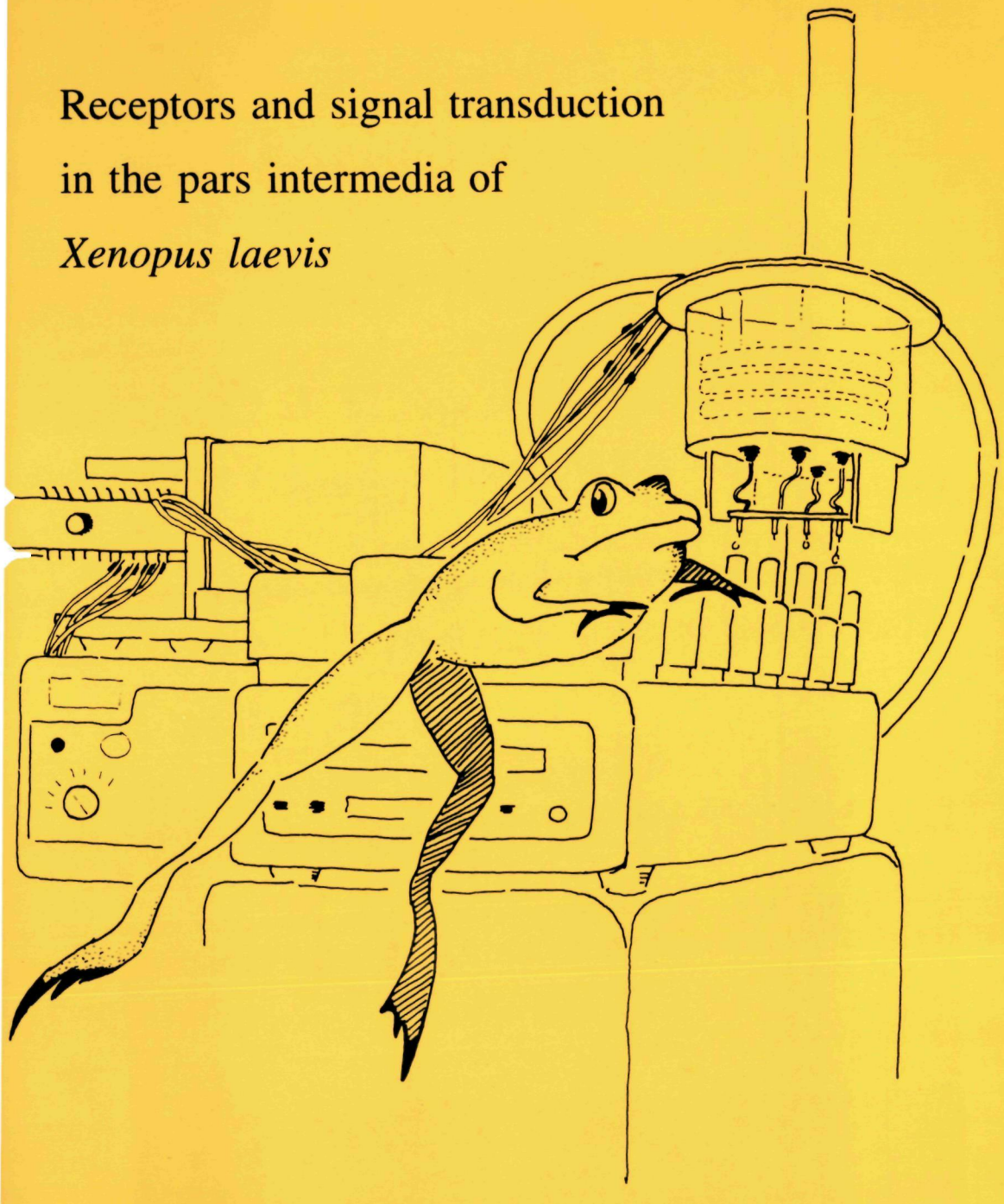
Receptors and signal transduction in the pars intermedia of *Xenopus laevis*



Harry de Koning

RECEPTORS AND SIGNAL TRANSDUCTION IN THE PARS INTERMEDIA OF *XENOPUS LAEVIS*

RECEPTORS AND SIGNAL TRANSDUCTION IN THE PARS INTERMEDIA OF *XENOPUS LAEVIS*

een wetenschappelijke proeve op het gebied van
de natuurwetenschappen,
in het bijzonder de biologie

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HENDRIK PIETER DE KONING

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Table I
overview of artificial receptor ligands used in this study

ligand	receptor	action
2-hydroxy-saclofen	$GABA_B$	antagonist
4-aminobutylphosphonic acid	$GABA_B$	antagonist
apomorphine	D_2	agonist
baclofen	$GABA_B$	agonist
bicuculline	$GABA_A$	antagonist
δ-aminovaleric acid	$GABA_B$	agonist
isoguvacine	$GABA_A$	agonist
phaclofen	$GABA_B$	antagonist
picrotoxin	$GABA_A$	antagonist
sulpiride	D_2	antagonist

Table II
overview of other bioactive agents used in this study

agent	target	action
4-aminopyridine	K^+-channel	inactivation
A23187	membranes	Ca^{2+}-ionophore
BAY-K8644	L-type VOCC	activation
cadmium chloride	VOCC	inactivation
cobalt chloride	VOCC	inactivation
forskolin	adenylate cyclase	activation
isobutylmethylxanthine	phosphodiesterase	inactivation
lithium chloride	Ins1,4,5-P_3 5-phosphatase	inactivation
nifedipine	L-type VOCC	inactivation
omega-conotoxin	N-type VOCC	inactivation
pertussis toxin	G_i and G_o	inactivation
tetraethylammonium chloride	K^+-channel	inactivation
thapsigargin	Ca^{2+}-ATPase	inactivation
verapamil	L-type VOCC	inactivation

GENERAL INTRODUCTION

Scope of the thesis

In all multicellular organisms communication between cells and organs is essential for the functioning of the organism as a whole. This holds for virtually all cells, but is most obvious for cells that constitute the organs belonging to the central nervous system and the endocrine system, the main systems responsible for information transfer. Both systems are involved in the perception, integration and export of various signals, enabling the organism to react adequately to the continuously changing environment. Communication does not only occur between neurons and endocrine cells with their respective peripheral targets (*e.g.* muscles, gonads and immune system) but also between neurons themselves as well as between neurons and endocrine cells. In some cases the intercellular communication process involves direct intercellular contacts (*e.g.* gap junctions, electrical synapses), but in most cases communication is effectuated by the release of a chemical ('first') messenger that traverses the extracellular space to couple to a receptor of the target cell. The receptor subsequently 'translates' the signal via a transduction mechanism into a cellular response (*e.g.* generation of electrical activity or secretory activity). As will be outlined below, first messengers may reach their targets in various ways and also signal transduction mechanisms may differ from one neuron or endocrine cell to another. This diversity in intercellular communication is most conspicuous in target cells that receive multiple input signals that are transduced simultaneously to form a complex cellular response. In this thesis the nature and functioning of such multiple signalling phenomena will be treated on the basis of research on signal transduction in the neuroendocrine melanotrope cells in the pituitary gland of the amphibian *Xenopus laevis*.

How first messengers reach their receptors

The classical image of the nervous system is that of a network formed by neurons and their processes, communicating through specialized contacts, the synapses. Neurons release a first messenger (neurotransmitter), such as acetylcholine or dopamine, which binds to a specific receptor in the plasma membrane of the target cell. The action of the neurotransmitter is confined to the immediate surroundings of the nerve terminal, the synaptic cleft. In contrast, endocrine first messengers, the hormones, always reach their target via the circulation and are thus capable of influencing more than one target. As a consequence, the action of hormones is much slower but more widespread than that of neurotransmitters. Endocrine regulation is in particular responsible for the control of long-term physiological processes such as growth, reproduction, development and adaptation. In this traditional view, the nervous and endocrine systems are two separate communication systems, each controlling distinct processes.

More recently, it has become clear that the communication both within and between the nervous and endocrine systems is far more elaborate than previously imagined. In contrast to Dale's Law (1935), which states that one neuron produces only one type of transmitter, it has been shown that most if not all neurons produce and secrete more than one chemical messenger (*e.g.* Hökfelt *et al.*, 1986). Probably, each messenger exerts a distinct physiological

action on the target cell, through a specific receptor. Since it has also been demonstrated that several messengers may coexist within the same nerve terminal, and even within the same synaptic vesicle, the question whether these messengers are released simultaneously or differentially is now receiving particular attention. This also holds for endocrine cells, many of which simultaneously produce a variety of first messengers. Examples of both co-release and differential release have been reported (*e.g.* Lundberg and Hökfelt, 1983; Viveros *et al.*, 1983; Lundberg *et al.*, 1988). The concept of functional cooperation between co-released messengers at the cellular and intracellular level is currently emerging from this field of study. Many of these messengers are peptides.

Another important change in the concept of the nervous system is the finding that communication between neurons is not only accomplished via direct, synaptic contacts but also in a hormone-like way. In this 'nonsynaptic' or 'paracrine' type of communication, messengers (particularly peptides) are not released into a synaptic cleft but into the extracellular space of the nervous system; they subsequently diffuse over considerable distances through the nervous system to bind eventually to their distant targets (*e.g.* Beaudet and Descarriers, 1978; Buma and Roubos, 1986).

In addition to this paracrine mode of neurotransmission, a second mode of communication exists in which neuronal messengers can reach distantly located targets. This is when the messenger, mostly a neuropeptide, is released into the circulation in a neurohaemal area, *e.g.* from the median eminence or from the neural lobe of the pituitary. In this case there is a convergence of the nervous and endocrine systems in that the neuronal messenger acts as a neurohormone. Well known examples of this type of hormone are vasopressin and oxytocin. The process is commonly known as 'neurosecretion'.

Not only can the nervous system release neurohormones, it also has a profound influence on the endocrine system. In turn, the endocrine system influences the nervous system. Communication between the two systems occurs in various ways. The endocrine cells in the periphery can act, directly or indirectly, on the central nervous system, by sending hormones capable of crossing the blood-brain barrier to the brain. These actions are mostly part of feedback loops that start with neurons that control the secretory activity of the endocrine cells.

Some of these neurons, primarily located in the hypothalamus, control the activity of the main endocrine gland, the pituitary, either via the blood (first described by Speidel, 1919; Scharrer, 1929) or by a direct synaptic contact (Bargmann *et al.*, 1967). The latter situation is best exemplified by the innervation of the pituitary gland in lower vertebrates. This complex organ consists of endocrine cells, axon terminals, and supporting cells, and is referred to as a neuroendocrine gland. The main supporting cell is a glial-like cell, called folliculo-stellate cell. This cell is present throughout the intermediate and anterior lobe of the pituitary, and its slender processes make intimate contacts with virtually all endocrine cells (Perryman, 1989). The recent observation that the stellate cell in the anterior lobe plays a role in the regulation of the secretory activity of the endocrine cells (Baes *et al.*, 1987; Gospodarowicz and Lau, 1989) adds yet an other level of complexity to the scala of communication mechanisms between the nervous and the endocrine system.

How receptors transduce signals

Changes in cellular activity are usually brought about by agents in the extracellular space, like neurotransmitters, neurohormones and hormones. These first messengers bind to specific receptors, either in the cell (as in the case of steroid hormones and thyroxin) or at the cell surface (*e.g.* neurotransmitters). In the latter case, the messenger does not enter the cell and its message will therefore have to be conveyed into the cell by the receptor. For that purpose, the receptor activates or inactivates enzymes that control key processes in the cell. This procedure, from receptor activation to intracellular response, is known as signal transduction. The receptor may control key enzymes directly, by intrinsic kinase activity (*e.g.* receptors for growth factors), or indirectly, either by activation of transmembrane ion fluxes (*e.g.* $GABA_A$ receptor, NMDA receptor, glycine receptor) or by activation of a G-protein (*e.g.* $GABA_B$ receptor, catecholamine receptors, most neuropeptide receptors). In the latter case, it is the G-protein that in turn activates or inactivates an ion channel or an enzyme that generates an intracellular messenger ('second messenger'; for review see *e.g.* Gilman, 1987).

The most important second messengers are probably adenosine 3',5'-cyclic monophosphate (cyclic-AMP), inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Cyclic-AMP is generated by the enzyme adenylate cyclase, a process that is stimulated by the G-protein G_s and inhibited by the G-proteins G_i and G_o (Gilman, 1984, 1987). The main function of cyclic-AMP is to activate protein kinase A (PKA), which phosphorylates multiple intracellular targets (Hemmings *et al.*, 1989). The second messengers IP_3 and DAG are generated by cleavage of the membrane phospholipid phosphatidylinositol 1,4-biphosphate. The cleavage is performed by phospholipase C (PLC), which is activated by the G-protein G_p (for review see *e.g.* Berridge, 1988). To date, no conclusive evidence has been reported that PLC is under both inhibitory and stimulatory control by G-proteins. The receptor for IP_3 is a Ca^{2+}-selective ion channel, mediating Ca^{2+} release from intracellular stores (Ferris *et al.*, 1989). After IP_3 binds the to receptor, the ion channel is opened and calcium ions enter the cytoplasm, activating various calcium-binding proteins such as calmodulin and protein kinase C. The kinase is activated by Ca^{2+} and DAG together and plays a pivotal role in the interaction between the various signal transduction pathways (*e.g.* Houslay, 1991). Other calcium-binding proteins that are important in the regulation of the secretory activity of endocrine cells are *e.g.* calmodulin and calmodulin/Ca^{2+}-dependent protein kinases (Hemmings *et al.*, 1989; Stojilković and Catt, 1992).

Among the targets for second messenger-activated protein kinases are plasma membrane ion channels (*e.g.* Hemmings *et al.*, 1989; Conn, 1990) that control intracellular calcium concentrations and the membrane potential of the plasma membrane. Thus, ion channel gating is controlled by the second messenger concentration. In addition, the gating of some channels may be regulated by direct coupling of G-proteins to the channel (*e.g.* Holz *et al.*, 1986; Sternweis and Pang, 1990). A single G-protein may bind multiple effector sites, so that activation of one receptor can activate and/or inactivate several intracellular signalling pathways. In this way, different receptors on one cell can converge their actions to one

common intracellular pathway to inhibit secretion, while, at the same time, each receptor can individually control one or more additional cellular processes, such as the biosynthesis and the intracellular transportation of secretory products. A simplified overview of some of the signal transduction pathways discussed above is provided in figure 1. In reality, the complexity of these pathways is often considerably higher. In order to characterize this complexity and to elucidate the cell physiological relevance of this complexity in terms of intercellular neuronal and endocrine communication, signal transduction has been studied in a neuroendocrine gland, the pituitary of *Xenopus laevis*.

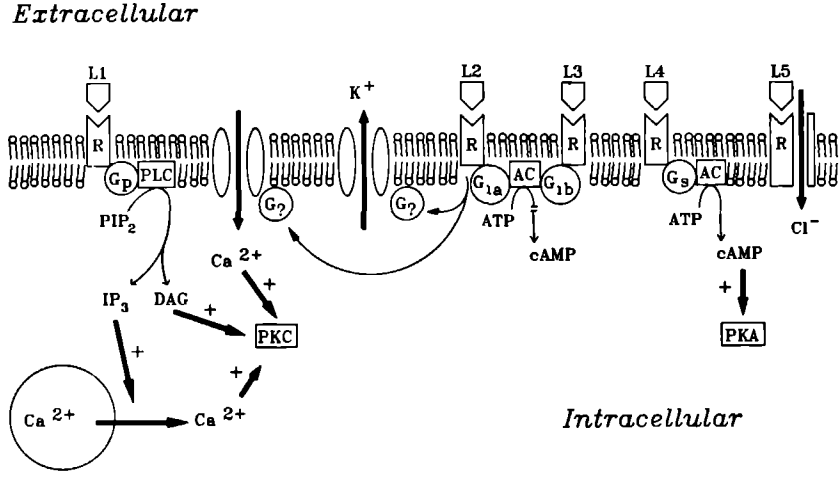

Fig. 1. *Some possible intracellular mechanisms involved in the regulation of α-MSH secretion. L = ligand; R = receptor; PKA = protein kinase A; PKC = protein kinase C; PLC = phospholipase C; AC = adenylate cyclase; DAG = diacylglycerol; IP_3 = inositol 1,4,5-triphosphate; cAMP = adenosine 3',5'-cyclic monophosphate.*

The melanotrope cells of *Xenopus laevis* as a model to study multiple signal transduction mechanisms

The functioning of a neuroendocrine gland can be considered at four levels: (1) the innervation of the endocrine tissue, (2) the receptors present on the endocrine cells, (3) the intracellular events integrating the neuronal messages and (4) the resulting endocrine output. This thesis describes the integration of a complex neuronal input, consisting of both classical neurotransmitters and neuropeptides, into an endocrine output. This has been studied in the pars intermedia of the aquatic toad *Xenopus laevis*. Particular attention has been given to the

characterization of the receptors involved and the mechanisms that couple receptor activation to a stimulation or inhibition of the release of the peptide hormone α-melanophore-stimulating hormone (α-MSH)

The pars intermedia of *Xenopus* contains one endocrine cell type, the melanotrope cell This cell releases α-MSH, which causes dispersion of the black pigment melanin in the dermal melanophores In this fashion, the concentration of α-MSH in the blood regulates skin colour, the skin changes from white to black with increasing α-MSH concentration (Bagnara and Hadley, 1973, Jenks, 1977, Van Zoest *et al*, 1989) In adapting its skin colour to match the background, the animal has to regulate the α-MSH concentration in its blood This is achieved mainly by inhibiting the spontaneous secretion of α-MSH from melanotrope cells when the animal perceives a light background The inhibition is caused by agents released by neurons, probably of hypothalamic origin, that innervate the pars intermedia (Jenks *et al*, 1988, Roubos, 1992) To date, three agents have been identified that probably are physiological α-MSH inhibiting factors in *Xenopus* Innervation with neurons containing dopamine, γ-aminobutyric acid (GABA) and Neuropeptide Y (NPY) has been identified in the pars intermedia (Verburg-van Kemenade *et al*, 1986c, 1987b, Van Strien *et al*, 1991, De Rijk et al, 1990a, 1992) All three messengers are potent inhibitors of α-MSH from neurointermediate lobes *in vitro* (Verburg-van Kemenade *et al*, 1986c,d, 1987b) For GABA two distinct receptor types have been shown to exist on *Xenopus* melanotropes, the $GABA_A$ receptor and the $GABA_B$ receptor (Verburg-van Kemenade *et al*, 1986a, 1987b) Two neuropeptides that are capable of stimulating α-MSH secretion have been identified thyrotropin-releasing hormone (TRH) and sauvagine, an amphibian peptide related to corticotropin-releasing hormone (CRH, Verburg-van Kemenade *et al*, 1987a,f) Finally, adrenalin inhibits α-MSH release *in vitro* (Verburg-van Kemenade *et al*, 1986b), but a physiological role in the regulation of melanotrope cell activity remains to be established for this biogenic amine

From the above it appears that the regulation of melanotrope cell activity is a very complex affair The question arises as to the physiological relevance of this complexity, and in particular, of at least four inhibitory receptors Two very likely possibilities are the release of distinct neuronal factors after specific physiological stimuli, and differential action of the individual inhibitors on cellular processes unrelated to α-MSH secretion This matter has become even more interesting now that it has been shown that all the inhibitory factors coexist in nerve terminals in the pars intermedia (De Rijk *et al*, 1992) The diversity of the neural input makes the neurointermediate lobe of *Xenopus laevis* an interesting system to study neuroendocrine regulation and the interactions between multiple regulatory factors at the cellular level

Aim and outline of this thesis

The aim of the studies described in this thesis was to investigate how the pars intermedia transduces multiple neuronal input to a coordinated endocrine output To obtain a thorough understanding of this intricate process, the following components of the process have been

investigated

(1) Identification of the *first messengers* involved in the regulation of melanotrope cell activity

(2) Determination of the *extracellular pathways* (directly via synapses, or indirectly) by which the first messengers act on the melanotrope cell

(3) Identification and characterization of the *receptors* through which the various first messengers act upon the melanotropes

(4) Identification and characterization of the *second messengers* that are responsible for the receptor-mediated signal transduction into a response (secretion) of the melanotrope cell

(5) Assessment of *interactions* between different second messenger systems within the melanotrope cell

The first component of the signal transduction system, the first messengers, has previously received much attention On the basis of *in vitro* superfusion experiments and of immunocytochemical techniques a number of potential physiological regulators was identified (*e g* Jenks *et al*, 1988, De Rijk *et al*, 1992) However, the action of one putative regulator of α-MSH release, *viz* α-MSH itself, was still unclear Early reports discussed this subject and presented evidence both *pro* (Kastin *et al*, 1971, Iturriza, 1973) and *contra* (Huntington and Hadley, 1974, Thornton and Geschwind, 1975) the possibility that α-MSH would exert a direct, negative feedback action on its own release from the melanotrope cell On the basis of a series experiments, described in Chapter 1, it is shown such a feedback mechanism does not exist

Chapter 2 deals with the second component of the signal transduction system in the melanotrope cell the extracellular pathways of the first messengers It has been known for some time that GABA, dopamine and CRH act directly on the melanotrope cell to regulate α-MSH secretion (Verburg-van Kemenade *et al*, 1986a, 1987a) but the mechanism by which NPY inhibits melanotrope cell activity was not known The experiments described in this chapter reveal that NPY acts on the folliculo-stellate cell to indirectly inhibit α-MSH secretion

Chapter 3 describes the pharmacological characterization of the $GABA_B$ receptor of *Xenopus* melanotrope cells At the beginning of this study, no specific tools were known to selectively stimulate or antagonize the $GABA_B$ receptor of endocrine cells, except for the traditional agonist baclofen In this study, several specific $GABA_B$ receptor antagonists have been identified and one of these agents was used to block the $GABA_B$ receptor *in vivo*, together with the dopamine receptor antagonist sulpiride In this way it was shown that the inhibition of α-MSH release in animals on a white background (see above) is not exclusively mediated by $GABA_B$ and dopamine receptors Treatment with sulpiride caused the white

animal to become darker, but only slightly. It was concluded that the *in vivo* inhibition of α-MSH release is accomplished by multiple neuronal messengers.

The identification and characterization of second messengers, is treated in the last five chapters of this thesis. Attention has been paid to three messenger types: calcium ions, IP_3 and cyclic-AMP. In Chapter 4 the most common intracellular second messenger in secretory cells, the calcium ion, is considered. It is demonstrated that mobilization of intracellular calcium does not contribute significantly to the process of spontaneous α-MSH release. This release appears to be sustained by influx of calcium ions through voltage-operated calcium channels, predominantly of the dihydropyridine-insensitive N-type. The second messenger IP_3 plays no evident role in the regulation of the secretory process, because regulatory factors hardly affect inositol phosphate metabolism (Chapter 5). However, there is a very clear difference in the rate of inositol phosphate production between animals on white *versus* black backgrounds. It is suggested that the inositol signalling mechanism plays a role in long-term adaptation processes rather than in short-term secretory processes. The emerging picture from Chapter 6 is that in addition to calcium ions, cyclic-AMP, and not IP_3, is involved in the regulation of the secretory process. Inhibitors of α-MSH release also inhibit cyclic-AMP production (dopamine, baclofen) and stimulators of α-MSH release also stimulate cyclic-AMP production (CRH and the related amphibian peptide sauvagine).

Finally, the second messenger systems in the melanotrope cell interact with each other (Chapters 7 and 8). This appears from the observation that the coupling between an increase of cyclic-AMP production and α-MSH secretion is not absolute: stimulation of the $GABA_A$ receptor by its agonist isoguvacine leads to a stimulation of cyclic-AMP production that is concomitant with inhibition of α-MSH secretion. Similarly, the inhibition of cyclic-AMP production is no prerequisite for the dopamine-induced inhibition of secretion. These data strongly suggest the involvement of interacting, multiple intracellular mechanisms in dopamine receptor-mediated signal transduction and inhibitory control of α-MSH secretion.

CHAPTER 1

Analysis of autofeedback mechanisms in the secretion of pro-opiomelanocortin-derived peptides by melanotrope cells of *Xenopus laevis*

With B.G. Jenks, W.J.J.M. Scheenen, P.H.M. Balm and E.W. Roubos

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Abstract. The secretion of most pituitary hormones is under the control of feedback mechanisms. The feedback control of α-melanophore-stimulating hormone (α-MSH) from melanotrope cells is controversial. The possible existence of an autofeedback exerted by α-MSH or other POMC-derived peptides on melanotrope cells of the amphibian *Xenopus laevis* has been investigated. α-MSH or its potent agonist 4-norleucine,7-D-phenylalanine-α-MSH has no effect on the release of radiolabelled POMC-derived peptides or immunoreactive β-endorphin from superfused neurointermediate pituitary lobes. Melanin concentrating hormone, previously reported to have an α-MSH-like effect on melanophores, did not affect α-MSH secretion Neurointermediate lobe superfusate, which contains a mixture of POMC-derived peptides, failed to affect the secretory activity of melanotropes. It is concluded that in *X. laevis* the secretory activity of melanotropes is not under the control of short-term autofeedback mechanisms involving α-MSH or other POMC-derived peptides.

INTRODUCTION

The secretion of many pituitary hormones is regulated by feedback mechanisms involvir hormones from peripheral endocrine target organs. Some pituitary hormones, such as prolacti and α-melanophore stimulating hormone (α-MSH), do not appear to activate peripher endocrine organs, so their secretion may not involve target organ feedback. For prolactin, thei is an autoregulatory mechanism, whereby the secreted hormone inhibits its own releas (Kadowaki *et al.*, 1984, Frawley and Clark, 1986) With regard to α-MSH, there is evidenc from pituitary transplant studies, for long-term effects of pars intermedia secretory products c the morphology of *in situ* melanotrope cells (Perryman, 1974; Gianoulakis and Gupta, 198 Ito *et al.*, 1986) However, evidence for direct short-term autofeedback mechanisms equivocal, with positive (Kastin *et al.*, 1971; Iturriza, 1973) and negative (Huntington ar Hadley, 1974, Thornton and Geschwind, 1975) findings. In the studies cited, bioassays with rather low specificity were used that also may monitor inhibitory factors, such a catecholamines, released from the neurointermediate lobe (Bagnara and Hadley, 1973 Furthermore, in these studies melanotropes may also have been affected by inhibitory factor such as dopamine and GABA, released from the (statically) incubated lobes. Consequently, th existence of an autofeedback mechanism controlling α-MSH release is still open to debate.

The present study addresses this question, focusing on α-MSH release from melanotrop cells of the aquatic toad *Xenopus laevis*. Since these cells display high rates of biosynthes and secretion of POMC-derived peptides (Loh and Gainer, 1977, Martens *et al.*, 1981a), the are suitable for the study of their response to inhibitory input in general and to putativ negative autofeedback in particular. In a tissue superfusion system, the *in vitro* effects of c MSH treatment on the release of radiolabelled POMC-derived peptides and of immunoreactiv β-endorphin, a POMC-derived peptide known to be cosecreted with α-MSH (Verburg-va Kemenade *et al.*, 1986a, 1987a,b), were examined In addition, the responses to a hyperactiv α-MSH analogue (4-norleucine,7-D-phenylalanine-α-MSH; NDP-MSH, Sawyer *et al.*, 1980) a

well as to melanin concentrating hormone (MCH), a hypothalamic peptide that has been suggested to bind to the α-MSH receptor in amphibians (Castrucci *et al*, 1987, 1989, Hadley *et al*, 1988), have been assessed Finally, to investigate whether POMC-derived peptides other than α-MSH exert a feedback to melanotropes, neurointermediate lobes were challenged with superfusate containing POMC-derived peptides

MATERIALS AND METHODS

Animals. Adult *X laevis* from laboratory stocks were adapted to a black background for 3 weeks and fed trout pellets (Trouvit, Trouw, Putten, The Netherlands) weekly Water temperature was 22 °C Animals were decapitated and their neurointermediate lobes removed

Analysis of release of radiolabelled peptides. Before superfusion, lobes were cultured for 20 h in culture medium (CM) consisting of 67% (v/v) L15 medium (Gibco, Renfrewshire, UK), 10% fetal calf serum (FCS, Gibco), 1% (v/v) kanamycin solution (Gibco), 1% (v/v) antibiotic/antimycotic solution (Gibco), 0 08 mg/ml $CaCl_2 2H_2O$ and 0 2 mg/ml glucose (pH 7 4) They were then incubated in 100 µl CM for 20 h in the presence of 200 µCi [^{3}H]-lysine (Amersham, Buckinghamshire, UK, 86 Ci/mmol) To obtain maximum specific activity of labelled protein, labelling was performed in lysine-free culture medium, using dialysed FCS (Gibco) and lysine-free L15 medium After labelling, lobes were washed three times and superfused with incubation medium (IM) containing 112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 15 mM HFPFS (pH 7 4, Calbiochem, La Jolla, CA), 2 mg/ml glucose and 0 3 mg/ml bovine serum albumin The superfusion system for neurointermediate lobes has been described before (Verburg-van Kemenade *et al*, 1987b) Lobes were superfused simultaneously at 22 °C, in separate 10 µl chambers, with IM, for at least 75 min before the first pulse with a potential secretagogue was applied The flow rate was 1 6 ml/h, an average of 3 2 ng α-MSH was released in this interval, which represents a concentration of 2 ng/ml ($1\,2*10^{-9}$ M) Synthetic α-MSH (Sigma, St Louis, MO), NDP-MSH (Sigma) and dopamine (Sigma) were added in various concentrations (see Results) Fractions were collected every 15 min, and submitted to solid phase extraction, using a Baker octodecyl-C18 column (see Van Zoest *et al*, 1990), to separate free radiolabelled lysine from labelled peptides Four ml of scintillation fluid (Scintillator 199, Packard, Groningen, The Netherlands) was added to the peptide containing column fractions and radioactivity was determined in a liquid scintillation counter (LKB 1216 Rackbeta)

The effects of neurointermediate lobe-enriched medium on the release of radiolabelled peptides were also examined The enriched medium was obtained as follows lobes were cultured for 7 days in CM (see De Koning *et al*, 1991) to remove regulatory neuronal inputs (see Discussion) and were then superfused in IM, which was collected on ice, for 90 min This medium was then placed in a waterbath at 22 °C for 15 min, and applied to [^{3}H]-lysine-labelled lobes in a parallel superfusion This method discriminates between immunoreactive α-MSH in the enriched medium and labelled α-MSH from the radiolabelled tissue The concentration of α-MSH in the enriched medium was determined by radioimmunoassay

HPLC analysis of radiolabelled peptides. To determine whether α-MSH had an effect on the profile of the secretory signal, superfusates were collected before and during administration of α-MSH and submitted to HPLC. Chromatography was performed as described previously (Martens *et al*, 1980), using a spherisorb 10 ODS column (Bischoff, Leonberg) with 0 5 M formic acid/0 14 M pyridine as the primary solvent and n-propanol as the secondary solvent Flow rate was 2 ml/min and 1 ml fractions were collected Of the superfusion fractions involved, a sample (25%) was used directly for HPLC analysis and the remainder was applied to a Baker column to determine the total amount of radioactivity released The HPLC fractions were mixed with scintillation fluid and their radioactivity was determined by liquid scintillation counting Peaks were identified according to Martens *et al* (1982)

Analysis of the release of immunoreactive α-MSH. Freshly dissected neurointermediate lobes were superfused as above. Fractions were collected every 7.5 min and submitted to radioimmunoassay for α-MSH (Van Zoest *et al.*, 1989), bound and free antibodies were separated by the polyethylene glycol/albumin method Cross-reactivity was 100% with desacetyl α-MSH but was <0.5% with ACTH (1-24) and ACTH (1-39). The limit of detection was 2 pg α-MSH per sample. MCH (synthesized by T. Matsunaga in the laboratory of Dr. V J. Hruby, Tucson) was applied at concentrations of 10^{-8} to 10^{-5} M. The possible cross-reactivity of MCH with the anti-α-MSH antiserum was determined in both the absence and presence of unlabelled α-MSH (see Results)

Analysis of release of immunoreactive β-endorphin. Superfusions were conducted as above and 7.5 min fractions were submitted to radioimmunoassay for β-endorphin, using a porcine β-endorphin antiserum kindly provided by Dr. H Vaudry (Rouen, France) The characteristics of the antiserum have been described (Jegou *et al*, 1983) and it has been successfully used to determine *Xenopus* β-endorphin (Verburg-van Kemenade *et al*, 1986a, 1987a,b). Since *Xenopus* intermediate lobe extracts do not dilute in parallel with porcine β-endorphin (Verburg-van Kemenade *et al.*, 1986a) all superfusion fractions were assayed at the same dilution. The data obtained thus refer to relative amounts of β-endorphin released Dopamine (10^{-5} M) and α-MSH ($3*10^{-11}$ M - $9*10^{-9}$ M) were added in 15 min pulses

Statistics. Data of cross-reactivity were tested for significance using the two-sided, unpaired Student's T-test A *P*-value <0 05 was considered to indicate significance

RESULTS

Effect of α-MSH and NDP-MSH on release of radiolabelled peptides. α-MSH did not affect the release of [^{3}H]-lysine labelled peptides from superfused neurointermediate lobes (Fig 1) Short (15 min) pulses of α-MSH ($3*10^{-9}$ - $9*10^{-9}$ M) and NDP-MSH ($2.4*10^{-9}$ M) had no effect on the release of these peptides. To assess the effect of longer-term exposure to α-MSH, pulses of 2 h ($6*10^{-9}$ M α-MSH) were given, but no effect on peptide release was seen (Fig 1) At the end of each superfusion, 10^{-5} M dopamine was shown to rapidly inhibit secretion (Fig 1). HPLC analysis of peptides released prior to and during α-MSH administration

showed that α-MSH treatment had no effect on the HPLC profile of the peptides in the secretory signal (Fig. 2).

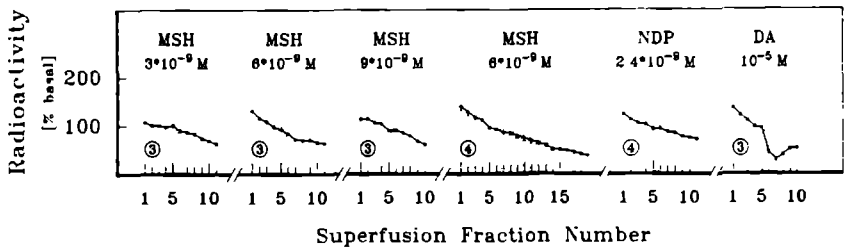


Fig. 1. *Effect of α-MSH and NDP-MSH on the release of [³H]-lysine-labelled peptides from superfused neurointermediate lobes. Data are the means of several independent experiments, indicated by the encircled numbers. Basal release (100 %) is defined as the average release in the three fractions preceding the pulse. Vertical bars represent -SEM. Fractions were collected every 15 min; DA is dopamine.*

Effects of MCH on release of immunoreactive α-MSH. MCH, in the range 10^{-8} to 10^{-6} M, had no effect on α-MSH release (Fig. 3); at 10^{-5} M a slight stimulation was seen. However, this may reflect the slight cross-reactivity of MCH of about 0.0015% with the anti-α-MSH serum used in the radioimmunoassay, which becomes evident at 10^{-5} M. The displacement of [^{125}]I-labelled α-MSH by MCH in a dilution curve is not parallel to α-MSH (Fig. 4).

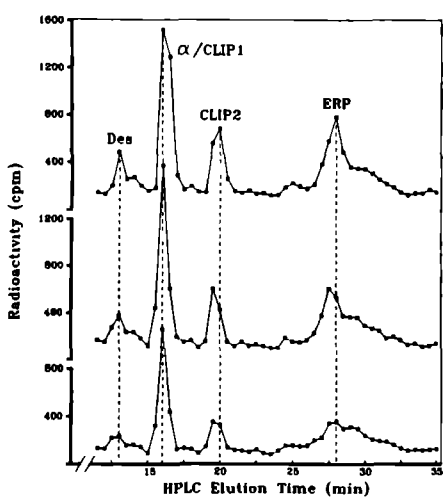


Fig. 2. *Reversed-phase HPLC-profile of radiolabelled peptides in superfusion fractions 5-6 (upper panel), 7-8 (middle panel) and 15-16 (lower panel) of the fourth superfusion shown in Fig. 1. Des is desacetyl α-MSH, α/CLIP1 is coeluting peptides α-MSH and corticotropin-like intermediate lobe peptide-1, CLIP2 is corticotropin-like intermediate lobe peptide-2 and ERP represents a mixture of endorphin-related peptides.*

Effect of α-MSH on release of immunoreactive β-endorphin. The release of immunoreactive β-endorphin-related peptides (ERP) from superfused neurointermediate lobes was unaffected by α-MSH (Fig. 5). In each experiment dopamine gave the normal inhibitory response.

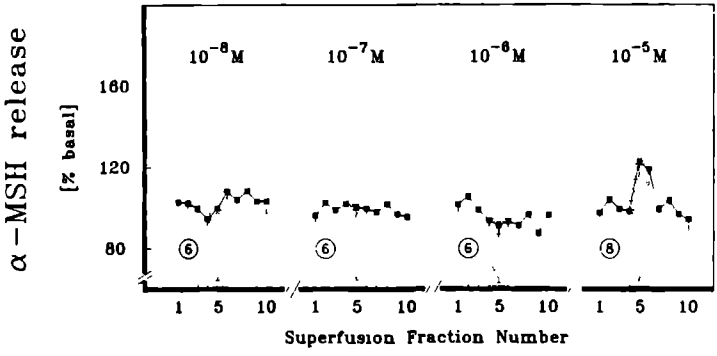


Fig. 3. Dose-response relation between MCH and the release of immunoreactive α-MSH from superfused neurointermediate lobes. Basal secretion is the average release in the four fractions preceding the pulse. Data are the means of several independent experiments, indicated by the encircled numbers. Vertical bars represent -SEM.

Effect of enriched medium on the release of radiolabelled peptides. The neurointermediate lobe-enriched medium had no effect on the release of radioactive peptides from superfused radiolabelled lobes (Fig. 5). The concentration of α-MSH in the medium was $1.18*10^{-10}$ M, which is within the physiological range of plasma α-MSH concentrations of black-adapted *Xenopus* (Van Zoest *et al.*, 1989; De Rijk *et al.*, 1990). A pulse of dopamine at 10^{-5} M gave a clear inhibitory response.

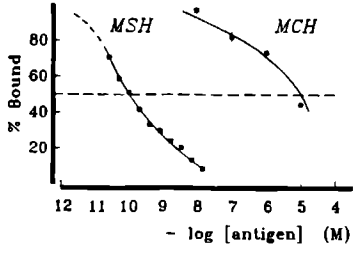


Fig. 4. Cross-reactivity of MCH with the α-MSH antiserum. Concentrations of 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M MCH in incubation medium (IM) were tested in the α-MSH radioimmunoassay. α-MSH-like immunoreactivity, compared to the IM control, was raised significantly by 10^{-7}, 10^{-6} and 10^{-5} M MCH ($P<0.05$, $P<0.01$ and $P<0.001$, respectively). Data are the means of four determinations and vertical bars represent SEM.

DISCUSSION

Previous studies have demonstrated that POMC-derived peptides are released from melanotrope cells of *Xenopus laevis* in a coordinate way (Martens *et al.*, 1981b; Verburg-van Kemenade *et al.*, 1986a, 1987b; Van Zoest *et al.*, 1990, De Koning *et al.*, 1991). Therefore, in the present study, analysis of release of radiolabelled POMC-derived peptides provided an appropriate marker for secretory events in the intermediate lobe tissue. Newly synthesized peptides are known to be released from *Xenopus* melanotrope cells in a biphasic pattern, the first involving release from a fast, rapidly depleted compartment and the second phase exhibiting a slower secretory rate (Van Zoest *et al.*, 1990). This complex release profile makes it very difficult to quantify actions of secretagogues. To overcome this problem, lobes were labelled to steady-state rendering relatively stable (and only slowly decreasing) release rates of radiolabelled POMC-derived peptides. The release of these peptides was clearly inhibited by dopamine, an established α-MSH inhibitory factor (Verburg-van Kemenade *et al.*, 1986b), but was unaffected by α-MSH. HPLC analysis revealed that α-MSH also had no qualitative effect on peptide composition of the secretory signal, thus providing evidence against a selective effect of α-MSH on release of individual POMC-derived peptides. NDP-MSH also had no effect on the secretion of POMC-derived peptides from melanotropes. NDP-MSH is a potent α-MSH receptor agonist with prolonged biological activity (Sawyer *et al.*, 1980); in the toad skin bioassay it is about 10 times more potent than α-MSH (Ferroni and Castrucci, 1987).

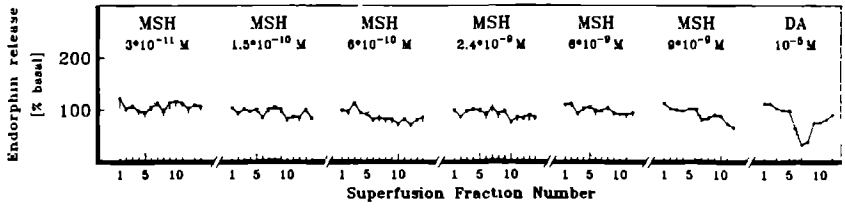


Fig. 5. *Effect of α-MSH on the release of β-endorphin from superfused neurointermediate lobes. Basal release (100 %) is the average release in the three fractions preceding the pulse. Data are the means and SEM of four independently superfused lobes. Fractions were collected every 7.5 min; DA is dopamine.*

Another peptide that may bind α-MSH receptors is MCH (Castrucci *et al.*, 1987, 1989; Hadley *et al.*, 1988) and therefore analysis of the action of this peptide on melanotrope cells was also included in the present study. This peptide is reported to exert, at high concentrations, an α-MSH-like effect on melanophores of amphibians (Wilkes *et al.*, 1984; Ide *et al.*, 1985; Ferroni and Castrucci, 1987) and teleosts (Hadley *et al.*, 1988, Castrucci *et al.*, 1989). While MCH appears to inhibit α-MSH secretion in fish (Barber *et al.*, 1987), the effect of this peptide on secretion of α-MSH from melanotropes of amphibians has not been

previously studied. Such an effect would seem to be feasible because MCH-immunoreactivity has been demonstrated in the neural lobe of the frog, *Rana ridibunda* (Andersen *et al.*, 1986). The effect of MCH on the release of immunoreactive α-MSH from *Xenopus* neurointermediate lobes, was analyzed using concentrations that have α-MSH-like effects on amphibian melanophores (Castrucci *et al.*, 1987, 1989; Hadley *et al.*, 1988). While no effect of MCH on α-MSH release was observed at 10^{-7} and 10^{-6} M MCH, 10^{-5} M MCH seemed to stimulate α-MSH secretion. However, analysis of the anti-α-MSH antiserum revealed a slight cross-reactivity with MCH, which was sufficient to account for the apparent stimulation. The occurrence of this cross-reactivity may reflect the notion that the tertiary structures of α-MSH and MCH are related (Castrucci *et al.*, 1989). It is concluded overall that MCH has no effect on α-MSH secretion from melanotrope cells of *Xenopus*.

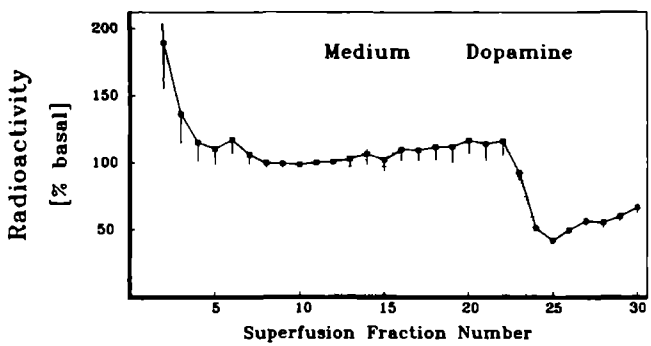


Fig. 6. Effect of neurointermediate lobe-enriched medium on the release of [^{3}H]-lysine-labelled peptides from superfused neurointermediate lobes. Basal release (100 %) is the average release in the three fractions preceding the pulse. Data are the means and SEM of four independently superfused lobes. Fractions were collected every 7.5 min. The concentration of dopamine was 10^{-5} M.

The present data suggest a lack of autofeedback by α-MSH in *Xenopus*, a contention supported by the analysis of immunoreactive β-endorphin release. It has been established that β-endorphin is cosecreted and coregulated with α-MSH (Verburg-van Kemenade *et al.*, 1986a, 1987a,b). In analyzing the effect of α-MSH on release of immunoreactive endorphin, various concentrations of α-MSH were administered, starting well within the physiological range of plasma α-MSH ($1.5*10^{-12}$ - $2.5*10^{-10}$ M, Van Zoest *et al.*, 1989; De Rijk *et al.*, 1990) but, because the local concentrations of α-MSH in the pituitary may be much higher than in the circulation, lobes were also challenged with higher doses, up to $6*10^{-9}$ M of α-MSH. That α-MSH had no effect on the release of β-endorphin supports the conclusion that α-MSH has no autofeedback effect on the secretory process of the *Xenopus* melanotrope.

In earlier reports it was presumed that melanotrope cell feedback involves α-MSH (Kastin *et al.*, 1971; Iturriza, 1973; Perryman, 1974; Ito *et al.*, 1986), but other released POMC-

derived peptides may be concerned. The effect of superfusate of cultured neurointermediate lobes on the release of radiolabelled peptides from neurointermediate lobes was therefore examined. The superfusate of cultured lobes contains the entire peptide secretory signal of melanotrope cells and the superfusates do not contain potentially interfering neurotransmitters and neuropeptides, because in such lobes all neuronal elements have fully degenerated (De Koning *et al.*, 1991). The fact that this medium had no effect on the secretory process indicates an absence of autofeedback mechanisms within the amphibian pars intermedia. The possibility that melanotrope cells control their own secretion in a more complex fashion, *e.g.* indirectly via control mechanisms in the hypothalamus (*cf.* Kastin and Schally, 1967; Lichtensteiger and Monnet, 1979), deserves further attention.

CHAPTER 2

Indirect action of elevated potassium and Neuropeptide Y on α-MSH secretion from the pars intermedia of *Xenopus laevis*: A biochemical and morphological study

With B.G. Jenks, W.J.J.M. Scheenen, E.P.C.T. de Rijk, R.T.J.M. Caris and E.W. Roubos

Neuroendocrinology 54:68-76 (1991)

Abstract. A number of neurochemical messengers have been shown to act directly on the melanotrope cells of the pars intermedia of *Xenopus laevis* to regulate α-MSH secretion. In the present study the possibility that the melanotropes are also indirectly controlled has been examined. For this purpose, the characteristics of α-MSH release from superfused intact lobes, cultured lobes and isolated melanotropes were compared after treatment with elevated potassium. Isolated melanotropes responded with an increased secretion of α-MSH, whereas intact lobes showed a profound inhibitory response, probably caused by potassium-induced release of inhibitory factors from nerve terminals. Cultured lobes displayed a biphasic response characterized by an initial activation followed by a strong inhibition; the stimulatory phase likely reflects a direct action of potassium on the melanotropes, before being overridden by an inhibitory mechanism. The inhibitory phase must originate from the action of non-neuroendocrine cells because the cultured lobes lack functionally active nerve terminals, as verified by immunocytochemistry and electron microscopy. The most likely candidates for this action are folliculo-stellate cells which are in intimate contact with the melanotropes and are innervated by neuropeptide Y-containing nerve terminals. Like elevated potassium, neuropeptide Y inhibited α-MSH secretion from fresh and cultured lobes but not from isolated melanotropes. This indicates that NPY acts indirectly, in a nonpresynaptic way, to inhibit α-MSH secretion.

INTRODUCTION

The melanotropes of the neurointermediate lobe of the amphibian *Xenopus laevis* produce α-melanocyte stimulating hormone (α-MSH), a peptide that causes darkening of the skin (Bagnara and Hadley, 1973). The release of α-MSH from melanotropes is under the control of various neurochemical messengers. On the basis of *in vitro* superfusion studies of neurointermediate lobes, dopamine, γ-aminobutyric acid (GABA), corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH) and neuropeptide Y (NPY) have been identified as potential regulators (Verburg-van Kemenade *et al.*, 1986c,d, 1987a,b,d,f); dopamine, GABA and NPY inhibit, whereas CRH and TRH stimulate α-MSH release. Axons and synaptic structures containing GABA, NPY, CRH and TRH are present in the *Xenopus* neurointermediate lobe, as shown by light and electron microscope immunocytochemistry (Verburg-van Kemenade *et al.*, 1987a,b,f; De Rijk *et al.*, 1990a). For some of these messengers the primary sites of action have been established: superfusion experiments with isolated melanotropes have shown that dopamine, GABA and CRH act directly on the melanotropes in regulating α-MSH secretion (Verburg-van Kemenade *et al.*, 1986a, 1987a). The action mechanism of NPY on *Xenopus* melanotropes has not been studied. In the frog, *Rana ridibunda*, NPY was reported to act directly on melanotropes (Danger *et al.*, 1986, 1990).

In addition to a direct action on the melanotropes, there are two possible mechanisms through which secretagogues might affect α-MSH secretion indirectly: (1) in a presynaptic way, *e.g.* by stimulating release of dopamine or GABA from nerve terminals within the

neurointermediate lobe or (2) through action on nonendocrine cells, such as the folliculo-stellate cells in the pars intermedia. These cells, together with the melanotropes, form the main constituent of the *Xenopus* pars intermedia. Possible involvement of the folliculo-stellate cells in the control of melanotropes has recently gained considerable attention (for a review, see Perryman, 1989) and the suggestion has been made that, in the rat anterior lobe, folliculo-stellate cells release a factor that inhibits the stimulated secretion of prolactin, growth hormone and luteinizing hormone (Baes *et al.*, 1987).

The present study deals with the possible existence of an indirect mechanism regulating α-MSH secretion from *Xenopus* melanotropes. For this purpose characteristics of potassium-induced α-MSH release from (1) intact, fresh neurointermediate lobes, (2) isolated melanotropes in primary culture and (3) tissue-cultured neurointermediate lobes were compared. The cultured lobes were used to exclude a possible involvement of presynaptic mechanisms; the absence of functionally active axon terminals as a result of degeneration in these cultured lobes was verified using immunocytochemistry and electron microscopy. The secretory dynamics of cultured lobes were compared to those of intact tissue, using pulse chase, high performance liquid chromatography (HPLC) and superfusion techniques. The effects of NPY on fresh and cultured neurointermediate lobes, as well as on isolated melanotropes, were analyzed to investigate whether this neuropeptide inhibits α-MSH release via an indirect mechanism.

MATERIALS AND METHODS

Animals. Adult *X. laevis* were taken from laboratory stock. They were adapted to a black background for three weeks. The animals were fed trout pellets (Trouvit) once a week. The water temperature was 22 °C.

Preparation of cultured neurointermediate lobes. Neurointermediate lobes were dissected and washed with culture medium containing 67% (v/v) L15 medium (Gibco), 1% (v/v) kanamycin solution (Gibco), 1% (v/v) antibiotic/antimycotic solution (Gibco), 10% fetal calf serum (FCS, Gibco), 0.08 mg/ml $CaCl_2.2H_2O$ and 0.2 mg/ml glucose (pH 7.4). Before use, the culture medium was sterilized by ultrafiltration using a Millex-GV 0.22 μm filter (Millipore). After washing 4 times with 1 ml of this medium, each lobe was cultured in 1 ml of culture medium in a multidish (Nunc) at 22 °C in an incubator for 1-7 days. Medium was refreshed daily.

Biosynthetic analysis of pro-opiomelanocortin peptides. Freshly dissected or cultured neurointermediate lobes were washed three times with 0.5 ml incubation medium (IM; 112 mM NaCl, 2 mM KCl, 15 mM HEPES, pH 7.4; Calbiochem, Ultrol Grade, 2 mM $CaCl_2$, 2 mg/ml glucose, 0.3 mg/ml bovine serum albumin) and incubated at 22 °C for 1 or 3 h with 30 μCi [^{3}H]-tryptophan (Amersham, 55 Ci/mM) in 30 μl IM/lobe. Lobes were then washed with 1 ml IM containing 1 mM tryptophan and chase-incubated for 4 h in this medium. Incubation was terminated by adding HCl to the medium to a final concentration of 0.1 N, after which the lobes

were homogenized in 1 ml 0.1 N ice-cold HCl Half of a homogenate and the chase-medium were submitted to reversed-phase HPLC using a Spherisorb 10 ODS column (Bischoff, Leonberg) with 0.5 M formic acid/0.14 M pyridine as the primary solvent and n-propanol as the secondary solvent; flow rate was 2 ml/min. One-millilitre fractions were collected and radioactivity measured in a scintillation detector (LKB 1216 Rack Beta) Peaks were identified according to Martens et al. (1980, 1982). The other half of the homogenate was lyophilized and resuspended in application buffer for sodium dodecyl sulphate (SDS) gel electrophoresis, according to Laemmli (1970), using 13% acrylamide gels Proteins were detected by fluorography (Bonner and Laskey, 1974). [^{14}C]-methylated molecular weight marker proteins were from Amersham (CFA626).

Superfusion of neurointermediate lobes. Superfusion of neurointermediate lobes was performed as described previously (Verburg-van Kemenade *et al.*, 1987b) The superfusion system allowed four lobes to be superfused simultaneously Volume of a superfusion chamber was 10 µl. Fractions were collected every 7.5 min At least 10 fractions were collected before 15 min pulses (2 fractions) of NPY (0.33-1.0 µM) or elevated potassium (10-60 mM KCl) were introduced In experiments where the concentration of KCl was varied, the concentration of NaCl was adjusted to maintain the osmolarity of medium.

Superfusion of cultured dispersed melanotropes. Lobes were washed with culture medium lacking FCS and incubated for 45 min in 2 ml of this medium containing 10 mg collagenase type V (Sigma) and 20 mg protease type IX (Sigma) at 22 °C. Then cells were dispersed by passing the tissue several times through a siliconized pasteur's pipette and filtered through a nylon gauze (pore size 150 µm) mounted on a syringe to remove undissociated tissue (including the complete neural lobe) The dispersed cells were washed, collected by centrifugation and transferred to a lymphocyte tube (Nunc), in the presence of 2 mg/ml Cytodex-3 beads (Pharmacia) The number of living cells isolated per lobe was 20,000-25,000, as determined by counting cells in a sample of the suspension, using a Bürker-Turk chamber, in the presence of trypan blue (Sigma). This represents a yield of 30-35% It was estimated by immunocytochemical analysis that over 90% of the cells were melanotropes; the remaining cell types included folliculo-stellate cells, endothelial cells and fibroblasts

The isolated cells were cultured for 2 days at 22 °C in 1 ml of complete culture medium to allow their attachment to the beads. It was found that inclusion of excess beads prevented cells from aggregating, thus precluding any reestablishment of contacts between melanotropes and stellate cells. The beads were washed in IM before transfer to superfusion chambers, which had a volume of 75 µl. The chambers were equipped with Millipore filters (HAWP02500, pore size 0 45 µm) to support the beads Each chamber contained cells derived from 4 lobes. The flow rate and the method of administration of high potassium were the same as described above

Radioimmunoassay. α-MSH concentrations were measured as described previously, using an anti-α-MSH serum produced in our laboratory (Van Zoest *et al.*, 1989). Bound and free antibodies were separated by the polyethylene glycol/albumin method Cross-reactivity with desacetyl α-MSH was 100%, but cross-reactivity with ACTH (1-24) or ACTH (1-39) was almost absent (<0 5%). Detection limit was 10 pg α-MSH per sample (Van Zoest *et al.*, 1989).

Immunocytochemistry. Immunocytochemistry for NPY and GABA was performed as described previously (De Rijk *et al.*, 1990a). In short, for analysis with antiserum to NPY, lobes were fixed in Bouin's fluid, dehydrated, embedded in paraffin and cut into sections of 5 µm. For analysis with antiserum to GABA, lobes were fixed in 5% glutaraldehyde and 1% paraformaldehyde in 0.05 M phosphate buffer (pH 7.4) for 2 h at 20 °C, and postfixed in 1% osmium tetroxide in buffer, for 1 h at 4 °C. They were then embedded in Epon and 1 µm sections were cut.

The sections were preincubated with 20% normal goat serum in Tris/sodium chloride buffer (TBS; pH 7.6) and incubated sequentially with anti-NPY (1:4000) or anti-GABA (1.1000) for 16 h at 4 °C, goat-antirabbit IgG (Nordic Immunology, Tilburg; 1.40) and rabbit peroxidase/ antiperoxidase (Nordic Immunology, Tilburg; 1:200). Finally, sections were treated with a solution of 0.02% 3,3' diaminobenzidine, 0.06% nickel ammonium sulphate and 0.005% H_2O_2 in TBS, for 10 min.

Analysis with antiserum to dopamine was performed according to Buijs et al (1984). In short, lobes were fixed in 5% glutaraldehyde and 1% $Na_2S_2O_5$ in 0.05 M phosphate buffer (pH 7.4) and kept in 20% sucrose and 1% $Na_2S_2O_5$ in the same buffer for 16 h at 4 °C. Cryosections (20 µm) were treated with normal goat serum in the presence of 1% $Na_2S_2O_5$ (10 min) and with antidopamine (dilution 1·4000) for 16 h at 4 °C. Control stainings were carried out after preabsorbing the antisera with the respective antigens. In the case of dopamine, absorbtion was performed in the presence of 1% $Na_2S_2O_5$. The specificity of the antisera has been described previously (Buijs *et al.*, 1984; Seguela *et al.*, 1984; Danger *et al*, 1985).

Electron microscopy. Neurointermediate lobes were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 15 min at 20 °C and postfixed in a mixture of 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, 2% osmium tetroxide and 5% potassium dichromate (1·1:1) for 1-1.5 h at 4 °C, as described previously (De Rijk *et al.*, 1990b). The lobes were then blockstained with 2% uranyl acetate, dehydrated and embedded in Spurr's resin. Ultrathin sections were stained with lead citrate and examined in a Jeol JEM 100CX II electron microscope.

Statistics. Percentages of stimulation and of inhibition of α-MSH secretion in response to secretagogue administration during superfusion were calculated on the basis of integration of the respective peak areas in the graphs, using defined fractions just before and during pulses with secretagogues (for fractions involved, see Results). The integrated areas were tested for significance using the paired Student's t test (α=5%).

RESULTS

Biosynthetic dynamics of neurointermediate lobes. The HPLC profiles of [^{3}H]-tryptophan-labelled newly synthesized peptides from freshly dissected and cultured neurointermediate lobes (1, 2, 5 and 7 days in culture) were very similar and highly reproducible. Profiles of freshly dissected lobes and lobes cultured for 5 days are shown in figure 1. Most prominent among the HPLC-resolved peptides were desacetyl α-MSH and α-MSH. In all cases, desacetyl α-MSH was the major tissue form of MSH (Fig. 1a), whereas α-MSH was the major form of

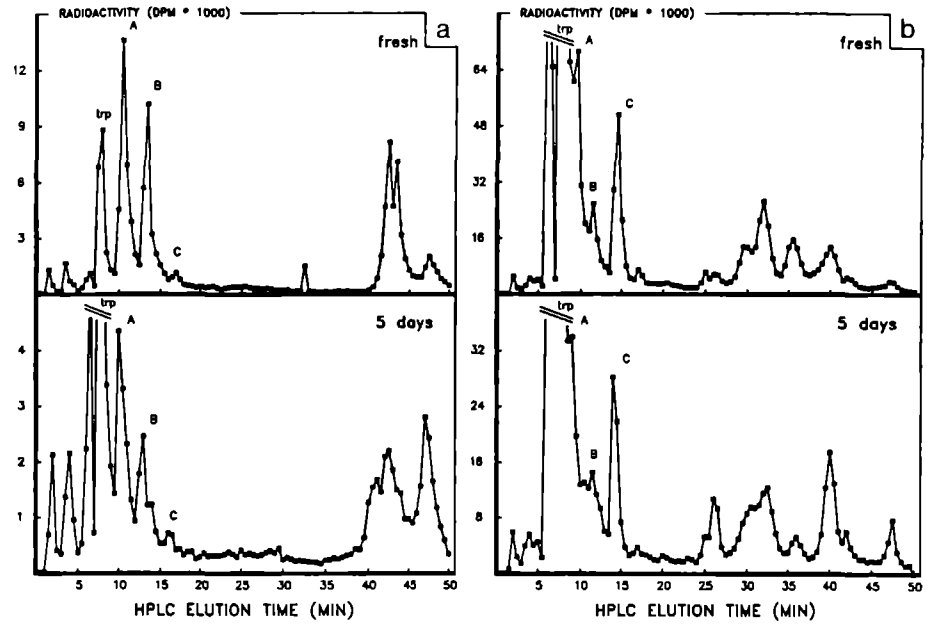


Fig. 1. HPLC profiles of radioactive peptides in tissue extracts (a) and chase medium (b) of freshly dissected neurointermediate lobes (upper panels) and lobes cultured for 5 days (lower panels) Lobes were pulsed for 3 h in medium containing [^{3}H]-tryptophan, followed by 4 h of chase incubation. Peaks represent: trp =[^{3}H]-tryptophan; A = coeluting peptides β-MSH and γ-MSH; B = desacetyl α-MSH; C = α-MSH.

the peptide released into the medium (Fig. 1b), an observation in keeping with the secretion-associated acetylation of this peptide (Martens *et al.*, 1981c; Vaudry *et al.*, 1983; Jenks *et al.*, 1985; Verburg-van Kemenade *et al.*, 1987c). The HPLC profiles do not show endorphins or corticotropin-like intermediate peptides because these peptides do not contain tryptophan. The products eluting after 20 min from the HPLC column have not yet been identified. In the tissue extracts there were three such products, which eluted between 40 and 50 min. In the medium these products were minor or absent, but there were some products eluting between 25 and 40 min, which were not represented in the tissue profiles.

Electrophoretic analysis showed that both the degree of radioactive labelling and the pattern of labelled proteins were very similar in freshly dissected and cultured lobes (Fig. 2). Dominant among the newly synthesized products were pro-opiomelanocortin (POMC) and pro-γ-MSH.

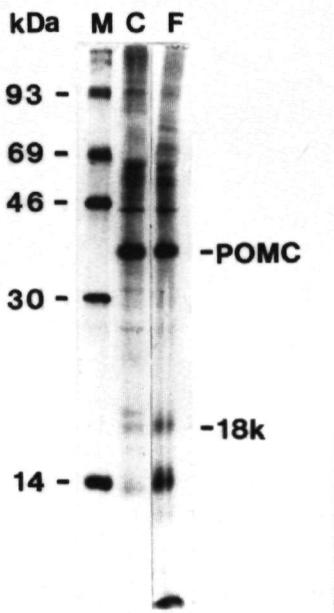


Fig. 2. SDS gel electrophoresis of tissue extracts of freshly dissected (lane F) and cultured (lane C) neurointermediate lobes after 3 h pulse with [^{3}H]-trp and 4 h of chase incubation. The electrophoretic mobilities of the radioactive markers are indicated (lane M). Lane F contains half of the tissue extract of the same freshly dissected neurointermediate lobe as used for HPLC analysis in figure 1a, upper panel. Lane C contains half of the tissue extract used for HPLC analysis in figure 1a, lower panel. POMC (36 kDa); 18k = pro-γ-MSH (18 kDa).

Structural integrity of cultured lobes. At the light microscope level both fresh and cultured neurointermediate lobes reveal normally shaped melanotropes and folliculo-stellate cells, in a proportion of about 10:1.

Immunocytochemistry showed that fresh neurointermediate lobes are innervated by dense networks of dopamine-, GABA- and NPY-immunopositive fibres. The immunostaining had a dotted appearance indicative of nerve varicosities and was largely confined to the intermediate lobe (Fig. 3a-c). In cultured lobes, the density of the networks and the number of varicosities decreased with increasing culture duration. After 7 days of culture dopamine, GABA and NPY immunoreactivities were absent. As an example, this is shown for GABA in figure 3d. In no case staining was found with any of the preabsorbed antisera.

At the ultrastructural level the general morphology of the cells of cultured lobes was not obviously different from that of fresh lobes. The melanotropes had a healthy appearance, showing large amounts of rough endoplasmic reticulum, numerous large mitochondria and a well-developed Golgi apparatus that formed many secretory granules (Fig. 4a,b). The folliculo-stellate cells also appeared normal, extending their long, slender processes between the melanotropes (figs. 4a,c) with which they were firmly connected by desmosome-like structures (Fig. 4d). The cultured lobes clearly differed from fresh ones, however, with respect to synapse-like contacts on melanotropes and folliculo-stellate cells. In fresh lobes synapse-like structures were filled with small electron-lucent and larger electron-dense vesicles (Fig. 4e), while nerve terminals in cultured lobes were characterized by empty vesicles of various shape and diameter, as well as by various types of lysosome-like structures (Fig. 4f). In the pars nervosa of cultured lobes, pituicytes appeared very active, having engulfed parts of granule-filled axon terminals. Although some axon terminals appeared normal, others showed clear signs of degeneration (Fig. 4g).

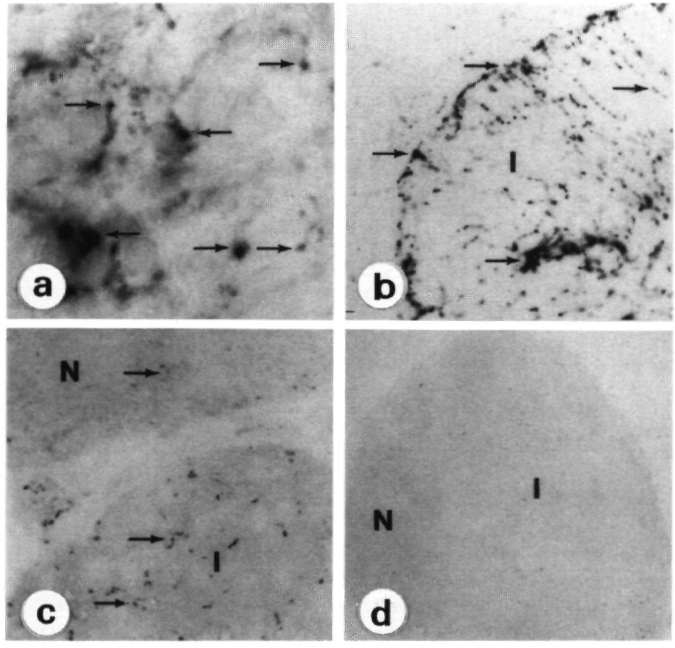
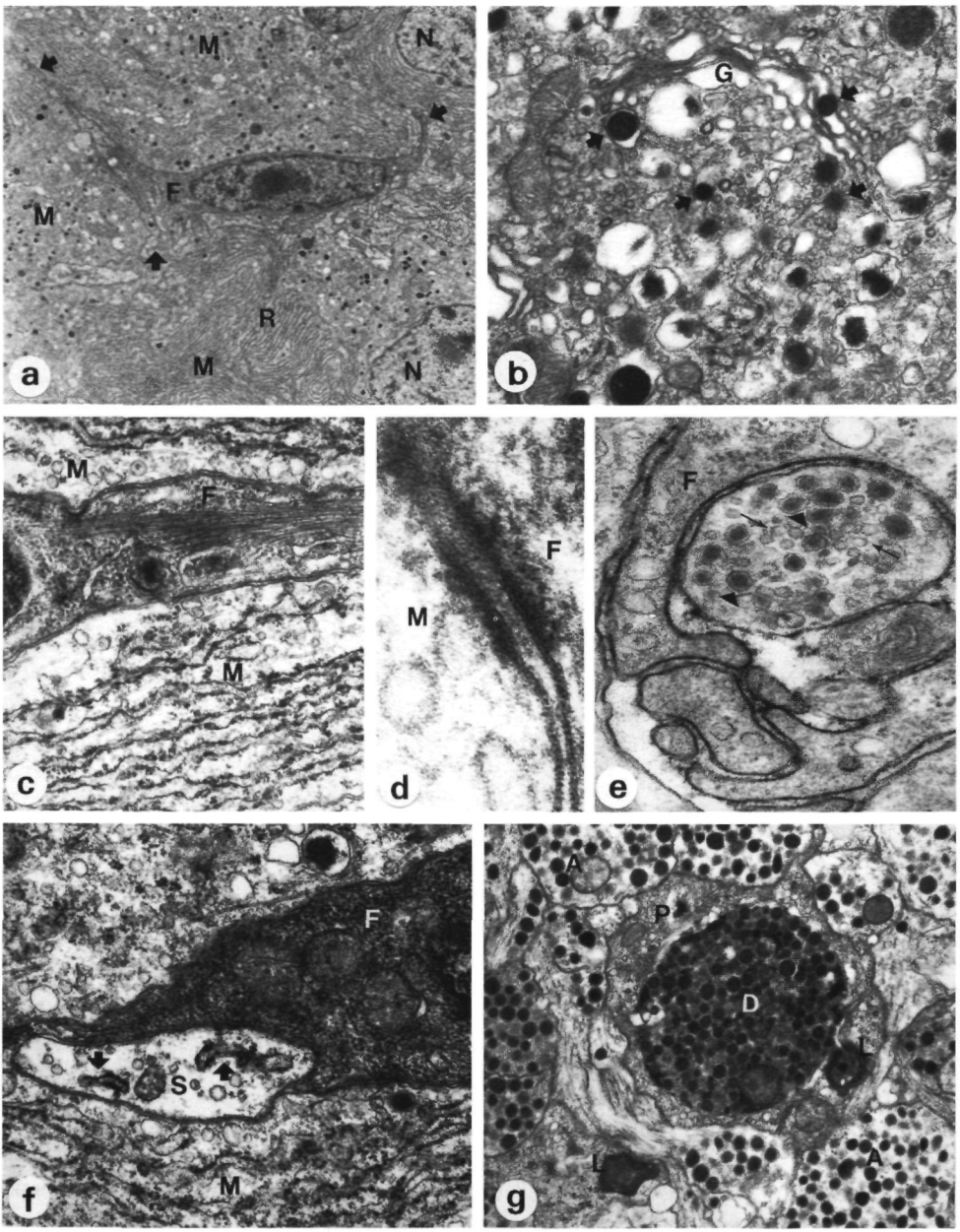


Fig. 3. *Immunocytochemistry of the neurointermediate lobe. Arrows indicate immunoreactive varicosities. I = pars intermedia; N = pars nervosa.* (***a***) *Fresh intermediate lobe, stained with antidopamine. x 2,200.* (***b***) *Fresh neurointermediate lobe, anti-NPY. x 1,000.* (***c***) *Fresh neurointermediate lobe, anti-GABA. x 900.* (***d***) *Neurointermediate lobe cultured for 7 days, anti-GABA. Note absence of immunoreactivity. x 1,000.*

Fig. 4. *Electron microscopy of 7 days cultured neurointermediate lobes* (***a-d,f,g***) *and a fresh lobe* (***e***). (***a***) *Folliculo-stellate cell (F) with slender processes (arrows) extending between melanotrope cells (M). N = nuclei of melanotropes; R = rough endoplasmic reticulum. x 8,000.* (***b***) *Highly active Golgi apparatus (G) forming secretory vesicles (arrows) in melanotrope cell. x 25,000.* (***c***) *Part of folliculo-stellate cell (F) flanked by melanotropes (M). x 30,000.* (***d***) *Desmosome-like connection between melanotrope (M) and folliculo-stellate cell (F). x 150,000.* (***e***) *Normal synaptic contact on folliculo-stellate cell (F) with small electron-lucent (arrows) and large electron-dense (arrowheads) synaptic vesicles. x 35,000.* (***f***) *Degenerative synaptic contact (S) associated with folliculo-stellate cell (F) and melanotrope (M), with lysosome-like structures (arrows) and empty vesicles of various shape and size. x 30,000.* (***g***) *Neural lobe showing axon terminals (A) and pituicyte (P) which has engulfed a degenerated terminal (D). L = Lysosomes. x 12,500.*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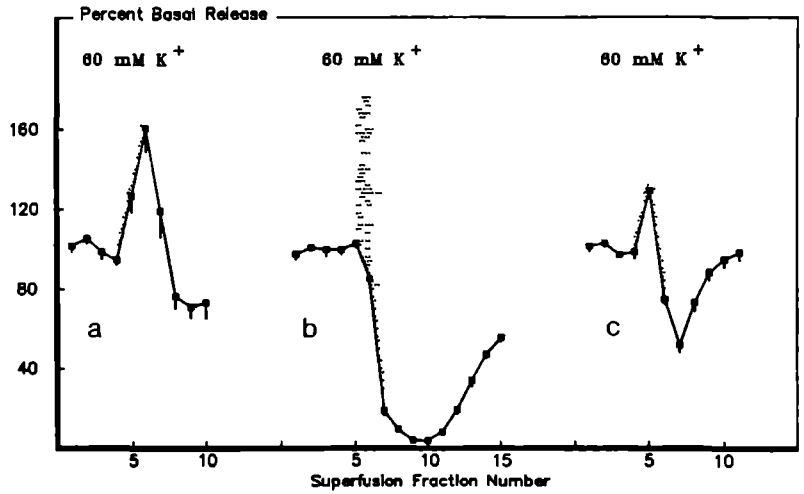


Fig. 5. Response to 60 mM KCl (15 min) during in vitro superfusion of isolated melanotropes (a), freshly dissected neurointermediate lobes (b) and neurointermediate lobes cultured for 7 days (c) Fractions were collected every 7 5 min The basal level of α-MSH release was calculated for each experiment as the average secretion in the four fractions preceding the pulse The results are the average of several independent experiments Bars represent -SEM K^+ stimulated α-MSH release from isolated melanotropes by 47 8 ± 7 44% (n=8, P<0 001, fractions 3+4 compared to fractions 5+6) and inhibited the release from fresh lobes by 48 2 ± 2 3% (n=10, P<0 001, fractions 4+5 compared to fractions 6+7) The release from cultured lobes was stimulated initially by 32 3 ± 6 5% in the first pulse fraction (n=6, P<0 01, fraction 4 compared to fraction 5) and subsequently inhibited by 35 2 ± 2 1% in the next two fractions (n=6, P<0 001, fractions 3+4 compared to fractions 6+7)

Secretory response to elevated potassium. Responses of isolated melanotropes, freshly dissected neurointermediate lobes and cultured tissue to elevated potassium clearly differed (Fig 5) Freshly dissected lobes showed a strong and long-lasting inhibition of α-MSH release In contrast, isolated melanotropes responded with a marked increase in α-MSH secretion Cultured lobes showed a biphasic effect Initially, there was a clear stimulation of release, then a sharp inhibition started, even while high potassium was still being administered This biphasic response was evident in lobes cultured for 1-7 days The dose dependency of the stimulatory and inhibitory phase of the effect of elevated potassium on cultured tissue and isolated melanotropes was also examined For cultured lobes, 20 mM KCl induced inhibition

but there was no stimulatory component, whereas at 40 and 60 mM KCl clear stimulatory components followed by marked inhibitions were observed (Fig. 6, upper panel). At all concentrations, the inhibitory component was stronger than the stimulatory component. The dose-response for KCl of isolated melanotropes did not show an inhibitory component; a slight stimulation was observed at 20 mM KCl, whereas at 40 and 60 mM stimulation of α-MSH release was marked (Fig. 6, lower panel). Responses of isolated cells were strongly delayed compared to those displayed by intact lobes, due to the larger volume of the superfusion chambers used for isolated cells.

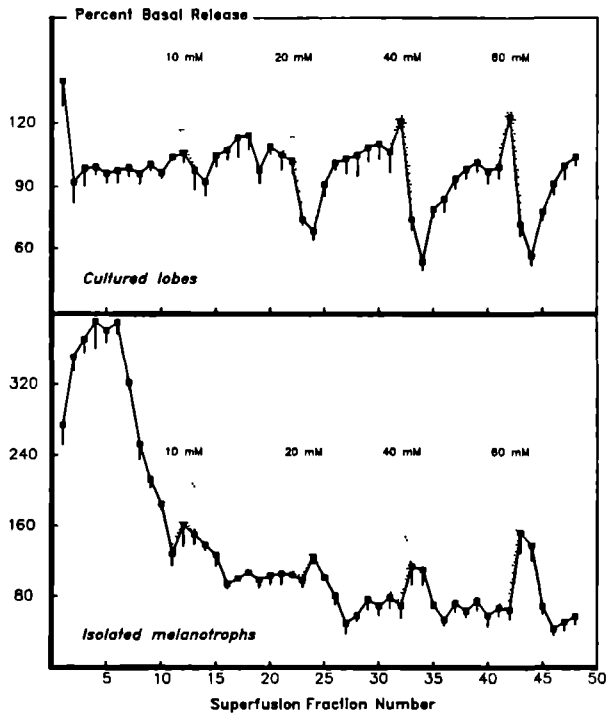


Fig. 6. *Dose-response of the effect of KCl on 7 days cultured neurointermediate lobes (upper panel) and on isolated melanotropes (lower panel). Each graph represents the average result from four lobes or cell suspensions. Basal level was calculated from the average release of the three fractions preceding the first pulse (fractions 9-11 for the cultured lobes and fractions 16-18 for the melanotropes). Bars indicate -SEM.*

Effect of NPY. NPY induced a marked, long-lasting inhibition of α-MSH secretion when applied to freshly dissected neurointermediate lobes but had no effect on α-MSH secretion from isolated melanotropes (Fig. 7a,b). On cultured lobes, NPY exhibited a very strong inhibitory effect (Fig. 7c), very similar to the effect on freshly dissected lobes; in both cases maximum inhibition was achieved 1-3 fractions after NPY treatment was terminated and secretion returned to basal levels only very slowly.

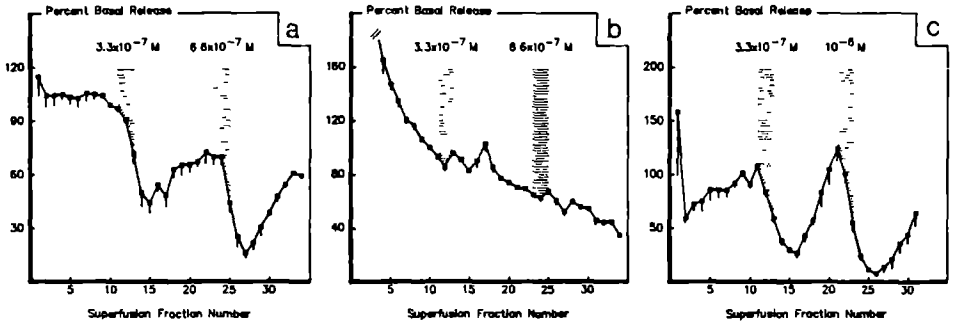


Fig. 7. Effect of NPY on superfused fresh neurointermediate lobes (a), isolated melanotropes (b) and neurointermediate lobes cultured for 7 days (c). NPY was added during two 15 min periods. Experiments shown in frame A and B were performed simultaneously, each preparation receiving the same medium. Each graph represents the average of 4 lobes or cell suspensions. Basal level was calculated from the average release of the 3 fractions preceding the first pulse (fractions 9,10,11). Bars represent - SEM.

DISCUSSION

The ultrastructural data indicate that the pars intermedia of cultured lobes was structurally intact, showing close contact between folliculo-stellate cells and melanotropes. Normal functioning of the melanotropes was reflected in the results of the biosynthetic analysis which demonstrated that synthesis, processing and release of POMC-derived peptides was unaffected by culturing. The nerve terminals within the cultured lobe, however, showed degenerative features, an observation in keeping with the fact that immunoreactivity to dopamine, GABA and NPY had disappeared. Altogether we conclude that the pars intermedia of cultured lobes is an actively secreting organ that is free from neural influences. Therefore the cultured lobe can be utilized to study involvement of indirect mechanisms in the regulation of α-MSH secretion.

The secretory responses of freshly dissected neurointermediate lobes, cultured lobes and cultured melanotrope cells to the depolarizing effect of elevated potassium were clearly

different. Cultured melanotropes responded with an increased release of hormone, as expected for isolated secretory cells. In sharp contrast, fresh lobes responded with strong inhibition of release, a phenomenon that can be attributed to release of endogenous inhibitory factors, such as dopamine, GABA or NPY, from depolarized nerve terminals within the neurointermediate lobe. Clearly, such inhibitory action overrides any stimulatory action K^+ might exert on the melanotropes. The biphasic response of cultured tissue to elevated K^+ indicates the presence of two K^+-sensitive mechanisms. The stimulatory phase may well be attributed to direct K^+-induced depolarization of the melanotrope plasma membrane. The inhibitory phase, however, cannot be due to release of endogenous neural factors, as cultured lobes apparently lack functionally active nerve terminals. Therefore, we conclude that the inhibitory phase must involve a K^+-induced activation of an inhibitory mechanism emanating from another cell type within the (cultured) lobe. Our results indicate that while this cell type reacts more slowly than melanotropes to elevated K^+, it is probably more sensitive to K^+ since 20 mM KCl induced a clear inhibitory response with no apparent stimulatory phase.

In our view the most likely cell type involved in the regulation of the secretory activity of the melanotropes is the folliculo-stellate cell. While the neurointermediate lobe contains endothelial cells lining blood vessels, the pars intermedia is poorly vascularized and thus endothelial cells have a very restricted distribution in the tissue. Similarly, the ependymal lining of the hypophyseal cleft consists of a single layer of cells, making this cell type an unlikely mediator of an inhibitory action on melanotropes. In contrast, folliculo-stellate cells are relatively abundant and distributed evenly throughout the pars intermedia, with their processes making intimate contact with virtually every melanotrope cell (Perryman, 1974, 1989; De Rijk *et al.*, 1990a). The concept that folliculo-stellate cells play a role in the regulation of pituitary hormone secretion finds precedent in the literature. Semoff and Hadley (1978) observed staining indicative of ATPase activity between melanotropes and folliculo-stellate cells and suggested that the stellate cells regulate the extracellular ionic environment of the melanotropes. This idea is supported by recent studies by Ferrara and Gospodarowicz (1988), who showed that bovine intermediate lobe folliculo-stellate cells are probably involved in ion transport. In addition, folliculo-stellate cells of the pars distalis have been reported to produce and secrete paracrine factors which regulate the secretory activity of endocrine cells in this organ (Baes *et al.*, 1987; Gospodarowicz *et al.*, 1989; Gospodarowicz and Lau, 1989). In view of the inhibitory action observed on the melanotrope in the present study, the report of a folliculo-stellate cell-derived factor inhibiting stimulated prolactin release is of particular interest (Baes *et al.*, 1987).

The indirect mechanism inhibiting α-MSH secretion from *Xenopus* melanotropes could very well be evoked by NPY because this peptide did not inhibit secretion from isolated melanotropes but had a clear inhibitory action on cultured lobes. Moreover, the inhibitory profile obtained by NPY, showing a prolonged inhibition and slow recovery to basal levels, is very similar to the profile of K^+-induced inhibition of neurointermediate lobes. In contrast, inhibition through dopamine and GABA, which have been shown to act directly on the melanotrope, are rapidly reversible (Verburg-van Kemenade *et al.*, 1986a). Involvement of the folliculo-stellate cells in NPY-induced inhibition is supported by the present observation that

NPY-containing nerve fibres are evenly distributed throughout the pars intermedia and from immunoelectron microscope studies showing that NPY containing varicosities make synaptic contacts with folliculo-stellate cells (De Rijk *et al.*, 1990a). Furthermore, specific [^{125}]I-Bolton-Hunter-NPY binding sites have been localized on folliculo-stellate cells of the *Xenopus* neurointermediate lobe (De Rijk *et al.*, 1991) and an antiidiotypic antibody against NPY, recognizing NPY receptors, binds to the intermediate lobe folliculo-stellate cell of this species (E.W. Roubos, unpubl. data). Interestingly, in contrast to the situation in *Xenopus*, in the frog *Rana ridibunda*, NPY has been reported to act directly on melanotropes. Continued studies on the location and pharmacological characteristics of NPY receptors will further our understanding of the control of the amphibian pars intermedia.

CHAPTER 3

Analysis of $GABA_B$ receptor function in the *in vitro* and *in vivo* regulation of α-MSH secretion from melanotrope cells of *Xenopus laevis*

With B.G. Jenks and E.W. Roubos

Endocrinology, in press

Abstract. The activity of many endocrine cells is regulated by γ-aminobutyric acid (GABA) The effects of GABA are mediated by $GABA_A$ and/or $GABA_B$ receptors While $GABA_B$ receptors in the central nervous system have now been extensively characterized, little is known of the function and pharmacology of $GABA_B$ receptors of endocrine cells. In the amphibian *Xenopus laevis*, GABA inhibits the release of α-melanophore-stimulating hormone (α-MSH) from the endocrine melanotrope cells through both $GABA_A$ and $GABA_B$ receptors. We have investigated the following aspects of the $GABA_B$ receptor of the melanotrope cells of *X. laevis*. (1) the pharmacology of this receptor, using antagonists previously established to demonstrate $GABA_B$ receptors in the mammalian central nervous system, (2) the relative contribution to the regulation of hormone secretion by the $GABA_A$ and $GABA_B$ receptors on melanotrope cells *in vitro*, and (3) the role of the $GABA_B$ receptor with respect to the physiological function of the melanotrope cell, *i.e.* regulation of pigment dispersion in skin melanophores in relation to background colour *in vivo* We show that phaclofen, 2-hydroxy-saclofen and 4-aminobutylphosphonic acid dose-dependently blocked the inhibition of α-MSH release by $GABA_B$ receptor activation but not $GABA_A$ receptor activation. The $GABA_B$ receptor antagonist δ-aminovaleric acid, appeared to be a selective agonist on the $GABA_B$ receptor of melanotrope cells The inhibitory secretory response to a low dose of GABA (10^{-5} M) was not affected by bicuculline, but was significantly reduced by phaclofen; a high dose of GABA (10^{-4} M) was not antagonized by phaclofen. These results indicate that at a low GABA concentration the $GABA_B$ receptor mechanism would dominate in inhibiting the melanotrope cells, whereas at high concentrations both GABA receptor mechanisms would be activated. Different thresholds of activation may form the basis for differential action of GABA acting through both GABA receptor types. The tonic inhibition of α-MSH release in animals on a white background was not affected by 4-aminobutylphosphonic acid, indicating that the $GABA_B$ receptor is not (solely) involved in the *in vivo* inhibition of α-MSH release in animals on a white background.

INTRODUCTION

The neurotransmitter γ-aminobutyric acid (GABA) is important in regulating hormone secretion from many endocrine tissues, including the anterior (Anderson and Mitchell, 1986a,b; Roussel *et al.*, 1990, Virmani, *et al.*, 1990) and intermediate (Tomiko *et al*, 1983; Demeneix *et al*, 1986; Verburg-van Kemenade *et al*, 1986c) lobe of the pituitary gland, the pineal gland (Rosenstein *et al.*, 1990), the adrenal medulla (Kataoka *et al.*, 1986; Castro *et al.*, 1989) and the endocrine pancreas (Gilon *et al.*, 1991). The action of GABA on endocrine cells is often mediated by the bicuculline-sensitive $GABA_A$ receptor. In some cases, however, an additional, bicuculline-insensitive $GABA_B$ receptor has been shown to be present. Among the endocrine cells with both $GABA_A$ and $GABA_B$ receptors are chromaffin cells (Castro *et al.*, 1989), gonadotropes (Anderson and Mitchell, 1986b), corticotropes (Anderson and Mitchell, 1986b), and melanotropes (Demeneix *et al.*, 1984, 1986, Verburg-van Kemenade *et al.*, 1987d) The

physiological significance of the coexistence of $GABA_A$ and $GABA_B$ receptors is unclear. $GABA_B$ receptors in the central nervous system (CNS) have been well characterized, using recently developed $GABA_B$ receptor antagonists such as phaclofen and 2-hydroxy-saclofen. In endocrine systems the $GABA_B$ receptors have only been identified on the basis of their sensitivity to baclofen and insensitivity to bicuculline.

A system suitable to study endocrine $GABA_B$ receptors is the intermediate lobe of the pituitary gland of the amphibian clawed toad, *Xenopus laevis*. This lobe contains only one endocrine cell type, the melanotrope cell. The *in vitro* release of α-melanophore stimulating hormone (α-MSH) from these cells is inhibited by administration of GABA (Verburg-van Kemenade *et al.*, 1986a) and by agonists of the $GABA_A$ receptor (*e.g.* isoguvacine) and of the $GABA_B$ receptor (baclofen) (Verburg-van Kemenade *et al.*, 1987d). The pars intermedia of *Xenopus* is strongly innervated by GABAergic neurons (De Rijk *et al.*, 1990a, 1992). These neurons form synaptic contacts with the melanotropes (De Rijk *et al.*, 1990a). The question arises as to the relative importance of the $GABA_A$ and $GABA_B$ receptors, because activation of either receptor type fully inhibits the secretory process (Verburg-van Kemenade *et al.*, 1987d). The receptors may be separately activated as a result of independent innervation by distinct GABAergic neurons, selectively forming $GABA_A$ or $GABA_B$ synapses. Alternatively, the two receptor types might have different thresholds of activation, so that a low GABA concentration would activate only one receptor type, as suggested for rat gonadotropes (Anderson and Mitchell, 1986b) and rat CNS neurons (Otis and Mody, 1992). To solve this problem selective antagonists to the $GABA_B$ receptor in endocrine cells are required. Therefore, we first examined the suitability of a number of established $GABA_B$ receptor antagonists in the CNS, to selectively antagonize the effects of the $GABA_B$ receptor agonist baclofen on α-MSH release. This was followed by an analysis of the relative effectiveness of $GABA_A$ and $GABA_B$ receptor antagonists to block GABA-induced inhibitory responses.

In addition, we studied the possible involvement of $GABA_B$ receptors in the *in vivo* regulation of the secretory activity of melanotrope cells. In *Xenopus*, α-MSH is strongly secreted in animals on a black background, stimulating dispersion of pigment in dermal melanophores thus leading to darkening of the animal (Bagnara and Hadley, 1973). On a white background, secretion is under tonic inhibition, and *in vitro* experiments have indicated that GABA, dopamine and NPY are potential physiological inhibitory factors (Verburg-van Kemenade *et al.*, 1986c,d, 1987b). To determine if the $GABA_B$ receptor is important in the regulation of the physiological process of α-MSH release in animals on a white background, the effect of *in vivo* treatment of *X. laevis* with a $GABA_B$ receptor antagonist (4-aminobutylphosphonic acid; 4-ABPA) was studied. Previously, indications were obtained that dopamine, acting via a sulpiride-sensitive D_2-receptor, is involved in the *in vivo* inhibition of α-MSH release from *Xenopus* melanotropes. To study whether the $GABA_B$ receptor acts in synergism with the D_2 receptor, the action of sulpiride on pigment dispersion in the skin has also been considered in the present study.

MATERIALS AND METHODS

Animals Young adult *Xenopus laevis* (8 months old) were taken from laboratory stock Three weeks prior to the experiments they were placed in black or white containers under constant illumination Water temperature was 22 °C For superfusion experiments, only black-adapted animals were used, as their stable, high level of α-MSH secretion permitted us to observe clear effects of inhibitory factors For the *in vivo* experiments, both black- and white-adapted *Xenopus* were used

Superfusion experiments. Neurointermediate lobes of *Xenopus laevis* were dissected out and placed immediately on a filter in a superfusion chamber (10 µl volume) Four chambers were superfused simultaneously with incubation medium (IM), containing 112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 15 mM HEPES (Calbiochem, La Jolla, CA), 0 3 g/l bovine serum albumin (Sigma, St Louis, MO), 2 g/l glucose, 1 mg/l ascorbic acid (pH 7 4 and carbogen-aerated), at a rate of 1 5 ml/h at 22 °C Fractions (7 5 min each) were collected and stored at -20 °C until assayed for α-MSH using a radioimmunoassay Lobes were superfused for at least 75 min with IM before receptor agonists and antagonists were introduced into the superfusion medium Basal release of α-MSH was calculated as the average secretion measured in the three fractions preceding the first application of ligand, and expressed as 100% Ligands tested, either alone or in combinations, were baclofen (Ciba-Geigy Ltd, Basel, Switzerland), isoguvacine (Cambridge Research Biochemicals Ltd, Cambridge, UK), phaclofen (Tocris Neuramin, Buckhurst Hill, UK), 4-aminobutylphosphonic acid (4-ABPA, Sigma), δ-aminovaleric acid (DAVA, Sigma), 2-hydroxy-saclofen (2-OH-saclofen, Research Biochemicals, Inc , Natick, MA) and bicuculline (Sigma) The concentrations of the ligands used and their administration protocols are given in the Results

Radioimmunoassay Radioimmunoassay for α-MSH were performed as described previously, using an α-MSH-antiserum raised and characterized in our laboratory (Van Zoest *et al*, 1989) The antiserum has equal affinity for the acetylated and non-acetylated forms of α-MSH and cross-reactivity with ACTH was less than 0 01 % Bound and free antibodies were separated by polyethylene glycol/albumin precipitation Detection limit is 2 5 pg α-MSH per sample of 50 µl All superfusion fractions were assayed in duplicate

In vivo experiments Black- or white-adapted animals were injected with receptor agonists and antagonists in 200 µl of saline (0 6% NaCl) Control animals were injected with 200 µl saline Melanophore indices (MI) were scored under a dissection microscope, using the criteria of Hogben and Slome (1931) An MI of 1 indicates complete aggregation of melanin and an MI of 5 indicates full dispersion

Calculations and statistics. In the figures data are graphically shown as means (n=4, unless otherwise indicated) + S E M (vertical bars) To calculate percentages of stimulation or inhibition of α-MSH release in the superfusion experiments, in the graphs peak areas (covering 2 - 4 fractions) before and during administration of ligands were integrated and compared The integrated areas were tested using the paired Student's T-test ($\alpha = 5\%$)

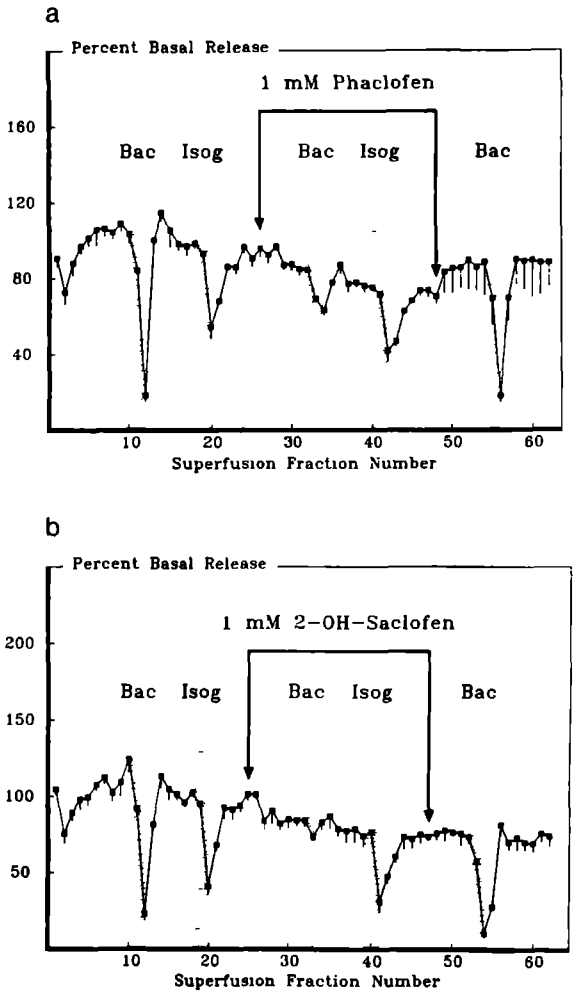


Fig. 1. The effects of phaclofen (a) and 2-OH-saclofen (b) on $GABA_A$ and $GABA_B$ receptor-mediated actions on α-MSH release from superfused neurointermediate lobes. Shaded areas indicate the administration of the indicated drug. Thick lines with arrowheads indicate start and end of antagonist administration. The concentrations of baclofen (Bac) and isoguvacine (Isog) were $3.3*10^{-6}$ M and $2*10^{-5}$ M respectively. Phaclofen reduced the baclofen-induced inhibition of release from 50.8 ± 1.9% to 21.4 ± 3.6% ($P<0.001$); 2-OH-saclofen attenuated the baclofen response from 52.2 ± 2.2% to 6.14 ± 1.7% ($P<0.001$). There was no effect of either antagonist on the response to isoguvacine.

RESULTS

Pharmacological profile of the $GABA_B$ receptor of Xenopus laevis melanotropes. The inhibition of α-MSH release induced by the $GABA_B$ receptor agonist baclofen was significantly attenuated in the presence of 1 mM phaclofen (Fig 1a) The response to the $GABA_A$ agonist isoguvacine was not affected by phaclofen The effect of phaclofen was reversible, as full recovery of the baclofen-response was observed after treatment with antagonist had been terminated A concentration of 1 mM 2-OH-saclofen antagonized the response to baclofen almost completely, without influencing the isoguvacine-induced inhibition of α-MSH secretion (Fig 1b) This effect of 2-OH-saclofen was reversible and dose-dependent, with 0 1 mM significantly reducing the strongly inhibitory effect of baclofen (Fig 2) Also, the action of baclofen was selectively and dose-dependently antagonized by 4-ABPA in the range of 0 1 - 1 mM (Fig 3) However, 4-ABPA itself caused a dose-dependent inhibition of α-MSH secretion (Fig 4a) The putative $GABA_B$ receptor antagonist δ-aminovaleric acid (DAVA) also inhibited α-MSH release, but with greater potency than 4-ABPA (Fig 4b) At 10^{-4} M, DAVA did not significantly reduce baclofen-induced inhibition of α-MSH release (not shown) The inhibitory responses to 4-ABPA and DAVA were significantly reduced by phaclofen (Fig 5a,b), but they were not by affected by 10^{-4} M of the $GABA_A$ receptor antagonist bicuculline (Fig 5c) Bicuculline completely antagonized the

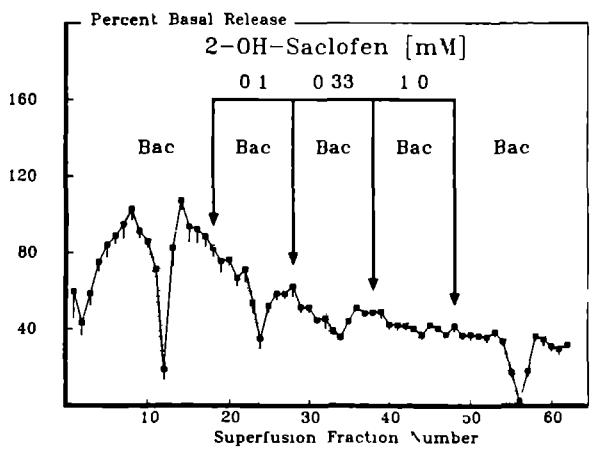


Fig 2. Dose-dependency of 2-OH saclofen antagonism of baclofen action on superfused neurointermediate lobes Baclofen concentration was $3\,3*10^{-6}$ M, 2-OH-saclofen concentrations are indicated in the figure Baclofen-induced inhibition of α-MSH release was 60 9 ± 5 0% This was reduced in the presence of 2-OH-saclofen, subsequently to 35 4 ± 5 5% (0 1 mM, P<0 02), 16 0 ± 4 3% (0 33 mM, P<0 001) and 7 7 ± 3 4% (1 mM, P<0 001)

inhibition of secretion induced by isoguvacine. The order of potencies for the $GABA_B$ receptor antagonists is: 4-ABPA = 2-OH-saclofen > phaclofen. In a concentration of 1 mM the ligands antagonized 3.3 µM baclofen by 93.6 ± 5.2% (n=4), 87.8 ± 3.3% (n=8) and 57.9 ± 7.2% (n=4), respectively.

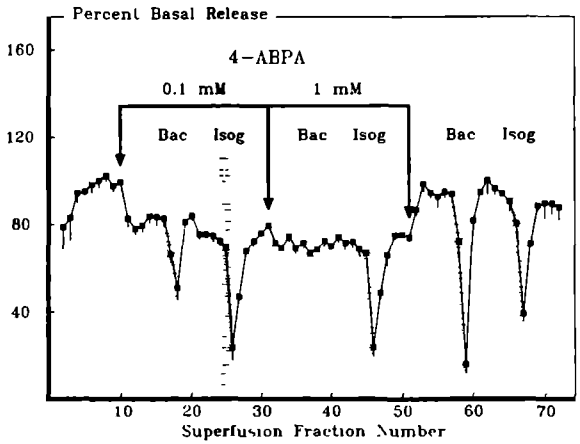


Fig. 3. Effect of 4-ABPA on $GABA_A$ and $GABA_B$ receptor-mediated actions on α-MSH release from superfused neurointermediate lobes. Concentrations of baclofen and isoguvacine were $3.3*10^{-6}$ M and $2*10^{-5}$ M respectively. Baclofen induced inhibition of α-MSH secretion (53.4 ± 4.4%) was attenuated by 4-ABPA to 28.7 ± 6.4 (0.1 mM, $P<0.05$) and 3.4 ± 2.8% (1 mM, $P<0.01$) respectively. Subsequent pulses with isoguvacine were not significantly affected by the presence of 4-ABPA.

Differential effects of GABA on $GABA_A$ and $GABA_B$ receptors in vitro. The release of α-MSH from superfused neurointermediate lobes was inhibited by 10^{-5} - 10^{-4} M GABA. This inhibition was not influenced by application of 10^{-4} M bicuculline (Fig. 6a,b), a treatment that completely blocks responses to $GABA_A$ receptor agonists (Fig. 5c). Phaclofen (1 mM) almost completely antagonized the inhibitory response evoked by 10^{-5} M GABA, but not the inhibition evoked by 10^{-4} M GABA (Fig. 6c,d). Also, administration of 2-OH-saclofen or 4-ABPA antagonized the inhibitory responses induced by 10^{-5} M GABA (not shown).

Involvement of the $GABA_B$ receptor in background adaptation. Intraperitoneal injection of baclofen in adult *Xenopus laevis* adapted to and remaining on a black background, leads to pigment aggregation. The MI decreased from about 5 to about 3 within 1 h and stayed at this value for at least 18 h (Fig. 7). When baclofen was injected together with 4-ABPA (10^{-5} mole/g body weight), the MI remained at control value (about 5) during the same period (Fig. 7). Injection of animals adapted to and remaining on a white background with the same dose

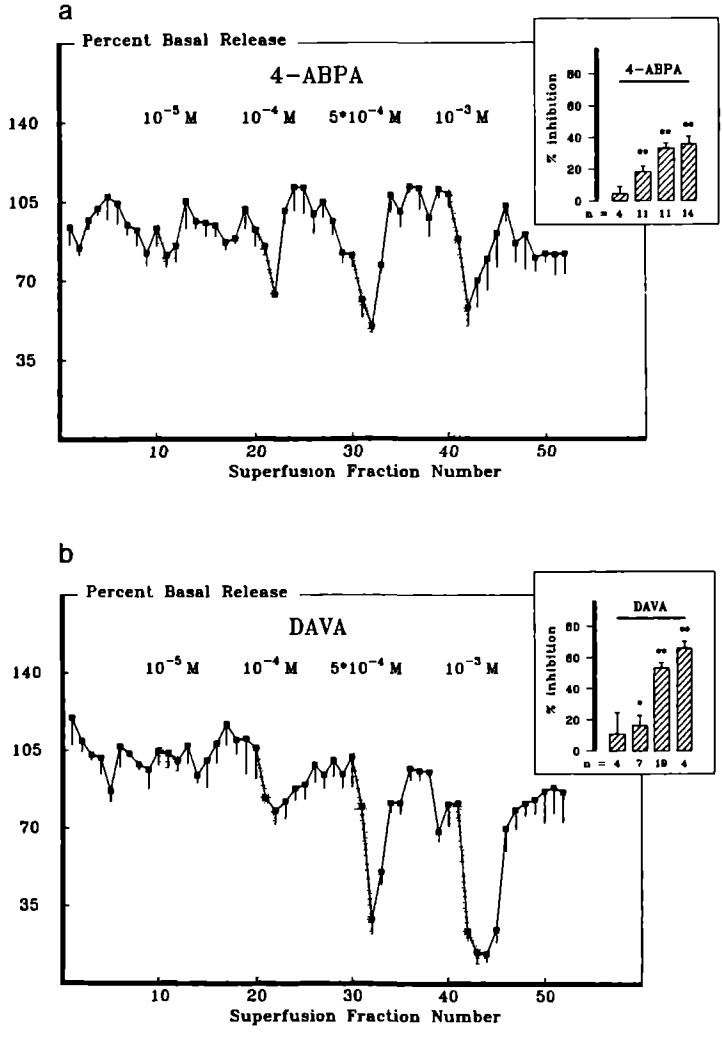


Fig. 4. *Effects of 4-ABPA (**a**) and DAVA (**b**) on α-MSH release from superfused neurointermediate lobes. Concentrations of 4-ABPA and DAVA are indicated in the figure. Insert: Percent inhibition of secretion induced by the same concentrations of 4-ABPA or DAVA, representing several experiments combined; n indicates number of experiments, * = P<0.05, ** = P<0.001.*

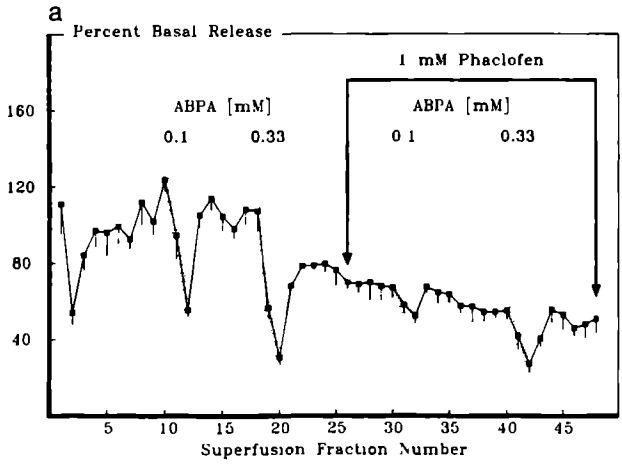
of 4-ABPA had no effect on MI, while sulpiride (10^{-7} mole/g body weight) caused a significant, but transient darkening of the skin. Coinjection of 4-ABPA and sulpiride resulted in a similar response (Fig. 8). Bicuculline (10^{-7} - 10^{-6} mole/g body weight) caused pigment aggregation in black animals. The MI significantly fell from 4.6 ± 0.1 to 3.2 ± 0.2 within 90 min.

DISCUSSION

Pharmacological profile of the $GABA_B$ receptor of melanotrope cells. To our knowledge, only one study to identify specific antagonists for $GABA_B$ receptors on endocrine cells has been published, by Shibuya *et al.* (1991). They also used melanotrope cells of *X. laevis* and found most antagonists tested, including phaclofen and 2-OH-saclofen, to be ineffective in antagonizing baclofen-induced inhibition of α-MSH release: at $3*10^{-4}$ M, 2-OH-saclofen was modestly effective and phaclofen was ineffective. The only effective antagonist was the novel ligand CGP 35-348 (Shibuya *et al.*, 1991); 4-ABPA was not studied. In contrast, we find that both phaclofen and 2-OH-saclofen, as well as 4-ABPA, selectively antagonize $GABA_B$ receptor-mediated inhibition of α-MSH secretion. While phaclofen, 2-OH-saclofen and 4-ABPA are probably less potent agonists than CGP 35-348, we show that these antagonists have similar potencies to antagonize $GABA_B$ receptor agonists as they have in the mammalian CNS (Kerr *et al.*, 1988; Kerr and Ong, 1991).

The antagonistic effects of phaclofen, 2-OH-saclofen and 4-ABPA are reversible, as can be concluded from the observation that baclofen-induced inhibition of α-MSH secretion was fully restored after treatment with the antagonist had been stopped. 2-OH-saclofen is a more potent antagonist than phaclofen, as is the case in the mammalian CNS (Kerr *et al.*, 1988; Harrison *et al.*, 1990; Kerr and Ong, 1991). Phaclofen, 2-OH-saclofen and 4-ABPA are widely accepted as selective $GABA_B$ antagonists in the CNS (for reviews see Bowery, 1989; Kerr *et al.*, 1990; Kerr and Ong, 1991). Our results suggest that these antagonists can also be used to characterize $GABA_B$ receptors in endocrine systems.

While most of our results conform with $GABA_B$ receptor pharmacology in the CNS, the $GABA_B$ receptor of *Xenopus* melanotrope cells shows some remarkable characteristics. The most obvious difference with the CNS $GABA_B$ receptor is the fact that DAVA has an agonist action. This appeared from the observation that DAVA-induced inhibition of α-MSH release was attenuated by phaclofen but not by the $GABA_A$ receptor antagonist bicuculline. While it has been reported that DAVA is a weak agonist of $GABA_A$ receptor (Bowery and Brown, 1974; Dickenson *et al.*, 1988; Bowery, 1989), it has been widely used as a $GABA_B$ receptor antagonist in the CNS and various other organs in mammals (Muhyaddin *et al.*, 1982; Ong and Kerr, 1983; Nakahiro *et al.*, 1985; Schwarz *et al.*, 1988; Hills *et al.*, 1991). The strong agonistic action of high concentrations of DAVA makes it hard to determine whether this substance has some antagonistic activity as well, but at low concentrations (10^{-4} M) no evidence for an antagonistic action was observed.


a
Percent Basal Release
1 mM Phaclofen
ABPA [mM]
0.1
0.33
ABPA [mM]
0 1
0.33
160
120
80
40
5
10
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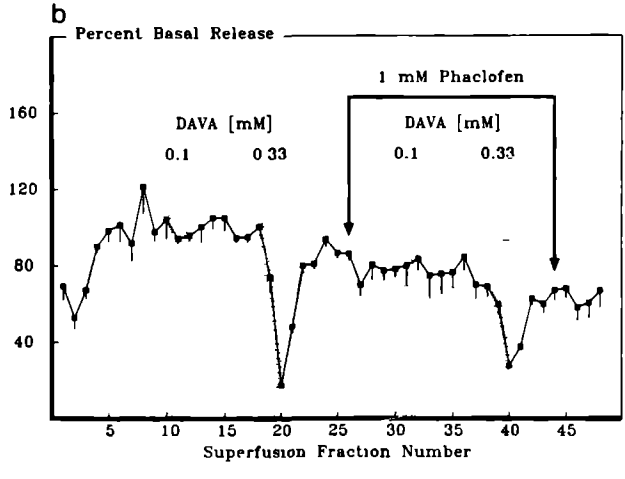
25
30
35
40
45
Superfusion Fraction Number


b
Percent Basal Release
1 mM Phaclofen
DAVA [mM]
0.1
0 33
DAVA [mM]
0.1
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Superfusion Fraction Number

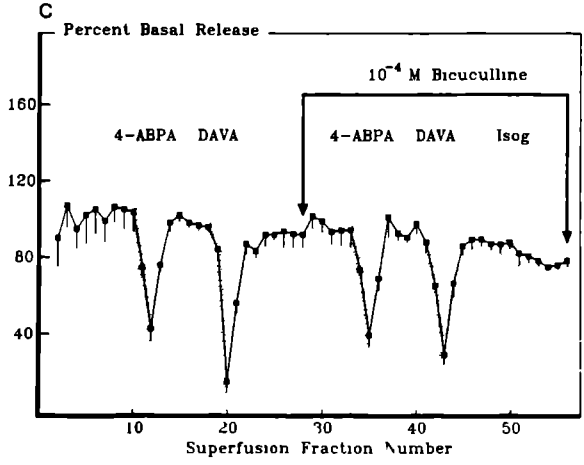
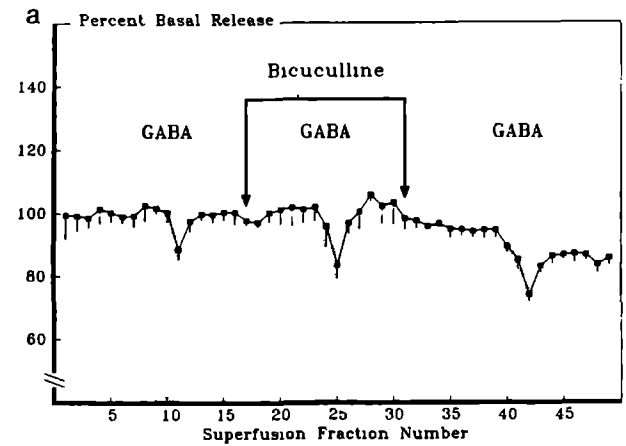


Fig 5. *Effect of GABA$_A$ and GABA$_B$ receptor antagonists on the inhibition of α-MSH release induced by 4-ABPA and DAVA* ***a,*** *effect of phaclofen on 4-ABPA response,* ***b,*** *effect of phaclofen on DAVA response,* ***c,*** *effect of bicuculline on 4-ABPA and DAVA response Concentrations are indicated in the figure Phaclofen attenuated the response to 0 1 mM 4-ABPA from 32 9 ± 6 0% inhibition to 17 2 ± 3 5% (NS) and to 0 33 mM 4-ABPA from 57 9 ± 6 2% to 37 9 ± 5 4% (P<0 05) The response to 0 33 mM DAVA was attenuated from 52 6 ± 4 4% to 36 1 ± 3 0% (P<0 05) by phaclofen In frame* ***c,*** *subsequent pulses with 4-ABPA inhibited α-MSH release by 41 1 ± 5 4% and 38 3 ± 2 2% and DAVA inhibited α-MSH secretion by 46 6 ± 5 0% and 46 8 ± 5 5% respectively*

Another difference with mammalian GABA$_B$ receptors concerns the action of 4-ABPA The slight inhibition of α-MSH release by 4-ABPA, which is attenuated by phaclofen, indicates that this antagonist, like DAVA, displays weak agonist action The observation that both 4-ABPA and DAVA show GABA$_B$ agonist activities may reflect a distinct characteristic of GABA$_B$ receptors on endocrine cells Possibly, the similar actions of 4-ABPA and DAVA, are based on their structural similarities

Differential activation of GABA receptor types by GABA. The presence of two GABA receptor types on the melanotrope cells of *X laevis*, raises two important questions (1) can these receptors be differentially activated and (2) if so, what are the functional implications? Concerning the first question, differential activation could be achieved by separate innervation of the receptors types, so that one GABAergic neuronal system could activate GABA$_A$ receptors, while another would activate GABA$_B$ receptors However, detailed immuno-electronmicroscopic studies have provided no evidence for such a separate innervation The


a
Percent Basal Release
Bicuculline
GABA
GABA
GABA
140
120
100
80
60
5
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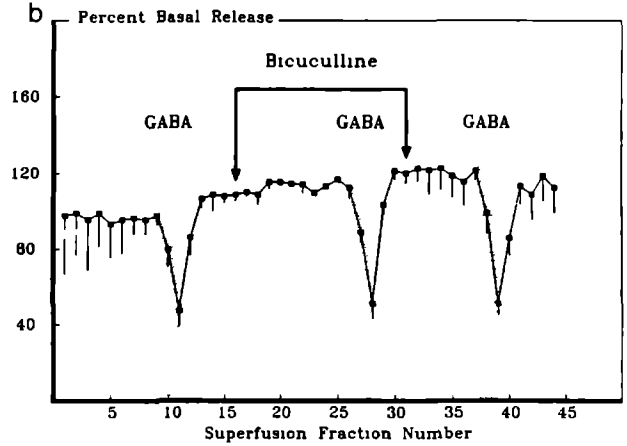
Superfusion Fraction Number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Percent Basal Release
Bicuculline
GABA
GABA
GABA
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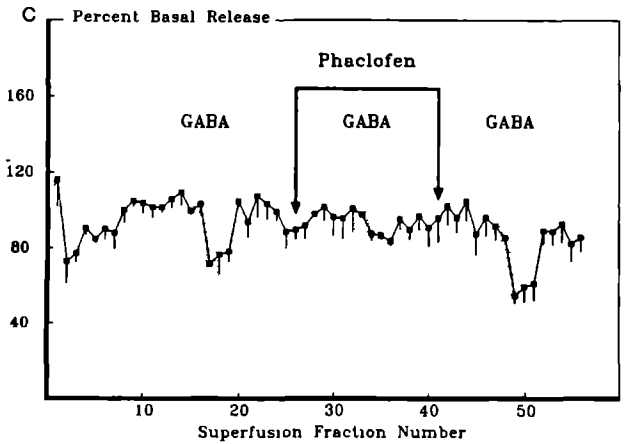
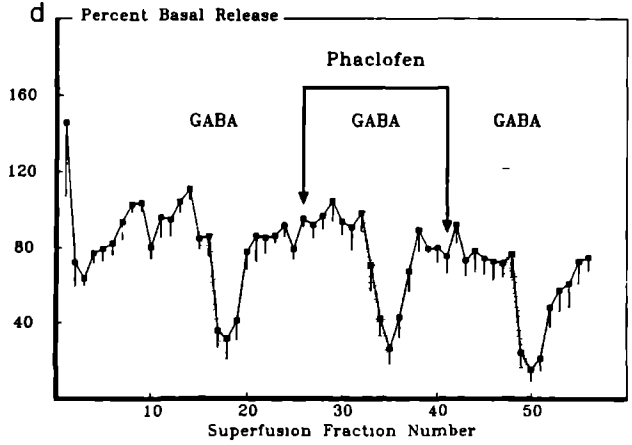
Superfusion Fraction Number


c
Percent Basal Release
Phaclofen
GABA
GABA
GABA
160
120
80
40
10
20
30
40
50
Superfusion Fraction Number


d
Percent Basal Release
Phaclofen
GABA
GABA
GABA
160
120
80
40
10
20
30
40
50
Superfusion Fraction Number

varicosities in the pars intermedia of *X. laevis* all have the same basic morphological characteristics (De Rijk *et al.*, 1990a), showing immunoreactivities to GABA, dopamine, and neuropeptide Y (De Rijk *et al.*, 1990a, 1992). Alternatively, differential activation could occur if the respective receptor mechanisms have different thresholds for activation. The present results show that the $GABA_B$ receptor mechanism has a lower threshold for activation by GABA than the $GABA_A$ receptor mechanism. This was evident from the observation that the *in vitro* response to a low dose of GABA was antagonized by $GABA_B$ receptor antagonists, but not by the $GABA_A$ receptor antagonist bicuculline. Thus, it seems feasible that at low GABA concentrations only the $GABA_B$ receptor mechanism is be activated while at high concentrations of GABA both $GABA_A$ and $GABA_B$ receptor mechanisms are activated. Apparently, at 10^{-4} M GABA, activation of either receptor type has a clear effect on α-MSH secretion. Therefore, blocking either receptor type does not significantly reduce the effect of a high concentration of GABA.

Differential activation of $GABA_A$ and $GABA_B$ receptors has previously been reported for rat gonadotropes and CNS neurons (Anderson and Mitchell, 1986b; Otis and Mody, 1992). In both cases, the $GABA_A$ receptor was more sensitive to GABA than the $GABA_B$ receptor, which is in contrast to the situation for melanotrope cells of *X. laevis*.

Differential activation of $GABA_A$ and $GABA_B$ receptor mechanisms could have important functional implications for the melanotrope cell, because the two receptors activate different intracellular mechanisms. In *Xenopus* melanotropes, $GABA_B$ receptor inhibits production of cyclic-AMP (Jenks *et al.*, 1991) whereas activation of the $GABA_A$ receptor causes influx of chloride ions. Though both receptors are involved in the inhibition of α-MSH release, the possibility that they have additional (differential) effects on other aspects of cell functioning (*e.g.* gene expression and posttranslational precursor processing) can not be ruled out.

The $GABA_B$ receptor and background adaptation. Multiple factors have been shown to regulate α-MSH secretion from *Xenopus* melanotrope cells *in vitro*, but it is not known which of these factors are controlling the secretory process *in vivo*. The melanotrope cell is spontaneously secreting and, *in vivo*, primarily controlled through tonic inhibition by factors of hypothalamic origin (Jenks *et al.*, 1988). Thus far, GABA, dopamine and NPY have been identified as potential inhibitory regulators, because they could be shown in nerve terminals in the pars intermedia (De Rijk *et al.*, 1990a, 1992) and are capable to inhibit α-MSH secretion

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*Fig. 6. The effects of bicuculline and phaclofen on GABA-induced inhibition of α-MSH secretion. **a**, Effect of 10^{-4} M bicuculline on the response to 10^{-5} M GABA; **b**, Effect of 10^{-4} M bicuculline on the response to $3.3*10^{-5}$ M GABA; **c**, Effect of 10^{-3} M phaclofen on the response to 10^{-5} M GABA; **d**, Effect of 10^{-3} M phaclofen on the response to 10^{-4} M GABA. Data are the average of four. In frames **a**, **b** and **d**, responses to GABA were not significantly attenuated in the presence of antagonist. In frame **b**, inhibition of α-MSH release by 10^{-4} M GABA was reduced from 26.4 ± 2.2% (average of pulse 1 and 3) to 5.7 ± 7.4% ($P<0.02$).*

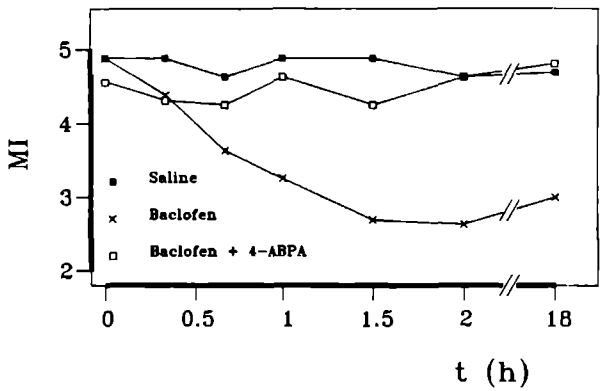

Fig. 7. The effect of 4-ABPA on baclofen-induced decrease in pigment dispersion in melanophores (MI) of black adapted Xenopus in vivo Baclofen (10^{-7} mole/g body weight) caused a clear aggregation of pigment in web melanophores This effect was significant (P<0 01) after 40 min as compared to control animals injected with saline and to animals injected with the same amount of baclofen in addition to 10^{-5} mole/g BW 4-ABPA The animals treated with baclofen and 4-ABPA displayed a lower MI than control animals throughout the experiment, but this difference was not statistically significant

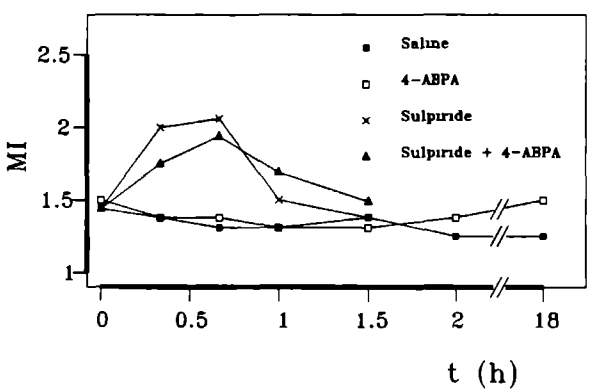

Fig. 8. The effects of receptor antagonists on pigment dispersion (MI) in melanophores of white-adapted Xenopus in vivo Concentrations were 10^{-7} mole/g body weight for sulpiride and 10^{-5} mole/g body weight for 4-ABPA The increase in MI after administration of sulpiride was significant after 20 and 40 min (P<0 05)

in vitro (Verburg-van Kemenade *et al.*, 1986c,d, 1987b). It has previously been shown that injection of the dopamine D_2 receptor antagonist sulpiride slightly increases pigment dispersion in white animals (Verburg-van Kemenade *et al.*, 1986d), indicating that a catecholamine is, in part, responsible for the tonic inhibition. We now show that 4-ABPA, which is capable of blocking baclofen-induced pigment aggregation in black-adapted animals, has no effect on pigment dispersion in melanophores of white-adapted animals. The use of 4-ABPA in our *in vivo* experiments has advantages over the use of phaclofen and 2-OH-saclofen in that 4-ABPA is highly soluble in saline, allowing high doses to be injected in relatively small volumes. The *in vivo* results indicate that the tonic inhibition of α-MSH release is not mediated by the $GABA_B$ receptor, or at least not exclusively. The tonic inhibition is also not the result of a simultaneous activation of a catecholamine and $GABA_B$ receptor, because coinjection of 4-ABPA with sulpiride was not more effective in causing pigment dispersion than sulpiride alone. *In vivo* injection experiments with the $GABA_A$ receptor antagonist bicuculline were not possible, because bicuculline reduced pigment dispersion by itself, probably reflecting its action on $GABA_A$ receptors within the CNS. From the results discussed above, it appears that the tonic inhibition of α-MSH release *in vivo* is caused by a mixture of neurochemical messengers. The exact composition of this mixture cannot be determined until suitable antagonists for each of the components are available.

In conclusion, we have characterized a $GABA_B$ receptor of *Xenopus* melanotrope cells *in vitro*, and identified several specific antagonists. One of these, 4-ABPA, is also useful in *in vivo* studies. In addition, we have shown that a different threshold of activation may lead to selective activation of a $GABA_B$ receptor mechanism in a system containing both $GABA_A$ and $GABA_B$ receptor types.

CHAPTER 4

The secretion of α-MSH from *Xenopus* melanotropes depends on calcium influx through N-type voltage-operated calcium channels

With W.J.J.M. Scheenen, B.G. Jenks, J.G.G. Borst, K.S. Kits and E.W. Roubos

Submitted for publication

Abstract. The secretory activity of endocrine cells largely depends on the concentration of free cytosolic calcium. We have studied which mechanisms are involved in supplying the calcium necessary for the secretion of α-melanophore-stimulating hormone (α-MSH) from melanotrope cells in the intermediate pituitary lobe of the amphibian *Xenopus laevis*. Using whole-cell voltage clamp, high voltage-activated calcium currents were observed, which were blocked by 0 1 mM $CdCl_2$. The currents showed a variable inactivation rate. The non-inactivating current was reminiscent of L-type voltage-dependent currents, the inactivating current resembled currents through N-type channels. No evidence was found for the presence of transient, low voltage-activated currents indicative of T-type channels. The secretion of α-MSH from superfused neurointermediate lobes was dependent on extracellular calcium, as administration of medium with low calcium concentrations (10^{-4} - 10^{-8} M) rapidly inhibited this process. Under these low calcium conditions, secretion was not affected by depolarizing concentrations of potassium chloride. The calcium ionophore A23187 increased secretion during low calcium conditions, but had no effect on spontaneous α-MSH release Treatment with $CoCl_2$, a general blocker of calcium channels, strongly inhibited the secretory process. These results suggest that spontaneous α-MSH release depends on influx of calcium through voltage-operated calcium channels Specific blockers of the L-type calcium channel, nifedipine and verapamil, had no effect on spontaneous secretion. Moreover, BAY-K8644, a specific agonist for this channel, did not affect α-MSH release under normal or low calcium conditions. The blocker of N-type voltage-operated calcium channels, ω-conotoxin, dose dependently inhibited α-MSH release. Thapsigargin, an agent that mobilises calcium from intracellular stores, had no effect on spontaneous α-MSH release under normal or low calcium conditions. It is concluded that the spontaneous release of α-MSH by melanotrope cells of *X. laevis* is maintained by calcium influx through voltage-operated N-type calcium channels and that mobilization of calcium from intracellular stores plays no major role in the regulation of this process, even when extracellular calcium is very low and, therefore, free calcium is rate-limiting.

INTRODUCTION

The amphibian melanotrope cell functions as a neuroendocrine transducer cell and is involved in the process of background adaptation. The cell integrates neuronal input into endocrine output, namely the secretion of α-melanophore-stimulating hormone (α-MSH) for the regulation of pigment dispersion in dermal melanophores (Bagnara and Hadley, 1973). The neuronal input includes GABA, dopamine and neuropeptide Y, which inhibit α-MSH secretion, and corticotropin-releasing hormone and thyrotropin-releasing hormone, which stimulate secretion (Jenks *et al.*, 1988, Tonon *et al*, 1988) These regulators activate distinct intracellular signalling pathways that ultimately converge to control secretion. The ultimate event in stimulus-secretion coupling is the regulation of the free intracellular calcium concentration. It can be predicted that regulatory factors functioning through transmembrane

signalling mechanisms directly or indirectly influence cytosolic calcium concentrations in order to regulate secretion (Rasmussen and Barrett, 1984) An understanding of the mechanisms controlling changes in cytosolic calcium levels linked to secretion will enhance our insight in the process of integration of multiple neuroendocrine inputs to secretory cells

The cytosolic free calcium concentration may be controlled by mobilization of calcium from intracellular stores, a process regulated by the second messenger inositol 1,4,5-triphosphate (Volpe *et al*, 1988, Mignery *et al*, 1989) Intracellular Ca^{2+} may furthermore be regulated by entry of extracellular calcium through calcium channels and, in excitable cells, especially through voltage-operated calcium channels (VOCC) (Hosey and Lazdunski, 1988, Scott *et al*, 1991) Free calcium levels may also be controlled by a combination of both mechanisms, each employed as circumstances require

The presence of VOCC in melanotrope cells has been investigated with electrophysiological techniques in several species (Douglas and Taraskevich, 1980, Cota, 1986, Taleb *et al*, 1986, Louiset *et al*, 1988, Taraskevich and Douglas, 1989, Keja *et al*, 1991) It appeared that these cells possess a mixed population of channels consisting of N- and L-type VOCC and, in some cases, also the presence of T-type VOCC has been shown (Taleb *et al*, 1986, Williams *et al*, 1990a, Keja *et al*, 1991) Relatively little attention has been paid to the possible involvement of such channels in the secretory process Only in mouse and frog melanotrope cells a minor role for L-type channels has been reported (Taraskevich and Douglas, 1986, Lamacz *et al*, 1988) The present study concerns a multidisciplinary study, involving electrophysiological, pharmacological and superfusion techniques, on the characteristics of VOCC and their role in the secretion of α-MSH from melanotrope cells of the amphibian *Xenopus laevis* These cells have been chosen because their basic secretory characteristics are well known and their secretory activity can be readily assessed under experimental conditions (see *e g* Ref 2) Furthermore, the possible involvement of intracellularly stored calcium ions in secretion has been examined

MATERIALS AND METHODS

Animals. Adult *Xenopus laevis* (7-10 months old) were taken from laboratory stock and kept in black containers for three weeks prior to the experiments Water temperature was 22 °C They were fed trout pellets (Trouvit, Trouw, Putten, The Netherlands) weekly

Cell cultures. Neurointermediate lobes from three animals adapted to a black background were incubated for 25 min in L15 medium (Gibco, Renfrewshire, UK) diluted 2 1 with distilled water and supplemented with 0 08 mg/ml $CaCl_2\,2H_2O$ and 0 5-1% protease (type IX, Sigma, St Louis, MO) Cells were dispersed by passing the lobes several times through a siliconized Pasteur's pipette and 8 ml of culture medium (CM) consisting of 66% L15 and 10% foetal calf serum (PAA, Austria) were added The cells were then filtered over a nylon gauze (pore size 30 μm) to remove undissociated tissue, and collected by centrifugation They were resuspended in CM and plated on glass cover slips coated with poly-L-lysine (MW 150,000 - 300,000, Sigma) in a density of 3,500-

5,000 cells per cover slip Trypan blue exclusion test showed that >95% of the cells were viable

Electrophysiological studies. To record action potentials in the whole cell configuration, non-selective pipette and bath solutions were used Medium in the pipette contained 100 mM KCl, 2 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES (pH 7 4) and 2 mM MgATP Non-selective extracellular medium contained 112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 15 mM HEPES (pH 7 4) and 10 mM glucose

Currents through calcium channels were recorded in whole-cell patch clamp configuration, at 22 °C, according to Hamill *et al.* (1981). Recordings were made from cells with a diameter of about 10 µm, which had been cultured for 0-9 days in CM The pipette solution contained 100 mM CsCl, 2 mM $CaCl_2$, 2 mM MgATP, 10 mM EGTA and 10 mM HEPES (pH 7 4, Merck, Darmstadt, Germany) The extracellular solution contained 96 mM tetraethyl ammonium chloride, 10 mM $BaCl_2$, 2 mM $MgCl_2$ and 15 mM HEPES (pH 7 4) Currents were recorded using a List EPC-7 patch clamp amplifier (Darmstadt, Germany), filtered at 3 kHz using the internal 3-pole Bessel filter and digitized at 5 kHz with a CED 1401 A/D converter (Cambridge Electronics, Cambridge, UK) Holding potential was -80 mV. The current-voltage relations (I/V) were obtained by applying test potentials of -70 to +60 mV, with steps of 10 mV, for 200 ms Interpulse interval was 10 s. Errors in the test potentials as a result of series resistance did not exceed 5 mV Measurements were started between 5 and 10 min after entering the whole cell configuration The effect of 0.1 mM $CdCl_2$ was tested by bath perfusion

In vitro superfusion. Neurointermediate lobes of *Xenopus laevis* were dissected out and placed in 10 µl superfusion chambers Four such chambers were superfused simultaneously with incubation medium (IM), consisting of 112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 15 mM HEPES (pH 7.4), 2 mg/ml glucose, 0 3 mg/ml bovine serum albumin and 1 mg/l ascorbic acid, at a rate of 1 5 ml/h, at 22 °C In order to establish a stable release, lobes were superfused for at least 75 min (10 fractions of 7 5 min) with IM before drugs (or different calcium conditions) were applied Basal (spontaneous) α-MSH release was defined as the average secretion in the three fractions preceding the first application Drugs tested were thapsigargin (Calbiochem, La Jolla, CA), A23187 (free acid, Sigma), BAY K8644 (Miles Pharmaceuticals, West Haven, USA), nifedipine (Sigma), verapamil (Laboratoires Biosedra, Malakoff, France) and ω-conotoxin GVIA (Peninsula Laboratories, Belmont CA, USA) For concentrations of drugs used and their administration protocols, see Results BAY K8644 and nifedipine were dissolved in ethanol. The ethanol concentration never exceeded 0 1% (v/v) A23187 (10-6 M) was dissolved in IM containing 0 1% dimethyl sulfoxide In experiments where the KCl and/or $CaCl_2$ concentrations in the medium were varied, the osmolarity was kept constant by adjusting the concentration of NaCl in the medium Low levels of $CaCl_2$ were buffered with EGTA

Radioimmunoassay. Radioimmunoassay for α-MSH was performed as described previously, using an antiserum raised in our laboratory (Van Zoest *et al*., 1989), which has equal affinity for the acetylated and non-acetylated forms of α-MSH Cross-reactivities with ACTH (1-24) and ACTH (1-39) were lower than 0 01% Bound and free antiserum were separated by polyethylene-glycol/ovalbumin precipitation Detection limit was 2 pg α-MSH per 50 µl sample

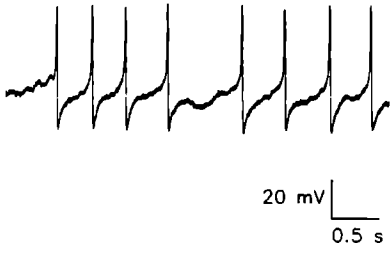

Fig. 1. Spontaneous action potentials in a whole-cell recording from a melanotrope cell. Membrane potential was approximately -35 mV.

Calculations and statistics. Results are shown as the average of four experiments ± SEM, unless stated otherwise. Percentages of stimulation and of inhibition of α-MSH-secretion in response to experimental treatment were calculated on the basis of integration of the respective peak areas in the graphs, using the fractions just before and during treatment. The integrated areas were tested for significance using the paired Student's T-test. A P value <0.05 was considered to indicate significance.

RESULTS

Patch-clamp recordings. In non-selective medium, melanotropes had membrane potentials between -50 and -30 mV. In general, they spontaneously fired action potentials, both in the cell-attached configuration (not shown) and in the whole cell configuration (Fig. 1). A series of initial whole cell voltage clamp experiments using barium-selective salines that blocked currents through sodium and potassium channels, provided evidence for the occurrence of high voltage-activated calcium channels in melanotropes. The barium current activated at potentials of -20 mV and higher. It had a peak amplitude of 465 ± 75 pA (n=9) at 0 or +10 mV. It was blocked completely by adding 0.1 mM $CdCl_2$ to the saline (n=3). The inactivation properties of the current varied strongly between cells. The extreme cases are shown in figure 2. Average inactivation at the end of a 200 msec test pulse was 31.7 ± 7.7% at +30 mV (n=9).

Effects of factors interfering with calcium influx. Application of medium with a low calcium concentration reduced the rate of α-MSH secretion from intact neurointermediate lobes. This effect was clear within 2 superfusion fractions (15 min) and reached its maximum after 3-4 fractions. In the presence of 10^{-8} M $CaCl_2$ the reduced rate of α-MSH release was not affected by a high (60 mM) concentration of KCl (Fig. 3). The calcium ionophore A23187 (10^{-6} M) had no effect on spontaneous secretion, but increased α-MSH release under low (10^{-4} M) calcium conditions (Fig. 4a,b).

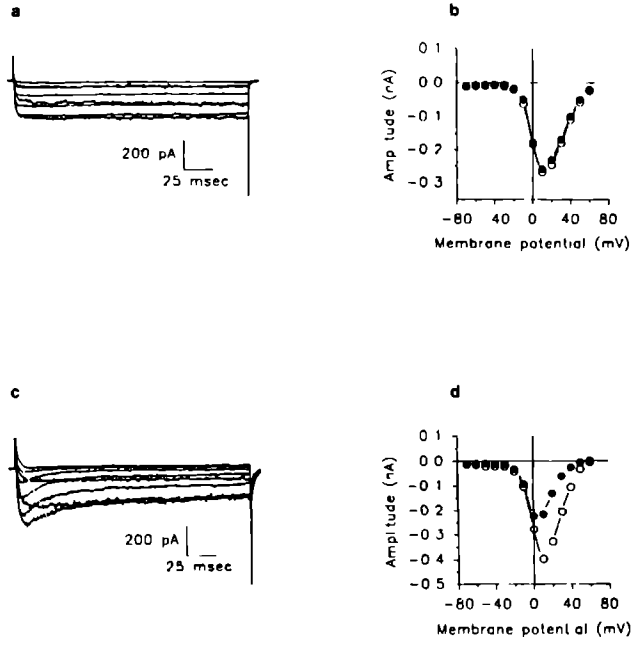

Fig. 2. *Whole-cell voltage clamp recordings of currents through calcium channels in melanotropes (**a**) Currents evoked by test pulses of 200 msec from a holding potential of -80 mV to potentials of -20 mV to +60 mV, with steps of 10 mV (**b**) Current-voltage (I/V) relation of experiment shown in (**a**) There is little inactivation of the barium currents (6% at +30 mV), since peak current (open circles) and the current at the end of the test pulse (filled circles) differ little (**c**) As (**a**), different experiment (**d**) I/V relation of experiment shown in (**c**), showing clear inactivation of the barium currents (70% at +30 mV)*

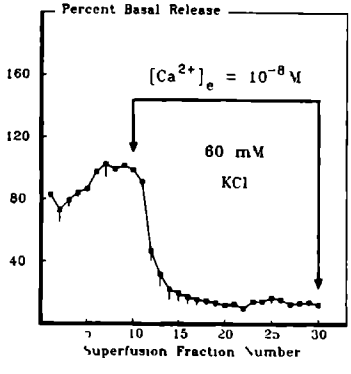

Fig. 3. *Response of superfused neurointermediate lobes to 60 mM KCl during low-calcium conditions (10^{-8} M) Start and end of treatment with low calcium medium is indicated with arrows Administration of medium containing 60 mM KCl is indicated by the shaded area. Data are the means of four lobes, superfused separately Basal release (100 %) is defined as the average release in the three fractions preceding the pulse Vertical bars represent -SEM*

Effect of drugs that affect voltage-operated calcium channels. Administration of $CoCl_2$, a general calcium channel blocker, produced a dose-dependent inhibition of α-MSH release (Fig 5) Verapamil (10^{-6} M), a specific phenylalkylamine antagonist for the L-type VOCC, and nifedipine (10^{-8} - 10^{-5} M), a potent 1,4-dihydropyridine blocker of L-type VOCC, did not change the rate of α-MSH secretion (Fig 6) The dihydropyridine agonist BAY K8644 also had no effect on α-MSH release under normal as well as under low calcium conditions, at concentrations up to 10^{-5} M (Fig 7a,b) The antagonist of N-type VOCC ω-conotoxin (10^{-7} - $5*10^{-6}$ M) induced a dose-dependent inhibition of α-MSH release (Fig 8a) The effect of conotoxin was not rapidly reversible and subsequent pulses of increasing doses of conotoxin caused a cumulative effect, reaching about 70% inhibition of secretion at $5*10^{-6}$ M After 15 min of treatment with conotoxin ($3.3*10^{-6}$ M) a stable level of secretion was established, which did not change upon adding 10^{-5} M nifedipine (Fig 8b)

The effect of thapsigargin on α-MSH secretion. Thapsigargin ($3.3*10^{-7}$ M), an agent known to mobilize free Ca^{2+} from intracellular stores by inhibiting microsomal Ca^{2+}-ATPase, had no effect on α-MSH secretion under normal and low (10^{-4} M) calcium conditions (Fig 9a)

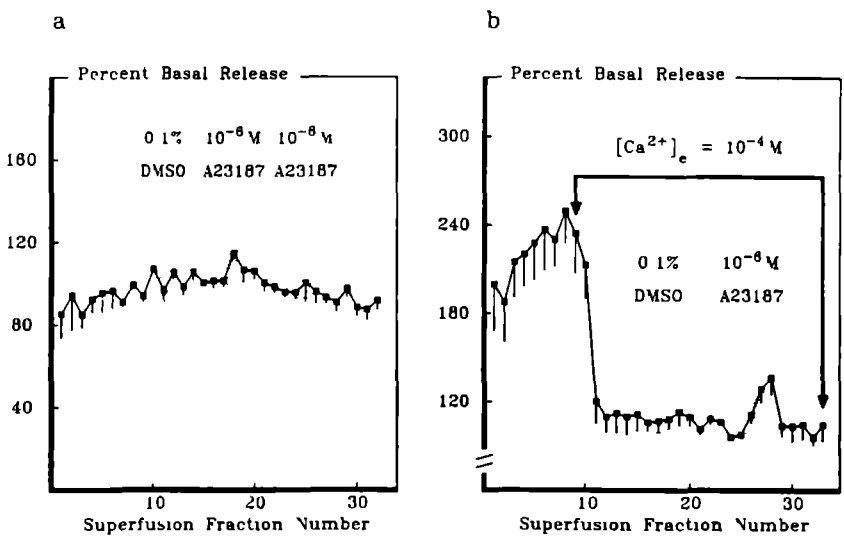

Fig. 4. The effect of A23187 on α-MSH release during (a) normal conditions and (b) low calcium conditions Data shown are the average of eight lobes The solvent of A23187, 0.1% DMSO, had no effect on secretion in either case A23187 (10^{-6} M) had no significant effect on basal release (9.6 ± 4.4 and 2.5 ± 6.5% stimulation of release respectively), but stimulated α MSH release during low calcium conditions by 19.1 ± 7.7% ($P<0.05$)

DISCUSSION

It is generally accepted that the cytosolic concentration of free calcium ions controls the secretory activity of secretory cells Regulation of free intracellular calcium levels is therefore considered the pivotal event in stimulus-secretion coupling (Rasmussen and Barrett, 1984, Scott *et al*, 1991) In the present study, we have investigated whether the mechanisms of calcium influx through VOCC and of calcium mobilization are involved in the spontaneous secretion of α-MSH from *Xenopus* melanotrope cells

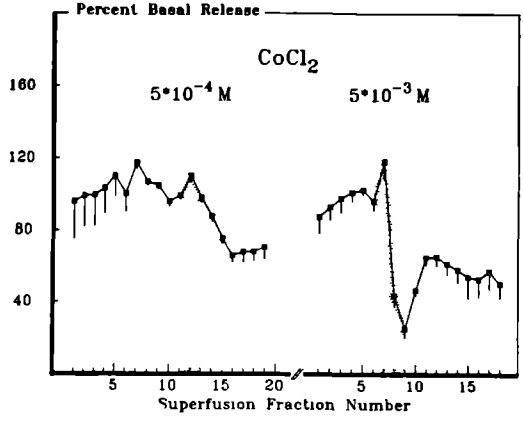

Fig. 5. The effect of cobalt chloride on α MSH release The different doses of $CoCl_2$ were applied in separate experiments, each the average of four lobes Application of $5*10^{-4}$ M $CoCl_2$ inhibited α-MSH release by 32 9 ± 5 1% after 30 min (P<0 02, fractions 8-11 compared to fr 16-19), $5*10^{-3}$ M $CoCl_2$ inhibited secretion rapidly by 45 0 ± 4 7% (P<0 01, fr 5-6 compared to fr 8-9), the secretory rate stabilizing on 64 7 ± 4 1% of basal level (P<0 01, fr 4-6 compared to fr 11-13)

A possible mechanism by which cytosolic calcium levels can be controlled is through activation of VOCC in the plasma membrane The involvement of such channels is plausible because melanotrope cells of various species display spontaneous action potentials, causing sufficient depolarizations to induce VOCC opening (Taraskevich and Douglas, 1979, 1989, Douglas and Taraskevich, 1980, Louiset *et al*, 1988) We now show that melanotrope cells of *X laevis* also display such action potentials Moreover, the presence of high voltage-activated calcium currents that might be activated by such action potentials to provide the calcium necessary for the spontaneous α-MSH release has been demonstrated The recorded currents display the characteristics associated with currents through VOCC, because they are carried by barium ions, can be blocked by Cd^{2+} and are voltage-dependent The observation that these currents display two distinct types of inactivation kinetics suggests that *Xenopus* melanotrope

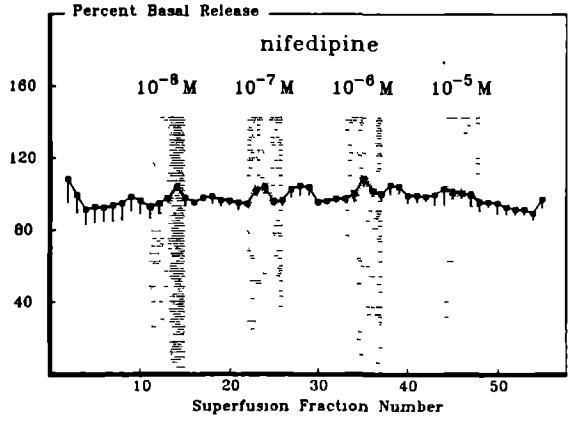

Fig. 6. Dose-response of the effect of nifedipine on α-MSH secretion. None of the nifedipine concentrations tested affected the secretory process significantly. Data are the average of four lobes.

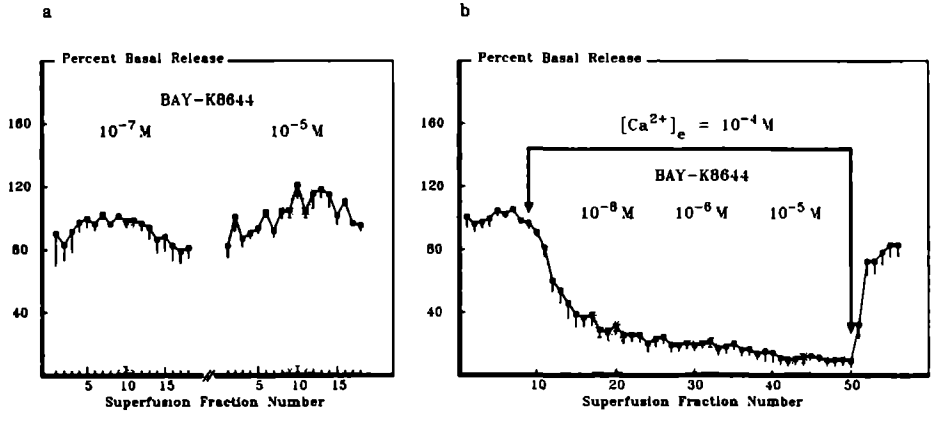

Fig. 7. The effect of BAY-K8644 on α-MSH secretion from superfused neurointermediate lobes during (a) normal and (b) low calcium conditions. BAY-K8644 had no significant effect on hormone release under either condition.

cells, like those of rat (Cota, 1986; Williams *et al.*, 1990a; Keja *et al.*, 1991), pig (Taleb *et al.*, 1986) and frog (Louiset *et al.*, 1988), possess a mixed population of VOCC. The voltage-dependence and the kinetic characteristics of the non-inactivating and inactivating currents are similar to those of L-type and N-type currents, respectively, as have been described in neurons (Tsien *et al.*, 1988; Scott *et al.*, 1991) and in melanotropes of the rat (Williams *et al.*, 1990a; Keja *et al.*, 1991) and the frog (Louiset *et al.*, 1988). It should be noted, however, that additional pharmacological data (see below) are necessary to identify these channels because the identification of high-voltage activated calcium channels solely on inactivation properties is difficult (Swandulla *et al.*, 1991). For example, in some cases N-type VOCC do not inactivate (Plummer and Hess, 1991). T-type channels, which are electrophysiologically characterized by activation at low voltage (-60 mV), and rapid inactivation (Tsien *et al.*, 1988; Scott *et al.*, 1991), have not been found in the present study.

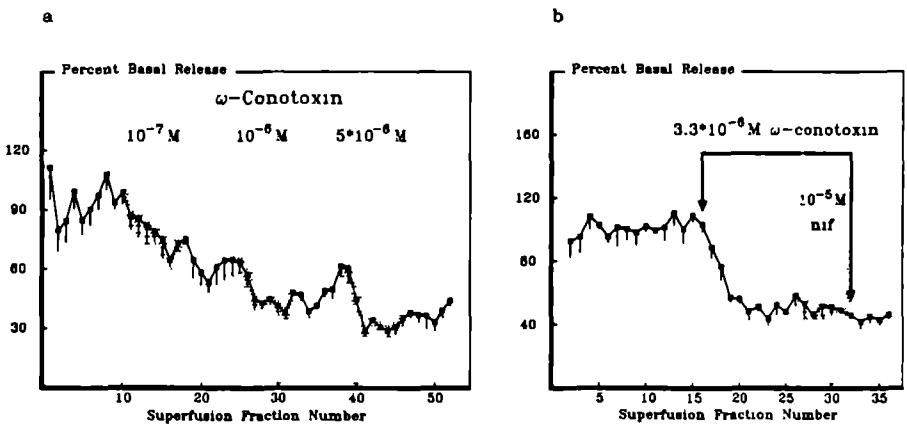

Fig. 8. *(a) Dose-response of the effect of ω-conotoxin on α-MSH release. Conotoxin progressively reduced α-MSH release to 70.8 ± 1.6% (10^{-7} M, P<0.001), 42.1 ± 3.2% (10^{-6} M, P<0.01) and 31.1 ± 2.2% ($5*10^{-6}$ M, P<0.001) of basal level, as calculated from the average secretion in the last four fractions of each conotoxin administration. (b) The effects of 10^{-5} M nifedipine on α-MSH release during conotoxin-induced inhibition of secretion. Conotoxin alone inhibited secretion to about 40% of basal secretion. Nifedipine had no additional effect on this process.*

The role of calcium in the spontaneous α-MSH secretion from melanotropes was investigated using *in vitro* superfusion techniques. The secretory process was found to depend on extracellular Ca^{2+}, as reported for melanotrope cells of mammals (Bower and Hadley, 1972; Schmitt *et al.*, 1979; Tomiko *et al.*, 1981; Tsuruta *et al.*, 1982) and lower vertebrates (Hopkins, 1970; Thornton and Geschwind, 1975; Lamacz *et al.*, 1988). The relatively rapid

inhibition of secretion as a result of lowering the external calcium concentration suggests that spontaneous α-MSH secretion is not primarily sustained by calcium ions mobilized from intracellular stores. The high spontaneous (*in vitro*) release rate of α-MSH found for melanotrope cells of many species, including *X. laevis*, most likely is the result of spontaneous membrane depolarizations in the form of action potentials mediated by inward currents of Na^+ and Ca^{2+} (Taraskevich and Douglas, 1979; Douglas and Taraskevich, 1980; Taleb *et al.*, 1986; Louiset *et al.*, 1988; Taraskevich and Douglas, 1989). In the absence of extracellular calcium, these membrane depolarizations must be ineffective in inducing α-MSH release, because administration of 60 mM KCl, which also depolarizes the membrane, does not appear to affect secretion under these circumstances. It follows that the mechanism underlying spontaneous secretion must be dependent on the influx of extracellular calcium ions.

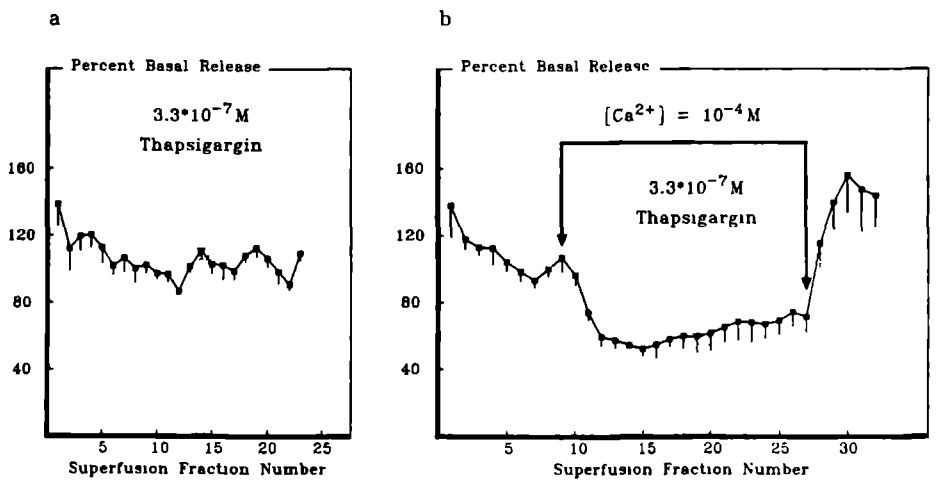

Fig. 9. *The effect of thapsigargin on α-MSH release from superfused neurointermediate lobes during (a) normal conditions and (b) low calcium conditions. Data are the average of eight in frame (a) and the average of four lobes in frame (b).*

The influx of extracellular calcium that is required to maintain a high level of α-MSH secretion by the *Xenopus* melanotrope cell is most likely mediated by VOCC. This is deduced from the experiments with $CoCl_2$: treatment with this agent caused a dose-dependent inhibition of α-MSH secretion. However, while $CoCl_2$ acts primarily on calcium channels (Hagiwara and Byerly, 1981) it may also have less specific effects on intracellular calcium-binding proteins (Thaw *et al.*, 1984) and it is not selective for a specific type of VOCC. Therefore, the effects of specific agonists and antagonists of VOCC have been examined.

The involvement of N-type channels in melanotrope secretory activity clearly appears from the studies with the blocker of N-type VOCC, ω-conotoxin, which dose-dependently inhibited

α-MSH release. There are some reports that conotoxin also acts on L-type VOCC (for reviews see Refs 7 and 8). As the following arguments will show, such action does not seem to be relevant in the present context, because L-type channels do not appear to be involved in the control of secretory activity of the melanotrope cells tested in this study.

Since blockers of the L-type VOCC, nifedipine (a 1,4-dihydropyridine) and verapamil (a phenylalkylamine), had no effect on α-MSH secretion, calcium influx through L-type channels does not seem to be essential in maintaining spontaneous release. Moreover, BAY-K8644 (a specific agonist of L-type VOCC) did not affect α-MSH release under normal and low calcium conditions. If L-type channels would be important for spontaneous release, activation of these channels with BAY-K8644 would have stimulated secretion, as reported for melanotrope cells of the frog *Rana ridibunda* (Lamacz *et al.*, 1988), the rat (Nemeth *et al.*, 1990) and the mouse (Taraskevich and Douglas, 1986, 1989). Even a minor role for this class of calcium channels could not be demonstrated, as nifedipine also had no effect during conotoxin treatment. Such an effect would have been expected if L-type channels, not blocked by conotoxin, would play a role in providing the Ca^{2+} necessary for the residual secretion found during conotoxin treatment. We conclude that, in *Xenopus* melanotrope cells, L-type VOCC play no significant role in the secretory process. This is in contrast to the situation in other species studied sofar (Taraskevich and Douglas, 1986; Lamacz *et al.*, 1988), where dihydropyridine-sensitive channels have been reported to play some role in α-MSH release (albeit that in these species dihydropyridine antagonists produce only a small reduction in basal secretion). Whether the *Xenopus* melanotrope cell also differs from other melanotropes with respect to the involvement of N-type channels in secretion, awaits conotoxin studies with the latter cells.

To investigate whether mobilization of calcium from intracellular stores is involved in α-MSH secretion, we have examined the effects of thapsigargin, an agent that is known to raise cytosolic free calcium (Thastrup *et al.*, 1990; Robinson and Burgoyne, 1991) including in *Xenopus* melanotrope cells (Scheenen *et al.*, 1992). Thapsigargin causes the release of calcium from intracellular stores by specifically inhibiting the Ca^{2+}-ATPase that pumps calcium into those stores, without affecting calcium pumps of the plasma membrane (Lytton *et al.*, 1991). Thapsigargin treatment can increase hormone secretion in cells that are, at least in part, dependent on intracellular calcium mobilizations, as shown by an analysis of luteinizing hormone secretion from chicken gonadotrope cells (Johnson and Tilly, 1991). In the present study, thapsigargin was ineffective in stimulating α-MSH secretion under normal or low calcium conditions. We conclude that mobilization of calcium from intracellular stores is not effective in stimulating α-MSH secretion in *Xenopus*, even under conditions when calcium is rate-limiting. In contrast, the calcium ionophore A23187 significantly stimulated secretion under low calcium conditions, most likely by causing an increased influx of extracellular calcium ions. The absence of a clear effect of A23187 on spontaneous release under normal calcium conditions again indicates that the intracellular calcium concentration is not rate-limiting under normal conditions.

In conclusion, the presence of high voltage-activated calcium currents, the strong inhibition of α-MSH release by low calcium conditions and by Co^{2+}, the absence of any effect of thapsigargin on secretion and the stimulation of secretion by A23187 all strongly suggest that

spontaneous α-MSH release from *Xenopus* melanotrope cells is sustained by influx of calcium through VOCC. This influx apparently occurs predominantly through N-type VOCC, as can be deduced from the absence of an effect of 1,4-dihydropyridines and the inhibition of release induced by ω-conotoxin.

CHAPTER 5

Analysis of inositol metabolism in neurointermediate lobes of *Xenopus laevis* in relation to background adaptation

With B.G. Jenks, P.M.J.M. Cruijsen, C.M.A. Mauger, E.W. Roubos, M.C. Tonon, L. Desrues and H. Vaudry.

Annals N. Y. Acad. Sci., in press

Abstract. The pituitary intermediate lobe melanotrope cells of the amphibian *Xenopus laevis* secrete α-melanophore-stimulating hormone (α-MSH), which causes dispersion of black pigment in dermal melanophores The biosynthesis and secretion of POMC-derived peptides in these cells is high in animals adapted to a black background and low in white background-adapted animals The present study concerns inositol phosphate metabolism in neurointermediate lobes of white and black-adapted animals in order to determine if the inositol phospholipid signalling system is involved in regulating melanotrope cell biosynthetic and/or secretory activity Lobes from black-adapted animals had a 2-fold higher rate of incorporation of [^{3}H]inositol into phospholipids than lobes from white-adapted animals and chromatographic analysis of the radiolabelled inositols revealed that the former lobes had up to a 4-fold higher level of phosphorylated forms of inositols Results of white to black background transfer experiments showed that the higher capacity of lobes of black-adapted animals to produce inositol phosphates was acquired only very slowly, the animals must be several days on black background before their melanotrope cells achieve the level of phosphorylation observed in lobes of long-term black-adapted animals After black- to white-background transfer the transition from the high- to the low-level of inositol phosphates also took several days to be established These slow transitions correlate well with the slow transitions known to occur with respect to the biosynthetic activity of the melanotrope cells as a result of background change They do not correlate with the fast changes that occur in melanotrope secretory activity Short-term treatment (up to 2h) with factors that inhibit α-MSH secretion, such as dopamine (acting on a D_2 receptor), baclofen (a $GABA_B$ receptor agonist) and isoguvacine (a $GABA_A$ receptor agonist), had no inhibitory effect on inositol phosphate production Therefore, there appears to be no direct linkage between the dopaminergic and GABAergic receptors and the inositol phosphate signalling system The observation that long-term treatment (3 days) with apomorphine (a dopamine D_2 receptor agonist) leads to inhibition of inositol phosphate production indicates that the action of the D_2 receptor on inositol phosphate production is slow and probably indirect We conclude that inositol phospholipid metabolism does not appear to be directly involved in the regulation of peptide release from melanotrope cells of *X laevis*, but is likely involved in the regulation of biosynthetic processes within these cells

INTRODUCTION

Melanotrope cells of the intermediate lobe of the pituitary gland synthesize proopiomelanocortin (POMC), a precursor protein for a number of bioactive peptides including α-MSH (Eipper and Mains, 1980) In amphibians this hormone regulates the dispersion of the black pigment melanin in dermal melanophores and, for the species *Xenopus laevis*, it has been shown that both the biosynthetic and secretory activity of melanotrope cells is much higher in black than in white background-adapted animals (Jenks *et al*, 1977, Loh and Gainer, 1977) The secretory activity of these cells is regulated by multiple factors (for review see

Jenks *et al*, 1988, Tonon *et al*, 1988), both classical neurotransmitters (dopamine and GABA) and neuropeptides (*e g.* neuropeptide Y and corticotropin-releasing hormone) The melanotrope cells integrate the various neuronal inputs to determine endocrine output A critical point of control in this integration is the regulation of adenylate cyclase activity Cyclic-AMP stimulates α-MSH secretion from *Xenopus* melanotrope cells (Verburg-van Kemenade *et al.*, 1987a) and production of the cyclic nucleotide has been shown to be under both stimulatory and inhibitory control (Verburg-van Kemenade *et al.*, 1987a; Jenks *et al.*, 1991). An important function for adenylate cyclase and cyclic-AMP in the regulation of α-MSH secretion has also been established for mammalian melanotrope cells (Tsuruta *et al*, 1982, Guild *et al*, 1986)

A role for phospholipid metabolism in the generation of second messengers in melanotrope cells is less well documented than that of the adenylate cyclase system Only in the case of the frog, *Rana ridibunda*, is there direct evidence for an involvement of receptor-regulated phosphoinositide turnover in the regulation of the secretory activity of melanotrope cells (Desrues *et al.*, 1990) Interestingly, in this species there have been no reports for a role of adenylate cyclase in the regulation of the secretory process To further our understanding of the physiological functioning of melanotrope cells we have now made an analysis of several aspects of inositol phosphate metabolism in melanotrope cells of *Xenopus laevis* First, we examined the rate of incorporation of radiolabelled inositol by neurointermediate lobes derived from fully black and fully white background-adapted animals (animals adapted to background for several weeks), we also determined the capacity of these lobes to phosphorylate the incorporated inositol The production of inositol phosphates was determined for lobes derived from animals undergoing the process of background adaptation in order to determine the speed with which melanotrope cells change their level of production of inositol phosphates. Finally, the effects of several regulators of α-MSH release on inositol phosphate production were determined, following both short-term (up to 2h) and long-term treatments (3 days).

MATERIALS AND METHODS

Animals. *Xenopus laevis* were bred in the aquarium facility of the Department of Animal Physiology, Nijmegen. Young-adult animals, maintained in black or white plastic containers, were used The animals were fed trout pellets (Trouvit, Trouw, Putten, The Netherlands) once a week. Water temperature was 22 °C

Analysis of inositol incorporation in lobes of white- and black-adapted animals. Neurointermediate lobe were taken from fully white- or black-background adapted animal (maintained on background for 3 weeks). The lobes were rinsed in sterile L15 medium which was made identical to commercially available L15 medium, except that no inositol was present The medium was diluted 2:1 with distilled water to obtain the appropriate ion concentrations for amphibian tissue culturing; dialysed fetal calf serum (GIBCO, Renfrewshire, UK) was added to the medium to a final concentration of 10%, antibiotic/antimycotic solution (GIBCO) and kanamycin solution (GIBCO) were added, both at a concentration of 1% (v/v), and the medium was

supplemented with $CaCl_2 2H_2O$ (0 08 mg/ml) and glucose (0 2 mg/ml) The dissected neurointermediate lobes were placed in siliconized glass vials, four lobes per vial, each vial containing 50 µl of modified L15 medium prepared as described above except that the dilution was with an aqueous [^{3}H]inositol preparation (Amersham, Buckinghamshire, UK, 2 96-4 44 Bq/mmol) The lobes were incubated at 22 °C for up to 24 hours They were then washed 5 times in Hepes-buffered Ringer's solution (pH 7 4) containing 1 mM inositol and then given a final wash in inositol-free Ringer's solution This was followed by a 10 min incubation in 500 µl Ringer's solution containing 10 mM LiCl The Li-ion blocks the conversion of inositol monophosphate to inositol, thus allowing for a build-up of phosphorylated inositols, which aids in their chromatographic analysis Following this incubation the individual lobes were homogenized by sonication (Vibra Cell, Sonics and Materials, Danbury, CT) in 1 ml of a 10% trichloroacetic acid (TCA) solution The homogenate was centrifuged (5 min at 12,000 g) and a 50 µl sample of supernatant was taken for scintillation counting to determine the total amount of radioactivity associated with inositol-labelled sugars The remainder of the supernatant was submitted to chromatographic analysis to determine the types of inositol phosphates present, as described below The lipids were extracted from the pellet with 50 µl chloroform-methanol (1 1) and the amount of radioactivity associated with the inositol-labelled lipids was determined by liquid scintillation analysis

Chromatographic analysis of inositol phosphates The analysis of the inositol phosphates was conducted by an established ion-exchange method (Batty *et al*, 1985) Each supernatant was washed 4 times with 2 volumes of water-saturated ether to remove the TCA, the pH was adjusted to 7 5 by adding $NaHCO_3$, and the sample was then loaded on a column containing 2 ml AGI-X8 resin (Biorad, Richmond, CA, 200-400 mesh, formate form) The resin was conditioned by washing 6 times with distilled water, 6 times with 0 1 M formic acid/2 M ammonium formate followed by a repeat of the wash in distilled water A fresh column was used for each sample The column was eluted step-wise with water (10 X 1 ml) to elute inositol, 0 1 M formic acid/0 2 M ammonium formate (10 X 1 ml) to elute inositol monophosphate, 0 1 M formic acid/0 45 M ammonium formate (10 X 1 ml) to elute inositol biphosphate, and finally with 0 1 M formic acid/0 8 M ammonium formate (10 X 1 ml) to elute inositol triphosphate Scintillation fluid (Optiphase 3, LKB, Loughborough, UK) was added to each column fraction and the radioactivity determined using a scintillation analyzer No radioactivity came from the columns when a final elution with 0 1 M formic acid/2 M ammonium formate was conducted and therefore this step was eliminated in our analysis

The amount of radioactive inositol or phosphorylated forms of inositol resolved by the chromatographic analysis were expressed as a percent of the total amount of radioactivity eluting from the columns

Analysis of inositol phosphate production during background adaptations. Groups of fully black-adapted animals (4 animals per group) were transferred to white background and, following 1, 2, 3 or 7 days on white background, the neurointermediate lobes were removed and incubated for 4 h in siliconized glass vials containing 50 µl modified L15 medium and [^{3}H]inositol They were then washed 5 times in Ringer's solution (pH 7 4) containing 1 mM inositol and than given a final

wash in inositol-free Ringer's solution Each lobe was then placed in an individual 1 5 ml conical plastic vial containing 250 µl Ringer's solution and at t=0 another 250 µl of Ringer's solution, containing 20 mM LiCl, was added. The lobes were incubated for 10 min, the incubation being terminated by adding 500 µl ice-cold 20% TCA solution The tissue was homogenized by sonication and the supernatant submitted to chromatographic analysis of inositol phosphates, as described above

Experiments were also conducted starting with fully-white adapted animals which were transferred to black background for 1, 2, 3 or 7 days before analyzing inositol phosphate production.

Analysis of the effect of secretagogue on inositol phosphate production in prelabelled neurointermediate lobes. Neurointermediate lobes of black-background adapted animals were labelled for 24 h in modified L15 containing [^{3}H]inositol The lobes were then washed 5 times in Ringer's solution containing 1 mM inositol and once in inositol-free Ringer's solution. Each lobe was placed in a vial containing 250 µl of Ringer's solution Treatment of the lobes started by adding either 250 µl of Ringer's solution containing 20 mM LiCl (control) or 250 µl LiCl containing Ringer's solution together with secretagogue Secretagogues tested were dopamine (Sigma, St Louis, MO), baclofen (Ciba Geigy, Basel, Switzerland), apomorphine (Sigma) and isoguvacine (Cambridge Research Biochemicals, Cambridge, UK), at concentrations given in the results The lobes were incubated for 5 min up to 2 h, at 22 °C. In some experiments the lobes were preincubated with secretagogue before LiCl treatment (see Results) Incubations were terminated by adding 500 µl of ice-cold 20% trichloroacetic acid (TCA) followed by sonification The supernatant was analyzed chromatographically for content of radiolabelled inositol phosphates

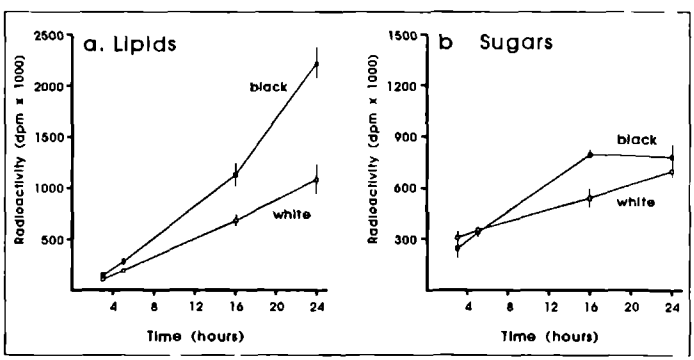

*Fig. 1. Incorporation of [^{3}H]inositol in (**a**) lipids and (**b**) sugars in neurointermediate lobes derived from black- (filled boxes) and white-adapted (open boxes) animals. Animals had been on black or white backgrounds for three weeks prior to the experiment. Data are the average of four lobes ± SEM.*

Analysis of effect of long-term secretagogue treatment on inositol phosphate production and pomc biosynthesis. Neurointermediate lobes from black background-adapted animals were cultured in modified L15 for three days in the presence or absence of apomorphine (10^{-6} M). The lobes were then washed in Ringer's solution and incubated in modified L15 containing [^{3}H]inositol for 4 h. Each lobe was then placed in a vial containing 250 µl of Ringer's solution and another 250 µl Ringer's solution containing 20 mM LiCl was added. The lobes were incubated for 10 min, the incubation being terminated by adding 500 µl ice-cold 20% TCA solution. The tissue was homogenized by sonication and the supernatant submitted to chromatographic analysis of inositol phosphates, as described previously.

Statistics. Results are presented as average and SEM, and tested for significance using Student's T-test. A value of $P<0.05$ was considered to indicate significance.

RESULTS

Incorporation of inositol and inositolphosphate production in black versus white-adapted animals. The amount of radioactive inositol incorporated into inositol-labelled lipids was higher in lobes derived from black- than from white-adapted animals (Fig. 1a). After 24 h of labelling there was approximately a 2-fold higher degree of labelling in the lipid fraction by the former tissue. There was no clear difference between black- and white-adapted animals when the total amount of radioactivity associated with inositol-labelled sugars was considered (Fig. 1b). Chromatographic analysis showed, however, that the percentage of labelled inositol in phosphorylated forms was much higher in lobes derived from black-adapted animals (Fig. 2). After 16 h of labelling, the level of inositol phosphates was more than 4-fold higher in tissue derived from black-adapted animals than in tissue from white-adapted animals. Most of this difference was due to differences in the level of inositol monophosphate; no significant difference was found in the amount of labelled inositol biphosphate and inositol triphosphate between the tissue from the black- and white-adapted animals (data not shown).

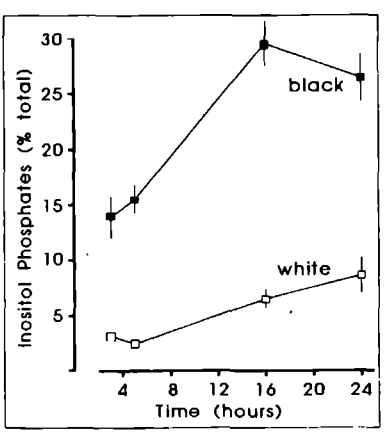

Fig. 2. Percentage of inositol phosphates in total radiolabelled inositol sugars in black- (filled boxes) and white-adapted (open boxes) animals. Labelling with [^{3}H]inositol was started at t=0. The relative amount of inositol phosphates in lobes of black-adapted animals was at all time points higher than in lobes of white-adapted animals. Data are the average of four lobes ± SEM.

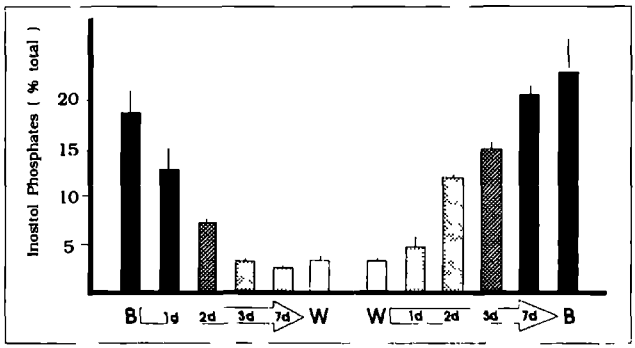

Fig. 3. Percentage of inositol phosphates in total radiolabelled inositol sugars after shift of background colour. Fully background-adapted animals were shifted from black to white background or vice versa and remained there for the indicated number of days before lobes were dissected and labelled for 4 h with [^{3}H]inositol. They were then treated for 10 min with 10 mM LiCl. B indicates three weeks black adapted; W indicates 3 weeks white adapted. Data are the average of four animals with SEM.

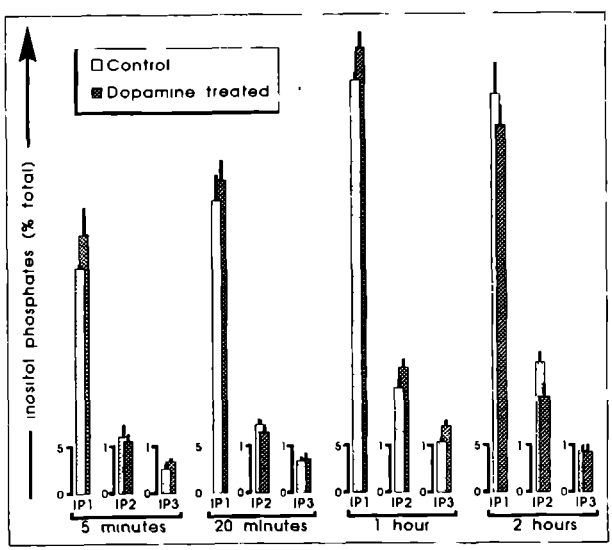

Fig. 4. Percentage of inositol phosphates in total radiolabelled inositol sugars after treatment of [^{3}H]inositol-prelabelled neurointermediate lobes with 10 mM LiCl (control) or LiCl plus dopamine for 5 min, 20 min, 1 h and 2 h. Concentration of dopamine was 10^{-5} M. Data are the average of four lobes and SEM. IP_1, IP_2 and IP_3 indicate inositolmono-, bi-, and triphosphate, respectively.

The phosphorylation of inositol during background adaptations. In fully black-adapted animals the relative level of radiolabelled inositol phosphates (expressed as a percentage of the total [^{3}H]inositol) was more than 4-fold higher than in white-adapted animals (Fig 3) The transition to the lower level of inositol phosphate seen in lobes of fully white-adapted animals took several days to be achieved following transfer of the animals from black to white background Likewise, in white to black background transfers, the transition from the level of white-adapted animals to the black-adapted level of inositol phosphate also took several days to be established (Fig 3)

Effects of α-MSH secretagogues on inositolphosphate production. Dopamine had no clear inhibitory effect and, in fact, had a tendency to stimulate production of inositol phosphates, except after 2h treatment (Fig 4) In general the action on the different forms of inositol phosphate was the same The dopamine D_2 receptor agonist apomorphine and the $GABA_B$ receptor agonist baclofen also had a tendency to stimulate inositol phosphate production (Fig 5), although only in case of 1 h treatment with baclofen was this significant ($P<0.05$, Fig 5b) The $GABA_A$ receptor agonist isoguvacine had no effect on inositol phosphate production (Fig 5a,b) Three-day treatment of neurointermediate lobes with apomorphine lead to a significant decrease in the production of inositol phosphates ($P<0.05$, Fig 5c)

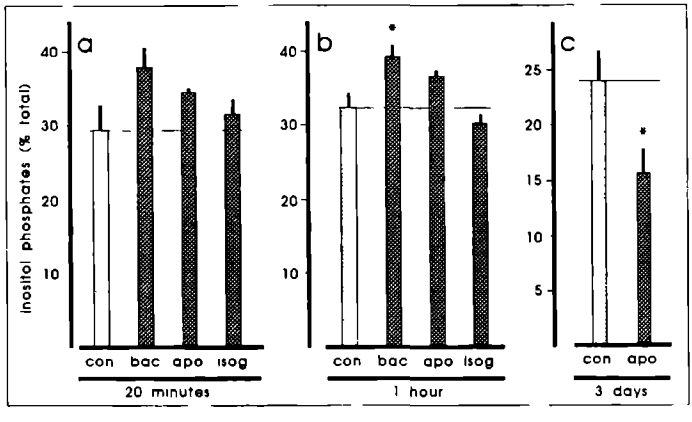

*Fig. 5. Effect of α MSH release-inhibiting agents on the percentage of inositol phosphates in total radiolabelled inositol sugars Neurointermediate lobes were prelabelled with [^{3}H]inositol, treated with secretagogues for the indicated times and then incubated for 10 min in 10 mM LiCl in the continued presence of secretagogue Data are the average of four lobes and SEM Con = control group not treated with secretagogue bac = baclofen (10^{-5} M), apo = apomorphine (10^{-6} M), isog = isoguvacine (10^{-4} M), * $P<0.05$*

DISCUSSION

To obtain a maximum specific activity of [^{3}H]inositol during radiolabelling we used an inositol-free L15 medium (commercially available L15 medium contains myo-inositol) and dialysed fetal calf serum (normal fetal calf serum also contains free inositol). Our methods were sensitive enough to measure inositol phosphate in individual neurointermediate lobes, which contain about 50,000 melanotrope cells (De Rijk *et al.*, 1990). The results show that there are clear differences between black- and white-adapted animals in both the rate of incorporation of inositol into phospholipids within the neurointermediate lobes as well as in the level of radiolabelled inositol phosphates in the cells. The higher rate of incorporation displayed by lobes of black background-adapted animals was not an unexpected result because the melanotrope cells of these animals are considerably larger than those of white-adapted animals (De Rijk *et al.*, 1990). The differences in inositol incorporation may, at least in part, simply reflect differences in plasma membrane surface area in these tissue preparations. However, the observation that the lobes of black-adapted animals had a higher percentage of [^{3}H]inositol in phosphorylated forms suggests a role for inositol phosphate metabolism in the regulation of melanotrope cell functioning in *X. laevis*.

We investigated whether the inositol phosphate metabolism of *Xenopus* melanotrope cells is involved in the regulation of biosynthesis and/or secretion of POMC-derived peptides. A comparison of the rate of changes in inositol metabolism with rates of changes in peptide secretion indicates an apparent dissociation between the inositol metabolism and the secretory process. The pars intermedia of the white-adapted animals is under tonic hypothalamic inhibition; when the lobe is removed and brought into the *in vitro* situation this inhibition is eliminated and consequently the lobe very rapidly (within minutes) displays a high rate of α-MSH secretion (Verburg-van Kemenade *et al.*, 1986a). It is apparent from the present results that changes in inositol phosphate metabolism occur much slower than these fast changes occurring in the secretory process. Even after 24 h of *in vitro* incubation, the lobes derived from white-adapted animals showed a lower percentage of inositol phosphates than that exhibited by corresponding lobes derived from black-adapted animals.

The analysis of inositol phosphate production during background adaptations gives further evidence for dissociation of inositol phosphate metabolism and the secretory process of the melanotrope cells. When black-adapted *Xenopus* are transferred to a white background the secretion of α-MSH is inhibited almost immediately, reflected by a rapid drop (within minutes) in plasma α-MSH (Van Zoest *et al.*, 1989) and an equally rapid aggregation of pigment in dermal melanophores. In sharp contrast, the present study shows that it takes several days on white background before the low level of inositol phosphorylation of long-term white-adapted animals is reached. This suggests that inositol phosphate metabolism is associated with slowly changing events in the melanotrope cells of *Xenopus*, such as the biosynthetic activity of the melanotrope cells. Indeed, the time course for the fall in the level of inositol phosphorylation during adaptation to white background is very similar to the time course for the fall in biosynthetic activity during white background adaptation established in earlier studies (Jenks *et al.*, 1977; Loh and Gainer, 1977). These same studies show that, after

transfer from a white to a black background, the biosynthetic activity of melanotrope cells increases very slowly. We now show that the capacity of these cells to produce inositol phosphates also increases only very slowly, which again suggests that inositol phosphate metabolism is associated with biosynthetic events in the melanotrope cells.

The results of our *in vitro* analysis of the effects of release-inhibiting factors on inositol phosphate production also indicates a dissociation between inositol phosphate metabolism and the release process. The factors, which included dopamine D_2 receptor agonists and GABA receptor agonists, have all been established in superfusion experiments to give rapid inhibition of α-MSH release from *Xenopus* melanotrope cells (Verburg-van Kemenade *et al.*, 1986b, 1987b). In treatments of up to 2h with these factors there was no evidence for any inhibition of inositol phosphate metabolism. Indeed, there was even a tendency for a slight increase in the amount of phosphorylated inositols produced, although in most cases this was not significant. These results contrast sharply with those obtained in similar experiments using melanotrope cells of another amphibian species, the frog *Rana ridibunda*. Dopamine treatment leads to a relatively rapid inhibition (within 20 min) in the production of inositol phosphates in *Rana* melanotrope cells (Desrues *et al.*, 1992). These differences could reflect inherent differences in mechanisms regulating the secretory process of melanotrope cells of the two amphibian species. In *Xenopus* adenylate cyclase is clearly involved whereas in *Rana* other mechanisms, including G-protein coupled ion channels (Valentijn *et al.*, 1991b), appear to be important.

We have previously shown that 3 day-treatment of *Xenopus* melanotrope cells with the D_2 receptor agonist apomorphine inhibits the biosynthesis of POMC (Ayoubi, 1991). We now show that this same tonic treatment leads to a decrease in inositol phosphate production. Together, these observation lend further support to the idea that inositol phosphate metabolism, while not being directly involved in the regulation of release, is important in the regulation of the biosynthetic activity of the melanotrope cells. The primary transmembrane signalling event activated by the D_2 receptor of *Xenopus* melanotrope cells involves an inhibition of adenylate cyclase (chapter 6, this thesis). The pathway coupling this event with the slow changes seen in both the inositol phosphate metabolism and the biosynthetic activity of the cells remains to be established.

CHAPTER 6

Dynamics of cyclic-AMP efflux in relation to α-MSH secretion from melanotrope cells of *Xenopus laevis*

With B.G. Jenks, B. Huchedé and E.W. Roubos

Life Sciences, in press

Abstract. An important factor in regulating secretion from endocrine cells is the cytoplasmic concentration of cyclic-AMP Many regulatory substances are known to either stimulate or inhibit the production of this second messenger through activation of their receptors. In the present study, we have monitored changes in cyclic-AMP efflux from melanotrope cells of *Xenopus laevis* in response to established neurochemical regulators of α-MSH secretion. *In vitro* superfusion of neurointermediate lobes allows for a dynamic recording of cyclic-AMP production in relation to hormone secretion Unlike α-MSH secretion, the efflux of cyclic-AMP was not dependent on the concentration of extracellular calcium, indicating that hormone release and cyclic-AMP efflux are mediated by different mechanisms The phosphodiesterase inhibitor IBMX and the adenylate cyclase activator forskolin stimulated cyclic-AMP efflux, but had no stimulatory effect on α-MSH release Treatment with IBMX for several hours slowly reduced the rate of hormone secretion. Therefore, we conclude that an increase in cyclic-AMP production in melanotrope cells does not increase the rate of α-MSH release but may even, in the long term, reduce secretion. Corticotropin-releasing factor stimulated cyclic-AMP efflux in a similar way as the amphibian peptide sauvagine, suggesting that the actions of these related peptides are mediated by the same receptor. Dopamine and the $GABA_B$ receptor agonist baclofen both inhibited cyclic-AMP efflux and α-MSH release, with similar dynamics of inhibition and similar dose-response relationships It is proposed that an inhibition of cyclic-AMP efflux is coupled to an inhibition of α-MSH secretion

INTRODUCTION

The secretion of α-melanocyte stimulating hormone (α-MSH) from melanotrope cells of the amphibian *Xenopus laevis* is regulated by various factors of hypothalamic origin. Corticotropin-releasing factor (CRF) (Verburg-van Kemenade *et al.*, 1987a) and thyrotropin-releasing hormone (Verburg-van Kemenade *et al.*, 1987f) stimulate α-MSH release whereas dopamine (Verburg-van Kemenade *et al*, 1986d), γ-aminobutyric acid (GABA) (Verburg-van Kemenade *et al*, 1986c, 1987d) and neuropeptide Y (Verburg-van Kemenade *et al.*, 1987b) inhibit this process. In view of the complexity of its regulatory input, the melanotrope is an interesting cell type to study the intracellular mechanisms by which multiple external stimuli are integrated to produce a coordinated secretory response Preliminary studies have indicated that cyclic-AMP plays an important role in mediating stimulatory and inhibitory actions on *Xenopus* melanotropes (Jenks *et al.*, 1991, Verburg-van Kemenade *et al*, 1987c). We have shown that these cells, like many other cell types (Barber and Butcher, 1983), release cyclic-AMP into the extracellular space (Jenks *et al*, 1991). The amount of cyclic-AMP efflux from *Xenopus* melanotrope cells seem to be proportional to the intracellular cyclic-AMP concentration (Jenks *et al.*, 1991) This relationship between intra- and extracellular cyclic-AMP appears to be a general phenomenon as it has been reported for a number of stimulated and unstimulated cell types and for brain slices (Barber and Butcher, 1981, 1983; Lazareno *et*

al, 1985, Stoof and Kebabian, 1981). It is therefore possible to use the extracellular concentration of cyclic-AMP as a parameter for cyclic-AMP production inside the cell, and hence, as a parameter for the effect of receptor activation on adenylate cyclase activity (Stoof and Kebabian, 1981) The production of second messengers is generally determined in tissue homogenates or in cell-free membrane preparations Monitoring cyclic-AMP efflux from endocrine tissues or cells allows for an analysis of the relationship between the dynamics of cyclic-AMP production and hormone release from living cells over a long period of time In the present study we have applied this approach to investigate the involvement of cyclic-AMP in the control of α-MSH release by several established regulators of the melanotrope cell To study the nature of the cyclic-AMP-dependent control mechanism(s), the effects of omission of extracellular calcium, and of treatment with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) and with forskolin, an activator of adenylate cyclase, on the cyclic-AMP-dependent control of α-MSH secretion were analyzed

MATERIALS AND METHODS

Animals. Adult *Xenopus laevis* were bred and reared in our aquarium facility. Before the experiments, they had been kept on a black background for three weeks, at 22 °C

Superfusion experiments. Superfusion of neurointermediate lobes was performed as described previously (Verburg-van Kemenade *et al*, 1987b), using 10 µl superfusion chambers The incubation medium (IM) contained 112 mM NaCl, 2 mM KCl, 15 mM HEPES (pH 7 4; Calbiochem, La Jolla, CA), 2 mM $CaCl_2$, 2 g/l glucose, 1 mg/l ascorbic acid and 0 3 g/l bovine serum albumin (Sigma, St Louis, MO). In experiments where the concentration of $CaCl_2$ was lowered to 10^{-5} M, the concentration of Ca^{2+} was buffered with EGTA. Superfusion fractions were collected every 15 min, for at least 75 min before pulses of regulatory factors were added These factors were dopamine (Sigma), baclofen (Ciba-Geigy, Basel, Switzerland), 3-isobutyl-1-methylxanthine (IBMX, Sigma), forskolin (Sigma), CRF (Bachem, Bubendorf, Switzerland) and sauvagine (Bachem) Flow rate was 1 6 ml per hour. Fractions were submitted to radioimmunoassays for α-MSH and cyclic-AMP In experiments in which IBMX was administered throughout the superfusion, lobes were incubated for 30 min prior to the superfusion with IBMX-containing IM.

Radioimmunoassays. The radioimmunoassay for α-MSH was applied as described previously, using an anti-α-MSH serum produced and characterized in our laboratory (Van Zoest *et al.*, 1989). Bound and free antibodies were separated by the polyethylene glycol/albumin method The antiserum has equal affinities for α-MSH and desacetyl α-MSH, whereas cross-reactivity with ACTH (1-24) and with ACTH (1-39) is less than 0 1%. Detection limit is 2 pg α-MSH per sample Radioimmunoassay of cyclic-AMP was carried out according to the manufacturer's instructions (RPA 509; Amersham, Buckinghamshire, England), with solutions of radioactive cyclic-AMP and of first and second antibodies diluted 4-fold, without loss of sensitivity or selectivity of the assay. Detection limit was 1 fmole per sample of 50 µl. The assay was performed using an Aspec

automatic pipettor (Gilson, Villiers le Bel, France)

Calculations and statistics. In the figures data are graphically shown as means (n=4, unless otherwise indicated) and S F M (vertical bars) To calculate percentages of stimulation or inhibition of α-MSH release in the superfusion experiments, peak areas in the graphs (before and during administration of ligands) were integrated and compared The integrated areas were tested using the paired Student's T-test A P value <0 05 was considered to indicate statistical significance

RESULTS

Extracellular calcium is not required for cyclic-AMP efflux. During superfusion with IM containing a very low concentration of $CaCl_2$ (10^5 M) the secretion of α-MSH became drastically reduced to 11 6 ± 2 9% of basal secretion At the same time, the rate of cyclic-AMP efflux was not affected (Fig 1)

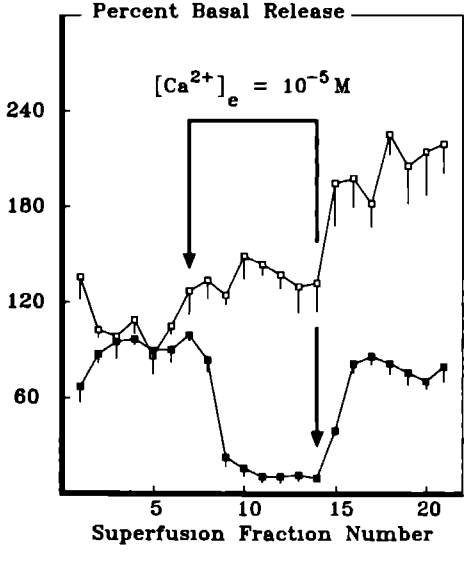

Fig. 1. The effect of low extracellular calcium concentration on α-MSH secretion (filled boxes) and cyclic-AMP efflux (open boxes) The concentration of IBMX was 10^{-4} M throughout the superfusion Basal release was the average secretion in fractions 5-7

The effect of IBMX and forskolin on α-MSH secretion and cyclic-AMP efflux. IBMX had a rapid stimulatory effect on cyclic-AMP efflux, but did not influence α-MSH secretion When neurointermediate lobes, pretreated with 10^{-4} M IBMX, were superfused in the presence of IBMX, the average cyclic-AMP content of the first 8 fractions was approximately 5 times as high (35 6 ± 6 4 fmole) as in the absence of IBMX (6 9 ± 3 0, Fig 2a) After the initial rapid stimulation of cyclic-AMP efflux during IBMX pretreatment, the cyclic-AMP efflux drifted

upward more slowly, taking several hours to reach a plateau level. Lobes not treated with IBMX displayed an almost constant rate of cyclic-AMP efflux up to 6.5 hours of superfusion (Fig. 2a). The rate of α-MSH release was also constant during this period. However, the α-MSH secretion from IBMX-treated lobes slowly declined during the experiment (Fig. 2b). Forskolin induced a strong stimulation of cyclic-AMP efflux, with a latency of 15 min. An elevated level of cyclic-AMP efflux persisted after forskolin treatment had stopped. Forskolin did not affect α-MSH secretion (Fig. 2c).

The effect of secretagogues on cyclic-AMP efflux. CRF and sauvagine stimulated cyclic-AMP efflux in a dose-dependent way but had no clear effect on α-MSH release (Fig. 3). The effect of CRF on cyclic-AMP efflux did not significantly differ from that of sauvagine. Also, the dynamics of the cyclic-AMP efflux during superfusion were similar for both peptides. The higher doses of these peptides induced a rapid and reversible increase in cyclic-AMP efflux. Near-maximum efflux was reached within the first 15 min fraction.

Dopamine and baclofen inhibited both α-MSH release and cyclic-AMP efflux, in a dose-dependent way (Fig. 4a,b). The dynamics of both inhibitory actions were very similar: an

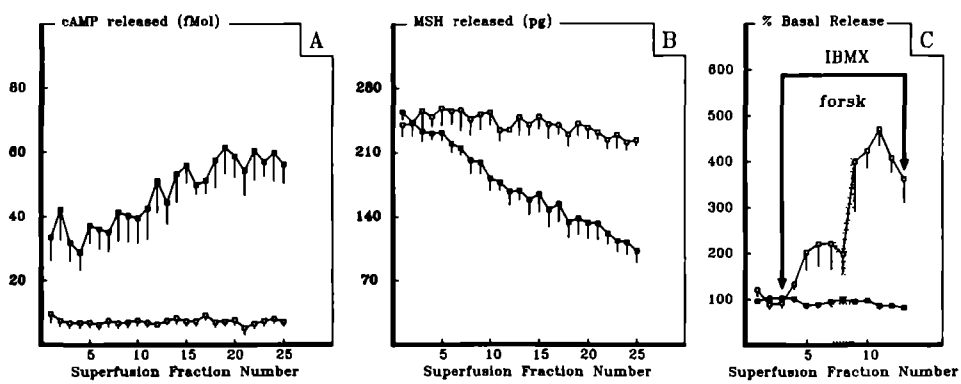

Fig. 2. The effects of IBMX and forskolin on cyclic-AMP efflux and α-MSH release. (a) The effect of IBMX on the efflux of cyclic-AMP and (b) release of α-MSH from superfused neurointermediate lobes. Four lobes were pretreated for 30 min and independently superfused in the presence of 10^{-4} M IBMX (filled boxes), while twelve lobes, distributed over four chambers, received the same treatment in the absence of IBMX (open boxes). Data are expressed as cyclic-AMP or α-MSH released per lobe. (c) The effect of 10^{-6} M forskolin on cyclic-AMP efflux (open boxes) and α-MSH release (filled boxes) in the presence of 10^{-3} M IBMX. Arrowheads indicate start and end of IBMX treatment; shading indicates forskolin (forsk) treatment. Basal release was calculated as the average release in the first three fractions.

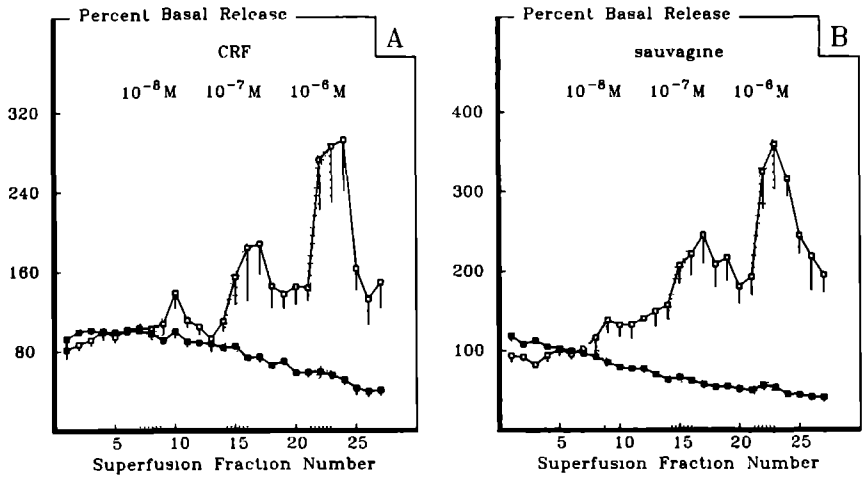

Fig. 3. The effect of CRF (a) and sauvagine (b) on cyclic-AMP efflux (open boxes) and α-MSH secretion (closed boxes). The concentration of IBMX was 10^{-4} M. Basal release was calculated from the three fractions preceding the first pulse. Shading indicates secretagogue administration.

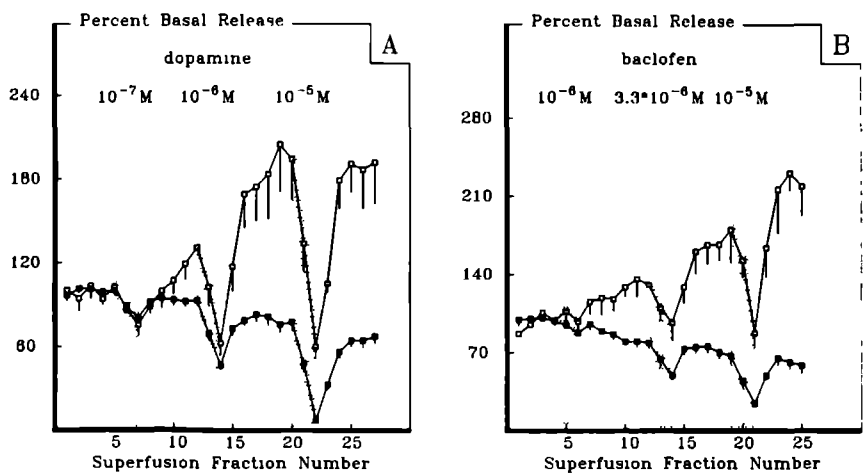

Fig. 4. The effect of dopamine (a) and baclofen (b) on cyclic-AMP efflux (open boxes) and α-MSH secretion (closed boxes). The concentration of IBMX was 10^{-4} M. Basal release was calculated from the three fractions preceding the first pulse.

almost linear decrease of release during the administration of dopamine or baclofen, returning to basal secretory rate within 30 min. During periods in which dopamine or baclofen were not present in the superfusion medium, the efflux of cyclic-AMP increased, apparently as a result of the presence of IBMX. The quantitative data on stimulation and inhibition of α-MSH release and cyclic-AMP efflux induced by the various factors are presented in Table 1.

DISCUSSION

The mechanism by which cyclic-AMP is released from cells is not well understood, though it is almost certainly an energy-dependent process (Barber and Butcher, 1983; Brunton and Heasley, 1988). Our study with low extracellular calcium indicates that cyclic-AMP release from melanotrope cells of *X. laevis* does not depend on extracellular calcium ions. The production and efflux of cyclic-AMP from melanotrope cells of the rat are also calcium-independent (Tsuruta *et al.*, 1982). Apparently, the mechanism of cyclic-AMP efflux is different from common secretory mechanisms, including that of α-MSH, which depend on the influx of calcium ions. The conclusion that the rate of cyclic-AMP efflux is not dependent on the secretory activity of the melanotrope cell but on the cytosolic concentration of cyclic-AMP is also based on the results of experiments with IBMX. This agent is commonly used to raise cyclic-AMP levels by inhibiting phosphodiesterase activity. We have studied the dynamics of IBMX action on both cyclic-AMP efflux and α-MSH release and found that the two are completely opposed: cyclic-AMP efflux increased, while α-MSH secretion decreased. An elevation of the intracellular concentration of cyclic-AMP is usually associated with an increased secretion of α-MSH from melanotropes (Tsuruta *et al.*, 1982; Verburg-van Kemenade *et al.*, 1986b, 1987c; Jenks *et al.*, 1991). It is possible that compartmentalisation of the melanotrope cell is at the basis of the decrease of α-MSH release during IBMX treatment. Treatment with IBMX would be expected to elevate cyclic-AMP levels throughout the melanotrope cell. Possibly, there are compartments within the cell where elevated cyclic-AMP levels work to the detriment of α-MSH secretion. The same explanation may hold for the results with forskolin treatment where, again, a large elevation in cyclic-AMP production was not accompanied by an increased level of α-MSH secretion.

In the present study we show that the physiological α-MSH releasing factors such as the neurohormone CRF, and the related amphibian peptide sauvagine (Verburg-van Kemenade *et al.*, 1987a), also increase cyclic-AMP efflux with only the highest doses inducing any stimulation of α-MSH release. The absence of a clear effect of CRF and sauvagine on α-MSH release in our present study is probably due to the presence of IBMX. The observation that CRF and sauvagine induced similar responses with respect to cyclic-AMP efflux, both in magnitude and dynamics, supports the notion that these peptides may act on the same receptor on the melanotropes (Verburg-van Kemenade *et al.*, 1987a).

The apparent absence of a coupling between stimulation of cyclic-AMP production and α-MSH secretion has prompted us to investigate the effect of inhibitory inputs to the melanotrope cell on cyclic-AMP efflux. Activation of the $GABA_B$ receptor or the dopamine D_2

Table 1

Effects of Secretagogues on α MSH Secretion and Cyclic AMP Efflux.

Secretagogue	Concentration [M]	MSH–secretion [% stimulation]	cAMP efflux [% stimulation]
CRF	10^{-8}	−3 9 ± 2 6 (NS)	2 0 ± 7 8 (NS)
	10^{-7}	0 4 ± 1 8 (NS)	61 3 ± 22 0 (•)
	10^{-6}	3 5 ± 1 6 (NS)	88 3 ± 17 9 (•)
sauvagine	10^{-8}	0 9 ± 1 4 (NS)	28 5 ± 18 2 (NS)
	10^{-7}	4 7 ± 2 7 (NS)	43 0 ± 11 5 (•)
	10^{-6}	10 1 ± 1 5 (••)	80 0 ± 7 6 (•••)
dopamine	10^{-6}	−15 5 ± 1 5 (•••)	−16 3 ± 6 5 (NS)
	$3\,3{*}10^{-6}$	−37 5 ± 2 5 (••••)	−33 3 ± 3 8 (•••)
	10^{-5}	−64 5 ± 2 2 (••••)	−50 6 ± 2 9 (••••)
baclofen	10^{-6}	−8 6 ± 1 4 (••)	−0 3 ± 9 3 (NS)
	$3\,3{*}10^{-6}$	−29 3 ± 3 1 (•••)	−22 2 ± 3 7 (••)
	10^{-5}	−49 9 ± 1 0 (••••)	−29 2 ± 4 9 (•)

Data shown are the average of four independent experiments Statistical significance of secretagogue effect on α-MSH secretion or on cyclic-AMP efflux is indicated (NS) not significant, (*) P<0 05, (**) P<0 02, (***) P<0 01, (****) P<0 001

receptor has been shown to inhibit cyclic-AMP production in melanotrope cells of *Xenopus laevis* (Verburg-van Kemenade *et al*, 1987c, Jenks *et al*, 1991) Our analysis of α-MSH secretion and cyclic-AMP efflux from superfused neurointermediate lobes shows that both cyclic-AMP efflux and α-MSH secretion are inhibited by the specific $GABA_B$ receptor agonist baclofen as well as by dopamine Baclofen and dopamine inhibited both processes with similar potency and dynamics, suggesting a causal relationship between the events Therefore, the inhibition of α-MSH secretion upon receptor activation could well be caused by a reduced activity of adenylate cyclase, the cyclic-AMP generating enzyme On the other hand, it may be that the G-protein that couples the D_2 and/or $GABA_B$ receptor to adenylate cyclase has additional intracellular targets that are (in part) responsible for the inhibition of hormone secretion Both receptor types have, in some cases, been reported to be coupled to plasma membrane channels for calcium or potassium ions (Robertsson and Taylor, 1986, Ogata, 1990a, Stack and Surprenant, 1991, Valentijn *et al*, 1991a)

CHAPTER 7

Evidence for different intracellular mechanisms controlled by $GABA_B$ and dopamine D_2 receptors in melanotrope cells of *Xenopus laevis*

With B.G. Jenks and E.W. Roubos

Submitted for publication

Abstract. Activation of both dopamine D_2 and $GABA_B$ receptors of melanotrope cells of *Xenopus laevis* leads to an inhibition of the release of a-melanophore-stimulating hormone (α-MSH) and a reduction of the production of cyclic-AMP This suggests that these receptors act by identical intracellular signal transduction mechanisms To test this hypothesis, the signal transduction mechanisms of both receptors were characterized in more detail The inhibitory responses to dopamine and the $GABA_B$ receptor agonist baclofen appeared to be strongly attenuated by pertussis toxin, indicating the involvement of a G-protein Inhibition of α-MSH release induced by baclofen was abolished by 8-bromo-cyclic-AMP, in contrast to inhibition by dopamine, which was only slightly attenuated by this cyclic-AMP analogue The phosphodiesterase inhibitor IBMX had no effect on the inhibitory secretory response to dopamine but markedly reduced the response to baclofen Dopamine-induced inhibition of α-MSH secretion does not seem to be primarily due to a reduction in the intracellular calcium concentration, because the calcium ionophore A23187 did not reverse this inhibition The involvement of potassium channels in the signal transduction mechanism of D_2 and $GABA_B$ receptors was investigated using tetraethyl ammonium chloride and 4-aminopyridine These agents, alone or in combination, caused a transient stimulation of α-MSH release, but had no effect on dopamine- or baclofen-induced inhibition of secretion In conclusion, activation of the $GABA_B$ receptor probably inhibits α-MSH release solely through inhibition of cyclic-AMP production, whereas dopamine-mediated inhibition appears to involve not only cyclic-AMP but also one or more other signal transduction mechanisms

INTRODUCTION

The secretory activity of the melanotrope cell in the pars intermedia of the pituitary gland of the amphibian *Xenopus laevis* is regulated by multiple inhibitory and stimulatory agents, including GABA, dopamine, neuropeptide Y, corticotropin releasing hormone and thyrotropin releasing hormone (Jenks *et al.*, 1988) We have previously shown that activation of both the dopamine D_2 and the $GABA_B$ receptors of these cells leads to inhibition of α-MSH secretion (Verburg-van Kemenade *et al*, 1986d, 1987d) as well as of cyclic-AMP production (Verburg-van Kemenade *et al*, 1987c, Jenks *et al*, 1991) This finding suggests that these receptors control melanotrope cell activity by the same (adenylate cyclase mediated) signal transduction mechanism This notion seems to be supported by the fact that the inhibitory effects of dopamine and of the $GABA_B$ agonist baclofen on α-MSH release are additive and not synergistic (Kongsamut *et al*, 1991, Jenks *et al*, 1992)

It would seem rather inefficient that two different neurotransmitters would exert identical actions on their target A possible rationale for this dual control would be that dopamine and GABA are differentially released to the melanotropes, mediating α-MSH inhibition under different states of neuronal activity Another possibility is that the dopamine and $GABA_B$ receptors effectuate their respective inhibitions of α-MSH release not only via a common adenylate cyclase system but act, moreover, via one or more additional receptor-specific signal

transduction systems. In this way, each transmitter would be able to control specifically, in addition to α-MSH release, cellular events like biosynthesis and processing of α-MSH. In the present study we have investigated this latter possibility, examining whether the dopamine D_2 and $GABA_B$ receptors act via different signal transduction systems.

It has been well established that dopamine as well as GABA (via the $GABA_B$ receptor) act via various signal transduction mechanisms, viz. by inhibiting adenylate cyclase through a G_i or G_o protein, activating potassium channels (Vallar and Meldolesi, 1989; Lledo *et al.*, 1990b; Cass and Zahniser, 1991) and inactivating calcium (Vallar and Meldolesi, 1989; Lledo *et al.*, 1990a; Keja *et al*, 1992) and sodium channels (Valentijn *et al.*, 1991a). In our *in vitro* superfusion study with neurointermediate pituitary lobes, first the involvement of G-proteins in the action of dopamine and baclofen has been determined, using pertussis toxin Then the relative importance of cyclic-AMP in dopamine- and baclofen-induced inhibition of α-MSH was investigated, using agents that increase intracellular cyclic-AMP levels, like 8-bromo-cyclic-AMP (8Br-cAMP) and IBMX. Finally, we analyzed the effects on the inhibition process of potassium channel blockers, tetraethyl ammonium chloride (TEA) and 4-aminopyridine (4-AP), and of a calcium ionophore, A23187.

MATERIALS AND METHODS

Animals. Adult *Xenopus laevis* (age 6-8 months) were bred in our aquatic facility and adapted to black background for three weeks just prior to the experiments. The toads were fed trout pellets (Trouvit, Trouw, Putten, The Netherlands) once a week. The water temperature was 22 °C

Incubation with pertussis toxin. Neurointermediate lobes were dissected out and washed four times with culture medium containing 67% (v/v) L15 medium (Gibco, Renfrewshire, UK), 1% (v/v) kanamycin solution (Gibco), 1% (v/v) antibiotic/antimycotic solution (Gibco), 10% fetal calf serum (FCS, Gibco), 0.08 mg/ml $CaCl_2.2H_2O$ and 0.2 mg/ml glucose (pH 7.4). Before use, the culture medium was sterilized by ultrafiltration using a disposable 0.22 μm filter (Type FP 030/3, Schleicher & Schuell, Dassel, Germany). Groups of four lobes were cultured in 1 ml of culture medium in a multidish (Nunc, Roskilde, Denmark) at 22 °C, in the presence or absence of 1 μg/ml of pertussis toxin (Sigma, St. Louis MO, U.S.A.) for 24 h. Before transferring the lobes to superfusion chambers, they were washed three times with incubation medium (IM) containing 112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 2 mg/ml glucose, 0.3 mg/ml bovine serum albumin (Sigma) and 15 mM HEPES (pH 7.4; Calbiochem, La Jolla, CA) .

Superfusion experiments. Neurointermediate lobes were superfused in 10 μl chambers with IM, as previously described (Verburg-van Kemenade *et al.*, 1987b). Fractions were collected every 7.5 min and at least for 75 min before pulses of potential secretagogues were introduced. Flow rate was 1.6 ml per hour. Potential secretagogues tested were dopamine (Sigma), baclofen (Ciba-Geigy, Basel, Switzerland), isoguvacine (Cambridge Research Biochemicals, Cambridge, UK), IBMX (Sigma), forskolin (Sigma), 8-bromo-cyclic-AMP (8Br-cAMP; Sigma), 4-aminopyridine (4-AP; Sigma), tetraethyl ammonium chloride (TEA; Sigma) and A23187 (free acid; Sigma).

Superfusion of cultured dispersed melanotropes. Suspensions of intermediate lobe cells were prepared as previously described (De Koning *et al.*, 1991), with minor modifications. Briefly, neurointermediate lobes were incubated with trypsin (Gibco) and subsequently dispersed mechanically. Dispersed cells were separated from undissociated tissue, including the complete neural lobe, by passing the suspension over a nylon gauze (pore size 30 µm). Cells were collected by centrifugation, washed and incubated for two days in culture medium to permit attachment to Cytodex-3 beads (Pharmacia, Uppsala, Sweden), at 22 °C. Then cells were washed in IM before transfer to superfusion chambers, equipped with Millipore filters (SSWP02500, pore size 3 µm; Millipore, Bedford MA, U.S.A.) to support the beads. Flow rate and method of administration of secretagogues were as described above.

Radioimmunoassay. α-MSH concentrations were measured as described previously, using an anti-α-MSH serum produced and characterized in our laboratory (Van Zoest *et al.*, 1989). Bound and free antibodies were separated by polyethylene glycol/albumin precipitation. The antiserum has equal affinity for α-MSH and desacetyl α-MSH, whereas cross-reactivity with ACTH (1-24) and with ACTH (1-39) is less than 0.1%. Detection limit is 2 pg α-MSH per sample of 10 ml.

Calculations and statistics. Data are expressed as percent of basal secretion and graphically shown as means (of four neurointermediate lobes, superfused separately) and S.E.M. (vertical bars). The basal level of α-MSH release was calculated as the average secretion in the three fractions preceding the first pulse To calculate percentages of stimulation or inhibition of α-MSH release in the superfusion experiments, peak areas in the graphs (before and during administration of ligands) were integrated and compared. Peak areas concerned are indicated in the legends of the graphs. The integrated areas were tested using Student's T-test. A P value <0.05 was considered to indicate statistical significance.

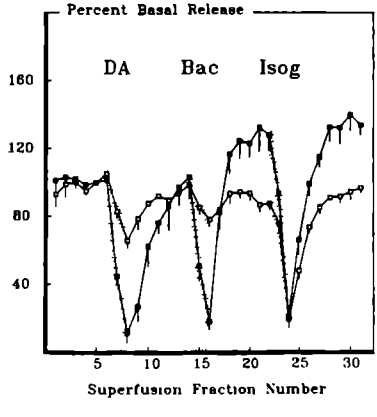

Fig. 1. The effect of pertussis toxin (PTx) pretreatment on the response of superfused neurointermediate lobes to inhibitory agents. Four lobes incubated for 24 h with 1 µg/ml of PTx (open boxes) and four control lobes (filled boxes), all superfused simultaneously in separate chambers, were challenged successively with 10^{-6} M dopamine, $3.3*10^{-6}$ M baclofen and $2*10^{-5}$ M isoguvacine. Fractions were collected every 7.5 min. The response to dopamine and to baclofen were significantly reduced in the PTx treated group, as compared to the control group (27.3 ± 4.2% versus 71.9 ± 4.9%, $P<0.001$ and 14.5 ± 3.2% versus 65.9 ± 2.9% inhibition, $P<0.001$ respectively). The response to isoguvacine was not significantly different in both groups (44.9 ± 4.4% versus 55.2 ± 2.11% inhibition).

RESULTS

The effect of pertussis toxin treatment on secretagogue-induced inhibition of α-MSH release. Dopamine, baclofen ($GABA_B$ receptor agonist) and isoguvacine ($GABA_A$ receptor agonist) induced rapid and strong inhibitions of α-MSH release from intact neurointermediate lobes. These inhibitions were rapidly reversible. The responses to dopamine and to baclofen, but not to isoguvacine, were greatly reduced by pretreatment with pertussis toxin (Fig. 1).

The effect of 8Br-cAMP on secretagogue-induced inhibition of α-MSH release. Treatment with 8Br-cAMP stimulated basal α-MSH secretion from intact neurointermediate lobes (Fig. 2a,b). A pulse of 8Br-cAMP during dopamine administration induced a small but significant attenuation of the inhibition induced by the dopamine treatment (Fig. 2a). Treatment with 8Br-cAMP during baclofen administration resulted in a clear stimulatory response, with α-MSH secretion returning to almost basal levels (Fig. 2b). The recovery of α-MSH release caused by 8Br-cAMP was significantly higher during baclofen than during dopamine treatment (78.5 ± 6.7 *versus* 33.3 ± 3.1% recovery, respectively, $P<0.01$), the response to cyclic-AMP before treatment with dopamine or baclofen was virtually identical (13.1 ± 0.9% and 13.0 ± 3.7%, respectively).

The effect of IBMX on secretagogue-induced inhibition of α-MSH release. The inhibition of the release of α-MSH from intact lobes by dopamine was not influenced by the introduction of 10^{-4} M IBMX into the medium (Fig. 3a). The inhibitory responses to $3.3*10^{-6}$ and 10^{-5} M baclofen were clearly attenuated by IBMX (Fig. 3b).

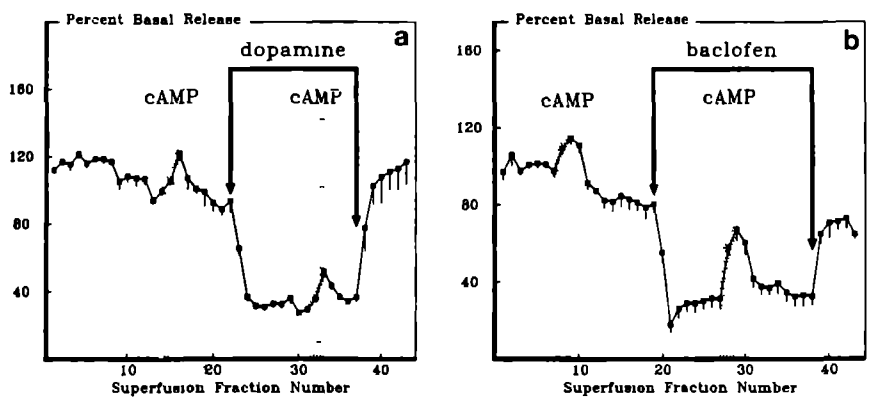

Fig. 2. The effect of 8Br-cAMP on α-MSH secretion inhibited by dopamine (a) and baclofen (b). 8Br-cAMP stimulated basal secretion from neurointermediate lobes by 13.0 ± 1.9% ($P<0.01$, n=8). Stimulation by 8Br-cAMP was 53.8 ± 4.8% ($P<0.01$, fractions 32-33 compared to fr. 30-31) during dopamine treatment and 107 ± 16% ($P<0.01$, fr. 28-29 compared to fr. 26-27).

Effect of A23187 on dopamine-induced inhibition of α-MSH release. Long-term treatment with dopamine decreased α-MSH release from intact lobes to a stable level of secretion at about 40 % of basal release. The calcium ionophore A23187 (10^{-6} or 10^{-5} M) had no effect on dopamine-induced inhibition of secretion (Fig. 4).

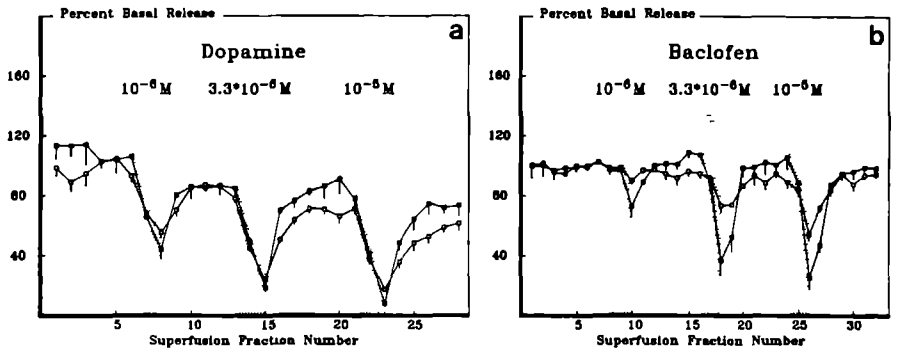

Fig. 3. The effect of IBMX on α-MSH release from neurointermediate lobes during administration of dopamine (a) and baclofen (b). Each graph represents the average of four lobes superfused in the absence (filled boxes) or presence (open boxes) of 10^{-4} M IBMX. The inhibition of α-MSH release induced by dopamine was not significantly changed by IBMX (subsequently 47.4 ± 2.9 versus 37.3 ± 4.6%, 59.7 ± 4.9 versus 57.4 ± 1.0% and 70.5 ± 2.0 versus 59.0 ± 5.7%). The responses to the higher concentrations of baclofen were significantly attenuated by IBMX (subsequently 14.9 ± 3.3 versus 6.2 ± 1.8% (NS), 41.8 ± 7.2 versus 13.7 ± 3.2% (P<0.05) and 43.2 ± 5.8 versus 25.0 ± 2.2% (P<0.05)).

Effect of potassium channel blockers. Secretion of α-MSH from isolated melanotropes during *in vitro* superfusion was inhibited by $3.3*10^{-6}$ M dopamine and by 10^{-5} M baclofen (Fig. 5a,b,c). The effect of baclofen on α-MSH release from isolated melanotrope cells was rapidly reversible, as it was on release from intact lobes. After baclofen treatment the rate of α-MSH secretion very rapidly but transiently increased above the level maintained prior to baclofen application. In contrast, dopamine-induced inhibition of α-MSH release was much prolonged after dopamine treatment had been stopped. The α-MSH release did not increase above the level before the start of the dopamine treatment (Fig. 5a,b,c). The responses evoked by baclofen and by dopamine were not affected by treatment with high doses of the potassium channel blockers 4-AP (Fig. 5a) and TEA (Fig. 5b), nor by simultaneous treatment with both blockers (Fig. 5c). However, application of each blockers resulted in a transient stimulation of α-MSH release. These stimulations attained a maximum within 7.5 min (1 fraction) and rapidly disappeared.

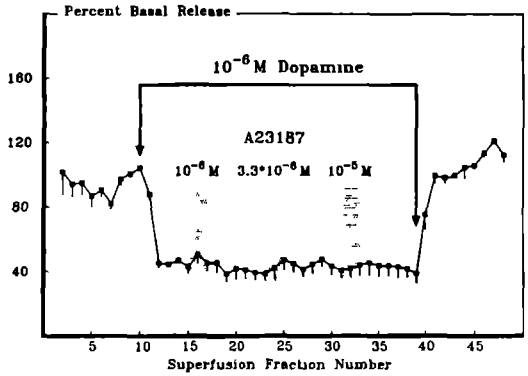

Fig. 4. The effect of A23187 on dopamine-induced inhibition of α-MSH release. A23187 was dissolved in DMSO, final concentration in the experiment not exceeding 0.1%. The calcium ionophore had no effect on α-MSH release (P>0.05).

DISCUSSION

The secretion of α-MSH from melanotrope cells of *Xenopus laevis* is inhibited by NPY (Verburg-van Kemenade *et al.*, 1987b; De Koning *et al.*, 1991), dopamine (Verburg-van Kemenade *et al.*, 1986d) and GABA (Verburg-van Kemenade *et al.*, 1986c). The action of the latter neurotransmitter is mediated by two receptors, the $GABA_A$ receptor and the $GABA_B$ receptor (Verburg-van Kemenade *et al.*, 1987d). In the present study we have investigated the possibility that the dopamine D_2 and the $GABA_B$ receptor control melanotrope cell activity via different signal transduction pathways. We have previously reported that activation of the D_2 and $GABA_B$ receptors inhibits cyclic-AMP production in melanotrope cells of *Xenopus laevis* (Verburg-van Kemenade *et al.*, 1987c; Jenks *et al.*, 1991). In addition, dopamine and baclofen inhibit the efflux of cyclic-AMP from these cells with very similar dynamics and potencies, which suggests that both messengers inhibit cyclic-AMP production via a common pathway (De Koning, unpublished observation). In accordance with these data, we now report that the responses to dopamine and baclofen, but not to the $GABA_A$ receptor agonist isoguvacine, can be reduced with pertussis toxin. Apparently, G proteins (viz. G_i or G_o) are components of the putative common mechanism.

Although both dopamine and baclofen clearly inhibit cyclic-AMP production in melanotrope cells, they do not affect α-MSH secretion in an identical way. This firstly appears from the analysis of the effects of the cyclic-AMP analogue 8Br-cAMP and of IBMX (agents that elevate the intracellular concentration of cyclic-AMP) on dopamine- and baclofen-induced inhibition of α-MSH release. Both IBMX and 8Br-cAMP appeared to antagonise the inhibitory action of baclofen but not that of dopamine. This results suggests that activation of the

$GABA_B$ receptor results exclusively in an inhibition of adenylate cyclase. In contrast, activation of the D_2 receptor must activate additional intracellular pathways, because the action of dopamine on α-MSH secretion appears, for a large part, independent of the intracellular cyclic-AMP concentration. This conclusion seems in contradiction with the traditional view (Barnes *et al.*, 1978; Stoof and Kebabian, 1981; McDonald *et al.*, 1984) that D_2 receptors inhibit secretory activity by decreasing the concentration of cyclic-AMP. It is, however, supporting evidence that more recently emerged from studies on mammalian pituitary cells that D_2 receptors also inhibit secretion in a cyclic-AMP independent fashion (Lafond *et al.*, 1986; Ray *et al.*, 1986; Boyd *et al.*, 1988).

Theoretically, the cyclic-AMP-independent inhibition of α-MSH release by dopamine may involve two mechanisms: (1) reduction of the intracellular free Ca^{2+} concentration or (2) an increase of potassium influx. These possibilities arise from electrophysiological studies on melanotrope cells of the rat and the frog, which have provided evidence that activation of D_2 receptors inhibits calcium (Williams *et al.*, 1990b; Stack and Surprenant, 1991; Valentijn *et al.*, 1991a; Keja *et al.*, 1992) conductances and increases a potassium current (Stack and Surprenant, 1991; Valentijn *et al.*, 1991a; Keja *et al.*, 1992). As to the first mechanism, our results show that it is unlikely that the dopamine-induced inhibition depends on the cytosolic concentration of Ca^{2+} because the calcium ionophore A23187 had no effect on the action of dopamine. It should be noted that treatment with A23187 is effective in raising intracellular Ca^{2+} levels in *Xenopus* melanotropes, because in a separate experiment, using low extracellular calcium conditions to inhibit secretion, A23187 was effective in stimulating secretion (De Koning, unpublished results). With respect to the second mechanism, the absence of any effect by TEA or by 4-AP on dopamine-induced inhibition indicates that activation of a potassium current is also not essential for the action of dopamine on α-MSH secretion. This absence of effect of TEA and 4-AP was not the result of ineffective drug application, because both 4-AP and TEA stimulated α-MSH secretion, apparently as a result of potassium channel inactivation and subsequent membrane depolarization. We propose that, while part of the dopamine D_2 receptor mediated inhibition of secretion is effectuated by reducing adenylate cyclase activity, also at least one other, cyclic-AMP independent and pertussis toxin-sensitive mechanism is involved in secretory inhibition. In contrast, the reduction in cyclic-AMP production is sufficient to fully account for the inhibition of secretion through the $GABA_B$ receptor. The lack of effect of 4-AP and TEA on baclofen-induced inhibition of α-MSH secretion action supports this conclusion. While $GABA_B$ receptor activation has been reported to inhibit cyclic-

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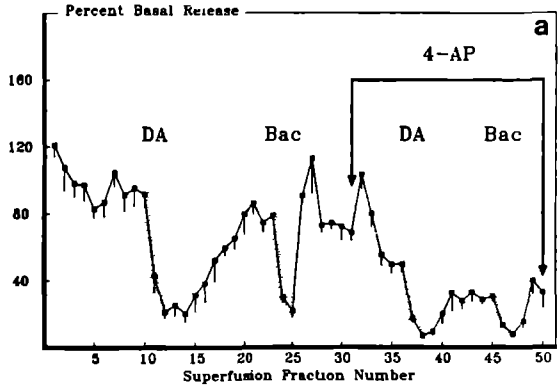
Fig. 5. The effect of 4-AP (a), TEA (b) and 4-AP + TEA (c) on baclofen- and dopamine-induced inhibition of α-MSH release from superfused melanotrope cells. Concentrations used were $3.3*10^{-6}$ M (dopamine), 10^{-5} M (baclofen), 10^{-3} M (4-AP) and $5*10^{-3}$ M (TEA). TEA and 4-AP had no significant effect on the inhibitory action of baclofen or dopamine, either alone or together. Secretion was stimulated by 51 ± 9 % by 4-AP ($P<0.02$), by 166 ± 26% by TEA ($P<0.01$) and by 347 ± 116% by the combination of the blockers ($P<0.02$).


Percent Basal Release
a
4-AP
160
120
80
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DA
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DA
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5
10
15
20
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30
35
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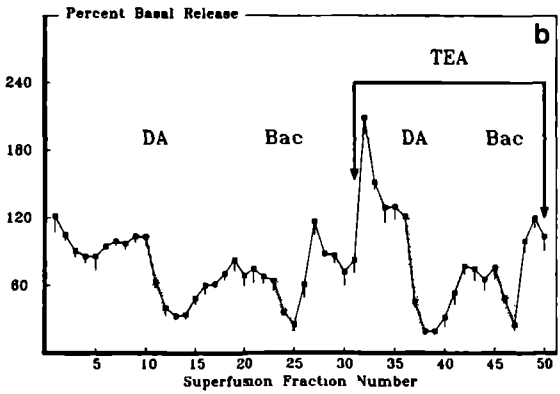
Superfusion Fraction Number


Percent Basal Release
b
TEA
240
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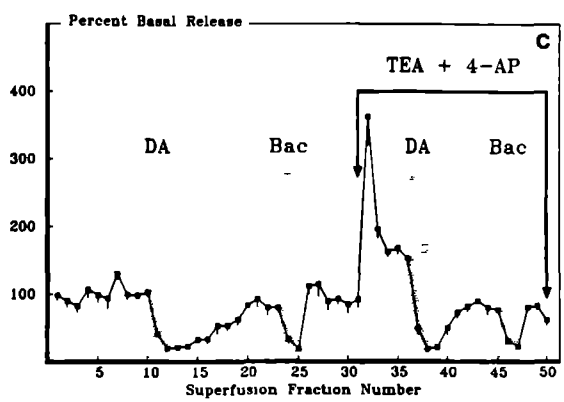
Percent Basal Release
c
TEA + 4-AP
400
300
200
100
DA
Bac
DA
Bac
5
10
15
20
25
30
35
40
45
50
Superfusion Fraction Number

AMP production in many cell types (Wojcik *et al.*, 1990) it has recently become known that this receptor often couples to 4-AP-sensitive potassium channels in neurones (Inoue *et al.*, 1985; Andrade *et al.*, 1986; Ogata, 1990a,b). In the present study this coupling has been investigated for the first time in an endocrine cell type and no evidence for an involvement of potassium channels in $GABA_B$ receptor-mediated signal transduction has been gained.

The conclusion that the D_2 receptor and the $GABA_B$ receptor control distinct intracellular pathways is also supported by the dynamics of their inhibition of α-MSH release from superfused isolated melanotrope cells. While the dynamics of the action of both agents on intact neurointermediate lobes are very similar (*e.g.* rapidly reversible), their actions on isolated cells clearly differ: in contrast to baclofen-induced inhibition, the action of dopamine is not rapidly reversible and strongly prolonged.

In conclusion, it appears that the dopamine D_2 receptor, unlike the $GABA_B$ receptor, independently activates multiple intracellular pathways, each pathway being capable to induce a drastic inhibition of α-MSH release. It is highly probable that the physiological relevance of the simultaneous presence of dopamine D_2 and $GABA_B$ receptors on melanotropes lies in the possibility for dopamine to control, in addition to α-MSH secretion, other important aspects of cellular functioning via additional signal transduction pathways.

CHAPTER 8

Dual action of $GABA_A$ receptors on the secretory process of melanotrope cells of *Xenopus laevis*

With B.G. Jenks and E.W. Roubos

Submitted for publication

Abstract. The effects of $GABA_A$ receptor activation on the secretion of α-melanophore stimulating hormone (α-MSH) from the superfused pars intermedia of the amphibian *Xenopus laevis* were examined The $GABA_A$ receptor agonist isoguvacine inhibited secretion of α-MSH from the pars intermedia, an action completely antagonized by the chloride channel blocker picrotoxin Isoguvacine stimulated secretion from picrotoxin-treated tissue This effect was blocked by the $GABA_A$ receptor antagonist bicuculline, indicating that it is a specific action via the $GABA_A$ receptor We conclude that, besides the inhibitory chloride channel, there is a stimulatory signalling property associated with the $GABA_A$ receptor Isoguvacine stimulated the production of cyclic-AMP, an action that was not blocked by picrotoxin This suggests that the stimulatory mechanism of the $GABA_A$ receptor involves, directly or indirectly, the generation of cyclic-AMP

INTRODUCTION

The $GABA_A$ receptor is the main member of the chloride channel class of receptors in the central nervous system Activation of the receptor leads to channel opening, an influx of Cl^-, and consequent hyperpolarization and inactivation of neurons Molecular studies have revealed that the $GABA_A$ receptor structure is very complex It has a multimeric structure with 5 known subunits, α, β, γ, δ and ε (for review see Burt and Kamatchi, 1991, Costa, 1991) Molecular analysis of cloned receptors indicates that different combinations of the various subunits can give rise to pharmacologically different receptor subtypes (Verdoorn *et al*, 1990) To which degree this fact is responsible for the occurrence of functionally different $GABA_A$ receptors in native effector systems remains to be established In view of the potential for multiple $GABA_A$ receptor types, functional studies of native $GABA_A$ receptor action take on additional significance Such studies may reveal unique transmembrane signalling properties reflecting receptor heterogeneity for this important class of ion channel receptor

Besides its central function, the $GABA_A$ receptor has been shown to have important neuroendocrine actions in the regulation of the secretion of pituitary hormones (Anderson and Mitchell, 1986, Virmani *et al*, 1990) The receptor is, for instance, directly involved in the regulation of α-MSH secretion from melanotrope cells of the pituitary pars intermedia, both in mammals (Tomiko *et al*, 1983, Demeneix *et al*, 1986) and amphibians (Adjeroud *et al*, 1986, Verburg-van Kemenade *et al*, 1987a, Louiset *et al*, 1990, Kongsamut *et al*, 1991) The secretion of α-MSH from superfused intermediate lobes can be easily measured and thus, by monitoring hormone secretion, the dynamics of integrated responses of this neuroendocrine transducer cell to receptor activation can be determined Therefore, the melanotrope cell is an ideal effector system to carry out functional studies of $GABA_A$ receptor action

As expected, we have found that the inhibitory action of the $GABA_A$ receptor agonist isoguvacine on α-MSH secretion from superfused neurointermediate lobes could be completely blocked by the chloride channel blocker picrotoxin More interesting was the observation that, following this treatment, isoguvacine induced stimulatory responses The results of these

functional studies are presented here, together with the results of studies to establish the receptor specificity of the stimulatory response. In addition, the effect of isoguvacine on the production of cyclic-AMP in superfused intermediate lobe tissue was analyzed to determine if the cyclic nucleotide could be involved in this stimulatory response of the melanotrope cell to $GABA_A$ receptor activation.

MATERIALS AND METHODS

Animals. Adult *Xenopus laevis* were bred in our aquatic facility and adapted to black background for three weeks prior to the experiments. The animals were fed trout pellets (Trouvit, Trouw, Putten, The Netherlands) once a week. The watertemperature was 22 °C.

Tissue superfusion methods. Freshly dissected neurointermediate lobes were rinsed in Ringer's solution, containing 112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 15 mM Hepes (pH 7.4), 0.3 mg/ml bovine serum albumin, 2 mg/ml glucose and 1 µg/ml ascorbic acid, and placed individually on a filter in a 10 µl superfusion chamber. Ringers solution was pumped through the chambers using a multichannel peristaltic pump at a rate of 1.5 ml/h and 7.5 min fractions were collected and submitted to radioimmunoassay for α-MSH and, in some experiments, also to a radioimmunoassay for cyclic-AMP. In these latter experiments IBMX (Sigma, St Louis, MO) was present in the superfusion medium at a concentration of 10^{-4} M. Factors tested for their effect on α-MSH secretion were introduced to the superfusion medium, either alone or in various combinations. These factors were: isoguvacine (Cambridge Research Biochemicals, Cambridge, UK), bicuculline (Sigma) and picrotoxin (Sigma). The concentrations of the factors used, and their administration protocols, are given in the Results.

Radioimmunoassay for α-MSH. The details of the assay have been reported earlier (Van Zoest *et al*., 1989). Bound and free label were separated using the polyethylene glycol precipitation method. The assay shows equal affinity for desacetyl-α-MSH and α-MSH and displays <0.01 % cross-reactivity with adrenocorticotropic hormone. Sensitivity of the assay was 2 pg per sample of 50 µl and each superfusion fraction was measured in duplicate.

Radioimmunoassay for cyclic-AMP. Radioimmunoassay of cyclic-AMP was carried out according to the manufacturer's instructions (RPA 509, Amersham, Buckinghamshire, UK), with solutions of radioactive cyclic-AMP and of first and second antibodies diluted 4-fold, without loss of sensitivity or selectivity of the assay. Detection limit was 1 fmole per sample of 100 µl. The assay was performed using an Aspec automatic pipettor (Gilson, Villiers le Bel, France).

Calculations and statistics. In the figures data are graphically shown as means (n=4) and S.E.M. (vertical bars) of percent basal release. The 100% basal release was defined, for each lobe, as the mean α-MSH value in the three superfusion fractions immediately preceding the first treatment with isoguvacine. In the case of the experiment with IBMX in the medium, 100% basal release of cyclic-AMP and α-MSH was defined, for each lobe, as the mean values in the three superfusion

fractions before each isoguvacine treatment. To calculate percentages of stimulation or inhibition of α-MSH release in the superfusion experiments, peak areas (before and during administration of ligands) were integrated and compared. The integrated areas were tested using Student's T-test. A P value <0.05 was considered to indicate statistical significance.

RESULTS

The effect of picrotoxin on isoguvacine-induced responses. Isoguvacine (10^{-4} M) had a strong inhibitory action on α-MSH secretion which could be completely blocked by picrotoxin (Fig. 1a). Picrotoxin itself had no clear effect on the secretion of α-MSH. However, a pulse of isoguvacine given after the picrotoxin treatment resulted in a small but significant increase in α-MSH secretion (Fig. 1a). Stimulatory responses to isoguvacine were also seen following a short pulse of picrotoxin (Fig. 1b). The effect of picrotoxin on isoguvacine responses was long lasting. The stimulatory response to isoguvacine was still evident two hours after picrotoxin treatment (second post-picrotoxin isoguvacine pulse; Fig. 1b) and, within the time of the experiment, a full inhibitory response to isoguvacine never materialized following picrotoxin treatment.

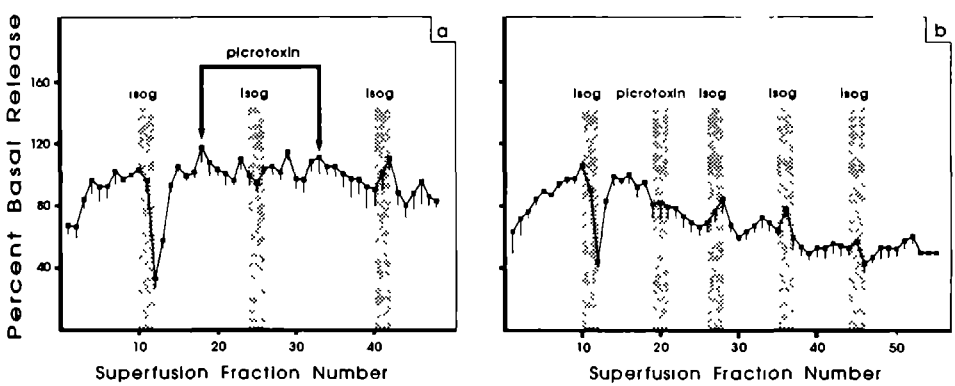

Fig. 1. The effect of picrotoxin on isoguvacine induced inhibition of α-MSH secretion form superfused neurointermediate lobes of X. laevis. The vertical hatched areas indicate those fraction where isoguvacine (10^{-4} M) was given. In (a) the isoguvacine was given during treatment with picrotoxin and in (b) the picrotoxin was given alone during 2 superfusion fractions. Picrotoxin concentration was $3.3*10^{-4}$ M. In frame (a) Isoguvacine treatment subsequently caused 36.2 ± 5.6% (P<0.02) and 5.1 ± 4.4% (NS) inhibition and 18.4 ± 5.9% (P<0.05) stimulation. In frame (b) isoguvacine initially inhibited secretion by 33.7 ± 1.9% (P<0.001). After picrotoxin treatment isoguvacine subsequently stimulated secretion by 18.8 ± 3.4% (P<0.02), 3.4 ± 5.9% and 3.0 ± 9.3%.

The effect of bicuculline on isoguvacine-induced responses. Isoguvacine (3.3×10^{-5} M) induced a strong inhibitory response which could be completely antagonized by bicuculline (Fig. 2). Because the stimulatory actions of isoguvacine following picrotoxin treatment were always small, we decided, in attempting to block the stimulation with bicuculline, to simultaneously conduct control experiments with lobes not treated with bicuculline. In this way direct comparisons could be made as to the degree of stimulation in the presence or absence of

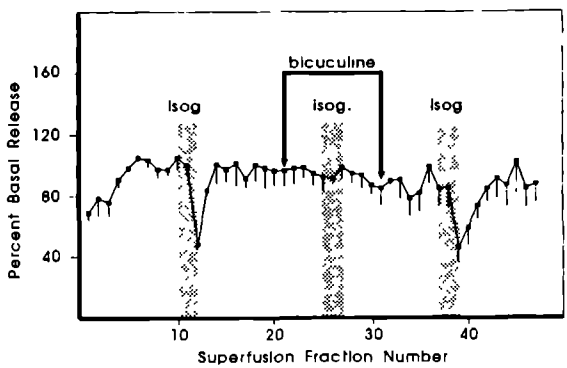

Fig. 2. The effect of bicuculline on isoguvacine induced inhibition of α-MSH secretion. Concentration of isoguvacine for each pulse was 3.3×10^{-5} M and concentration of bicuculline was 10^{-4} M.

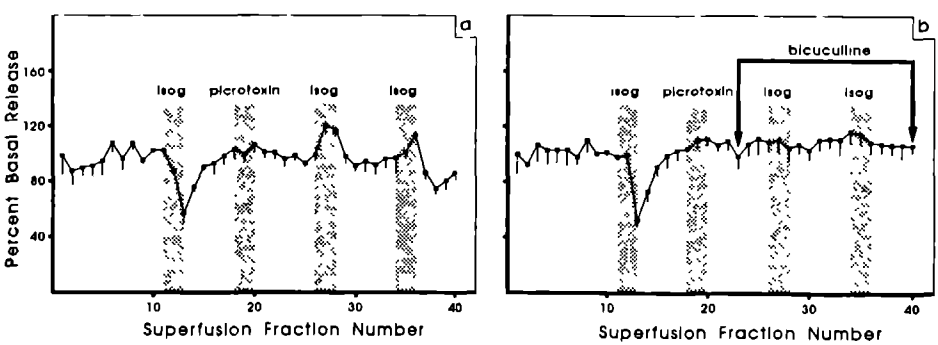

Fig. 3. The effect of (a) picrotoxin and (b) picrotoxin followed by bicuculline treatment on isoguvacine action on α-MSH secretion. The two experiments were conducted simultaneously. The concentration of isoguvacine was in each case 2×10^{-5} M, picrotoxin 10^{-4} M and bicuculline 10^{-4} M. In frame (a), the first post-picrotoxin pulse with isoguvacine caused a stimulation of 19.8 ± 6.7% ($P<0.05$). The second pulse had no significant effect on secretion (7.6 ± 6.0%). In frame (b), the post-picrotoxin pulses with isoguvacine had no effect on α-MSH secretion.

bicuculline. The control set of lobes gave an inhibitory response to the isoguvacine pulse given before picrotoxin treatment and stimulatory responses to each of the two isoguvacine pulses given after picrotoxin treatment (Fig. 3a). The first stimulatory response was significantly above basal level of secretion ($P<0.05$). These post-picrotoxin stimulatory responses were completely lacking in the bicuculline treated tissue (Fig. 3b).

The effect of isoguvacine on the production of cyclic-AMP. Isoguvacine induced a clear increase in the efflux of cyclic-AMP to the superfusion medium (Fig. 4, upper profile). The isoguvacine treatment simultaneously induced an inhibition in the secretion of α-MSH (Fig. 4, lower profile). Following picrotoxin treatment isoguvacine induced a slight but significant increase in cyclic-AMP efflux but had little or no effect on α-MSH secretion (Fig. 4, upper and lower profiles, respectively).

DISCUSSION

It is generally known that activation of the $GABA_A$ receptor induces a chloride current across the plasma membrane. The current view is that the subunits of the $GABA_A$ receptor complex together form a Cl^--channel that opens upon receptor activation. In *Xenopus* melanotrope cells, $GABA_A$ receptor activation leads to an inhibition of α-MSH release (Verburg-van Kemenade *et al.*, 1987a), most likely through chloride ion influx and, subsequently, membrane hyperpolarization. In accordance with this hypothesis, the chloride channel blocker picrotoxin antagonized the inhibition of α-MSH secretion induced by the $GABA_A$ receptor agonist isoguvacine. The stimulatory response induced by isoguvacine following picrotoxin treatment was our first indication that this receptor agonist has the potential to induce a stimulatory signalling event in *Xenopus* melanotrope cells. Apparently the effect of picrotoxin was long-lasting because it took may hours following a single pulse of the toxin before there was any indication of a return of the inhibitory action of isoguvacine. It is unclear why stimulatory responses to isoguvacine appeared stronger in tissue pretreated with the toxin rather than when the receptor agonist was given together with the toxin.

The stimulatory action of isoguvacine on the secretory process of *Xenopus* melanotropes would appear to be a specific action on the $GABA_A$ receptor. This conclusion is based on the observation that the specific $GABA_A$ receptor antagonist bicuculline blocks the isoguvacine-induced stimulatory responses. The observed stimulations do not appear to be equivalent to the very short, transient stimulations reported for $GABA_A$ receptor action on melanotropes of *Rana* (Adjeroud *et al.*, 1986; Tonon *et al.*, 1989). This latter stimulation is blocked by picrotoxin treatment and therefore it has been suggested that it could be the result of an efflux of Cl^- causing cell depolarization. In contrast, picrotoxin treatment was a prerequisite to obtain stimulatory responses from *Xenopus* melanotrope cells. We conclude that by blocking the strong inhibitory action of the $GABA_A$ receptor, through picrotoxin treatment, a stimulatory component of $GABA_A$ receptor action has come to the forefront.

The stimulations induced by isoguvacine may very well be associated with production of

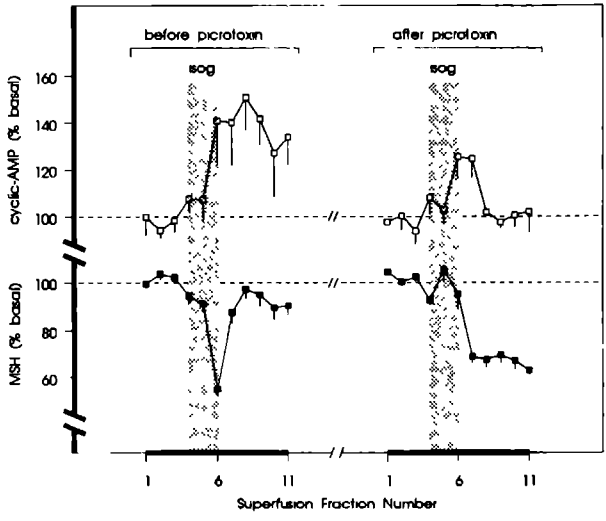

***Fig. 4.** The effect of picrotoxin on the efflux of cyclic-AMP and secretion of α-MSH from superfused Xenopus neurointermediate lobes. The concentration of isoguvacine was $2*10^{-5}$ M and picrotoxin $3.3*10^{-4}$ M. Cyclic-AMP production was stimulated by 19.2 ± 7.6% (P<0.05) and 10.8 ± 3.6% (P<0.05).*

cyclic-AMP. This cyclic nucleotide is known to stimulate α-MSH secretion from melanotrope cells of *X. laevis* (Verburg-van Kemenade *et al.*, 1986). Moreover, as was previously reported (Verburg-van Kemenade *et al.*, 1987b), cyclic-AMP production, measured using a cyclic-AMP protein binding assay, is stimulated by isoguvacine in statically incubated *Xenopus* neurointermediate lobes In the present study we measured the efflux of cyclic-AMP as an indication for the intracellular cyclic-AMP concentration. It is generally considered that this concentration is reflected by the amount of released cyclic-AMP (Barber and Butcher, 1983). Previous studies in our laboratory show that this is indeed the case for melanotrope cells of *X. laevis* (Jenks *et al.*, 1991). Recently, we have conducted an extensive analysis of the appropriateness of efflux measurements to follow the dynamics of cyclic-AMP production (Chapter 6, this thesis). Using this approach we have now confirmed that isoguvacine treatment leads to an increase in the production of the cyclic nucleotide. The observation that isoguvacine still induced cyclic-AMP production after picrotoxin treatment indicates that the stimulation of cyclic-AMP production is effectuated independently from isoguvacine action on the Cl^- ion channel domain of the receptor. The fact that the increase in cyclic-AMP seen in these experiments was not accompanied by an increase in the secretion of α-MSH probably reflects the fact that these experiments were conducted in the presence of IBMX, a phosphodiesterase inhibitor which is used to increase basal levels of cyclic-AMP thereby

permitting reliable measurements of the cyclic nucleotide. This treatment has previously been shown to render the *Xenopus* melanotrope cells insensitive to regulatory factors which stimulate secretion through activation of the adenylate cyclase system (Jenks *et al.*, 1991). This refractoriness is probably based on the fact that the intracellular cyclic-AMP levels in IBMX-treated cells is sufficiently high to fully support the secretory process.

Our studies are the first to associate $GABA_A$ receptor activation with an increased production of cyclic-AMP. The exact mechanism coupling the *Xenopus* intermediate lobe $GABA_A$ receptor to cyclic-AMP production is unknown. It is clear that the action of the receptor on cyclic-AMP production is independent of its functioning as a Cl^- channel, and therefore we consider this action to reflect an independent signalling property of the receptor. $GABA_A$ receptors have no structural or functional similarity to receptors known to be directly involved in the regulation of adenylate cyclase, the so-called G-protein coupled receptors. Therefore, it would appear highly unlikely that the effect of the *Xenopus* intermediate lobe $GABA_A$ receptor on cyclic-AMP is through a direct coupling to the adenylate cyclase enzyme complex. A full explanation for this unique property of the *Xenopus* $GABA_A$ receptor awaits the structural characterization of the receptor.

Concerning the potential physiological significance of the stimulatory component of the dual action of isoguvacine on α-MSH release, it is improbable that it would be directly involved in the control of α-MSH secretion. We observed this stimulation only when the inhibitory action of the receptor was blocked by a toxin. As no physiologically relevant mechanism to block Cl^- channel function is known, the stimulatory action on secretion would always be overshadowed by the more dominant inhibitory action of the receptor functioning as a Cl^- ion channel. Possibly, the cyclic-AMP generated through activation of the $GABA_A$ receptor has a function in the melanotrope cell that is not directly involved in regulating secretion, such as modulation of cellular functions related to long-term adaptational changes in biosynthetic or metabolic activity.

GENERAL DISCUSSION

The studies described in this thesis are concerned with the regulation of the activity of a neuroendocrine transducer cell, the melanotrope cell of the amphibian *Xenopus laevis* and with the mechanisms involved in this regulatory process Particular attention has been given to the characterization of the receptors involved in regulating the release of the main secretory product of the melanotrope, α-MSH, and of the second messenger systems that are controlled by these receptors

Previous work (reviewed by Jenks *et al*, 1988) has identified a large number of receptors on *Xenopus* melanotrope cells Activation of these receptors causes an inhibition (dopamine D_2 receptor, $GABA_A$ receptor, $GABA_B$ receptor and receptor for NPY) or a stimulation (receptors for CRH, sauvagine and TRH) of α-MSH secretion While the presence of these receptors in the pars intermedia was evident, there has been much debate as to the question of whether α-MSH receptors on the melanotrope cell exist (Kastin *et al*, 1971, Iturriza, 1973, Huntington and Hadley, 1974) These receptors would be involved in autofeedback of α-MSH release In the experiments described in Chapter 1, specific radioimmunoassays for α-MSH and β-endorphin (like α-MSH derived from POMC) as well as analysis of radiolabelled peptides have been carried out, to quantify the secretory response of melanotrope cells to α-MSH It is proven that a short-loop autofeedback system for α-MSH does not exist, though it remains possible that secretory products of melanotrope cells control their secretion through more complex feedback loops

Most of the endogenous regulators of α-MSH secretion are known to act directly on the melanotrope cell (Verburg-van Kemenade *et al*, 1986a, 1987a) Such action could not be demonstrated for NPY (Chapter 2) It was shown that NPY inhibits α-MSH release from cultured neurointermediate lobes, which do not contain presynaptic nerve terminals Apparently, NPY does not act in a presynaptic way on melanotrope cells The involvement of the folliculo-stellate cell of the pars intermedia in the indirect action of NPY on α-MSH secretion was therefore highly probable Indeed, receptors for NPY are present on *Xenopus* folliculo-stellate cells (De Rijk *et al*, 1991) The present study provided the first evidence that folliculo-stellate cells of the pars intermedia are involved in the regulation of an endocrine secretory process

Chapter 3 addresses the question of the importance of the two GABA receptors in regulating α-MSH secretion For this purpose, a $GABA_B$ receptor antagonist, acting both *in vivo* and *in vitro*, was required Because no $GABA_B$ receptors of endocrine cells have been pharmacologically characterized, a survey way has been made of the effects of $GABA_B$ receptor antagonists used in non-endocrine systems, like the mammalian nervous system It appears that the pharmacological profile of the $GABA_B$ receptor of *Xenopus* melanotrope cells is rather similar to that of most $GABA_B$ receptors, though some differences are obvious From the use of the newly characterized antagonists, it is clear that, *in vitro*, the $GABA_B$ receptor is more sensitive to stimulation with GABA than the $GABA_A$ receptor Using the $GABA_B$ receptor antagonist *in vivo*, it appears that the $GABA_B$ receptor is either not involved or not essential in the tonic inhibition of α-MSH release in animals adapted to a white background Most likely, a mixture of inhibitory agents is involved in the *in vivo* process of melanotrope

cell-controlled background adaptation.

The analysis of the signal transduction mechanisms controlled by the receptors that regulate α-MSH secretion constitutes a large part of this thesis. In Chapters 4 and 5, the calcium/IP_3 pathway was examined. It was found that the secretory process depends on the influx of extracellular calcium ions through voltage-operated calcium channels. From the observation that these channels are activated at high voltage (patch-clamp) and from their pharmacological profile (release studies) it is concluded that these channels are N-type voltage-operated calcium channels. In contrast to studies using frog and mouse melanotrope cells (Taraskevich and Douglas, 1986; Lamacz *et al.*, 1988), no evidence has been obtained for a role of the dihydropyridine-sensitive L-type calcium channel in the regulation of secretion. It is concluded that the exclusive involvement of N-type channels in the regulation of secretory activity of *Xenopus* melanotropes is a species specific phenomenon. Obviously, the *Xenopus* melanotrope may serve as a suitable model to study the significance of N-type channels for the control of endocrine secretory processes.

The mobilization of Ca^{2+} from intracellular stores does not seem to play an important role in the regulation of α-MSH secretion, since thapsigargin, an agent known to mobilize calcium ions from intracellular stores, does not affect secretion. This notion is supported by the observation that established regulators of α-MSH secretion have no clear effect on IP_3 production. This second messenger is an endogenous mobilizer of intracellular calcium (*e.g.* Berridge, 1987). The IP_3/Ca^{2+} mechanism in the melanotrope cell may be involved in long-term changes in cellular activity occurring as a result of background adaptation (*e.g.* biosynthesis of secretory products). This is evident from the following data: (1) The IP_3 mechanism is much more active in black-adapted animals than in white-adapted animals. (2) After a shift in background colour, black animals retained the high level of inositol phosphates for several days, like white animals retained their low level of inositol phosphates. (3) The slow change in inositol metabolism coincides with the slow changes in biosynthesis of POMC mRNA (Ayoubi, 1991).

Apart from calcium and IP_3, the most important second messenger involved in the regulation of melanotrope cell activity is cyclic-AMP. Early studies had established that cyclic-AMP production in *Xenopus* melanotropes is inhibited by dopamine and the $GABA_B$ receptor agonist baclofen and stimulated by sauvagine (Verburg-van Kemenade *et al.*, 1987c; Jenks *et al.*, 1991). In these studies, static incubations were applied and, in part, a rather insensitive cyclic-AMP-binding assay. In Chapter 6, a new method for the simultaneous and dynamic recording of α-MSH release and cyclic-AMP production is described. The method is based on the assumption that efflux of cyclic-AMP from melanotrope cells is proportional to the intracellular concentration of the second messenger. This assumption is valid for cells in general (Barber and Butcher, 1981, 1983) and also for *Xenopus* melanotrope cells (Jenks *et al.*, 1991). It is therefore possible to measure cyclic-AMP and α-MSH in the same superfusion fractions (using radioimmunoassays) and to study the dynamics of cyclic-AMP efflux in relation to α-MSH secretion. One of the most interesting findings is that an increase in cyclic-AMP production is not necessarily coupled to an increase in α-MSH release. Probably, cyclic-AMP plays an important role in secretion, but the stimulatory action of high cyclic-AMP

levels can be overruled by changes in the plasma membrane potential or the concentration of free cytosolic Ca^{2+}. Most likely, cyclic-AMP controls other cellular processes besides the secretory activity. In this way, cyclic-AMP may have a coordinating function with respect to different aspects of the complicated changes in cellular activity during background adaptation.

Dopamine and baclofen inhibit both α-MSH release and cyclic-AMP efflux. Both processes are inhibited with the same dynamics, suggesting a direct coupling between receptor and adenylate cyclase. However, adenylate cyclase may not be the only effector coupled to dopamine or $GABA_B$ receptors of melanotrope cells. In Chapter 7, it is shown that the inhibition of release induced by baclofen, but not by dopamine, can be reversed by agents that increase the intracellular concentration of cyclic-AMP. This strongly suggests that the $GABA_B$ receptor is coupled to adenylate cyclase only, while the D_2 receptor is coupled to at least one other mechanism capable of fully inhibiting α-MSH release in a cyclic-AMP-independent way. Electrophysiological studies have shown that dopamine receptors of melanotrope cells may activate potassium channels and inactivate calcium channels in the plasma membrane (Williams *et al*., 1990b, Stack and Surprenant, 1991, Valentijn *et al*., 1991a,b, Keja *et al*., 1992). However, the calcium ionophore A23187 and blockers of potassium channels do not affect dopamine-induced inhibition of α-MSH secretion. It has to be concluded that the D_2 receptor directly controls multiple signal transduction mechanisms through a pertussis toxin-sensitive G-protein. Each of these mechanisms is probably sufficient to completely inhibit α-MSH secretion, but may have additional intracellular effects unrelated to secretion.

In Chapter 8, the signal transduction mechanisms of the $GABA_A$ receptor have been examined. It is shown that the inhibitory action of this receptor is, as expected, mediated by an influx of chloride ions. The inhibition of α-MSH release induced by the $GABA_A$ receptor agonist isoguvacine is completely antagonized by the chloride channel blocker picrotoxin. In fact, isoguvacine stimulated secretion after picrotoxin, indicating the presence of a second signal transduction mechanism controlled by the $GABA_A$ receptor, which is independent of the chloride channel. This second mechanism is somehow coupled to the cyclic-AMP pathway, as isoguvacine stimulates the efflux of cyclic-AMP from the cells, both before and after treatment with picrotoxin. This study is the first to associate $GABA_A$ receptor activation with a stimulation of cyclic-AMP production, but more study is required to establish by what mechanism the $GABA_A$ receptor interferes with the cyclic-AMP concentration.

It thus appears that all the inhibitory receptors have their own unique (set of) signalling mechanisms: the NPY receptor acts on the folliculo-stellate cells to inhibit α-MSH secretion, the $GABA_B$ receptor inhibits adenylate cyclase, the dopamine receptor also inhibits cyclic-AMP production, but has additional signalling mechanisms (probably couples to ion channels), and activation of the $GABA_A$ receptor induces a chloride influx and a stimulation of cyclic-AMP production. It is therefore probable that the physiological rationale of the multiple inhibitory receptors of *Xenopus* melanotrope cells is that each receptor has, besides its effects on secretion, differential actions on additional cellular processes. This possibility is currently under investigation in our department, and particular attention is being paid to differential effects on processes related to background adaptation, like biosynthesis and processing of POMC, acetylation of POMC-derived peptides and the composition of the secretory signal. A

model of the intracellular mechanisms controlled by the various receptors of *Xenopus* melanotrope cells is provided in figure 1

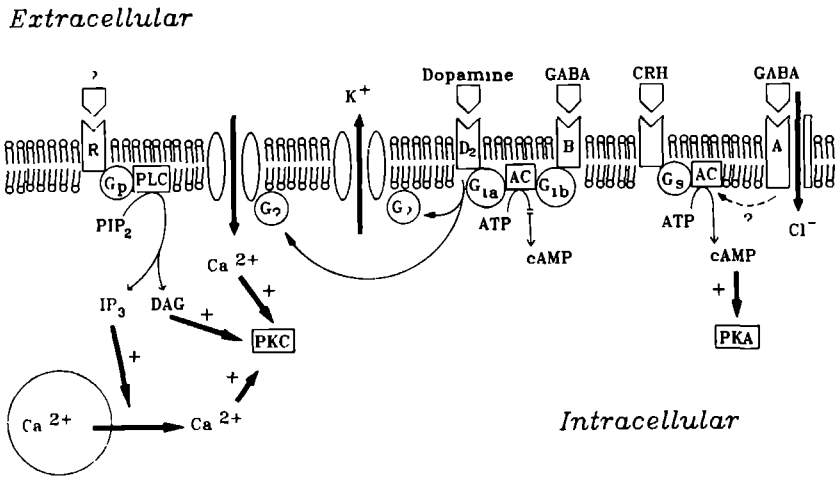

Fig. 1. *Overview of receptor-activated receptor mechanisms in melanotrope cells R = receptor, PKA = protein kinase A, PKC = protein kinase C, PLC = phospholipase C, AC = adenylate cyclase, DAG = diacylglycerol, IP_3 = inositol 1,4,5-triphosphate, cAMP = adenosine 3',5'-cyclic monophosphate, ATP = adenosine 5'-trisphosphate, D_2 = dopamine D_2 receptor, B = $GABA_B$ receptor, A = $GABA_A$ receptor*

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SAMENVATTING

De twee belangrijkste regelsystemen in dierlijke organismen worden gevormd door het zenuwstelsel en het endocriene systeem Het zenuwstelsel verwerkt de zintuiglijke informatie en reageert daarop met signalen naar perifere organen, b v spieren en spijsverteringsorganen Endocriene cellen produceren hormonen, die in het algemeen aan het bloed worden afgegeven en op die manier naar doelwit-organen worden getransporteerd

In alle dierlijke organismen is de samenwerking tussen het zenuwstelsel en het endocriene systeem van het grootste belang voor het optimaal functioneren van het organisme Door deze communicatie kan het dier niet alleen zijn gedrag, maar ook lange-termijn processen, aanpassen aan veranderende omstandigheden Voorbeelden van zulke processen zijn groei en voortplanting

Over de wijze waarop de interactie tussen zenuwstelsel en endocrien systeem verloopt bestaat nog veel onduidelijkheid Wel is duidelijk dat de communicatie in beide richtingen verloopt het zenuwstelsel reguleert de activiteit van veel endocriene cellen en veel hormonen werken (mede) op het zenuwstelsel In sommige gevallen vindt een directe innervatie van de endocriene klier plaats en wordt de secretoire activiteit van de endocriene cel bepaald door de hoeveelheid neurale boodschappers die door de zenuweinden worden afgegeven De neurale boodschappers, onder te verdelen in klassieke neurotransmitters en neuropeptiden, oefenen een stimulerende of juist een inactiverende invloed uit op de endocriene cel Dit proefschrift behandelt de vraag hoe de secretoire activiteit van een bepaalde endocriene cel, de melanotrope cel in de pars intermedia van de hypofyse van de amfibie *Xenopus laevis*, gereguleerd wordt door de neurale boodschappers

De melanotrope cellen van *Xenopus* produceren het melanoforen-stimulerend hormoon (α-MSH) Dit hormoon werkt op de pigment-bevattende cellen in de huid, de melanoforen, en zet aan tot dispersie van het zwarte pigment melanine Hierdoor krijgt de huid een zwart aanzien Voor het overleven van deze amfibie is het van belang dat zijn huidskleur wordt aangepast aan de kleur van de achtergrond Op een donkere achtergrond moet het dier dus een hoge concentratie α-MSH in zijn bloed hebben, en op een lichte achtergrond een lage concentratie De kleur van de achtergrond wordt door het dier waargenomen en in het zenuwstelsel vertaald in activiteit van bepaalde neuronen in de hypothalamus Deze neuronen innerveren de hypofyse en geven daar hun boodschappers af die de activiteit van de melanotrope cellen bepalen Tot dusver zijn twee boodschappers geidentificeerd die de afgifte van α-MSH stimuleren corticotropin-releasing factor (CRF) en thyrotropin-releasing hormone (TRH) Ten minste drie andere boodschappers inhiberen de hormoon afgifte γ-aminoboterzuur (GABA), dopamine en neuropeptide Y (NPY) De neurale boodschappers binden aan een voor die receptor specifieke receptor op het celoppervlak Het signaal dat de neurale boodschapper vertegenwoordigt wordt door de receptor naar binnen doorgegeven door middel van een intracellulaire boodschapper ("second messenger") Activering van de receptor zal dus leiden tot een toename of afname van de concentratie van intracellulaire boodschappers zoals calcium ionen, adenosine cyclisch 3',5'-monofosfaat (cyclisch-AMP) of inositol 1,4,5-trifosfaat (IP_3) Dit proces wordt signaaltransductie genoemd

Om meer inzicht te verkrijgen in de manier waarop het zenuwstelsel de α-MSH afgifte reguleert, is in dit onderzoek aandacht geschonken aan de volgende vragen 1) Wordt de secretoire activiteit van melanotrope cellen uitsluitend bepaald door neurale boodschappers of is er ook een endocriene component betrokken bij de regulatie, mogelijk via een

terugkoppeling van het eigen secretieproduct op de afgifte ervan? 2) Werken de neurale boodschappers direct op de melanotrope cellen of zijn er andere hypofysecellen die als intermediair optreden? 3) Welke receptoren worden door de verschillende boodschappers gebruikt en wat zijn de farmacologische eigenschappen van die receptoren? 4) Welke intracellulaire processen worden in werking gesteld door de binding van de neurale boodschappers aan hun receptoren?

ad 1) Uit de experimenten beschreven in hoofdstuk 1 blijkt dat α-MSH geen effect heeft op de afgifte van peptiden uit de melanotrope cellen Ook het volledige door melanotrope cellen afgegeven endocriene signaal (mengsel van peptiden) had geen invloed op het secretie proces Hieruit volgt dat de melanotrope cel niet gereguleerd wordt door middel van een direct terugkoppelingsmechanisme

ad 2) De neurointermediaire lob bestaat uit een neuraal gedeelte (pars nervosa) en een endocrien gedeelte (pars intermedia) De pars intermedia wordt voor het overgrote deel gevormd door de melanotrope cellen, met daarnaast een kleine hoeveelheid glia-achtige stellaatcellen Van de meeste van bovengenoemde neurale boodschappers is aangetoond dat ze direct aangrijpen op de melanotrope cel Voor NPY kon dit niet worden aangetoond (hoofdstuk 2) NPY remde wel de α-MSH secretie door gekweekte neurointermediaire lobben, die geen functionerende zenuweinden maar wel stellaatcellen bevatten Kennelijk inhibeert NPY de α-MSH secretie op indirecte wijze, via de stellaatcel

ad 3) Hoofdstuk 3 behandelt de receptoren voor een van de andere neurale boodschappers, GABA De melanotrope cel heeft twee verschillende receptoren voor deze neurotransmitter, de $GABA_A$ receptor en de $GABA_B$ receptor Activatie van de A of B receptor induceert een remming van de α-MSH secretie Om de bijdrage van elk type GABA receptor in de regulatie van de α-MSH secretie te onderzoeken was de farmacologische karakterisering van beide receptoren noodzakelijk De farmacologie van de $GABA_A$ receptor is reeds eerder beschreven, maar voor $GABA_B$ receptoren van endocriene cellen was geen antagonist bekend Met behulp van de in dit hoofdstuk geïdentificeerde antagonisten werd vastgesteld dat de $GABA_B$ receptor gevoeliger is voor stimulatie met GABA dan de $GABA_A$ receptor Verder werd gevonden dat activatie van de $GABA_B$ receptor *in vivo* niet essentieel is voor de inhibitie van α-MSH secretie in dieren die zijn geadapteerd aan een witte achtergrond Waarschijnlijk is een mengsel van meerdere inhiberende neurale boodschappers verantwoordelijk voor de inhibitie van de secretie

ad 4) De hoofdstukken 4-8 behandelen diverse aspecten van de intracellulaire processen van de melanotrope cel Aandacht wordt gegeven aan zowel de basale condities van de ongestimuleerde cel *in vitro* (hoofdstukken 4-6) als aan de effecten van activatie van specifieke receptoren op die intracellulaire processen (hoofdstukken 5-8)

In hoofdstukken 4 en 5 wordt de rol van het Ca^{2+}/IP_3 systeem van signaaltransductie behandeld Met behulp van electrofysiologische technieken werd vastgesteld dat de melanotrope cellen van *Xenopus* spontane actiepotentialen vuren en voltage-afhankelijke calciumkanalen bezitten De rol van deze kanalen werd verder onderzocht met superfusie technieken Het blijkt dat influx van Ca^{2+} door met name N-type calciumkanalen essentieel is voor de α-MSH secretie Het vrij maken van calcium ionen uit intracellulaire opslag lijkt weinig bij te dragen aan de secretie Dit is in overeenstemming met de bevinding dat de concentratie van de intracellulaire boodschapper IP_3, die Ca^{2+} vrij maakt uit intracellulaire depots, niet of nauwelijks beinvloed wordt door activatie van receptoren voor dopamine en GABA Mogelijk is intracellulair opgeslagen calcium betrokken bij andere cellulaire processen

zoals biosynthese. Dit wordt gesuggereerd door de langzame veranderingen in IP_3 concentratie nadat dieren die geadapteerd zijn aan een zwarte achtergrond op een witte achtergrond geplaatst worden.

Hoofdstukken 6-8 behandelen onder meer de rol van de intracellulaire boodschapper cyclisch-AMP in de regulatie van de α-MSH secretie. Er werd vastgesteld dat deze boodschapper een belangrijke rol speelt in de regulatie van de afgifte van α-MSH. Activering van de dopamine receptor of de $GABA_B$ receptor leidt tot een sterke daling in de produktie van cyclisch-AMP, die samenvalt met de remming van het secretieproces. Bovendien veroorzaken sauvagine en CRH (peptiden die de α-MSH afgifte stimuleren) een sterke stijging van de cyclisch-AMP produktie. De correlatie tussen intracellulaire cyclisch-AMP concentratie en secretie is echter niet absoluut, zoals blijkt uit experimenten met agentia die wel de cyclisch-AMP produktie verhogen, maar niet de α-MSH afgifte stimuleren. Ook het feit dat activatie van de $GABA_A$ receptor, hetgeen leidt tot remming van de secretie, een lichte stijging van de cyclisch-AMP produktie tot gevolg heeft, duidt er op dat de cyclisch-AMP concentratie en secretie-activiteit niet altijd direct gekoppeld zijn. Deze experimenten en het feit dat het effect van dopamine niet afhankelijk is van de intracellulaire cyclisch-AMP concentratie, bewijzen dat de cyclisch-AMP concentratie een belangrijke factor is bij het reguleren van de secretie, maar dat onder sommige omstandigheden bijvoorbeeld de potentiaal van de celmembraan of de calcium concentratie bepalend is.

Het onderzoek heeft aangetoond dat de neurale boodschappers die de secretoire activiteit van de melanotrope cellen van *Xenopus laevis* bepalen, dit elk op een unieke wijze doen. Hierbij zijn de stellaatcellen betrokken en een aantal verschillende signaaltransductie systemen. Vervolgonderzoek zal moeten aantonen of de verschillende boodschappers dus ook verschillende cellulaire processen reguleren, afgezien van het effect op α-MSH secretie. Dit zal dan een antwoord geven op de vraag waarom er zoveel boodschappers betrokken zijn bij de regulatie van de melanotrope cel en mogelijk leiden tot een groter inzicht in de fysiologische betekenis van de secretieproducten van de melanotrope cel.

september 1992

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CURRICULUM VITAE

Harry de Koning werd geboren op 25 oktober 1961 in Rotterdam en verhuisde kort daarop naar Bleiswijk, waar hij tot zijn zesde jaar woonde. In de hierop volgende periode woonde hij in Zevenaar. Na in 1980 het einddiploma VWO te hebben gehaald aan het Christelijk Lyceum te Arnhem begon hij zijn studie scheikunde aan de Vrije Universiteit in Amsterdam. Het kandidaatsexamen werd behaald in februari 1985. In januari 1988 volgde het doctoraalexamen, met als hoofdvak Biochemie (onder leiding van Dr. T.T.A.L. El-Baradi en Prof. Dr. R.J. Planta) en als bijvak Organische Chemie in de groep van Prof. Dr. G.W. Klumpp en Dr. J.L. van der Baan. Vanaf april 1988 tot april 1992 was hij werkzaam als Assistent in Opleiding bij de Vakgroep Experimentele Dierkunde van de Katholieke Universiteit Nijmegen. In deze periode werd onder leiding van Dr. B.G. Jenks en Prof. Dr. E.W. Roubos het onderzoek verricht dat in dit proefschrift is beschreven. Daarnaast werd een bijdrage geleverd aan het doctoraal onderwijs van studenten Biologie. De schrijver van dit proefschrift is momenteel werkzaam als post-doctoraal onderzoeker in het laboratorium van Prof. Dr. P.M. Conn te Iowa City.

RECEPTIE
NA AFLOOP VAN DE PROMOTIE IN HET
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