644 was an effective stimulator of secretion.

However, in AtT-20 cells the currents and secretion were much more sensitive to nifedipine compared to melanotrophs. This also is supportive evidence that perhaps only one type of calcium channel is present in AtT-20 cells whereas the melanotrophs may have more than one channel. In AtT-20 cells, partial inhibition of the calcium current that was purely "L" would be more effective at reducing calcium influx than partial inhibition of the current of which only a portion of that current was the "L" type

With respect to quinpirole, the D_2 receptor has been shown to be coupled not only to the voltage-dependent calcium current but also to potassium currents (see Section 3.1.4). A good relationship existed between the membrane hyperpolarization and the inhibition of secretion in the melanotroph by quinpirole (Figure 3.33). These data suggest that the primary mechanism of inhibition of secretion by quinpirole may be an

indirect, voltage-dependent inhibition of calcium currents by hyperpolarization of the membrane.

A major problem involved in future studies of the role of the potassium current activated by quinpirole in stimulus-secretion coupling is that there currently are no agents that are selective in blocking this potassium current (see North, 1989; Surprenant & North, 1988). There are many non-selective potassium channel blockers, such as high concentrations of tetraethylammonium (TEA), cesium, and barium, but all of these non-selective potassium channel blockers also significantly increase hormone release (Figure 2.5 and 3.14; Douglas, Taraskevich & Tomiko, 1983). This means that the same type of experiments carried out herein, in which actions of calcium channel antagonists on both calcium current and secretion were measured, are unlikely to yield meaningful data when carried out with the currently available potassium channel blockers. Nevertheless, in view of the findings that both AtT-20 cells and melanotrophs show spontaneous action potential activity at rest, and based on the lack of evidence to support a direct role for voltage-dependent calcium currents in inhibition of secretion, it seems reasonable to conclude that the potassium current activated by quinpirole in melanotrophs (Stack, Allen & Surprenant, 1987) as well as that activated by SRIF in AtT-20 cells (Pennefather, Heisler & MacDonald, 1988) may be the primary means whereby inhibition of β-endorphin secretion by these agonists occurs.

To summarize, these studies were based on the hypothesis that voltage-dependent calcium currents are involved in the regulation of the stimulus-secretion coupling response in pituitary cells. The data presented here showed no consistent relationship between the inhibition

of the voltage-dependent calcium current and the inhibition of secretion. Therefore it is concluded that inhibition of voltage-dependent calcium currents by cadmium, nifedipine, SRIF, and quinpirole is not the primary mechanism responsible for the inhibition of the secretion in these cells.

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